Novel collagen-based wafers as a drug delivery method for local analgesia in deer antlers

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Overview

The overarching goal of this PhD is to prepare novel nanocomposite local anaesthetic formulations of collagen, which, when applied to the open wound, would slowly release the local anaesthetic drug and enhance the wound healing process.

The initial stage of this research focused on using collagen as a vehicle to deliver lidocaine and bupivacaine for a prolonged period. The formulations thus obtained were tested using an *in vitro* drug release test (IVDRT) to measure the rate and amount of drug release. IVDRT compared the drug release characteristics of collagen extracted with different extraction methods and determined the effects of crosslinking of collagen fibres with nanoparticles on drug release. IVDRT aided in the selection of the collagen and local anaesthetics formulation to be tested in the animal study. The animal study (*in vivo*) used velvet antler removal in deer as a model to investigate the safety and efficacy of the topical formulation containing collagen and local anaesthetic after its application to the wound resulting from cutting the velvet antler in deer.

The pilot study was conducted on three groups of 6 deer each. In all three groups, the antlers were removed following the standard industry protocol (local anaesthetic ring block in a hydraulic crush). The control group did not receive any post-operative treatment. Treatment group 1 received a topical application of a collagen local anaesthetic nanocomposite formulation, while in treatment group 2, a collagen local anaesthetic formulation prepared without any crosslinking was used. Blood samples were collected from the treatment groups to measure the systemic absorption of both the local anaesthetics from the formulations.

The mechanical nociceptive thresholds (Newtons) or MNT in all the animals were tested by using a handheld algometer applied around the pedicle. The wound healing aspect of the treatment groups was measured by using the FLIR (Forward Looking Infrared) camera, which measured the temperature of the wound both at its centre and periphery and could detect inflammation. The collagen formulation in treatment group 2 animals (non-crosslinked collagen) did not adhere appropriately. In contrast, 3 of the six animals in treatment group 1 had the formulation until the end of the study period (48 hours). The results of mechanical pain testing showed that the collagen nanocomposites crosslinked with nanoparticles were able to provide more prolonged pain relief as compared to the control group. The MNTs thresholds were significantly different after 2 hours of the removal of antlers. Thermal imaging results did not show any significant difference between the groups. The adherent properties of the formulation on the wound can be enhanced by modifying collagen fibres and making them look more porous and spongier, with

a higher potential to absorb blood from the wound. This change in the chemistry of collagen resulted in changes in the drug release properties, tested using IVDRT, followed by the second-year animal studies.

In the second year of study, the first stage involved the validation of algometry and investigating the baseline reading in forty animals, and it was found that the heavier animals and the deer with longer antlers lengths tolerate pain induced by the noxious mechanical stimulus to a greater degree. In this study, we also developed a grading system for the MNT readings, and we confirmed 50 N as the cut-off point for our second *in vivo* study.

The *in vitro* study comprised of 5 groups including the control group, a group with collagen nanocomposite wafers without nanoparticles and three other groups of collagen wafers with 5%, 10%, and 25% nanoparticles. The results proved that by increasing the percentage of nanoparticles, particularly in 25% involvement, the release of the local anaesthetics is sustained.

For the *in vivo* study, forty animals, which had previously been enrolled in algometry validation testing were assigned to 4 treatment groups. The groups consisted of control with no wafers, treatment groups with non-modified wafers, and two treatments involving modified wafers with nanoparticles which contained 5% and 25% nanoparticles. The results showed that the treatment groups with modified collagen wafers had a longer-lasting adherence to the antler wounds and delivered pronged analgesia compared to the other groups. Wound healing in the 5% treatment group was comparative to the control, and none of the groups compromised the wound healing process after two months. Modified collagen wafers with 25% adhered for a longer time as compared to control and 5% and thus effectively provided analgesia for 10 hours of analgesia post-velvet removal. can be explored further for

There is great scope for this research, and we propose this technique to be implemented in the deer industry to help deer experience less or even no pain after velvet antler removal.

Abstract

Introduction

This study provided a practical and novel solution for post-operative pain mitigation and wound management after velvet antler removal in red deer *(Cervus elaphus)*. Currently, there are no topical methods to mitigate pain for an extended period of time in deer following surgical removal of antlers. The current methods licensed in New Zealand provide only peri-operative analgesia with short-term effects and raise animal welfare concerns about whether animals are still in pain when the effect has worn off, especially in the deer industry in which a large number of animals are being managed.

Materials and methods

In vitro study: In vitro drug release test (IVDRT) was conducted using the Franz diffusion cell to assess the drug release rates of lidocaine and bupivacaine in two different phases of the pilot and main studies. The pilot *in vitro* study contained 9 treatment groups and 3 control groups (n=3), which were classified based on collagen extraction technique, whether modified with zinc oxide-polyvinylpyrrolidone (ZnO-PVP) nanoparticles and the difference in the order of adding local anaesthetics and ZnO-PVP nanoparticles. The main *in vitro* study was comprised of 4 treatment groups of 5%, 10%, and 25% ZnO-PVP nanoparticles (n=6) proportional to dry collagen weight and a control group. In both pilot and main *in vitro* studies, the samples were taken every 15 minutes in the first hour and every 2 hours up to 12 hours. LC-MS and HPLC were used for the quantification of the samples in the pilot and main *in vitro* studies, respectively.

MNT validation study: Forty male deer (stags) were assigned for the MNT validation study on three alternative days. A handheld algometer (Wagner FPX50™) was used for mechanical nociceptive threshold (MNT) assessment of four antler sites (cranial, medial, caudal, lateral) in both right and left antlers. Animal body weight (kg) and antler length (cm) were recorded to investigate the correlation with MNT. The MNT readings from three days were compared with each other. In addition, the MNT reading from all four antler sites and the right and left antlers were compared with each other.

In vivo study: Eighteen stags sorted into three groups of 6 animals in each (2 treatment groups and 1 control group) for the pilot *in vivo* study, and forty yearling age stags assorted into four groups of 10 animals (three treatments and one control), were used in the main *in vivo* study. All animals had both antlers removed after administration of local anaesthesia. The control group in both pilot and main *in vivo* studies received a ring block of 4% articaine hydrochloride only, whilst the treatment groups received modified (with ZnO-PVP) or non-modified collagen composite wafers to the wound sites. The modified collagen composite wafers had 50% ZnO-PVP for the pilot *in vivo* study and had 0%, 5% or 25% ZnO-PVP proportional to dry collagen weight for the main *in vivo* study. A handheld algometer (Wagner FPX50™), was used for mechanical nociceptive threshold (MNT) assessment at different time points (0, 4, 24, 72 hrs, 7 days and 14 days). Thermal imaging with a forward-looking infrared (FLIR) camera was performed for the detection of temperature differences between the groups. Digital photography of the wounds was performed for further quantitative wound healing analysis.

Pharmacokinetic study: Blood samples were drawn from deer after the application of collagen composite wafers at time points t0, t1, t2, t4, t6, t8, t12, and t24 hours for the pilot study and at time points t0, t1, t2, t4, t6, t8, and t24 hours for the main *in vivo* study. The plasma was

analysed with LC-MS to calculate pharmacokinetic parameters with the non-compartmental method such as C_{max}, T_{max}, AUC, AUMC, half-life, the volume of distribution and clearance.
Statistical analysis: Higuchi model was mainly incorporated to calculate drug release rates for the *in vitro* studies. For *in vivo* studies, the statistical analyses were performed with a linear model for repeated measurements that accounted for the fixed effects of day, antler, location within antler or antler sites, antler length and weight of deer as covariates, and the random effect of animals.

Results

IVDRT did not show any statistically significant difference between the treatment groups; however, the treatment groups had significantly slower release compared to the control group in the pilot *in vitro* study. IVDRT in the main *in vitro* study showed the slowest release rate in the treatment group with 25% ZnO-PVP compared to the other groups for both lidocaine and bupivacaine. The control group had the most rapid drug release rates compared to the treatment groups, particularly for lidocaine. Furthermore, lidocaine showed a considerably slower release compared to bupivacaine when zinc oxide nanoparticles were incorporated, and the results significantly differed. MNT validation results showed that antler length (cm) and animal body weight (kg) are directly and positively correlated with the baseline MNT readings. The MNT readings from four sites of antlers, including cranial, medial, caudal and lateral aspects, did not have any significant difference from each other. In addition, the MNT readings from the right and left antlers did not show any significant difference from each other.
In vivo results in the pilot study showed a lack of collagen composite wafer adherence for the non-modified wafers (PT2) and 50% adherence for the modified wafers (PT1) in the pilot study. As a result of the main *in vivo* study, 90%, 70%, and 45% were in group 25%NP (T1), 5%NP (T2), and 0%NP (T3) to the wounds, respectively. A significant difference was observed in the recovery rates of PT1 compared to the control group ($P<0.0001$) for the pilot study. For the main *in vivo* study, all three treatment groups also showed a significant difference compared to each other: T1 vs. T2 ($P<0.01$), T1 vs. T3 ($P<0.05$), and T2 vs. T3 ($P<0.0001$). In addition, the treatment groups showed a significantly slower recovery rate from analgesia compared to the control group ($P<0.0001$ for all). All the treatment groups in the main study demonstrated analgesia beyond 6 hrs and up to 10 hrs.
The pharmacokinetics study showed significantly smaller C_{max} for T1 and T2 compared to T3 only for bupivacaine. T_{max} showed significantly smaller values for T1 compared to T2 for only bupivacaine. Both $AUC_{(0-24)}$, $AUC_{(0-\infty)}$, and $AUMC_{(0-\infty)}$ showed smaller values for T1 and T2 compared to T3.

Conclusion

The physically modified collagen composite wafer with zinc oxide-PVP nanoparticles, containing a short-acting (lidocaine) and a long-acting (bupivacaine) local anaesthetic, is a novel method to sustain drug delivery of local anaesthetics after the surgical removal of velvet antlers. Our suggested treatment can deliver analgesia to the wounded antler for up to 10 hours and is a safe and convenient method to use by farmers in the deer industry. Furthermore, the collagen wafer is very adhesive to the wound and can help facilitate wound healing of deer antlers.
KEYWORDS: Sustained Release, Pain, Lidocaine, Bupivacaine, Articaine, Collagen, Composite, Analgesia, Algometry, Mechanical Nociceptive Threshold, Local Anaesthesia, Red Deer

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Lastly, I would like to acknowledge all may not be cited.

List of Publications and Patents

1) Sahebjam F. July 22nd, 2021, Oral presentation (as a delegate), ***Australian/NZ College of Veterinary Scientists*** (***ANZCVS***), Science week, Gold Coast, Australia "Pressure algometry as a method to quantify pain and the efficacy of articaine hydrochloride in Red Deer (*Cervus elaphus*)".

2) Sahebjam F, Kongara K, Chambers JP, Walker RE, Naffa R, Lopez-Villalobos N, Singh PM. Pressure algometry validation and determination of efficacy of articaine hydrochloride ring block in antler removal in Red Deer (*Cervus elaphus*). ***Animals***. 2020; 10(11): 2023.

3) Sahebjam F. July 4th, 2019, Oral presentation (as a delegate), ***Australian/NZ College of Veterinary Scientists*** (***ANZCVS***), Science week, Gold Coast, Australia "Collagen nanocomposites for postoperative pain management in velvet antler removal of red deer".

4) This project was granted an Australian-NZ patent in 2021-22.

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Abbreviations

AUC	*Area under the curve*
AUMC	*Area under the moment curve*
Col	*Collagen*
COX	*Cyclooxygenase*
HCl	*Hydrochloric acid*
HPLC	*High-performance liquid chromatography*
IVDRT	*In vitro drug release test*
LCMS	*Liquid Chromatography/Mass Spectrometer*
LLQ	*lower limit of quantification*
LOD	*Limit of detection*
MNT	*Mechanical nociceptive testing*
MRT	*Mean resident time*
NaCl	*Sodium Chloride*
NaOH	*Sodium hydroxide*
NP	*Nanoparticle*
PVP	*Poly (vinylpyrrolidone)*
UV	*Ultra Violet*
SC	*Subcutaneous*
SPE	*Solid phase extraction*
RSD	*Relative standard deviation*
$T_{1/2}$	*Half life*
TiO_2	*Titanium oxide*
TPLO	*Tibial-Plateau-Levelling Osteotomy*
Vd	*Volume of distribution*
ZnO	*Zinc oxide*

To my loving family

Askar Sahebjam

Shirin Hashemy Soodmand

Solmaz Sahebjam

Farhad Sahebjam

My devoted fiancé

Ruth Ellen Walker

Our adorable fur babies

Franco, Yashar, Keyvan, and Fandy

And our little daughter

Lillian Maia

And finally

To all animal kind

Chapter 1

General Introduction

1.1. Background

This research focused on assessing a novel formulation to provide pain relief and enhance wound healing in deer whose antlers are surgically removed, for either commercial purposes or in the case of young stags, before their slaughter (Wilson *et al.*, 2001). The current industry practice is to remove the antlers under a lidocaine ring block applied around the base of each antler (Wilson *et al.*, 2001). Lidocaine has a rapid onset and relatively short duration of action ranging from 60 to 90 minutes (Wilson and Stafford, 2002, Wilson *et al.*, 2001). Pollard *et al.* 1992a and 1992b first described evidence of painful behaviour in deer 2 hours after antler removal (Pollard *et al.*, 1992a, Pollard *et al.*, 1992b). Additionally, there are numerous reports of postoperative infections at the site of antler removal in deer inspected at slaughter plants (Killorn and Heath, 1993, Lloyd, 2002). This suggests that there is a need to improve wound management and the pain associated with infected wounds.

To date, postoperative pain management (as opposed to the duration of drugs to treat operative pain) has not been investigated in deer. Various analgesic drugs are employed in humans (Kokki, 2003), and in other animal species, such as non-steroidal anti-inflammatory drugs (NSAIDs) in cats (Slingsby and Waterman-Pearson, 2000), dogs (Leece *et al.*, 2005) and cattle (Anderson and Edmondson, 2013), α2 agonists such as xylazine in cattle (Anderson and Edmondson, 2013) and opioids in humans and animals (Johnson *et al.*, 2005, Duke-Novakovski, 2013); however, to date, they have not been investigated in deer (Walsh and Wilson, 2002). Moreover, analgesic drugs such as non-steroidal anti-inflammatory drugs (NSAIDs) (Lazzaroni and Bianchi Porro, 2004, Clark-Price, 2013), opioids (Johnson *et al.*, 2005, Duke-Novakovski, 2013), and α2 agonists such as xylazine (Seddighi, 2013) have significant adverse effects in humans and other mammalian species. NSAIDs such as ketoprofen or meloxicam can induce gastrointestinal damage and ulceration by suppressing COX-1 and COX-2 enzymatic activities, interfering with prostaglandin

formation, and altering gastrointestinal mucosal defence in most species (Wallace and Vong, 2008). NSAIDs may also have variable potency and may not provide sufficient analgesic effect for painful conditions in large animals (Anderson, 2012).

In ruminants, opioids are less effective than other analgesics, such as local anaesthetics, as they require high dosing and have a short duration of action (Chambers, 2011). The occurrence of ileus due to decreased GI motility, respiratory depression (Pattinson, 2008), and sedation (Duke-Novakovski, 2013) exist as the potential adverse effect.

Alpha-2 adrenergic agonists such as xylazine are known to cause hypoxemia in small ruminants as a result of ventilation-perfusion mismatch (Celly *et al.*, 1997a, Celly *et al.*, 1997b). Nausea and vomiting have been reported in small animals such as dogs and cats (Granholm *et al.*, 2006, Vähä-Vahe, 1989).

Combining local anaesthetics such as lidocaine with bupivacaine or mepivacaine may be beneficial in providing quick and more extended pain relief for disbudding of calves and dehorning of cattle as opposed to using local anaesthetics individually (Wilson and Stafford, 2002, Sutherland *et al.*, 2002, Petrie *et al.*, 1996). However, these techniques have not been researched in deer.

This research topic explores a knowledge gap in animal welfare within the deer industry and aims to find a suitable treatment for postoperative pain in deer. The research seeks to achieve this in a way that delivers analgesia for an extended period, enhances the wound healing process, and makes treatment practical on farms as it is non-invasive. There is currently no topical formulation available that can provide long-lasting analgesia and assist in the management of wound-healing for velvet antler removal. According to Animal Welfare (Care and Procedures) Regulations 2018, velvet removal is classified as a surgical procedure. Currently, postoperative pain is not addressed as part of 58C. However, according to Haigh *et al.* (2001), mitigating

postoperative pain should be included as part of the procedure to address animal welfare, and this field requires further research (Haigh *et al.*, 2001), and more importantly, veterinarians are responsible for the welfare of animals under their care and this involves treating pain. Similarly, American Veterinary Medical Association (AVMA) in 2009 and in relation to post-operative pain management re-stated concerns previously addressed by Walsh and Wilson (2002), which identified that there is a need for more research to discover a safe and effective method for postoperative pain management (Walsh and Wilson, 2002).

1.2. Anatomy of the deer antler

i. Growing antler tissue and velvet

Antlers are produced in most species of deer, and with the exception of reindeer, only in male animals (Kawtikwar *et al.*, 2010). Antlers differ from horns, as they have the capability to develop branched architecture. In addition, antlers are not permanent structures, unlike horns, and can regenerate and re-grow from the base annually (Goss, 1983). Antler growth is significantly impacted by testosterone, and as such is considered a secondary sex characteristic. Each season, antlers develop and are shed, and their character and shape is influenced by the seasonal levels of testosterone in male deer. The antler growth begins in spring and peaks to its maximum at the end of summer, with minimal further growth occurring during autumn (Goss, 1983).

Velvet antlers are predominantly made of cartilage and skin, rich in collagen and hydroxyapatite calcium (Gomez *et al.*, 2013). The growing antler is comprised of densely aggregated mesenchymal cells, which differentiate into cartilage and bone. Osteoclastic activity can be altered by circulating sex hormones, particularly testosterone: increased testosterone levels increase the inhibition of the RANKL signalling pathway in the bone cells, resulting in more antler growth (Shevde *et al.*, 2000, Huber *et al.*, 2001). The existing cartilage eventually calcifies and ossifies (Kierdorf and Kierdorf, 2002). The cartilage and bone are covered by a perichondral (periosteal) layer, and the entire antler is encased by "velvet", which is a modified skin covered by hair follicles producing short hairs which resembles velvet. The velvet layer contains numerous sebaceous glands; however, it lacks erector pili muscles and sweat glands (Li and Suttie, 2000). Each antler is connected to the skull by the frontal protuberance, located close to the parietal suture (Kierdorf and Kierdorf, 2002). Refer to Figure 1.1 for more details about the antler structure.

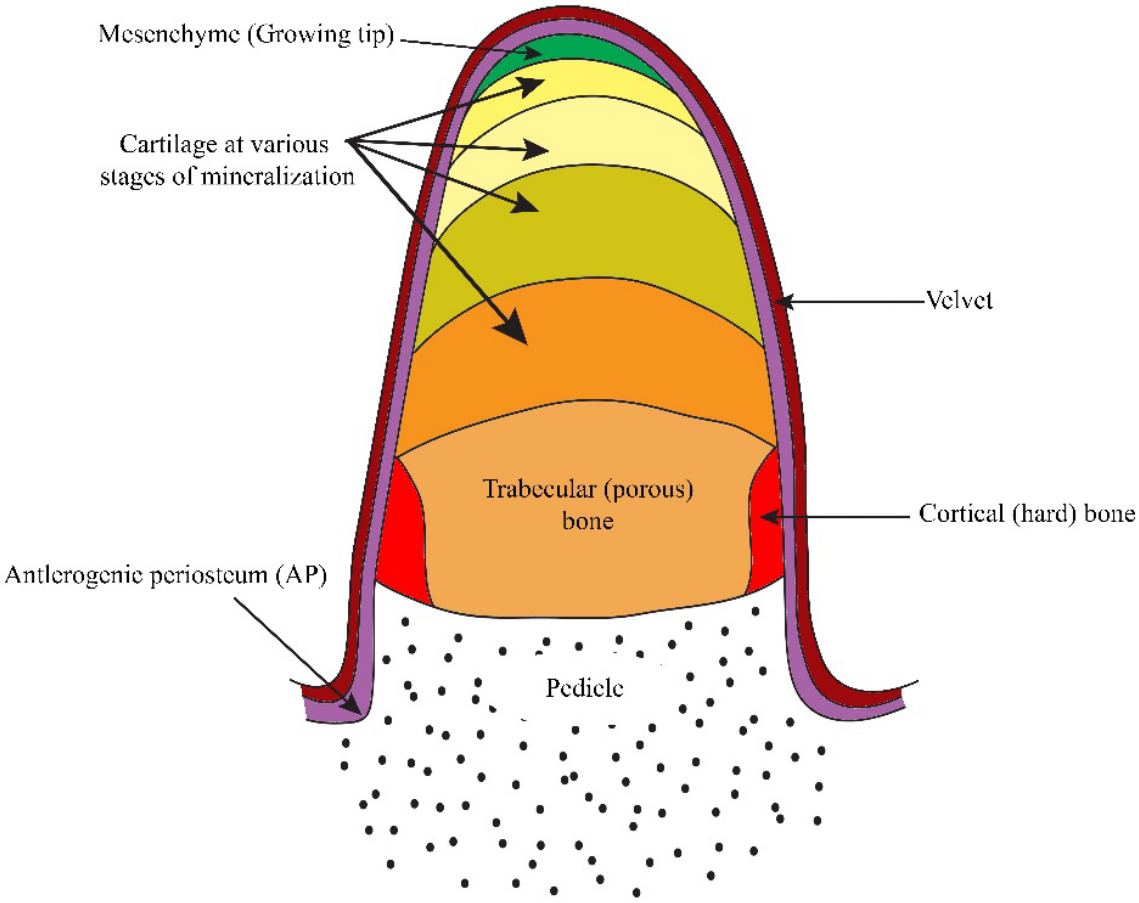


Figure 1.1 Schematic illustration of deer antler growth and its mineralization (modified from (Flint, 2012)).

ii. Histology of the velvet antler

Velvet arises from the epidermis, which overlies a thick dermis. The dermal layer is comprised of the outer dermis and inner dermis. The outer dermis consists of many hair follicles and sebaceous glands. Below the dermal layer lies the vascular layer, which contains highly vascular loose connective tissue (Wislocki and Singer, 1946, Vacek, 1955, Pita-Thomas *et al.*, 2010, Li *et al.*, 2005, Barling *et al.*, 2005), as well as nerve fibre bundles which project through the vascular layer to the more superficial layers above. The nerve fibres may also be observed in both the dermal and epidermal layers, in addition to the periosteal and perichondral layers (Wislocki and Singer, 1946, Vacek, 1955, Li *et al.*, 2005). Antler nerves contain both myelinated and non-

myelinated fibres (Wislocki and Singer, 1946, Vacek, 1955). The nerves converge at the base of the antler and become isolated at the tip (Nieto-Diaz *et al.*, 2012).

iii. Nerve Supply

The anatomy of antler innervation in deer was first described in the 19th century (Berthold, 1831), and later studies continued on Virginia deer (*Odocoileus virginianus*) (Wislocki and Singer, 1946), red deer (*Cervus elaphus*) (Adams, 1979) wapiti (*Cervus canadensis*) (Woodbury and Haigh, 1996), and fallow deer (*Dama dama*) (Woodbury and Haigh, 1996). According to these studies, the supraoptic (infratrochlear) nerve, a branch of ophthalmic division, and the zygomaticotemporal nerve, a branch of the maxillary division of the trigeminal nerve (CN V), innervate the antlers (Figure 1.2). The supraoptic branch exits from the skull under the orbital edge as a single bundle (close to the medial canthus in red deer) and courses medially towards the medial angle of the eye and over the dorsal rim (Adams, 1979, Woodbury and Haigh, 1996). Then, it courses medially through the orbicularis oculi muscle and branches into several nerves that pass caudally to the base of the antler, around the pedicle. From the other side, the zygomaticotemporal nerve (a branch of the maxillary nerve) emerges on the scalp close to the zygomatic arch, which lies at the caudal margin of the zygomatic process of the frontal bone. The zygomaticotemporal nerve then divides into a number of branches that travel towards the base of antlers and ears. Those branches which innervate the antlers also pass through the periorbital fat, under the frontalis muscle, caudo-dorsally.

These nerves branch in the space between the orbit and pedicle and track around the lateral and caudal aspects of the antler pedicle, and finally on the antlers themselves. Both branches of the trigeminal nerve lie close to the medial and lateral coronary branches of the superficial temporal artery (which itself is the branch of the external carotid artery), and these vessels are responsible for the blood supply of the deer antlers (Wislocki and Singer, 1946, Woodbury and Haigh, 1996). Additionally, the zygomatic branch of the auriculo-palpebral nerve, a branch of cranial nerve VII

(facial nerve), also courses towards the pedicle from the medial aspect (Adams, 1979, Woodbury and Haigh, 1996) (Figure 1.2).

The complexity of the nerve supply to the antlers means that it is more effective to apply local analgesia around the antler as a ring block than applying a specific nerve block (Walsh and Wilson, 2002). Wilson *et al.* (1999) showed that high dose ring block is effective and has a faster onset than the high dose regional nerve block in stags (Wilson *et al.*, 1999).

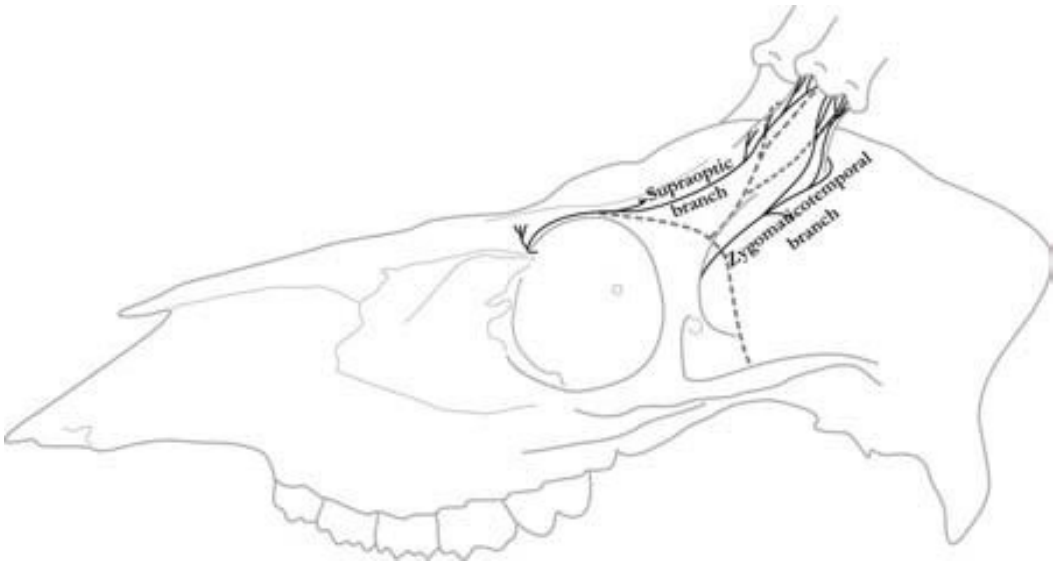


Figure 1.2 The illustration of deer antler nerve supplies by Nieto-Diaz et al. (2012). The solid lines are branches of the Trigeminal nerve, and the dashed line is the superficial temporal artery.

iv. Vascular supply

Deer antler is a highly vascularized tissue. The superficial temporal artery, a branch of the external carotid artery, continues into the lateral and medial arteries at the base of the pedicle before continuing rostrally. Another component of the superficial temporal artery, the intermediate artery, connects both the lateral and medial arteries, thus forming a vascular ring at the base of the pedicle. This ring gives rise to a number of branches that extend distally to the end of the antler (Adams, 1979) (also see Figure 1.3). The venous return is by the superficial temporal vein formed by converging lateral and medial veins draining the tip of the antler. The bony structure in the middle of the antler also contains blood vessels, and they are prone to

bleed after antler removal (Flint, 2012). Understanding the vasculature and the nature of bleeding from the surgical wounds is essential to create a topical product that mitigates blood loss but is sufficiently absorbent to allow drug transfer from a drug delivery vehicle to the wound surface.

Figure 1.3 Left lateral view of the vascular supply in red deer (Cervus elaphus) skull and antlers. Courtesy of Allan Nutman, School of Veterinary Science, Massey University, New Zealand.

1.3. Deer antler removal

i. The commercial importance of deer antler

Deer antler has been removed and consumed in Asian countries such as China and Korea for centuries (Kawtikwar *et al.*, 2010). However, in western countries such as New Zealand, commercial deer farming is a relatively new industry, first established in 1970 (Kawtikwar *et al.*, 2010). In New Zealand, the primary purpose of commercial deer farming is to produce and export venison, antler, and by-products. According to the Deer Industry New Zealand (DINZ) Annual Report 2018-19, the farm gate value of velvet produced in New Zealand was over 100 million NZD, more than double the farm gate value ten years prior. The production of velvet antlers surpassed 800 tonnes, a 12% increase in the previous year and almost doubled the amount of the production in 2010-11 (Flint, 2012, DINZ, 2019). The majority of harvested velvet antlers were sold to East Asian countries (94%), including Korea (43%), China (41%) and Hong Kong (10%). A small amount (2%) was exported to the United States of America (Flint, 2012).

Once the deer antler becomes calcified, the velvet is shed, and the hard antler casts naturally; however, since the cast antlers lack the velvet covering, the market has no interest in purchasing them. Therefore, the deer velvet industry focuses on harvesting the antler during its growing stage (Kawtikwar *et al.*, 2010).

Velvet antlers are highly valued, particularly within Asian cultures, where they have been used in traditional medicines for more than 2000 years. In the last fifty years, from the beginning of deer farm legalization, there has been a considerable amount of genetic selection for antlers, resulting in heavier antlers and higher yields; thus, greater financial benefits. A stag can grow up to 5 kg of velvet antler annually, with an average price of 80 NZD per kilogram (2019), ie, 400 NZD per year per stag. This market easily competes with other agriculture commodity markets in value and economic returns. However, the market is also subjected to financial fluctuations that make it unpredictable. For example, in 1990, the prices increased to 247 NZD per kilogram for premium quality antlers and dropped to 40 NZD per kilogram in 1999. Nevertheless, the

convenience of storage, maintenance, and low health concerns has made turned velvet antlers a suitable and beneficial product for farmers (Flint, 2012).

The FDA in the United States of America has approved velvet antler use in treating arthritis and enhancing performance among athletes (Gilbey and Perezgonzalez, 2012, Sleivert *et al.*, 2003). In addition, a recent report suggested that pharmaceutical manufacturers use more than a thousand kilograms of deer antlers annually, which has made this product unique among other traditional medicines in China (Wu *et al.*, 2013). This increase in demand and interest has led to rapid growth in deer farming in New Zealand (www.deernz.org).

ii. Transport

Commercially reared yearling stags destined for venison production are transported to venison processing plants after their antlers are removed in the first year. The industry recommendations states that velvet antlers should be removed before March and should not exceed more than 110 mm prior to transportation (Code of welfare transport in New Zealand, year 2016, page 13, issued under Animal Welfare Act 1999), and they should be removed with proper analgesia due to the risk of trauma to themselves or the other deer or handlers (Goddard, 2019, MPI, 1999, MPI, 2016).

iii. Current practice on deer antler removal

Velvet antlers are removed from live animals by either veterinarians or farmers acting under veterinary supervision and following the Animal Welfare (Care and Procedures) regulation (2018). Velvet antlers are removed surgically under lidocaine ring block using a sharp saw. A tourniquet is applied at the base of the antler before injecting the local anaesthetic below the tourniquet to reduce its distribution to the antler (Wilson and Stafford, 2002, Wilson *et al.*, 2001). All efforts should be made to ensure no pain and stress during the harvesting procedure (Kawtikwar *et al.*, 2010). Correct harvesting of velvet antlers is not known to have any long term negative impact on the regrowth of the antlers, the physiology of the animals, or their social or behavioural needs (Flint, 2012).

An alternative harvest method utilises NaturO™ rings, which are strong rubber rings applied around the base of the pedicle. They provide some local desensitization and control of bleeding (Woodbury *et al.*, 2002). The application is limited to yearling deer (1-year-old) that require their antlers to be removed before slaughter (Flint, 2012). The duration of NaturO™ ring application must not exceed 72 hours due to the heightened risk of tissue necrosis (DeerQA, 2019). The preferred method of application developed by Nicol *et al.* (2009) and repeated by Flint (2012) was called the cable-tie method. By this method, the free end of the cable tie was passed through a rubber band and tied onto itself, creating two links, one being the NaturO™ ring and the other, the cable tie. The cable-tie link is inverted to create a loop with one side a double rubber band and the opposite side a double cable tie. The loop is passed over the antler tip and brought to the antler base, just above the pedicle, at which point the cable tie is pulled tight, resulting in a double-thickness band of the NaturO™ ring encompassing the antler. After 1 hour, there is sufficient analgesia to remove the antlers (Matthews and Suttie, 2001, Flint, 2012, Nicol *et al.*, 2009), a nick test (saw test) for reassurance of sufficient analgesia is performed, and antlers are removed using handheld meat saw (Nicol *et al.*, 2009). However, pain relief is temporary

and inferior to that provided with local anaesthesia (Woodbury *et al.*, 2002, Flint, 2012), and the deer might feel discomfort during application (Flint, 2012).

1.4. Pain

i. The importance of postoperative analgesia after antler removal

Velvet antler removal using current industry practice can result in haemorrhage and pain (Pollard *et al.*, 1992a, Pollard *et al.*, 1992b). Under New Zealand Legislation and animal welfare regulations (2018), this procedure requires appropriate surgical methods and pain management (Agriculture, 2018, MPI, 2018). Unfortunately, there is little research evidence concerning pain management in deer after their antlers are removed. Pain management options in other ruminant species and pigs include the administration of NSAIDs such as ketoprofen and meloxicam (Viscardi and Turner, 2018, Cagnardi *et al.*, 2017); however, these drugs are not registered for use in deer. Although still no evidence in ruminants yet, the non-selective nature of NSAIDs has the potential disadvantage of causing adverse gastrointestinal effects (Walsh and Wilson, 2002) or resulting in renal papillary necrosis (Stern *et al.*, 2010). The only pain management technique developed thus far is the local ring block, which in the case of lidocaine injection only lasts for a mean of 90 minutes, and in the case of bupivacaine can last from 4 to 7 hours, depending on the formulation (Bartels, 2002, Bartels *et al.*, 2001). Webster and Matthews (2006) studied velveting under lidocaine injections compared to restraint only. Surprisingly, they found that the deer with lidocaine injections showed more behavioural indications of pain irritation than the controls. However, these reactions disappeared after 7 hours. In summary, their findings proved that lidocaine injected as a ring block does not provide long-term pain relief after antler removal in deer (Webster and Matthews, 2006). There is thus a requirement to find a method of producing postoperative analgesia which is effective and has a duration of action superior to that currently employed. According to Pollard in 1992 and 1993, the behavioural and heart rate differences were noticed comparing two groups, the one with antlers were removed and the ones with intact antlers.

ii. Mechanism of nociception and pain

Pain is universal, and all mammals can experience it in similar ways, although the intensity of the feeling might be different in each individual (Beecher, 1957) . The International Association for the Study of Pain (IASP) describes pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage"(Marskey *et al.*, 1979). Pain perception is crucial to react to noxious stimuli from the environment and protect the animal from injuries. However, it is important to distinguish between pain and nociception, where activation of a nociceptor does not necessarily produce a pain sensation (Julius and Basbaum, 2001, Muir III and Woolf, 2001, Smith and Lewin, 2009). The nociceptive information has to be transmitted to various centres of the brain in order to perceive it as pain, and the processing includes: transduction, transmission, modulation, projection and perception (Shilo and Pascoe, 2014). At first, tissue and nerve damage occurs by the external stimulant (Pace *et al.*, 2006, Zhang and An, 2007). Then cytokines including Tissue Necrosis Factor alpha (TNFα) and Interleukin-1 and Interleukin-6 are produced and released by the damaged nervous tissue in the affected area (Pace *et al.*, 2006, Zhang and An, 2007). Interleuking-6 has particularly been shown to be responsible in neuronal reaction when injury occurs to the nervous tissue (Hirota *et al.*, 1996, Zhang and An, 2007). This is where the process of transduction converts the noxious stimulus to an action potential, During this process, it is important that the noxious stimulus depolarizes the nerve endings sufficiently to stimulate the voltage-gated sodium channels to open and cause a rapid influx of sodium ions (Na^+); thereby forming action potentials that propagate along the neuron (Cummins *et al.*, 2007, Schaible *et al.*, 2011). After transduction, transmission propagates the action potentials towards the spinal cord via primary afferent neurons. For peripheral nerves, the dorsal root ganglion (DRG) contains the cell bodies responsible for collecting nociceptive inputs and sending them to laminae 1, 2, and 5 of the dorsal horn of the spinal cord via a second-order neuron. However, in the case of deer antlers, which are innervated by branches of the cranial nerves (CN V), the first-order

neurons that are depolarized by a noxious stimulus reach the trigeminal ganglia (TG) instead of DRG and extend to trigeminal subnucleus caudalis in the caudal medulla oblongata via second-order neurons (Nieto-Diaz *et al.*, 2012, Westlund, 2005, Smith and Lewin, 2009, Dubin and Patapoutian, 2010). The nerve fibres in deer antler are devoid of specialised sensory function; instead, they end freely in the tissue (Wislocki and Singer, 1946, Vacek, 1955). The free fibre endings consist of A-beta, A-delta, and C fibres. A-beta fibres transmit tactile information created by low-intensity mechanical stimuli, which travel through cutaneous mechanoreceptors. The A-delta fibres respond to pressure, touch and cold temperature, transmitting sharp pain. The C- fibres are peptidergic and transmit warmth, slow pain, burning pain and itch and are involved in inflammation and thus wound healing (Nieto-Diaz *et al.*, 2012). In deer antler, A-beta and A-delta fibres can accurately localise the site of damage and stimulus, especially in the case of growing antler (Li *et al.*, 2007). This causes extreme sensitivity and a withdrawal reflex, thus providing better protection of the velvet antlers.

The first synapse occurs at the dorsal horn, where modulation either enhances or inhibits the passage of nociceptive information. Modulation was reviewed by Melzack and Wall (1965) who proposed the gate control theory of pain, in which the interneurons in the substantia gelatinosa control the onward flow of nociceptive information to the ascending pathways and brain. The dorsal horn of the spinal cord contains multiple receptors and neurotransmitters, with either excitatory or inhibitory actions (Shilo and Pascoe, 2014). The excitatory receptors include AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (N-methyl-D-aspartate) which are activated by glutamate, and NK1 (neurokinin-1) receptors which are mainly activated by substance-P (SP). SP is a neuropeptide found in the unmyelinated primary afferent neurons and is released as a consequence of noxious stimulus (Besson, 1999). The main mechanism that involves nociception includes surface receptor interaction with guanine nucleotide-binding proteins (G-proteins)(Molliver, 2009). By the activation of G-protein inside the cells, a reduction in potassium ion (K^+) current and an increase in calcium ion (Ca^{2+}) current occurs (Shilo and

Pascoe, 2014). Although NK1 receptors are excitatory by nature, they co-exist with opioid receptors, which have inhibitory effects (Xiao *et al.*, 2016). Recently, Bowman *et al*. (2015) found that SP may cross-regulate and re-sensitize mu-opioid receptors via NK1 receptors in trigeminal ganglion (TG) neurons. The inhibitory receptors include two classes of GABA (Gamma-aminobutyric acid) receptors, $GABA_A$ and $GABA_B$ which are predominantly present in the superficial laminae; three opioid receptors, μ, δ and κ, which are mainly present in the primary sensory neurons; α2 adrenergic receptors, which co-localize with opioid receptors on the primary afferent fibres in the dorsal horn of the spinal cord, and finally, strychnine-sensitive glycine receptors (GlyRs), which act as synaptic inhibitors (although glycine itself is also a co-agonist of glutamate in NMDA receptors which is excitatory) in the spinal cord and brain stem (Millan, 2002, Shilo and Pascoe, 2014, Dutertre *et al.*, 2012). $GABA_A$ receptors are present at the post-synaptic membrane of inhibitory synapses, and they increase the chloride (Cl^-) permeation into the cell, whilst $GABA_B$ receptors are located at the presynaptic terminals and they cause G-protein activation, which results in the inhibition of adenylyl cyclase (AC), increase in intracellular potassium ions (K^+) and decrease in calcium ions (Ca^{2+}) (Millan, 2002, Shilo and Pascoe, 2014). The activation of opioid and α2 adrenergic receptors increases intracellular K^+ and decreases $Ca^{2+,}$ which as a result, reduces neuronal excitatory action (Millan, 2002, Shilo and Pascoe, 2014). Finally, glycine receptors play a role when the post-synaptic membrane is hyperpolarized by fast inhibitory postsynaptic potentials, resulting in increasing Cl^- influx and inhibition of neuronal firing (Dutertre *et al.*, 2012, Shilo and Pascoe, 2014). Modulation of nociceptive information can also happen in the brain via either opiate receptors mainly located in the anterior cingulate cortex (ACC), or via non-opiate-mediated modulation such as the effects of dopamine (Shilo and Pascoe, 2014).

Projection conveys the information towards the supraspinal structures such as the brainstem, thalamus and cortex through the spinal cord. At this level, when the information is integrated to comprehend pain, perception occurs**.** Perception is a phenomenon that results in the

conscious and emotional experience of pain after the integration of nociceptive information (Shilo and Pascoe, 2014). There are three components for perception: sensory-discriminative, motivational-affective, and evaluative-cognitive, which coincide (Melzack, 1999, Treede *et al.*, 1999, Dubin and Patapoutian, 2010, Zhuo *et al.*, 2011). During the sensory-discriminative process, which is predominately located in S1, S2, and the lateral thalamus, basic sensory inputs such as quality, location or intensity of pain are analysed. The motivational-effective process, which is mainly located in the anterior cingulate cortex (ACC), reticular structures and limbic systems, encompasses the emotional reaction to pain, and during the evaluative-cognitive process, which occurs in the posterior parietal cortex and ACC, learning behaviours and memories from previous painful experiences are analysed (Melzack, 1999, Melzack and Katz, 2002, Treede *et al.*, 1999, Hofbauer *et al.*, 2001, Masedo and Esteve, 2002, Gustin *et al.*, 2011). Recent research in pain perception discovered a group of regions located in the brain which are responsible for analysing pain information and have been labelled as "pain matrix", which includes the thalamus, primary (S1) and secondary (S2) somatosensory cortices, ACC, insular cortex, and amygdala (Heinricher *et al.*, 2009, Basser, 2012, Hofbauer *et al.*, 2001, Brooks and Tracey, 2005, Henry *et al.*, 2011). Among all these centres, S2 and the insular cortex are the only ones that react most immediately and strongly to a noxious stimulus by producing pain perception (Peyron *et al.*, 2000, Ostrowsky *et al.*, 2002, Brooks and Tracey, 2005). The descending pathways which receive information from the ACC, anterior insular cortex and amygdala, are also responsible for the modulation of nociception which could be either facilitatory or inhibitory. Examples of these regions include the midbrain, such as the periaqueductal grey (PAG), and in the medulla oblongata: rostral ventromedial medulla (RVM), dorsal reticular nucleus, Nucleus Raphe Magnus (NRM), which is a serotonergic nucleus, and the ventrolateral medulla (VLM). The RVM contains on-cells that have a role in bursts of excitatory activity, off-cells that temporarily reduce nerve firing rates and neutral cells, which have no role in nociceptive information (Roeder *et al.*, 2016). These centres can modulate the pain pathway

by either inhibiting or facilitating the nociceptive transmission. There are other centres in the pons such as the locus coeruleus and sub-coeruleus which contain catecholaminergic neurons, and the parabrachial complex, which are involved in descending pain modulation (Roeder *et al.*, 2016, Shilo and Pascoe, 2014). Glial cells and lymphocytes can also regulate transmission at the level of the dorsal horn by secreting acetylcholine-binding proteins and interfere in the modification of NMDA receptors, thereby controlling glutamatergic function. These cells secrete a series of factors such as proinflammatory cytokines, e.g., tissue necrosis factor (TNF-α) and interleukins 1β, 4, 6; and anti-inflammatory cytokines such as tissue growth factor (TGF- β1), interleukins 10, and other factors such as nitric oxide (NO), histamine, prostaglandins (PG), glycine, glutamate and ATP, which influence nociception processing (Vanderwall and Milligan, 2019, Millan, 2002).

We should remember that the body has a built-in opioid system, called stress-induced analgesia (SIA), located in the descending pain pathway and suppresses pain sensation by regulating nociceptive signalling (Ferdousi and Finn, 2018, Stein, 2016). This system is influenced by age, sex, species, breed, severity, and type of stimulus. It is proven that SIA is predominantly triggered by acute and robust stimuli (Ferdousi and Finn, 2018). According to Bolles and Fanselow (1980), this innate system has evolved to protect the animal from natural danger and set the body on survival mode as a component of the fight-or-flight response (Bolles and Fanselow, 1980). The stress-induced analgesia has not been investigated in deer yet, but the information from other species can be extrapolated about this mechanism.

iii. Pain assessment techniques

According to Weilburg (1996), behavioural and physiological measurements can assess pain related to deer antler removal. The interpretation of these behavioural reactions are complicated because of other factors such as environment and social grouping. In addition, stress always exists while handling and restraining animals, which can also affect behaviour and pain assessment. The definition of stress in this context, whether it is biological or physiological, is the response of the animal to a stressor such as the environmental conditions (Rozenbaum, 2019). For this reason, stress and pain assessment related to deer antler removal requires a range of measurements (Wilson and Stafford, 2002). For example, Matthews *et al.* (1990) claimed that no difference in behaviour was detected between deer which were restrained, and their velvet antlers were removed compared to deer with intact antlers which were restrained only (Matthews *et al.*, 1990). However, in a later study, they observed higher mean heart rates in deer whose antlers were removed with no analgesia provided, compared to the ones which received analgesia before velveting (Matthews and Suttie, 2001). Mathews *et al.* (1994) also believed that some behavioural changes up to 24 hours later were due to the increase in pain sensation after the effect of local anaesthetics applied to the antler base wore off (Matthews *et al.*, 1994). Pollard *et al.* (1992) concluded that those deer which received postoperative analgesics displayed slight pain-related behavioural movements, such as ear flicking and grooming in the first two hours after antler removal as compared to the control group without any postoperative analgesics (Pollard *et al.*, 1992a, Pollard *et al.*, 1992b). There was no difference in cortisol levels in deer which were velveted with analgesics compared to those without any analgesia (Matthews, 2000), which could be due to handling stress or isolation during the procedure (Matthews, 2000). All these findings clarify the variability in results found by using different methods.

Pain can be assessed by changes in behaviour or physiology, or reversion to normal with analgesics. An experiment to measure pain response in deer was conducted by Matthews *et al.*

(1992 and 2001) and involved an electrical stimulus applied to antlers. In this technique, two electrodes were placed on the main tines, and the voltage of the device was manually increased until the deer felt pain as evidenced by behavioural changes, when the voltage was recorded. Wilson *et al*. (2000) used this method in red deer and scaled the behavioural response from 1 up to 10, with a voltage from 0 to 75 V. They assessed analgesia after lidocaine HCl (2%) ring block around the circumference of antlers before velvet removal and compared the response in different groups: high dose (1 ml/cm), low dose (0.4 ml/cm), and at two different anatomical sites: high site, and low site (5 ml per injection site), and concluded that high dose and high site analgesia had the best efficacy with rapid analgesia compared to low dose and low site analgesia in red deer stags. The high site included subcutaneous injection in the soft tissue located in between the ridge of the frontal bone. The low site included subcutaneous injection in the fat pad located behind the zygomatic arch (Wilson *et al.*, 2000). More recently, Electroencephalography (EEG) has been a suggested method of quantification of pain in deer (Murrell and Johnson, 2006, Johnson *et al.*, 2008, Flint, 2012); however, the necessity of general anaesthesia is a limiting factor, and for that reason, it is only suitable for acute pain assessment.

To date, no other techniques have been developed to assess pain in deer. Consequently, this study explored mechanical nociceptive threshold (MNT) testing in deer for the first time, as this has proved already to be effective in several animal species, including large animals (Chambers *et al.*, 1994), such as dairy cows (Raundal *et al.*, 2014), calves (Krug *et al.*, 2018), pigs (Di Giminiani *et al.*, 2016) piglets (Janczak *et al.*, 2012), and horses (Menke *et al.*, 2016, Chambers *et al.*, 1990, Rainger *et al.*, 2021), and small animals including dogs (Lane and Hill, 2016, Harris *et al.*, 2018), and cats (Dixon *et al.*, 2007, Steagall *et al.*, 2007).

- *Algometry*

Algometry (dolorimetry) means measurement of pain sensitivity according to the Merriam-Webster dictionary. The stimulus can be mechanical, chemical, or electrical. Mechanical or pressure algometry describes the procedure whereby the minimum pressure of a mechanical stimulus required to provoke reaction or discomfort is determined (Fischer, 1987). The term algometer (dolorimeter) describes a tool that is designed to measure pain sensitivity. The earliest algometer was a round head screw attached to kitchen scales (Dundee and Moore, 1960). However, the upgraded prototype after two decades had a body with an emerging metal rod at one end, and the rod had a detachable rubber disc to minimise trauma. The force was transmitted through the rod to the body of the algometer, moving the indicator needle on a scale, showing the force applied. The indicator needle stayed at the detected force until the device was re-zeroed (Fischer, 1987). Recent algometers have a similar mechanism; however, they are designed to measure the force digitally. The Wagner™ hand-held algometer is an excellent example of modern digital algometers. They possess a soft-grip handle and a stainless-steel piston at the end to which a strain gauge transducer is connected. The algometer has a flat circular rubber tip probe with a 1-cm diameter. The probe transmits the force to the load cell, which is part of a Wheatstone bridge and alters its resistance. This produces a voltage output proportional to the force applied, which is shown on the LCD display in the units selected such as Newtons (N), lbf, kgf or ozf. The displayed force represents the highest force applied before the animals reacted to the noxious stimulus (Kaka *et al.*, 2015). However, there are a number of limitations such as the response to the algometer which can potentially vary depending on how rapidly it is applied.

1.5. Local anaesthetics

i. Mechanism of action of local anaesthetics

Nociceptive signals transmitted by afferent nerve fibres can be efficiently blocked (Strichartz, 1990) providing local analgesia sufficient for surgery. In mammals, local anaesthetics act by changing the conformation of proteins in the voltage-gated sodium channels located in nerve cell membranes (Scholz, 2002). The sodium channels become less permeable to sodium ion influx, thereby inhibiting depolarization and the propagation of action potentials along the axons. Small bupivacaine doses are sufficient to block action potentials in small C fibres and Aδ-fibres without affecting large neurons such as Aβ fibres (Hartrick, 2004).

ii. Pharmacology of lidocaine, bupivacaine, and articaine

Bupivacaine and lidocaine (see Figure 1.4) are amide local anaesthetics that block nerve conduction (Sweetman, 2009). Bupivacaine has a longer duration of action compared to lidocaine (lignocaine). Local anaesthetics are usually injected around the nerve or neurons intended to be blocked, penetrate the neurons and block the voltage-gated sodium channels. Their local analgesic effect ends when they are absorbed into the systemic circulation and metabolised (Sweetman, 2009). Articaine (see Figure 1.4) is a local anaesthetic that acts very similarly to lidocaine and bupivacaine (Oertel *et al.*, 1997, Chandrasekaran *et al.*, 2021). The difference with articaine is its thiophene ring instead of benzene ring which gives greater lipid solubility properties (Oertel *et al.*, 1997, Chandrasekaran *et al.*, 2021, Malamed *et al.*, 2000, Maruthingal *et al.*, 2015). This allows the drug to diffuse relatively better than other local anaesthetics through the nerve membranes (Oertel *et al.*, 1997, Chandrasekaran *et al.*, 2021). Articaine also has an extra ester linkage in the structure which allows it to be hydrolysed by plasma esterase in the body (Malamed *et al.*, 2000, Maruthingal *et al.*, 2015). Articaine can block sodium channels with lower concentrations than lidocaine (Borchard and Drouin, 1980).

Plasma concentrations decrease rapidly after an intravenous injection of articaine, with an elimination half-life of 1 to 2 hours; however, the half-life may be prolonged if infusions exceed 24 hours especially if the hepatic blood flow is considerably reduced (Sweetman, 2009). Lidocaine binds to plasma proteins such as α1-acid glycoprotein (AAG), and any alteration in binding protein concentration can change the concentration of lidocaine in plasma (Sweetman, 2009). Regarding bupivacaine, the two main binding proteins are α1-acid glycoprotein, which binds predominantly at low concentrations of bupivacaine, and albumin, which plays the major role at high concentrations of bupivacaine (Sweetman, 2009). Plasma pH also alters the level of binding; a reduction in pH from 7.4 to 7.0 reduces the affinity of the α1-acid glycoprotein for bupivacaine but does not affect the affinity towards albumin (Sweetman, 2009). The protein binding of articaine and articainic acid is about 70%, similar to lidocaine (Oertel *et al.*, 1997).

Every local anaesthetic is unique in terms of onset time, duration of action and potency, and these factors depend on their physio-chemical structure (Weiniger *et al.*, 2012). One example is the aromatic ring structure and hydrocarbon chain length, which is essential in lipid solubility, and thus their potency. Molecular structures can influence the protein binding ability of local anaesthetics, for example, bupivacaine has 95% (Sweetman, 2009) and lidocaine has 66% (Sweetman, 2009) ability to bind to the plasma proteins. Duration of action is also dependant on the affinity of the drugs to the proteins within the neuronal sodium channels (Becker and Reed, 2012). Consequently, bupivacaine with a higher affinity to bind to these proteins has a longer duration of action than lidocaine (Fournier *et al.*, 2010, Becker and Reed, 2012).

Regarding articaine, its half-life is 20 minutes, and the elimination pattern is almost exponential (Oertel *et al.*, 1997). The time that articaine reaches its maximum concentration in the body is 45 minutes after submucosal injection, and its maximum concentration is 2000 µg/l (Oertel *et al.*, 1997). A study found that articaine injected for regional anaesthesia peaked in plasma less than lidocaine with numbers of 1.85 and 8.5 µg/ml, respectively. (Simon *et al.*, 1997).

Comparatively, articane has a faster onset; its effect on sensory and motor neurons is shorter and nerve blockade is more successful than lidocaine and bupivacaine (Simon *et al.*, 1997, Vree *et al.*, 1997).

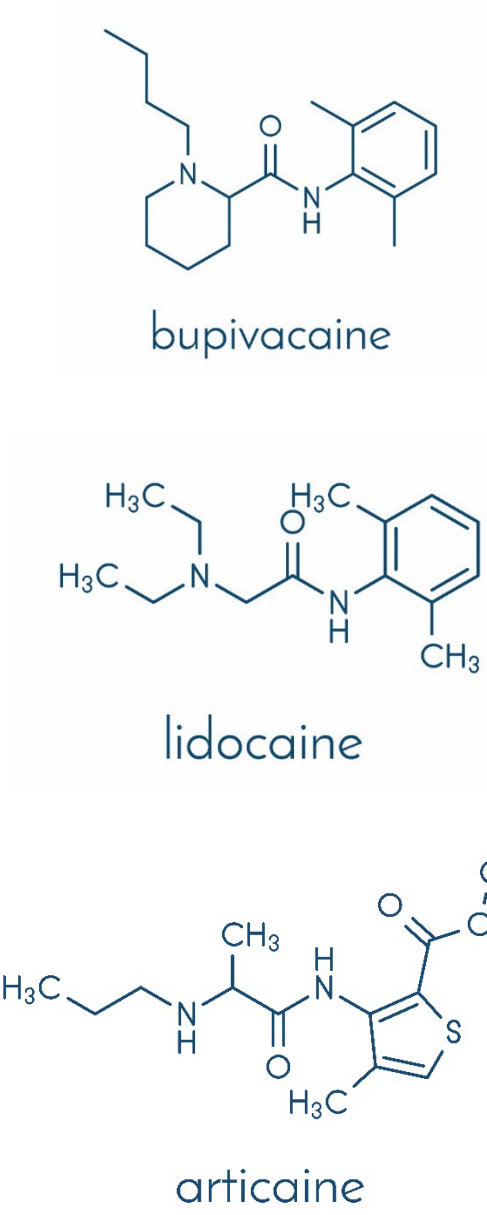


Figure 1.4 The chemical structure of bupivacaine, lidocaine, and articaine (Image source: Studio Molekuul, Illustrator / Vector Artist Videographer, Belgium)

iii. Adverse effects of lidocaine, bupivacaine, and articaine

Adverse effects of local anaesthesia are usually caused by excessive concentrations reaching the systemic circulation. This can be due to overdose, accidental intravenous injection or rapid absorption through a wound or inflamed tissue (Sweetman, 2009). Patients may develop adverse effects of the cardiovascular system, particularly myocardial depression and peripheral vasodilatation, which leads to hypotension and bradycardia. In addition, arrhythmias and cardiac arrest are possible with high doses (Sweetman, 2009). Langerman *et al.* (2001) studied the lethal dose (LD50) of five different local anaesthetics, including tetracaine, bupivacaine, ropivacaine, lidocaine and procaine, and concluded that the values were 0.137, 0.143, 0.198, 0.982 and 2.622 mmol kg^{-1}, respectively. Relative to procaine, they calculated the LD50 as 19:18:13:2.7:1 respectively. As a result, although bupivacaine did not show any significant difference with tetracaine, its LD50 was significantly lower than ropivacaine, lidocaine and procaine. Lidocaine showed the second highest LD50 among all. These observations demonstrate that lidocaine has a wider safety margin compared to bupivacaine (Langerman *et al.*, 2001). Factors such as lipophilicity could have a great influence on the permeation and toxicity of local anaesthetics such as bupivacaine. Onizuka *et al.* (2011) concluded that there is a positive correlation between LD50 and lipophilicity of the local anaesthetics. In addition, they stated that local anaesthetics with high lipophilicity tend to cause tissue necrosis; whilst, the local anaesthetics with low pKa tend to increase the risk of apoptosis in tissue (Onizuka *et al.*, 2011).

A similar order of potency is observed in the concentrations which cause 50% inhibition of action potentials (IC50). IC50 is lower in more potent local anaesthetics, such as bupivacaine which is 0.21 µM. IC50 for ropivacaine is 4.2 µM, and 56 µM for lidocaine, which means they have less potency as compared to bupivacaine (Bräu *et al.*, 1995). IC50, in general, is a measure for the potency of the drugs and represents the concentration of drugs that can inhibit the biological process by 50% (Aykul and Martinez-Hackert, 2016). The efficacy of the local anaesthetics can vary due to pH levels at the site. For example, when bupivacaine is exposed to decreased

extracellular pH levels from 7.4 to 6.6, its efficacy diminishes; however, in increased pH levels such as 8.2, its efficacy enhances (Bräu *et al.*, 1995). Another important factor that can assist in better understanding the toxic levels of local anaesthetics is the oil: water partition coefficient (P). Partition coefficient is the ratio of the concentration of a drug in one medium or phase (C1) as compared to a concentration in a second medium or phase (C2) when both concentrations are at equilibrium (Johanson and McQueen, 2010). Langerman *et al.* (2001) stated an inverse correlation between partition coefficient and the toxicity levels of local anaesthetics (Langerman *et al.*, 2001). For example, the partition coefficient of mepivacaine, lidocaine, ropivacaine, bupivacaine and are 21, 43, 115, 346, and 136, respectively (Lagan and McLure, 2004). Therefore, mepivacaine is expected to have the least toxicity and bupivacaine the most toxicity levels in this list.

Articaine's toxicity is very low due to its fast metabolism and rapid breakdown to non-toxic compounds in the body, short half-life, and fewer cumulation effects when administered repeatedly compared to many other local anaesthetics such as lidocaine and bupivacaine (Oertel *et al.*, 1997). 5-10 percent of articaine breaks down in the liver, and 90-95 percent is metabolised in the blood (Maruthingal *et al.*, 2015).

iv. The onset of action in local anaesthetics

The concentration of local anaesthetic molecules in a non-ionized state, dosage, dissociation constant (pKa) and the pH of the tissue determine the onset of action. The closer the pKa is to physiological pH levels (7.4), the faster the onset of action of local anaesthetics (Weiniger *et al.*, 2012, Columb and MacLennan, 2007). The reason why bupivacaine has a slower onset compared to lidocaine is that more bupivacaine molecules remain in the ionized form (86%) compared to lidocaine (72%) under physiological conditions at pH 7.4 (Columb and MacLennan, 2007). This is because ionised drug forms cannot move through the lipid bilayer of the cellular membranes; they must be in the non-ionised form to passively enter the cells and bind to the sodium channels from inside. Columb and MacLennan (2007) suggested using bicarbonate to increase the free

base concentration of local anaesthetics such as bupivacaine; therefore, increasing the onset of action (Columb and MacLennan, 2007, Kashyap *et al.*, 2011, Schumacher and Boone, 2021).

The onset of action for articaine is 1 to 3 minutes following injection in submucosa, which is more rapid than lidocaine with about 2 to 3 minutes and bupivacaine with about 5 minutes (Oertel *et al.*, 1997, Maruthingal *et al.*, 2015, Cousins *et al.*, 2009, Facts and Comparisons. 2011).

v. Duration of action in local anaesthetics

The duration of action is different among local anaesthetics as their affinity for plasma proteins differ (Becker and Reed, 2012). In general terms, local anaesthetics with a high affinity for protein-binding tend to have longer analgesic effects (Becker and Reed, 2012). For example, bupivacaine exhibits longer analgesic effects than mepivacaine due to its 95% protein binding affinity compared to mepivacaine's 55% (Becker and Reed, 2012). Lidocaine is a good example of an intermediate-acting (Lagan and McLure, 2004) local anaesthetic, which reduces its efficacy by dilating the regional vessels, thereby removing the drug molecules from the applied site. Articaine's duration of action was proved to last about 30 to 120 minutes for pulp anaesthesia in human dentistry and about 4 hours to mandibular blocks (Winther and Patirupanusara, 1974, Malamed *et al.*, 2001, Martin *et al.*, 2021). Recent experiments aimed to find novel techniques to achieve prolonged blockade in sensory nerves, of a few days to weeks, instead of multiple injections or infusions (Liu *et al.*, 2015).

1.6. Drug delivery systems for a prolonged duration of analgesia

Prolonged duration local anaesthesia (PDLA) is a topic that has recently become very important in all species. PDLA requires maintenance of high concentrations of drugs at the target site, the nerve, for an extended period of time without increasing drug concentrations to toxic levels systemically (Santamaria *et al.*, 2017, McAlvin and Kohane, 2014). According to McAlvin and Kohane (2014), and later, Santamaria *et al*. (2017), the properties for a suitable PDLA formulation should consider the following features:

- Simple administration techniques
- Being safe and reliable
- Initiation by a single injection
- Adequate shelf life
- Biodegradability of the vehicle which is very similar to depletion of the drug
- No requirements for any general anaesthesia or surgery for the application
- Prolonged duration of action in blocking the nerve
- Relative zero-order release
- Minimal motor blockade compared to sensory blockade
- Acceptable inflammatory response
- No myotoxicity or neurotoxicity
- No systemic toxicity (locally effective and systemically non-toxic)
- Completely reversible (in terms of the effect on nerve function)

- Easy production
- Reasonable price and cost-effectiveness

The applications of these formulations could vary from days to weeks based on the duration of pain (acute or chronic). As mentioned above, the drug delivery vehicle should not stay in the body tissue for a prolonged period of time after the drug has been completely delivered. This is due to the fact that the residues of the vehicle might inhibit uptake of the drug at the site by their presence if a second dose is administered (Santamaria *et al.*, 2017, McAlvin and Kohane, 2014).

i. Loading of the drug on vehicle

Selection of the desired properties and compounds of each PDLA formulation requires careful consideration of the chemical activities of each component when they are combined with each other. The conventional local anaesthetic agents have a common structure that contains a lipophilic aromatic ring, which is linked by an ester or amide to a terminal amine (McLure and Rubin, 2005).

When protonation occurs under acidic pH conditions, local anaesthetic drugs become more hydrophilic. In clinical procedures, they are most often dispensed in aqueous solutions with acidic pH levels. Acidification, and thus ionisation, makes the local anaesthetics more water soluble, allowing them to be successfully loaded into the aqueous cores inside liposomes or hydrogels. Alternatively, the hydrophobic free base component of the local anaesthetics, which is also able to dispense in organic solvents, which are commonly used in drug delivery vehicles, or hydrophobic particles (Santamaria *et al.*, 2017). Another aspect to consider is potential synergistic effects when local anaesthetics are combined with other drugs. The efficacy of local anaesthetics could be dramatically enhanced by adding other drugs such as dexamethasone

(Colombo *et al.*, 2005, Castillo *et al.*, 1996, Dräger *et al.*, 1998), tetrodotoxin and saxitoxin (Kohane *et al.*, 2003).

ii. The rationale for the drug delivery system

Various factors can be modified to increase drug duration of action by reducing release rates such as: particle size, viscosity, encapsulation using lipids, hydrogels or modification of these structures.

Increased particle size can lead to reduced release rate of drugs. These large particles have the capacity to remain in the tissue and degrade slowly over an extended period; consequently, they would have a greater potential to prolong nerve block (Santamaria *et al.*, 2017). Furthermore, viscosity plays a very important role in release rate, in which increased viscosity reduces the drug release rate within the tissue. However, the caveat is that highly viscous materials are difficult to inject in clinical practice (Paavola *et al.*, 1998, Jia *et al.*, 2004). Consequently, highly viscous substances are used as implants rather than injectable formulations (Masters *et al.*, 1993, Cusack *et al.*, 2012a, Cusack *et al.*, 2012b, Cusack *et al.*, 2013). Lipid-based encapsulations such as liposomes have been proven to be safe in painful surgeries such as bunionectomy, hemorrhoidectomy, breast augmentation, hernia repair, and total knee arthroplasty in humans (Viscusi *et al.*, 2014). Additionally, Ilfeld *et al*. (2014), proved the effectiveness of the combination of bupivacaine and liposomes in prolonging analgesia for femoral nerve block in humans (Ilfeld *et al.*, 2013). For veterinary use, Sahebjam *et al*. (2019) concluded that the injectable combination of soybean lipids (Intralipid) and bupivacaine is effective in prolonging the analgesic effects of bupivacaine injected to the forefeet of sheep (Sahebjam *et al.*, 2019). The lipid bilayer used in drug encapsulation is formulated from biocompatible components such as phospholipids and cholesterol (Santamaria *et al.*, 2017). The bilayer structure protects the drug and reduces its bioavailability; thus, the drug effect only takes place when the layer is breached (Allen and Cullis, 2013). Hydrogels are also very effective at holding local anaesthetics

in the drug delivery vehicle. They are water-soluble, can swell when exposed to any type of liquid, and are extremely versatile in texture and form. Hydrogels can be produced in solid, elastic, gel or even film forms, and their absorption capacity can be modified. Hydrogels maintain moisture in healing tissues and could be an ideal vehicle for drug delivery systems. Hydrogels incorporating collagen are some of the most popular for biomedical purposes and are ideal for wound dressing (Morgan, 1999, Boateng *et al.*, 2008, Vashist *et al.*, 2014, Drury and Mooney, 2003, Slaughter *et al.*, 2009, Varghese and Jamora, 2012). Consequently, in this research, the aim was to harness local anaesthetics in a biocompatible collagen hydrogel that could offer pain mitigation and increase wound healing.

1.7. Biocompatibility and toxicity

In the context of drug delivery systems, how the vehicle or excipients act in the tissue is very important. For PDLA formulations, the active drugs themselves can also result in complications after tissues are exposed to them for extended periods. Adverse effects such as myocardial toxicity and neurotoxicity can occur when local anaesthetics are inadvertently administered intravascularly or given in excessively high doses; lesser adverse effects such as localized inflammation can occur when the drug remains in the tissue for a long period of time after conventional application (Padera *et al.*, 2008, Epstein-Barash *et al.*, 2009, Kohane *et al.*, 2002, Neal *et al.*, 2016, Kalichman *et al.*, 1993). Consequently, this could be considered one of the main challenges of sustained-release local anaesthesia (Kohane and Langer, 2010). Secondly, the vehicles which are utilised for drug delivery during surgery could produce an inflammatory response greater than that of the surgery itself. The intensity of the inflammatory reaction is dependent on the anatomical region where the biomaterials are used. For example, poly(lactic-co-glycolic) acid (PLGA) can cause a mild reaction; however, when it is applied in a soft tissue surrounded by nerves, it can cause more significant reactions by attracting neutrophils and macrophages to the site. When the reaction persists up to weeks, more macrophages move to the region, in addition to lymphocytes (Anderson, 1994, Kohane and Langer, 2010). Particle size

is also an important factor in tissue reaction. The particles either remain at the site of application or become phagocytosed. For example, larger particles become engulfed by giant cells in the tissues (Kohane and Langer, 2010, Kohane, 2007).

The risk of systemic toxicity also requires consideration as high concentrations of local anaesthetics are usually being incorporated into the vehicles of PDLA formulations. There is always a chance of a vehicle delivering too much at once, such as a "burst" release of local anaesthetics being systemically absorbed, which can easily compromise animals' health by severe toxicity (Kohane and Langer, 2010).

1.8. Encapsulation of the local anaesthetics

Biodegradable compounds can be utilised as transport vehicles whose role is to encapsulate high doses of local anaesthetics within the body and prolong the release of the drug molecules to extend the duration of analgesia. The analgesic effect persists as long as the drug molecules are available in sufficient concentration to bind to the sodium channels and perform their action of blocking signal transmission in the nerves. Encapsulating drug molecules is one of the effective ways drug doses greater than the therapeutic level can be locked and stored inside the body when it is administered (Le Corre *et al.*, 2002, Shikanov *et al.*, 2007). If drug release occurs with a sudden initial explosive burst or "burst release", this could be very toxic to the body. This phenomenon can happen due to physical or chemical changes in the carrier structure and should be thoroughly anticipated before application to avoid any harm to the animal (Sokolsky-Papkov *et al.*, 2009, Weiniger *et al.*, 2010).

1.9. Development of a novel formulation of local anaesthetics

There are numerous medications available for postoperative pain in various species in injectable and topical forms. The only FDA approved long-acting injectable bupivacaine formulation approved so far is EXPAREL. This product is formed using a multivesicular system known as DepoFoam™ technology, which encapsulates bupivacaine (Ye *et al.*, 2000). EXPAREL cannot be

administered conjunctly with other local anaesthetics but may be administered at the same site following lidocaine administration, provided 20 minutes has elapsed. After administration of EXPAREL, no additional local anaesthetic drugs can be administered for 96 hours as this can reduce the effect and duration of action (Mont *et al.*, 2018, Gorfine *et al.*, 2011, Patel *et al.*, 2020, Baker *et al.*, 2018). The development of EXPAREL revolutionised postoperative analgesia in human surgeries reducing the need to use opioids; thereby reducing adverse effects in humans (Nedeljkovic *et al.*, 2020). In animals, Lascelles *et al.* (2016) investigated the effects of injectable liposomal bupivacaine in dogs and compared them to the control group, which only received normal saline. They found that this suspension could prolong analgesia for up to 72 hours after stifle surgery (Lascelles *et al.*, 2016).

In the case of topical forms of analgesia, one example would be liposomal lidocaine which has been used successfully in human infants which showed less cutaneous pain sensation, compared to the control who did not receive the formulation, followed by catheterization (Taddio *et al.*, 2005). The use of liposomal lidocaine has also been proven to be safe in cats. Fransson *et al.* (2002) investigated the systemic absorption of topically applied liposomal lidocaine cream (15 mg/kg) in six cats. They found that the transdermal absorption of lidocaine varies in animals when applied via liposomal formula, and the concentration in plasma is much lower than toxic levels (Fransson *et al.*, 2002).

The products currently available on the market provide short-term pain relief and may cause adverse effects, but a higher treatment cost could be prohibitive in postoperative pain management, particularly in deer velvet applications. Additionally, as of yet, the majority of available treatments are not licensed to be used in deer.

There are several available polymers that are broadly used as a vehicle in sustained-release drug delivery, including chitosan and alginate, which are both highly biocompatible and biodegradable (Bhattarai *et al.*, 2005). An example of a chitosan vehicle combination with local anaesthetics

includes chitosan combined with lidocaine which was utilised as a transdermal technique for delivering pain relief for an extended period (Zhang *et al.*, 2016). Xaracoll®, which is an FDA-approved collagen-based implant, contains collagen as the vehicle and bupivacaine as a local anaesthetic (FDA, 2020). Xaracoll® was successful in prolonging pain relief in humans after abdominal hysterectomy for a median of 12 hours and in inguinal hernioplasty patients for up to 72 hours post operation (Cusack *et al.*, 2012).

The most comparable product in the market that has been used in animals is Tri-Solfen® which has been available since 2006. Tri-Solfen® is a gel-based topical formula containing bupivacaine (4.2 g/L) and lidocaine (40.6 g/L) as the local anaesthetics, cetrimide (5 g/L) for antisepsis, and adrenaline (24.8 g/L) for haemostasis. Lomax *et al*. (2008) investigated the effects of Tri-Solfen® on merino lambs undergoing the mulesing procedure, which coincides with castration and tail docking and is routinely performed to prevent flystrike. They found that the application of Tri-Solfen® had a rapid onset of action and prolonged analgesia compared to the placebo that did not receive Tri-Solfen® treatment (Lomax *et al.*, 2008). Later in 2010, Lomax *et al.* conducted a trial of analgesia after hot-iron tail docking and castration in lambs and found that Tri-Solfen® prolonged pain relief up to 4 hours compared to the placebo.

They also addressed the challenge they encountered during the experiment being bleeding from the site of application which prevented Tri-Solfen® from adhering and delivering its maximum benefit (Lomax *et al.*, 2010). In 2013, Lomax and Windsor conducted an investigation on pain after castration on Angus bull calves. They concluded that Tri-Solfen® elicits a rapid onset with significantly prolonged analgesia for up to 24 hours compared to the animals which did not receive Tri-Solfen® (Lomax and Windsor, 2013). There is no evidence of Tri-Solfen® use in deer to date.

1.10. Collagen structure and uses in drug delivery

Collagen as a vehicle in drug delivery is exceptionally biocompatible and biodegradable (Jardelino *et al.*, 2010, Panduranga Rao, 1996), and its abundance has made it unique. Collagen matrices can be used in various forms (Petrisor *et al.*, 2012, Purna and Babu, 2000), such as a pad (Lewis *et al.*, 2014), gel (Tabata *et al.*, 1994) or sprays (Chen *et al.*, 2005). The attractiveness of collagen as a biomaterial is due to its low antigenicity (Meena *et al.*, 1999). The other advantage is that collagen can be extracted from the by-products of the leather industry (cattle hide), which makes it abundant and easily accessible (Zhang *et al.*, 2006).

Many drugs such as antibiotics, anticoagulants, cholinergic, immunosuppressants, steroids, and local anaesthetics have been incorporated into collagen-based systems (Nandi *et al.*, 2009). Previous studies have proven the successful combination of local anaesthetics in collagen, resulting in sustained drug delivery. Petrisor *et al*. studied the effects of a delivery system using collagen with lidocaine as a local anaesthetic (Petrisor *et al.*, 2012). They concluded that the physical state of the delivery system has a significant influence on the behaviour of drug release rates when various drugs are incorporated (Petrisor *et al.*, 2012). Additionally, they found that crosslinked collagen matrices can delay the delivery of lidocaine *in vitro* (Petrisor *et al.*, 2012).

1.11. Collagen

Collagen is the most abundant protein in the body of mammals, comprising some 25% of the total protein mass (Fleck and Simman, 2010). It is present in many tissues, including skin, bone, tendon, cartilage, and cornea (Bhattacharjee & Bansal, 2005). Inherent binding sites on collagen molecules and the scaffolding architecture of certain collagen types hold potential for drug delivery and tissue repair applications (Gelse *et al.*, 2003). Collagen has many functions, such as providing structural support and stability to a variety of tissues and organs (An *et al.*, 2016, Gelse *et al.*, 2003).

Knowledge of collagen has increased significantly since the first studies in the 1950s, and today, 28 distinct genetic types of human collagens have been described, which can be assigned to one of two broad categories; fibrillar and non-fibrillar collagen (An *et al.*, 2016). Fibrillar collagens are the most prevalent, comprising 90% of the collagen found in tissues (Gelse *et al*., 2003). Non-fibrillar collagens can be further distinguished into sub-categories depending on their distinctive properties, such as additional domains and variations in the structure, which gives them unique functions (See Table 1.1) (Hulmes, 2008, Gelse *et al.*, 2003)

Collagen is produced intracellularly, and whilst the majority is produced inside fibroblast cells, the initiation of biosynthesis is regulated depending on the cell type and is influenced by cytokines and various growth factors (Gelse *et al.*, 2003). Biosynthesis is complex. Transcription and translation steps are followed by post-translational modification in the endoplasmic reticulum (Gaut and Hendershot, 1993, Gelse *et al.*, 2003). Once the procollagen has undergone processing and assembly, the triple helix molecules are packaged by the Golgi body into transport vesicles and released from the fibroblast into the extracellular matrix (Gelse *et al.*, 2003), where they are activated (Fleck and Simman, 2010). Assembly of the procollagen into fibrils occurs spontaneously after proteolytic processing, and larger bundles or supramolecular structures are formed by cross-linking, which impart tensile strength and support to the extracellular matrix (An *et al.*, 2016, Fleck and Simman, 2010).

The most important collagens from a biomedical perspective are the fibrillar collagens. This is due to the tensile strength and torsional stability that not only provide necessary integrity to the native extracellular matrix but can be harnessed for other medical purposes (An *et al.*, 2016, Gelse *et al.*, 2003). The major characteristic of fibrillar collagen is its conformation. Collagen molecules are comprised of 3 long, rod-like procollagen α chains that display a left-handed helix conformation (Fleck and Simman, 2010) with a pitch of 18 amino acids per turn (Gelse *et al.*, 2003). The three peptide chains are staggered in the triple helix by one residue (An *et al.*, 2016).

The three procollagen chains then assemble into a right-handed triple helix conformation, which is stabilised by hydrogen bonding between the chains to form procollagen molecules.

In the case of fibrillar collagen, each collagen α chain contains glycine repeated at every 3rd residue in the sequence with other amino acids (Xaa and Yaa). Xaa and Yaa residues are prone to interact as they are exposed on the surface of the triple helix, whilst glycine is embedded in the core (Brodsky and Ramshaw, 1997). The stability of the collagen triple helix is influenced by the composition of amino acids, and research conducted in the 1960s demonstrated that when Xaa-Yaa are proline and/or hydroxyproline this would form a stable triple helix (Persikov *et al.*, 2005). Additionally, stability is partly determined by the degree of intramolecular hydrogen bonding, and the prevalence of 4-hydroxyproline is an essential component (Gelse *et al.*, 2003). The most stable form known is (Pro-Hyp-Gly) provided the chains are a minimum of 6 repeated units in length (Persikov *et al.*, 2005). In the case of Collagen type I, the α chains are heterotypic, and type I consists of two identical α1 chains and a third distinct α2 chain (Hulmes, 2008). Type I along with type II and III, are flanked on each end of the triple helix by propeptides at both the C and N termini. The N-propeptide is believed to regulate fibril diameter, whilst the C-propeptide is thought to have a role in the initiation of the triple helix. The propeptides undergo cleavage during biosynthesis to leave shorter telopeptides which are involved in bonding both to other collagen molecules and other structures within the extracellular matrix. This process occurs extracellularly to avoid bonding between collagen fibrils within the cell (Lodish *et al.*, 2000).

Animal tissues such as bovine hide, fish skin and porcine gastrointestinal tract are common sources of collagen type 1 (An *et al.*, 2016). Extracted collagens can be re-purposed into various forms, including implants, sponges, hydrogels or films, that can be used for haemostasis, tissue scaffolds, and for the purpose of wound healing or drug delivery (Fleck and Simman, 2010, An *et al.*, 2016, Hoare and Kohane, 2008). See Figures 1.5 and 1.6 for collagen fibre and its components.

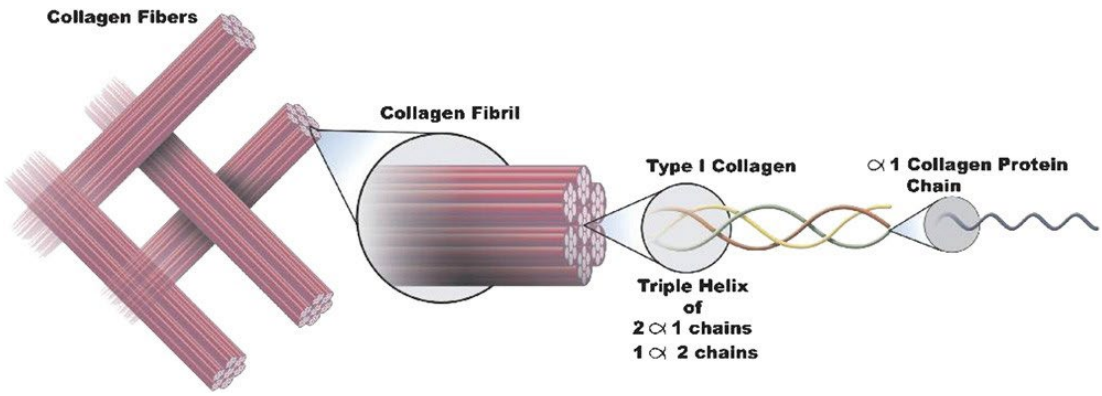


Figure 1.5 Collagen fibres and their components (Fleck and Simman, 2010).

Table 1.1 Types of collagen and its tissue distribution (Gelse et al., 2003)

TYPE	TISSUE DISTRIBUTION
Fibril-forming collagens	
I	bone, dermis, tendon, ligament, cornea
II	cartilage, vitreous body, nucleus pulposus
III	skin, vessel walls, reticular fibres of lungs, liver, spleen
V	lung, cornea, bone, foetal membranes
XI	cartilage, vitreous body
Non-fibril forming collagens	
Basement membrane collagens	
IV	basement membranes
Microfibrillar collagen	
VI	dermis, cartilage, placenta, lungs, vessel wall, intervertebral disc
Anchoring fibrils	
VII	skin, dermal-epidermal junctions, oral mucosa, cervix
Hexagonal network-forming collagens	
VIII	endothelial cells, Descemet's membrane
X	hypertrophic cartilage
Fibril associated collagens (FACIT collagens)	
IX	cartilage, vitreous humour, cornea
XII	perichondrium, ligaments, tendon
XIV	dermis, tendon, vessel wall, placenta, lungs, liver
XIX	human rhabdomyosarcoma
XX	corneal epithelium, embryonic skin, sternal cartilage, tendon
XXI	blood vessel wall
Transmembrane collagens	
XIII	epidermis, hair follicle, endomysium, intestine, chondrocytes, lungs, liver
XVII	dermal-epidermal junctions
Multiplexins	
XV	fibroblasts, smooth muscle cells, kidney, pancreas
XVI	fibroblasts, amnion, keratinocytes
XVII	lungs, liver

i. Collagen structure and its alteration by nanoparticles

A collagen molecule is composed of three left-handed polypeptide alpha chains, right-hand twisted into a triple helical structure (Shoulders and Raines, 2009).

Figure 1.6 The collagen triple helix formed from (ProHypGly)4–(ProHypAla)–(ProHypGly)5. (Photo:(Shoulders and Raines, 2009))

Collagen is broadly used as a nanocomposite, especially with the addition of nanoparticles. Nanoparticles are particles that have a size between 1-100 nm (Scenihr, 2007). Nanocomposites are a new class of materials that contain multi-phases with at least one phase having a size smaller than 100 nm in one or more dimensions (Aimé and Coradin, 2012). Nanoparticles can be incorporated into collagen fibres to enhance their tensile strength, mechanical stability across different pH and temperatures (Kołodziejczak-Radzimska and Jesionowski, 2014) and to prolong

their retention in the tissue whilst inhibiting their absorption. This slows the degradation of collagen hydrogels and provides a vehicle for drug delivery purposes (Grant *et al.*, 2014).

There are several nanoparticles that are used in the modification of collagen matrices, including zinc oxide (Agban *et al.*, 2016), titanium oxide (Agban *et al.*, 2016), gold (Castaneda *et al.*, 2008), silver (Mandal *et al.*, 2015) and organic compounds. Zinc oxide was chosen in this study as the main modifier because of its proven antimicrobial effects, usefulness on open wounds, and its ability to stabilise and gelate the collagen structure efficiently for drug delivery (Lian *et al.*, 2016). In summary, nanoparticles improve mechanical strength compared to collagen without nanoparticles. This is important for tissue engineering products.

ii. Collagen hydrogel fabrication

Type 1 collagen is used in most collagen hydrogels (Abraham *et al.*, 2008, Wolf *et al.*, 2009) due to accessibility of raw materials with low contamination by other proteins or types of collagens (Antoine, 2014). Various factors can significantly alter the quality and properties of the extracted collagen hydrogels, including collagen source and its solubilization, polymerization pH, polymerization temperature, collagen concentration and ionic strength. Sources include bovine, porcine, avian (Parenteau-Bareil *et al.*, 2011), and fish skins (Sadowska *et al.*, 2003) and rat tail tendon (Wolf *et al.*, 2009). Most of the commercially available collagen products for medical purposes vary significantly (Kreger *et al.*, 2010, Abraham *et al.*, 2008). For example, although rat tail tendon yields pure collagen type 1, it contains poorly crosslinked fibres and is susceptible to denaturation even though acid solubilization is sufficient (Antoine *et al.*, 2014). However, other collagen sources, including bovine and porcine skins, which are comprised from highly crosslinked collagen (Antoine *et al.*, 2014), require a combination of salt solution with proteolytic enzymes such as pepsin for denaturation (Parenteau-Bareil *et al.*, 2010, Wolf *et al.*, 2009, Walters and Stegemann, 2014). It has been shown that the pepsin-digested collagen matrix, particularly from bovine tendon and porcine skin sources, results in longer fibres and bigger pores (3-5 μm

diameter) compared to the acid-extracted collagen matrix (containing telopeptides), which leaves shorter fibres with small pores (1-2 μm diameter) (Wolf *et al.*, 2009). This variation mainly occurs due to telopeptide removal in collagen hydrogels (Wolf *et al.*, 2009). pH is also an important factor for the fabrication of collagen hydrogels, and it has a significant impact on hydrogel structure and its properties (Raub *et al.*, 2008, Yang *et al.*, 2010, Gobeaux *et al.*, 2008). Yamamura *et al.* (2007) and Raub *et al.* (2008) found that by changing pH levels between 5 and 10 and 5.5 and 8.8, respectively, there is an ability to make collagen structure more or less robust (Yamamura *et al.*, 2007, Raub *et al.*, 2008). For example the higher the pH levels, the stiffer the produced collagen (Raub *et al.*, 2008). Collagen fibre size and its organization is also a factor that can be manipulated by altering pH levels, particularly in ranges between 7.1-8.3 and 6-12 (Gobeaux *et al.*, 2008, Sung *et al.*, 2009). Most of the available collagen hydrogels have been designed to deliver cells and let cells move freely inside the structure. For this reason, they usually possess pH levels between 7.4 to 8.4 (Antoine, 2014), which can result in an improved wound healing process.

iii. Hydrogels as a method of drug delivery

When local anaesthetics are administered alone, one complication is high systemic concentrations causing adverse effects. Multiple injections will make this worse. The necessity for multiple injections is usually rapid absorption, metabolism and elimination (Liechty *et al.*, 2010, Langer, 1998). Slow release drug delivery systems are advantageous due to five factors; 1) maintenance of drug levels within their therapeutic range, 2) reduction of adverse effects by aiming the drug toward the target cell type, organ or tissue, 3) reduced consumption of drugs, 4) fewer attempts and less invasive techniques in the administration of the drugs, 5) facilitating the administration of drugs that have short half-lives (Langer, 1998). Achieving these factors has challenges such as the cost of a specifically designed drug delivery vehicle, the potential toxicity and safety levels of the material employed, the danger of rapid drug release, and finally, the discomfort they might cause to the recipient (Langer, 1998). There are three mechanisms by which drugs can be delivered by a polymer or lipid drug delivery system: diffusion from the delivery vehicle, cleavage from the vehicle by enzymes, or swelling of the system by osmosis (Langer, 1998). Since the 1950s, drug delivery systems that incorporate nanoparticles, membranes, hydrogels and liposomes to create a controlled-release system have been investigated (Tiwari *et al.*, 2012, Tibbitt *et al.*, 2016).

Hydrogels, in particular, are popular and have been used in areas such as oncology (Fakhari and Subramony, 2015), cardiology (Rodell *et al.*, 2016), immunology (Wei *et al.*, 2019), pain management (Bagshaw *et al.*, 2015) and wound healing (Lokhande *et al.*, 2018). Hydrogels typically have a water content of 70-99 percent, which allows them to be an efficient delivery vessel for hydrophilic drugs. Additionally, their high-water content increases their biocompatibility at the site of application (Li and Mooney, 2016). The polymer network and water holding capacity give rise to a porous structure that allows hydrophilic drug molecules to move through the hydrogel (Li and Mooney, 2016). This movement can be influenced by chemical or physical interactions between the drugs and polymer chains (Li and Mooney, 2016, Narayanaswamy and Torchilin, 2019, Hoare and Kohane, 2008). Specific modifications of the structure of hydrogels can be used to design an effective system for a particular application (Li and Mooney, 2016, Hoare and Kohane, 2008). The type of application depends on site constraints and the ability to reach therapeutic levels of the drug whilst being maximally efficient and with ease of administration (Rodriguez-Tenreiro *et al.*, 2007, Hoare and Kohane, 2008). When designing a suitable drug delivery system, it is important to ensure the hydrogels degrade over time so that subsequent surgical removal is not required (Li and Mooney, 2016, Hoare and Kohane, 2008, Lee *et al.*, 2002).

The affinity of drugs to bind to the hydrogel network plays an important role in sustained release (Li and Mooney, 2016, Hoare and Kohane, 2008, Wang and von Recum, 2011). Any binding, including electrostatic interaction, covalent linkage or hydrophobic association, can change the delivery of a drug to the target tissue (Li and Mooney, 2016, Lee *et al.*, 2002, Hoare and Kohane, 2008, Zarzycki *et al.*, 2010). The materials in the hydrogels are critical. The hydrogels can be fabricated from either synthetic polymers such as poly (hydroxyalkyl methacrylate) and poly(vinyl alcohol) (Thorn *et al.*, 2006) or non-synthetic polymers such as collagen (Pandit *et al.*, 1999), alginate (Momoh *et al.*, 2015), or chitosan (Jayakumar *et al.*, 2011).

In addition to the interaction between the polymer and drugs, pore size (mesh size) (see Figure 9.1 and 9.2) is crucial to both load drugs into the matrix and subsequently allow their passage through the hydrogel (Lin and Metters, 2006, Hoare and Kohane, 2008). The pore size for which effective drug delivery occurs ranges from 5 to 100 nm (Li and Mooney, 2016). The pore size in manufactured hydrogels varies depending on the type of polymer, the crosslinker employed and their relative concentrations, and temperature and pH levels during formation (Li and Mooney, 2016). The pore sizes in the hydrogel matrix determine the degree of drug diffusion and steric interactions occurring between the drug and the polymer chains (see Figure 1.7 and appendix, which shows the Scanning Electron Microscopy (SEM) images from collagen matrices produced in this study). In cases where a pore is larger than a drug molecule, diffusion plays the main role in drug release. When the pore and drug sizes are very similar, steric hindrance plays the main role in limiting drug release. In this situation, the movement of drug molecules occurs over a longer path than usual (Amsden, 1998). Finally, when the pore size is much smaller than the drug molecule, drugs become entirely immobilised in the hydrogel structure due to strong steric hindrance. They can only be released when the hydrogel undergoes degradation or the pore size becomes enlarged (Li and Mooney, 2016).

Hydrogels can be reversibly compressed to facilitate storage and administration. For example, hydrogels may be compressed to form solid spongiform composites by techniques such as freeze-drying (Hassan and Peppas, 2000) without any permanent damage to the structural properties. Then, after application, the freeze-dried hydrogel absorbs exudate and gelatinises at the site, re-forming as a drug-delivery vehicle and allowing drug release. The reversibly compressible nature of hydrogels also allows them to be injected directly into the body tissue and regain their drug delivery properties (Bencherif *et al.*, 2012).

Figure 1.7 Pore sizes and drug diffusion in the hydrogel matrix (Li and Mooney, 2016).

iv. Available products containing collagen and local anaesthetics

Xaracoll® (INL-001), which is a collagen-based implant consisting of collagen as the vehicle and bupivacaine as the local anaesthetic, is registered for use in humans; however, the application of this combination differs from the application which is proposed in this study. These collagen-based implants are administered during repair of the abdominal wall and can be placed underneath the skin immediately prior to wound closure during inguinal hernioplasty in humans (Cusack *et al.*, 2012a, Cusack *et al.*, 2012b). Thus, it is not directly applied to the bleeding wound. Additionally, Xaracoll® does not contain lidocaine or zinc oxide nanoparticles, and its effect when applied directly to wounds has not been studied previously, especially in animals (Cusack *et al.*, 2013). On the contrary, there is a vast amount of literature on collagen and its effects on wound

healing (Chattopadhyay and Raines, 2014, Lazovic *et al.*, 2005, Madden and Peacock Jr, 1971, Ruszczak, 2003, Mian *et al.*, 1992), which makes collagen a suitable delivery vehicle for sustained-release of local anaesthetics whilst promoting wound healing.

1.12. Wound healing

In this section the aim is discussing classical model of wound healing which could differ from velvet antler rapid regeneration over a period of time in weeks following removal due to the involvement of stem cells. That is why velvet antlers are unique. Li *et al.* (2005) have stated that wound healing in red deer antlers occurs when growth centres located anteriorly and posteriorly to the wound start growing and leaving pressure on the main wound. Eventually these buds grow to form new antlers and they stretch the main wound in between them (Li *et al.*, 2004, Li *et al.*, 005). For better understanding of wound, its types and its healing process in general terms and due to limited amount of publications in this area, the rest of the material will focus on classical wound healing.

A wound is a break or defect in the skin, and it is caused by various physical or thermal insults (Boateng *et al.*, 2008). The Wound Healing Society defines a wound as a result of "disruption of normal anatomic structure and function" (Lazarus *et al.*, 1994). Wounds can be acute or chronic.

Acute wounds usually heal within 8 to 12 weeks, and healing is generally accompanied by a small amount of scar formation (Percival, 2002). These wounds are typically caused by frictional contact between the skin and a surface, producing tears and abrasion. Other wound categories, such as penetrating wounds caused by sharp objects, gunshots, surgical incisions, and thermal and chemical burns, are also classified as acute wounds (Boateng *et al.*, 2008).

Chronic wounds arise from injuries that have protracted healing, and a wound may be classified as chronic when the healing process extends beyond 12 weeks. This failure in healing could be due to repeated trauma or some other physiological conditions, such as inadequate primary

treatments or persistent infections in the wounded area (Boateng *et al.*, 2008, Harding *et al.*, 2002, Moore *et al.*, 2006).

A wound may also be categorized based on the impact on different layers of the skin (Bolton, 1991, Krasner *et al.*, 1993). Wounds may be classified as superficial wounds (in the epidermal skin), partial-thickness wounds (epidermis, deep dermal layers, blood vessels, sweat glands, and hair follicles) and full-thickness wounds (subcutaneous fat and deeper tissues in addition to the epidermis and dermal layers) (Boateng *et al.*, 2008).

i. The physiology of wound healing

Wound healing is related to the growth of cells and tissue regeneration through a series of overlapping and reliant steps. This phenomenon occurs when the cells and matrix components perform in unison to renew and replace the lost tissue by retaining the integrity of the main structure after injury or trauma (Rothe and Falanga, 1989, Shakespeare, 2001, Boateng *et al.*, 2008). Wound healing can be divided into five different stages; haemostasis, inflammation, migration, proliferation, and maturation (Schultz, 1999). Today, new techniques in wound healing and wound dressing attempt to increase the natural healing processes to enhance tissue regeneration and healing rates (Debra and Cheri, 1998).

ii. Haemostasis and inflammation

Haemostasis plays a crucial role in successful wound healing. However, bleeding, which occurs before haemostasis, aids in flushing out debris, antigens, and bacteria from the injury site (Martin, 1997). Bleeding also helps activate haemostasis, which begins after activation of clotting factors and exudate formation. Fibrinogen is the initiating factor that promotes the production of fibrin and the commencement of coagulation in the wound, eventually stopping the bleeding. The clot which develops in the wound makes a suitable scaffold to support and strengthen the injured site (Martin, 1997, Boateng *et al.*, 2008).

Inflammation and haemostasis usually occur concurrently and sometimes within in few minutes of the injury up to 24 hours, and in some cases may last about three days. First, arteriolar vasoconstriction occurs within seconds primarily by neurogenic mechanisms. The vasoconstriction may be enhanced by the release of endothelium-derived factors such as endothelin. The next phase is primary haemostasis which occurs by the aggregation and adherence of platelets to stop bleeding from the injury site, and it is facilitated by the exposed von Willebrand factor (vWF) and collagen. These factors change the nature of the platelet shape and surface, which increases their adherence. Secondary haemostasis assists the primary phase

by the expression of a membrane-bound glycoprotein known as the tissue factor. Tissue factor is found in the smooth muscles cells and fibroblasts and activates coagulation factor VII. The activation of factor VII initiates the coagulation cascade, which concludes in the production of thrombin. Thrombin cleaves the circulating fibrinogen to fibrin, essential for forming platelet plugs (Mitchell, 2020).

During the inflammatory phase, protein-rich exudate is released, and this phenomenon causes vasodilatation and, eventually, the release of histamine and serotonin. Together, these processes attract phagocytes to the site, which begin engulfing and removing the necrotic tissue from the wounded area (Eccleston, 2007, Boateng *et al.*, 2008).

iii. Cell Migration

The migration phase happens when epithelial cells and fibroblasts migrate to the damaged site to replace the tissue. The regeneration process takes place from the wound margins, and it grows under the formed scab or clot, which has already dried after exposure to the air (Boateng *et al.*, 2008).

iv. Cell Proliferation

In the proliferation phase, which happens at about the time of migration (starting from day 3), the basal cells start multiplying, which takes about 2 to 3 days. The next step is the development of granulation tissue, which is due to the formation of small blood vessels and lymphatic vessels. Fibroblasts are responsible for the formation of collagen fibres which provide a strong and solid structure facilitating the formation of vessels. The peak of this process occurs on about the 5th day, in which the blood vessels and granulation tissue are fully formed. The final step, epithelial thickening, occurs when collagen becomes widespread on the wounded area, and this phenomenon allows for regeneration. This proliferation and regeneration (mostly fibroblast proliferation and collagen formation) occurs over two weeks. Eventually, blood vessels regress and oedema starts to disappear (Boateng *et al.*, 2008).

v. Cell Maturation

In the maturation phase, which is also called the remodelling phase, cellular connective tissue starts its formation. Eventually, over several months and up to a period of almost two years, the granular tissue changes its structure from a cellular structure to an acellular structure (Boateng *et al.*, 2008).

Figure 1.8 illustrates these phases of wound healing in damaged tissue over the first 100 days.

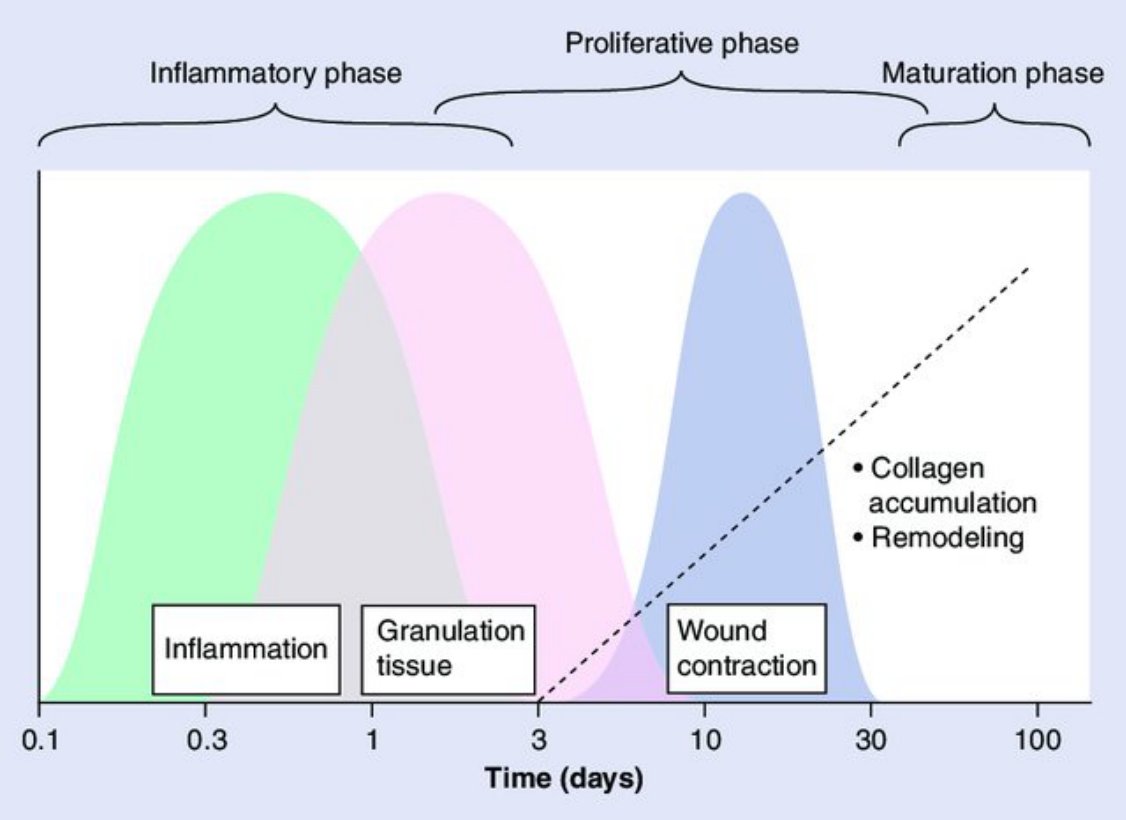


Figure 1.8 Phases of wound healing (Kumar et al., 2005)

The following table (Table 1.2) illustrates different wound types and their appearance in wound healing. It also explains which cells play a role in each section (Boateng *et al.*, 2008, Eccleston, 2007).

Table 1.2 Classification of wounds based on appearance. Each wound type represents the phases that a single wound may go through as it heals (Eccleston, 2007, Boateng et al., 2008).

Wound type	Appearance	Stage of wound healing affected
Necrotic	Often black or olive green due to dead devitalized tissue that is dry, thick and leathery to touch. Common with pressure sore.	Under favourable conditions, dead tissue in a wound such as pressure sore will usually separate spontaneously from the healthy tissue beneath. This occurs as a result of autolysis and presumably involves macrophage activity and the action of proteolytic enzymes, which act at the interface of the necrotic and healthy tissue. A dry environment prevents autolytic and proteolytic action of macrophages and enzymes.
Sloughy	Fluid, moist, loose and stringy rehydrated necrotic tissue that is typically yellow in colour	Associated with excess exudate during inflammatory phase. Slough leads to wounds getting stuck in the late inflammatory stage leading resulting in delayed wound healing.
Granulating	Significant quantities of granulation tissue, generally red or deep pink in colour. May produce excess exudate.	Proliferative phase
Epithelializing	Pink in colour with new formation of epidermis.	Involves both migratory and proliferative phases. Final stages of wound healing.
Infected and malodorous	Red, hot inflamed tissue, pus present. Infection with anaerobic bacteria causes unpleasant odour.	Inflammatory response, collagen synthesis, epithelialisation, Infection. Prolongs the inflammatory process, which prolongs wound healing.

vi. pH in skin layers and wound

Skin surface pH is usually referred to as ss-pH; however, this measurement does not account for the variability in pH across the different layers of skin. As the depth of the skin increases, the pH level also increases gradually (Ohman and Vahlquist, 1994). However, there is still no consistent average pH described in the literature. The lowest recorded is 4 to 4.5 (Dikstein and Zlotogorski, 1994), and in general, the pH ranges from 4 to 6.3 (Lambers *et al.*, 2006), and in medical courses, the ss-pH is described as equalling 5.4 to 5.9 (Braun-Falco and Korting, 1986). There are three different sources of hydrogen ions on the skin:

1. *cis-urocanic acid from filaggrin*

2. *Phospholipase A2 catalysed generation of free fatty acids originated from phospholipids*

3. *Energy-dependent sodium proton exchanger or NHE1*

A wound should always be categorised differently from intact skin since the stratum corneum, and other deeper layers are absent (Schreml *et al.*, 2010). The loss of stratum corneum resulted in higher pH levels in the wound-bed with a mean of 7.44 in acute wounds and 7.42 in chronic wounds (Krien and Kermici, 2000, Behne *et al.*, 2002), which is considerably higher than what is believed to be the average pH on the skin surface (Schneider *et al.*, 2007).

pH is crucial in wound healing, and some of the important wound healing factors, matrix metalloproteinases (MMPs), might be negatively affected by pH changes. This can result in impairment of the wound healing process (Behne *et al.*, 2002).

iv. Hydrogels as a method for wound healing

Injury to skin epithelial tissue triggers a chain of events starting with a loss in integrity in the epidermal tissue and vessels (Han, 2015). When a tissue is injured by surgery, the healing process is more rapid than for unwanted insults. The healing process can occur broadly in three different ways: primary, secondary, and tertiary intention. In primary intention healing, the wound is closed by various techniques such as sutures or adhesive materials; in secondary intention closure, which is mainly associated with acute trauma or disease, the dermal matrix is lost, and the wounded area fills with granulation tissue (Martin, 2013). In tertiary intention, which is also called delayed primary intention, the wound margins remain un-apposed until the necrotic tissue and any infection is removed (Beldon, 2010).

In order to protect the wound surface and prevent exposure to pathogens, wound dressings can be applied to help facilitate the healing process (Moura *et al.*, 2013). Traditional wound dressings such as dry gauze or bandages are affordable and work by passively helping in wound healing. They do not actively change the condition of the wound bed or rate of wound healing; however, they can protect it from any possible infections and keep the wound moist. Historically wound management involved the use of dry dressings on the wound until the 1960s when George Winter investigated the concept of moist wound healing and concluded that the epithelialization of the skin increases under moist conditions (Winter, 1962). During the same period, Hinman and Maibach experimented with differences in wound healing when covered with polyethylene film compared to controls without an occlusive dressing. They found that the occluded wounds had an increased rate of re-epithelialization compared to air-exposed wounds (Hinman and Maibach, 1963). Today, a vast array of moist wound dressings are available, including hydrocolloids, foams, films, and hydrogels (Gupta *et al.*, 2019). Examples of commercially available products include Adaptic Touch® (silicone dressing), ALGICELL® and Algosteril® (alginate dressings), BIOSTEP™ (collagen dressing), and Drawtex® (hydro-conductive dressing) (Gupta *et al.*, 2019).

Hydrogels are considered one of the most versatile compounds to be employed for wound dressings (Varghese and Jamora, 2012). Hydrogels consist of polymeric networks that are porous in nature and have a high affinity to water (Gupta *et al.*, 2016, Capanema *et al.*, 2018, Fan *et al.*, 2016). Polar functional groups such as amide, amino, carboxyl and hydroxyl contribute to the hydrophilicity of hydrogels (Kaith *et al.*, 2015, Pal *et al.*, 2009). The high affinity towards water results in hydrogels containing up to 99.5 percent water, and consequently, they have poor adherence to surfaces. This can be advantageous as it reduces damage to the healing wound bed during the removal and replacement of dressings. Another benefit is that they strongly resemble living tissues, making them bio-compatible when applied (Moura *et al.*, 2013, Capanema *et al.*, 2018, Caló and Khutoryanskiy, 2015). Also, the ability of hydrogels to reversibly swell and shrink makes them unique for drug delivery and wound management (Gupta *et al.*, 2019).

Collagen dressings can be used in many wound applications, such as covering fasciotomy wounds, forming mesh grafts, or repairing defects after tumour removal (Pallaske *et al.*, 2018). Another advantage is that collagen dressings can be applied and removed easily at the application sites (Fleck and Simman, 2010) and can safely remain adhered to the wound for up to 7 days, which reduces the need for multiple applications of dressings (Pallaske *et al.*, 2018). Collagen dressings can also optimise tissue repair by helping to deposit and organise collagen newly formed by the injured tissue (Fleck and Simman, 2010). Furthermore, wound healing is accelerated as collagen dressings have been shown to activate chemotaxis of fibroblasts and macrophages (Fleck and Simman, 2010). With collagen hydrogel, the raw collagen source must undergo a process of solubilisation using salts, acids or enzymes (Piez, 1967), which eventually turns the collagen into a foam or suspension form (Chvapil *et al.*, 1973, Doillon *et al.*, 1986). In addition, the chemical and mechanical processing for solubilization mainly using NaOH, effectively reduces bacterial and viral activity in the collagen matrix (Forest *et al.*, 2007).

v. Zinc oxide nanoparticles

Nanoparticles can improve the safety and efficacy of drugs in drug delivery systems (Hamidi *et al.*, 2008). Collagen on its own lacks the desired mechanical stability for biomedical purposes. Collagen modifiers such as zinc oxide nanoparticles can considerably improve the structure to increase stability and reduce biodegradation at the site of application (Wallace and Rosenblatt, 2003). Modification of collagen can also alter the pore sizes (see Figure 9.1 and 9.2), and thus the release of drugs (Wallace and Rosenblatt, 2003). Zinc oxide (ZnO) is an inorganic compound which can kill *Escherichia coli* by damaging the cell membrane (Liu *et al.*, 2009). ZnO can remain in epithelial cells for extended periods, making it ideal for wound treatments, particularly in cases of protracted healing such as chronic wounds and burns. Topical zinc oxide has been demonstrated to reduce inflammation and bacterial growth and improve wound re-epithelialization. Zinc itself acts as a co-factor for matrix metalloproteinases (MMPs) which are imperative for the regeneration of extracellular matrix (Rajendran *et al.*, 2018). However, the wound healing properties of zinc can alter based on the nanoparticle size and concentration. These nanoparticles have regulatory effects on auto phagocytosis and keratinocyte migration which is crucial for wound healing (Rajendran *et al.*, 2018). The smaller the size of the nanoparticle, the more bactericidal effects due to the high ratio of surface area compared to volume (Rajendran *et al.*, 2018). Păunica-Panea *et al*. (2016) concluded that by creating a collagen or dextran matrix with 50% embedded ZnO nanoparticles, the wound healing and antimicrobial effects reached an optimum level for the application. They also concluded that 75% ZnO included in composites had a negative effect on the integrity of collagen and introducing collagen to the enzymatic process on the wound surface (Păunica-Panea *et al.*, 2016). It is possible to increase the effects of collagen matrix using lower concentrations of ZnO by incorporating the nanoparticles in bio-compatible composites (Rajendran *et al.*, 2018).

1.13. The objective of this research

1. Develop a novel collagen-based nanocomposite of local anaesthetics.

2. Test the efficacy of such formulation for analgesia and wound healing using antler removal in red deer (*Cervus elaphus*) as a model.

3. Validate the use of mechanical nociceptive threshold (MNT) for pain assessment and quantification post antler removal.

1.14. Hypotheses

1. Modified collagen-based local anaesthetic formulation will increase the duration of action of local anaesthetics.

2. Modified collagen-based local anaesthetic formulation will enhance wound healing in red deer after antler removal compared to control deer in which antlers are removed without any postoperative wound management protocol.

Chapter 2

Pilot study: In vitro study using Franz Diffusion Cells

Abstract

The first phase consisted of *in vitro* investigations of collagen-local anaesthetic composite wafers for sustained release of local anaesthetics. Collagen was extracted from lime split leather waste using three different techniques, and the resultant wafers comprised of collagen modified with zinc oxide-PVP nanoparticles with a ratio of 2:1. Local anaesthetics bupivacaine hydrochloride and lidocaine hydrochloride were used in this study. The *in vitro* drug release test (IVDRT) used Franz diffusion cells to investigate the release rates of bupivacaine and lidocaine from the composites in 9 treatment groups (T1- T9) and 3 control groups (C1-C3).

The IVDRT results for both the local anaesthetics demonstrated significant differences in the rate of release between treatments (T1-T9) compared to the control groups. None of the treatment groups showed a significant difference from each other. The results from treatment groups for both drugs in the *in vitro* showed extended drug release compared to the control groups.

2.1. Introduction

An *In vitro* drug release test (IVDRT) is one of the essential techniques for studying the release of the drugs from various drug delivery systems (D'Souza, 2014). The Franz diffusion cell method is the industry-standard protocol for conducting IVDRT (Saadatmand *et al.*, 2019). These cells consist of a donor chamber containing the test drug and a receiver chamber containing collecting media. The chambers are separated by the membrane of choice, such as dialysis membrane (Herrera *et al.*, 2012) or commercially produced skin-like material (Bolla *et al.*, 2020). Validation studies conducted by Ng *et al.* (2010) demonstrated that Franz cell experiments resulted in a considerable decrease in the variability of *in vitro* permeation data (Ng *et al.*, 2010). This evidence suggests that Franz cells are a reliable method for *in vitro* drug release trials.

This *in vitro* study compared the effects of collagen preparation, modification with ZnO-PVP nanoparticles, and the order in which local anaesthetics and ZnO-PVP nanoparticles were added to the collagen hydrogel. The objective of the *in vitro* experiment was to select the best local anaesthetic collagen nanocomposite formulation for the animal study based on the morphology of collagen and the release rate of bupivacaine and lidocaine from the collagen nanocomposite.

Collagen was extracted from lime-split cattle hides, a by-product of the leather industry. The Food and Agriculture Organization (FAO), estimated the availability of cattle hide at 5.6 million tonnes in developing countries and 1.2 million tonnes in developed countries between 2012 and 2014 (FAO, 2016a). Besides abundance, collagen extracted from cattle hide has increased hydroxyproline levels and a higher denaturation temperature compared to collagen from marine sources (Aberoumand, 2012, Noorzai *et al.*, 2020). Collagen derived from pigs is not acceptable in Jewish and Muslim cultures, making cattle hide waste an ideal source for these markets (Schmidt *et al.*, 2016). The final destination for leather industry waste leather off-cuts is typically landfill; however, this has environmental implications due to various chemical residues which can contaminate sites (Noorzai *et al.*, 2020). Therefore, apart from helping to reduce

environmental impacts, the abundance of sources, zero purchase-cost and high quality make the use of extracted collagen from cattle hide attractive.

Collagen may be extracted using different organic acids such as acetic acid, chloroacetic acid, citric acid and lactic acid, and from inorganic acids including hydrochloric acid (Skierka and Sadowska, 2007). The collagen yield depends on various factors such as animal species, animal age and the extraction technique used (Skierka and Sadowska, 2007, Noorzai *et al.*, 2020). Although factors including mechanical, chemical and enzymatic pre-treatments can improve collagen yield (Skierka and Sadowska, 2007), there is still very limited literature about yield, particularly from cattle hide. During collagen solubilization, the covalent bonds, particularly where lysine and hydroxylysine are located, as well as the ester bonds and any bonding with saccharides, are required to be removed (Skierka and Sadowska, 2007). Another strategy to increase the collagen yield is the inclusion of proteolytic enzymes such as pancreatin, pepsin, trypsin (Higheberger, 1961, Nishihara, 1962), papain, ficin or bromelain in the extraction procedure (Hochstadt *et al.*, 1960). These enzymes cleave the telopeptides, which are located at the non-helical terminal regions (producing atelocollagen); thus, increasing solubilization to a greater degree than that which can be achieved with acid treatment alone (Hickman *et al.*, 2000, Bailey and Light, 1989). Compared to extraction with acid, the other different characteristic is that when treated with proteolytic enzymes, collagen still maintains its helical structures (Lodish, 2000, Holmes *et al.*, 2017). Another reason why enzymatic treatment with pepsin 10% (1 gram pepsin/10 gram cattle hide) was selected for this study was to reduce antigenicity (Michaeli *et al.*, 1969, Lynn *et al.*, 2004) of the end product.

The primary objective of the pilot *in vitro* study was to select a lidocaine/bupivacaine collagen composite formulation based on the release rates of local anaesthetics from the formulation.

The null hypotheses for the pilot *in vitro* study were as follows:

i) The collagen extraction technique has no effect on the release rate of lidocaine and bupivacaine.

ii) The order of adding the local anaesthetics and the nanoparticle has no effect on the release rate of lidocaine and bupivacaine.

iii) The addition of zinc-oxide PVP nanoparticles has no effect on the release rate of lidocaine and bupivacaine.

2.2. Materials and methods

2.2.1. Collagen/local anaesthetic/zinc oxide-PVP composite preparation

i. Collagen extraction

Three methods of collagen extraction were evaluated to produce wafers for sustained release of lidocaine and bupivacaine. The three methods were:

1) extraction with acetic acid,
2) with acetic acid and precipitated with NaCl,
3) with acetic acid and pepsin and precipitated with NaCl.

- *Method 1) Collagen extracted by only acetic acid*

Frozen lime splits from cattle hides stored at -20°C were sourced from the New Zealand Leather and Shoe Research Association (LASRA). The hides were cut using a scalpel blade into 0.5 cm^2 pieces. Twenty-five grams of cattle hide was added to 250 ml of acetic acid 0.5 M in a 500 ml beaker to give a 10% w/v mixture. The resulting mixture was mechanically agitated using an overhead stirrer (Changzhou Ronghua, Instrument Manufacturing Co. Ltd., China) at 4°C for 48 hours. Once the mixture was homogenized, it was aliquoted to 50 ml tubes and centrifuged using an ultra-speed centrifuge (Thermo-Fischer Scientific, USA) at 10,000 RPM for 30 minutes. The supernatant was then collected and frozen at -20°C in disposable plastic cups for 6 hours and transferred to a freeze-dryer (EZ DRY, FTS Systems, New York, USA) (-60°C) for 5 days. Once freeze-drying was complete, the freeze-dried collagen wafers were removed from the cups and stored in re-sealable polythene bags. Silica gel beads were added to the storage bags to maintain a moisture-free environment (see Figure 2.1).

- *Method 2) Collagen extracted by acetic acid and precipitation with NaCl salt*

Frozen lime splits were treated as in method 1. After centrifugation, the supernatant was transferred to a 500 ml beaker, and NaCl 4 M was added and mixed with an overhead stirrer at 4°C for 48 hours, forming a collagen precipitate. The resulting mixture was transferred to 50 ml tubes and placed in an ultra-speed centrifuge at 10,000 RPM for 30 minutes. After centrifuging,

the supernatant was discarded, and the pellets were collected and transferred to a 250 ml beaker and liquified using acetic acid 0.5 M. The resulting collagen hydrogel was transferred to a dialysis membrane (Visking tubing 84, The Scientific Instrument Centre Ltd., UK), and placed in a 2-litre beaker of distilled water. It was agitated with a magnetic stirrer (Cimeron™, Magnetic Lab Stirrer) until the water was no longer conductive (Hanna Instrument HI-98304 Conductivity Meter, USA). Once dialysis of the collagen hydrogel was complete, it was placed in disposable plastic cups and frozen at -20°C for 6 hours and then transferred to a freeze-dryer (-60°C) for 5 days. Once freeze-drying was complete, the freeze-dried collagen wafers were removed from the cups and stored in re-sealable zipper storage bags. Silica gel beads were added to the storage bags to maintain a moisture-free environment (see Figure 2.1).

- *Method 3) Collagen extracted by acetic acid and pepsin and precipitated with salt*

In this method, instead of supernatants, the pellets were collected and placed in a 500 ml beaker, and acetic acid 0.5 M was added with the ratio of (9:1). 2.5 grams of pepsin was also added to the pellet-acetic acid mixture and mixed with an overhead stirrer at 4°C for 48 hours. The resulting mixture was transferred to 50 ml tubes and placed in an ultra-speed centrifuge at 10,000 RPM for 30 minutes. After centrifuging, the pellets were discarded, and the supernatant was collected and transferred to a 250 ml beaker; NaCl 2 M was added to the mixture and mixed with an overhead stirrer at 4°C for 48 hours forming a collagen precipitate. The resulting mixture was transferred to 50 ml tubes and placed in an ultra-speed centrifuge at 10,000 RPM for 30 minutes. The supernatant was discarded, and the remaining collagen hydrogel pellets were transferred to a beaker and liquified using 300 µl acetic acid. The liquified collagen hydrogel was transferred to a dialysis membrane and placed in a 1-litre beaker of distilled water then agitated with a magnetic stirrer until the water was no longer conductive. Once dialysis of the collagen hydrogel was complete, it was placed in disposable plastic cups and frozen at -20°C for 6 hours and then transferred to a freeze-dryer (-60°C) for 5 days. Once freeze-drying was complete, the freeze-dried collagen wafers were removed from the cups and stored in re-sealable zipper

storage bags. Silica gel beads were added to the storage bags to maintain a moisture-free environment (see Figure 2.1).

ii. Zinc oxide-PVP nanoparticle preparation

Zinc oxide-polyvinylpyrrolidone (ZnO-PVP) was manufactured in three steps (Figure 2.2) and mixed with collagen hydrogel to produce crosslinks. 1.0975 g of zinc acetate dihydrate ($C_4H_{10}O_6Zn$) (Sigma Aldrich, Germany) was dissolved in 100 mL of ethanol (C_2H_6O) in a 500 ml flask to form a 0.05 M solution, then, 0.3334 g of polyvinylpyrrolidone (Sigma Aldrich, Germany) (PVP, MW 55,000, molar ratio of Zn^{2+}: PVP=5.3) was added to the zinc acetate solution. The resulting mixture was heated at 60°C using a hot plate (IKA® C-MAG HS7, Sigma Aldrich, Germany). 0.561 g of potassium hydroxide (Univar Solutions Inc., USA) was dissolved in 50 mL of ethanol and added dropwise to the mixture using a drip funnel under vigorous stirring using a magnetic stirrer. Stirring continued for 2 hours at 60°C. Upon completion, UV luminescence was used to qualitatively detect the presence of fluorescing ZnO nanoparticles in a dark room (see Figure 2.1). The nanoparticles were purified by adding 100 ml of n-hexane (Panreac®, Panreac Qu ímica S.L.U., Barcelona, Spain) to 50 ml of ZnO-PVP solution in 250 ml flask to form a precipitate within 5-10 min. The solution was centrifuged at 10,000 RMP for 10 minutes, and the precipitate was collected, then dispersed in 50 ml ethanol and centrifuged for a further 10 minutes. The final precipitate was collected after rotary evaporation (Rotavapor R-3, Büchi Labortechnik AG, Switzerland) by scraping the dried zinc oxide -PVP nanoparticles from the vessel. The resulting white, odourless powder from the purification procedure was stored at 4°C for further use (see Figure 2.2).

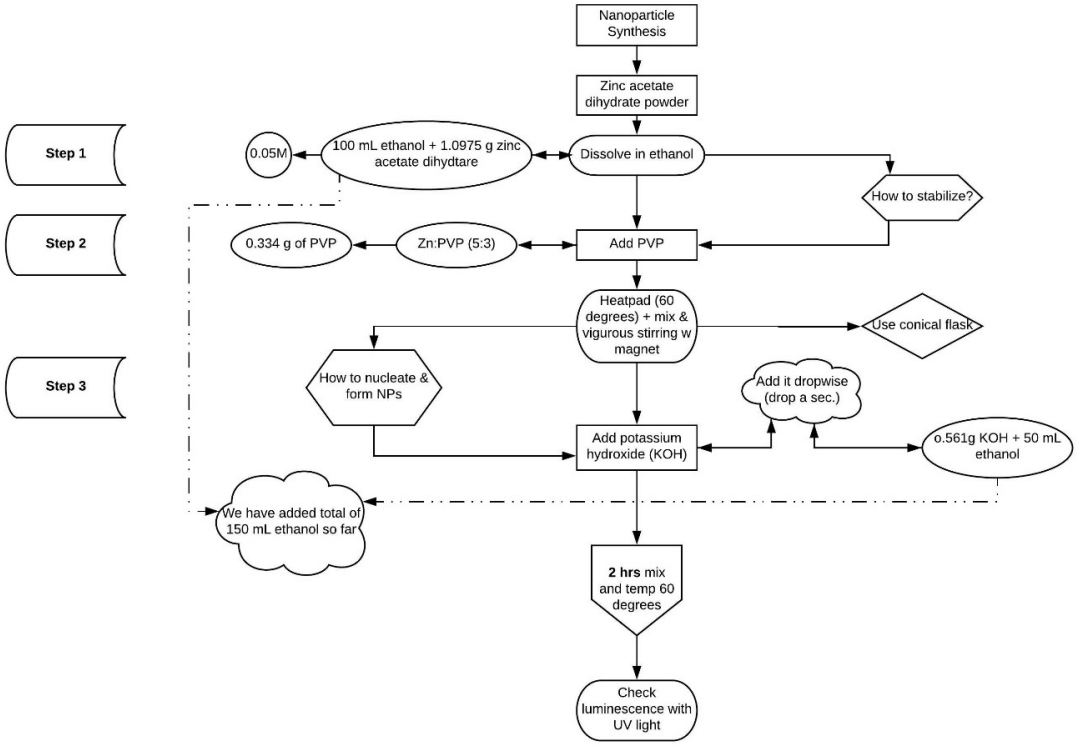


Figure 2.1 The three steps of the ZnO-PVP nanoparticle synthesis procedure used in this study.

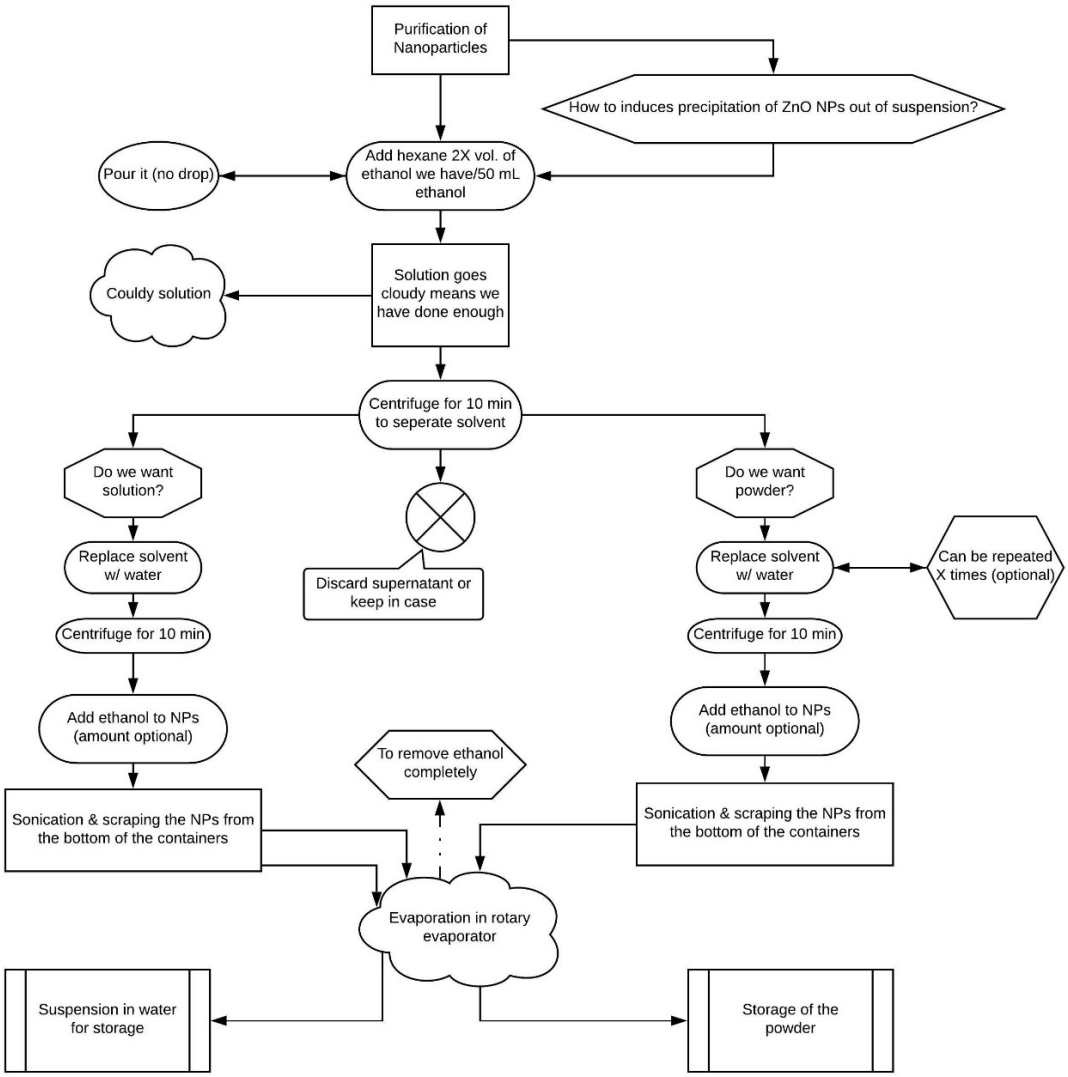


Figure 2.2 Steps of the ZnO-PVP nanoparticle purification procedure used in this study.

iii. Preparation of collagen composite with local anaesthetics

Stored freeze-dried collagen was dissolved with hydrochloric acid (HCL) 0.01M, forming a ratio 5:1 mg/ml. The solution was thoroughly mixed with an overhead stirrer (Changzhou Ronghua, Instrument Manufacturing Co. Ltd., China) for 2 hours at 4°C. After mixing the solutions, they were placed in a rotator (Mini Labroller™ Dual Format Rotator H5500, USA) and kept at 4°C for 12 hours in the fridge to develop a homogenous collagen hydrogel. ZnO-PVP nanoparticles were prepared according to the protocol by Lian *et al.* (Lian *et al.*, 2016), and added to the collagen with a proportion of 1:2 ZnO-PVP: freeze-dried collagen by weight. The solution then consisted of 50 mg of collagen, and 25 mg of ZnO-PVP. 2 mg of bupivacaine HCl (Hunan Russel Chemicals Technology Co. Ltd, China) and 2 mg of lidocaine HCl (Hunan Russel Chemicals Technology Co. Ltd, China) in powder form, were added to the collagen hydrogel (Table 2.1).

Table 2.1 Components of collagen composites in the pilot study for in vitro trial.

Components of the collagen composites for the pilot study					
	Collagen	*ZnO-PVP*	*Bupivacaine*	*Lidocaine*	*Total*
In vitro	50 mg	25 mg	2 mg	2 mg	80 mg

The final hydrogels were freeze-dried for 48 hours, and treatment wafers were stored at room temperature in re-sealable zipper storage bags for the trials.

iv. Methods of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on a gradient separating gel consisting of 10% polyacrylamide with a 3% stacking gel. Collagen samples and standard collagen (Sigma Aldrich®, Germany) were mixed with a 1.5 M Tris–HCl buffer with pH 6.8. This buffer contained 10% SDS and 11.14% 2-mercaptoethanol, 40% glycerol and 0.02% bromophenol blue. Ten µg of each collagen sample, standard collagen (Sigma Aldrich®, Germany), and protein marker (Sigma Aldrich®, Germany) were loaded into an individual well and were electrophoresed (EC135, E-C Apparatus Corporation, Florida, USA) at 150 V for 40 minutes in the SDS-PAGE apparatus (Bio-Rad Laboratories Inc., California, USA).

Next, the gels were fixed using a fixing solution (Sigma Aldrich®, Germany) for 15 minutes and then stained with Colloidal Coomassie dyeing solution (Sigma Aldrich®, Germany) for three hours on the orbital shaker (KJ-201BD, Orbital Shaker, Yuesen Med, Guangzhou, China). After staining, the gels were laid on transparent sheets for scanning and comparing the protein bands of the samples to standard collagen for purity assessment.

v. Collagen wafer treatment groups

In total, the study comprised 9 collagen wafer (collagen-local anaesthetic composite +/- nanoparticles) treatment groups (T1-T9) (Figure 2.3) and 3 drug-only control groups (dry powder only, C1-C3) placed inside the donor chambers for IVDRT. Each group was allocated an individual Franz cell for the duration of the experiment and replicated three times (n=3). The collagen wafers for each of the treatment groups were manufactured as follows:

- *Treatment groups with non-crosslinked collagen (no zinc oxide-PVP nanoparticles)*

T1- A 50 mg aliquot of freeze-dried collagen produced by Method 1 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once liquified, 2 mg each of lidocaine and bupivacaine were added to the hydrogel and mixed with the orbital agitator.

T2- A 50 mg aliquot of freeze-dried collagen produced by Method 2 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once liquified, 2 mg each of lidocaine and bupivacaine were added to the hydrogel and mixed with the orbital agitator.

T3- A 50 mg aliquot of freeze-dried collagen produced by Method 3 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once liquified, 2 mg each of lidocaine and bupivacaine were added to the hydrogel and mixed with the orbital agitator.

- *Treatment groups with cross-linked collagen nanocomposite (crosslinked with zinc oxide-PVP)*

T4- A 50 mg aliquot of freeze-dried collagen produced by Method 1 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once the hydrogel formed, ZnO-PVP nanoparticles (25 mg) were added, and the mixture was agitated for 10 minutes. After thorough mixing, 2 mg each of lidocaine and bupivacaine were added to the mixture and mixing continued for further 2 hours.

T5- A 50 mg aliquot of freeze-dried collagen produced by Method 1 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once the hydrogel formed, 2 mg each of lidocaine and bupivacaine were added to the mixture and agitated for 10 minutes. After thorough mixing, ZnO-PVP nanoparticles (25 mg) were added and mixing continued for further 2 hours.

T6- A 50 mg aliquot of freeze-dried collagen produced by Method 2 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once the hydrogel formed, ZnO-PVP

nanoparticles (25 mg) were added, and the mixture was agitated for 10 minutes. After thorough mixing, 2 mg each of lidocaine and bupivacaine were added to the mixture and mixing continued for further 2 hours.

T7- A 50 mg aliquot of freeze-dried collagen produced by Method 2 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once the hydrogel formed, 2 mg each of lidocaine and bupivacaine were added to the mixture and agitated for 10 minutes. After thorough mixing, ZnO-PVP nanoparticles (25 mg) were added and mixing continued for further 2 hours.

T8- A 50 mg aliquot of freeze-dried collagen produced by Method 3 was reconstituted in 0.01M HCl(aq) (5mg/mL) to produce a 10 ml hydrogel. Once the hydrogel formed, ZnO-PVP nanoparticles (25 mg) were added, and the mixture was agitated for 10 minutes. After thorough mixing, 2 mg each of lidocaine and bupivacaine were added to the mixture and mixing continued for further 2 hours.

T9- A 50 mg aliquot of freeze-dried collagen produced by Method 3 was reconstituted in 0.01M HCl(aq) (5mg/mL) to give us a 10 ml hydrogel. Once the hydrogel formed, 2 mg each of lidocaine and bupivacaine were added to the mixture and agitated for 10 minutes. After thorough mixing, ZnO-PVP nanoparticles (25 mg) were added and mixing continued for further 2 hours.

- ***Control Groups***

I. Bupivacaine HCl powder (200 mg)

II. Lidocaine HCl powder (200 mg)

III. Bupivacaine HCl and Lidocaine HCl powder combination (each 100 mg)

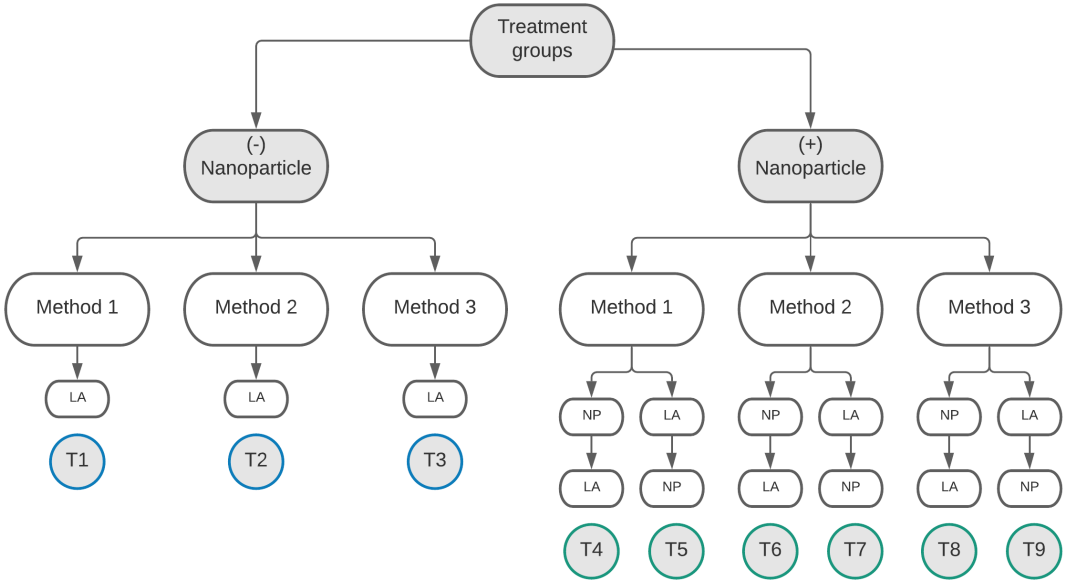


Figure 2.3 The in vitro study comprised of 9 treatment groups as shown above. The first subdivision categorizes the groups by their combination with ZnO-PVP nanoparticles, and the second division classifies different collagen samples extracted with different methods. The last subdivision shows whether ZnO-PVP nanoparticles were added before or after the addition of the local anaesthetics. (-)=without, (+)=with, NP=ZnO-PVP nanoparticle, LA=local anaesthetic.

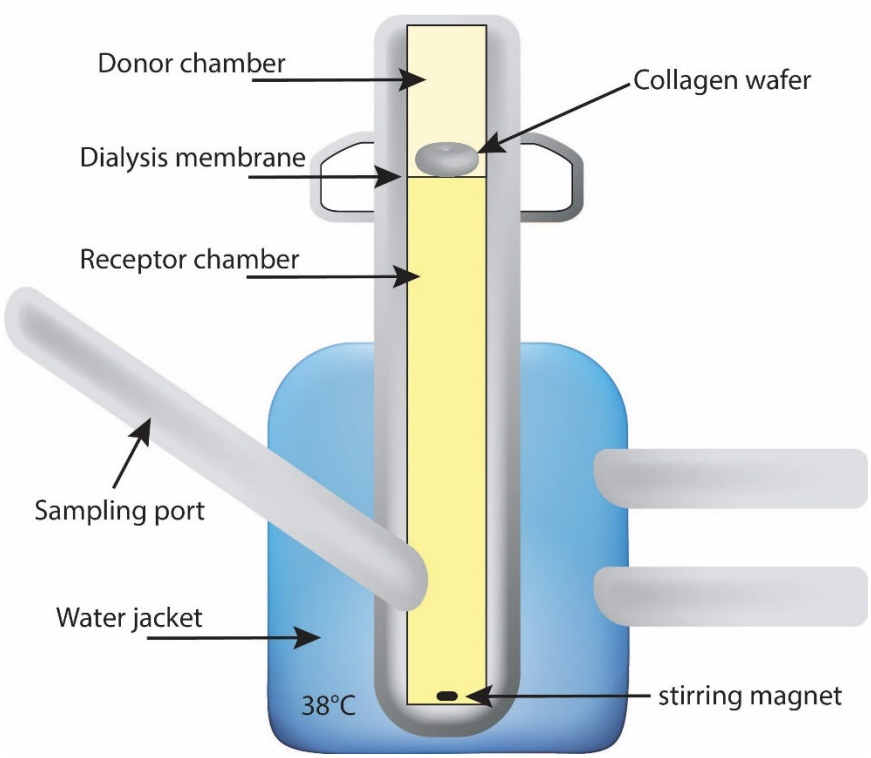
2.2.2. *Drug release study*

Collagen and ZnO-PVP nanoparticles were manufactured using the methods outlined in the previous sections. Bupivacaine hydrochloride and lidocaine hydrochloride in powder form were sourced from Hunan Russel Chemicals Technology Co. Ltd, China and used in all the study groups. The control and treatment groups were assessed using Franz Diffusion Cells, and samples taken at set time-points were evaluated using Liquid Chromatography Mass-Spectrometry (LC-MS) to measure the amount of drugs diffused through the membrane.

i. IVDRT methodology

Franz Diffusion Cells (PermeGear, Hellertown, PA, USA) consisted of two separate chambers, the upper chamber being the donor and the lower chamber being the receptor. Each receptor chamber had a sampling port and had a water jacket for temperature control. The chambers were divided by placing a Strat-M® membrane (Transdermal Diffusion Test Model 25mm, Merck, Darmstadt, Germany) between the donor and receptor chambers (see Figure 2.4). Prior to the placement of the membrane, a stirring magnet was added to the receptor chamber. The membrane was sealed with foam rings on either side and clamped into position. Each receptor chamber of the diffusion cells was carefully filled to the 8 ml mark on the sampling port with Milli-Q water, using a 20G needle attached to a 60 ml syringe. The Franz Cells were then placed into individual holders of the V9-CB Stirrer (PermeGear, Hellertown, PA, USA) (see Figure 2.5). The Franz Cell jackets were connected to a water bath, and the temperature was set at 38°C to simulate deer body temperature and allowed to run for 20 minutes to ensure that the temperature of the Milli-Q water within the cell had equilibrated. After temperature equilibration, 4 drops of deer plasma (weighing ~ 247 mg) were added to the donor chamber using a disposable Pasteur pipette. The plasma was added to both prime the membranes and simulate wound conditions. After priming the membranes, the collagen wafers for the treatment groups and dry local anaesthetic powders for the control groups were added using stainless-steel

forceps to make sure they were soaked with plasma. Once the wafers were added to the cells, timing was started. Samples were drawn from the sampling port at time zero (t0), and then every 15 minutes for the first hour (until t60 min), followed by t120 min and then every 2 hours until 12 hours had elapsed. 0.5 ml aliquots were obtained using a 23G needle attached to a 2.5 ml syringe. The samples were then placed into individual 0.5 ml safe lock tubes (Eppendorf®, Hamburg, Germany). After each sampling, the receptor chamber was filled up to 8 ml mark with milli-Q water. At the end of the experiment, the samples were placed in a refrigerator at 4°C until further analysis. IVDRT was repeated three times for all nine treatment groups and three control groups.



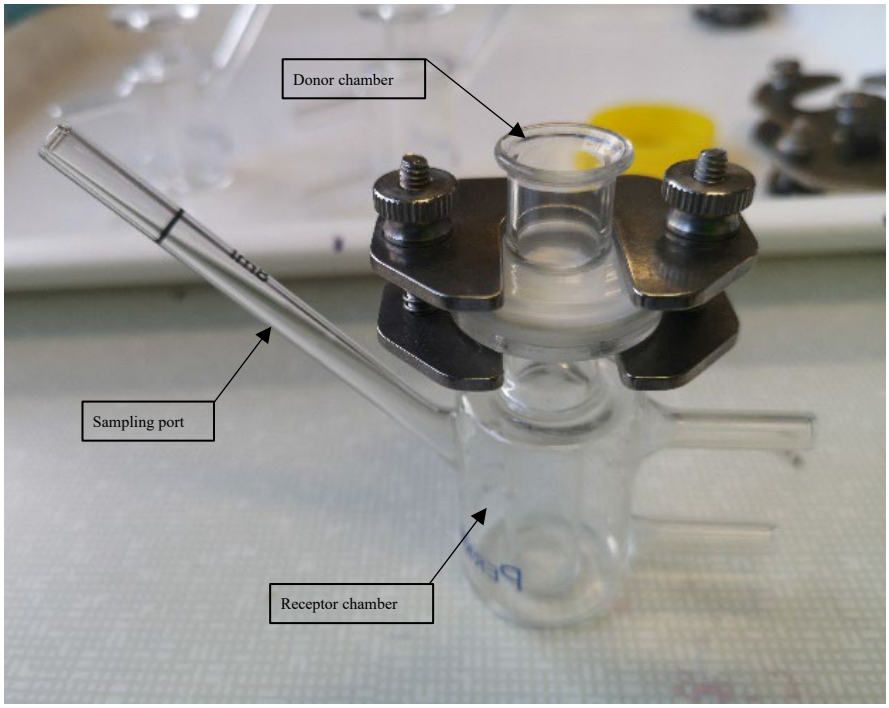


Figure 2.4 Franz Diffusion Cell as used in IVDRT, and its components. A collagen wafer was placed in the donor compartment, and a membrane was placed between the donor and receptor chambers in all groups. The water was maintained at 38°C and a magnetic stirrer was used to maintain homogeneity of contents in the receptor chamber.

Figure 2.5 Franz Diffusion Cells in the V9-CB Stirrer (PermeGear).

ii. Liquid Chromatography-Mass Spectrophotometry (LC-MS) analysis

The samples from the receptor chambers in the previous section were analysed for local anaesthetics using LC-MS. Liquid chromatography was carried out using an ultra-high-performance liquid chromatography system equipped with a quaternary pump, a vacuum degasser, a column compartment and an autosampler (Dionex Ultimate 3000 System; Thermo Scientific, Germering, Germany). Chromatographic separations were achieved using a 2.6 µm particle size C-18 column (100 mm × 2.1 mm; Accucore, Auckland, NZ), coupled with a security guard column (Defender Guard Column; Accucore, Auckland, NZ) and set at a temperature of 25°C. The mobile phase consisted of 0.1% formic acid (100% formic acid, Fisher Chemical™, Fischer Scientific®, Belgium) and acetonitrile 70:30 v/v (Optima™, LC-MS Grade, Fisher Chemical™, Fischer Scientific®, Belgium) and was delivered at a flow rate of 0.3 mL/minute. Mass spectrometric detection was performed on a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer; Thermo Scientific™, Bremen, Germany) with an electrospray-ionisation interface, positive ion mode. The precursor ions of lidocaine (m/z 235.18) and bupivacaine (m/z 288.43) were included in the target list and were fragmented into their respective daughter ions using a collision energy of 35 eV and detected using a resolution of 35,000 FWHM (full width at half maximum). Data processing was performed using the Windows®-based Xcalibur™ data system (Thermo Scientific™, Waltham, MA, USA). The linearity of the LC-MS method was determined by linear regression analysis of calibration samples which were prepared by dilution of both bupivacaine HCl and lidocaine HCl in LC-MS water (Optima™, LC-MS Grade, Fisher Chemical™, USA). Calibration curves were constructed using three replicates of both bupivacaine HCl and lidocaine HCl in the same sample in LC-MS water with concentrations between 10 to 1000 ng/ml. Intra-day and inter-day precision and accuracy of the method was determined by processing three replicates.

iii. Sample preparation

0.5-ml aliquots of Franz Cell samples were centrifuged at 12,000 RPM for 10 min (Thermo Fischer Scientific™, Heraeus™, Megafuge™ 8 small Benchtop, UK). Following centrifuging, 5 µl of the supernatant was injected into the chromatography column for analysis. For Liquid Chromatography-Mass Spectrophotometry (LC-MS), samples from 3 control groups and nine treatment groups were analysed using a validated LC-MS assay. Linearity, the standard curves, the limit of detection and the limit of quantification were assessed after multiplying the diluted samples by 20. This procedure was performed for a more accurate assessment. To avoid residues on the LC-MS, 50 µL samples were spiked in 950 µL LC-MS water to reduce the concentration to 1/20 (The dilution was mainly performed to avoid deposition of large amounts of drugs within the LC-MS instrumentation, then final results were multiplied by 20 to have a rough estimation of actual numbers).

iv. Statistical analysis

Statistical analyses were performed by SAS (version 9.4, SAS Institute Inc., Cary, NC, USA). Data collection was performed using Microsoft Excel (version 365) and graph designs were created by GraphPad Prism (version 8.3.0, GraphPad Software Inc., San Diego, USA). The dependent variable, drug concentration (ng/ml), for the *in vitro study* was analysed in SAS with the MIXED procedure using a linear mixed model for repeated measures. For assessment of the drug release rate, a simplified version of the Higuchi model (Paul, 2011, Siepmann and Peppas, 2011) for drug release kinetics of treatment groups (T1 – T9) and the control groups (C1 – C3) was used here.

$$K_H = Q / \sqrt{t}$$

where Q is the amount of drug released over a period of time (t), K_H is the release rate variable of Higuchi, and t is time in minutes. The release rate (K_H) was calculated by transforming the x

axis values (time in min) to their square roots and dividing the drug concentration over time ((ng/ml) √min^{-1}).

2.3. Results

2.3.1. Collagen purity assessment with SDS-PAGE

The collagen samples extracted from all three extraction techniques showed high purity comparable to the purchased pure collagen (See Figure 2.6 for details).

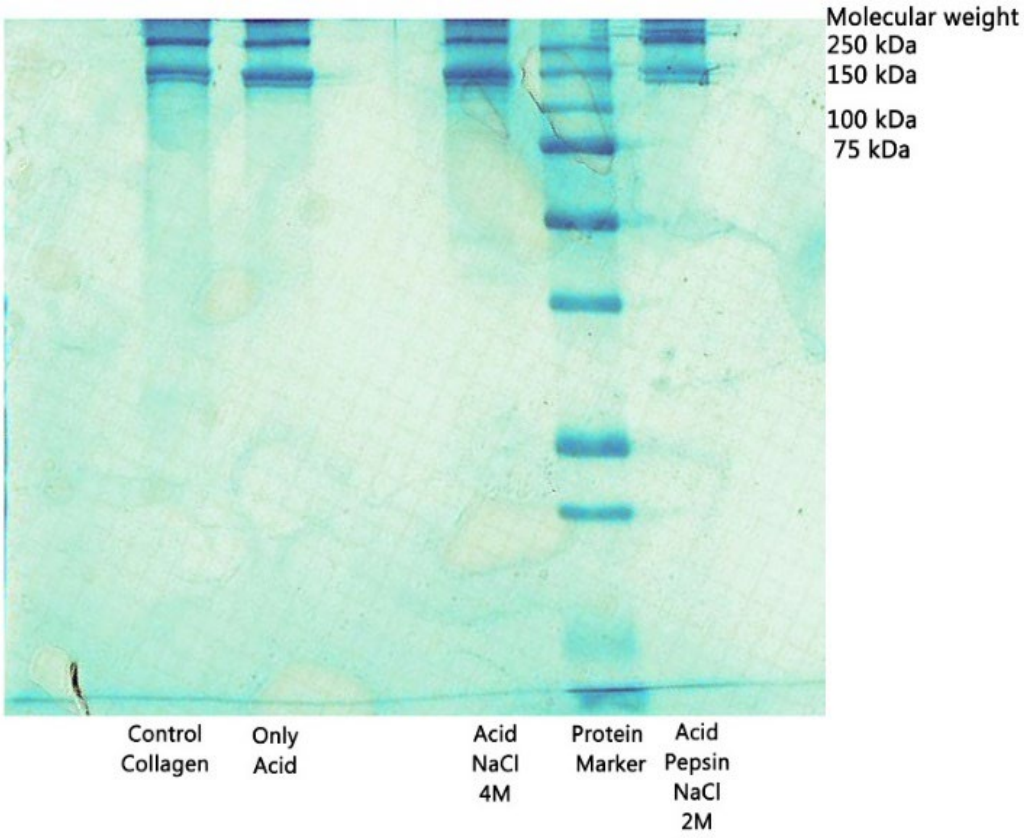


Figure 2.6 SDS-PAGE bands for standard collagen, collagen extracted only with acetic acid, collagen extracted with acetic acid and precipitated with salt, collagen extracted with acetic acid and pepsin, and the protein marker to identify molecular weight.

2.3.2. In vitro study

i. LC-MS method validation

The standard curves for both local anaesthetics (lidocaine and bupivacaine) were linear from 3.125 to 50 ng/ml, with $R^2 > 0.99$ correlation coefficient. The lowest limit of detection for lidocaine and bupivacaine was 1 ng/ml. The lowest limit of quantification for both local anaesthetics was 3.125 ng/ml (see Table 2.2).

Table 2.2 Inter-day and intra-day variation of LC-MS method for analysis of bupivacaine (a) and lidocaine (b) standard solutions. Solutions of known concentrations were analysed in triplicate on three different testing days. The mean and relative standard deviation (RSD%) was calculated from the actual concentrations measured for both inter-day and intra-day.

2.2a Bupivacaine: LC-MS

Concentration Spiked (ng/ml)	Inter-day Concentration Measured (ng/mg)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%
50	62.55	62.02	4.24	9.30
25	28.03	31.03	8.33	2.97
12.5	11.78	13.57	6.49	8.23
6.25	5.58	8.45	13.71	13.73
3.125	2.34	3.91	7.52	12.41

2.2b Lidocaine: LC-MS

Concentration Spiked (ng/ml)	Inter-day Concentration Measured (ng/mg)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%
50	63.52	62.17	2.89	0.82
25	30.66	35.28	10.75	9.17
12.5	16.99	15.42	5.96	5.76
6.25	8.59	8.38	13.93	13.34
3.125	3.83	3.53	11.70	10.59

ii. IVDRT for lidocaine and bupivacaine

Figures 2.7-2.10 show the drug release patterns for the 12 study groups, comprising 9 treatment groups and 3 control groups, for lidocaine and bupivacaine. Error bars (see appendix) were removed from the diagrams for better clarity and comparison between groups. T1-T3 represent the treatment groups (see Figure 2.3) with non-modified collagen wafers, and T4-T9 represent the modified collagen treatment groups (see Figure 2.3). As observed in Figures 2.7-2.10, the control group released significantly higher amounts of lidocaine (C2 and C3) and bupivacaine (only C3) compared to the treatment groups. It should be noted that the concentrations for both lidocaine and bupivacaine were higher than the designed wafers. In the treatment groups for lidocaine, the drug release pattern for the modified collagen composite wafers (T4-T9) showed a more sustained release than the treatment groups with no modification in collagen (T1-T3). T3 from the non-modified collagen groups (T1-T3), and T8 and T9 from the modified collagen groups (T3-T9), which were extracted by pepsin, showed the most sustained drug release trend for lidocaine (Figure 2.8). Similarly, for bupivacaine, the drug release pattern for the modified collagen composite wafers (T4-T9) showed a more sustained release than non-modified collagen composite wafers (T1-T3). T3 showed the most sustained-release trend among the non-modified collagen composites, and T7 and T8 had the most sustained trend among the modified collagen composite treatment groups (Figure 2.1).

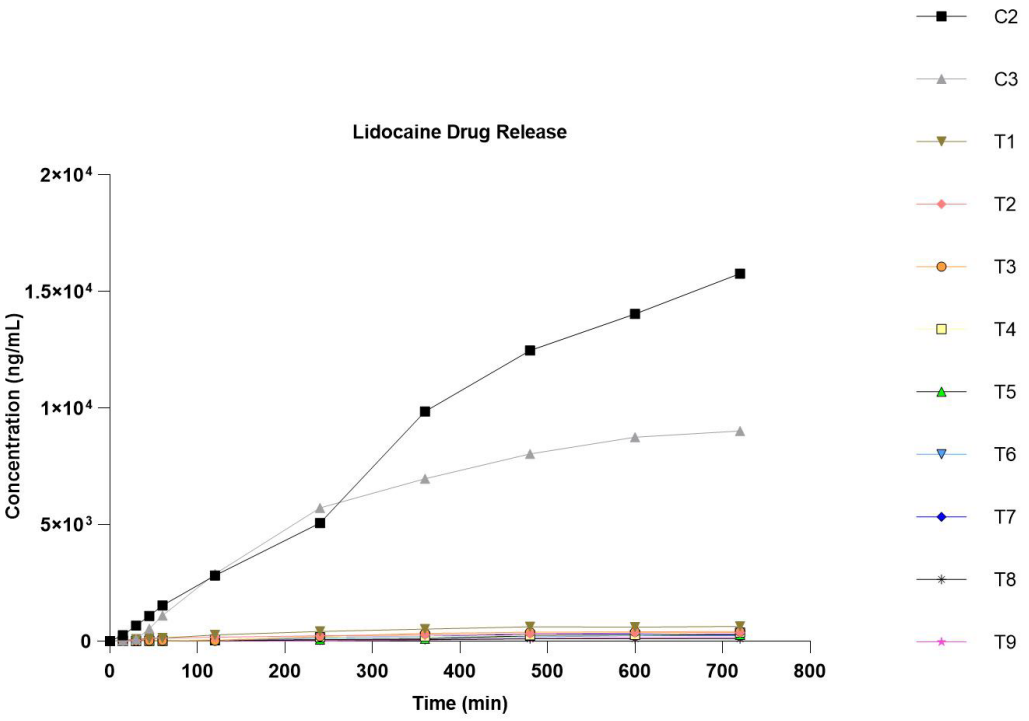


Figure 2.7 Lidocaine concentrations (ng/ml) over time (min) for each of the 9 treatment and 3 control groups in the in vitro study. C2 and C3 showed significantly higher concentrations compared to all the treatment groups (P<0001) (n=3).

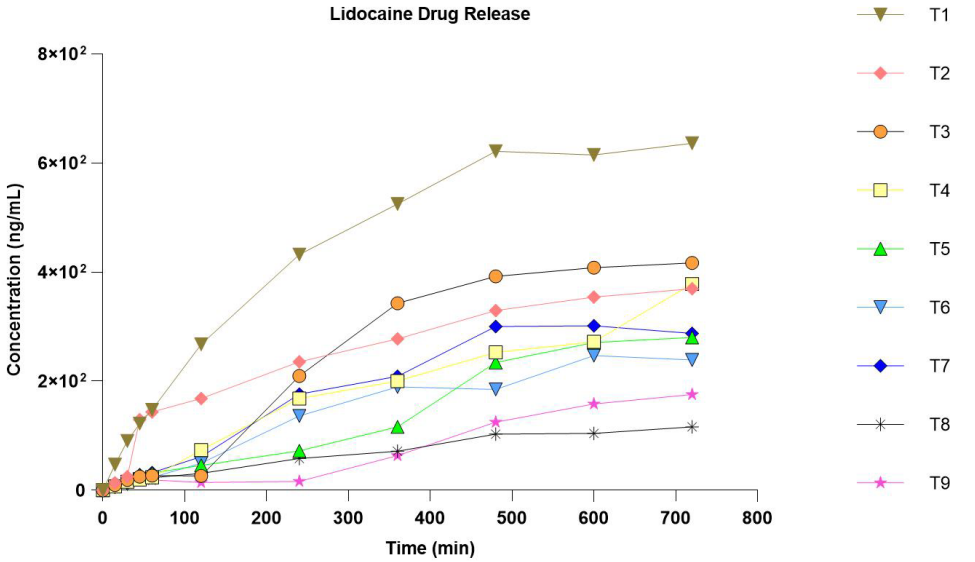


Figure 2.8 Lidocaine concentrations (ng/ml) over time (min) for each of the 9 treatment groups in the in vitro study. None of the treatment groups showed statistically significant differences compared to each other (n=3).

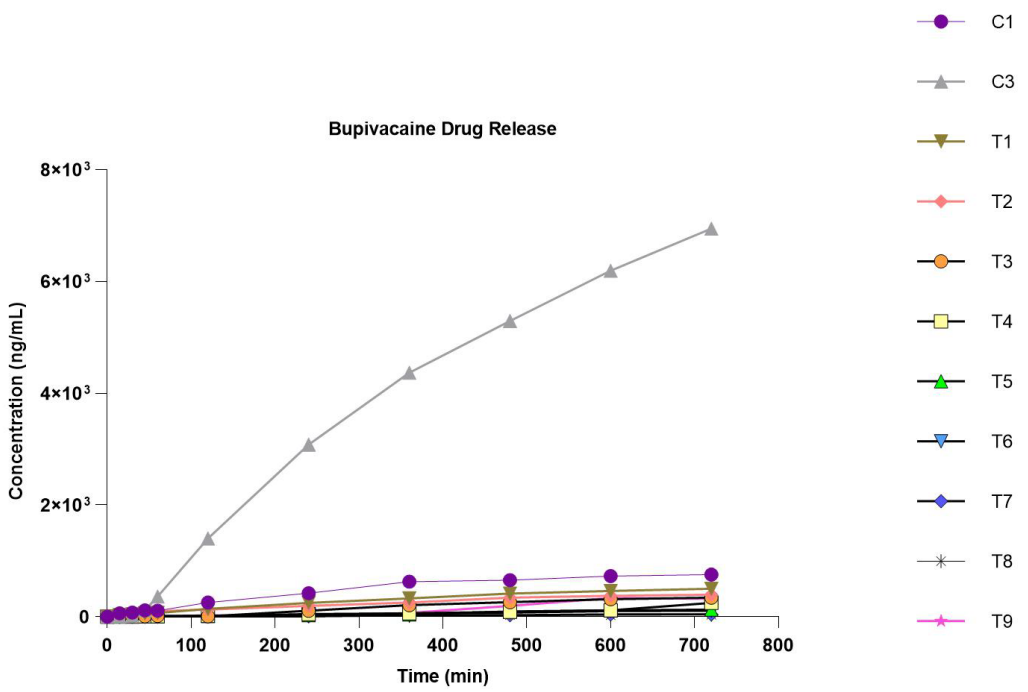


Figure 2.9 Bupivacaine concentrations (ng/ml) over time (min) for each of the 9 treatments and 3 control groups in the in vitro study. C3 showed significantly higher concentrations compared to all the treatment groups (P<05), and C1 showed lower concentrations compared to C3 (P<0.05) (n=3).

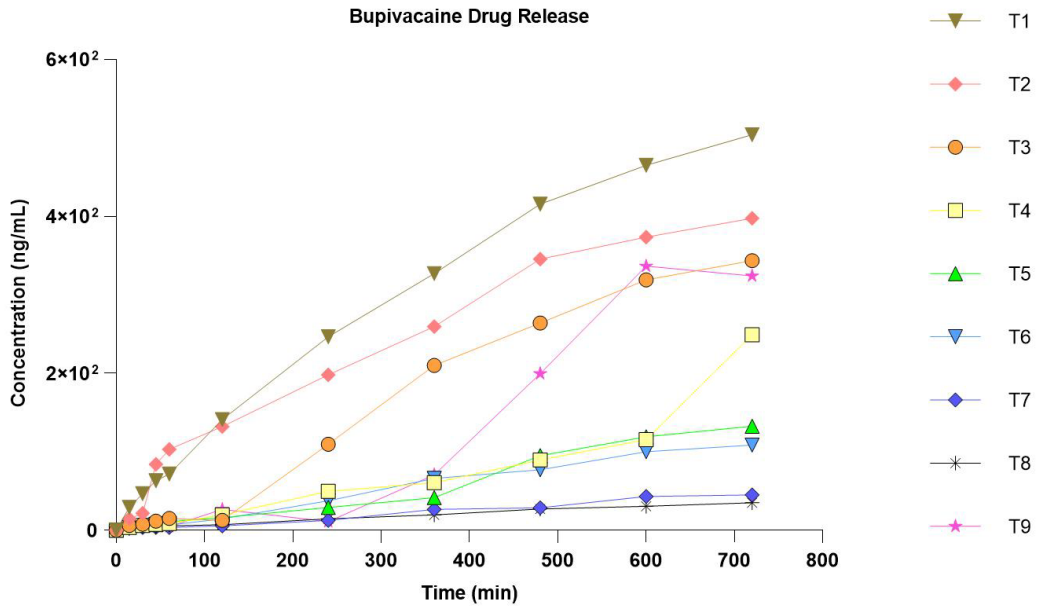


Figure 2.10 Bupivacaine concentrations (ng/ml) over time (min) for each of the 9 treatment groups in the in vitro study. None of the treatment groups showed statistically significant differences compared to each other (n=3).

iii. Drug release rates by Higuchi model

ANOVA showed a significant difference between the K_H values for bupivacaine and lidocaine (P<0.0001 and P<0.0001) between groups. Table 2.3 shows the calculated K_H values (±SEM) for bupivacaine and lidocaine and the statistical differences between them. Figures 2.11 and 2.12 illustrate the regression lines representing the release rates for both bupivacaine and lidocaine. As observed in Figure 2.11, C3 showed the highest release rate ((ng/ml) $\sqrt{min^{-1}}$) compared to the other groups for bupivacaine. As observed in Figure 2.12, C2 and C3 showed the highest release rate ((ng/ml) $\sqrt{min^{-1}}$) compared to the treatment groups for lidocaine. For bupivacaine, the K_H value of C3 was significantly higher than all the other groups (P<0.0001). For lidocaine, the K_H value of C3 was significantly higher than the treatment groups and the control group C2 (P<0.0001, and P<0.01, respectively) (see Table 2.3).

Table 2.3 Rate of release for Bupivacaine and Lidocaine in the IVDRT study following Higuchi model. The alphabetical superscript shows the statistically significant difference between the groups (in columns) for each row. For bupivacaine, C3 had a significantly higher KH value compared to C1 (P<0.0001) other groups (P<0.0001). Similarly, for lidocaine, C3 had a significantly higher KH value compared to C2 (P<0.01) other groups (P<0.0001) (n=3).

	Study groups											
	C1	**C2**	**C3**	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
Bupivacaine ***((ng/ml) √min^{-1})*** ***±SEM***	*33.9^{b}*	*N/A*	*324.6^{a}*	*21.9^{b}*	*16.9^{b}*	*16.2^{b}*	*7.9^{b}*	*5.5^{b}*	*4.8^{b}*	*2.1^{b}*	*1.4^{b}*	*14.3^{b}*
	±30.9		*±30.9*	*±30.9*	*±30.90*	*±30.9*	*±30.9*	*±30.9*	*±30.9*	*±31.4*	*±30.9*	*±30.9*
Lidocaine ***((ng/ml) √min^{-1})*** ***±SEM***	*N/A*	*710.5^{bc}*	*441.9^{ad}*	*28.2^{bd}*	*14.9^{bd}*	*21.3^{bd}*	*15.4^{bd}*	*12.2^{bd}*	*11.5^{bd}*	*14.3^{bd}*	*4.8^{bd}*	*7.1^{bd}*
		±59.6	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*	*±59.6*

IVDRT study for bupivacaine and lidocaine with Higuchi model

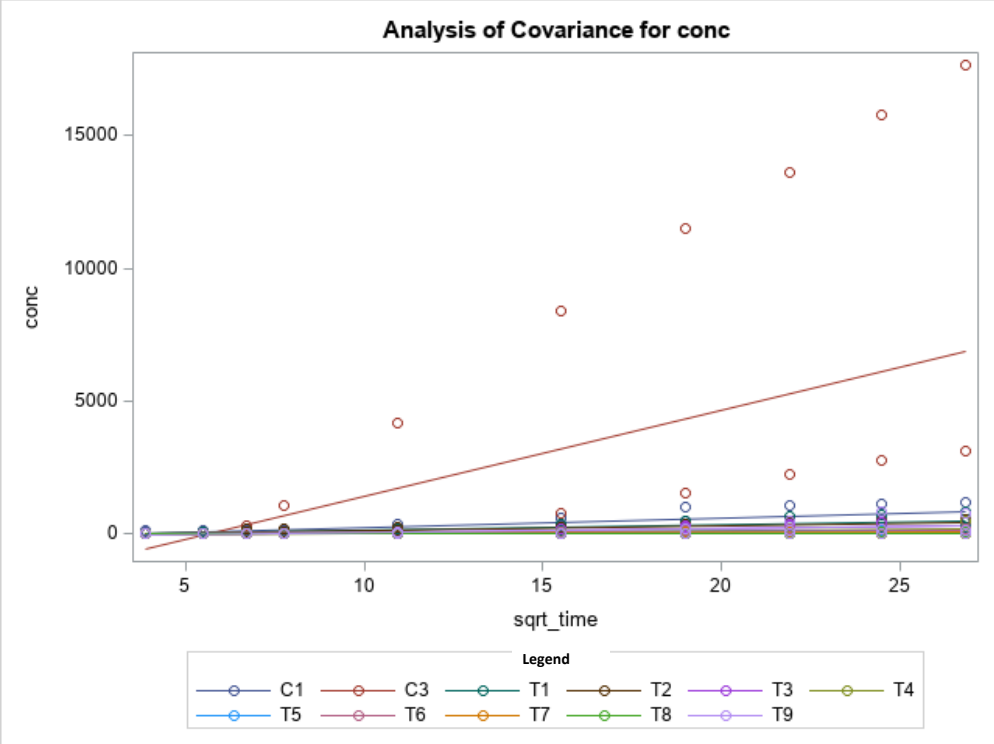


Figure 2.11 Relationship between drug bupivacaine concentration (Q) and the square root of time ($\sqrt{t}$) in 9 treatment and 2 control groups. C3 (red) showed significantly higher release rate ((ng/ml) √min-1) compared to the other groups (p<0.0001) (n=3).

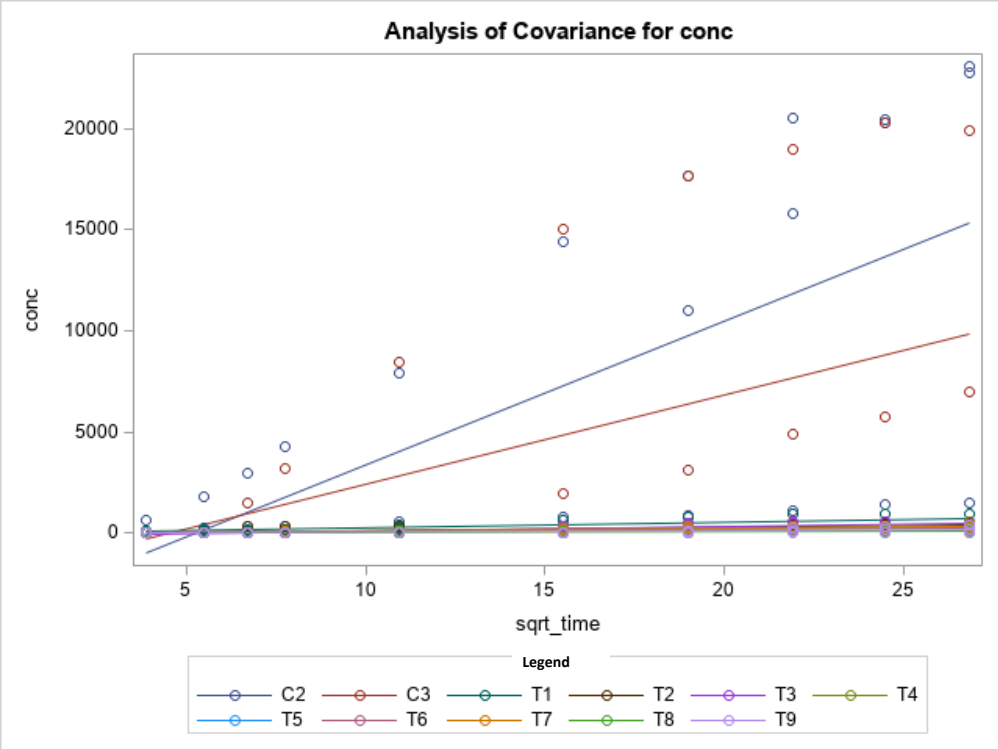


Figure 2.12 Relationship between drug lidocaine concentration (Q) and the square root of time ($\sqrt{t}$) in 9 treatment and 2 control groups. C3 (red) showed significantly higher release rate ((ng/ml) √min-1) compared to the treatment groups and C2 (navy blue) (p<0.0001 and P<0.01, respectively) (n=3).

2.4. Discussion

This pilot study aimed to investigate for the first time the release rate of lidocaine and bupivacaine *in vitro* from different collagen composites extracted using three different techniques. It is known that acetic acid and pepsin extraction techniques for collagen manufacture effectively increase its yield (Tomio, 1962, Skierka and Sadowska, 2007). However, there is a lack of knowledge in determining the effect of extraction techniques on the release rate of drugs.

Collagen hydrogels were successfully extracted using three different extraction techniques in the collagen preparation process. The main differences between the techniques were the inclusion of enzymatic breakdown and / or sodium chloride (NaCl) precipitation steps.

Recently, Noorzai *et al*. (2020) investigated collagen quality using three different extraction techniques and concluded that collagen extracted with acetic acid 0.5 M and precipitated with NaCl salt 2.5 M had 3 and 7 times less yield compared to the other two groups which contained pepsin as part of the extraction protocol (Noorzai *et al.*, 2020). The latter had a very high yield due to major modifications in temperature, exposure time, the concentration of acetic acid, as well as more repetitions and purification steps (Noorzai *et al.*, 2020). Combining insights from previous studies, in addition to those from the recent research by Noorzai *et al.,* extraction protocols were developed for use in this study. Another advantage of this study was determining the salt precipitation effect on the drug release parameters of the collagen wafers. Salt precipitation is an inevitable step to increase yield and purify collagen. More than half a century ago, Bowes *et al.* (1955) concluded that solubility increases with small amounts of salt. In their study, two-thirds of proteins extracted were identified as collagen when using a citrate buffer followed by salt precipitation, and the remaining one third comprised of albumin and globulins (Bowes *et al.*, 1955).

SDS-PAGE analysis was performed on collagen hydrogels samples from the three different extraction techniques compared to a collagen standard (Sigma-Aldrich, USA) (see Figure 2.12). All collagen hydrogel samples demonstrated a high degree of purity compared to the collagen standard, as all samples showed clear protein bands at 150kDa and 250kDa. The collagen hydrogels produced in this study were frozen at -20°C for 8 hours prior to transferring to a freeze-dryer for lyophilization. During the first freezing cycle (-20°C), the forming water crystals expand, which causes the collagen matrix fibres to retract and form pores and tracts within the structure. Further studies are required to quantify collagen yield in our three extraction techniques as there is still a lack of literature in this area.

Hydrochloric acid 0.01 M was used to reconstitute the bulk freeze-dried collagen prior to adding the nanoparticles and local anaesthetics. HCl was used due to its compatibility with lidocaine and bupivacaine and its ability to solubilize collagen. Research on collagen extraction from marine sources has shown that HCl solubilisation provides the lowest collagen yield (only 10%) compared to acetic acid (90% yield) (Skierka and Sadowska, 2007). In this study, HCl was only used to reconstitute freeze-dried collagen to form a hydrogel to mix with the other components such as nanoparticles, and local anaesthetics, and yield was not a concern at this step.

The zinc oxide nanoparticles which were synthesized and purified showed an excellent form, observed as a very bright yellow colour under UV luminescence. Polyvinylpyrrolidone capped zinc oxide was used in this study to create crosslinks in the collagen matrix due to its advantages in delivering sustained release of the drugs (Agban *et al.*, 2016). Polyvinylpyrrolidone (PVP) has an effect on the growth direction of the ZnO nanoparticles, determining the particle shapes (Yang *et al.*, 2019). PVP has also been shown to create hydrogen bonds with collagen (Sionkowska, 2003). Such interactions help to reduce fibrotic tissue formation and induce a better pattern in wound healing by improving tissue architecture (Furuzawa-Carballeda *et al.*, 1999). Therefore, ZnO-PVP was selected to help in both drug delivery and wound healing.

Although this pilot *in vitro* study supported the null hypothesis that collagen extraction did not affect the drug release rate, the groups' release trend was observable. Pepsin extracted collagen composites had the slowest trend in releasing lidocaine and bupivacaine; therefore, this was selected as the best candidate to investigate effects *in vivo*. Additional groups were included to investigate drug release rates from modified and non-modified collagen composites and clarify the effect of mixing orders on drug release rates. Modification of the collagen matrix with zinc oxide-PVP nanoparticles appears to increase the ability to entrap local anaesthetics based on the results obtained from IVDRT, in which greater sustained-release of lidocaine and bupivacaine was observed in modified collagen composites compared to non-modified composites.

Similarly, this study supported the null hypothesis that the order of adding the local anaesthetics and the nanoparticle does not influence the release rate of lidocaine and bupivacaine. No significant difference was observed between the treatment groups suggesting that drugs could be added at any time during the manufacture of modified collagen composite without concerns about changing the drug delivery properties.

Although this study supported the null hypothesis that the addition of zinc-oxide PVP nanoparticles had no effect on the release rate of lidocaine and bupivacaine, the trends of drug release showed a more sustained-release in all treatments with modified collagen composite compared to the groups with non-modified collagen composite. The calculation of the release rate (K_H) was performed by using the Higuchi model, and slopes for each treatment group were created and compared after the transformation of time (t) effect to the square root of time (Siepmann and Peppas, 2011, Paul, 2011). The Higuchi model was selected in this study because it was assumed that the drugs were released by a first-order process (Dash *et al.*, 2010). Other first-order drug release models include Hixson-Crowell, Korsmeyer-Peppas, Bakar-Lonsdale, Weibull, Hopfenberg, and Gompertz (Dash *et al.*, 2010). Hixson-Crowell calculates drug release kinetics by plotting the square root of concentration percentage remained in the matrix over time (Dash *et al.*, 2010). In this case, the amount of drug remaining in the matrix on completion

of the IVDRT remained unknown. In the Korsmeyer-Peppas model, the log of concentration is plotted over the log of time (Dash *et al.*, 2010). The Bakar-Lonsdale model, which is a modified version of the Higuchi model, is used to study the drug release from microspheres and microcapsules, and it is calculated by plotting the amount of drug in time t over time infinity versus inverse square root of time (Dash *et al.*, 2010). This model was not appropriate for analysing IVDRT in this study as the compounds of interest were not encapsulated. The Weibull model is suitable for drug release from a matrix source and is calculated by knowing the time when 50% and 90% of the drug is released (Dash *et al.*, 2010). The Hopfenberg model studied the drug release from oil spheres and counts the geometrical structure of the applied matrix to calculate drug release (Dash *et al.*, 2010). The Gompertz model is used in highly soluble systems and accounts for the dissolution profile (Dash *et al.*, 2010). For this study, the Higuchi model was preferred because of its fast and easy calculation. For calculation of the release rates, the slopes were the representatives of drug release rate or constant K_H ((ng/m)/√t). The reason for the modification of the dataset was to increase the accuracy of comparison between groups. To better understand any interaction between both local anaesthetics, three control groups were used; C1 contained lidocaine only, C2 contained bupivacaine only, and C3 contained both drugs. The results showed that the collagen matrix in the treatment groups significantly decreased the release rate of bupivacaine and lidocaine compared to C3 which contained both drugs but no collagen. Similarly, lidocaine showed no statistical difference among the treatment groups (T1 – T9); however, all the treatment groups had a significantly slower release rate compared to the control group C3. Comparison of the drug release rate for lidocaine showed that when both drugs were combined (C3), the release rate was slower than only lidocaine (C2); however, for bupivacaine, a combination of the drugs in (C3) showed a greater release rate compared to bupivacaine alone (C1). This raised the assumption that bupivacaine is more suitable for sustained-release when it is used alone, as opposed to in combination with lidocaine, and lidocaine has more effective sustained-release properties when it is combined with bupivacaine.

The trends in the release rates showed more sustained release of both lidocaine and bupivacaine when they were used in modified collagen composites compared to the non-modified collagen composites.

The bandings from SDS-PAGE analysis showed high collagen purity similar to the standard across all three extraction techniques of Method 1, Method 2 and Method 3. In addition, zinc oxide - PVP nanoparticles were successfully produced and integrated with collagen structures in crosslinked treatment groups, turning them into a more robust structure. The techniques used in this study showed a promising method to extract collagen from cattle hide and incorporate purified collagen as a dressing for wounds.

2.5. Conclusion

The treatments with modified collagen composites had greater sustained-release properties than the controls for lidocaine and bupivacaine *in vitro*. Additionally, the modified collagen composites, particularly pepsin extracted collagen composites, were more successful in prolonging the release of local anaesthetics compared to non-modified treatment and control groups.

Chapter 3

Pilot in vivo study of the efficacy of collagen composite wafers for postoperative analgesia and wound healing in red deer (Cervus elaphus)

Abstract

This *in vivo* study used 18 male yearling red deer divided into two treatment groups (PT1 and PT2) and a control group (C) with 6 animals in each. All groups received 4% articaine hydrochloride subcutaneous ring blocks prior to velvet antler removal. PT1 had modified collagen wafers with lidocaine and bupivacaine applied after velveting, PT2 had non-modified collagen with lidocaine and bupivacaine, and the control received the 4% articaine hydrochloride ring block only. Analgesic efficacy was assessed using mechanical nociceptive threshold (MNT) measurement at 4 locations including cranial, medial, caudal, and lateral on both the left and right antlers. A Wagner® handheld algometer with a 2mm diameter tip recorded the force required to elicit a response in Newtons (N). MNT was performed at 6 time points after antler removal for the treatment groups (PT1 and PT2) and at 4 time points for the control group. Wound healing efficacy was monitored by thermography on antler wounds at 6 time points over the first 14 days after velvet antler removal. Statistical analyses were performed with a linear model for repeated measurements that accounted for the fixed effects of time after velvet antler removal, antler, location of MNT measurement as covariates, and the random effect of the animal.

The overall least-squares means (±SEM) of MNT in the control and treatment group PT1 were 29.61 ±1.68 N, and 33.98 ±1.52 N, respectively. No significant difference was seen between the thermography values for control and treatment group PT1. PT2 was excluded from further analysis due to the non-adherence of the treatment wafers to the antler wounds. The MNT results from the treatment group in this study showed a more prolonged analgesic effect than in the control group.

3.1. Introduction

Pain assessment in non-verbal animals has been a challenge because of the physiological complexity of pain (Morton and Griffiths, 1985). As mentioned in chapter 1, Electroencephalography (EEG) has been a suggested method of quantification of pain in deer (Murrell and Johnson, 2006, Johnson *et al.,* 2008, Flint, 2012); however, the necessity for general anaesthesia is a severely limiting factor, and for that reason it is only suitable for acute pain assessment. Pollard *et al.* assessed behavioural changes in deer (struggling, ear flicking, head shaking, and grooming) following acetylsalicylate injections and recorded these changes for 4 hours after velveting (Pollard *et al.*, 1992b). Behavioural assessment for many hours requires the animals to be penned and monitored in an artificial environment, which may cause stress and confound results. It is possible to assess behavioural changes in paddocks; however, monitoring behaviour with large sample sizes and long hours can be challenging, particularly during darkness. As an alternative to EEG and behavioural assessment, mechanical nociceptive threshold (MNT) testing can be used to quantify response to a painful stimulus. MNT has previously been established and applied quantification in human pain studies by Henry Beecher (Benedetti, 2016, Beecher, 1957). The MNT is the amount of force required to elicit an avoidance response by the subject (Krug *et al.*, 2018). MNT has been used in several research studies, including humans (Potter *et al.*, 2006) and animals. Large animal studies (Chambers *et al.*, 1994) include dairy cows (Raundal *et al.*, 2014, Fitzpatrick *et al.*, 2013, Dyer *et al.*, 2007, Heinrich *et al.*, 2010, Troncoso *et al.*, 2018), calves (Krug *et al.*, 2018), sheep (Stubsjøen *et al.*, 2010), pigs (Di Giminiani *et al.*, 2016) piglets (Janczak *et al.*, 2012), and horses (Pongratz and Licka, 2017, Chambers *et al.*, 1990). Small animal studies include dogs (Lane and Hill, 2016) rats (Möller *et al.*, 1998), and cats (Steagall *et al.*, 2006, Steagall *et al.*, 2007). However, there are no published reports available on the use of MNT to assess pain sensitivity in deer.

There are many different methods used to monitor wound healing both in humans and animals (Lazarus *et al.*, 1994). In humans, numerous non-invasive and quantitative techniques are

available, such as wearable biosensors (Pasche *et al.*, 2008), optical coherence tomography (OCT) (Cobb *et al.*, 2006) and digital photography (Bon *et al.*, 2000). However, methods such as infrared thermography with a forward-looking infrared camera (FLIR) may be more suitable in animals. A FLIR camera can detect wound temperature from a distance. Increased wound temperature is one of the cardinal signs of inflammation and possible infection at the site; therefore, its measurement may be beneficial to quantify the assessment and treatment of wounds (Horzic *et al.*, 1996, Dini *et al.*, 2015). This technique has been used in cows to assess the surface temperature of wounds, and also confirmed vasoconstriction followed by sympathetic sprouting (the sprouting of sympathetic efferent fibres into dorsal root ganglion or DRG when peripheral nerve injury occurs) caused by traumatic nerve injury in the skin (Troncoso *et al.*, 2018). It has been used in the early detection of mastitis in dairy cows (Sinha *et al.*, 2018), monitoring the depth of burn injuries in pigs (Miccio *et al.*, 2016, Eicher *et al.*, 2006) and wound temperature of horses to verify the presence of impaired blood flow in limb wounds developing exuberant granulation tissue (Celeste *et al.*, 2013).

The primary objective of this study was to determine the duration of analgesia provided by the topical application of novel collagen/local anaesthetic wafers on the wound after velvet antler removal in deer. The secondary objective of this study was to evaluate the effects of the formulation on wound healing and whether modified and non-modified collagen composite wafers remained adhered on the wounded antlers differently.

<u>The null hypotheses for the study were:</u>

i) The application of collagen composite treatment wafer does not prolong analgesia compared to the control group after velvet antler removal in deer.

ii) Collagen composite treatment wafer containing nanoparticles does not prolong analgesia compared to the other groups after velvet antler removal in deer.

iii) Collagen composite treatment wafers have no effect on wound temperature by thermal imaging.

iv) Modification of collagen wafers has no effect on their adherence to the wound.

3.2. Materials and methods

3.2.1. *In vivo* study

i. Study design

The animal study was approved by Massey University Animal Ethics Community (MUAEC) protocol number 18/86.

The experiment was conducted at Massey University Deer Unit, Palmerston North, New Zealand, between the 28th of November 2018 and the first week of January 2019.

This study investigated the efficacy of two different collagen composite wafer treatments containing local anaesthetics to provide prolonged analgesia after velvet antler removal. The selection of the deer from the herd was based on antler maturity in order to prevent wastage of the harvested velvet antler tissue and mimic a commercial situation. As a result, all deer were at approximately the same body weight and antler maturity whilst undergoing treatment. Eighteen healthy 1-year-old male red deer (*Cervus elaphus*) weighing between 110–130 kg were enrolled into the study and divided into three treatment groups (see Table 3.1). All deer received a 4% articaine HCl ring block 4 cm around the base of the pedicle to desensitize the antler prior to velvet removal. The collagen composite wafer treatments were applied to wounds after velveting and compared to the deer who did not receive treatment wafers. The reduction in pain sensitivity around the wound was assessed using MNT algometry. Finally, wound healing was monitored with thermography using a Forward-Looking Infrared Camera (FLIR).

Table 3.1 The pilot in vivo study groups, including the control and treatment groups PT1 (Pilot Treatment 1) and PT2 (Pilot Treatment 2). n=the number of animals in each group. All the groups received 4% articaine hydrochloride ring blocks. PT1 and PT2 received collagen composite wafers, and the control received ring block only.

	In vivo study groups		
	Control (n=6)	***PT1 (n=5)***	***PT2 (n=6)***
Ring Block	*4% articaine HCl*	*4% articaine HCl*	*4% articaine HCl*
Treatment	*No wafer*	*modified collagen wafer* *25 mg lidocaine* *25 mg bupivacaine*	*Non-modified collagen wafer* *25 mg lidocaine* *25 mg bupivacaine*

All animals were clinically inspected by a veterinarian for demeanour, lameness, or any current injuries around the head before the experiment. All deer were kept in paddocks throughout the study period and had *ad libitum* pasture and water. On the day of treatment, all deer were mustered into indoor pens at the deer unit. Deer body weight was measured using Tru-Test load bars connected to a Tru-Test XR5000 (Datamars Limited, Auckland, New Zealand) while passing through the handling facility. The deer were placed into holding pens, each containing six deer. Once all deer were inside, two deer were guided carefully from the holding pen to the hydraulic crush (Heenan Work Room, Farmquip, New Zealand). The hydraulic crush enabled the safe handling of deer by confining them between padded walls, with the operator positioned next to the animal. The sides of the hydraulic crush had curtains above the walls, which could be closed to reduce stimulation and exposure to stressors while allowing the operator quick and easy access for manipulations on the animals. After each treatment or recording was performed, the deer were released back into a holding pen. While indoors, they had access to water and concentrated feed (Multi-feed nuts, Sharpes Stock Feeds, Carterton, New Zealand). At the completion of each treatment day, all animals were returned to the paddock.

The treatment groups received modified collagen wafers containing ZnO-PVP nanoparticles at a ratio of collagen: nanoparticle, 2:1 plus 25 mg lidocaine and 25 mg bupivacaine (PT1), and non-

modified collagen wafers containing 25 mg lidocaine and 25 mg bupivacaine (PT2). The control group did not receive any collagen wafer treatment (Control) (see Table 3.1). Each deer was physically restrained in the hydraulic crush (Figure 3.1), and a tourniquet was placed just below the pedicle to reduce excessive bleeding after velvet antler removal. The tourniquet was removed after 2 hours. The pedicle circumference of each antler was measured using a measuring tape, and 4% Articaine HCl (SCI Pharmtech, Taoyuan, Taiwan) was injected subcutaneously (SC) using a 20G needle attached to a 20 ml syringe at a dose rate of 1 ml/cm of pedicle circumference (4–5 injections) to provide a complete ring block around the base of both the antlers. The efficacy of local anaesthesia was assessed by pressing the 2-mm tip of a handheld algometer (Wagner® FPX 25, Wagner Instruments, Greenwich, CT, USA) as a noxious stimulation 10mm above the pedicle, followed by a "nick test" where a small experimental cut was made with a saw. If there was no response to these, the antlers were removed with a sharp handsaw, and the time was recorded as time zero (t0). Since the main unit used in the study was in hours, the decision was to name the start of effective local anaesthesia as t0ii and at the time when baseline MNTs before performing local blocks t0i. Just to mention that these are just labels and solely mean before nerve block and after effective nerve block. For consistency and more convenient data analysis, 10 min after injection of local block was allocated to measure all antlers for certainty of reaching a complete nerve block. The treatment groups (PT1 and PT2) received a topical application of 3.8 cm diameter collagen composite wafers (Figure 3.2) on the wound immediately after the removal of the antler. The control group animals did not receive any wafer treatment after velvet antler removal (Table 3.1).

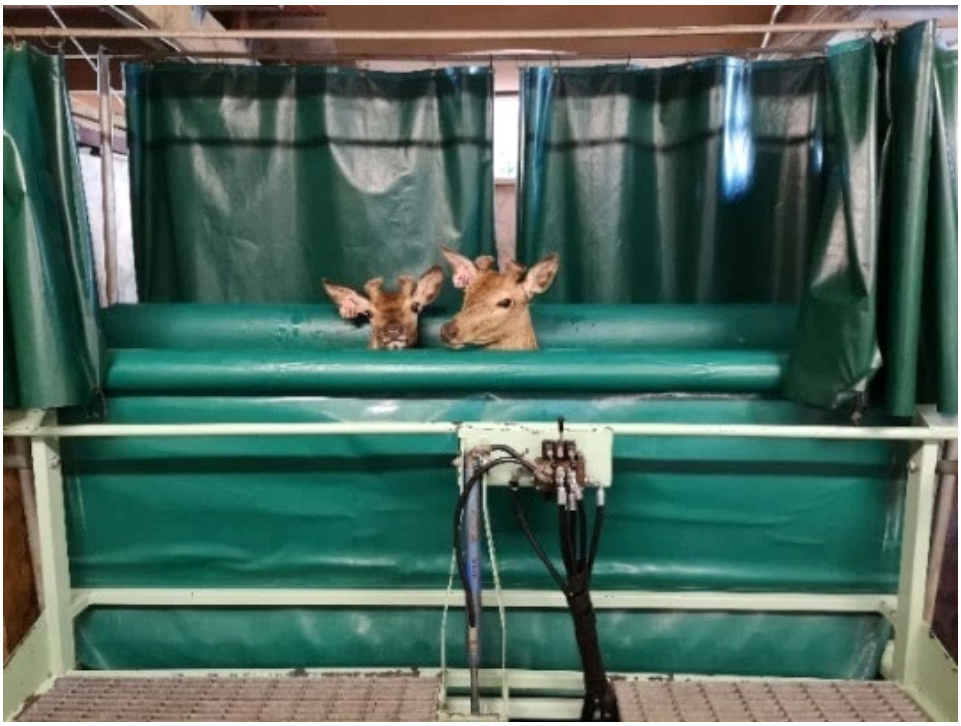


Figure 3.1 Heenan Work Room Hydraulic crush (Farmquip, New Zealand) to restrain deer in the study. For each procedure in the experiment, two deer were guided through the device to restrain them safely. The curtains were closed before their entry to reduce visual stimulations and minimise stress in animals.

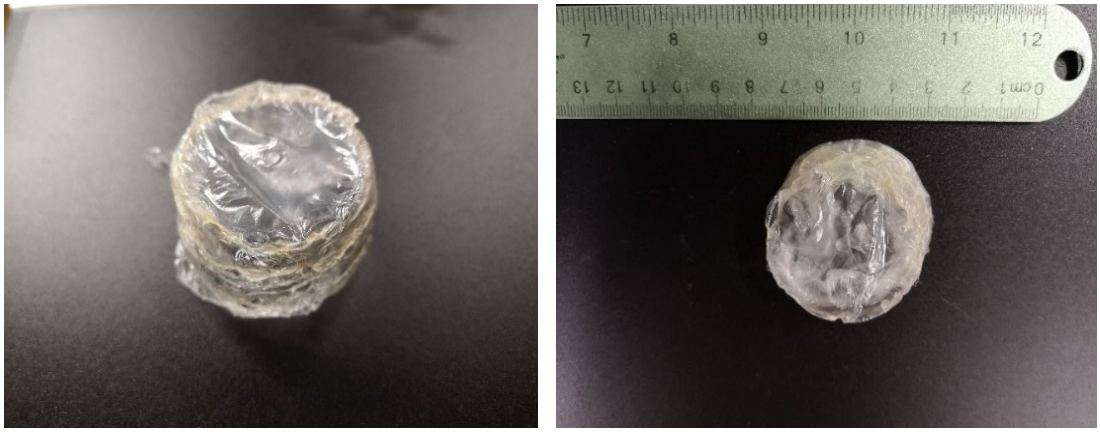


Figure 3.2 Left: non-modified collagen composite treatment group. Right: modified collagen composite treatment group. The diameter of the wafers was 3.8 cm.

ii. Mechanical nociceptive threshold (MNT)

A hand-held algometer (FPX 25, Wagner Instruments, Greenwich, CT, USA) with a two mm-diameter round stainless-steel probe tip was used to measure the MNT of deer. MNT assessments were performed at t0 (baseline reading), t10 min (10 minutes after ring block); then, t2 hr, t4 hr, t6 hr, and t8 hr after antler removal. A single, experienced investigator measured the mechanical nociceptive thresholds of all study animals. He was not blinded to assess the study. The probe tip of the algometer was placed on cranial, caudal, medial, and lateral sites around the base of the antler, 1 cm below the pedicle (the site of antler growth) (Figures 3.3 and 3.4) and a gradually increasing force applied for 1 second. The behavioural response to the threshold being reached was head shaking, and the algometer reading was automatically recorded then in newtons (N). Half of the deer were tested on the right antler first, followed by the left antler and vice versa. This was to minimise the animal's anticipation of the procedure. The cut-off point for pressure algometry was set at 50 N to minimise any tissue damage and extra discomfort in animals (Stubsjøen *et al.*, 2010). Values were recorded in a table shown in the appendix section (Figures 9.2-9.3).

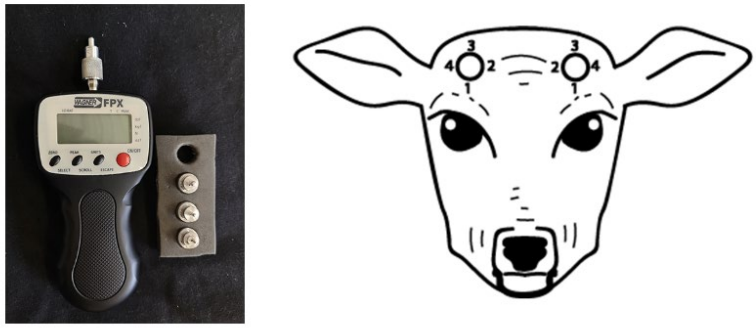


Figure 3.3 Left: Wagner hand-held algometer (FPX 20) with a 2-mm-diameter round steel probe. Right: Sites of algometer probe placement around the base of the antler to measure nociceptive thresholds. 1 = cranial, 2 = medial, 3 = caudal and 4 = lateral.

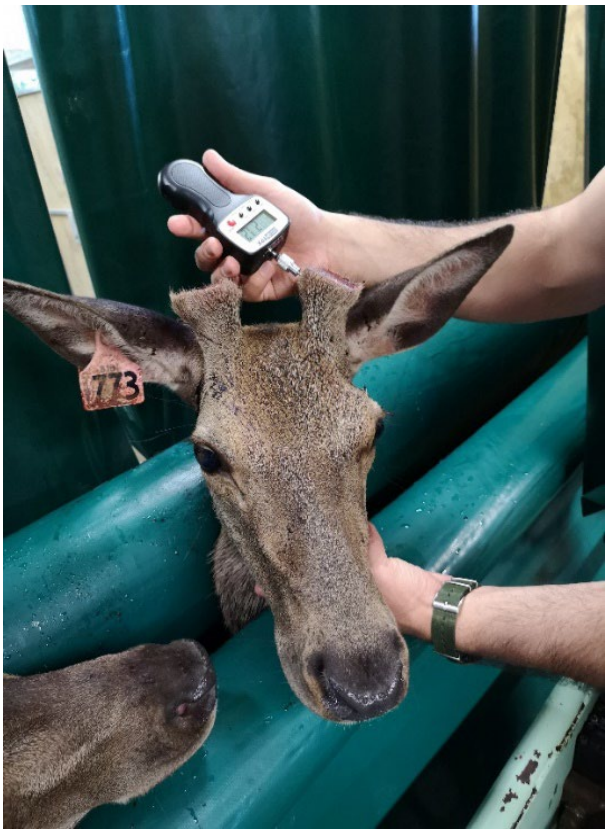


Figure 3.4 Figure shows positioning for MNT measurement at the medial aspect of the left antler. MNT is measured using a Wagner® handheld algometer 4 hours after the antlers have been surgically removed. No tourniquet is visible at this stage as they were removed two hours post-surgery. For consistency, the device was handled as shown in the image.

iii. Thermal imaging

Thermal imaging or thermography was conducted using a Forward-Looking Infrared (FLIR) camera (FLIR T6XX Series, Model T650sc, FLIR Systems AB, Sweden). The FLIR camera was used to measure both the maximum temperature on the cut surface of the antler and the temperature at the centre of the wound. The camera automatically detected the location of the highest temperature; however, the target symbol was manually set at the centre of the wound for that measurement. A distance of about 50 cm from the wound to the camera was used. Thermography measurements were collected at times 0, 4, 24, 72 hrs, 7 days and 14 days after antler removal for both treatment and control groups. Time zero (t0) measurements were taken immediately after surgical removal of the velvet antlers. Values were recorded in a previously prepared table (Figure 9.1).

iv. Statistical analysis

The MNT (N) in the control group of this study were not recorded at time points 360 min (t6) and 480 min (t8) because they had returned to baseline. This created an incomplete design, and so the analyses were performed in two steps. First, the main effects were evaluated with a model that included the fixed effect of treatment and time and the random effect of animal to account for repeated measurements on the same animal. Least-squares means were calculated for the variables, treatment and time, and used for multiple comparisons using the Fisher's least significant difference as selected in the option LSmeans (Least Squares Means) of the MIXED procedure. Second, the interaction between time and treatment was evaluated with the same model as before but including the fixed effect of interaction between time and treatment. The time values were transformed from hours to minutes for consistency within the statistical analysis; for example, t2 was represented as t120. Measurement of the antler (right or left), site on the antler base test sites (cranial, medial, caudal and lateral) were as covariates, and the random effect of deer (between-animal variation; $^{2}_{a}$) and residual (within-animal variation; $^{2}_{e}$) was accounted for. The repeated measurements on the same deer were modelled with a compound symmetry error structure. The compound symmetry error structure was determined as the most appropriate residual covariance structure based on Akaike's information criterion. For the analysis of the recovery rate of analgesia in the *in vivo* study, slopes were created (MNT/time) starting from t10 minutes to compare the value between the control and PT1. Estimates with $P<0.05$ were considered significant.

3.3. Results

3.3.1 In vivo study

i. Adherence of the collagen wafers

In this study, 6 out of 10 wafers remained adhered to the wounds in the first 24 hours and by the end of the experiment in day 14, five out of 10 wafers were remained adhered (Table 9.21).

ii. Mechanical Nociceptive Threshold (MNT) testing

From a total of 18 animals enrolled in the *in vivo* study, six animals from the non-modified collagen composite group (PT2) were eliminated from sampling due to the loss of wafers during the treatment period, and one animal was excluded due to trialling the adherence of a novel formulation, leaving a total of 11 deer. Of the 11 deer available for the study, 6 deer were from the control group and 5 deer from the modified wafer treatment group (PT1). A total of 432 algometer force test results (treatment=242, control=192) were obtained across 6 time-points for PT1 and 4 time-points for the control group. MNTs were recorded across the range of 7.4 N to a maximum of 50 N for the control and PT1 groups. The MNT assessments of the PT1 group were continued until 8 hours (t8) from t0; however, the MNT assessment for the control ceased at 4 hours (t4) since the values obtained at t4 hours were similar to the baseline measurements (t0), and any further testing was likely to induce more discomfort and pain to the animals. During the experiment some of the study deer presented tonic immobility which was one of the main complications in the study. On some occasions, the assessors had to wait and even undo the hydraulic crush so animal would return back to their normal condition for the second attempt.

The least-squares means (±SEM) of MNT in the control and PT1 groups were 29.61 ±1.68 N, 33.98 ±1.52 N, respectively. The trendlines demonstrate the change of MNT over time recorded from all four different aspects of antlers in the control and treatment groups (Figures 3.5-3.8). No significant difference was observed for MNT readings between the antler test sites (cranial, medial, caudal and lateral). Comparison of MNT readings of the cranial and medial aspects of

the treatment group compared to the control group showed significantly higher MNT readings in the treatment group at t120 and t240 (see Table 3.2 for *p*-values).

Comparison of MNT readings of caudal and lateral aspects of the treatment group compared to the control group only showed significantly higher readings in the treatment group at t240 (see Table 3.2 for *p*-values).

Table 3.2 p-values of the MNT readings (N) in the pilot in vivo study comparing the control and treatment groups considering all aspects of the antler and combined. Statistically significant differences are shown with asterisk.

MNT±SEM (N) *p*-values for control vs. PT1					
Time (min)	**Cranial**	**Medial**	**Caudal**	**Lateral**	**All aspects**
0	0.5	0.5	0.6	0.3	0.3
10	1.0	1.0	1.0	1.0	1.0
120	<0.05*	0.001*	0.07	0.1	<0.001*
240	<0.001*	<0.01*	<0.01*	<0.01*	<0.0001*

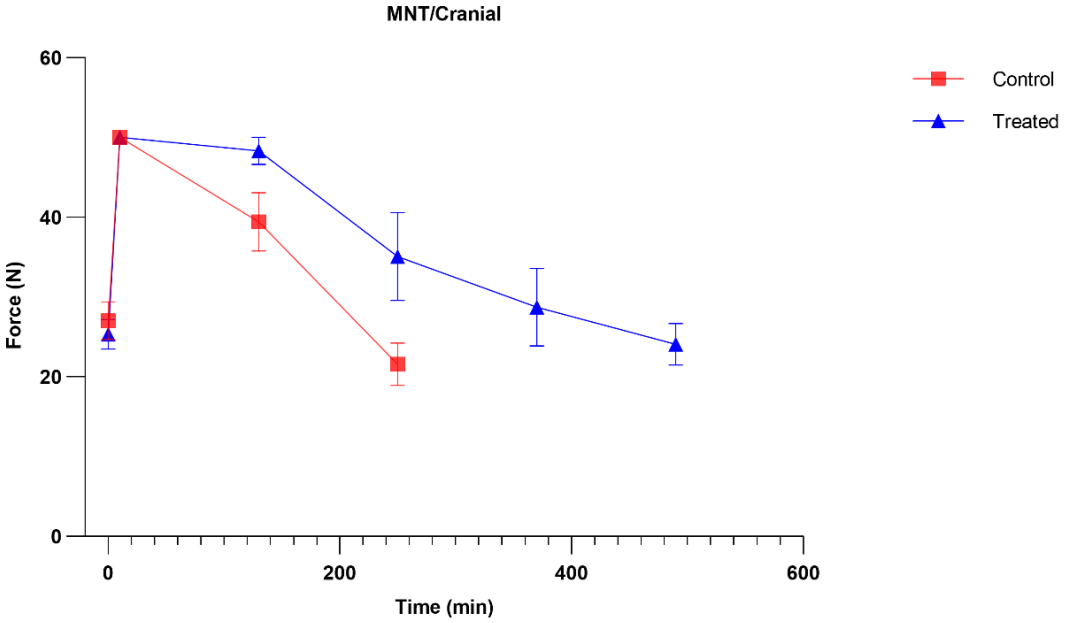


Figure 3.5 The least-squares of MNT±SEM (N) for the control (red) and treatment group (PT1) (blue) at different time points from the cranial aspect of deer antler. The Y-axis represents MNT measurements as force (N), and X-axis represents time in minutes. There was a significant difference between control and treatment groups in time-points t120 (P<0.05) and t240 (P<0.001) (control (n=6), treatment (n=5)).

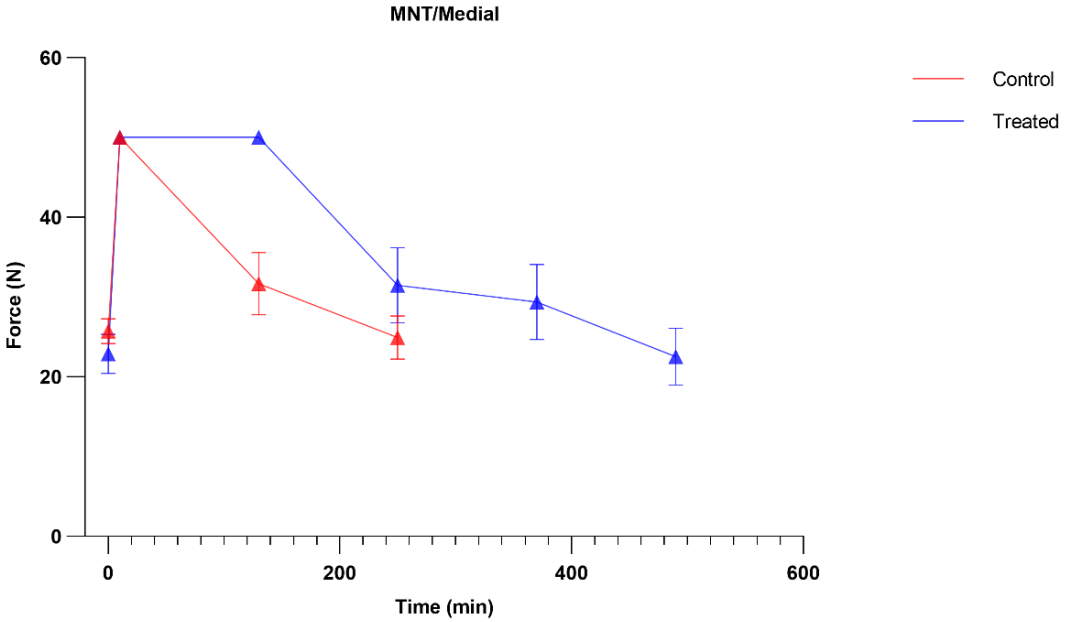


Figure 3.6 The least-squares of MNT±SEM (N) for the control (red) and treatment group (PT1) (blue) at different time points from the medial aspect of deer antler. The Y-axis represents MNT measurements as force (N), and X-axis represents time in minutes. There was a significant difference between control and treatment groups in time-points t120 (P=0.001) and t240 (P<0.01) (control (n=6), treatment (n=5)).

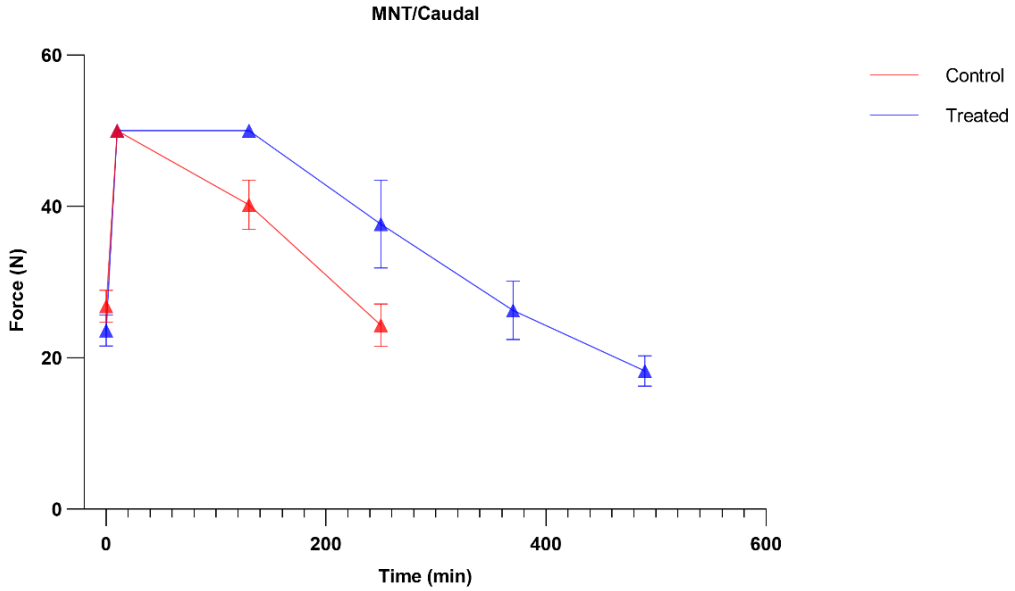


Figure 3.7 The least-squares of MNT±SEM (N) for the control (red) and treatment group (PT1) (blue) at different time points from the caudal aspect of deer antler. The Y-axis represents MNT measurements as force (N), and X-axis represents time in minutes. There was a significant difference between control and treatment groups in time-point t240 (P<0.01) (control (n=6), treatment (n=5)).

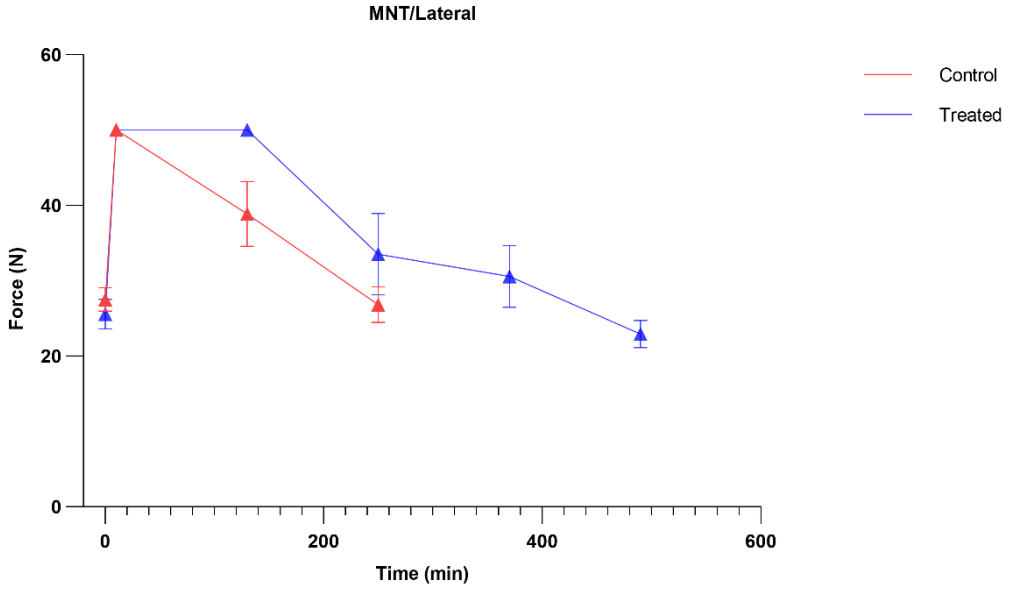


Figure 3.8 The least-squares of MNT±SEM (N) for the control (red) and treatment group (PT1) (blue) at different time points from the lateral aspect of deer antler. The Y-axis represents MNT measurements as force (N), and X-axis represents time in minutes. There was a significant difference between control and treatment groups in time-point t240 (P<0.01) (control (n=6), treatment (n=5)).

iii. Least-squares means of MNT

The MNT (N) measurements stopped at 240 min in the control group due to the measurements reaching the baseline MNT force. However, the MNT measurements in the treatment group PT1 were continued up to 480 min, when the baseline was reached (see Tables 3.3 and 9.6).

*Table 3.3 Least-squares means of MNT (±SEM) (N) between the treatment group (PT1) and the control group up to 8 hours. As seen in the table, measurements in the control group ceased at t4. The means of MNT for the control group (t120) was significantly lower than the treatment group (PT1) (P<0.001). The means of MNT for the control group (t240) was significantly lower than the treatment group (PT1) (P<0.0001). * Statistically significant difference.*

	MNT±SEM (N)	
Time (min)	**Control group**	**Treatment group (PT1)**
0 (baseline)	26.62±1.18	23.85±1.29
10	50±1.18	50±1.29
120	37.48±1.18*	46.07±1.29*
240	23.69±1.18*	35.25±1.29*
360		27.22±1.29
480		21.37±1.29

iv. The comparison of recovery rates from analgesia

To calculate slopes that represent the recovery rate from analgesia, the MNT (force) measurements (N) were plotted against time (min). The calculated recovery rate for the control group was -0.11 N $min^{-1,}$ and for the treatment group, PT1 was -0.06 N min^{-1}. Regarding the comparison of rates of recovery, the control group showed a more rapid trend returning to the baseline MNT readings compared to the treatment group PT1 (P<0.0001) (Figure 3.9).

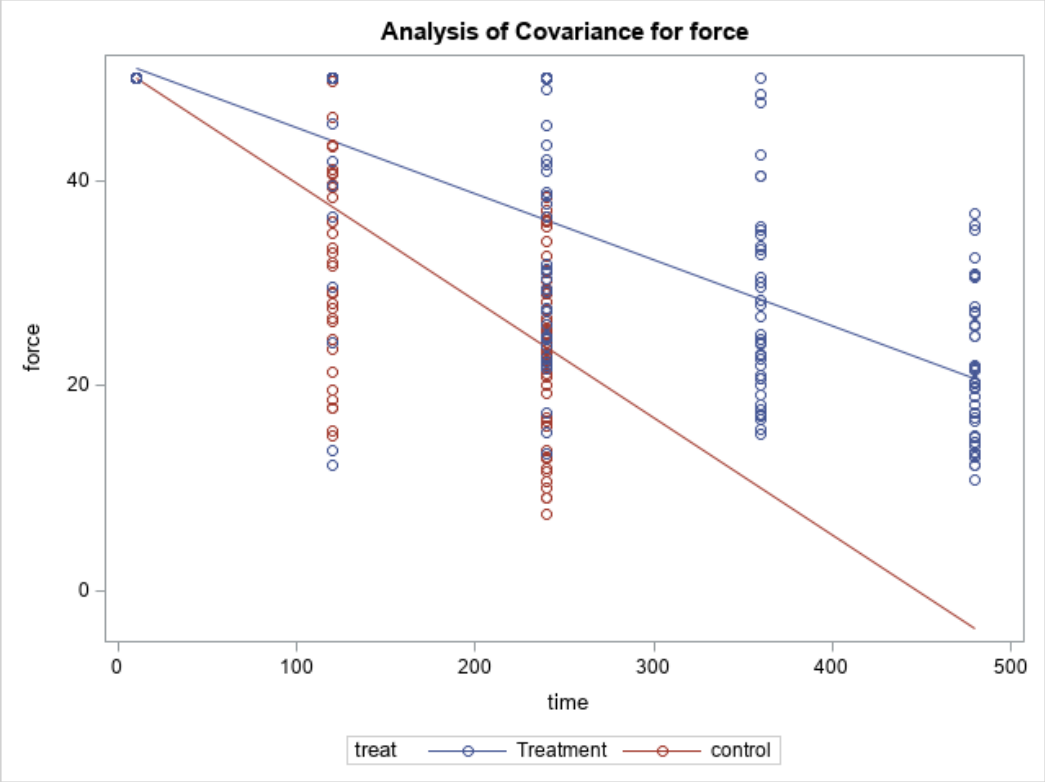


Figure 3.9 The slopes illustrate the recovery rate (N min^{-1}) between the control and treatment (PT1) groups (after t10 min). The measurements in the control group ceased after t= 240 minutes and the trendline extended until t480. The PT1 group MNT measurements ceased after t= 480 minutes. The control group showed a more rapid recovery rate compared to the treatment group (PT1) (P<0.0001) (control (n=6), treatment (n=5)).

v. FLIR and thermal imaging results

In thermography, a total of 264 (maximum heat=132, central heat=132) observations were recorded for both maximum and central heat readings at six different time points: 0, 4, 24, 72, 168 and 336 hours, in the control and PT1 groups. The target symbols on the thermographs below illustrate the location where the temperature of the wound surface area was measured by the FLIR camera and recorded for further analysis (Figures 3.10 and 3.11). Refer to appendix for mean temperatures in a table (Table 9.7).

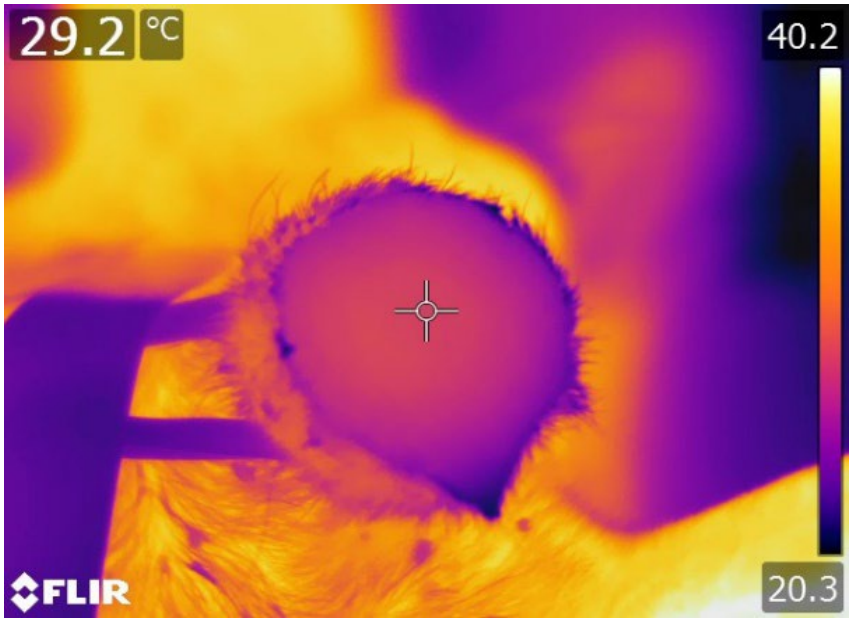


Figure 3.10 Thermography by selecting the central area of the wound in removed deer antler. The temperature in the top left corner equals the temperature at the target sign located in the centre of the screen. The scale on the right-hand side shows the minimum and maximum temperature detected in the image frame and the colour bar showing white or yellow as hot and blue as cold.

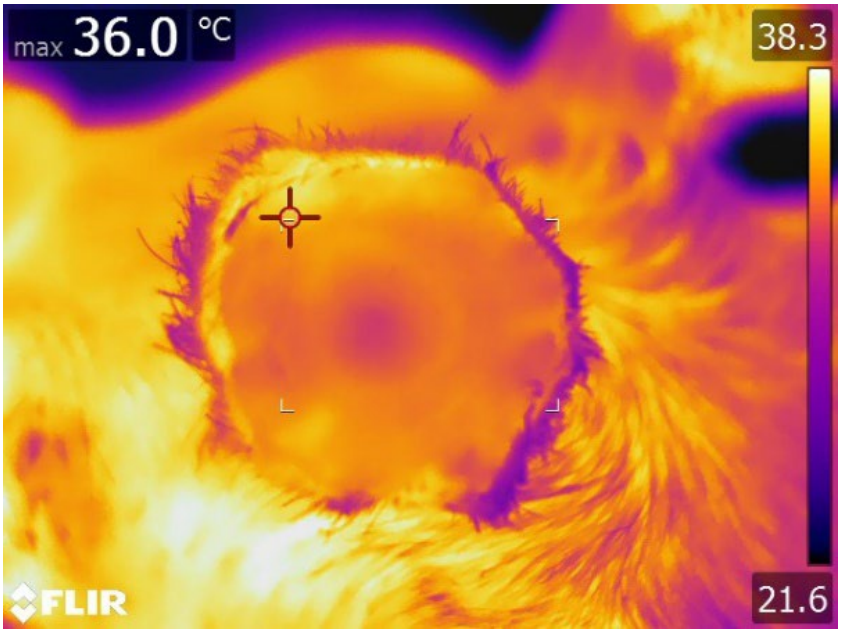


Figure 3.11 The thermograph shows the automatic setting on the FLIR camera for detection of maximum heat on the surface of the wound of the deer antler. The temperature in the top left corner equals the temperature at the target sign located at the hottest point on the wound. The scale on the right-hand side shows the minimum and maximum temperature detected in the image frame and the colour bar showing white or yellow as hot and blue as cold.

vi. Least-squares means of thermography

The least-squares means (±SEM) of central and maximum temperatures measured in the control and treatment (PT1) groups were calculated as follows; control central temperature = 34.11 ±0.29 °C, control maximum temperature = 37.26 ±0.29 °C, treatment (PT1) central temperature = 34.45 ±0.31 °C, and treatment (PT1) maximum temperature = 37.32 ±0.31 °C. Tables 3.4 and 3.5 depict the temperature (°C) recordings for central and maximum modes in the control and treatment groups at time points; 0, 4, 24, 72, 168 and 336 hours after velvet antler removal.

Table 3.4 The central heat detection least-squares means (±SEM) in control and treatment (PT1) groups in 6 different time points. No statistically significant difference was seen between the control and treatment groups (PT1).

Time-point (hr)	Control group		Treatment group (PT1)	
	LSmeans	**±SEM**	**LSmeans**	**±SEM**
0	31.79	0.82	34.18	0.89
4	33.06	0.59	33.23	0.65
24	34.77	0.39	34.64	0.42
72	33.87	0.30	34.66	0.33
168	35.30	0.45	33.61	0.49
336	35.87	0.41	36.38	0.45

Table 3.5 The maximum heat detection least-squares means (±SEM) in control and treatment (PT1) groups in 6 different time points. No statistically significant difference was seen between the control and treatment groups (PT1).

Time-point (hr)	Control group		Treatment group (PT1)	
	LSmeans	**±SEM**	**LSmeans**	**±SEM**
0	34.25	0.82	36.16	0.90
4	37.58	0.59	36.81	0.65
24	37.62	0.39	37.17	0.42
72	37.90	0.30	37.55	0.33
168	38.14	0.45	37.85	0.49
336	38.06	0.41	38.38	0.45

vii. The comparison of the least-squares means of thermography

The total least-squares means (combining central and maximum temperature) in the control group did not significantly differ from the treatment group (P=0.59). The overall least-squares means calculated from the central thermography compared to the maximum thermography showed a significant difference regardless of the group (P<0.0001). The least-squares means obtained from thermography in control and treatment groups in both central and maximum modes did not show any significant difference compared to each other (Table 3.6).

*Table 3.6 The comparison of the least-squares means (±SEM) in control and treatment (PT1) groups in two modes of thermography, central and maximum heat detection. * Statistically significant difference.*

Group	Mode	Group	Mode	Estimate	±SEM	*P*-value
control	**central**	**control**	**maximum**	-3.15	0.27	<.0001*
control	**central**	**treatment**	**central**	-0.34	0.43	0.43
control	**central**	**treatment**	**maximum**	-3.21	0.43	<.0001*
control	**maximum**	**treatment**	**central**	2.81	0.43	<.0001*
control	**maximum**	**treatment**	**maximum**	-0.06	0.43	0.89
treatment	**central**	**treatment**	**maximum**	-2.87	0.30	<.0001*

Figures 3.12 and 3.13 illustrate the least-squares means (±SEM) of central and maximum modes of thermography in two groups, control, and treatment (PT1), at six different time points.

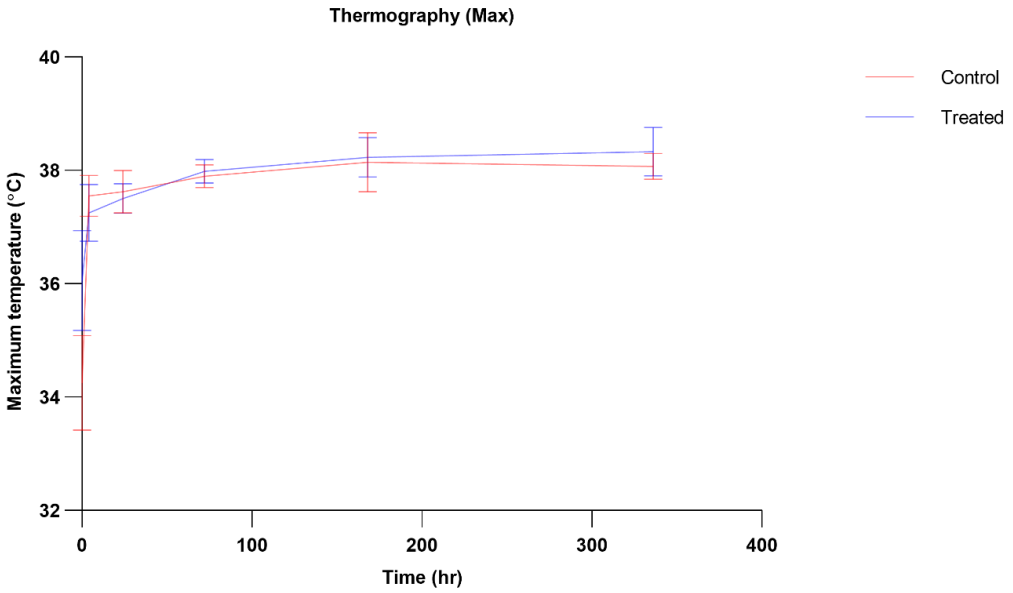


Figure 3.12 Means (±SEM) of maximum temperature (°C) obtained by FLIR camera in the control and treatment (PT1) groups from t0 to t336. No statistically significant difference was observed between the groups (control (n=6), treatment (n=5)).

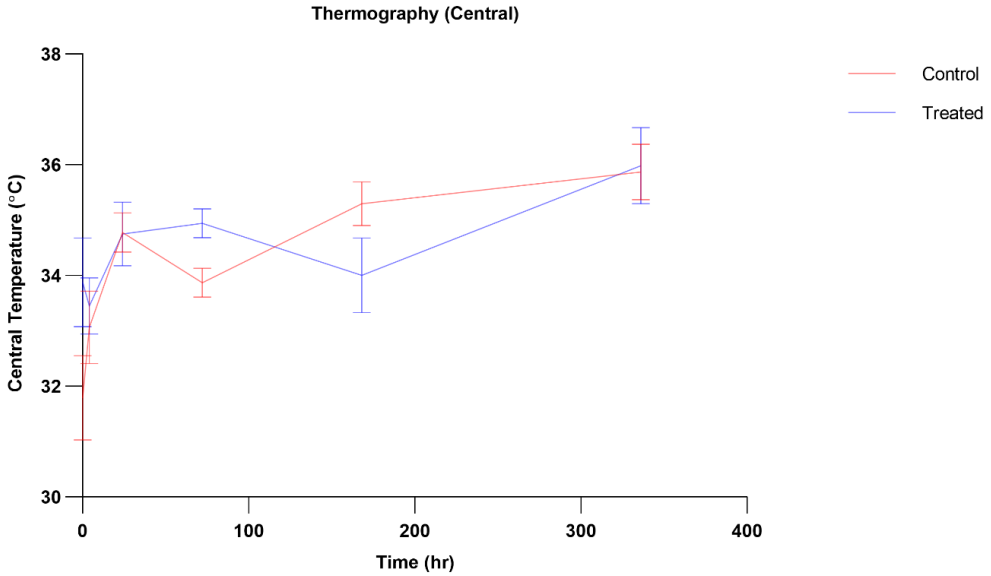


Figure 3.13 Means (±SEM) of central temperature (°C) obtained by FLIR camera in the control and treatment (PT1) groups from t0 to t336. No statistically significant difference was observed between the (control (n=6), treatment (n=5)).

3.4. Discussion

This pilot *in vivo* study showed that the application of a collagen composite treatment wafer prolonged analgesia compared to the control group after velvet antler removal in deer, allowing the null hypothesis to be rejected. Until this study no similar formulation has been prepared for use on deer after velvet antler removal and no other preparations have been designed to cover the wound and provide analgesia both regionally and postoperatively.

The results obtained from the in vitro study showed that pepsin extracted collagen composites were the best candidate for use in the in vivo study. Consequently, both modified and non-modified pepsin extracted collagen composites were included to create two different treatment groups. The assumption that modified and non-modified collagen treatment wafers would have different adherence on the wound was tested. In this study which was more aimed to be a pilot procedure, modified collagen wafers only had 50% success in adherence. This obliged us to experiment and design another set of modified collagen wafers that could adhere on the velvet antler wounds to be able to successfully release the local anaesthetics.

In the *in vivo* study, assessments at time points 6 and 8 hrs were not performed for the control group to reduce any discomfort and stress from repetitive algometry as they were already exhibiting obvious withdrawal behaviour at earlier time points.

For a better comparison of MNT between the groups, regression lines from the time point t10 (10 minutes after velvet antler removal) were created, and the slopes, as the representative of recovery rate from local anaesthesia, were compared between the groups. The results demonstrated that the treatment group with modified collagen wafers (PT1) had a significantly slower recovery rate from analgesia compared to the control ($P<0.0001$). The extended analgesia observed in the PT1 treatment group lasting up to almost 6-8 hours suggested that it was a good candidate for further research.

This study supported the null hypothesis and showed that collagen composite treatment wafers had no effect on wound temperature measured by thermal imaging compared to the control

group. Thermal imaging or thermography with a forward-looking infrared camera (FLIR) was successfully performed without causing any discomfort or additional trauma to the deer. The purpose of using this technique was solely to investigate the presence of prolonged or extensive inflammation at the wound site, which could potentially limit wound healing in animals. The results showed no significant difference between the treatment and control groups and led to a better understanding about the safety of the collagen wafers. In essence, the results of this part of the study suggested that the wafers, while prolonging analgesia, did not affect normal wound healing or cause inflammation. There are two further possibilities. One would be that wafers were thermally transparent when thermal imaging was performed, and the other possibility wound be that the traditional inflammatory response associate with wound healing is either absent in deer or of extremely short duration, such that it could not be captured in the selected time period. However, given that the sample size was small, it was decided that this should be investigated further, using a larger sample size.

During the pilot *in vivo* study, limitations were encountered, including failure in the adherence of some of the wafers to the antler wound surface, particularly in the treatment group, which included non-modified collagen composites. Since this was a pilot study, only a small number of groups and animals were used which limited the options for investigating more factors and possibilities.. The other limitation during the study was lack of staff that made it impossible to "blind" the MNT assessor as the same person was directly involved in preparing the formulations as well. Tonic immobility or freezing during the assessments prolonged the procedure time for some animals throughout the study. This made it difficult to have algometry measurements, consequently no readings were recorded for those periods.

3.5. Conclusion

The results demonstrated the capacity for a modified collagen composite using zinc oxide - PVP to provide extended analgesia without causing any additional visible inflammation, and the potential for improving red deer welfare. Additional studies are required to determine the optimum proportion of zinc oxide -PVP nanoparticles needed for modification and to compare their effect on the release of lidocaine and bupivacaine, and on wafer adhesion.

Chapter 4

Main study: In vitro study using Franz Diffusion Cells

Abstract

In this *in vitro* study, collagen was extracted using an acid/pepsin technique from lime-split leather biowaste. The resultant wafers comprised collagen modified with zinc oxide-PVP nanoparticles in different ratios (4:1, 10:1, and 20:1) and non-modified collagen wafers. The local anaesthetics bupivacaine hydrochloride and lidocaine hydrochloride were used in this study. There were four treatment groups (0NP, 5NP, 10NP and 25 NP) (NP stands for nanoparticles) and one control group (Control). Franz Diffusion Cells were used to investigate the release rate of bupivacaine and lidocaine.

Bupivacaine demonstrated a significantly slower release rate in 25NP compared to all other groups. All the treatment groups (0, 5, 10, 25 NP) released lidocaine significantly slower than the control group. There were significant differences between each treatment group, and 25NP showed the slowest release rate for lidocaine compared to the other groups.

This study showed that collagen composites could successfully provide sustained release of lidocaine and bupivacaine and have the potential for prolonging analgesia in animals such as deer.

4.1. Introduction

The objective of this study was to investigate the release rate of lidocaine and bupivacaine from collagen composite wafers designed for the main study, using Franz Diffusion Cells. There were also improvements in this study compared to the pilot study, including the change in medium whilst performing IVDRT and using a dialysis membrane in the Franz Cells. Furthermore, the replications were increased in each group (n=6) for a more reliable outcome.

The primary objective of this *in vitro* study was to select a lidocaine/bupivacaine collagen composite formulation for *in vivo* study based on the release rates of local anaesthetics from the formulation.

The null hypotheses for this study were as follows:

i) The addition of zinc-oxide PVP nanoparticles has no effect on lidocaine and bupivacaine release rates.

ii) The amount of zinc-oxide PVP nanoparticle proportional to collagen dry-weight has no effect on the release rate of lidocaine and bupivacaine.

4.2. Materials and methods

4.2.1. Collagen/local anaesthetic/zinc oxide-PVP composite preparation

Following the promising results obtained from the pilot *in vitro* and *in vitro* study, the pepsin extraction technique (Method 3, Chapter 2) was selected for the manufacture of treatment collagen wafers for the main study (see Figure 2.1 for methodology). Similarly, zinc oxide – PVP nanoparticles for incorporation into the treatment wafers were produced as per section 2.2.1.

i. Study design – manufactured collagen wafer treatment groups

Bulk freeze-dried collagen produced by method 3 was reconstituted using HCl 0.01 M (5mg/mL) and agitated (EOM5, Ratek Orbital Mixer, Ratek Instruments Pty Ltd, Victoria, Australia) for 2 hours. The reconstitutions occurred with the addition of local anaesthetics (lidocaine HCl and bupivacaine HCl) and with or without the addition of nanoparticles to produce a treatment wafer. The volume of hydrogels before freeze-drying was 1.2 ml and the final hydrogels were freeze-dried (FreeZone® Freeze Dryer 4.5 litre, Labconco™, USA) in plastic cell culture moulds (JET Biofil®, Tissue Culture Plate, 6 Well, Australia) for 48 hours at -80°C, with the end-product consisting of a white circular wafer measuring 17mm in diameter and 5mm in depth. The treatment wafers were removed from the mould with stainless steel forceps and stored at room temperature in re-sealable zipper storage bags with silica beads to maintain product integrity for future trials during the whole experiment year (unknown actual shelf-life at this stage) (Figure 4.2).

See Table 4.1, which contains details about the *in vitro* trial of the main study. Also, see Figure 4.1, which schematically illustrate the steps of treatment group preparation.

Table 4.1 Components of collagen composites in the main study for in vitro trial.

Components of the collagen composites for the main study						
	Group	***Collagen***	***ZnO-PVP***	***Bupivacaine***	***Lidocaine***	***Total***
In vitro	T1	10 mg	0 mg	17 mg	17 mg	44 mg
	T2	10 mg	0.5 mg	17 mg	17 mg	44.5 mg
	T3	10 mg	1 mg	17 mg	17 mg	45 mg
	T4	10 mg	2.5 mg	17 mg	17 mg	46.5 mg

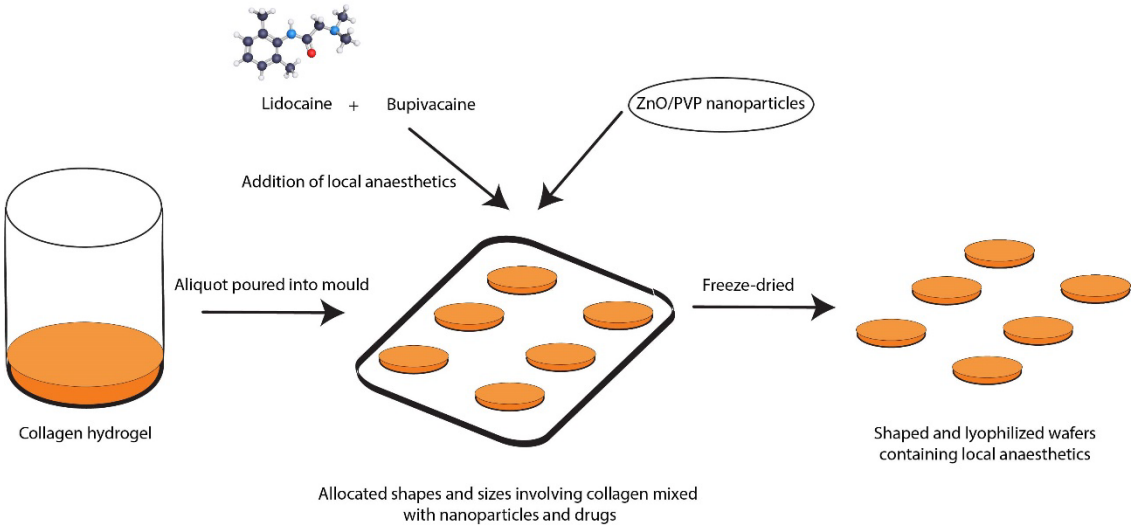


Figure 4.1 The collagen/drug/nanoparticle wafer preparation process for modified collagen groups. The non-modified collagen groups did not contain nanoparticles.

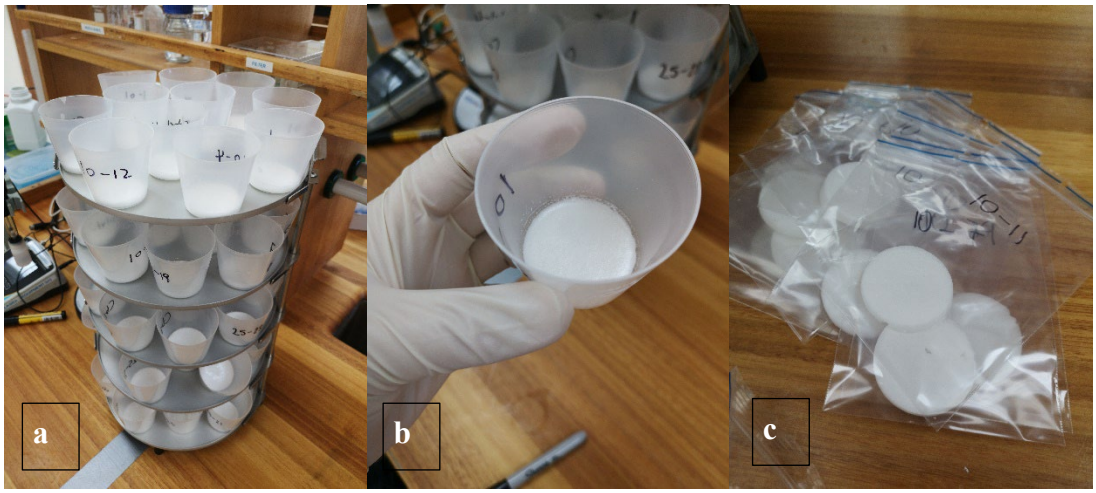


Figure 4.2 Freeze-dried collagen wafers in the assigned moulds (a and b) and storage in re-sealable zipper storage bags (c).

4.2.2. In vitro study

Th*is* study compared the effects of different concentrations of ZnO-PVP nanoparticles in collagen composite wafers on the drug release rate compared to an unmodified collagen control group. Collagen and ZnO-PVP nanoparticles were manufactured using the methods outlined in section 4.2.1 (refer to chapter 2, section 2.2). In powder form, dried bupivacaine hydrochloride and lidocaine hydrochloride were sourced from Hunan Russel Chemicals Technology Co. Ltd, China and were used in all the study groups.

The control group consisted of a solution containing 20 mg of bupivacaine HCl and 20 mg of lidocaine HCl in 1 ml of milli-Q water.

The four treatment groups (T1-T4) consisted of three groups with ZnO-PVP and 1 group without ZnO-PVP (Table 4.1). All four treatment groups contained 10 mg of freeze-dried collagen and 17 mg of each lidocaine HCl and bupivacaine HCl.

The control and treatment groups were assessed using a Franz Diffusion Cell, and samples taken at set time-points (replicated six times, n=6), were assayed using High-Performance Liquid Chromatography (HPLC) to measure the amount of drugs diffused through the membrane.

i. IVDRT methodology

Franz Diffusion Cells (PermeGear, Hellertown, PA, USA) were used for IVDRT as described in Chapter 2. The chambers were divided by placing a dialysis membrane (MWCO 14,000, average width 43 mm, Dialysis Tubing Cellulose Membrane, Sigma Aldrich, New Zealand) between the donor and receptor chambers (see Figure 2.5). Each receptor chamber of the diffusion cells was carefully filled to the 8 ml mark on the sampling port with phosphate-buffered saline (PBS) water instead of only Milli-Q water (which was used in Chapter 2) using a 20G needle attached to a 60 ml syringe.

Phosphate-Buffered Saline (PBS) was manufactured using PBS tablets (Gibco®, Thermo Fisher Scientific, USA) dissolved in Milli-Q water until the solution reached a pH of 7.45. The Franz Cells

were then placed into individual holders of the V9-CB Stirrer (PermeGear, Hellertown, PA, USA) (see Figure 2.6). The Franz Cell jackets were connected to a water bath, and the temperature was set to 38°C to simulate deer body temperature and allowed to run for 20 minutes to ensure that the temperature of the PBS solution within the cell had equilibrated. After temperature equilibration, four drops of deer plasma (weighing ~ 247 mg) was added to each membrane using a disposable Pasteur pipette. Blank deer plasma was collected from healthy 1-year-old deer from Massey University Deer Unit. The plasma was added to both prime the membranes and simulate wound conditions. After priming the membranes, the treatment groups were added using stainless-steel forceps to make sure they were soaked with plasma. The control groups were placed dropwise (1 ml) using a 1 ml syringe. Once the test wafers were added to the cells, it was considered time zero. Samples were drawn from the sampling port at time zero (t0), every 15 minutes for the first hour (until t60 min), followed by t120 min and then every 2 hours until 12 hours had elapsed. 0.5 ml aliquots were obtained using a 23G needle attached to a 2.5 ml syringe. The samples were then placed into individual 2 ml safe lock tubes (Eppendorf®, Hamburg, Germany). After each sampling, the receptor chamber was filled up to 8 ml with PBS solution. At the end of the experiment, the samples were placed in a refrigerator at 4°C until further analysis. IVDRT was repeated six times for all four treatment groups and one control group (n=6).

ii. High-Performance Liquid Chromatography (HPLC)

- *HPLC instrumentation*

The HPLC system consisted of LC-20AD pumps (Shimadzu, Japan), an SIL-20AC HT auto-injector (Shimadzu, Japan), a diode array (DA) detector SPD-M20A (Shimadzu, Japan), a CTO-20A column oven (Shimadzu, Japan) and a DGU-20A3 degasser (Shimadzu, Japan). All chromatograms were analysed by LabSolutions software (Shimadzu, Japan). The analytical column used was Phenomenex C18A (Luna® 5 µm C18 100 Å, LC column 150 x 4.6 mm internal diameter, 5µm particle size).

- *Mobile Phase*

The mobile phase consisted of a 75% buffer solution containing potassium phosphate 30 mM and 0.016% triethylamine (adjusted to pH 4.9 with orthophosphoric acid and NaOH 1N), 25% acetonitrile (Optima™, LC-MS Grade, Fisher Chemical™, Fischer Scientific®, Belgium) as the organic solvent. Potassium phosphate solution was prepared using milli-Q water (Milli-Q PFplus system, Millipore Cooperation, USA). The flow rate was set at 1 ml/min, and the separation was achieved under isocratic conditions at 40°C. The DA (Diode Array) detector was set at 210 nm wavelength throughout the experimental procedure.

- *Accuracy and precision*

A stock solution containing 1 mg/ml of each drug was prepared, and serial dilutions were made by firstly adding 50 µl of the stock solution to 900 µl of milli-Q water using 200 µl and 1000 µl, single-channel microlitre pipettes (Eppendorf Research®, Plus IVD fix, Hamburg, Germany) to make a final drug concentration of 100 µg/ml. 100 µl of the diluted solution was mixed with 900 µl milli-Q water to form a 10 µg/ml. Serial dilutions were made using the 10 µg/ml solution to create 7 different concentrations (1000, 500, 250, 125, 62.5, 31.25, 16.12 ng/mL). The linearity of the measurements was checked by running duplicate runs of each of the selected drug concentrations three times in the mobile phase, every day for six consecutive days. The data

were analysed by linear regression in Microsoft Excel (version 2019). The precision test confirms the sensitivity of the proposed HPLC procedure and increases the understanding of the lowest reliable drug concentration that can be detected in future tests. The values of less than 10 RSD% for intra-day and less than 15 RSD% was determined, and for the LLD, the RSD% of less than 20 was determined. LLD means the smallest amount of an analyte that can reliably be detected (it has to be distinguished from a blank sample). RSD means Relative Standard Deviation and is calculated in percentage. It is calculated by multiplying Standard Deviation (SD) by 100 and dividing by average (also known as coefficient variance).

- *The lowest limit of detection and Lowest limit of quantification*

HPLC was selected due to the use of PBS as a medium in IVDRT and its ability to detect very low concentrations of the selected drugs. The purpose of this testing procedure was to check the accuracy of the HPLC measurement and its sensitivity to detecting very low concentrations of lidocaine and bupivacaine obtained from the receptor chambers. Furthermore, it was important to ascertain that the pipetting process produced repeatable and reliable results. The lower limit of detection (LLD) in the mobile phase was measured by running a series of low concentrations of bupivacaine HCL and Lidocaine HCL and mixed bupivacaine and lidocaine standard (1000, 500, 250, 125, 62.5 and 31.25 and 16.12 ng/mL). LLD was performed to be able to define the lowest reliable concentration. The lower limit of quantification (LLQ) was confirmed by running a series of low concentrations of bupivacaine HCL and Lidocaine HCL and mixed bupivacaine and lidocaine standard (1000, 500, 250, 125, 62.5 ng/mL). The LLD was set at the lower concentration showing a signal to noise ratio of 3:1, and LLQ was set at the lower concentration showing a signal to noise ratio of 10:1. The signal to noise ratio (S/N) is calculated by peak height divided by baseline noise.

- *Sample preparation for HPLC*

0.5-ml aliquots of Franz Cell samples were centrifuged at 14,000 RPM for 10 min (Thermo Fischer Scientific™, Heraeus™, Megafuge™ 8 small Benchtop, UK), then 180 µl of the supernatant was transferred to HPLC autosampler vials (Thermo Fisher Scientific™, MA, USA) and loaded into the autosampler. The linearity formula obtained from the intra-day and inter-day analyses were used to calculate the concentration in nanograms.

iii. Statistical analysis

The data was collected and recorded on Microsoft Excel (version 365), then statistical analyses were performed using a SAS software (version 9.4, SAS Institute Inc., Cary, NC, USA), and graphs were created, by GraphPad Prism (version 8.3.0, GraphPad Software Inc., San Diego, CA, USA). The dependent variable, drug concentration (ng/ml), for the *in vitro* study, was analysed in SAS with the MIXED procedure using a linear mixed model for repeated measures. A Kolmogorov-Smirnov test indicated that the dependent variable followed a normal distribution, and consequently, data was analysed in the nominal scale without numerical transformation. For the *in vitro* drug release assessment refer to chapter 2 methodology.

4.3. Results

4.3.1. Collagen preparation and freeze-dryer differences

The freeze-dryer used in the main study showed more spongiform, porous, and consistent collagen wafers (FreeZone® Freeze Dryer 4.5 litre, Labconco™, USA) compared to the pilot study.

4.3.2. In vitro study

i. HPLC method validation

The run time for HPLC analysis for each sample was 15 minutes, and the results from the chromatograms showed the retention time (RT) for lidocaine was approximately 5 minutes and for bupivacaine, approximately 11.5 minutes (see Figure 4.3). The absence of any other peak at the same time reduced the possibility of any interference due to contamination. The standard curves for lidocaine and bupivacaine were linear from 3.125 to 50 ng/ml, with an $R^2 > 0.99$ correlation coefficient. For bupivacaine, the inter-day variation for this method ranged from 2.27 to 5.18 percent, and the and intra-day variation ranged from 1.57 to 12.93 percent. For lidocaine, the inter-day variation for this method ranged from 0.41 to 2.56 percent, and the intra-day variation ranged from 1.56 to 7.48 percent. For the Lower Limit of Quantification (LLQ), the variation from the mean of values was less than RSD<10% for intra-day and RSD<15% for inter-day, which indicated from the dataset that LLQ for this method was 62.5 ng/ml. For the Lower Limit of Detection (LLD), the RSD<20% indicated from the dataset that the LLD for this method was 16.12 ng/ml (see Table 4.2). See table for raw data regarding area readings directly obtained from HPLC machine (Table 9.22-9.25).

Table 4.2 Inter-day and intra-day variation of HPLC method for analysis of bupivacaine (a) and lidocaine (b) in milli-Q water. Solution of known concentrations was analysed in triplicate on three different testing days. The mean and percent relative standard deviation (RSD) was calculated from the actual concentrations measured for both inter-day and intra-day.

4.2a Bupivacaine: HPLC

Concentration Spiked (ng/ml)	Inter-day Concentration Measured (ng/mg)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%
1000	976.52	1051.65	0.27	2.86
500	499.89	485.77	0.54	5.06
250	230.32	213.59	1.57	2.71
125	111.62	105.96	1.60	9.59
62.5	51.95	49.38	5.19	17.79

4.2b Lidocaine: HPLC

Concentration Spiked (ng/ml)	Inter-day Concentration Measured (ng/mg)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%
1000	891.12	870.80	0.42	1.56
500	474.16	446.96	2.56	3.55
250	247.50	231.83	1.28	6.34
125	82.28	121.51	0.61	6.39
62.5	38.94	54.69	2.37	7.49

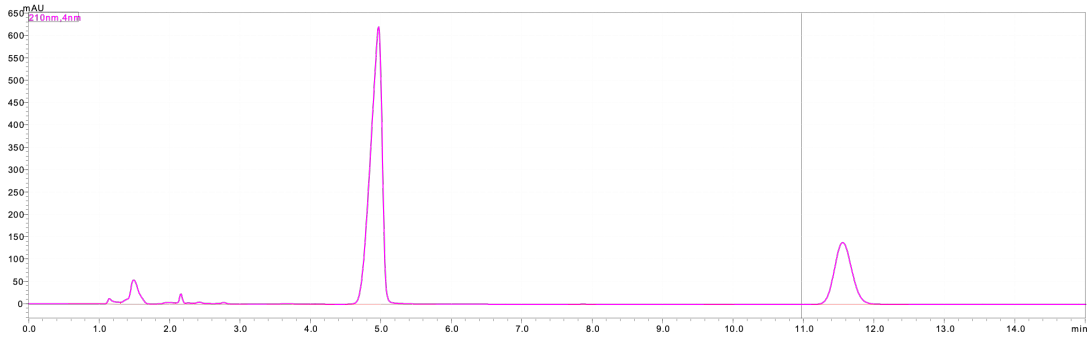


Figure 4.3 Chromatograph showing lidocaine and bupivacaine peaks after approximately 5 and 11.5 minutes after applying collagen wafers in the IVDRT experiment.

ii. IVDRT for lidocaine and bupivacaine

In total, 600 results were obtained for five groups across ten sampling timepoints excluding t0 hrs, with six replications for each drug. Only the values for groups 25NP, 10NP, 5NP and 0NP were normally distributed. Figures 4.4 and 4.5 show graphical drug release patterns for the 5 study groups. The error bars were removed from the diagrams for better clarity and better comparison between the groups. As observed in Figures 4.4 and 4.5, the aqueous control group released significantly more lidocaine and bupivacaine than the other groups. A sudden release was observed in the control group after 360 minutes for bupivacaine and after 240 minutes for lidocaine. Before this burst, there was minimal drug release in the control group compared to the treatment groups in both lidocaine and bupivacaine. Groups 0NP, 5NP, 10NP and 25NP showed a gradually increasing release until 360 minutes for both drugs, after which the release rates plateaued. The drug release pattern appeared inversely proportional to the amount of nanoparticles in the wafers (Figures 4.4 and 4.5, Tables 9.5 and 9.26).

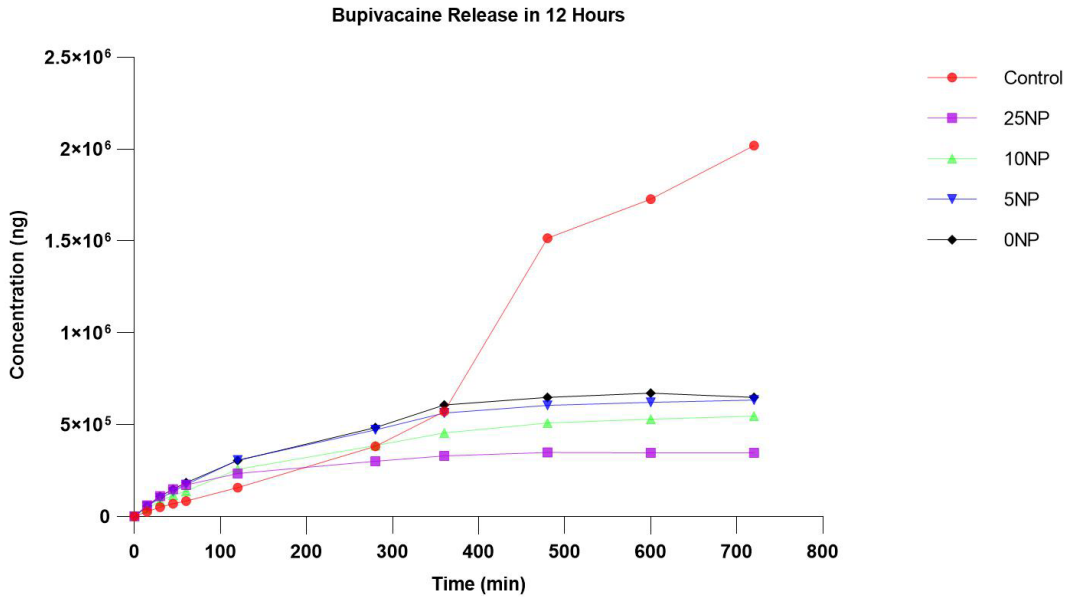


Figure 4.4 Bupivacaine concentrations (ng/ml) over time (min) for each of the four treatment groups and one control group in the in vitro study. None of the treatment groups showed statistically significant differences compared to each other (n=6).

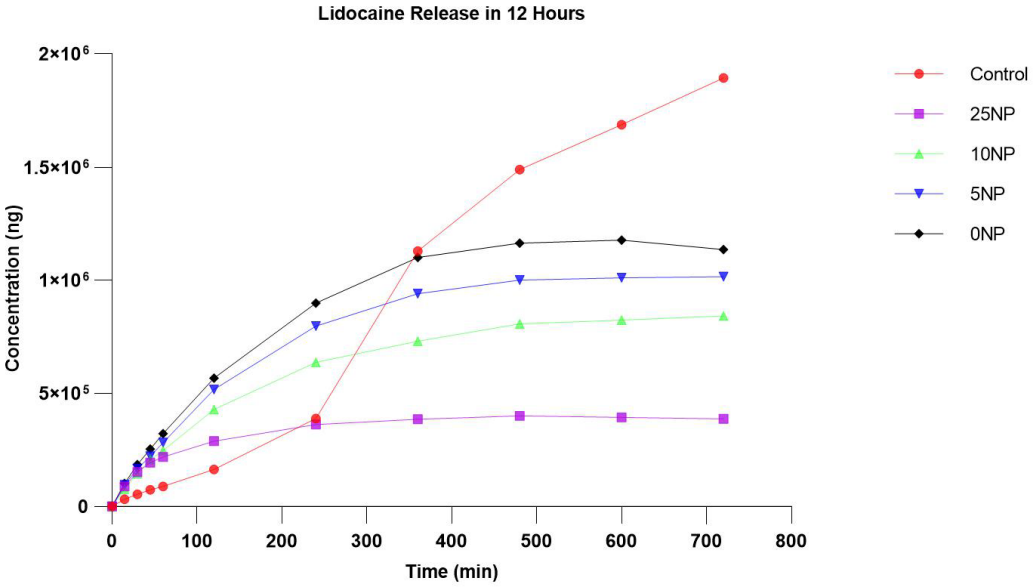


Figure 4.5 Lidocaine concentrations (ng/ml) versus time (min) for each of the four treatment groups and one control group in the in vitro study. The control group showed significantly higher concentrations compared to treatment groups 0NP (P<0001), 5NP (P<0.001). 25NP showed significantly lower concentrations compared to 0NP (P<0.0001), 5NP (P<0.001) and 10NP (P<0.0001). 0NP showed significantly higher concentrations compared to 10NP (P<0.05) (n=6).

iii. Drug release rates by Higuchi model

ANOVA showed a significant difference between the slopes of study groups for bupivacaine and lidocaine (P<0.0001 and P<0.0001). Table 4.3 shows the calculated K_H values (±SEM) for bupivacaine and lidocaine and the statistical differences between them. Figures 4.6 and 4.7 illustrate the regression lines representing the release rates for bupivacaine and lidocaine. As observed in Figure 4.6, the control group showed the fastest release rate ((ng/ml) √min^{-1}) compared to the other groups for bupivacaine, and treatment group 25NP had the slowest release rate ((ng/ml) √min^{-1}) compared to the other groups. Similarly, for lidocaine, as observed in Figure 4.7, the control group showed the fastest release rate ((ng/ml) √min^{-1}) compared to the treatment groups, and group 25NP showed the slowest release rate ((ng/ml) √min^{-1}) compared to the other groups (see Table 4.3). The analysis of the results only showed a significant difference in drug release rate ((ng/ml) √min^{-1}) of bupivacaine between 25NP and the rest of the treatment groups; however, 5NP and 10NP showed no significant difference with 0NP for bupivacaine. In contrast, all the treatment groups showed significant differences in drug release rate ((ng/ml) √min^{-1}) between each other (see Table 4.3). The bupivacaine release rate ((ng/ml) √min^{-1}) for treatment group 25NP was almost two times slower than that of other treatment groups and 4 times slower than the control group. In comparison, the release rate ((ng/ml) √min^{-1}) for lidocaine was 4 times slower than the other treatment group and the control group (see Table 4.3).

Table 4.3 Drug release rates (±SEM) (ng/ml/√min) of bupivacaine and lidocaine in treatment groups and control group in the main study (n=6). The alphabetical icons denote the statistically significant difference between groups in columns for each local anaesthetic. For bupivacaine, the control group had a significantly higher K_H value compared to 0NP (P<0.0001), 10NP (P<0.0001), and 25NP (P<0.0001). Furthermore, 25NP had a significantly lower K_H value compared to 0NP (P<0.0001), 5NP (P<0.0001) and 10NP (P<0.01). For lidocaine, the control group had a significantly higher K_H value compared to 0NP (P<0.01), 5NP (P<0.0001), 10NP (P<0.05), and 25NP (P<0.0001). Furthermore, 25NP showed a lower K_H value compared to treatment groups 0NP (P<0.0001), 5NP (P<0.0001), and 10NP (P<0.0001). 0NP had a significantly higher K_H value compared to 5NP (P<0.05) and 10NP (P<0.01). 10NP had a significantly lower K_H value compared to 5NP (P<0.01) (n=6).

IVDRT study for bupivacaine and lidocaine with Higuchi model

	groups				
	Control	**0NP**	**5NP**	**10NP**	**25NP**
Bupivacaine ***((ng/ml) vmin^{-1}) ±SEM***	44695.0 ±2587.9^{bc}	29100.1 ±2471.3^{bd}	27511.0 ±2471.3^{b}	23531.68 ±2471.34^{bd}	12298.93 ±2471.34^{a}
Lidocaine ***((ng/ml) vmin^{-1}) ±SEM***	42281.4 ±2105.7^{bc}	51602.8 ±2002.8^{bdfh}	44626.8 ±2002.8^{bdfg}	35623.8 ±2002.8^{bde}	12745.5 ±2002.8^{a}

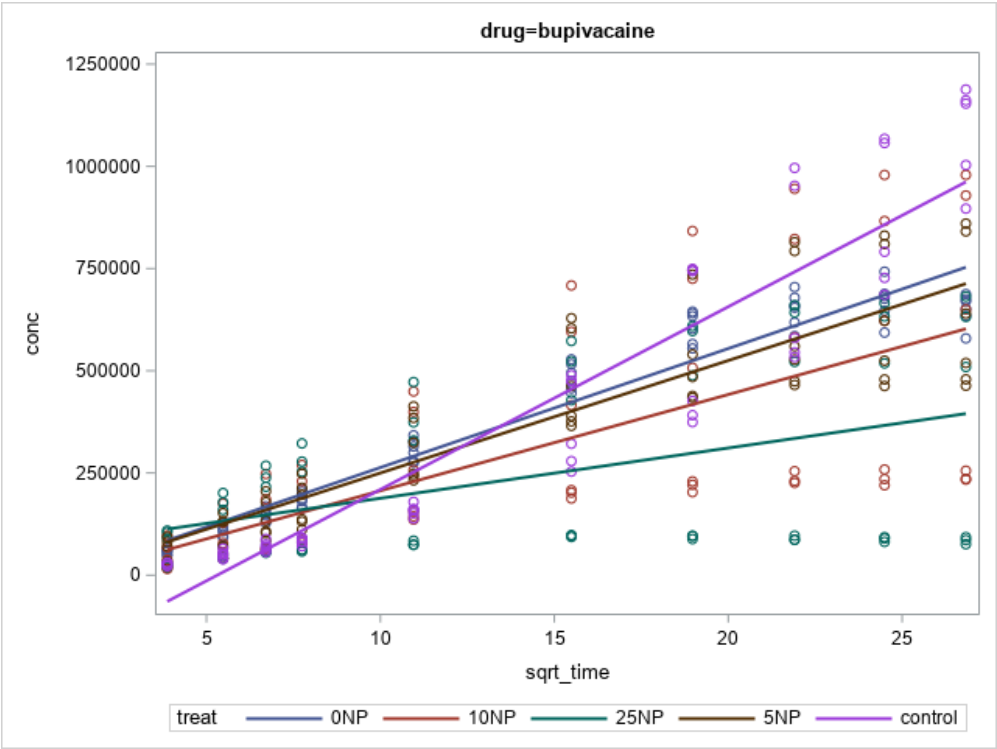


Figure 4.6 Regression line of the concentration (Q) square root of time ($\sqrt{t}$) curve for IVDRT for bupivacaine in 4 treatment groups (0NP, 5NP, 10NP, 25NP) and control group starting from t15 min until time t720 min (12 hours) using Higuchi model. For bupivacaine, the control group had a significantly higher K_H value compared to 0NP (P<0.0001), 10NP (P<0.0001), and 25NP (P<0.0001). Furthermore, 25NP had a significantly lower K_H value compared to 0NP (P<0.0001), 5NP (P<0.0001) and 10NP (P<0.01). (n=6).

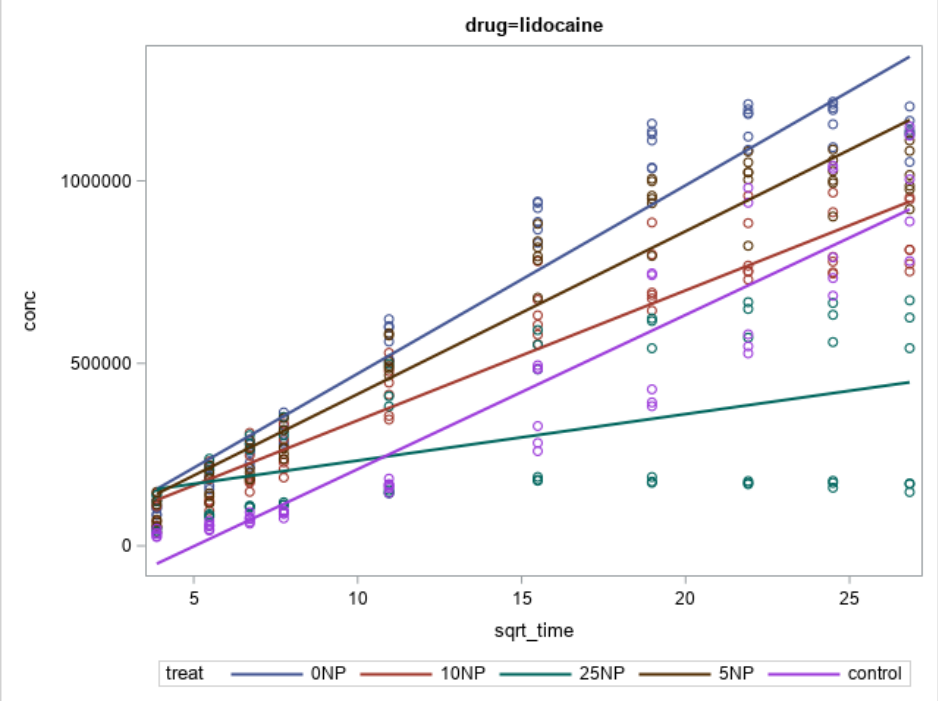


Figure 4.7 Regression line of the concentration (Q) square root of time ($\sqrt{t}$) curve for IVDRT for lidocaine in 4 treatment groups (0NP, 5NP, 10NP, 25NP) and control group starting from t15 min until time t720 min (12 hours) using Higuchi model. For lidocaine, the control group had a significantly higher K_H value compared to 0NP (P<0.01), 5NP (P<0.0001), 10NP (P<0.05), and 25NP (P<0.0001). Furthermore, 25NP showed a higher K_H value compared to treatment groups 0NP (P<0.0001), 5NP (P<0.0001), and 10NP (P<0.0001). 0NP had a significantly higher K_H value compared to 5NP (P<0.05) and 10NP (P<0.01). 10NP had a significantly lower K_H value compared to 5NP (P<0.01) (n=6).

4.4. Discussion

The *in vitro* study using Franz Diffusion Cells confirmed a sustained release of lidocaine and bupivacaine from modified collagen wafers, making them a suitable candidate for investigation in red deer after velvet antler removal in the following *in vivo* study.

The collagen hydrogels produced in the pilot and the current studies were frozen at -20°C for 8 hours prior to transferring to a freeze-dryer for lyophilization. Two different freeze-dryers, EZ Dry (FTS Systems, New York, USA) and (FreeZone® Freeze Dryer 4.5 litre, Labconco™, USA), were used to create wafers for this thesis. The former was used in the pilot study, and the latter was used in the main study. During the first freezing cycle (-20°C), the forming water crystals expand, which causes the collagen matrix fibres to retract and form pores and tracts within the structure (Doillon *et al.*, 1986). The more rapid the freezing process occurs, the more rapidly ice crystals grow, which in itself becomes self-limiting, and prevents the water crystals from growing in size; therefore, smaller crystals form (Doillon *et al.*, 1986). In contrast, larger water crystals form when the freezing process is slow. Smaller pore sizes and channel formation occurs in rapid freezing, and large pore sizes with missing channels occur when the freezing process is slow (Doillon *et al.*, 1986). During freeze-drying, the ice crystals sublimate by the negative pressure created within a sample chamber of the freeze-drying machine; thus, it leaves a porous structure (Davidenko *et al.*, 2012).

Uniformity and an isotropic porous structure occur when the collagen hydrogel samples have the same conditions in freezing (O'Brien *et al.*, 2004). We observed that the wafers had a more uniform, spongy texture when produced with FreeZone® Freeze Dryer 4.5 litre (Labconco™, USA). The lack of sponginess observed in the collagen composites created using the older machine could be due to insufficient negative pressure, therefore causing the structure to absorb moisture and collapse at the time of freezing, leaving sheet-like structures.

The *in vitro* study rejected the null hypothesis that the addition of zinc-oxide PVP nanoparticles has no effect on the drug release rate. The trend of release between the groups was significantly different. Furthermore, the *in vitro* study rejected the null hypothesis that the amount of zinc-oxide PVP nanoparticle proportional to collagen dry-weight has no effect on the lidocaine and bupivacaine release rates. Phosphate-Buffered Solution (PBS) was used in the IVDRT study to simulate a medium as close as possible to the normal pH of extracellular fluid (ECF).

Due to precipitate formation using PBS as a medium, High-Performance Liquid Chromatography (HPLC) was used in favour of LCMS to analyse the dynamicity of both lidocaine and bupivacaine and the rate of their passage through the collagen matrix and into the receptor chamber. Another improvement in methodology was the use of a dialysis membrane as opposed to a Strat-M® transdermal simulation membrane. Strat-M® is formulated to simulate intact skin in order to assess dermal applications; however, in the case of a wound, the dermal layer is no longer intact. Consequently, dialysis membranes were incorporated to mimic open wound conditions.

The control groups in this study contained lidocaine and bupivacaine in solution; however, in the pilot *in vitro* study (Chapter 2), lidocaine and bupivacaine in powder form were used. In the pilot study, the order of adding the ingredients, local anaesthetics and ZnO-PVP, and the comparisons of three different collagen extraction techniques were investigated. However, in this study, the aim was to compare the rate of drug release from collagen composites modified using four different concentrations of ZnO-PVP: 0%, 5%, 10%, and 25%. The results showed that with an increasing percentage of ZnO-PVP, a reduced rate of release of lidocaine and bupivacaine occurred. In addition, disregarding the group effect, the total amount of drug released for lidocaine was significantly greater compared to bupivacaine ($P<0.0001$). A difference in the pattern of drug release was observed between lidocaine and bupivacaine, with lidocaine showing a sudden and rapid release after 4 hours and bupivacaine showing a similar rapid release after 6 hours only in the aqueous controls. A more consistent drug release pattern (less variance in

standard deviation) was observed for bupivacaine compared to lidocaine among all groups, making bupivacaine release in this study more predictable than lidocaine. This indicated that chemical structures and lipophilicity might alter drugs' passage through the porous structure of the collagen composites (Velioglu *et al.*, 2020, Hoare and Kohane, 2008, Santamaria *et al.*, 2017).

The Higuchi model has been considered the gold standard model for more than fifty years to analyse drug release from permeable matrices (Paul, 2011, Siepmann and Peppas, 2011). Table 4.3 demonstrates the least-squares means (±SEM) of slopes (constant K_H) for lidocaine and bupivacaine, which represents the release rate ((ng/ml)/√min). In the Higuchi model, group 25NP, which represents the treatment group with modified collagen (25% ZnO-PVP), showed the slowest release rate for lidocaine and bupivacaine, and the control group had the fastest release rate. Furthermore, it was observed that as increasing amounts of nanoparticles were used to modify collagen matrices, the more capable they were of slowly releasing the drugs. This could be because of the entrapment of more drugs in between the collagen fibres, as nanoparticles physically modify the structures.

Some variations noticed in this chapter compared to the pilot in vitro study might have been due to improved methods used in the second experiment. The pilot study wafers may have been produced by non-homogenous mixing (Mauri *et al.*, 2018, Faudzi and Hamzah, 2020). Parts which have a higher concentration of nanoparticles physically entrap the drugs, and in the rest of the matrix where there are fewer or no nanoparticles, drugs move freely and are released (Mauri *et al.*, 2018). Although it is known that the higher the stability of a collagen composite structure, the more sustained the drug release, drug delivery depends on other factors such the method used to load the drug on the hydrogel matrix in the presence of nanoparticles. The stability of the structure of the vehicle is important when it is exposed to the body fluid and that interaction plays the main role in how the drugs are released at site (Mauri *et al.*, 2018). It is known that the movement of the nanoparticles through the hydrogel can be influenced by

electric charges (Mauri *et al.*, 2018). Therefore, the application of an electric or magnetic field during preparation could be an option to make the dispersion of the particles more even.

The results obtained from this study showed that modification of the collagen matrix could slow the release of both lidocaine and bupivacaine; however, for better consistency, future studies are required to investigate various methods to evenly spread nanoparticles throughout the hydrogel matrix to produce a more consistent drug delivery system.

Various parameters such as the differences in duration of freezing, the rate of freezing, and the temperature of freezing could also be investigated to optimise the best method to produce more uniform collagen wafers with optimal pore size (see Figure 9.1 and 9.2). To assure an optimal wafer structure, monitoring the micro-structure of the collagen matrix would be of great value. Various techniques such as scanning electron microscopy (SEM), confocal microscopy and light microscopy can be helpful in procedures such as pore size measurements to find out which pore size is the optimum for sustained release of lidocaine and bupivacaine.

Research is required to investigate ways to sterilize collagen wafers and into their antimicrobial effects, particularly when collagen is modified with ZnO-PVP. It would be of great value to study the effect of other nanoparticles, such as silver (Sondi and Salopek-Sondi, 2004), which have antimicrobial effects similar to zinc oxide (Rajendra *et al.*, 2010).

4.5. Conclusion

This study showed that collagen composite wafers containing ZnO-PVP nanoparticles slowed the release of lidocaine and bupivacaine, with 25% nanoparticles showing the most sustained release.

Chapter 5

Main study: In vivo study to investigate the efficacy of collagen composite wafers for postoperative analgesia, and assessment of wound healing and antler re-growth in red deer (Cervus elaphus)

Abstract

For this study, forty 1-year-old (yearling) male red deer were used. All stags received 4% articaine hydrochloride subcutaneous ring blocks prior to velvet antler removal. Mechanical nociceptive thresholds (MNT) were measured at four sites around each antler using a Wagner® handheld algometer at 6 time points after velvet antler removal.

Digital photographs were taken of the antler wounds, and assessment of wound surface area (mm^2) was performed using ImageJ software. Three collagen wafer treatment groups (T1, T2, and T3), containing different amounts of zinc oxide - PVP nanoparticles (25%, 5%, and 0%) in addition to a fixed amount of lidocaine and bupivacaine, were compared to the control group (no wafers).

The least-squares means (±SEM) of MNT in control, T1, T2, and T3 were 26.93±1.56, 40.83±1.37, 43.21±1.37, 41.39±1.37 N, respectively. A significantly slower recovery rate from local anaesthesia was observed in treatment groups compared to the control ($P<0.0001$ for each treatment group). Wound healing measurements from T2 showed a comparable healing rate to that of the control group. T1 and T3 significantly reduced the healing rate in the first 30 days. All treatment groups showed a significantly slower re-growth rate than control ($P<0.0001$). Modified collagen wafers with zinc oxide-PVP nanoparticles in concentrations of 5 and 25 percent showed prolonged local anaesthesia in deer for up to 10 hours after surgical removal of antlers. Antler wounds from all groups were visibly healed by two months; however, antler re-growth was significantly reduced in the T1 and T2 compared to the other groups. This novel method shows promise with regard to postoperative pain management in deer.

5.1. Introduction

The objective of this study was to determine the analgesic efficacy of collagen composite wafers containing lidocaine and bupivacaine using MNT and the effect of such treatments on wound healing and antler re-growth.

The pharmacokinetics of lidocaine (Bagonluri *et al.*, 2005) and articaine hydrochloride (Venkatachalam *et al.*, 2018) have been reported in deer after applying a ring block around the velvet antler prior to surgical removal. However, there is no drug or local anaesthetic formulation licensed for use in deer as a means of providing postoperative pain relief, and there is no literature available on the pharmacokinetics or efficacy of any such pain management practice. This research explored a knowledge gap in animal welfare within the deer industry and aimed to investigate a novel method for analgesia after the surgical removal of velvet antlers in red deer. The research aimed to achieve analgesia for an extended period and enhance the wound healing process in a practical and affordable way.

The primary objective of this study was to demonstrate the duration of analgesia provided by the topical application of a novel collagen/local anaesthetic formulation on the wound after velvet antler removal in deer. The secondary objectives of this study were to evaluate the effects of this formulation on wound healing and antler re-growth.

<u>The null hypotheses were:</u>

i) Collagen composite treatment wafer containing nanoparticles does not prolong analgesia compared to the other groups after velvet antler removal in deer.

ii) The application of collagen composite treatment wafer without nanoparticles does not prolong analgesia compared to the control group after velvet antler removal in deer.

iii) The modified collagen composite treatment wafer with zinc-oxide PVP does not adhere to the wounded deer antler better than the non-modified collagen composite treatment wafer.

iv) Collagen composite treatment wafers do not increase wound healing more than the control group.

v) Collagen composite treatment wafers do not increase antler re-growth more than control.

5.2. Materials and methods

5.2.1. In vivo study

i. Study design

This animal study was approved by Massey University Animal Ethics Community (MUAEC) protocol number 19/70.

This experiment was conducted over summer 2019/2020. The groups were divided into ten animals at first, on 30/10/2019, then six animals on 12/11/2019, six on 20/11/2019, six on 27/11/2019, six on 04/12/2019, and six on 02/01/2020. The first ten animals constituted the control group since the wafers were not ready then, and the study had to start when the antlers were ready to be harvested to avoid their calcification and reduction in price in the market. The study was not designed to be blinded as the laboratory provided the wafers on different days after the experiment had started.

The experiment was conducted at Massey University Deer Unit. The selection of the deer from the herd was based on antler maturity to prevent wastage of the harvested velvet antler tissue and to mimic a commercial situation. As a result, all deer were at approximately the same body weight and antler maturity whilst undergoing treatment. Forty healthy 1-year-old male (stag) red deer (*Cervus elaphus*) mean weight (±SD) 116.6 ± 11.38 kg were enrolled into the study and divided into four groups, each containing ten deer (Table 5.1). These were the same animals that were used in the MNT validation study in Chapter 3. All deer received a 4% articaine HCl ring block 4 cm below the pedicle to produce peri-operative analgesia prior to velvet antler removal. The collagen composite wafer treatments were applied to the wounds after surgical removal of velvet antlers and compared to deer who did not receive post-surgical collagen wafers. Duration of analgesia was assessed using MNT algometry (Chapter 3) and wound healing was monitored with digital photography.

Table 5.1 Table depicting the main in vivo study groups. All the groups received 4% articaine hydrochloride ring blocks. T1, T2, and T3 received collagen composite wafers, and the control received ring block only. NP=nanoparticles (n=10 in each group at the start).

	In vivo study groups			
	Control	***T1 (n=10)***	***T2 (n=10)***	***T3 (n=10)***
Ring Block	*4% articaine HCl*	*4% articaine HCl*	*4% articaine HCl*	*4% articaine HCl*
Treatment	*No wafer*	*Modified collagen wafer (25% NP)*	*Modified collagen wafer (5% NP)*	*Non-modified collagen wafer (0% NP)*

All the animals were clinically examined by a veterinarian for demeanour, lameness and any injuries around the head before the experiment. The deer were kept as a herd in paddocks at the Massey University Deer Unit and had *ad libitum* access to pasture and water throughout the study period. They were moved into the indoor deer handling facility and put in holding pens on the study days in groups of six. They were weighed using Tru-Test load bars connected to a Tru-Test XR5000 (Datamars Limited, Auckland, New Zealand) while passing through the handling facility. Two deer were guided carefully from the holding pen into the hydraulic crush (Heenan Work Room, Farmquip, New Zealand) (Figure 5.1) with closed curtains to reduce their exposure to stressors as much as possible. After each treatment or recording was performed, the deer were released back into a holding pen. Whilst inside the handling facility, deer had access to water and multi feed nuts (Sharpes Stock Feeds, Carterton, New Zealand). At the end of the day's testing, all the deer were released back to the paddock.

The treatment groups consisted of modified collagen wafers containing ZnO-PVP nanoparticles at a ratio of collagen: nanoparticle, 4:1 plus 136 mg lidocaine and 136 mg bupivacaine (T1); 20:1 plus 136 mg lidocaine and 136 mg bupivacaine (T2); non-modified collagen wafers containing 136 mg lidocaine and 136 mg bupivacaine (T3); and the control group which did not receive any collagen wafer treatment (Control) (Table 5.1 and 5.2).

Table 5.2 Components of collagen composites in the main study for in vivo experiment.

Components of the collagen composites for the main study						
	Group	***Collagen***	***ZnO-PVP***	***Bupivacaine***	***Lidocaine***	***Total***
In vivo	T1	80 mg	0 mg	136 mg	136 mg	362 mg
	T2	80 mg	4 mg	136 mg	136 mg	366 mg
	T3	80 mg	20 mg	136 mg	136 mg	382 mg

Each deer was physically restrained in a hydraulic crush similar to details explained in chapter 3 (also see Figure 5.1). Following removal, the treatment groups (T1, T2 and T3) had 3.8 cm diameter collagen composite wafers (Figure 5.2) applied to the wound immediately. The control deer did not receive any wafer treatment. The deer were restrained in the hydraulic crush for each assessment and sampling procedure. Experiment time points were allocated to avoid unnecessary handling of the animals. This also provided adequate time for each experimental component and avoided overlaps between measurements.

ii. Mechanical nociceptive threshold (MNT)

A hand-held algometer (FPX 25, Wagner Instruments, Greenwich, CT, USA) with a two mm-diameter round stainless-steel tip was used to measure the MNT of deer (Chapter 3). The MNT assessments were scheduled at 0, 2, 4, 6, 8 and 10 hours. A single, experienced, unblinded investigator measured the mechanical nociceptive thresholds of all study animals. The tip of the algometer was placed on cranial, caudal, medial and lateral sites around the base of the antler, 1 cm below the pedicle (Figures 5.1 and 5.2). The force was applied over 1 second by counting 1001. The behavioural response to reaching the threshold was head shaking, and the algometer reading was automatically held at that response. The reading was recorded in Newtons (N). Half of the deer were tested on the right antler first, followed by the left antler and vice versa. This was performed to minimise the animal's anticipation of the procedure and to reduce bias. The cut-off point force for algometry was set at 50 N to minimise any tissue damage and extra discomfort in animals (Stubsjøen *et al.*, 2010). This procedure created a grading system for MNT measurements based on trends in the data and indicated a cut-off point for MNT readings.

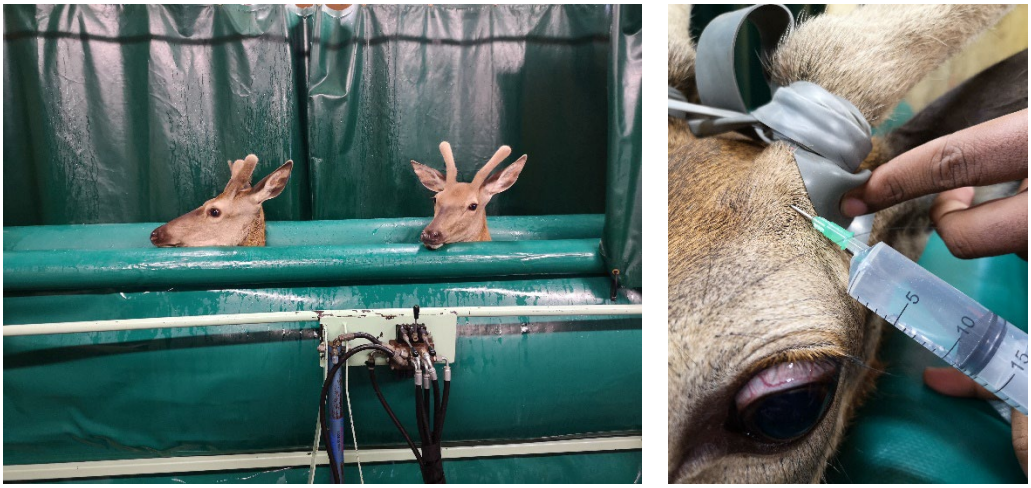


Figure 5.1 Left: Each time, two deer were restrained in Heenan Work Room Hydraulic crush (Farmquip, New Zealand), as shown in the picture. Right: Ring block local anaesthesia using 4% articaine HCL injections subcutaneously (SC), below the tourniquets (4 cm below pedicle), at the base of the antlers.

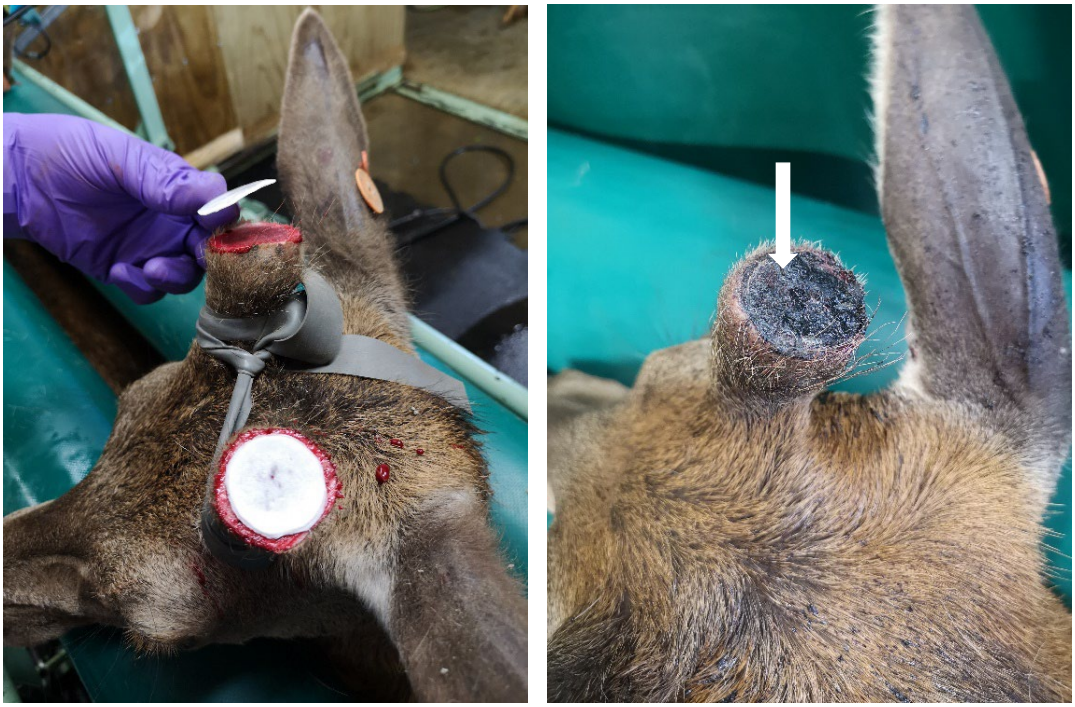


Figure 5.2 Left: The application of collagen wafers on the surgical site after velvet antler removal. Right: Collagen wafer (treatment group T1) adhered to the right antler at t10 hours after velvet antler removal, showing absorbed wound exudate (white arrow).

ii. Wound healing and antler re-growth

For wound healing and antler regrowth analysis, images were taken using Huawei P20 Pro mobile phone camera with a built-in Leica lens and filed for further analyses. A ruler was positioned adjacent to the wound surface (Figure 5.3) for calibration measurements after transferring the images to ImageJ software (version 1.52r, Wayne Rasband, National Institute of Health, USA). The wound healing measurements were collected at 0, 3, 7, 14, 21 and 30 days after antler removal. Time zero (t0) measurements were taken immediately after surgical removal of the velvet antlers. The same operator made all the measurements for consistency. They were then transferred to Microsoft Excel for further analysis.

Antler re-growth was recorded using a ruler positioned at the midline, adjacent to each of the left and right antlers, measuring the length of the main beams. Antler re-growth measurements were collected at times 0, 3, 7, 14, 21, 30, and 60 days after antler removal for both treatment and control groups. Time zero (t0) was immediately after removal of the antlers. The measurements from each sampling day were recorded and transferred to Microsoft Excel for further analysis. Refer to appendix for more information regarding the calculated means (±SD) of antler re-growth length (cm) in 2 months of study (Table 9.42). For minimum and maximum recorded antler re-growth lengths (cm) in different time points among the groups refer to Table 9.43.

iv. Statistical analysis

Data was collected using Microsoft Excel (version 365), statistical analyses were performed using SAS (version 9.4, SAS Institute Inc., Cary, NC, USA) and GraphPad Prism (version 8.3.0, GraphPad Software Inc., San Diego, CA, USA) was used to create graphs.

The measurements of the MNT j(N) in the control group were not recorded at times 480 min (t8), 720 (t10). This created an incomplete design, and as a consequence, the analyses were performed in two steps. First, the main effects were evaluated with a model that included the fixed effect of treatment and time and the random effect of animal to account for the repeated measurements on the same animal. Least-squares means and standard errors (SEM) were calculated for the variables, treatment and time, and used for multiple comparisons using the Fisher's least significant difference as selected in the option LSmeans (Least Squares Means) of the MIXED procedure in SAS with a Bonferroni adjustment. Second, the interaction between time and treatment was evaluated with the same model as before but including the fixed effect of interaction between time and treatment. The time values were transformed from hours to minutes for consistency within the statistical analysis; for example, t2 was represented as t120. Measurement of antlers (right or left) and site within the antler (cranial, medial, caudal and lateral) were set as covariates, and the random effect of deer (between-animal variation; $\frac{2}{a}$) and residual (within-animal variation; $\frac{2}{e}$) was accounted for. The repeated measures on the same deer were modelled with a compound symmetry error structure. Least-square means for the different combinations of time and treatment were obtained and used for multiple comparisons of means using Fisher's least significant difference. The compound symmetry error structure was determined as the most appropriate residual covariance structure based on Akaike's information criterion. For the analysis of the recovery rate of analgesia in the *in vivo* study, slopes were created (MNT/time) starting from t10 minutes to compare the value between the treatment and control groups. For the assessment of wound healing, the day of measurement was considered a fixed effect, and wound surface area (mm^2) was considered the

dependent variable. The KS test was used to assess normal distribution. Least-square means for the different combinations of time and treatment were obtained and used for multiple comparisons of means using Fisher's least significant difference. Surface area values were transformed to logs, and slopes of wound healing rate were compared. For antler re-growth, the KS test was used. Least-square means for the different combinations of time and treatment were obtained and used for multiple comparisons of means using Fisher's least significant difference. For deer body weight (kg), the least-squares means were calculated and compared between the treatment and control groups. ANOVA test was performed prior to one-by-one comparison analysis to detect any significant difference among the treatment groups. Animals that were subjected to euthanasia were excluded from further analyses. Estimates with $P<0.05$ were considered significant from zero.

The surface area of the wound was calculated after manually selecting the visible wound margins using ImageJ software and recorded in mm^2. A ruler was used in photographing to be able to calibrate and convert sizes to the software for accurate measurements of the surface areas (Figure 5.3).

The regrowth of the antlers was assessed by placing a stainless steel ruler at the parietal region of the head between two antlers and were angled in a way to be able to measure the length of newly grown antlers from the pedicles and not the wound itself.

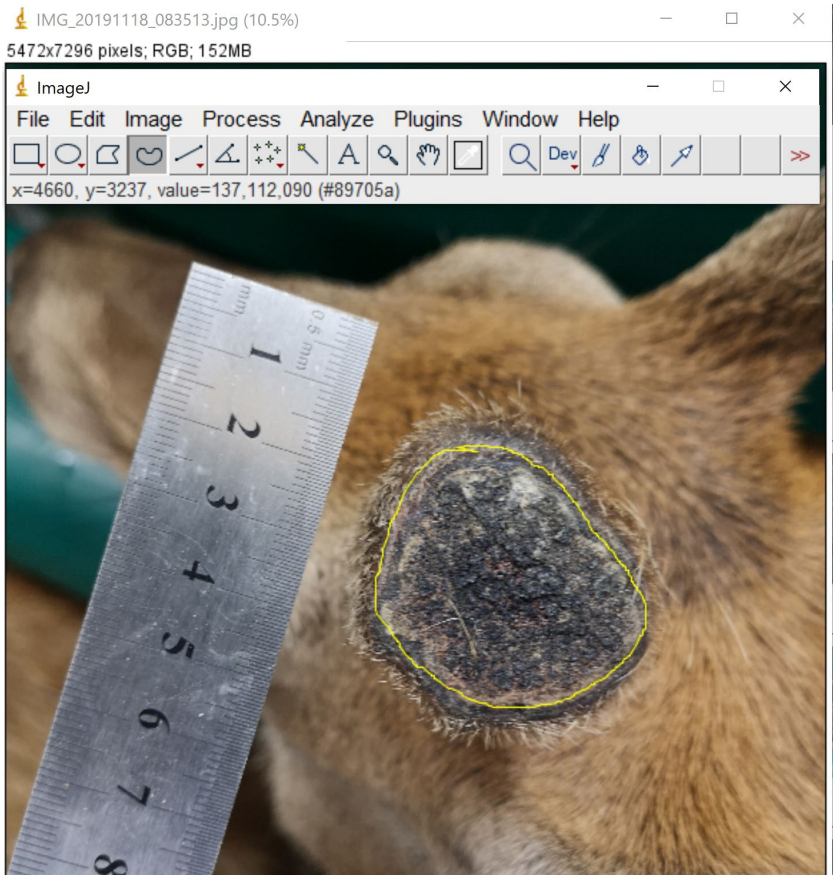


Figure 5.3 Antler wound surface area measurements using ImageJ software. The surface area was manually selected and then calculated in mm^2 using a stainless-steel metric ruler for scale, as shown in the photo.

5.3. Results

Forty animals were enrolled on day 1. One antler was removed on day 3 due to severe trauma to the right antler pedicle, and the left antler remained in the study. One deer was removed on day 7 of the experiment due to a non-salvageable injury to the hind limb, and the animal was humanely euthanised.

i. Bodyweight

The studied deer weighed a mean (±SD) of 116.58 ±11.51, minimum and maximum of 84.5 and 145.5, respectively.

ii. Adherence of the collagen wafers

The majority of the treatment wafers across all the treatment groups remained adhered up to the completion of the 2-month study period (see Figures 5.4 and 5.5). T3 (0% NP), in which the wafers were devoid of modification with nanoparticles, showed increased fragility and had the least wound adherence compared to the modified treatment groups (T1 and T2). In the first 24 hours of the experiment, incomplete adherence was observed, and in the case of antlers with only partial wafer remaining, these were excluded from further adherence analysis. The adherence of the treatment wafers up to 24 hours was greatest for T1 with 18 out of 20 (90%) wafers remaining adhered, T2 showed the second greatest adherence with 14 out of 20 wafers remaining adhered (70%), and T3 showed the least adherence with 9 out of 20 wafers remaining adhered (45%). The least-squares means (±SEM) of the overall period of wafer adherence for the three treatment groups: T1, T2, and T3, were 46.27±6.24 days, 50.13±6.24 days, and 35.13±6.24 days, respectively. Over the two months of observations, T1 showed the most adherence in the first 24 hours (90%); however, T2 had the longest attachment period to the antler surfaces. The tables in the appendix show the adherence of all the wafers (Tables 9.27-9.29).

Figure 5.4 This picture shows the high adherence of the modified collagen composite wafer (T1) applied to the post-surgical wound on the left antler and its capability of absorbing exudate within the porous structure after two hours of application.

Figure 5.5 The adherence of the collagen wafers in the treatment group (T1), at a) 24 hours, b) day 3, c) day 14, d) 1 month after application.

iii. Mechanical Nociceptive Threshold (MNT)

A total of 2005 algometer MNT results were measured, equating to 4 positions on each antler measured at 7 time-points for animals in each of the treatment groups and 5 time-points for animals in the control group. Baseline MNTs were measured at t0, during which some animals displayed behaviour similar to tonic immobility in response to the algometry (Sahebjam *et al*. 2020). Repeat readings were obtained in one animal in the control group, three aspects of one animal in T2, and 2 aspects of a deer in T3. However, in each case, these deer displayed tonic immobility for a second time and were excluded from baseline data analysis because they had MNT higher than our set cut-off point (50 N), and they were recorded as "No Response (NR)". Kolmogorov-Smirnov (KS) tests showed that the values from all the study groups were normally distributed. Baseline MNT readings measured at t0 ranged from a minimum of 5.8 N to a maximum of 48.8 N. Subsequent MNT readings in the treatment groups ranged from a minimum of 5.80 N to a maximum of 50 N. MNT assessments in the treatment groups were continued from t0 until 10 hours after antler removal (t10) and for six hours in the control group, by which time the readings had reached baseline. The least-squares means (±SEM) of overall MNT, from t0 to t6 in the control group was 26.93±1.56 N, and from t0 to t10 in the treatment groups: T1, T2, and T3, were 40.83±1.37 N, 43.21±1.37 N, 41.39±1.37 N, respectively.

ANOVA analysis showed a significant difference ($P<0.05$) between the study groups. The comparison of the least-squares means of MNT measurements showed a significantly higher MNT in T2 compared to T1 and T3 ($P<0.0001$ for both). In addition, the treatment groups T1, T2, and T3 showed significantly higher MNT compared to the control group ($P<0.01$, $P<0.01$, $P<0.01$, respectively) across the study period. Figure 5.6 illustrates the MNT recovery pattern and the trendline between the groups. The trendlines for all groups rise to 50 N from the baseline MNT (at t0) after 10 minutes due to the analgesic effect of 4% articaine ring block in all groups. The control group declined sharply from 50 N recorded at t10 min to approximately 22 N at t6 hrs. In contrast, the treatment groups maintained the MNT readings above 43 N up to t6 hrs. Refer

to Table 5.2 to see the comparison in the least-squares means of every time point between the groups. For comparison between all recorded forces (N) refer to appendix (Tables 9.30 – 9.40).

iv. The comparison of recovery rates from analgesia

The slopes depicting recovery from local anaesthesia were -0.084±0.003, -0.023±0.001, -0.016±0.001, and -0.029±0.001 for control, T1, T2, and T3, respectively (Figure 5.7). ANOVA tests showed significant differences between the groups ($P<0.0001$). After the exclusion of data obtained from the baseline MNT readings, comparison for slopes of recovery of local anaesthesia showed that the control had a significantly more rapid trend of recovery (N min^{-1}) compared to the treatment groups T1, T2 and T3 (Figure 5.7) ($P<0.0001$ for all groups). All three treatment groups also showed a significant difference compared to each other: T1 vs. T2 ($P<0.01$), T1 vs. T3 ($P<0.05$), T2 vs. T3 ($P<0.0001$). In addition, all the treatment groups demonstrated analgesia beyond 6 hrs and up to 10 hrs.

v. Development of grading system for MNT in red deer

Based on the obtained results in force records in the control group (only 4% articaine HCl ring block), a scaling system was established, which determined; 0.1–20 N as baseline (B) or grade 1, 20–30 N as Low (L) or grade 2, 30–40 N as medium (M) or grade 3, and 40–50 N as High (H) or grade 4. Therefore, the forces measured at time point zero were considered baseline or B, 10 minutes and 2 hours were deemed to be high or H, and time points 4 and 6 were considered L or low in force detection scaling (Table 5.3).

Table 5.3 Least-squares means of MNT (±SEM) (N) between treatment groups (T1, T2, and T3) and the control group up to 10 hours. As seen in the table, measurements in the control group ceased at t6 hours. The alphabetical superscript shows the significant differences between groups. The LSmeans of MNT baseline readings in the control group was significantly less than the treatment groups (P<0.0001). The LSmeans of MNT baseline reading in T3 was significantly higher than T1 and T2 (both P<0.05). The LSmeans of MNT (t120) for the control group was significantly less than T1 (P<0.01), T2 (P<0.01), and T3 (P<0.001). The LSmeans of MNT (t240) for the control group was significantly less than T1 (P<0.0001), T2 (P<0.01), and T3 (P<0.001). Furthermore, the LSmeans of MNT (t240) for T1 was significantly less than T2 (P<0.05). The LSmeans of MNT (360) for the control group was significantly less than T1 (P<0.0001), T2 (P<0.0001), and T3 (P<0.0001). Furthermore, the LSmeans of MNT (t360) for T1 was significantly less than T2 (P<0.05). The LSmeans of MNT (480) for T2 was significantly higher than T3 (P<0.05). MNT measurements of 0.1–20 N was considered as baseline (B) or grade 1, 20–30 N was considered as Low (L) or grade 2, 30–40 N was considered as medium (M) or grade 3, and 40–50 N was considered as High (H) or grade 4 (n=10 deer). The superscripts are comparing the values in columns for each time point.

MNT±SEM (N)				
Time (min)	**Control**	**T1**	**T2**	**T3**
0 (baseline)	16.4±1.6^{a} (B)	27.1±1.5^{bd}(L)	26.1±1.5^{bd}(L)	31.7±1.5^{bc}(M)
10	50.0±1.6^{ab}(H)	50.0±1.5^{ab}(H)	50.0±1.5^{ab}(H)	50.0±1.5^{ab}(H)
120	42.0±1.6^{a}(H)	48.3±1.5^{b}(H)	49.1±1.5^{b}(H)	49.5±1.5^{b}(H)
240	27.1±1.6^{a}(L)	45.2±1.5^{bc}(H)	49.6±1.5^{bd}(H)	47.9±1.5^{b}(H)
360	22.2±1.6^{a}(L)	43.2±1.5^{bc}(H)	47.7±1.5^{bd}(H)	44.1±1.5^{b}(H)
480		40.4±1.5^{ab}(H)	43.4±1.6^{a}(H)	38.2±1.6^{b}(M)
600		35.8±1.6^{ab}(M)	40.2±1.6^{ab}(H)	33.1±1.6^{ab}(M)

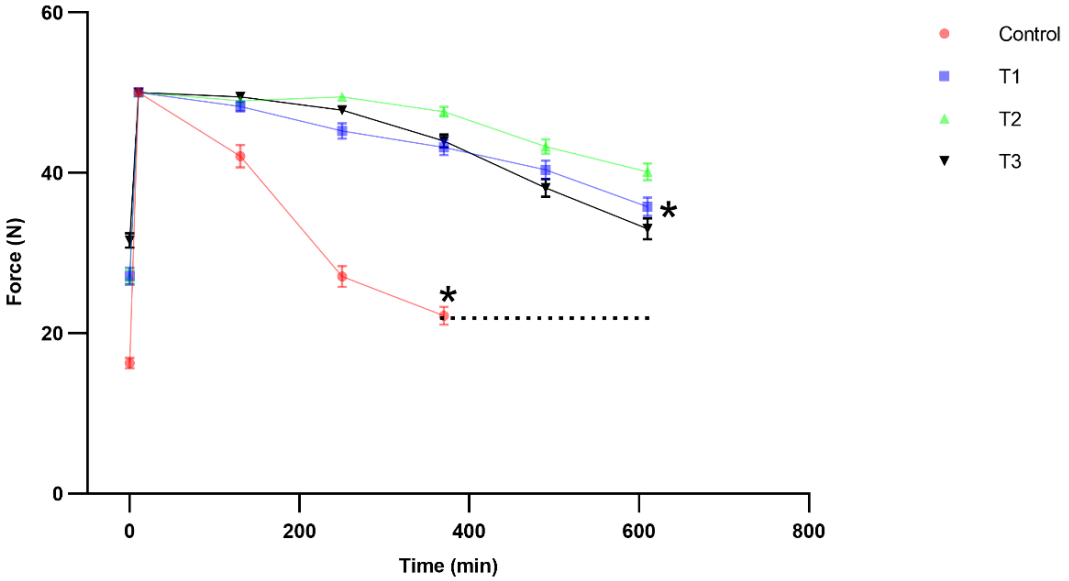


Figure 5.6 The least-squares means (±SEM) shows the obtained MNT (N) at different time points (min) in four study groups; T1 (blue), T2 (green), T3 (black) and control (red). The asterisk symbols represent the endpoints in each study. The dotted line represents the cessation of the measurements in the control group from 360 minutes and has no statistical value, and is only for better visualisation. The LSmeans of MNT baseline readings in the control group was significantly less than the treatment groups (P<0.0001). The LSmeans of MNT baseline reading in T3 was significantly higher than T1 and T2 (both P<0.05). The LSmeans of MNT (t120) for the control group was significantly less than T1 (P<0.01), T2 (P<0.01), and T3 (P<0.001). The LSmeans of MNT (t240) for the control group was significantly less than T1 (P<0.0001), T2 (P<0.01), and T3 (P<0.001). Furthermore, the LSmeans of MNT (t240) for T1 was significantly less than T2 (P<0.05). The LSmeans of MNT (360) for the control group was significantly less than T1 (P<0.0001), T2 (P<0.0001), and T3 (P<0.0001). Furthermore, the LSmeans of MNT (t360) for T1 was significantly less than T2 (P<0.05). The LSmeans of MNT (480) for T2 was significantly higher than T3 (P<0.05). Each data point represents measurements from 10 animals.

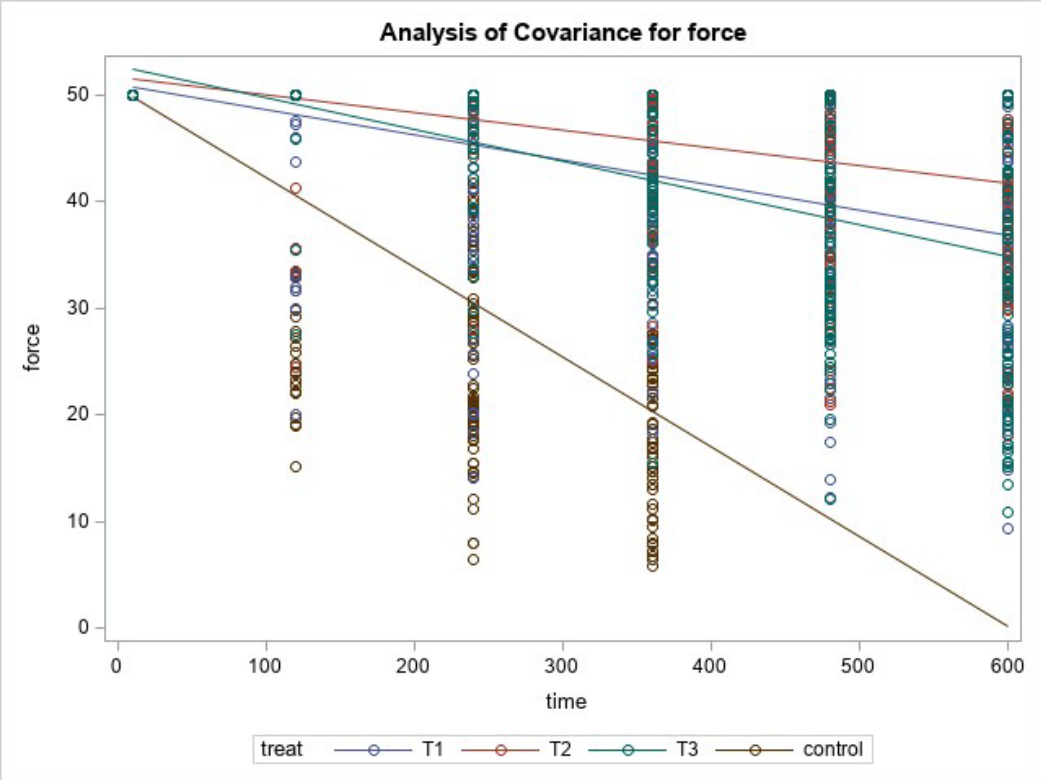


Figure 5.7 The slopes illustrate the rate (MNT or Force (N min^{-1})) of the return of pain recognition for the control group (olive) and 3 treatment groups: T1 (blue), T2 (red), and T3 (green) from t10 min (time≥10 minutes). The measurements in the control group ceased after t=360 minutes, and the plot was extended for a better visual comparison. Treatment groups ceased after t= 600 minutes. The control had a significantly more rapid trend of recovery (N min^{-1}) compared to the treatment groups T1, T2 and T3 (P<0.0001 for all groups). All three treatment groups also showed a significant difference compared to each other: T1 vs. T2 (P<0.01), T1 vs. T3 (P<0.05), T2 vs. T3 (P<0.0001) (n=10 animals in each group).

vi. Wound Healing

A total of 472 observations were used for the assessment of wound healing. The values in all study groups were normally distributed (KS test). None of the wounds showed any detectable evidence of abnormal tissue growth, myiasis (flystrike), pus/exudate, erythema or inflammation to the naked eye at the completion of the study period (day 60). All the wounds had completely healed at the end of the study. In the control group, where there was no wafer applied to the antler wound, rapid wound healing was observed between days 7 and 14 ($P<0.0001$) and again between days 14 and 21 ($P<0.0001$), in which the wound surface area approximately halved in size between each time-point. Early changes were not visible in the treatment groups because the wafers were obscuring the visibility of any underlying wound healing process. However, most collagen wafers naturally disintegrated from the edges as the wound was healing. Therefore, the only option was the measurement of remained wafers on the wounds. Comparisons of wound surface areas (mm^2) between time points showed a trend of significantly decreasing surface area from day 21 onwards in T1 ($P<0.01$), from day 14 onwards in T2 ($P<0.05$), and from day 21 onwards in T3 ($P<0.05$) compared to previous time points. Refer to appendix for more information regarding mean (±SD) of antler wound surface area (mm^2) for 2 months of wound healing assessment (Table 9.41).

vii. Rate of wound healing

Slopes encompassing measurements for the observed period (day1 to day 30) were calculated for better understating the wound healing rate as all cases by day 30 were completely healed. The slopes represented the rate of wound healing as a result of declining wound surface area and were: -47.83±3.15, -21.16±3.25, -26.20±3.15, and -14.01±3.15 for the control group, T1, T2, and T3, respectively. For improved analysis of slopes, the wound values were transformed to log10 of wound values (Figure 5.8), and the slopes post-transformation were -0.059±0.0047, -0.038±0.0049, -0.058±0.0048 and -0.027±0.0048 for control, T1, T2, and T3, respectively. For a better clarification, this technique created lines as slopes to include more possible values closer

to the lines, therefore making comparison more accurate. ANOVA test showed significant differences among the groups (P<0.0001). The slope of the control group was significantly more negative than T1 (P<0.01) and T3 (P<0.0001) and almost paralleled T2. T2 showed a more negative slope compared to T1 (P<0.01) and T3 (P<0.0001) (see Figure 5.8).

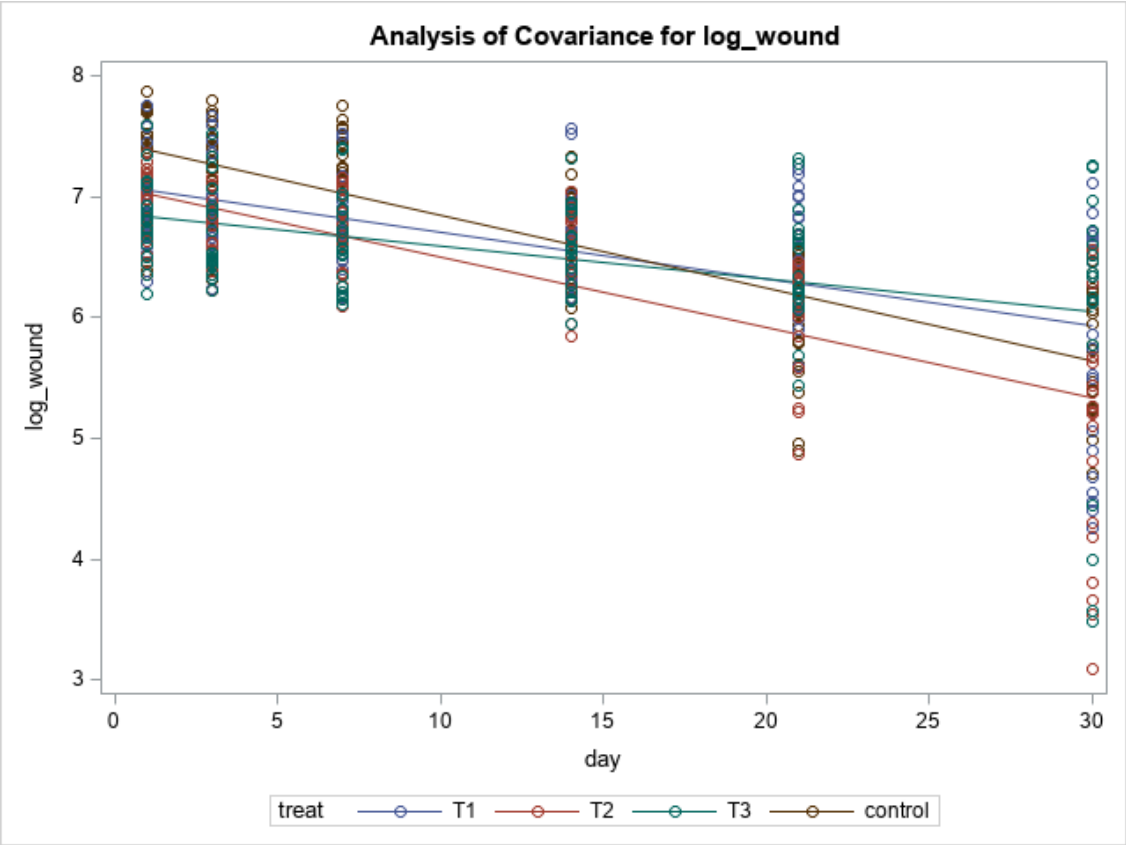


Figure 5.8 The rate of wound healing (log of wound area ((mm2) day^{-1}) between 4 different study groups: T1 (blue), T2 (red), T3 (green) and the control group (olive). The Y-axis represents the log of wound surface area, and X-axis represents the time point (day) in the main in vivo study. The control group had a significantly faster wound healing rate ((mm2) day^{-1}) compared to T1 (P<0.01) and T3 (P<0.0001). Furthermore, T2 had a significantly faster wound healing rate ((mm2) day^{-1}) compared to T1 (P<0.01) and T3 (P<0.0001) (n=10 animals in each time point).

viii. Antler re-growth

A total of 534 observations were used for the assessment of antler re-growth (right antler=265, left antler=269) over 2 months. As a reminder, only the newly grown antlers from the pedicles were assessed in this part irrespective of whether the wafers stayed on or lost adherence. On day 60, eight animals were eliminated because the antlers were cut prior: 1 animal from T1, one animal from T2, 4 animals from T3 and one animal from the control group. Observation occurred on days 1, 3, 7, 14, 21, 30, and 60. The minimum and maximum values recorded were 5 cm and 36 cm, respectively. The values across all study groups were normally distributed (K-S test). The least-squares mean (±SEM) of measured antlers for the assessment period in the right and left antlers disregarding group effect, were 9.8±0.2 cm and 9.7 ±0.2 cm, respectively, and there was no significant difference seen between left and right antler lengths. The pattern of antler re-growth showed a slow increase until day 30 and followed by a period of rapid antler re-growth from day 30 to day 60 (Figure 5.10). The overall least-squares means (±SEM) of measured antler length for control, T1, T2, and T3 without considering repeated measures were 11.2 ±0.2 cm, 9.5 ±0.2 cm, 9.5 ±0.2 cm, and 8.6 ±0.2 cm, respectively (Table 5.4, Figure 5.9). ANOVA test showed a significant difference between the groups ($P<0.0001$). On day 60, the control group had significantly longer antler length (cm) compared to T1 ($P<0.0001$), T2 ($P<0.001$), and T3 ($P<0.0001$). Furthermore, T2 showed longer antler length (cm) compared to T1 ($P<0.01$) and T3 ($P<0.0001$). The least-squares mean (±SEM) of each group considering the repeated measures (application of PROC MIXED on SAS software which results in a more accurate result) in different days and the antler growth trend are seen in Table 5.4 and Figure 5.9.

Table 5.4 Deer antler re-growth and the least-squares means (±SEM) of antler length (cm) for the control, T1, T2, and T3 groups on different days and for all days considering the repeated measure analysis. The alphabetical superscript shows the significant difference between the groups (comparing columns for each day). The LSMeans of total antler re-growth length (cm) was significantly longer in the control group compared to T1 (P<0.0001), T2 (P<0.0001), and T3 (P<0.0001). Furthermore, T3 showed significantly shorter total antler re-growth length (cm) compared to T1 (P<0.001) and T2 (P<0.0001). There was no significant difference until day 7, in which antler re-growth length (cm) was significantly longer in T1 compared to T2 (P<0.05) and T3 (P<0.05). On day 14, the control group showed significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.05). Furthermore, T1 had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.05). On day 21, the control group had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.01). Furthermore, T1 had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.01). On day 30, the control group had significantly longer antler length (cm) compared to T2 (P<0.01) and T3 (P<0.01). On day 60, The control group had significantly longer antler length (cm) compared to T1 (P<0.0001), T2 (P<0.001), and T3 (P<0.0001). Furthermore, T2 showed longer antler length (cm) compared to T1 (P<0.01) and T3 (P<0.0001).

		Least-Squares Means of antler re-growth on different days (±SEM)			
Day	**N**	**Control (cm)**	**T1 (cm)**	**T2 (cm)**	**T3 (cm)**
1	80	7.1 ±0.2^{ab}	7.2 ±0.2^{ab}	6.6 ±0.2^{ab}	6.6 ±0.2^{ab}
3	80	7.1 ±0.2^{ab}	7.2 ±0.2^{ab}	6.6 ±0.2^{ab}	6.6 ±0.2^{ab}
7	78	7.1 ±0.2^{ab}	7.4 ±0.3^{a}	6.8 ±0.2^{b}	6.7 ±0.2^{b}
14	77	7.8 ±0.2^{a}	7.6 ±0.3^{c}	6.9 ±0.2^{bd}	6.8 ±0.2^{bd}
21	77	8.4 ±0.3^{a}	7.9 ±0.3^{c}	7.0 ±0.3^{bd}	6.9 ±0.3^{bd}
30	77	9.9 ±0.5^{a}	9.2 ±0.6^{ab}	7.8 ±0.5^{b}	7.9 ±0.5^{b}
60	65	32.5 ±1.3^{a}	20.6 ±1.4^{bd}	26.1 ±1.3^{bc}	16.8 ±1.6^{bd}
Total		**11.4 ±0.2^{a}**	**9.6 ±0.2^{bd}**	**9.7 ±0.2^{bd}**	**8.3 ±0.3^{bc}**

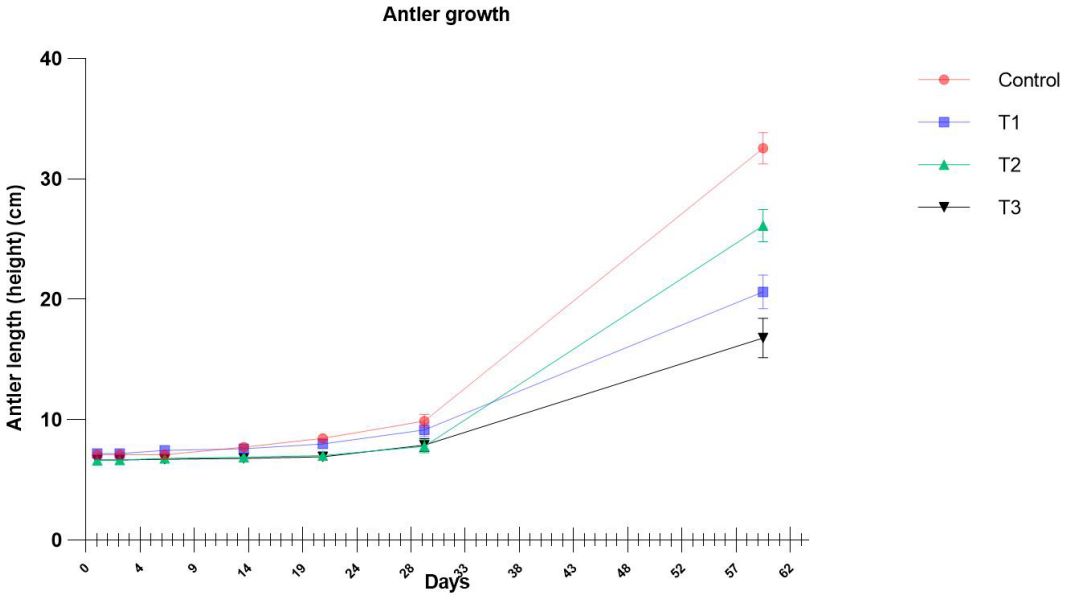


Figure 5.9 Antler re-growth and the least-squares means (±SEM) of antler length (cm) for the control, T1, T2, and T3 groups on different days. The Y-axis represents the length of antler re-growth (cm), and X-axis represents the time point (day) in the main in vivo study. The total antler re-growth length (cm) was significantly longer in the control group compared to T1 (P<0.0001), T2 (P<0.0001), and T3 (P<0.0001). Furthermore, T3 showed significantly shorter antler re-growth length (cm) compared to T1 (P<0.001) and T2 (P<0.0001). There was no significant difference until day 7, in which antler re-growth length (cm) was significantly longer in T1 compared to T2 (P<0.05) and T3 (P<0.05). On day 14, the control group showed significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.05). Furthermore, T1 had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.05). On day 21, the control group had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.01). Furthermore, T1 had significantly longer antler length (cm) compared to T2 (P<0.05) and T3 (P<0.01). On day 30, the control group had significantly longer antler length (cm) compared to T2 (P<0.01) and T3 (P<0.01). On day 60, The control group had significantly longer antler length (cm) compared to T1 (P<0.0001), T2 (P<0.001), and T3 (P<0.0001). Furthermore, T2 showed longer antler length (cm) compared to T1 (P<0.01) and T3 (P<0.0001). (n=originally 20 antlers in each group; however, it became 16 antlers for T1, 18 for T2, and 12 for T3 after 60 days due to exclusion of some animals. Control groups remained as 10 deer with 20 antlers until the end of 60 days).

The least-squares means (±SEM) at day 60 for antler re-growth length were compared, and all the treatments showed significantly shorter antler lengths (cm) compared to the control group (P<0.0001, P<0.0001, P<0.0001, for T1, T2, and T3, respectively). The least-squares mean (±SEM) for total antler re-growth length (cm) of T1 was significantly longer compared to T3 (P<0.05), and shorter antler growth (cm) compared to the control group (P<0.0001). The least-squares mean (±SEM) of T2 showed a significantly longer antler length (cm) compared to T3 (P<0.05) and a significantly shorter length compared to the control group (P<0.0001) (Table 5.5).

*Table 5.5 Differences in deer antler regrowth after removal and comparing the least-squares means (±SEM) of antler length between control, T1, T2, and T3 after 60 days. *Significant difference **very significant difference.*

Difference of Least-Squares Means (±SEM) for antler re-growth measured as length (cm)

Group	Group	Estimate	Standard Error	P-value
T1	**T2**	-0.10	0.32	0.74
T1	**T3**	1.24	0.35	0.0004*
T1	**Control**	-1.81	0.32	<0.0001**
T2	**T3**	1.34	0.34	<0.0001**
T2	**Control**	-1.70	0.31	<0.0001**
T3	**Control**	-3.05	0.34	<0.0001**

5.4. Discussion

The pilot study (Chapter 3) showed that using a collagen composite on the wound surface of red deer antlers can prolong pain relief. This study aimed to investigate and resolve the limitations of the use of collagen composites by including a greater number of red deer and focusing on treatments that showed promising results from the pilot study.

The key finding of this study was the successful and effective pain mitigation in red deer for more than 10 hours in the treatment groups and up to 6 hours in the control group. The effective, sustained release of lidocaine and bupivacaine confirmed from IVDRT, ease of application of wafers in the animals, adhesiveness, minimal discomfort displayed by deer during the application confirmed that the proposed modified collagen wafers are a suitable candidate to be used in deer after velvet antler removal. Furthermore, the application of wafers to the antler tissue wound did not elicit any observable inflammatory reaction, thus safe to use on wounds.

In this study, a circular shaped wafer was created in order to cover the cut surface area of the deer antlers. The wafers with zinc oxide -PVP nanoparticles had more integrity and were more resistant to damage during storage, handling, and transport. All the collagen wafers used in this study were white, sponge-like and odourless. They showed better integrity, more flexibility, and a more uniform porous structure than those in the pilot study. Perhaps the most feasible technique would be the measurement of water absorption capacity (WAC) which previously has been incorporated by Huang *et al*. (Huang *et al.*, 2016), or swelling measurements using the teabag method introduced by Pourjavadi and Kurdtabar (Pourjavadi and Kurdtabar, 2007)The assessment of wound healing and antler re-growth analyses were added in this study to investigate potential problems with this technique. Thermography with FLIR was not included in this study due to the lack of evidence of any additional visible inflammation or reaction by the tissue to the treatment wafers using this method in the pilot study.

An important practical consideration is the ability of collagen wafers to adhere to the wounded antlers, to maintain the contact with the wound surface and to ensure the local anaesthetic is

delivered to the wound tissue. There is no reference in the literature analysing adhesiveness of dressings to antler wounds in deer. A key part of this study has been to identify and develop a lasting and effective dressing. In addition, the ability of any dressings that successfully absorb wound exudates has been found to be important to reduce bacterial growth on the wound and tissue since bacteria use cytokines and chemokines to grow (Boateng *et al.*, 2008, Saghazadeh *et al.*, 2018). According to Jiménez-castellanos *et al*. (1993), bio-adhesion is a term which refers to the attachment of a drug delivery vehicle to a specific biological location (Jiménez-castellanos *et al.*, 1993). Smart *et al*. tested adhesiveness *in vitro* and the authors stated that extra hydration could lead to gel formation of the polymer which becomes slippery and can lose attachment from the tissue surface (Smart *et al.*, 1984). This phenomenon was also found in this study. In cases where bleeding was excessive, the collagen wafers were formed a gel making them prone to lose attachment.

The relevance of adhesiveness and drug delivery has mainly been studied in humans and in the context of hydrogels and transdermal drug delivery systems (Jung *et al.*, 2020). Numerous studies looked at the importance of adhesiveness in dressings such as chitosan sponges (De Castro *et al.*, 2012), in which they tested adhesiveness by determining detachment weight and contact surface area. In this study on deer antlers, adhesiveness was studied by observing whether the wafers were still attached. This was to some extent subjective rather than objective; the strength of the wafers was measured via lab assessments. However, the modified wafers including 5% and 25% nanoparticles remained adhered during the first critical 24 hours (70% and 90% of wafers) as compared to the non-modified wafers, was sufficient to consider adhesiveness a success. This period is crucial as ideally delivery of both local anaesthetics should occur to cause analgesia in antlers and reduce discomfort in animals. In general, almost all the wafers which survived the first 2 hours remained attached to up to 2 months.

Treatments that do not stick to the wound cannot provide an effectively prolonged pain relief as they don't physically exist to deliver the rest of the drugs. It is highly possibly some amount

become released when exposed to bleeding velvet antlers as wafers collapse on the wounds. It was useful to show that modified collagen composite treatment wafers with zinc-oxide PVP adhere better to the wounded deer antler than non-modified collagen composite treatment wafers with no nanoparticles. The adherence ability of the wafers presented a challenge in the pilot *in vivo* study. None of the non-modified wafers and only 50% of the modified collagen wafers remained attached to the antler wound surface. Problems identified in the pilot study necessitated the use of an updated model of freezer-dryer to create the wafers in the main study. The study design was improved by increasing the number of treatment groups and number of replicants.

Minimal manipulation of the deer's head, and sufficient restraint, was used to minimise the risk of losing wafers. In this study, there was an increase in adherence of the collagen wafers to the antler wounds compared to the pilot study. In the first 24 hours of this study, the adherence of wafers in T1 was 90%, and in T2 was 70%, whilst the least adherence was achieved in the non-modified treatment group (T3) in which less than 50% remained adhered. Regarding the duration of adherence across the 60-day study period, the treatments which contained modified collagen wafers (T1 and T2) persisted longer on the wound surface compared to wafers with no modification. T1 and T2 persisted for up to 50 and 46 days, respectively; in contrast, T3 wafers only persisted up to 35 days. There was no evidence of residual wafer material after 60 days. Analysis of adhesiveness concluded that collagen wafers modified with ZnO-PVP nanoparticles are better candidates for drug delivery in deer due to increased adhesiveness in the whole study.

MNT results in this study prove that bupivacaine and lidocaine loaded collagen wafers modified with zinc oxide-PVP could be a potential future product in the deer industry to deliver postoperative analgesia after velveting for up to 10 hours post-surgery. The extended analgesia could reduce the need to use systemic pain relief medications such as NSAIDS and opioids,

avoiding their systemic side effects. Locally applied, slowly released drugs would provide the deer with more targeted analgesia avoiding the possible complications of systemic drugs.

The *in vivo* study rejected the null hypothesis that the application of a collagen composite treatment wafer does not prolong analgesia, as measured by increased MNT, compared to the control group after velvet antler removal in deer. All the treatment groups: T1, T2, and T3, had significantly longer analgesia than the control group ($P<0.0001$). Furthermore, we rejected the null hypothesis that the application of a collagen composite treatment wafer containing nanoparticles does not prolong analgesia compared to the other groups after velvet antler removal in deer. The treatment groups which involved modified collagen composite (T1 and T2) prolonged analgesia significantly longer than T3, and T2 showed the highest statistical difference when compared to T3 ($P<0.0001$). The inclusion of 10 animals in each group resulted in a high statistical power (0.9), increasing the reliability of the results.

The cut-off point for MNT (50 N) was determined by measuring baseline readings in unmedicated healthy deer (see chapters 3 and 4). The reason for using a cut-off point was to minimise any damage to the skin from repeated applications of the algometer. Higher MNT readings represent reduced pain perception, whereas low MNT readings represent higher pain perception. MNT assessments ceased at 6 hours in the control group as measurements approached baseline readings. The MNT measurements showed significant differences between the treatment groups and the control group, which was aligned with the results from the *in vitro* study that showed a significantly slower release of local anaesthetic from the wafers in the treatment groups compared to the control group. For a more accurate analysis, the slopes of regression lines starting from t10 min were created and compared. The slopes represented the rate at which animals regained pain sensation as measured by MNT or force (N) over time (min). Slopes with a lower rate of change represented a slower return to normal sensation and more prolonged pain relief. The comparison of the slopes between the groups showed a significant

difference between the treatment groups, and all treatment groups had more prolonged analgesia than the control group. The treatment group with the slowest rate of change and thus, most prolonged pain relief was T2 (the only treatment group that remained in grade "H" after 10 hours in the scaling system developed in the study) (see section 5.3.2), in addition, T2 showed significantly higher MNT readings throughout the 10-hour assessment period. The results confirmed the *in vitro* study results, as the treatment groups with nanoparticles showed more prolonged analgesia. However, there was no dose response correlation: the treatment group modified with 5% ZnO-PVP nanoparticles (T2) showed more prolonged analgesia than that with 25% ZnO-PVP nanoparticles (T1). This could be due to the entrapment of drugs in T1, reducing the amount available to deliver analgesia at the application site. The more sustained release in the group with 25% nanoparticles was evident in the *in vitro* study via using Franz Cells (Chapter 4).

To the author's knowledge, no study has been published which investigates the analgesic effects of collagen-based lidocaine or bupivacaine or both in combination in animals. This study investigated its effects and potential usefulness in veterinary medicine using red deer velveting procedure as a model. Since collagen is a proven biomaterial which enhances wound healing, thus wound healing after velvet antler removal was a secondary objective alongside pain assessment to observe possible changes when wafers were applied on the wounded velvet antlers. Further investigation on wound healing properties of velvet antlers particularly in an *in vitro* level is encouraged.

The *in vivo* study concluded that antler wounds treated with collagen composite treatment wafers healed very well at the end of the study with no apparent difference among the groups. There is no standard method to evaluate antler wound healing in deer. It was assessed by measuring the wound area over time in the ImageJ software program. For example, assessment by histopathological analysis of the wound tissue is an invasive method which would have caused welfare issues as the samples would have to be harvested at different time points. Therefore,

digital photography was selected as a safe, non-invasive and affordable technique to monitor the wound healing process. The treatment and control groups did not show any significant difference in the rate of wound healing compared to each other, which suggested that T2 did not promote or hinder the wound healing process compared to the control group. All wounds showed complete healing at 30 days. The advantage of T2 over the control group was that the wounded area was covered by a collagen composite wafer, protecting the wound from environmental exposure.

The study accepted the null hypothesis that collagen composite treatment wafers do not increase antler re-growth more than the control group. However, this study was not ideal for antler re-growth analysis since other aspects of antler growth, such as forking the antlers, were not included, and consistent recording was not performed from 30 days to 60 days. Antlers were harvested between 30th October and 2nd January, and deer were assigned to the study groups in the following order: control, T1, T2 and T3. As a result, an environmental effect on antler regrowth cannot be excluded as antlers in the control group re-grew under conditions of increasing photoperiod and spring pastures with a high plane of nutrition. In contrast, T3 antlers re-grew under conditions of decreasing photoperiod and late summer pastures, which may have had a lower plane of nutrition. During the trial, the re-growth of antlers was monitored by measuring at set time-points during the first 60 days post-harvest to better understand the effect of collagen composite wafers compared to the control group. Analysis of re-growth measurements (cm) showed no difference between the right and left antlers regardless of the group effect.

Consequently, further analysis was conducted by collating data from both antlers. It became evident that antler re-growth did not follow a linear pattern; instead, it showed a general trend of slow re-growth up to 30 days, followed by a period of rapid re-growth from day 30 to day 60. The analysis and comparison of the least-squares means for re-growth until 60 days showed a significant difference between the groups, except for T1 and T2. The greatest re-growth

occurred in the control group, followed by T2. The least re-growth occurred in T3; however, the results could vary because eight antlers were excluded from the study. This created a bias towards slower growing antlers in this group as the more rapidly growing antlers reached a commercial length and were subsequently harvested between day 30 and day 60.

Similarly, two from each of T1 and T2, which reached the commercial length, were removed between day 30 and day 60. The results showed a treatment effect with adding a collagen composite wafer reducing antler re-growth between days 1 to 30 and days 30 to 60 for T1, T2, and T3 compared to the control group. During the first 30 days, the control group on average re-grew by 2.8 cm, whereas treatment groups T1, T2 and T3 showed reduced re-growth with averages of 1.9 cm, 1.2 cm, and 1.2 cm, respectively. Re-growth lagged between days 30 and 60 for the treatment groups, particularly in T1 and T3, where re-growth was approximately half that of the control group. T2 showed a very similar re-growth rate compared to the control group, with only a 3.5 cm difference on day 60. The treatment effect could be due to the presence of a collagen composite wafer delaying or altering the complete healing of the wound during antler re-growth. In future re-growth studies, it is recommended a comparison be made of every animal with its own control group rather than between animals due to a considerable variation between individuals in this study.

There were a number of limitations to this study. These could be categorised into study design and methodology constraints, environmental, animal factors, MNT measurements, wafer adhesion, wound healing and antler lengths.

The study was designed in such a way that animals were randomly selected for the whole study; however, bias occurred when the animals were assigned to treatment groups. All the deer were weighed on a single day before the commencement of the study, and it was found that heavier deer had more mature antlers (see chapter 4). No further weight measurements were recorded during the four-month study period. It is unclear whether this relationship existed until the end of the study. Bias was towards deer with antlers that had attained commercially harvestable

length, and these were assigned in priority over a period of 8 weeks. Deer were assigned to the study groups in the following order: control, T1, T2 and T3; i.e., non-randomised. Every experimental day was limited to six animals due to time constraints and human resources. This limited the observation period to 10 hours after antler removal. As mentioned above, the study was designed to be based on a clinical trial with a statistically acceptable number of deer in each group. However, a placebo containing wafers with no medication would have been beneficial to be able to generate a blinded study. This is where we also required a study group with no wafers involved to investigate and compare wound healing to the other groups that contained wafers. We had to choose one of these study groups due to limitations in the number of deer. Future studies would be beneficial to add a placebo wafer to investigate the analgesic effects in a double blinded and randomised manner.

Environmental and temperature changes may have affected the study since the study period was several months long. There is a chance that rises or falls in the temperature influenced MNT, wound healing and antler regrowth results. To avoid such environmental factors influencing the wound healing and regrowth results it is recommended that all of the study group be tested and treated in as shorter a time as possible. However, it was not feasible in this study due to limited personnel, the fact that only two deer could be restrained in the hydraulic crush at a time, which increased the experimentation time, and the number of measurements and sample collected from each animal.

Although the number of deer in each group was sufficient for statistically acceptable results, the animals were difficult to restrain and handle during the study. Even with the use of a hydraulic crush and the help of an experienced technician, it was very difficult to conduct the study using red deer as the animal model because they are less accustomed to such handling than other more domesticated animals such as sheep. Using more docile animal models would help investigate the effect of wafers in more depth and detail. Furthermore, as it was not possible to study all of the groups of animals in parallel, this could increase the possibility of the study bias.

For example, if an animal became more accustomed to the study conditions and procedures, this could have affected the MNT measurement outcome as they might have become less reactive and more comfortable. Conversely, they might become more reactive because they anticipate the stressful situation.

In the first two hours after velvet removal, the deer were subjected to blood sampling (for Chapter 7), algometry and digital photography of the wounds in addition to moving the deer in and out of the crush. The process of completing all the measurements and collecting samples while causing minimal stress to deer and assessors or overlap in testing periods was challenging. In addition, stress caused by animal handling and confinement increased the chance of fight, flight and freezing in the animal; therefore, increasing the likelihood of less MNT measurement and exclusion from the study. Even with additional human resources, algometry was limited to one operator to maintain consistency of the measurements, and the deer could not be restricted indoors, off-pasture for an extended period. In addition, analysis of the treatment effect did not extend to behavioural observations due to the study design and inclusion of confounding factors such as blood sampling from the treatment groups. The other issue with MNT measurements using algometry was the anticipation of the animal when approaching its antlers. This anticipation could have caused inaccuracy in some of the measurements which can be eliminated in the future by using pneumatic or remotely controlled algometers.

Regarding wafer adhesion to the deer antlers, the main challenge we encountered was at the beginning of each experiment day. As discussed earlier, extreme hydration such as bleeding could make the collagen polymers more slippery, therefore less adhesive. This was one of the main reasons why tourniquets were removed after 2 hours from application. The lack of adhesiveness was most evident in the non-modified wafers with no inclusion of nanoparticles. The study groups with nanoparticle inclusion showed better adhesion to the wounds. More study is required to investigate the optimum thickness of the wafers, the best time to remove tourniquets and the inclusion of more compounds to make wafer adhesion even more effective.

Although all the study groups showed complete wound healing at the end of the study, there were limitations in assessing healing during the study. Whilst the presence of collagen wafers as a dressing delivering local anaesthetics on wounded antlers was important, it obscured the healing process occurring underneath making visual assessment very difficult. It was only possible to measure the surface area of the remaining wafers as representative of the actual wound. In the future, obtaining biopsies for histopathological study or creating a reliable multi-factorial scoring system would be an additional method for analysing the wound healing process. Antler regrowth and measurement of its length over time was a feasible procedure by using a stainless-steel ruler from the base of the antlers and measuring the longest growing antler. The study groups had deer in different stages of antler development. However, the antlers had to be at a commercially harvestable length to be saleable. For this reason, some of the deer in the study groups' antlers had to be removed earlier which caused data limitations. In addition, not all the collagen wafers were ready to be applied at first; so, the study started with the control group with no wafers on. In the future, storing all the wafers ready for application for the *in vivo* study would be beneficial, and randomising the groups would eliminate any possible confounding factor. Future studies can be performed by evaluating the effect of treatment on antler regrowth only at the time of commercial harvesting which is usually 55 to 65 days after antler buttons have been cast (according to deernz.org and www.deerfarming.com.au).

It has already been shown in humans that an additional collagen matrix can help in wound healing; however, a further study in deer would be of great benefit to understand the histological details after applying the collagen composites proposed in this study to wounded deer antlers. Detailed wound healing at the cellular level, either by cell culture or histopathology, would be of great value for researchers to study the effects in the future. The study design for antler re-growth had some limitations, and the re-growth was observed to be highly variable in its architecture. Consequently, only the length of the main beam was selected for measurements due to its growth exceeding that of the brow tine. The treatment effect on antler branching was

not assessed during this study, and there is no conclusive knowledge as to whether the collagen composite wafers distorted the behaviour of the antler re-growth.

In some cases, the wafers remained adhered until 60 days, and antler re-growth occurred around its perimeter (Figure 5.10). Furthermore, there were no antler re-growth measurements between day 30 and day 60, which retrospectively coincided with the period of rapid growth. Consequently, it is unclear when the turning point from a slow to a rapid rate occurred during antler re-growth.

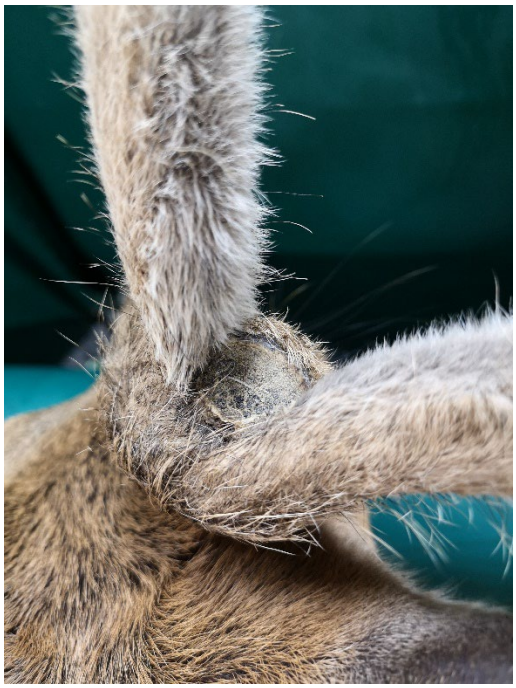


Figure 5.10 Antler re-growth at day 60 in group T2 showing adhered remnant of collagen composite wafer in the centre of the pedicle with main beam and brow tine growing from the periphery.

Future studies are encouraged to investigate the use of modified collagen wafers with both 5% and 25% nanoparticles in various species particularly in farm animals and the equine field. Additionally, further projects could study the mechanical properties of collagen wafers in detail, such as measuring pore sizes and consistency of pore sizes in each produced collagen wafer batch. This consistency would help in mass producing the product in the future and in better anticipation of the results. Another crucial factor would be finding the most suitable way to sterilise the collagen wafers before application such as using gamma radiation. However, investigations are encouraged to study the possible changes in the structure of collagen wafers after sterilisation and sending to the market for use. It is still not clear whether this product should be freely accessible by the farmers or pet owners or should only be dispensed via veterinary prescription. Furthermore, it is suggested that a comprehensive study would be beneficial for the future wafer producers to study the marketing for this product by creating

surveys, questionnaires and direct approaches to the companies, clinics, farmers and even practicing veterinarians in each field.

In terms of improving the infrastructure of the study prototype, future studies should investigate the effects of local anaesthetics in deer, particularly when combined with other biomaterials such as chitosan or chitin related material to provide analgesia for an extended period. There is a lack of extensive research in postoperative pain management in deer after surgical removal of velvet antlers. The MNT obtained from a hand-held algometer for pain response assessment proved successful in this research. However, remotely controlled, or pneumatic algometers which eliminate the anticipation factor by the animal when applying force would be of great value to obtain more objective MNT values with better accuracy. Other techniques such as detecting pain-related cytokines or heat shock proteins could help find a more objective way of measuring pain response. Future study designs that account for environmental effects and extend beyond 10 hours post-velvet antler removal are warranted.

5.5. Conclusion

This study concluded that the collagen wafers, particularly those modified with zinc oxide/PVP nanoparticles, remained attached to the antler wounds more successfully than non-modified collagen wafers for the entire study period. Furthermore, the modified collagen composite wafers, particularly T2, which contained 5% ZnO-PVP, showed the most prolonged analgesia and showed a wound healing rate similar to the control. In addition, all treatment collagen composite wafers reduced antler re-growth in deer compared to the control group.

Chapter 6

Pharmacokinetics of lidocaine and bupivacaine after application of collagen wafers to red deer

Abstract

The pharmacokinetics of lidocaine and bupivacaine, released from a collagen composite wafer, were analysed after application to the wound left after velvet removal. Collagen was extracted from lime split leather biowaste using acetic acid and pepsin extraction, and the resultant wafers comprising of collagen were either modified with zinc oxide-PVP nanoparticles or were free of nanoparticles.

This experiment was conducted in two parts: in the pilot study, 11 yearling stags were given 4% articaine hydrochloride subcutaneous ring blocks before velvet antler removal. Group PT1 (5 deer) had modified (ZnO/PVP nanoparticles) collagen wafers with lidocaine and bupivacaine applied and PT2 (6 deer) had non-modified collagen with lidocaine and bupivacaine applied. The ratio of collagen to zinc oxide-PVP was 2:1 (50%). For the main study, the groups contained collagen composites modified with 25% nanoparticles (T1) and 5% nanoparticles (T2) and control (no nanoparticles) T3. For the pilot study, blood was sampled at 0, 1, 2, 4, 6, 8, 12, 24 hr, and for the main study, blood was sampled at 0, 1, 2, 4, 6, 8, 24 hr after the application of the collagen wafers. The pharmacokinetic parameters maximum concentration (C_{max}), time of maximum concentration (T_{max}), area under the curve to both 24 hours and infinity ($AUC_{(0-24)}$), $AUC_{(0-\infty)}$, area under the first moment curve ($AUMC_{(0-\infty)}$), half-life, the volume of distribution, and clearance were analysed using PKsolver add-in macro for Microsoft Excel. The results from the pilot study were not analysed due to lack of adhesion of the wafers to most of the antlers. For the main study, treatment groups modified with zinc oxide-PVP nanoparticles (T1 and T2) exhibited lower C_{max} (-415.89 ng/ml for T1, and -27.14 ng/ml for T2 for lidocaine, and -1172.56 ng/ml for T1, and -773.92 ng/ml for T2 for bupivacaine), $AUC_{(0-24}$ (-5347.58 ng/ml*hr for T1, and -4192.28 ng/ml*hr for T2 for lidocaine, and -12832.82 ng/ml*hr for T1, and -13993.73 ng/ml*hr for T2 for bupivacaine), $AUC_{(0-\infty)}$ (-5717.57 ng/ml*hr for T1, and -3585.21 ng/ml*hr for T2, and -2090.07

ng/ml*hr for T1, and -802.63 for T2 for bupivacaine), for both lidocaine and bupivacaine compared to T3 values (no nanoparticles). Furthermore, T1 and T2 showed higher levels for the volume of distribution and clearance parameters compared to T3.

These results show that the novel treatment method for red deer containing the local anaesthetics, lidocaine and bupivacaine in a modified collagen composite with 5% or 25% concentrations of ZnO-PVP have a sustained-release and slow systemic absorption with much lower C_{max} than unmodified collagen wafers. The low C_{max} indicates that systemic side effects of local anaesthetics are unlikely.

6.1. Introduction

Local anaesthetics are preferred over systemically injected analgesics due to decreased probability of side effects by reduced systemic absorption as compared to drugs injected. Knowledge of C_{max} after local administration is essential for comparison with known toxicity levels. The amount of local anaesthetic which can be applied to the site of action can be increased if the release from the application site is slow and leads to low plasma concentrations.

Lidocaine's analgesia lasts only for the mean duration of 60 to 90 minutes in case of antler removal in deer (Wilson and Stafford, 2002, Wilson *et al.*, 2001), whilst the long-acting local anaesthetics such as bupivacaine HCL, do not exceed more than 8 hours in human and veterinary practice after a single administration (Jirasiritham *et al.*, 2004, Chester *et al.*, 1990, Møiniche *et al.*, 2002, Sahebjam, 2017). One of the main determinants of the duration of action of local anaesthetics is persistence of drugs at the site of action rather than whole body and their protein binding affinity. The higher the affinity to proteins, the longer the local anaesthetic is bound to nerve membranes; thereby resulting in more prolonged analgesia. As an example, the affinity of the local anaesthetic is high with serum proteins such as α1 acid glycoproteins or other proteins, which reduces the amount of free drug circulating in the animal (Buckenmaier and Bleckner, 2005). Since, lidocaine has a shorter duration of action, as stated earlier, a need for an analgesic system to extend the period in which local anaesthetics can remain active and effective at the target site.

Lidocaine is more short-acting than bupivacaine and has a faster onset of action (Valvano and Leffler, 1996) due to its lower p*K*a compared to bupivacaine, which means that onset is reduced in higher pH levels. Furthermore, the more closely the p*K*a approximates tissue pH levels, the faster the onset of action provided by local anaesthetics (Weiniger *et al.*, 2012, Stevens *et al.*, 1989). Therefore, in theory, a combination of a fast-acting local anaesthetic with a short onset of action (such as lidocaine) with a long-acting local anaesthetic with a moderate onset (such as bupivacaine) could lead to faster onset and longer acting analgesia (Valvano and Leffler, 1996).

The pharmacokinetics of lidocaine and bupivacaine (individually and not given together) has been extensively investigated in various species.

Mather *et al*. (1994) compared the pharmacokinetic parameters of bupivacaine enantiomers across three dosing regimens in adult Merino ewes: bolus injection (30 mg in 6 sec); infusion of 60 mg in 15 min; and infusion of 60 mg in15 min, followed by constant rate infusion of 60 mg/hr for 4 hrs (Mather *et al.*, 1994). They found that the overall clearance in the R(+) form was higher than the S(-) form and was higher in bolus injection compared to infusion. Bupivacaine molecule is a racemic mixture of (R)- and (S) stereoisomers. R in terminology means Rectus which in Latin means right and S means Sinister which in latin means Left. Molecules that rotate the plane polarised light to right is said as R isomer. In chemistry, any molecule that rotate the plane polarised light to left is said as S isomer. Mather *et al.* also found that the apparent volume of distribution for R(+) was significantly higher than S(-) bupivacaine. Though no significant difference was seen between the half-lives of the enantiomers, and the pharmacokinetic parameters obtained were independent of dose (Mather *et al.*, 1994). In their later study, Mather *et al.* (1998) investigated the differences in pharmacokinetic parameters of bupivacaine enantiomers in adult Merino ewes after bolus intravenous administrations of either 1 minute at a low adverse risk dose or 3 minutes at a high adverse risk dose (Mather *et al.*, 1998). Similar to their previous study, they concluded that pharmacokinetic parameters were not dose-dependent in either enantiomer of bupivacaine. Their analyses resulted in a significantly higher clearance in R(+) bupivacaine compared to S(-) bupivacaine (Mather *et al.*, 1998).

There is a paucity of pharmacokinetic data for local anaesthetic use in deer. Recently, Venkatachalam *et al.* (2018) studied the pharmacokinetic parameters of articaine hydrochloride and its metabolite, articainic acid, in red deer following subcutaneous administration. They quantified the pharmacokinetic parameters, including the mean maximum concentration of articaine (1013.9 ng/ml), time of mean maximum concentration (0.17 hr), and elimination half-life (1.12 hr) (Venkatachalam *et al.*, 2018).

i. Lidocaine and Bupivacaine formulations:

There are many different formulations of local anaesthetics. Examples include EMLA® topical cream, which contains lidocaine and prilocaine in an emulsion comprising of macrogol-glycerol-hydroxy-stearate, carboxy-poly-methylene, sodium hydroxide, and purified water (Van Oostrom and Knowles, 2018), EXPAREL® which contains bupivacaine as a liposomal injectable solution (Hu *et al.*, 2013), and Xaracoll® a bupivacaine collagen implant (Cusack *et al.*, 2013), which has been developed to manage postoperative pain. Liposome-encapsulated lidocaine (Mashimo *et al.*, 1992) can be formulated in egg yolk phosphatidylcholine and cholesterol. In Mashimo's study, the prolonged analgesic effect of liposomal lidocaine was studied following epidural blocks in dogs, and they concluded that the mean T_{max} of liposomal lidocaine occurred seven minutes later than free lidocaine (Mashimo *et al.*, 1992). Similarly, EMLA, which consists of a combination of lidocaine and prilocaine in a topical cream, was tested in dogs of different breeds, and their reaction to placement of an IV catheter was investigated. They discovered that EMLA cream could reduce the aversive behaviour of catheter placement for up to 60 minutes (Van Oostrom and Knowles, 2018). Although this result showed the effectiveness of topical anaesthetics over a short period of time, it is not a suitable candidate for long term pain management. A study conducted by Hu *et al.* (2013) investigated the different pharmacokinetic parameters between four different bupivacaine formulations: injectable liposomal formulation (EXPAREL), bupivacaine HCL, bupivacaine HCL in combination with adrenaline, and placebo (normal saline). Patients undergoing four different surgical procedures (inguinal hernia repair, knee arthroplasty, bunionectomy, and haemorrhoidectomy) were randomly given one of the four bupivacaine formulations. Their results demonstrated that liposomal bupivacaine administration provided sustained drug release for up to 96 hours after application. The half-life of liposomal bupivacaine was 12 hours, compared to bupivacaine hydrochloride 0.6 hours. This is a result of the encapsulation of bupivacaine with liposome which limits the drug uptake and thus provides a sustained release over time. It was noticed that, in the inguinal hernia repair group, EXPAREL

(266 mg bupivacaine) produced a C_{max} of 365 ng/ml compared to Marcain® (100 mg bupivacaine) which produced a C_{max} of 336 ng/ml. Although the calculated C_{max} was very similar across both EXPAREL and Marcain groups for the inguinal hernial repair, the area under the curve (AUC), measured up to four times greater in the liposomal form compared to bupivacaine HCL. The liposomal bupivacaine assessed on four different surgical procedures displayed a C_{max} of 935 ng/ml, which was below the toxic threshold range of 2000-4000 ng/ml. This formulation appeared unlikely to cause any unwanted cardiotoxic and neurotoxic side effects (Hu *et al.*, 2013).

With regard to lidocaine, the adverse effects are expected to occur at 6000 ng/ml; however, they are mainly observed when plasma levels exceed 10000 ng/ml (Scott, 1975, Tucker and Mather, 1979, Richard *et al.*, 2011a). A study conducted on sixty pigs investigated the interaction of lidocaine with EXPAREL after subcutaneous administration (Richard *et al.*, 2011a). Their study consisted of 20 groups with three pigs in each, and each group received either a combination treatment, EXPAREL or lidocaine (4 mg/kg). Their control groups consisted of groups 1 and 2, which only received EXPAREL treatment, and groups 3 and 4 only received lidocaine (0.2 ml/kg). The drug combination groups received 0.2 ml/kg lidocaine followed by a repeated dose of EXPAREL 0.27ml/kg at 5, 10, 20 and 40 minutes. The drug dosage was similar across the three treatment groups at five time points including time zero. The mean systemic plasma concentration of lidocaine and bupivacaine did not significantly change when EXPAREL was administered 20 min or longer compared to lidocaine or EXPAREL when they were injected alone. However, there was a significant increase observed in bupivacaine levels when lidocaine administration was followed by EXPAREL within 5 and 10 minutes compared to the control groups. For example, a 1640% increase for C_{max} and a 48% increase for $AUC_{0\text{-}24}$ was calculated in lidocaine-EXPAREL® compared to the lidocaine injections (Richard *et al.*, 2011a).

XaraColl® is the latest FDA approved (20th August 2020) product that contains collagen encapsulated bupivacaine and is licensed for pain relief in people after implantation in the

abdominal region (Cusack *et al.*, 2013). The primary objective for producing XaraColl® was to deliver postoperative analgesia after hysterectomy in women. They found that XaraColl® could prolong analgesia up to 72 hours after the surgery. The pharmacokinetic study revealed a biphasic release pattern of bupivacaine in this trial and in their previous preliminary trials. The peaks occurred after 2 hours and after 12 -24 hours, with higher concentrations observed in the second peak. The researchers stated that the biphasic pattern or double-peak phenomenon was potentially helping to replenish and boost local anaesthetic levels in blood circulation in the following hours. They also found the following pharmacokinetics for bupivacaine released from collagen implants; Cmax = 0.22 (µg/ml) (=220 ng/ml), tmax = 12.5 hours, t1/2 = 10.1 hours, and AUCinf = 6.36 (µg*hr/ml) (Cusack *et al.*, 2013). The Xaracoll study is the closest match to the intention for drug release from collagen composites investigated in this study. Liu *et al*. (2021) also noticed the double-peak phenomenon using poly (D, L-lactic acid-co-glycolic acid) (PLGA) microspheres to deliver palonosetron, and antiemetic medication in cancer patients and the study group which showed the double-peak phenomenon also had a great prolonged release (Liu *et al.*, 2021d).

The objective of this study was to investigate the pharmacokinetic parameters for lidocaine and bupivacaine released from a collagen-nanoparticle composite after its application on the wound created by surgical antler removal in red deer.

6.2. Materials and methods

6.2.1. Pilot Study

i. Animals

The pilot study was conducted from late October 2018 until January 2019 at Massey University Deer Unit, Palmerston North, New Zealand. The study was approved by Massey University Animal Ethics Community (MUAEC) (18/86).

The study design was as described in Chapter 3. Whole blood was sampled from the treatment groups, at 0, 1, 2, 4, 6, 8, 12, 24 hr after applying collagen wafers on the cut surface of the antlers. The samples were collected from the jugular vein via an 18-gauge needle into 10 ml BD vacutainer® lithium heparin blood collection tubes (Becton, Dickinson and Company, New Jersey, USA). All the samples were immediately centrifuged at 12000X RPM for 5 minutes (Thermo Fischer Scientific™, Heraeus™, Megafuge™ 8 small Benchtop, UK), and plasma samples were aliquoted using disposable Pasteur Pipettes into the 2 ml safe lock tubes (Eppendorf®, Germany) and stored at -80°C until liquid chromatography-mass spectrometry (LC-MS) analysis.

Eighteen healthy 1-year-old male red deer were selected from the Deer Unit population based on the maturity of their antlers. Only yearling stags with harvest-ready antlers (commercial length) were used to prevent wastage. The deer weighed between 110–140 kg and were kept as a single herd for the entire research period. They had *ad-libitum* food and water whilst in the paddock. The deer were herded into the handling facility for each experiment day; the herd was divided into small groups of six for each experimental day for ease of animal handling and safety. During the experiment, two animals were guided through a hydraulic crush with side curtains (Heenan Work Room, Farmquip, New Zealand) for safe physical restraint. The deer were pre-acclimatised to the handling facility and the hydraulic crush from previous treatments such as vaccination and ear tagging.

ii. Study design

The pedicle circumference of each antler was measured using a cloth measuring tape and tourniquets were applied at the base of the antlers. These were removed after 2 hours in all animals. All the deer received a ring block with articaine hydrochloride (SCI Pharmtech, Taoyuan, Taiwan) (40mg/mL) at the base of both antlers at a dose of 1 ml/cm of pedicle circumference (Figure 6.1). Atricaine was used to provide analgesia without interfering with the assays. The efficacy of local anaesthesia was tested by using the 2-mm round stainless-steel tip of the handheld algometer (FPX 25, Wagner Instruments, Greenwich, CT, USA) as a noxious stimulus at the site of impending antler removal. A nick test was performed to confirm that analgesia was adequate immediately before removal, and the antlers were surgically removed by using a saw (Wells handsaw, New Zealand). The treatment groups were subjected to an additional procedure of applying collagen wafers (Figure 6.2) containing lidocaine and bupivacaine for postoperative pain management immediately after the removal of the antlers (see chapters 2 and 5 for the formulation methods). One treatment group (PT1) contained modified collagen wafers with zinc oxide-PVP nanoparticles, and the other treatment group (PT2) included collagen wafers with no nanoparticles, and the control group did not receive any wafer treatment at all.

iii. Sample collection

Only treatment groups were recruited for the pharmacokinetic study. Whole blood was sampled from the treatment groups, at 0, 1, 2, 4, 6, 8, 12, 24 hr after applying collagen wafers on the cut surface of the antlers. The samples were collected from the jugular vein via an 18-gauge needle into 10 ml BD vacutainer® lithium heparin blood collection tubes (Becton, Dickinson and Company, New Jersey, USA). All the samples were immediately centrifuged at 12000X RPM for 5 minutes (Thermo Fischer Scientific™, Heraeus™, Megafuge™ 8 small Benchtop, UK), and plasma samples were aliquoted using disposable Pasteur Pipettes into the 2 ml safe lock tubes (Eppendorf®, Germany) and stored at -80°C until liquid chromatography-mass spectrometry (LC-MS) analysis.

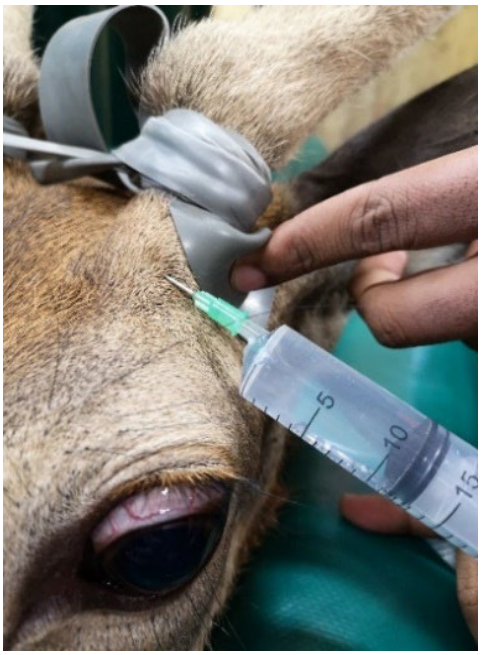


Figure 6.1 Articaine (4%) hydrochloride subcutaneous ring-block injections in deer for local anaesthesia of the antler before antler removal.

Figure 6.2 manufacturing collagen-nanoparticle wafer prototype containing lidocaine and bupivacaine, and application on the wound surface area of deer antler in our study. The tourniquets, shown in the picture, were removed after 2 hours in all animals.

6.2.2. Main study

i. Animals

The second animal study was conducted from late October 2019 to February 2020 at the Massey University Deer Unit located in Palmerston North, New Zealand. The application was approved by MUAEC (19/70).

As described for Chapter 6 pilot study for blood sample except that the 12h sample was omitted. Thirty healthy 1-year-old male red deer weighing (mean ±SD) 113.35 ±10.02 kg, minimum 84.4 and maximum 136.5 kg. These thirty animals were also used in the other studies involved in the PhD project. The animals were kept in paddocks at Massey University Deer Unit for the entire research period, and they were herded into the handling facility for each experiment day. The deer had *ad-libitum* to food and water whilst staying in the paddocks. During the experiment, two animals were guided at a time through a hydraulic crush with side curtains (Heenan Work Room, Farmquip, New Zealand) for safe physical restraint.

ii. Study design

The animals were divided into three groups; T1 (collagen wafer modified with 25% zinc oxide-PVP), T2 (collagen wafer modified with 5% zinc oxide-PVP), and T3 (collagen wafer with no nanoparticles involved). The study design and sample collection was the same as the pilot study, except that the 12h sample was omitted.

6.2.3. Chemicals and reagents

Lidocaine HCl and bupivacaine HCl standard powders were purchased from Hunan Russel Chemicals Technology Co. Ltd, (China), and procainamide HCl was purchased from Sigma Aldrich® (Germany). The lidocaine HCl and bupivacaine HCl standard solutions were made in ultrafiltered, deionised water (Milli-Q PFplus system, Millipore Cooperation, USA), and procainamide HCl standard solution was made in methanol. All the standards were made daily. Formic acid (0.1%) was prepared by mixing 1000 µl formic acid (100% formic acid, Fischer Scientific®, Belgium) in 1-litre mass spectrometry water (Fischer Scientific®, Belgium). Acetonitrile (Optima™, LC/MS Grade, Fisher Chemical™, Fischer Scientific®, Belgium) was used as an organic solution purchased from Sigma Aldrich® (Germany) to be used in mobile phase.

6.2.4. Sample preparation

Plasma sample preparation was performed using a positive pressure (Biotage®, PRESSURE+, Uppsala, Sweden), solid-phase extraction (SPE) procedure with Phenomenex Strata-X™ 60 mg/3 ml Polymeric Reversed-Phase SPE cartridges (Phenomenex). A 200 µl aliquot of plasma was allocated for t0 and t24 samples due to their predicted low drug concentrations. For t1, t2, t4, t6, t8, and t12, a 20 µl aliquot of plasma was diluted with 180 µl blank plasma to reduce the concentrations to 10% in order to fit in the limits of the machine. The samples were vigorously vortex-mixed with 200 µl 10% trichloroacetic acid (TCA). The samples were then centrifuged at 13000 RPM for 10 minutes. The supernatant sample was separated and loaded into the SPE cartridge that had been pre-conditioned with 1 ml acetonitrile and then equilibrating with 1 ml Milli-Q water. The next step was loading the centrifuged samples (350 µl) into the SPE tubes and washing them with 1 mL methanol: water (20:80), followed by drying under high pressure for 10 minutes. The samples were eluted with 1 ml acetonitrile into glass tubes. They were evaporated until dry using a Thermo Scientific Speedvac™ system (approximately 1 hour) and were reconstituted with 200 µl Milli-Q water. After final centrifugation (13000 RPM, 5 min), the

samples were transferred to the autosampler, with an injection volume of 5 µl in triplicate into a Liquid-Chromatography Mass Spectrometry system (LC-MS).

6.2.5. Liquid-Chromatography-Mass-Spectrophotometry (LC-MS)

i. Instrumentation

Liquid chromatography was carried out using an ultra-high-performance liquid chromatography system equipped with a quaternary pump, a vacuum degasser, a column compartment, and an autosampler (Dionex Ultimate 3000 System: Thermo Scientific, Germering, Germany). Mass spectrometric detection was performed on a hybrid quadrupole-orbitrap mass spectrometer (Q Exactive Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer; Thermo Scientific, Bremen, Germany) with an electrospray-ionisation interface, positive ion mode.

ii. Chromatographic conditions

Chromatographic separations were achieved using a 2.6 µm particle size C-18 column (100 mm × 2.1 mm; Accucore, Auckland, NZ) coupled with a security guard column (Defender Guard Column; Accucore) maintained at a temperature of 25°C. The mobile phase consisted of 0.1% formic acid and acetonitrile (70:30, v/v) and was delivered at a flow rate of 0.3 mL/minute. The precursor ions of lidocaine (235.18 g/mol), bupivacaine (288.43 g/mol), and procainamide (235.32 g/mol) were included in the target list. They were fragmented into their respective daughter ions using a collision energy of 35eV, which were detected using a resolution of 35,000 FWHM. Data processing was performed using the Xcalibur™ data system (Thermo Scientific™, USA).

iii. Linearity

The linearity of the measurements was assessed by running five different concentrations of lidocaine and bupivacaine in Milli-Q water; 50, 25, 12.5, 6.25, and 3.125 ng/ml in triplicate. Pre-spiked samples were produced by spiking the plasma with six concentrations from stock solution, and post-spiked (rehydration by Milli-Q water and spiking it with different drug concentrations) were prepared in deer plasma. The term pre-spiking means adding standards and internal

standards prior to sample preparation, whereas, post-spiking means adding standards and internal standards after sample preparation. These data were subjected to linear regression in Xcalibur™ software and were analysed using Microsoft Excel software (version 365).

iv. Accuracy and Precision

The linearity of the LC-MS method was determined by linear regression analysis of the samples, which were prepared by dilution of bupivacaine and lidocaine in blank deer plasma. Procainamide was used as an Internal Standard (IS) in all the samples, using 20 µl from a 1000 µg/ml stock solution to reach a final concentration of 100 µg/ml in deer plasma.

Calibration curves were constructed using three replicates of bupivacaine and lidocaine in LC-MS water with concentrations between 3.12 to 50 ng/ml. Intra-day and inter-day precision and accuracy of the method were determined by processing three replicates.

v. Specificity and Recovery

Blank plasma from 10 untreated red deer was extracted and analysed to assess the specificity of the SPE and LC-MS method. The extraction recovery was assessed by comparing the pre and post spiked samples with bupivacaine, lidocaine and, procainamide as an internal standard.

6.2.6. Statistical analysis

i. Pharmacokinetic parameters

For both the animal studies, PK solver (version 2.0) (Zhang *et al.*, 2010) as an add-in software in Microsoft Excel (version 365) was used for the analysis of the pharmacokinetics of lidocaine and bupivacaine in the treatment groups. The pharmacokinetic parameters were calculated using a non-compartmental analysis approach, using the plasma concentration over time curve, parameters such as maximum concentration (Cmax) of lidocaine and bupivacaine in deer plasma, and time to reach Cmax (Tmax). The terminal phase (λz) rate constant was calculated by linear regression of the logarithmic plasma concentration in six time points, and the terminal half-life was calculated as $t_{1/2\lambda z} = \ln_2 / \lambda z$.

The area under the curve (AUC) and the area under the first moment curve (AUMC) were determined using the linear trapezoidal method. The mean residence time was calculated as:

MRT = AUMC/AUC

The concentration-time curves were created with semi-log plots, having one axis plotted on a logarithmic scale and the other linearly. This method could lead us for a more accurate result.

ii. Comparison of pharmacokinetic parameters

Statistical analyses were performed by transferring calculated values by PkSolver addon on Microsoft Excel into SAS (version 9.4) and using GraphPad Prism LLC, version 8.3.0 to create graphs. The dependent variable, drug concentration (ng/ml) was analysed using MIXED procedure on SAS and incorporating a linear mixed model for repeated measures as samples were collected from the same animal in different time points. The main effects were evaluated with a model that included the fixed effect of treatment and time and the random effect of the animal to account for repeated measures on the same animal. The interaction between time and treatment was evaluated with the same model as before but including the fixed effect of interaction between time and treatment. The repeated measures on the same deer were modelled with a compound symmetry error structure. Outliers were discarded when they were three standard deviations away from the mean. Estimates with $P<-0.05$ were considered significant from zero.

6.3. Results

6.3.1. Wafer adhesiveness

i. Pilot study

Only one deer out of 12 from the treatment groups had collagen wafers on both antlers. Thus, no PK analysis was performed.

ii. Main study

Out of 30 animals, 18 animals were subjected to the pharmacokinetic analysis across three treatment groups (T1, T2, T3) as only the animals which had wafers on the antlers for the planned timeline were included. In T1 (25% NP), 18 wafers out of 20 successfully remained adhered on the antlers (90% adhesiveness), in T2 (5% NP), 14 wafers out of 20 successfully remained adhered on the antlers (70% adhesiveness), and in T3 (0% NP or no nanoparticles involved), nine wafers out of 20 successfully remained adhered on the antlers (45% adhesiveness). However, only the deer which had both wafers on the antlers for entire sample period were included in the pharmacokinetic study; T1 = 9 deer, T2 = 6 deer, and T3 = 3 deer. Therefore, any deer that lost wafers from either left or right or both antlers were excluded from the study to avoid any bias or error.

6.3.2. LC-MS method development and sample preparation

The standard curves for both local anaesthetics (lidocaine and bupivacaine) were linear for concentrations between 3.125 ng/ml and 50 ng/ml (correlation coefficient or $R^2 > 0.99$). The lowest limit of detection (LOD) for lidocaine and bupivacaine was 1 ng/ml for both drugs. The lowest limit of quantification (LOQ) for both local anaesthetics was 3.125 ng/ml. Table 6.1 depicts the results obtained using the mobile phase, and table 6.2 shows the results obtained from deer plasma, including the recovery rate (%) calculated by the formula below:

Extraction recovery (%) =

$$\frac{pre{-}spikedconcentrations(addedconcentrationsbeforeSPEpreparation)}{Post{-}spikedconcentrations(spikedconcentrationsafterSPEpreparation)}$$

Table 6.1 Inter-day and intra-day variation of LC-MS method for analysis of bupivacaine (a) and lidocaine (b) standard solutions. Solution of known concentrations was analysed from the absorbance measurements (mAu) in triplicate on three different testing days. The mean and relative standard deviation (RSD%) was calculated from the actual concentrations measured for both inter-day and intra-day. mAu= milli-absorbance unit.

6.1a Bupivacaine (mobile phase): LC-MS

Concentration Spiked (ng/ml)	Inter-day Area (mAu)	Intra-day Area (mAu)	Inter-day Variation RSD%	Intra-day Variation RSD%
50	7599.2	7534.8	4.2	9.3
25	3405.3	3769.8	8.3	2.9
12.5	1431.1	1648.6	6.5	8.2
6.25	677.9	1026.6	13.7	13.7
3.125	284.3	475.0	7.5	12.4

6.1b Lidocaine (mobile phase): LC-MS

Concentration Spiked (ng/ml)	Inter-day Area (mAu)	Intra-day Area (mAu)	Inter-day Variation RSD%	Intra-day Variation RSD%
50	11401.8	11159.5	2.9	0.8
25	5503.5	6332.8	10.7	9.2
12.5	3049.7	2767.9	5.9	5.8
6.25	1541.9	1504.2	13.9	13.3
3.125	687.5	633.6	11.7	10.6

Table 6.2 Inter-day and intra-day variation of LC-MS method for analysis of bupivacaine (a) and lidocaine (b) in deer plasma. The mean and % relative standard deviation (RSD) was calculated from the actual concentrations measured from three alternating days. The extraction recovery rate (%) was calculated by dividing measured pre-spiked (added concentrations before SPE preparation) sample concentrations over the measured post-spiked standard concentrations (spiked concentrations after SPE preparation).

6.2a Bupivacaine (plasma): LC-MS

Concentration spiked (ng/ml)	Measured post-spiked concentration (ng/ml)	Measured pre-spiked concentration (ng/ml)	Inter-day Concentration Measured (ng/ml)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%	Recovery Rate (%)
50	45.2	48.6	41.4	49.1	2.6	3.8	93.2
25	23.4	26.9	21.8	24.9	3.8	6.8	86.8
12.5	12.8	17.5	11.2	14.4	4.2	1.5	73.0
6.25	7.6	12.5	6.4	8.9	2.5	5.7	60.7
3.125	5.2	8.8	5.8	4.6	19.7	19.3	59.7

6.2b Lidocaine (plasma): LC-MS

Concentration Spiked (ng/ml)	Measured post-spiked concentration (ng/ml)	Measured pre-spiked concentration (ng/ml)	Inter-day Concentration Measured (ng/mg)	Intra-day concentration Measured (ng/ml)	Inter-day Variation RSD%	Intra-day Variation RSD%	Recovery Rate (%)
50	53.9	49.9	58.0	49.7	3.6	6.9	107.8
25	26.9	26.5	29.8	24.0	8.6	5.2	101.5
12.5	13.9	16.7	14.4	13.3	5.4	3.7	82.9
6.25	10.7	13.0	12.4	9.2	7.5	5.9	83.0
3.125	6.8	9.8	7.9	5.7	6.0	10.5	69.1

6.3.3. Pharmacokinetic analysis

For the main study, the semi-log plot of the concentration-time curve for lidocaine and bupivacaine in three treatment groups after applying collagen wafers are shown in Figures 6.3 and 6.4. The means of the pharmacokinetic parameters are shown in tables 6.3, and 6.4 (refer to details of each individual animal in tables 9.11-9.17). For the concentration-time curve of each individual animal before conversion to semi-log plot refer to appendix B (Figures 9.3-9.5). Refer to Table 9.19 and 9.20 for the least-square means (±SEM) of bupivacaine and lidocaine concentrations across treatment groups in the main *in vivo* pharmacokinetic study without considering the lost wafers.

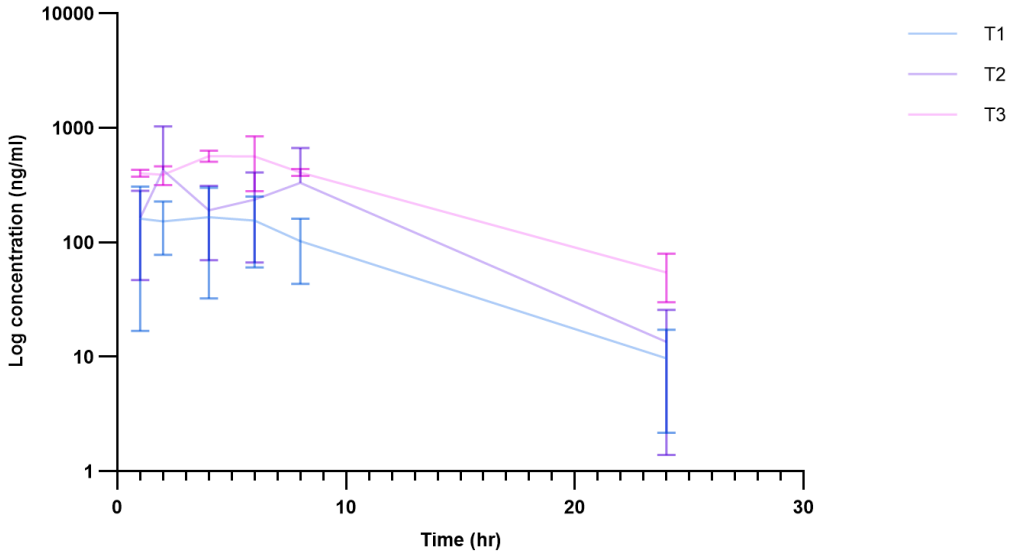


Figure 6.3 Semi-log plot of plasma concentration-time (log (ng/ml)/hr) curve with ±SD for lidocaine after applying collagen wafers at 136 mg/wafer in the main study in red deer (just a reminder that wafers included both drugs with identical concentration). T1=treatment group with 25% ZnO-PVP, T2=treatment group with 5% ZnO-PVP, T3=treatment group with no ZnO-PVP. Each data point represents 9 animals for T1, 6 for T2, and 3 for T3.

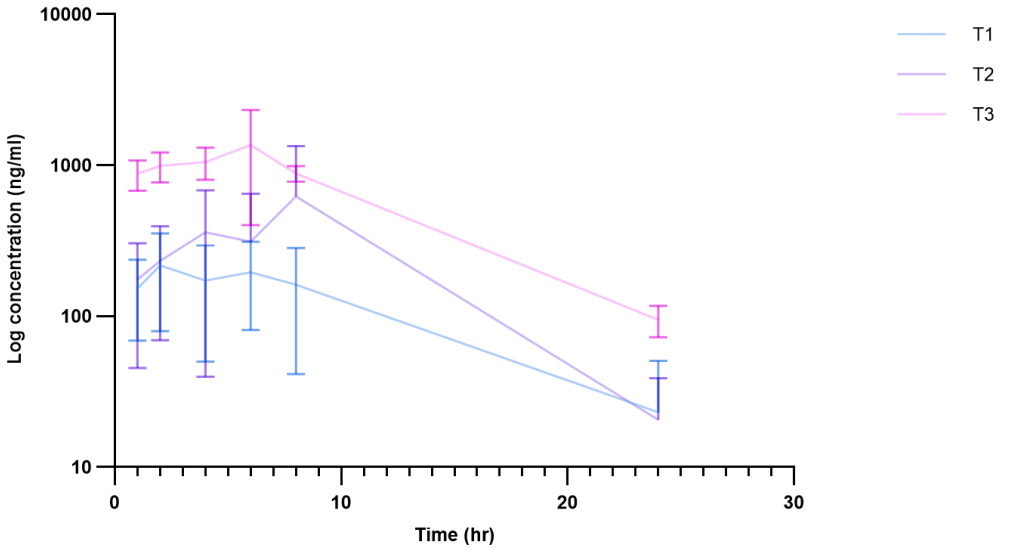


Figure 6.4 Semi-log plot of plasma concentration-time (log (ng/ml)/hr) curve with ±SD for bupivacaine after applying collagen wafers at 136 mg/wafer in the main study in red deer (just a reminder that wafers included both drugs with identical concentration). T1=treatment group with 25% ZnO-PVP, T2=treatment group with 5% ZnO-PVP, T3=treatment group with no ZnO-PVP. Each data point represents 9 animals for T1, 6 for T2, and 3 for T3.

In the main study, means (±SD) of body weight for T1, T2, and T3 were 119.28 ±2.31, 108.69 ±2.33, and 107.92 ±2.40, respectively. Lidocaine C_{max} did not show any statistically significant difference between the groups. However, for bupivacaine, the C_{max}, in T1 was less than T3 (P<0.001), and in T2, less than T3 (P<0.05). Lidocaine T_{max} did not show any statistically significant difference between the groups. Bupivacaine T_{max} of T1 was significantly less than T2 (P<0.05). For AUC $_{(0\text{-}24)}$ three outliers were excluded from lidocaine and bupivacaine. Lidocaine AUC $_{(0\text{-}24)}$ in T1 was significantly less than T3 (P<0.0001), and T2 was significantly less than T3 (P<0.001). Bupivacaine AUC $_{(0\text{-}24)}$ in T1 was significantly less than T3 (P<0.0001), and T2 was significantly less than T3 (P<0.0001). For AUC $_{(0\text{-}\infty)}$ three outliers were excluded from both lidocaine and bupivacaine. Lidocaine AUC $_{(0\text{-}\infty)}$ in T1 was significantly less than T3 (P<0.0001), and T2 was significantly less than T3 (P<0.05). Bupivacaine AUC $_{(0\text{-}\infty)}$ in T1 was significantly less than T3 (P<0.0001), and T2 was significantly less than T3 (P<0.0001). Lidocaine AUMC $_{(0\text{-}\infty)}$ in T1 was significantly less than T3 (P<0.05). Bupivacaine AUMC $_{(0\text{-}\infty)}$ in T1 was significantly less than T3 (P<0.001), and in T2 was significantly less than T3 (P<0.001). None of the other parameters such as half-life, mean residence time, the volume of distribution, and clearance showed any statistically significant difference between T1, T2 and T3.

Table 6.3 Noncompartmental analysis of lidocaine in the main study after applying the collagen wafers on the wound surface area of deer antlers. Each wafer contained 136 mg drug (lidocaine and bupivacaine). These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, LSMeans=Least-squares means, T1=treatment group with 25% ZnO-PVP, T2=treatment group with 5% ZnO-PVP, T3=treatment group with no ZnO-PVP (n=9 deer in T1, n=6 deer in T2, n=3 deer in T3).

Group	*Parameters*								
	C_{max}	T_{max}	$AUC_{(0-24)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	*Cl*
Units	*ng/ml*	*hr*	*ng/ml*hr*	*ng/ml*hr*	*ng/ml*hr^2*	*hr*	*hr*	*ml/kg*	*ml/hr/kg*
T1	236.8	3.9	2061.0	2089.9	15308	4.74	7.4	12896	1800
Mean ±SD	±152.2	±0.8	±1286.6 [b]	±604.3 [b]	±10049 [a]	±1.0	±1.3	±2844.8	±373.5
T2	625.5	5.8	3216.4	4222.2	27667	5.27	7.7	14845	1806
Mean ±SD	±186.4	±1.0	±1011.7 [b]	±128120 [b]	±21317 [ab]	±2.1	±2.7	±6805.2	±893.5
T3	652.7	4.7	7408.6	7807.4	66727	5.39	8.5	2532	326
Mean ±SD	±263.6	±1.4	±2206.7 [a]	±1046.7 [a]	±17405 [b]	±1.7	±2.2	±5505.1	±722.8

Table 6.4 Noncompartmental analysis of bupivacaine in the main study after applying the collagen wafers on the wound surface area of deer antlers. Each wafer contained 136 mg drug. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, LSMeans=Least-squares means, T1=treatment group with 25% ZnO-PVP, T2=treatment group with 5% ZnO-PVP, T3=treatment group with no ZnO-PVP (n=9 deer in T1, n=6 deer in T2, n=3 deer in T3).

Group	Parameters								
	C_{max}	T_{max}	$AUC_{(0-24)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
Units	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*hr^2	hr	hr	ml/kg	ml/hr/kg
T1 Mean ±SD	369.3 ±152.2 [b]	4.1 ±0.8 [a]	3105.2 ±1286.7 [b]	3372.1 ±685.2 [b]	39255 ±11394 [b]	6.6 ±1.1	9.4 ±1.4	8204 ±3225.7	965 ±423.5
T2 Mean ±SD	767.2 ±186.4 [b]	7.0 ±1.0 [b]	1944.3 ±1011.7 [b]	2084.6 ±1282.0 [b]	14200 ±21317 [b]	4.2 ±2.1	6.8 ±2.7	8486 ±6805.2	1300 ±893.5
T3 Mean ±SD	1541.1 ±263.6 [a]	5.3 ±1.4 [ab]	15938 ±2206.7 [a]	17452 ±1282.0 [a]	138391 ±21317 [a]	5.0 ±2.1	8.0 ±2.7	1103 ±6742.4	150 ±885.3

6.3.4 Clinical observations

No deer showed any adverse effects at any time.

6.4. Discussion

This is the first study reporting the pharmacokinetic parameters of lidocaine and bupivacaine after their application on the wound created after antler removal. The current practice of antler removal in New Zealand involves surgical removal of deer antlers under lidocaine local anaesthesia (Wilson *et al.*, 1999, Wilson *et al.*, 2000). The current recommendations do not include postoperative pain management or wound healing management. The objective of the pilot study was to evaluate the adhesiveness of the collagen wafers and whether the local anaesthetics were released from the collagen matrix when exposed to blood. The purpose of the study (pilot and main studies) was to evaluate the release and extent of systemic absorption of lidocaine and bupivacaine from the collagen nanocomposite matrix to assess the likelihood of systemic toxicity. In both the studies (pilot and main studies), the antlers were removed under articaine local anaesthesia, which is a deviation from the approved protocol of velvet antler removal in New Zealand. Articaine was chosen to avoid interference during the LCMS analysis, as the articaine chromatographic peak does not interfere with lidocaine or bupivacaine peaks.

Eighteen animals out of thirty from the study were included in calculating pharmacokinetic parameters. These animals had collagen wafers adhered to both the antlers for 24 hours. The group with the highest concentration of nanoparticles (T1; 25% ZnO-PVP) had the lowest C_{max} and highest T_{max} both for lidocaine and bupivacaine. In the case of bupivacaine, the T1 group had significant differences for both C_{max} and T_{max} compared to T2 (5% ZnO-PVP) ($P<0.05$) and T3 (0% ZnO-PVP) ($P<0.001$). The findings from T_{max} analysis showed slower drug release, which remained in the safety range and did not show any sign of sudden release. These results are of valuabale, specifically for bupivacaine, which can be cardiotoxic at more than 2000 ng/ml and neurotoxic when it exceeds 4000 ng/ml (Macfarlane *et al.*, 2021, Yu *et al.*, 2017, Rice *et al.*, 2017). The C_{max} for lidocaine was much lower (653ng/mL in T3) than documented toxic concentrations (5300 ng/ml (Mehra *et al.*, 1998)). The results from T1 showed concentrations approximately 7 to 10-

fold less than thresholds at which toxic signs are anticipated to occur. These results align with the second *in vitro* study in which drug release was slower (12298, and 12745 (ng/ml) √min^{-1}, for bupivacaine and lidocaine, respectively) when the collagen composites were modified with higher concentrations of zinc oxide-PVP. The T_{max} in T1 was shorter than that of T2 and T3, but most of the concentration / time curves had double peaks, so the relevance of this is unclear. This release pattern could be due to the large surface area to volume ratio in the wafers due to the nanostructure, which leads to initial desorption of a proportion of drugs at the surface of the modified polymers (Nkansah *et al.*, 2008, Hammady *et al.*, 2009). Both the nanoparticle groups (T1 and T2) had double peaks, which could be due to the release of unbound drug which would be sitting outside the matrix. This is commonly observed in the pharmacokinetics of slow and extended-release formulations (Cusack *et al.*, 2013) which is the closest formulation to the collagen wafers designed in this study. Cusack *et al.* (2013) considered the double peak phenomenon using Xaracoll (containing collagen as a vehicle) for drug delivery of bupivacaine to be a suitable release pattern for sustained drug delivery which cause better analgesia compared to normal release patterns. Liu (2021) *et al.* also noticed the double peak phenomenon but in an antiemetic drug called palonosetron with a PLGA. Their results showed that one of their treatment groups containing a water-oil-water PLGA microsphere which had a double peak phenomenon and showed a suitable sustained-release (they also called it double depo sustained release) of palonosetron *in vitro* (Liu *et al.*, 2021d). The more consistent release in T2 and T3 indicated better candidates for sustained release. These results showed that bupivacaine, as a longer-acting local anaesthetic compared to lidocaine, reached its maximum concentration at a later timepoint in T2 compared to T1 and T3. The C_{max} occurring later in T2 correlated with a slower recovery rate from analgesia.

The area under the plasma concentration curve, $AUC_{(0\text{-}24),}$ is directly equal to the amount of drug absorbed (Price and Patel, 2020). This parameter represents the extent of systemic body exposure to a drug until the last plasma samples were collected at 24 hours (see Tables 6.3 and

6.4). Bupivacaine and lidocaine both showed higher $AUC_{(0-24)}$ in T3 compared to T2 and T1, and T1 had the lowest value. Proportionally, $AUC_{(0-24)}$ in T3 was approximately 8 times higher than that of T2 and 5 times higher than T1 for bupivacaine (see Tables 6.3 and 6.4). For lidocaine, the $AUC_{(0-24)}$ for T3 was approximately 3.5 times higher than T1 and two times higher than that of T2 (see Tables 6.3 and 6.4). These results demonstrate a significantly lower amount of lidocaine and bupivacaine absorbed in T1 compared to the other groups. In other terms, lidocaine and bupivacaine in this drug delivery system were meant to remain locally at the wound to create the most effective analgesia possible. Systemic absorption of the drugs would eliminate lidocaine and bupivacaine from the site and decrease their regional concentrations. Since the aim was minimal systemic absorption, low AUC indicates suitability for local drug release. However, parameters such as clearance also affect AUC, which in this case were higher in T1 and T2 than T3. The other hypothesis for lower measurement of AUC could be the retention of the drugs in the collagen wafers that could not be released over the course of experiment. In previous studies, in products such as liposomal bupivacaine (bupivacaine with DepoFoam as a vehicle), the AUC was increasingly higher when higher doses were added to the carrier; however, the AUC was approximately at similar levels when bupivacaine was used without any carrier. The higher AUC levels for DepoFoam was explained to be due to increasing C_{max} and T_{max} levels (Bramlett *et al.*, 2012). In another study, the AUC for a novel pro-liposomal ropivacaine oil was investigated in flank incision sites of pigs, and this treatment elicited a much higher AUC as compared to ropivacaine-only systemically (Davidson *et al.*, 2016). This increase could also be explained by a much longer T_{max} in the novel formulation compared to the plain ropivacaine, the region where the formulation was applied, and the oil nature of the formulation (Davidson *et al.*, 2016). In our study, the T_{max} was not much different between the groups; however, C_{max} was much lower in the nanoparticle groups (T1 and T2) compared to T3. There is still a lack of evidence whether decreased AUC levels are caused by a slower release of local anaesthetics at the site and whether

the antler tissue exhibits a different release pattern than those applied to other tissue sites, for which the majority of formulations have been investigated.

The area under the curve was extrapolated to infinity ($AUC_{(0-\infty)}$) using the last measured plasma concentrations of lidocaine and bupivacaine divided by the terminal slope. The values calculated for $AUC_{(0-\infty)}$ in the main study groups were also close to the value obtained from $AUC_{(0-24)}$. The results showed the estimation of less than 2% difference comparing $AUC_{(0-\infty)}$ to $AUC_{(0-24)}$, supporting the primary feature of the study design that the last time point of blood collection to be 24 hours in all treatment groups. The values calculated for the area under the moment curve ($AUMC_{(0-\infty)}$) showed that lidocaine in T1 was significantly lower than T3. In terms of bupivacaine, T1 and T2 were significantly lower than T3. There were no statistical differences in the terminal half-life between the treatment groups of either of the drugs in this study which could be explained because of the very large standard deviation in the measurements. T1 and T2 showed higher clearance for both lidocaine and bupivacaine compared to T3, which means that both drugs were eliminated from the body more rapidly in T1 and T2 as compared to T3 (see Table 6.3 and 6.4). The only difference between T1 and T2 against T3 was the incorporation of ZnO-PVP nanoparticles. The phenomenon that increased clearance of the local anaesthetics, , whether the clearance of lidocaine and bupivacaine might be impacted by the presence of zinc oxide nanoparticles requires more investigation in the future.

Regarding the volume of distribution (Vd) and drug clearance (Cl), no significant difference was observed between the groups but T1 and T2 were very similar and T3 trended lower (see tables 6.3 and 6.4). The higher Vd levels in T1 and T2 may indicate that lidocaine and bupivacaine had a higher tendency to move to extravascular compartments (Mansoor and Mahabadi, 2020) as compared to T3. As discussed above, lower C_{max} levels in T1 and T2 might have been due to higher Vd values in T1 and T2. In this study, due to the aim of local drug delivery and local analgesia, the volume of distribution had minimal effect on the efficacy of lidocaine and bupivacaine at the level of deer antlers but could influence systemic adverse effects.

One of the main limitations of this study was the blood collection procedure in deer. Blood collection from a relatively unhandled domesticated animal is complicated and needs sufficient skills, equipment, and patience. Animal welfare was considered paramount at all times. Catheter placement in the facial vein was initially used but movement in the hydraulic crush, such as head rubbing against the walls, made it impossible to keep the catheter in place until the end of the blood collection (24 hrs). Therefore, the blood collection method was replaced by intermittent venepuncture of the jugular with a Vacutainer. Deer were herded into the hydraulic crush every time we needed to collect blood samples, causing stress to the animals. Therefore, the number of blood collections was reduced to six collections between 0 and 8 hrs, followed by a final blood sample at 24 hours. This 16-hour gap in blood collection could potentially comprise valuable information in elimination kinetics, and a further study performed on fewer animals with more frequent collections may be warranted.

This study suggests that using a novel formulation that releases the local anaesthetics slowly is very unlikely to compromise safety. Local anaesthetics are ideal candidates for postoperative pain management in animals such as deer; however, to maximise their analgesic effects and reduce their potential toxic side-effects, they need to be contained in a formulation that does not allow them to disperse rapidly to the body. Collagen modified with zinc oxide-PVP nanoparticles successfully reduced the maximum plasma concentrations of lidocaine and bupivacaine to well below toxic levels, indicating slowed systemic absorption. In particular, the collagen composites modified with 25% and 5% of the ZnO -PVP nanoparticles (T1, and T2) showed more favourable pharmacokinetic parameters compared to the group with no modification (T3). T2 had longer T_{max} for lidocaine and particularly for bupivacaine which could explain the longer analgesia measured by MNT in the *in vivo* study. More research is required to study the pharmacokinetic parameters of lidocaine and bupivacaine to provide increased knowledge of their systemic absorption, distribution and excretion in red deer, particularly when combined with matrices such as collagen to achieve a sustained drug release. Furthermore, the collagen matrix can be

improved to hold the drug for a more extended period, or a higher dose of local anaesthetic can be loaded into the matrix to enhance the duration of analgesia to 24 hours or more.

However, slow release of drugs from analgesic wafers applied to the antler wounds is likely to result in slower depletion of residues from meat. This may make this strategy unsuitable for spikers which are commonly slaughtered soon after velvet removal. Residue studies would be required before this type of use.

6.5. Conclusion

The pharmacokinetic parameters of lidocaine and bupivacaine released from a collagen-zinc oxide PVP composite to the wounded deer antler surface area were described. The groups which received collagen composites modified with 25% (T1) and 5% nanoparticles (T2) exhibited a better safety profile with lower C_{max} values, although all measured C_{maxs} were well below published toxic concentrations. Decreased values of $AUC_{(0-24)}$, $AUC_{(0-\infty)}$, and $AUMC_{(0-\infty)}$ for both lidocaine and bupivacaine for T1 and T2 as compared to T3 could be explained by increased levels of clearance and retention of the drugs within the collagen wafers. High levels of Vd in T1 and T2 could be the reason for much lower C_{max} levels in T1 and T2, indicating a greater safety margin. These results suggest that lidocaine and bupivacaine in a modified collagen composite with 5% or 25% concentrations of ZnO-PVP has a reliable sustained release with low C_{max} levels in red deer.

Chapter 7

General discussion

i. Main results:

Velvet antler removal of Red Deer is an important significant surgical procedure in the New Zealand deer farming. Currently, only two techniques are used within the industry. The chemical method is the administration of subcutaneous local anaesthetic, lidocaine HCl; a short acting local anaesthetic, as a ring block around the base of antlers, which results in analgesia for 1-2 hrs (Wilson and Stafford, 2002, Wilson *et al.*, 2001). The non-chemical method utilises the NaturO™ analgesic system, produced and patented by AgResearch, New Zealand. This system uses a specialised rubber ring and cable tie to induce analgesia by pressure on the nerves and vascular system before removal of velvet in one-year-old stags prior to slaughter. Both techniques have been shown to be effective for perioperative pain management. Furthermore, NaturO™ is only permitted to be applied on the antlers within 72 hours prior to slaughter (DeerQA, 2019).

The surgical method of velvet antler removal poses a problem of post operative pain and wound management. The current research attempted to address these two problems by application of a nanocomposite collagen wafers containing lidocaine and bupivacaine on the wound surface. This novel method provided a long-lasting analgesia ranging from 8 to 10 hours after application to wounded antlers, particularly with the nanocomposite collagen wafers as compared to the control group with no application on wound surface. Controlled drug delivery systems can reduce the need for frequent or high dosing. It has been suggested for some time that the future of drug delivery will focus on targeted local delivery (Langer, 1998, Li and Mooney, 2016, Zewdu *et al.*, 2021, Kim and Woo, 2021, Alruwaili *et al.*, 2021, Liu *et al.*, 2021b).

One of the main priorities was finding a composite material that was biodegradable, biocompatible, weakly antigenic, easily applicable, affordable, and effective. Collagen was selected in this study due to its high biocompatibility with mammalian tissues (Shantha and Panduranga Rao, 1993, Panduranga Rao, 1996) and it's biodegradable nature (Panduranga Rao,

1996), which makes this matrix an optimal platform for drug delivery system. In addition, collagen is the most abundant mammalian protein, making up approximately 30% of all body proteins.

Collagen as a biopolymer has been a reliable source for biomedical applications since the 1970s and 1980s (Friess, 1998), and it has a versatile structure that can be modified with a minimal cost into many forms such as hydrogel, gel, wafer, patch, dressing etc. for various purposes (Friess, 1998). Biopolymers can be derived from renewable sources and are considered more suitable for wound dressings than synthetic products (Mogoşanu and Grumezescu, 2014). In particular, collagen (particularly collagen type 1) can be extracted from the waste products of industries such as the leather industry by using chemical and enzymatic hydrolysis (Zavareze *et al.*, 2009).

The adhesiveness of collagen wafers on the wound surface improved with the addition of ZnO nanoparticles. Zinc oxide nanoparticles are known for their adhesive properties and are commonly incorporated into medical adhesive dressings (Gao *et al.*, 2017). The adhesion of the collagen wafers was not 100% as one and three animals lost wafers in 25% and 5% ZnO groups, respectively. In contrast, less than 50% of the treatment group with non-modified collagen composite wafers remained adhered to the wounds. Sufficient adhesion and contact surface area against the wound surface area are crucial in order to simultaneously absorb exudate and release local anaesthetics to the tissue and provide analgesia. Further work is required to improve the adhesive of these wafers to be accepted at the farm level.

The results from the pharmacokinetic analysis showed a biphasic peak (double peak phenomenon) in drug release, which was particularly evident in the treatment groups with modified collagen composite (5% and 15% ZnO-PVP). Furthermore, the biphasic peak was more pronounced in the modified collagen composite group with 5% ZnO-PVP (T2) compared to 25% ZnO-PVP. Previously, Cusack *et al.* (2013) stated that the biphasic nature could be considered an advantage in sustained drug release as it replenishes levels of local analgesia. The maximum plasma concentration (C_{max}) following application of the modified collagen wafer confirmed that the release of lidocaine and bupivacaine were far less than toxic limits. The difference between

groups was more pronounced when comparing C_{max} of bupivacaine. C_{max} was the lowest for the treatment with 25% ZnO-PVP (T1) and highest in the non-modified treatment group (T3). Low levels of maximum drug concentration achieved in this study allow future studies to confidently increase the amount of drugs applied in order to reach an optimum therapeutic level or help in delaying T_{max}.

ii. Limitations

The *in vivo* study experimental design presents several limitations, such as difficulty in conducting a double-blind study as there were not enough animals available to include a placebo treatment group. The control group did not have any wafers applied to the wounded antlers; therefore, making the study blind for the pain assessor was difficult.

The torniquet is applied before the injection of local anaesthetic and removed after 5 to 10 minutes of antler removal. In the current research the torniquet remained for 2 hours post antler removal due to the nature of study design. Therefore, it is important to test the efficacy of these wafers in the real farm conditions.

Another possible confounding factor is that there could be overlaps between the analgesic effects of articaine, tourniquets and the actual collagen wafers. All the groups apart from the control group that did not have any collagen wafers, experienced these factors, which removed the confounding effect throughout the study. It is not ethically acceptable to conduct a painful procedure without perioperative pain management. The best control for these sorts of experiments is current best treatment, i.e., ring block with a local anaesthetic. Although we used articaine to avoid interference with lidocaine assays, this should have had minimally different effects on post operative analgesia. Having an analgesic overlap was better for the animals, and the effort in this study was to minimise discomfort. It is recognised that this overlap of effects potentially masked some of the MNT responses attributed to the bupivacaine and lidocaine's effects in the shorter term.

The assessment of wound healing in the current study was difficult. The collagen composite wafers covered the wound surface area, obscuring the ability to observe the ongoing healing process underneath. In addition, the persistence of the wafer made it challenging to capture digital images of the wound healing process for further analyses. This prevented the effective measurement and analysis of wound healing during the whole experiment. Although there were problems measuring wound healing, it was fast in all groups.

iii. Future studies

The collagen wafers developed in the current research needs further improvements to increase the adhesiveness, and duration of analgesia.

The adhesiveness can be improved by incorporating biodegradable glues in these wafers or using other application methods on the wound surface. The duration of analgesia can be increased by increasing the crosslinking in the wafers to further reduce the rate of drug release or increase the concentration of the local anaesthetics, which is more feasible in large animals such as deer, cattle and horses.

Future studies are required to test the efficacy of these wafers in other species including small and large animals. All the animals get superficial wounds, and these collagen wafers could be an excellent first aid treatment for such wounds. These wafers will reduce the blood loss while providing pain relief to the affected animal.

General conclusion

In vitro studies proved that adding lidocaine and bupivacaine to a modified collagen matrix containing ZnO PVP nanoparticles resulted in sustained drug release compared to the control group containing the drug only. Further assessment of the effect of nanoparticles on drug release resulted in three levels of ZnO PVP being selected for *in vivo* trials.

The results from the *in vivo* experiment showed that a modified collagen wafer containing 5 and 25% ZnO PVP nanoparticles and local anaesthetics lidocaine and bupivacaine could provide haemostasis and produce superior extended pain relief, as measured by pain thresholds, when compared to other treatment groups.

Velvet antler production requires deer to undergo a painful procedure, and currently, there is no long-acting pain relief available for the postoperative period. This raises concerns regarding animal welfare and places the industry at risk of public scrutiny. The proposed modified collagen composite wafer, which contains lidocaine and bupivacaine, provides a postoperative pain relief solution for up to 10 hours after removal of the velvet antlers. The proposed collagen wafer also has potential in human emergency medicine. The modified collagen composite wafer can be stored conveniently at room temperature and is easy to apply in a farm setting immediately after the velvet antler has been removed. It is hoped the promising results obtained in this study will lead to additional studies to further extend analgesia with a better adherence for deer antler removal as well as explore other market opportunities. The proposed collagen composite wafer in this study has the potential for mass-production and could be adopted by the deer industry in New Zealand to improve animal welfare.

Appendix A

Plasma sample preparation techniques

Plasma sample preparation techniques

➢ *Pilot studies for plasma method development*

These pilot studies aimed to investigate the most suitable method of plasma precipitation to increase linearity and the percentage of the drug extraction. In all the methods, one set of standards (1000, 500, 250, 125, 62.5 ng/ml) were used, and one set of plasma (450 µL) spiked with bupivacaine and lidocaine (50 µL) to prepare the total of 500 µL solutions. They were vigorously vortex mixed for 10 seconds.

- *Method 1: Protein precipitation with TCA 10% and Phenomenex StrataX SPE tube*

Protein precipitation was achieved by adding 2 ml of 10% trichloroacetic acid (TCA). The mixtures were shaken for 10 minutes on the shaker and the tubes were centrifuged with 4500 RPM for 10 minutes. The supernatant (2.2 ml) was collected and added to StrataX tubes (Phenomenex®, Strata™-X 33 µm Polymeric Reversed Phase, 60 mg / 3 mL, Tubes, 50/Pk) on the vacuum system. The recommended StrataX Solid Phase Extraction (SPE) method provided by Phenomenex® was used in filtration of the plasma through the SPE tubes. The steps consisted of the following:

1) Condition: 1 mL(s) of Acetonitrile
2) Equilibrate: 1 mL(s) of Water

3) Load: Sample (2.2 ml of supernatant containing TCA, plasma and drugs)

4) Wash: 1 mL(s) of (20:80) Methanol: Water

5) Dry: 10 mins under full vacuum

6) Elute: 1 mL(s) of Acetonitrile

Then, the elutes were moved to a Thermo Scientific Speedvac™ system for drying the samples. Then, 200 ml of mobile phase was added to the tubes to reconstitute the samples, and they were centrifuged at 4500 RPM for 5 minutes, and the aliquots were moved to the HPLC (Shimadzu, Japan) autosampler injection.

- *Method 2: SPE with Phenomenex StrataX SPE tube, and no protein precipitation*

Samples were taken from both the wash stage and elute stage for investigation of drug loss. No drug was found at the wash stage. The spiked plasma samples and the blank sample were mixed with 2mL sodium acetate 100 mM (pH = 5.3) before loading. The following steps were performed:

1) Condition: 2 mL(s) of Acetonitrile

2) Equilibrate: 2 mL(s) of Water

3) Load: Sample (plasma mixed with sodium acetate 100 mM) in addition to washing the walls of the StrataX SPE tubes with 1 ml of sodium acetate 100 mM

4) Wash: 1 mL(s) of (20:80) Methanol: Water

5) Dry: 10 mins under full vacuum

6) Elute: 1 mL(s) of Acetonitrile

Then, the samples were centrifuged at 14000X RPM for 10 minutes, and, finally, the supernatants were transferred to the autosamplers of the HPLC.

- *Method 3: Only protein precipitation with HCL 1M and TCA 100%*

Vortex-mixing the samples each for 10-15 seconds and centrifuging the samples at 4000X for 10 minutes. Then, transferring the supernatants to SpeedVac and finally re-diluting them with 500 µl of NaOH 1M. The samples were transferred to the autosampler for HPLC analysis.

- *Method 4: Liquid/Liquid protein precipitation with Diethyl Ether*

Addition of 250 ml NaOH 1M, then 3 ml Diethyl Ether and vortex mixed 3-4 times for 5 minutes and centrifuge with 4000X RPM for 30 minutes. Then, carefully collect the liquid at the upper phase of diphasic liquid formation and transfer them to SpeedVac. Then, re-dilution was performed by adding 500 µl of mobile phase and transferring them to the autosampler for HPLC analysis.

- *Method 5: Protein precipitation with perchloric acid (PCA)*

First, 450 µl plasma sample was spiked with 50 µl of drug from the stock solution. Then, 200 µl perchloric acid 4M (ice cold) was added to the spiked plasma and was vortex mixed and centrifuged with 4000X RPM for 30 min. The supernatant was drawn, and KOH 2M (ice cold) was added slowly, every time 10 µl up to 60 µl (the generation of bubbles was stopped, and salt precipitated). Then the supernatant was centrifuged again and moved to the speedvac. Eventually, the dried samples were rehydrated by adding 500 µl of mobile phase before sending to the HPLC autosampler.

- *Method 6: The effect of pH on SPE*

The groups were divided into four including lidocaine and bupivacaine with pH~3 by adding 25 µl HCL 1M to 500 µl spiked water; and lidocaine and bupivacaine with pH~11 by adding 25 µl NaOH 1M to 500 µl spiked water. The results from the sample step, wash step and elute step with acetonitrile, and at the end, with formic acid, 2% in acetonitrile were analysed. The steps were performed as follows:

1) Condition: 2 mL(s) of Acetonitrile

2) Equilibrate: 2 mL(s) of Water

3) Load: Sample (water spiked with drug)

4) Wash: 2 mL(s) of (20:80) Methanol: Water

5) Dry: 10 mins under full vacuum

6) Elute 1: 1 mL(s) of Acetonitrile

7) Elute 2: 1 mL of Formic acid 2% in acetonitrile

- *Method 6: Protein precipitation with HCL 1M*

After making sure acidity has more recovery in the elutes, HCL 1M was used in this method. 25 µl HCL was added to the spiked plasma samples (50 µl drug from the stock solution to 450 µl plasma); they were vigorously vortex-mixed and sonicated for 10 minutes. Then, the samples

were centrifuged in 4000X RPM for 10 minutes, and the supernatants were used for SPE (with StartaX SPE tube). The elutes were delivered to the SpeedVac, and the samples were hydrated with 500 µl mobile phase. Finally, the samples were sent to the autosampler for HPLC analysis.

- *Conclusion and discussion*

For protein precipitation and extraction, various methods were incorporated, and most of them failed to yield a high recovery rate for spiked plasma samples. In the early steps, the yield after protein precipitation and drying the samples by SpeedVac (the original volume of 500 spiked plasma) trichloroacetic acid or TCA (10%) showed the highest rate of recovery (more than 100%), then methanol (100%) (recovery of about 60%) and finally acetonitrile (100%) (recovery of about 10%) was the least. In addition to TCA, HCl also showed a great result in improving the recovery rate using SPE (almost 100%).

Comparing two different Phenomenex SPE tubes, plasma samples that were passed through Phenomenex StrataX® showed higher drug recovery compared to Phenomenex Phree® tubes and direct centrifuged plasma spiked with drugs. Regarding the SPE StrataX, the steps were as follows: the pre-treatment step with sodium acetate buffer 100 mM with pH 5.8, the conditioning step with acetonitrile 100%, the equilibrate step with water, loading the sample, the wash step with Methanol 20% and collecting the elutes with acetonitrile in glass tubes. Based on these experiments, the drugs were spiked in water, and the same method used in plasma was performed. The samples were collected from three SPE steps being sample, wash and elute. The pre-treatment step was eliminated from this experiment since using buffer lowered the recovery dramatically in our previous tests. The results obtained from the experiments showed that although no drug was found in the sample step, the wash step with methanol 20% had the loss of drug to some extent, and the elute had the highest concentration of the drugs as expected. The chromatograms showed that very low pH (achieved either by HCl or TCA) necessitated higher recovery using the SPE method. Furthermore, TCA had the advantage of optimum protein

precipitation, which was not observed when HCl was used. Therefore, TCA was the best candidate for increasing the recovery rates.

Appendix B

Figures

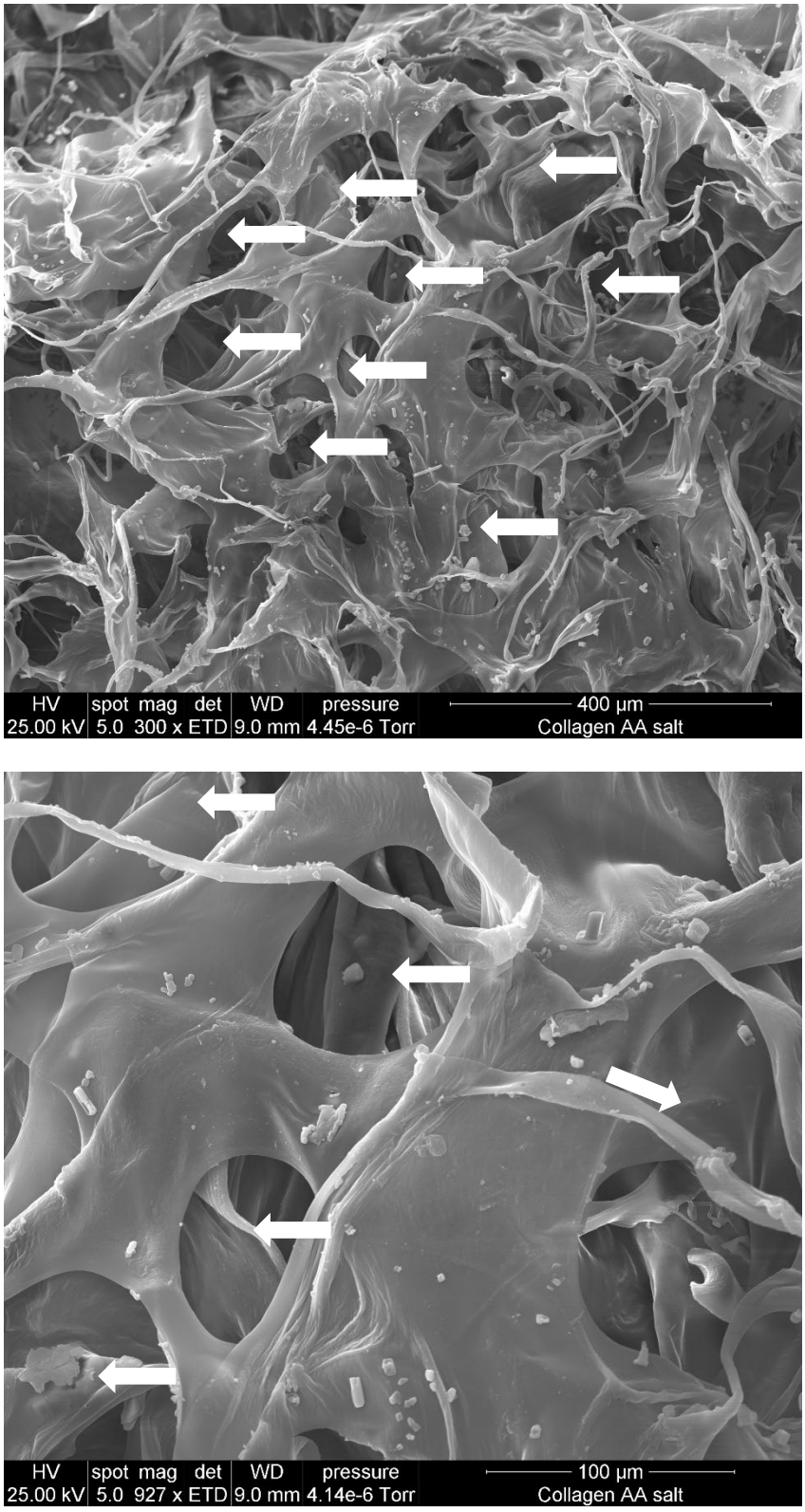


Figure 8.1 Microstructure and the pores (shown with white arrows) in collagen matrix with two magnifications. The collagen matrix structure was extracted by acetic acid and precipitated by salt for the pilot study. The scale bars are located at the right bottom of each image.

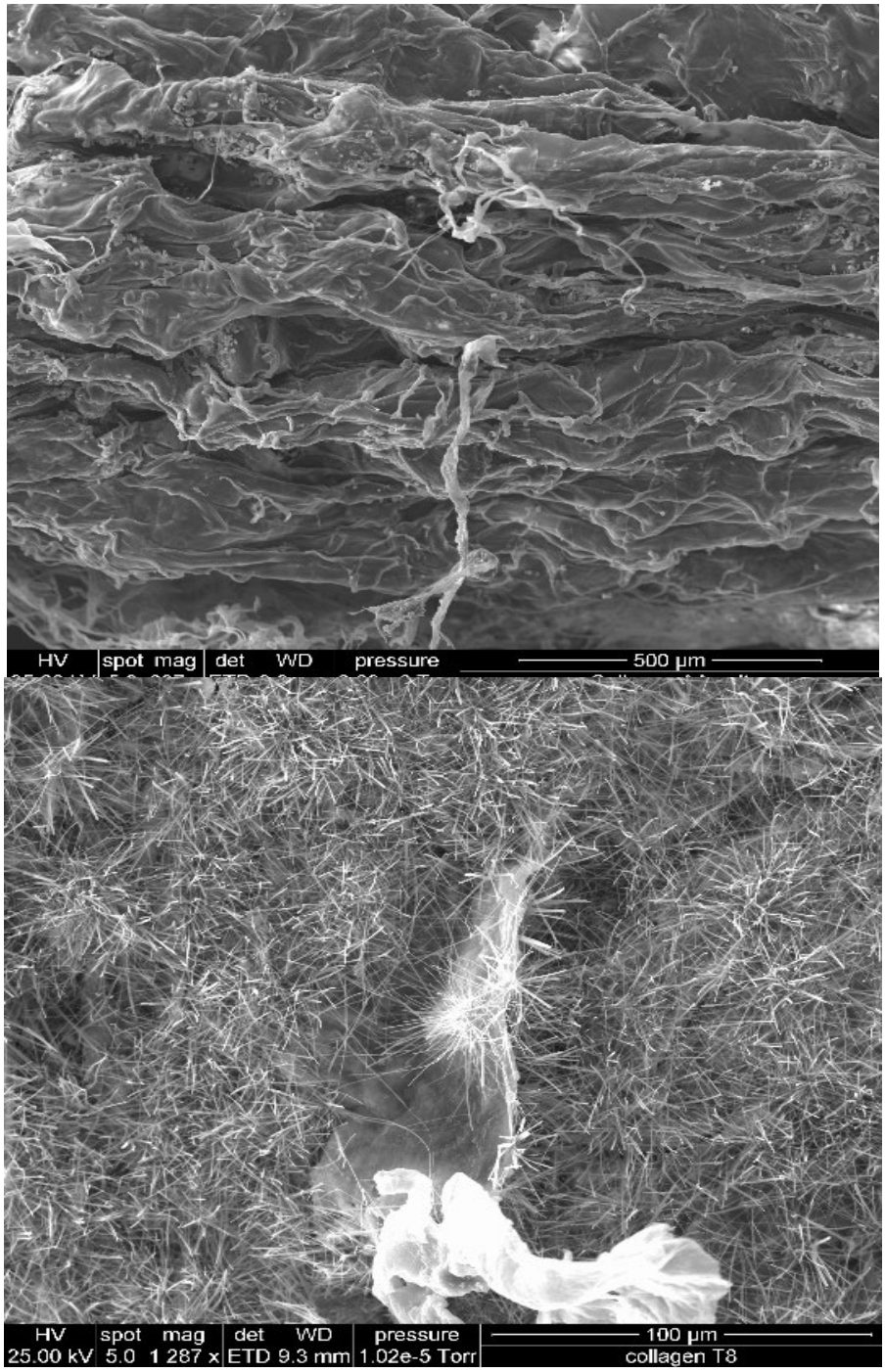


Figure 8.2 The image above illustrates the intact collagen. The image below depicts the denatured and changed structure after exposure to deer plasma, proving the drug release from the microstructures.

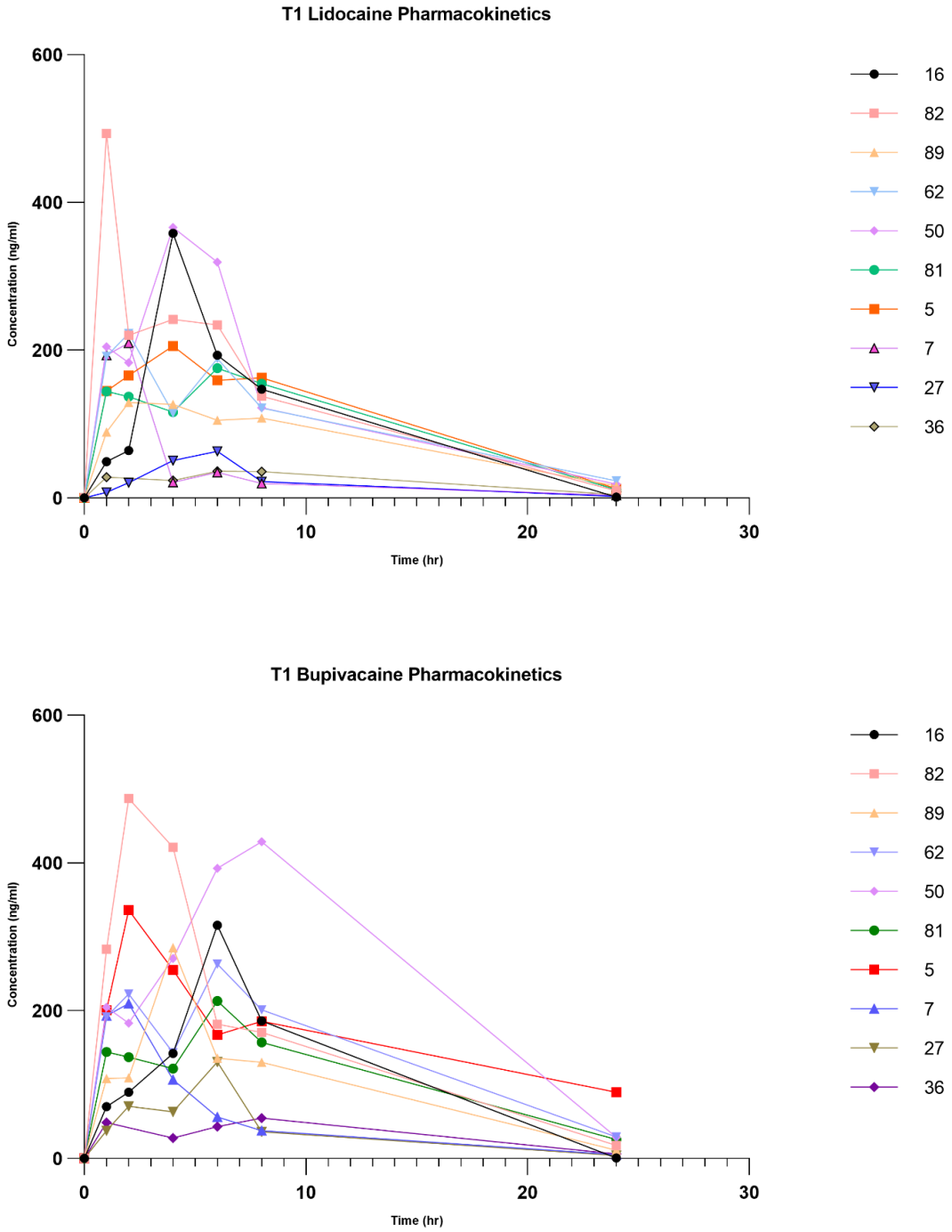


Figure 8.3 Concentration time curve for lidocaine and bupivacaine after applying treatment wafers in group T1 with 25% zinc oxide-PVP. Each colour represents an individual animal identification tag number, and each data point represents the concentration of drug present in a plasma sample taken at a given time point.

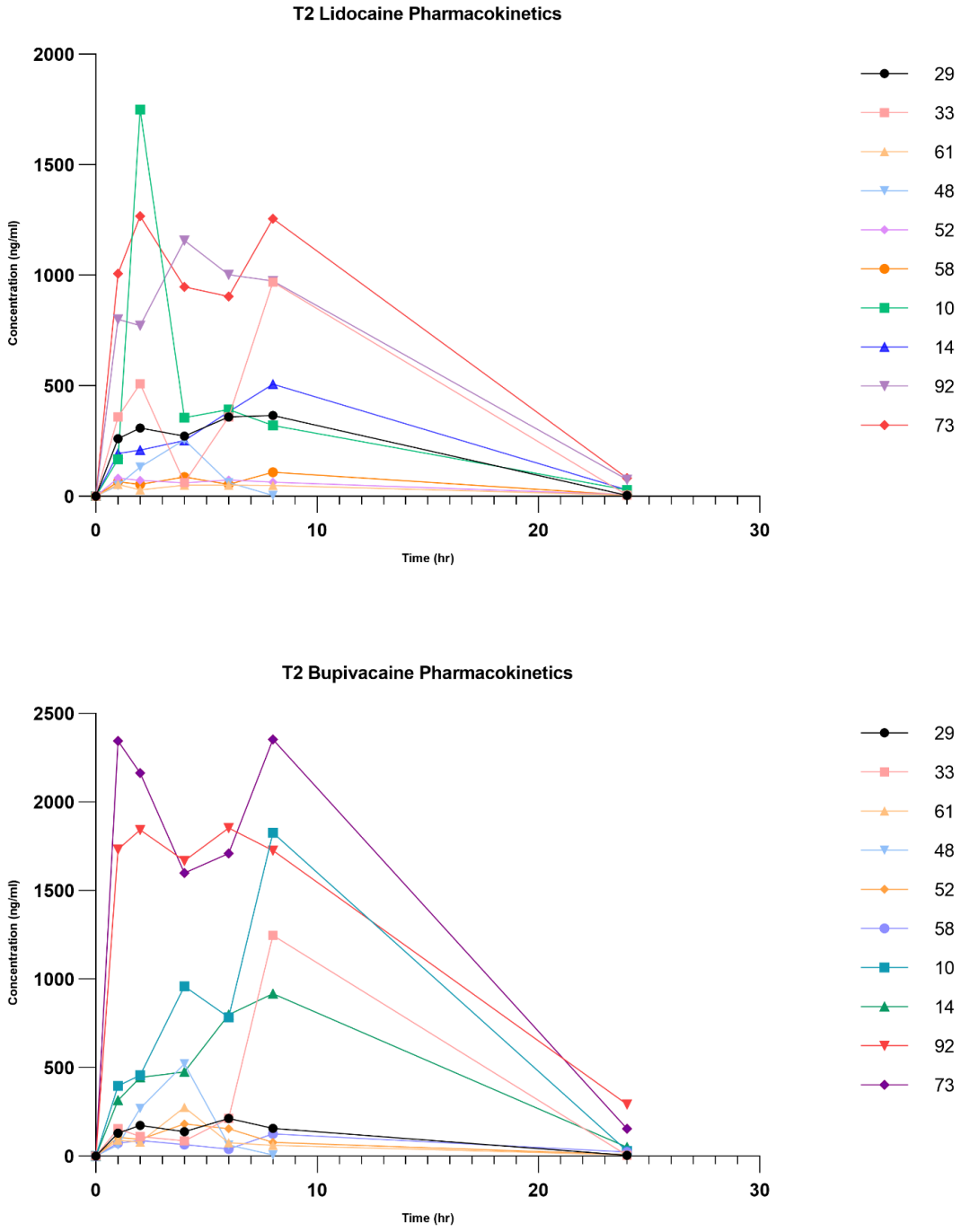


Figure 8.4 Concentration time curve for lidocaine and bupivacaine after applying treatment wafers in group T2 with 5% zinc oxide-PVP. Each colour represents an individual animal identification tag number, and each data point represents the concentration of drug present in a plasma sample taken at a given time point.

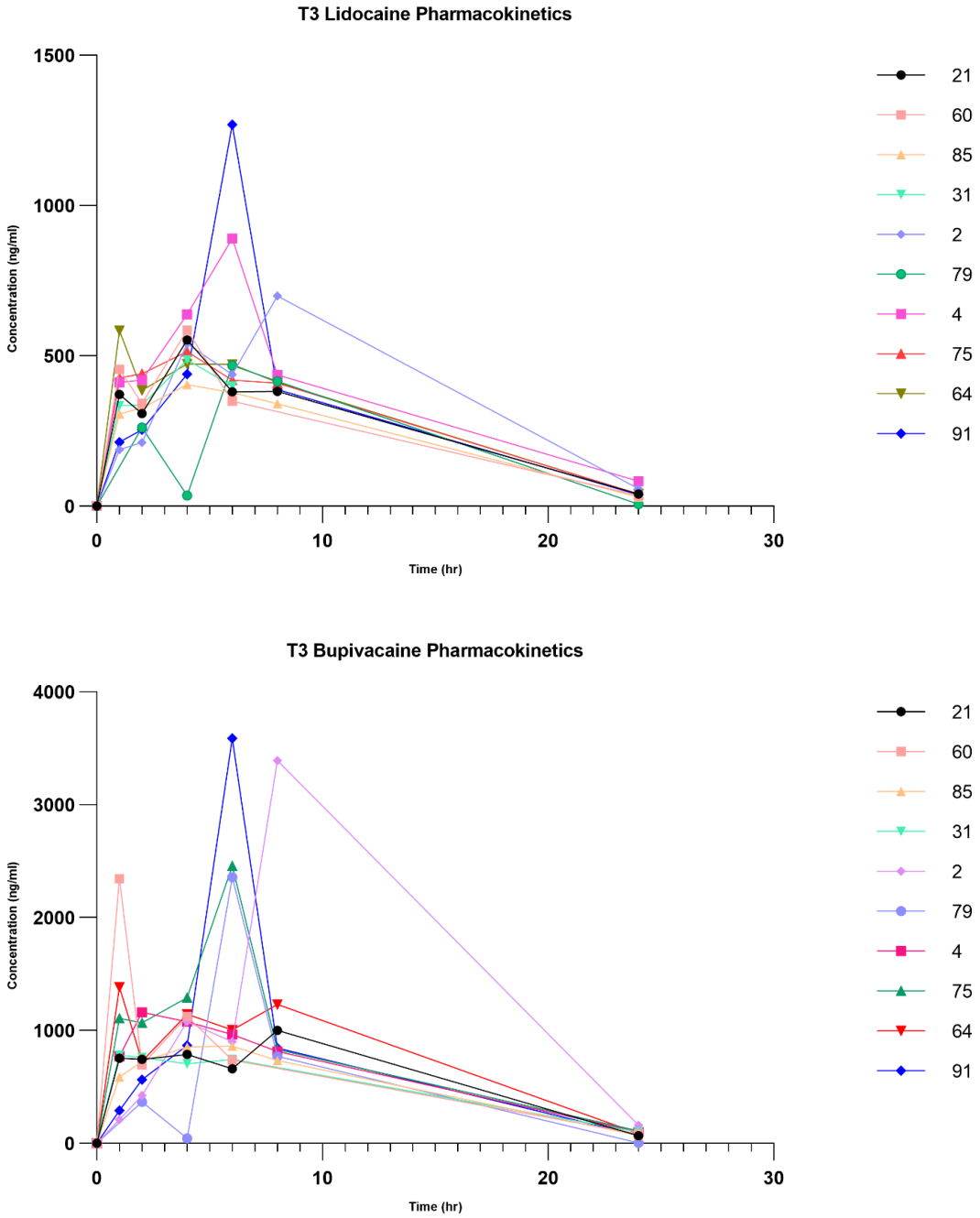


Figure 8.5 Concentration time curve for lidocaine and bupivacaine after applying treatment wafers in group T3 with 0% or no zinc oxide-PVP. Each colour represents an individual animal identification tag number, and each data point represents the concentration of drug present in a plasma sample taken at a given time point.

Appendix C

Tables

Table 8.1 Datasheet for recording thermography with Forward-Looking Infrared (FLIR) camera. R=Right antler, Left=Left antler. Centre=temperature recorded at the centre of the frame, Max=Maximum temperature recorded in the frame.

FLIR Data Sheet	DATE
Case Group Number:	
Time of LA injection:	**EAR TAG**
Time of Treatment:	

TIME		T0	T4	T24	DAY 3	DAY 7	DAY 14	DAY 21	DAY 28
DATE									
L	CENTRE								
	MAX								
R	CENTRE								
	MAX								

Table 8.2 Data sheet for recording MNT validation data. Cra=Cranial, Med=Medial, Cau=Caudal, Lat=Lateral. 1=day1, 2=day2, 3=day3. R=Right antler, Left=Left antler.

Animal #	Antler	Date	MNT assessment with algometer (N)											
			Cra 1	Cra 2	Cra 3	Med 1	Med 2	Med 3	Cau 1	Cau 2	Cau 3	Lat 1	Lat 2	Lat 3
	R													
	Antler	**Date**	**MNT assessment with algometer (N)**											
			Cra 1	Cra 2	Cra 3	Med 1	Med 2	Med 3	Cau 1	Cau 2	Cau 3	Lat 1	Lat 2	Lat 3
	L													

Table 8.3 Datasheet for in vivo study records.

Time of LA injection:	Date:
	Ear Tag:
Time of pad application:	

Algometry (N)										
Time		T0	T-10min	T-2hr	T-4hr	T-6hr	T-8hr	T-10	T-12hr	T-14hr	T-16hr
Date											
R	**Cra**										
	Med										
	Caud										
	Lat										
L	**Cra**										
	Med										
	Caud										
	Lat										

Blood Collection								
Time	T0	T-1hr	T-2hr	T-4hr	T-6hr	T-8hr	T-12hr	T-24hr

Photography of the wound									
Time & Date Unit (cm)		Day 1	Day 3	Day 7	D 14	Day 21	Month 1	Month 2	Month 3
	R								
	L								

Photography and length of the antlers									
Time & Date Unit (cm)		Day 1	Day 3	Day 7	D 14	Day 21	Month 1	Month 2	Month 3
	R								
	L								

Comments:

Table 8.4 Least-squares means (±SEM) of the concentrations (ng/ml) for the pilot in vitro drug release test (IVDRT) for treatment and control groups containing bupivacaine and lidocaine (n=3). The alphabetic superscripts indicate the statistically significant difference comparing values in columns.

	Bupivacaine		***Lidocaine***	
Group	***LSmeans***	***±SEM***	***LSmeans***	***±SEM***
C1	225.15 [b]	352.09		
C2			3101.57 [a]	352.09
C3	1281.35 [a]	352.09	2298.58 [c]	352.09
T1	131.03 [b]	352.09	222.94 [bd]	352.09
T2	119.26 [b]	352.09	142.95 [bd]	352.09
T3	52.69 [b]	352.09	87.10 [bd]	360.55
T4	26.02 [b]	352.09	70.22 [bd]	352.09
T5	21.59 [b]	352.09	55.44 [bd]	352.09
T6	19.84 [b]	352.09	56.57 [bd]	352.09
T7	8.79 [b]	354.08	68.19 [bd]	358.61
T8	8.90 [b]	352.09	33.46 [bd]	352.09
T9	34.82 [b]	352.09	29.21 [bd]	352.09

Table 8.5 Least-squares means (±SEM) of the concentrations (ng/ml) in the main in vitro drug release test (IVDRT) for treatment and control groups containing bupivacaine and lidocaine in the main study (n=6). LSmeans=Least-squares means. 25NP=collagen composite wafer modified with 25% ZnO-PVP, 10NP= collagen composite wafer modified with 10% ZnO-PVP, 5NP= collagen composite wafer modified with 5% ZnO-PVP, 0NP= non-modified collagen composite wafer.

	Bupivacaine		***Lidocaine***	
Group	***LSmeans***	***±SEM***	***LSmeans***	***±SEM***
Control	660791.26	83841.68	700510.12	83841.68
25NP	240280.44	83841.68	287704.68	83841.68
10NP	306399.13	83841.68	494928.64	83841.68
5NP	366694.90	83841.68	605253.04	83841.68
0NP	386862.63	83841.68	690838.96	83841.68

Table 8.6 Mean (±SD) of MNT or force (N) in the pilot in vivo for treatment group (PT1) comprising of modified collagen composite wafer with 50% ZnO-PVP, and control group in deer. N=6 for the control group and N=5 for PT1.

Group	***time (min)***	***Force (N)***	
		Mean	***±SD***
Treatment	0	23.85	6.12
	10	50.00	0.00
	120	46.07	9.60
	240	35.25	11.74
	360	27.22	9.63
	480	21.37	7.18
control	0	26.62	6.45
	10	50.00	0.00
	120	37.49	11.84
	240	23.69	8.88

Table 8.7 Mean (±SD) of temperature (°C) recorded with FLIR camera in pilot in vivo for treatment group (PT1) comprising of modified collagen composite wafer with 50% ZnO-PVP, and control group in deer. N=6 for the control group and N=5 for PT1.

Group	***time (min)***	***Temperature (°C)***	
		Mean	***±SD***
control	0	33.02	2.98
	4	35.32	2.91
	24	36.20	1.91
	72	35.88	2.21
	168	36.72	2.13
	336	36.96	1.73
treatment	0	35.17	2.71
	4	35.02	2.75
	24	35.90	1.72
	72	36.10	1.69
	168	35.73	2.61
	336	37.38	1.74

Table 8.8 MNT validation in three days in red deer (Cervus elaphus)- Mean (±SEM), minimum and maximum MNT or force (N) in three alternate days and between right and left antlers (n=40).

			Measured force (N)			
Day	***antler***	***site***	***Mean***	***±SD***	***Minimum***	***Maximum***
1	***Left***	***Caudal***	15.22	7.89	5.00	42.30
		Cranial	15.25	6.01	3.10	27.10
		Medial	15.77	6.06	5.60	34.50
		Lateral	13.91	6.53	4.10	29.10
	Right	***Caudal***	17.08	7.17	6.80	38.20
		Cranial	15.22	5.65	4.10	28.00
		Medial	16.47	8.12	5.40	46.40
		Lateral	14.76	6.03	3.80	29.60
2	***Left***	***Caudal***	15.38	5.69	5.80	25.90
		Cranial	13.78	4.92	4.20	22.40
		Medial	14.59	5.03	5.60	26.10
		Lateral	13.89	4.94	3.70	23.80
	Right	***Caudal***	15.35	6.56	5.50	28.80
		Cranial	13.83	6.12	3.40	35.60
		Medial	12.77	4.10	3.60	21.50
		Lateral	13.41	5.56	3.10	26.50
3	***Left***	***Caudal***	18.15	7.19	5.60	32.40
		Cranial	19.34	6.08	5.10	36.10
		Medial	19.06	5.77	5.60	29.80
		Lateral	18.48	5.83	6.10	28.90
	Right	***Caudal***	18.99	5.73	6.10	31.90
		Cranial	19.81	5.85	9.50	31.80
		Medial	18.08	6.21	5.10	32.00
		Lateral	17.98	5.71	4.80	29.70

*Table 8.9 MNT validation in three days in red deer (Cervus elaphus)- the differences of the Least-squares means (±SEM) of MNT (N) among three days and between right and left antlers, in addition to adjusted p-values with Bonferroni test. *Statistically significant difference (n=40).*

Differences of Least squares means

Effect	*antler*	*site*	*Day*	*antler*	*site*	*Day*	*±SEM*	*Pr>|t|*	*Adjusted P-value*
Day			***1***			***2***	0.43	0.0022	0.0067*
Day			***1***			***3***	0.42	<.0001	<.0001*
Day			***2***			***3***	0.43	<.0001	<.0001*
Antler	***Left***			***Right***			0.35	0.84	0.85

Table 8.10 Noncompartmental analysis of lidocaine after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 25 mg of drug in the pilot study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, LSMeans=Least-squares means (n=5 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
				ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
Pilot	705	Lidocaine	110 -140Kg	972.21	1	11159.44	38406.88	2804988	51.66	73.03	97031.58	13.01.85
	715			740.05	12	12691.71						
	731			927.82	8	9046.45	13401.36	272475.5	11.26	20.33	60622.59	3730.96
	758			799.76	6	10152.66	18187.6	521731.4	19.16	28.68	76007.46	2749.12
	789			380.32	1	7135.66	27656.05	2097202	51.42	75.83	134131.5	1807.92
LSMeans				**764.03**	**16.78**	**10037**	**24413**	**1424099**	**33.38**	**49.47**	**1377121**	**273576**
±SEM				**262.01**	**7.20**	**2819.39**	**3053.89**	**161308**	**3.02**	**4.07**	**1654114**	**257603**

Table 8.11 Noncompartmental analysis of bupivacaine after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 25 mg of drug in the pilot study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve T1/2λz =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, Cmax= maximum concentration, Tmax=time of maximum concentration, LSMeans=Least-squares means (n=5 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
				ng/ml	*hr*	*ng/ml*hr*	*ng/ml*hr*	*ng/ml*h^2*	*hr*	*hr*	*ml*	*ml/hr*
Pilot	705	Bupivacaine	110 -140Kg	1531.84	12			24584.14				
	715			1891.62	1	18506.1	27651.19	574101.4	11.27	20.76	29406.94	1808.24
	731			1171.60	8	10594.79	13344.88	194868.9	8.55	14.60	46215.86	3746.75
	758			1338.53	1	14519.85	17452.88	239772.5	9.12	13.74	37711.62	2864.86
	789			572.85	6	7487.05	13534.89	382202	18.30	28.24	97488.58	3694.16
LSMeans				**1301.29**	**16.78**	**12777**	**17996**	**283106**	**11.81**	**19.33**	**1342187**	**275117**
±SEM				**262.01**	**7.19**	**3152.18**	**3053.89**	**144278**	**3.02**	**4.07**	**1659390**	**258436**

Table 8.12 Noncompartmental analysis of lidocaine in treatment T1 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$=half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
T1	16	Lidocaine	114	358.14	4	2582.86	2586.79	16357.43	2.39	6.32	363255.9	105149.5
	82		120.5	493.15	1	3094.09	3151.09	19402.77	4.03	6.16	501811.6	86319.37
	89		117	129.20	2	1853.06	2009.03	18928.08	6.59	9.42	1288197	135388.4
	62		128.5	222.80	2	2414.63	2621.73	24081.3	6.20	9.18	928528.5	103748.1
	50		136.5	366.00	4	3098.84	3225.99	23809.04	4.72	7.38	574714.3	84315.16
	81		130	175.97	6	2416.68	2488.33	19069.68	4.41	7.66	696215.9	109310.1
	5		111	205.80	4	2689.75	2777.78	21073.59	4.84	7.58	683596.1	97919.94
	7		121.5	209.53	2	829.80	862.81	4653.88	6.01	5.39	2731841	315246.6
	27		114	63.13	6	487.27	501.18	3709.38	4.14	7.40	3243872	542722.5
	36		113	36.36	6	551.18	593.10	5618.46	5.95	9.47	3935141	458609.4
LSMeans			**119.28**	**226.01**	**4.39**	**2001.82**	**2081.78**	**15670**	**4.9299**	**7.60**	**1573129**	**220418**
±SEM			**2.31**	**185.27**	**0.98**	**1993.61**	**1931.45**	**102020**	**1.9104**	**2.58**	**251134**	**34508**

Table 8.13 Noncompartmental analysis of bupivacaine in treatment T1 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
T1	16	Bupivacaine	114	315.97	6	2804.68	2807.42	18928.22	2.11	6.74	294952.8	96885.94
	82		120.5	487.07	2	3900.46	4035.22	27284.97	5.20	6.76	505881.5	67406.44
	89		117	285.26	4	2375.58	2455.98	18403.12	4.85	7.49	775720.3	110750
	62		128.5	368.28	1	3738.58	3981.30	33653.69	5.72	8.45	563875.1	68319.29
	50		136.5	428.58	8	6137.41						
	81		130	757.63	2	3662.09	3884.21	29544.66	5.98	7.60	604617.9	70027.04
	5		111	336.07	2	3938.00	6242.76	150456.3	17.83	24.10	1121252	43570.45
	7		121.5	444.99	2	1690.41	1733.75	8319.00	5.49	4.80	1243579	156885.1
	27		114	130.62	6	892.76	919.71	6595.67	4.18	7.17	1784390	295743.8
	36		113	54.79	8	797.72						
LSMeans			**119.28**	**360.93**	**4.79**	**2993.77**	**3257.55**	**36648**	**6.42**	**9.14**	**941272**	**130471**
±SEM			**2.31**	**185.27**	**0.98**	**1993.61**	**2159.43**	**114062**	**2.14**	**2.88**	**276914**	**37889**

Table 8.14 Noncompartmental analysis of lidocaine in treatment T2 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve T1/2λz =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, Cmax= maximum concentration, Tmax=time of maximum concentration, lid=lidocaine, bup=bupivacaine, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	*ng/ml*	*hr*	*ng/ml*hr*	*ng/ml*hr*	*ng/ml*h^2*	*hr*	*hr*	*ml*	*ml/hr*
T2	29	Lidocaine	117.5	365.45	8	5313.94						
	33		116	968.74	8	10734.8						
	61		101	54.83	1	790.14	844.41	7646.94	5.82	9.05	2707508	322118.7
	48		126	256.49	4	888.64	892.30	3303.95	0.66	3.70	290169.7	304831.6
	52		109.5	82.41	1	1108.30	1172.60	9941.49	5.52	8.48	1846815	231962.7
	10		84.5	1748.68	2	7402.74	7600.05	47686.1	4.72	6.27	243677	35789.23
	14		119	506.79	8	6574.03						
	92		106	1157.90	4	15661.93	16167.98	124682.3	4.63	7.71	112378.5	16823.38
	73		115	1267.73	2	18573.04	19128.48	146526	4.69	7.66	96331.58	14219.63
	58		106	108.63	8	1456.22						
LSMeans			**108.69**	**651.77**	**5.31**	**6850.38**	**7634.30**	**56631**	**4.34**	**7.15**	**979320**	**174654**
±SEM			**2.33**	**185.27**	**0.99**	**1993.61**	**2493.49**	**131707**	**2.47**	**3.33**	**321936**	**44142**

Table 8.15 Noncompartmental analysis of bupivacaine in treatment T2 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, lid=lidocaine, bup=bupivacaine, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
T2	29	Bupivacaine	117.5	212.52	6	2563.90	2591.62	17595.94	3.40	6.79	514359.9	104953.4
	33		116	1245.94	8	12180.71						
	61		101	275.58	4	1527.48	1577.62	10803.16	5.11	6.85	1272174	172411.3
	48		126	522.60	4	1646.68	1654.53	5944.49	0.66	3.59	158094.3	164396.6
	52		109.5	181.46	4	1698.35	1755.21	12766.11	4.54	7.27	1016424	154967.1
	10		84.5	1825.19	8	21243.61						
	14		119	917.81	8	12202.67						
	92		106	1852.70	6	29423.59	32206.62	316686	6.56	9.83	80024.68	8445.47
	73		115	2352.80	8	34620.06						
	58		106	125.98	8	1741.42						
LSMeans			108.69	951.26	7.11	11885	7957.12	72759	4.06	6.8673	705411	141544
±SEM			2.33	185.27	0.99	1993.61	2731.48	144278	2.70	3.6467	348779	47657

Table 8.16 Noncompartmental analysis of lidocaine in treatment T3 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$=half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, lid=lidocaine, bup=bupivacaine, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
T3	21	Lidocaine	114.5	553.08	4	6456.18	6764.39	55238.26	5.31	8.17	307965.8	40210.57
	60		112.5	585.03	4	5951.04	6211.99	42944.39	5.12	6.91	323752	43786.27
	85		115.5	404.21	4	5668.99	5879.87	45935.01	4.80	7.81	320734.4	46259.54
	31		111	486.97	4	2213.18						
	2		113.5	699.28	8	9218.57						
	79		100	468.57	6	5327.69	5356.06	39002.61	2.85	7.28	209153.5	50783.57
	4		100.5	890.15	6	8699.02	9388.48	87174.26	5.73	9.28	239738.3	28971.67
	75		110	514.73	4	6965.70	7269.45	57769.29	5.14	7.94	277519.1	37416.86
	64		104	584.62	1	7092.34	7389.05	58026.09	4.99	7.85	265314.5	36811.22
	91		112.5	1269.22	6	7815.54	8035.12	61596.07	3.96	7.67	193247.7	33851.38
LSMeans			**107.92**	**645.59**	**5.47**	**6540.83**	**7036.80**	**55961**	**4.74**	**7.86**	**370363**	**61534**
±SEM			**2.40**	**185.27**	**1.01**	**1993.61**	**2159.43**	**114062**	**2.14**	**2.88**	**288968**	**40040**

Table 8.17 Noncompartmental analysis of bupivacaine in treatment T3 after applying the collagen wafers on the wound surface area of deer antlers without considering the lost wafers. Each pad contained 136 mg drug in the in vivo study. These pharmacokinetic parameters were calculated for each sample. AUC=area under the curve, AUMC=area under the moment curve $T_{1/2\lambda z}$ =half-life, Cl=clearance, Vd=volume of distribution, MRT=mean residence time, C_{max}= maximum concentration, T_{max}=time of maximum concentration, lid=lidocaine, bup=bupivacaine, LSMeans=Least-squares means (n=10 deer).

Group	tag	drug	Bodyweight	C_{max}	T_{max}	$AUC_{(0-t)}$	$AUC_{(0-\infty)}$	$AUMC_{(0-\infty)}$	$T_{1/2\lambda z}$	$MRT_{1/2\lambda z}$	Vd_{area}	Cl
			Kg	ng/ml	hr	ng/ml*hr	ng/ml*hr	ng/ml*h^2	hr	hr	ml	ml/hr
T3	21	Bupivacaine	114.5	1002.55	8	14351.62						
	60		112.5	2346.42	1	13763.24	14431.19	97148.36	5.50	6.73	149584.3	18848.07
	85		115.5	863.27	6	12298.64	12799.85	102106.5	4.90	7.97	150058.5	21250.25
	31		111	782.18	1	11687.72	12682.41	108897.5	6.78	8.58	209768.5	21447.03
	2		113.5	3392.02	8	36629.04						
	79		100	2357.02	6	12536.44	12549.86	87022.16	2.05	6.93	64232.41	21673.54
	4		100.5	1162.01	2	14760.9	15572.79	128778.1	5.48	8.27	138135.6	17466.36
	75		110	2458.69	6	18597.92	19331.07	148003.6	4.51	7.65	91639.59	14070.62
	64		104	1386.46	1	18583.75	19184.08	148998	4.72	7.77	96659.08	14178.42
	91		112.5	3588.94	6	18235.2	18606.09	137451.8	3.59	7.39	75667.18	14618.87
LSMeans			**107.92**	**1933.96**	**5.27**	**17144**	**15645**	**119801**	**4.69**	**7.66**	**227307**	**40171**
±SEM			**2.40**	**185.27**	**1.01**	**1993.61**	**2159.43**	**114062**	**2.14**	**2.88**	**290190**	**40257**

Table 8.18 The least-square means (±SEM) of bupivacaine and lidocaine concentrations for the pilot in vivo pharmacokinetic study treatment group (n= 5 animals), without considering the lost wafers.

Drug	Time (hr)	Concentration (ng/ml)	±SEM
Bupivacaine	1	2857.29	942.47
	2	2337.73	967.88
	4	2459.52	942.47
	6	2266.67	939.64
	8	2885.78	942.47
	12	2631.57	930.04
	24	2294.78	942.47
Lidocaine	1	2362.74	942.47
	2	2325.93	967.88
	4	2307.95	942.47
	6	2310.20	942.47
	8	2335.63	942.47
	12	2318.54	930.04
	24	2213.35	942.47

Table 8.19 The least-square means (±SEM) of bupivacaine and lidocaine concentrations across treatment groups in the main in vivo pharmacokinetic study without considering the lost wafers (n= 10 animals for each treatment group).

Drug	***Treatment***	***Concentration (ng/ml)***	***±SEM***
Bupivacaine	T1	89.74	62.79
	T2	456.04	63.88
	T3	671.00	66.45
Lidocaine	T1	34.46	62.77
	T2	253.12	63.28
	T3	269.61	66.11

Table 8.20 The least-square means (±SEM) of concentrations (ng/ml) for bupivacaine and lidocaine in treatment groups, in different time points (hrs) of the main study, without considering the lost wafers (n= 10 animals for each treatment group).

Drug	Treatment	Time (hr)	Concentration (ng/ml)	±SEM
Bupivacaine	T1	1	191.63	143.00
		2	318.01	143.36
		4	177.92	108.22
		6	184.28	138.67
		8	153.08	138.00
		24	16.23	15.87
	T2	1	541.31	143.01
		2	580.22	143.39
		4	591.27	108.23
		6	584.57	138.68
		8	844.19	138.01
		24	58.63	16.67
	T3	1	909.66	150.74
		2	717.82	136.05
		4	891.45	108.27
		6	1182.51	146.17
		8	883.32	164.98
		24	80.27	16.19
Lidocaine	T1	1	148.97	143.00
		2	144.39	143.36
		4	156.83	108.22
		6	145.23	138.67
		8	97.56	138.00
		24	4.64	15.87
	T2	1	303.37	143.01
		2	505.88	136.02
		4	344.49	108.23

		6	358.17	138.68
		8	455.91	138.01
		24	21.53	16.67
	T3	**1**	359.79	150.74
		2	322.32	136.05
		4	460.66	108.27
		6	540.06	138.71
		8	428.79	154.30
		24	34.83	17.03

Table 8.21 Collagen composite wafer adhesiveness in the pilot in vivo study - Table depicting the successful and failed collagen composite wafers applied on deer antlers after velvet antler removal in 14 days. Right= right antler, Left= left antler. Green sign=Successfully adhered wafer, Red sign=Failed wafer.

Case #	Time 0	Time 2	Time 4	Time 6	Time 8	Day 1	Day 3	Day 7	Day 14
1 Right	✓	✓	✓	✓	✓	✓	✓	✓	✓
1 Left	✓	✓	✓	✓	✓	✓	✓	✓	✓
2 Right	✓	✓	✗	✗	✗	✗	✗	✗	✗
2 Left	✓	✓	✓	✓	✓	✓	✓	✗	✗
3 Right	✓	✗	✗	✗	✗	✗	✗	✗	✗
3 Left	✓	✓	✓	✓	✓	✓	✓	✓	✓
4 Right	✓	✓	✓	✓	✓	✓	✓	✓	✓
4 Left	✗	✓	✗	✗	✗	✗	✗	✗	✗
5 Right	✓	✓	✗	✗	✗	✗	✗	✗	✗
5 Left	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 8.22 HPLC Inter-day variation of bupivacaine group in 5 different concentrations diluted in Milli-Q water. RSD=Relative Standard Deviation. SD=Standard deviation.

	Concentration 1		***Concentration 2***		***Concentration 3***		***Concentration 4***		***Concentration 5***	
	Concentration	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***
	1000	128906	**500**	59238	**250**	26379	**125**	13060	**62.5**	5774
	1000	129400	**500**	59663	**250**	26493	**125**	13169	**62.5**	6396
	1000	128711	**500**	59867	**250**	25731	**125**	12767	**62.5**	6005
Mean	**1000**	129005	**500**	59589	**250**	26201	**125**	12998	**62.5**	6058
SD	**1000**	355.14	**500**	320.90	**250**	411	**125**	207.89	**62.5**	314.41
RSD	**1000**	0.27	**500**	0.53	**250**	1.56	**125**	1.59	**62.5**	5.18

Table 8.23 HPLC Intra-day variation of bupivacaine group in 5 different concentrations diluted in Milli-Q water. RSD=Relative Standard Deviation. SD=Standard deviation.

	Concentration 1		***Concentration 2***		***Concentration 3***		***Concentration 4***		***Concentration 5***	
	Concentration	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***
	1000	123671	**500**	62880	**250**	23191	**125**	14109	**62.5**	5774
	1000	121570	**500**	54731	**250**	23423	**125**	12288	**62.5**	7084
	1000	119473	**500**	59238	**250**	24541	**125**	11958	**62.5**	5359
	1000	118794	**500**	57618	**250**	24315	**125**	13060	**62.5**	5594
	1000	120645	**500**	57290	**250**	25712	**125**	12342	**62.5**	5524
	1000	122892	**500**	58122	**250**	26154	**125**	11419	**62.5**	7034
Mean	**1000**	121174	**500**	58313	**250**	24556	**125**	12529	**62.5**	6061
SD	**1000**	1906.88	**500**	2687.37	**250**	1191.23	**125**	941.22	**62.5**	784.20
RSD	**1000**	1.57	**500**	4.60	**250**	4.85	**125**	7.51	**62.5**	12.93

Table 8.24 HPLC Inter-day variation of lidocaine group in 5 different concentrations diluted in Milli-Q water. RSD=Relative Standard Deviation. SD=Standard deviation.

	Concentration 1		***Concentration 2***		***Concentration 3***		***Concentration 4***		***Concentration 5***	
	Concentration	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***
	1000	164388	**500**	84433	**250**	42947	**125**	22860	**62.5**	10394
	1000	163730	**500**	85951	**250**	43998	**125**	22697	**62.5**	10002
	1000	163014	**500**	81700	**250**	43809	**125**	22975	**62.5**	10450
Mean	**1000**	163711	**500**	84028	**250**	43585	**125**	22844	**62.5**	10282
SD	**1000**	687.20	**500**	2154.24	**250**	560.26	**125**	139.68	**62.5**	244.09
RSD	**1000**	0.41	**500**	2.56	**250**	1.28	**125**	0.61	**62.5**	2.37

Table 8.25 HPLC Intra-day variation of lidocaine group in 5 different concentrations diluted in Milli-Q water. RSD=Relative Standard Deviation. SD=Standard deviation

	Concentration 1		***Concentration 2***		***Concentration 3***		***Concentration 4***		***Concentration 5***	
	Concentration	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***	***Concentration***	***Area***
	1000	167953	**500**	94587	**250**	44470	**125**	15113	**62.5**	6740
	1000	170652	**500**	89096	**250**	48926	**125**	16849	**62.5**	7500
	1000	164388	**500**	87942	**250**	49986	**125**	14234	**62.5**	7962
	1000	164655	**500**	87059	**250**	46320	**125**	15983	**62.5**	6759
	1000	170009	**500**	87028	**250**	42947	**125**	15162	**62.5**	7647
	1000	167522	**500**	90092	**250**	45556	**125**	15180	**62.5**	7768
Mean	**1000**	167530	**500**	89142	**250**	46530	**125**	15468	**62.5**	7322
SD	**1000**	2615.68	**500**	3158.24	**250**	2950.01	**125**	989.29	**62.5**	548.31
RSD	**1000**	1.56	**500**	3.54	**250**	6.34	**125**	6.39	**62.5**	7.48

Table 8.26 Main study in vitro study results – Least-squares means (±SEM) of drug concentrations (ng/ml) for IVDRT in 4 treatment groups and the control group for both bupivacaine and lidocaine (n=6).

Drug	***Group***	***LSMEAN***	***±SEM***
bupivacaine	***0NP***	386862.63	83841.68
	10NP	306399.13	83841.68
	25NP	240280.44	83841.68
	5NP	366694.90	83841.68
	control	660791.26	83841.68
lidocaine	***0NP***	690838.96	83841.68
	10NP	494928.64	83841.68
	25NP	287704.68	83841.68
	5NP	605253.04	83841.68
	control	700510.12	83841.68

Table 8.27 Collagen composite wafer adhesiveness in the main in vivo study for the treatment group (T1) - Table depicting the successful and failed collagen composite wafers applied on deer antler after velvet antler removal in 60 days. T1 = collagen composite wafer modified with 25% ZnO-PVP. Right= right antler, Left= left antler. Green sign=Successfully adhered wafer, Red sign=Failed wafer, blue circle= euthanasia/lost antler during the study.

Groups	t0	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs	24 hrs	3 days	7 days	14 days	21 days	1 Month	2 Months
T1-1R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-1L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-2R	✓	✓	✓	✓	✓	✓	✓						
T1-2L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-3R	✓	✗	✗	✗	✗	✗	✗	✗					
T1-3L	✓	✗	✗	✗	✗	✗	✗	✗					
T1-4R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-4L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-5R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-5L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-6R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
T1-6L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✗	✗
T1-7R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-7L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-8R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

T1-8L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-9R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-9L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-10R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T1-10L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 8.28 Collagen composite wafer adhesiveness in the main in vivo study for treatment group (T2) - Table depicting the successful and failed collagen composite wafers applied on deer antler after velvet antler removal in a 60-day period. T2 = collagen composite wafer modified with 5% ZnO-PVP. Right= right antler, Left= left antler. Green sign=Successfully adhered wafer, Red sign=Failed wafer, ½ red sign=half of the wafer was lost, blue circle= euthanasia/lost antler during the study.

Groups	t0	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs	24 hrs	3 days	7 days	14 days	21 days	1 Month	2 Months
T2-1 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-1 L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-2 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-2 L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-3 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-3 L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-4 R	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T2-4 L	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T2-5 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-5 L	✓	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗
T2-6 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-6 L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-7 R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-7 L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-8 R	✓	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗

T2-8L	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T2-9R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-9L	✓	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗	1/2 ✗
T2-10R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T2-10L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

Table 8.29 Collagen composite wafer adhesiveness in the main in vivo study for treatment group (T3) - Table depicting the successful and failed collagen composite wafers applied on deer antler after velvet antler removal in a 60-day period. T3 = non-modified collagen composite wafer modified. Right= right antler, Left= left antler. Green sign=Successfully adhered wafer, Red sign=Failed wafer, ½ red sign=half of the wafer was lost, ¼ red sign=a quarter of the wafer was lost, blue circle= euthanasia/lost antler during the study.

Groups	t0	2 hrs	4 hrs	6 hrs	8 hrs	10 hrs	24 hrs	3 days	7 days	14 days	21 days	1 Month	2 Months
T3-1R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-1L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-2R	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-2L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-3R	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-3L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-4R	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-4L	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-5R	✓	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗
T3-5L	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-6R	✓	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗	½✗
T3-6L	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-7R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-7L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-8R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

T3-8L	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-9R	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-9L	✓	✓	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗	✗
T3-10R	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
T3-10L	✓	✓	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗	1/4 ✗

*Table 8.30 Comparisons between the least-squares means (±SEM) of MNT or forces (N) at different time points for T1 comprising collagen composite wafer modified with 25% ZnO-PVP. *Statistically significant difference (n=10).*

Group	Time (min)	Time (min)	Mean difference	±SEM	P-value
T1	0	10	-22.86	1.21	<.0001**
	0	120	-21.16	1.21	<.0001**
	0	240	-18.10	1.21	<.0001**
	0	360	-16.04	1.21	<.0001**
	0	480	-13.23	1.21	<.0001**
	0	600	-8.64	1.21	<.0001**
	10	120	1.02	1.22	0.40
	10	240	0.48	1.22	0.69
	10	360	2.34	1.22	0.05
	10	480	6.72	1.22	<.0001**
	10	600	9.84	1.22	<.0001**
	120	240	3.06	1.21	0.0113*
	120	360	5.12	1.21	<.0001**
	120	480	7.93	1.21	<.0001**
	120	600	12.52	1.21	<.0001**
	240	360	2.06	1.21	0.09
	240	480	4.87	1.21	<.0001**
	240	600	9.46	1.21	<.0001**
	360	480	2.80	1.21	0.0201*
	360	600	7.40	1.21	<.0001**
	480	600	4.60	1.21	0.0001**

*Table 8.31 Comparisons between the least-squares means (±SEM) of MNT or forces (N) at different time points for T2 comprising collagen composite wafer modified with 5% ZnO-PVP. *Statistically significant difference (n=10).*

Group	Time (min)	Time (min)	Mean difference	±SEM	P-value
T2	0	10	-22.97	1.23	<.0001**
	0	120	-21.94	1.23	<.0001**
	0	240	-22.47	1.23	<.0001**
	0	360	-20.61	1.23	<.0001**
	0	480	-16.23	1.23	<.0001**
	0	600	-13.12	1.23	<.0001**
	10	120	1.04	1.23	0.40
	10	240	0.50	1.23	0.68
	10	360	2.36	1.23	0.05
	10	480	6.74	1.23	<.0001**
	10	600	9.86	1.23	<.0001**
	120	240	-0.53	1.23	0.66
	120	360	1.33	1.23	0.28
	120	480	5.71	1.23	<.0001**
	120	600	8.82	1.23	<.0001**
	240	360	1.86	1.23	0.1300
	240	480	6.24	1.23	<.0001**
	240	600	9.35	1.23	<.0001**
	360	480	4.38	1.23	0.0004*
	360	600	7.49	1.23	<.0001**
	480	600	3.11	1.23	0.0114*

*Table 8.32 Comparisons between the least-squares means (±SEM) of MNT or forces (N) at different time points for T3 comprising non-modified collagen composite. *Statistically significant difference (n=10).*

Group	Time (min)	Time (min)	Mean difference	±SEM	P-value
T3	0	10	-18.42	1.22	<.0001**
	0	120	-17.89	1.22	<.0001**
	0	240	-16.23	1.22	<.0001**
	0	360	-12.41	1.22	<.0001**
	0	480	-6.54	1.22	<.0001**
	0	600	-1.42	1.22	0.24
	10	120	0.53	1.22	0.66
	10	240	2.18	1.22	0.07
	10	360	6.00	1.22	<.0001**
	10	480	11.87	1.22	<.0001**
	10	600	16.99	1.22	<.0001**
	120	240	1.65	1.22	0.17
	120	360	5.48	1.22	<.0001**
	120	480	11.34	1.22	<.0001**
	120	600	16.47	1.22	<.0001**
	240	360	3.82	1.22	0.0018*
	240	480	9.69	1.22	<.0001**
	240	600	14.81	1.22	<.0001**
	360	480	5.87	1.22	<.0001**
	360	600	10.99	1.22	<.0001**
	480	600	5.12	1.22	<.0001**

*Table 8.33 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time points (min) for the control group that only received a 4% articaine HCl ring block before velvet antler removal. *Statistically significant difference (n=10).*

Group	Time (min)	Time (min)	Mean difference	±SEM	P-value
Control	0	10	-33.63	1.27	<.0001**
	0	120	-25.70	1.27	<.0001**
	0	240	-10.72	1.27	<.0001**
	0	360	-5.81	1.27	<.0001**
	10	120	7.92	1.27	<.0001**
	10	240	22.91	1.27	<.0001**
	10	360	27.81	1.27	<.0001**
	120	240	14.99	1.27	<.0001**
	120	360	19.89	1.27	<.0001**
	240	360	4.90	1.27	0.0001*

*Table 8.34 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t0 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***0***	***T2***	***0***	0.09	1.22	0.94
T1	***0***	***T3***	***0***	-4.43	1.21	0.0003*
T1	***0***	***Control***	***0***	10.81	1.24	<.0001**
T2	***0***	***T3***	***0***	-4.53	1.22	0.0002*
T2	***0***	***Control***	***0***	10.71	1.25	<.0001**
T3	***0***	***Control***	***0***	15.24	1.24	<.0001**

*Table 8.35 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t10 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***10***	***T2***	***10***	-0.02	1.22	0.99
T1	***10***	***T3***	***10***	0.01	1.21	0.99
T1	***10***	***Control***	***10***	0.04	1.24	0.97
T2	***10***	***T3***	***10***	0.03	1.22	0.98
T2	***10***	***Control***	***10***	0.06	1.25	0.96
T3	***10***	***Control***	***10***	0.03	1.25	0.98

*Table 8.36 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t120 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***120***	***T2***	***120***	-0.68	1.22	0.57
T1	***120***	***T3***	***120***	-1.16	1.21	0.34
T1	***120***	***Control***	***120***	6.26	1.24	<.0001**
T2	***120***	***T3***	***120***	-0.48	1.22	0.69
T2	***120***	***Control***	***120***	6.95	1.25	<.0001**
T3	***120***	***Control***	***120***	7.43	1.24	<.0001**

*Table 8.37 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t240 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time***	***Group***	***Time***	***Mean difference***	***±SEM***	***P-value***
T1	***240***	***T2***	***240***	-4.28	1.22	0.0005*
T1	***240***	***T3***	***240***	-2.57	1.21	0.03*
T1	***240***	***Control***	***240***	18.19	1.24	<.0001**
T2	***240***	***T3***	***240***	1.71	1.22	0.16
T2	***240***	***Control***	***240***	22.47	1.25	<.0001**
T3	***240***	***Control***	***240***	20.76	1.25	<.0001**

*Table 8.38 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t360 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***360***	***T2***	***360***	-4.48	1.22	0.0002**
T1	***360***	***T3***	***360***	-0.80	1.21	0.51
T1	***360***	***Control***	***360***	21.03	1.24	<.0001**
T2	***360***	***T3***	***360***	3.67	1.22	0.0028*
T2	***360***	***Control***	***360***	25.51	1.25	<.0001**
T3	***360***	***Control***	***360***	21.84	1.25	<.0001**

*Table 8.39 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t480 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***480***	***T2***	***480***	-2.90	1.22	0.02*
T1	***480***	***T3***	***480***	2.26	1.21	0.06
T2	***480***	***T3***	***480***	5.16	1.23	<.0001**
T1	***480***	***Control***	***360***	18.22	1.24	<.0001**
T2	***480***	***Control***	***360***	21.13	1.25	<.0001**
T3	***480***	***Control***	***360***	15.97	1.25	<.0001**

*Table 8.40 Comparisons of the least-squares means (±SEM) of MNT or forces (N) at different time-point t600 (min) between the treatment and control groups. T1=Collagen composite wafer modified with 25% ZnO-PVP, T2= Collagen composite wafer modified with 5% ZnO-PVP, T3= Non-modified collagen composite wafer, control=only 4% articaine HCl ring block. *Statistically significant difference (n=10).*

Group	***Time (min)***	***Group***	***Time (min)***	***Mean difference***	***±SEM***	***P-value***
T1	***600***	***T2***	***600***	-2.90	1.22	0.02*
T1	***600***	***T3***	***600***	2.26	1.21	0.06
T2	***600***	***T3***	***600***	5.16	1.22	<.0001**
T1	***600***	***Control***	***360***	-11.87	1.22	<.0001**
T2	***600***	***Control***	***360***	18.02	1.25	<.0001**
T3	***600***	***Control***	***360***	10.85	1.24	<.0001**

Table 8.41 Mean (±SD) of antler wound surface area (mm^2) for 2 months of wound healing assessment (n=10 animals with 20 antlers in each group).

	Control		***T1***		***T2***		***T3***	
Day	***Mean***	***±SD***	***Mean***	***±SD***	***Mean***	***±SD***	***Mean***	***±SD***
1	1589.4	571	1089.1	437.2	1035.4	291	963.7	348.5
3	1496.8	532.5	1064.9	443	861.3	220.7	887.9	364.8
7	1405.2	491	960.1	370.3	875.4	247.7	828.6	343.8
14	853.8	312	931.4	389.7	738	226.3	754.1	269.4
21	438	195	770.7	350.5	464.9	170.2	676.2	331.9
30	340	154	422.2	358.5	225.8	212.9	519.4	431.7
60	0	0	0	0	0	0	0	0

Table 8.42 Mean (±SD) of antler re-growth length (cm) in 2 months (n=10 animals with 20 antlers in each group).

Group	***Length (cm)***	
	Mean	***±SD***
T1	9.24	5.06
T2	9.33	6.67
T3	7.71	4.25
control	11.13	8.73

Table 8.43 Mean (±SD), and minimum and maximum recorded antler re-growth lengths (cm) in 2 months (n=10 animals with 20 antlers in each group).

		Antler length (cm)			
Group	**day**	**Mean**	**±SD**	**Minimum**	**Maximum**
T1	**1**	7.05	1.37	6.00	11.00
	3	7.05	1.39	5.00	11.00
	7	7.33	1.60	6.00	11.00
	14	7.44	1.34	6.00	11.00
	21	7.85	1.62	6.00	12.00
	30	9.03	4.26	6.00	24.50
	60	20.47	4.72	9.50	28.00
T2	**1**	6.50	0.46	5.50	7.00
	3	6.52	0.47	5.50	7.00
	7	6.65	0.40	6.00	7.00
	14	6.75	0.52	6.00	8.00
	21	6.90	0.57	6.00	8.00
	30	7.67	0.96	6.00	9.50
	60	26.00	4.11	16.00	30.00
T3	**1**	6.52	0.77	5.00	8.00
	3	6.52	0.77	5.00	8.00
	7	6.60	0.84	5.00	8.00
	14	6.67	0.80	5.00	8.00
	21	6.80	0.85	5.00	8.00
	30	7.77	1.60	5.00	11.00
	60	16.71	10.28	6.50	30.00
control	**1**	6.95	1.07	5.00	9.00
	3	6.95	1.07	5.00	9.00
	7	6.95	1.07	5.00	9.00
	14	7.60	1.38	5.50	10.00
	21	8.32	1.55	6.00	11.50

	30	9.77	1.86	7.50	14.50
	60	32.42	3.27	24.00	36.00

Appendix D

Published articles

animals

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Article

Pressure Algometry Validation and Determination of Efficacy of Articaine Hydrochloride Ring Block in Antler Removal in Red Deer (*Cervus elaphus*)

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Simple Summary: Red deer (*Cervus elaphus*) are farmed in New Zealand for the production of velvet antler. Velvet is harvested as living antler tissue, and currently lignocaine is the only licensed local anaesthetic approved for deer antler removal in New Zealand. The use of lignocaine is not without consequences, including drug residues in harvested velvet antler tissue and its short acting analgesic effect. This study was designed to determine the effect of local anaesthetic, articaine hydrochloride as an alternative treatment and to establish the baseline measurements of mechanical nociceptive threshold in 40 male yearling red deer. Ten of the forty enrolled deer were selected for the articaine efficacy study. The mechanical nociceptive thresholds were measured using a handheld algometer applied at 4 points; the cranial, medial, caudal and lateral aspects of the base of each antler. The force applied, which resulted in a movement by the animal, was recorded in newtons (N). This study showed that nociceptive threshold response in deer could be reliably measured, and articaine proved to be a promising alternative for velveting the deer antlers.

Abstract: New Zealand deer farming centres on the production of meat and velvet antler. Velvet antler removal is a painful procedure and currently, New Zealand Animal Welfare regulations dictate surgical removal of velvet antlers under lignocaine anaesthesia. To improve our knowledge on the efficacy and duration of other local anaesthetics to mitigate pain after antler removal, it is important to accurately assess and quantify pain arising from antler removal. Therefore, the current study was designed to validate mechanical nociceptive threshold (MNT) testing using a Wagner hand-held algometer, and to apply this methodology to assess the efficacy and duration of action of articaine for antler removal in deer. Baseline force (N) required to elicit the nociceptive response was recorded in 40 yearling male red deer on three alternate days. Ten of the 40 animals were selected for antler removal after administration of 4% articaine hydrochloride as a ring block. The duration of analgesic efficacy of articaine was assessed by algometry across 5 time points. There was a significant difference in MNTs among the three days (day 3 versus day 1 ($p < 0.0001$), day 2 versus day 1 ($p < 0.0001$), and day 1 versus day 2 ($p < 0.01$)). Positive correlations were observed between weight, antler length and thresholds. The MNT values remained above 20N for 6 h after removal of velvet antlers under the articaine ring block. This study provides valuable information about the use of MNT in red deer. These findings lay a foundation for future studies in the topics of peri-operative and postoperative pain management in deer antler removal, and a possible alternative use for articaine.

Keywords: algometry; articaine hydrochloride; deer; local anaesthetic; mechanical nociceptive threshold; pain

1. Introduction

Pain assessment related to deer antler removal was assessed by both behavioural and physiological measurements [1]. The interpretation of painful behaviour is difficult when deer are removed from their normal environment and their social grouping [2]. Most physiological parameters assess stress, which is attributed to pain, but handling stress and restraint can effect behaviour and pain assessments [2]. There were no differences in cortisol levels in the deer, which had antlers removed with analgesics compared to those removed without any analgesia [3], probably due to handling stress or isolation during the procedure [3]. Physiological parameters such as heart rate can be an indicator of stress; it increased following antler removal [4] but was found to be an unreliable measurement for pain assessment. There was no difference observed in the behaviour between stags that had antlers removed and those left intact [5]. In the same study, the authors observed increased walking as a result of both antler removal and restraint only. In other studies, a significant difference in behaviour was demonstrated in deer after antler removal with and without analgesia [3,6]. Deer, being a prey species, evolved to not exhibit the visible signs of pain [7].

Mechanical nociceptive threshold testing (MNT) or algometry is used extensively for assessment of pain and analgesia in animals. Sheep in pain had lower mechanical nociceptive thresholds as compared to healthy animals [8], which aligned with the findings in sheep with footrot [9]. Pressure algometry was successful in detecting and screening on-farm mastitis [10] and lameness in dairy cows [11]. MNT scores at the tail stump were lower in cows with docked tails, as compared to undocked tails, suggesting hyperalgesia [12]. Janczak et al. (2012) performed an extensive study on validation of MNT in piglets and suggested its use for testing analgesic drugs for surgical procedures in pigs, but only after an acclimatisation period of 2 to 3 weeks of repeated testing in normal subjects [13]. Subsequently, MNT was used in testing the efficacy of various analgesic drugs for treating lameness in experimentally induced lameness in sows [14,15]. All of the above-mentioned studies demonstrate the reliability of algometry as a method to objectively measure mechanical nociception.

The Wagner hand-held algometer is a commercially available mechanical nociceptive threshold testing device that was validated for use in various species. The analgesic efficacy of meloxicam for calf dehorning was tested using a Wagner FPX50™ algometry device (Wagner Instruments, Riverside, CT, USA), with significantly increased pain thresholds recorded in calves receiving meloxicam treatment, as compared to the control group [16]. The comparison of analgesia provided by topical anaesthetic spray (Tri-Solfen, Bayer Animal Health, Pymble, NSW, Australia) versus meloxicam administered subcutaneously, for calf disbudded with and without xylazine sedation, was investigated by using the Wagner FPX50™ device. MNT assessment was recorded in 4 different sites around each horn bud from the first hour, until 24 h after disbudding [17]. They reported a significant increase in nociceptive thresholds in calves under sedation, following treatment with meloxicam or Tri-solfen, as compared to the unsedated calves [17]. The same device was also validated and used in horses [18] and dogs [19]. Currently lignocaine hydrochloride is the only licensed local anaesthetic for deer in New Zealand, which is being used as a ring block around the antlers, before the surgical removal of velvet antlers [20,21]. Velvet antler is mainly consumed by people for its medicinal effects [22]. Previously, Woodbury et al. showed concerns about the presence of lignocaine in antler tissue [23]; similarly, Nelson et al. stated the presence of lignocaine metabolites called 2,6-dimethylaniline (DMA), after consumption of velvet antlers in people [24], which is considered to be carcinogenic and toxic [25]. Therefore, there is a need to evaluate a safer local anaesthetic such as articaine. The pharmacokinetics and efficacy of articaine hydrochloride was recently studied in red deer after administration of 4% articaine HCL as a ring block around the base of the deer antler [26,27]. Venkatachalam et al. demonstrated a dose of 1 mL/cm around the antler circumference, at the region of the pedicle, was sufficient to induce analgesia in 3–5-min in red deer. Articaine rapidly metabolises to its inactive metabolites by plasma esterases, thus making it less toxic, as compared to lignocaine or bupivacaine [26,28]. Rapid pain relief, fast elimination, and minimal residues in the antlers, suggests that articaine hydrochloride would be the best candidate for local anaesthesia use in red deer antlers, in the future.

The objective of this study was to establish a mechanical nociceptive threshold testing technique using a hand-held algometer in farmed red deer (*Cervus elaphus*). The validation results would enable the use of an algometer in testing the efficacy of analgesic and local anaesthetic drugs such as articaine hydrochloride, for antler removal in male deer. In addition, this study aimed to investigate the duration of the analgesic effect after articaine administration, via a ring block, using a hand-held algometer.

2. Materials and Methods

2.1. Animals

The animal study was approved by the Massey University Animal Ethics Committee (refer to the end of the manuscript), and at the completion of the study, the enrolled deer were returned to the farm pastures for venison/meat production. Forty yearling male deer weighing 116.6 ± 11.38 kg from the Massey University Deer Unit were recruited for validation assessment of algometry and measurement of baseline MNT. Baseline measurements of body weight and antler length were recorded at the start of the study. Deer body weight was calculated in kilograms using the Tru-Test load bars connected to a Tru-Test XR5000 (Datamars Limited, Auckland, New Zealand), while passing through the handling facility. Antler length was measured by placing a stainless-steel metric ruler alongside the medial edge of each antler. The length was recorded in centimetres from the skull-base to the antler tip. Ten out of 40 animals were selected for the assessment of the duration of action for articaine hydrochloride after surgical antler removal. The animals were selected based on appropriate maturation and antler size determined by the market demand. Based on our pilot study, preliminary results, and including the following assumptions; medium effect size of 0.25, number of cases (40 animals), and number of groups and covariates, the statistical priori power was calculated to be above 0.9, by the G-power software program (version 3.1, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). All animals were clinically inspected by a veterinarian for demeanour, lameness, or any current injuries around the head before the experiment. All deer were kept in paddocks throughout the study period and had free access to pasture and water. On the day of treatment, all deer were mustered into an indoor handling facility on the farm. The deer were placed into holding pens, each containing six deer. Once all deer were inside, two deer were guided carefully from the holding pen to the hydraulic crush (Heenan Work Room, Farmquip, New Zealand). The hydraulic crush enabled the safe handling of deer by confining them between padded walls, with the operator positioned next to the animal. The sides of the crush had curtains above the walls, which could be closed to reduce stimulation and exposure to stressors, while allowing the operator quick and easy access for manipulations on the animals. After each treatment or recording was performed, the deer were released back into a holding pen. While indoors, they had access to water and concentrated feed (Multi-feed nuts, Sharpes Stock Feeds, Carterton, New Zealand). At the completion of each treatment day, all animals were returned to the paddock.

2.2. Pressure Algometry

A hand-held algometer (FPX 25, Wagner Instruments, Greenwich, CT, USA) with a 2 mm-diameter round stainless-steel tip was used to measure the MNT of deer. A single, experienced investigator measured the mechanical nociceptive thresholds of all study animals. For the validation of the hand-held algometer, the MNT measurement was conducted across three alternate days in one week (every second day for 3 test days). The tip of the algometer was placed on cranial, caudal, medial and lateral sites around the base of the antler, 1 cm below the pedicle (the site of antler growth) (Figures 1 and 2). The force was applied (each time for a duration of 1 s by counting 1001) by the operator 1 cm below

the pedicle, which corresponded to the antler root at each of the four sites. The behavioural response indicating the attainment of threshold was head shaking and the algometer reading was automatically held at that response. The reading was recorded in newtons (N). Half of the deer were tested on the right antler, followed by the left antler and vice versa. This was performed to minimise the animal's anticipation of the procedure and reduce bias. The cut-off point for pressure algometry was set at 50 N, to minimise any tissue damage and extra discomfort in animals [8].

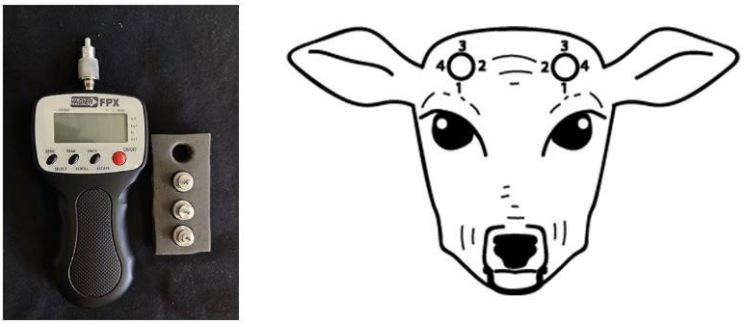

Figure 1. Left: Wagner hand-held algometer (FPX 20) with 2 mm diameter steel probe. Right: Sites of algometer probe placement around the base of the antler to measure nociceptive thresholds. 1 = cranial, 2 = medial, 3 = caudal and 4 = lateral.

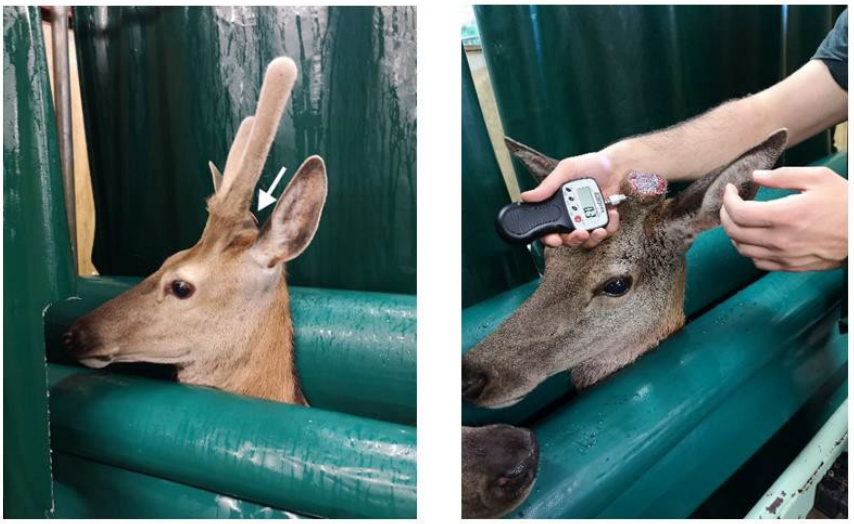

Figure 2. Left: Deer yearling showing early antler growth. Note the acute angle between the antler and the poll of the head. The white arrow indicates the site that the hand-held algometer was placed for the MNT measurements. **Right**: Pressure algometry in red deer using Wager® hand-held algometer below the level of velvet antler removal. This picture illustrates the method used to measure force (N) at the cranial aspect of antler.

2.3. Local Anaesthesia with Articaine Hydrochloride

Articaine hydrochloride (99.9%) was purchased from SCI Pharmtech (Taoyuan, Taiwan) and 40 mg/mL, or 4% injectable solution was prepared from standard powder. The dosage was calculated at 1 mL/cm around the antler pedicle circumference. Before any injections, the base of the antlers was measured in centimetre, using a cloth tape to calculate the volume (mL) of the 4% articaine hydrochloride required. Using a 20-gauge needle attached to a 20-mL syringe, articaine hydrochloride (4%) was administered subcutaneously (SC) as a complete ring block (4–5 injections), 2–3 cm below the pedicles for each antler. The baseline readings of the MNT were recorded prior to the injections. Then, 10 min later, MNT was re-measured followed by a nick test (rubbing saw blade gently against

the velvet antler) to ensure sufficient analgesia before antler removal. Tourniquets were applied before injections to control excessive bleeding from the major antler vessels around the antlers. Then, MNT assessment was performed 2, 4, and 6 h after the surgical removal of antlers (5 time-points in total). The force reading on the device froze with every sudden head shake (withdrawal response followed by stimulated mechanical pain), and the output on the screen was recorded in a data collection sheet. A single MNT test was performed at each time point. In any case of misalignment of the tip of the algometer to the antler surface, the measurement was repeated. Half of the deer were tested on the right antler first, followed by the left antler, and vice versa. This was performed to minimise the animal's anticipation of the procedure and reduce confounding factors. Any MNT measurement above 50 N was recorded as 50 N, to minimise any tissue damage and discomfort in the animals.

2.4. Statistical Analysis

The statistical analyses were performed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism 8.3.0 (GraphPad Software Inc., San Diego, CA, USA). The dependent variable, which was force in newtons, was analysed with the MIXED procedure, using a linear mixed model for repeated measures. The Kolmogorov-Smirnov (KS) test indicated that the dependent variable followed a normal distribution, and data were analysed on the nominal scale without a numerical transformation. The model included the fixed effects; day of measurement (1, 2 and 3), antler (right or left), antler site within antler (cranial, medial, caudal and lateral) and as covariates, antler lengths (cm) and deer body weight (kg). The repeated measures on the same deer were modelled with a compound symmetry error structure, which was determined as the most appropriate residual covariance structure, based on Akaike's information criterion. Least-squares means and standard errors (SEM) for the fixed effects were obtained and used for multiple mean comparisons, using the Fisher's least significant difference, as implemented in the LSMEANS (Least Squares Means) of the MIXED procedure, with a Bonferroni adjustment. Pearson correlation coefficients among antler lengths (cm), animal body weight (kg) and MNT (N) measurements were obtained using the CORR (Correlation) procedure. Estimates of correlation coefficients with $p < 0.05$ were considered significant from zero.

3. Results

All forty animals enrolled were used in the study, and none of the animals were excluded for injury, discomfort, disease or no response to the nociceptive threshold assessment. For the validation of hand-held algometer, a total of 946 out of 960 force test results from a minimum of 3.1 N to a maximum of 46.4 N were obtained in the threshold assessments. Fourteen recordings out of 960 measurements, measured in four animals, were excluded from data analysis, since they did not respond, and they were recorded as "No Response (NR)" with a reading force of above 50 N, which was the cut-off point.

All ten animals that were selected for analgesia with articaine hydrochloride (4%), showed no side-effects or reactions followed by the treatments.

3.1. Hand-Held Algometer Validation

The least-squares means (±SEM) for MNT on days 1, 2 and 3 were 15.49 ± 0.55, 14.18 ± 0.55, 18.74 ± 0.55 newtons, respectively. The estimates of variance components were $\sigma a2 = 8.58$, and $\sigma e2 = 28.55$, and total variance = $\sigma a2 + \sigma e2 = 37.13$, indicating that variation between the animals was 23.2% of the total variation.

The results showed that the force measurements in day 3 of the experiment were significantly higher, as compared to days 1 and 2 ($p < 0.0001$ for both). In addition, the forces measured on day 1 were statistically lower, as compared to day 2 ($p < 0.01$). Figure 3 shows the nociceptive thresholds (N) in three different days of experiments.

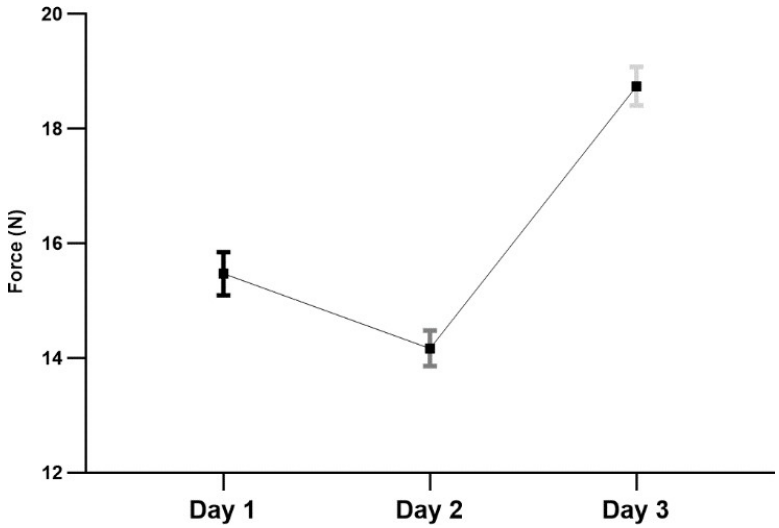

Figure 3. The least-squares means (±SEM) of MNT (N) over three alternate days. The values between all days were significantly different from each other ($p < 0.01$ for Day 1 vs. Day 2, $p < 0.0001$ for Day 1 vs. Day 3, $p < 0.0001$ for Day 2 vs. Day 3).

3.2. MNT Measurements from Left and Right Antlers, and the Testing Sites

The least-squares means (±SEM) of MNTs between the right and left antlers were not significant (right; 16.17 ± 0.52 and left; 16.10 ± 0.52 N). The least-squares means (±SEM) for the cranial, medial, caudal and lateral aspects of the antlers were measured 16.23 ± 0.67, 16.17 ± 0.67, 16.72 ± 0.67 and 15.44 ± 0.67 N, respectively, with no statistical difference.

3.3. The Relationship between Antler Length and Weight with Pain Thresholds in Deer

In general, there was a moderate and positive correlation between body weight (kg) and antler length (cm) with r = 0.6170 and $p < 0.0001$. The results showed a low and positive correlation between antler lengths (cm) and the pain thresholds (r = 0.1580, $p < 0.001$, and r = 0.1025, $p < 0.05$, for right and left antler, respectively). Furthermore, the results showed a low and positive correlation between body weights (kg) and the pain thresholds (N) (r = 0.1746, $p < 0.0001$, and r = 0.1356, $p < 0.01$, for right and left antler, respectively).

3.4. MNT with Articaine Hydrochloride 4% Ring Block

The least-squares mean (±SEM) of body weight was measured 125.01 ± 3.20 kg for ten animals, and the MNT values were normally distributed. The least-squares mean (±SEM) of MNT was measured as 26.93 ± 1.56 newtons. The least-squares means at all time-points were significantly higher compared to each other ($p < 0.0001$) and MNT at time 240 was significantly different, as compared to 360 ($p = 0.0001$), suggesting up to 6 h of the effective analgesia in deer antlers after administration of articaine hydrochloride (4%) (Figure 4).

Based on the obtained results in force records, a scaling system was established in our study, which determined; 0.1–20 N as baseline (B) or grade 1, 20–30 N as Low (L) or grade 2, 30–40 N as medium (M) or grade 3, and 40–50 N as High (H) or grade 4. Therefore, the forces measured at time point zero was considered baseline or B, time points 10 min and 2 h were considered high or H, and time points 4 and 6 were considered L or low in force detection scaling (Table 1).

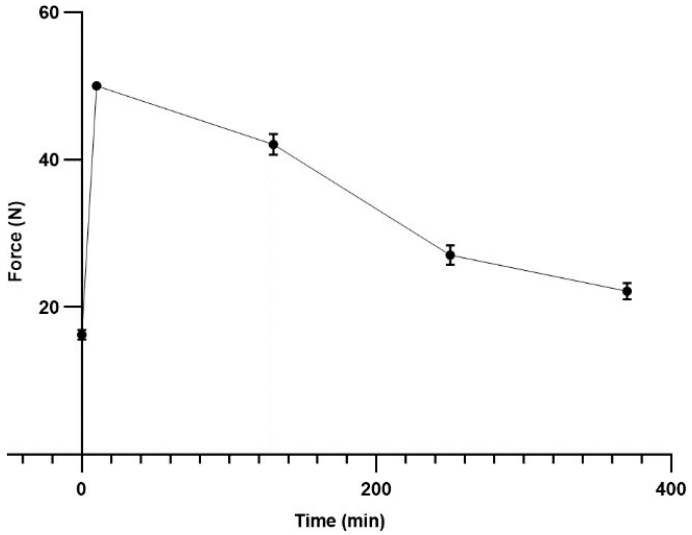

Figure 4. The least-squares means (±SEM) shows the obtained nociceptive thresholds or forces (N) at different time points after ring block with articaine hydrochloride 4% (Each data point represents 10 animals).

Table 1. The least-squares means (±SEM) of MNT (N) for algometry validation and articaine study. The values below depict MNT disregarding antler side and site. N = 40 animals for the validation study, and 10 for articaine study.

Time	Pressure Algometry Validation			Articaine Study
	Day 1	Day 2	Day 3	
Baseline	15.49 ± 0.55 [a] (B)	14.18 ± 0.55 [b] (B)	18.74 ± 0.55 [c] (B)	15.74 ± 0.95 * (B)
10 min				49.37 ± 0.95 * (H)
2 h				41.45 ± 0.95 * (H)
4 h				26.46 ± 0.95 * (L)
6 h				21.55 ± 0.95 * (L)

* statistically significant difference, [a–c] statistically significant difference. B = baseline force, H = High force, M = Medium force, L = Low force.

4. Discussion

The aim of this study was to validate the use of a hand-held algometer for the measurement of mechanical nociceptive thresholds in deer. Algometry is considered as an objective [29] and a highly repeatable [30] method to quantify pain. Mechanical nociceptive threshold testing or MNT is one of the commonly used technique to assess pain and the efficacy of analgesic drugs in research animals [31,32]. In this study, the least-square means of MNT (±SEM) between right and left antlers, and between the antler sites did not show any significant difference between each other, suggesting that antler side and aspect did not affect the MNT measurements. For MNT validation, the cut-off point was set at 50 newtons to minimise discomfort in animals and reduce tissue trauma; thereby, readings of the algometer that exceeded this value were labelled as "No Response" or "NR" to avoid any additional discomfort to the animals and any harm to the site of the stimulus. However, in the study for the assessment of the efficacy for articaine hydrochloride, any value of more than 50 N was considered 50 N. In the MNT validation study, animals that were recorded as "No Response" showed tonic immobility behaviour, which is an evolutionary adaptation for self-preservation and anticipation of any possible danger in nature [33,34]. In such cases, the hydraulic crush was opened, thereby releasing the immobilised deer and was gently re-closed in an attempt to reduce stress and make them more

responsive to the pain assessment. Our experiment cut-off force (50 newtons) was approximately three-fold of the average threshold readings (~16 N), which also aligned with a previously published study by Carroll (1959), as cited in Le Bars et al. (2001), which determined the cut-off point as three-times the threshold of the controls to prevent tissue damage [8,35].

There was an observable acclimatisation to the MNT measurements in deer over the course of this study. Deer are naturally apprehensive and try to avoid handling about the antlers, by moving their heads. By the third day of the study, the enrolled deer stood quietly with predictable behaviour. Stress, anxiety, and diversions of attention are known to change the perception of noxious stimuli [36]. This reflects why the mechanical nociceptive thresholds must always be recorded with minimal restraint, and in a quiet area. In humans, skin bruising is reported when repeated measurements were applied at the same site over 3 consecutive days [37]. Therefore, in this study, we performed our experiment on three alternate days (every other day) to minimise tissue damage. Furthermore, the results of this study suggest that the pain threshold increases significantly on day three of the study compared to days 1 and 2. The frequency of animals with no response (due to tonic immobility) was less on the third day, as compared to the first two days. Additionally, only those measurements acquired on day three showed normal distribution compared to days one and two. This finding suggests a decrease in animal distress and reduced anticipation during the procedure, which could be due in part to the habituation of the animals to the handling facilities, personnel, and procedures, resulting in a more relaxed posture and less tonic immobility. These findings aligned with those of Stubsjøen et al. (2010), which showed that habituation is crucial in order to reduce the variability of mechanical nociceptive thresholds in animals [8]. Furthermore, gentle stroking of the head of the animal and slight occlusion of the vision by lightly cupping the hand near the ipsilateral eye, prior to the algometer placement, helped in obtaining consistent readings.

Some research studies investigating pain assessment showed a decrease in MNT when measured over consecutive days. Stubsjøen et al. (2010) demonstrated a decrease in MNT in sheep over three consecutive days, and Musk et al. (2017) found a decreased MNT in bull calves tested over six days [38]. In contrast, an increase in MNT measurements was observed in the current study. This could be species variation, or the procedure followed in this study did not cause any inflammation around the site of application, which might increase sensitivity to mechanical nociceptive thresholds. Therefore, it is important to validate the test and acclimatise animals prior to conducting pain assessment during clinical research studies, in order to decrease confounding factors.

The reliability of the assessments might be increased by introducing and re-exposing the animals to novel situations. During each assessment day, only one person recorded pain using algometry to decrease the chance of variability in the measurements. The algometer was applied at a regular interval to maintain consistency. It was observed that when the algometer was applied rapidly after initial touch, the deer reacted quickly in a more predictable manner. This could be explained by the sensory nerve pathways arising from the trigeminal nerve (CNV) in growing antlers and A-delta fibres, which are the receptors for pressure and which transmit pain information from an acute noxious stimulus [39,40]. Alternatively, if the algometer is applied slowly, it is more likely that the reaction would be due to A-beta fibres that transmit information from skin receptors and C fibres, which are slow pain receptors [39,40].

The MNT testing was performed at four sites around the base of the antlers; cranial, medical, caudal and lateral, on each of the left and right antlers. The results demonstrated a non-significant difference between the left and right antlers, which indicate that these could be treated as repeatable subjects in future studies. MNT results from all four different sites were normally distributed in the case of cranial, medial and lateral locations; however, the caudal site was not normally distributed. This could be due to the topographical location and acute angle of the developing antler (Figure 2), which limits access for the perpendicular placement of the algometer tip, compared to the other sites.

A positive correlation was demonstrated between animal body weight and MNT, between antler length and MNT, and between animal body weight and antler length, which means that, the heavier yearling deer had longer antlers. These results suggest that larger animals with increased body weight or animals with increased antler length are more tolerant of a painful stimulus applied to the velvet. It is likely that the increased tolerance could be a result of tissue changes within the antler as it develops. For example, the network of sensory nerve fibres in the vascular layer and those penetrating the superficial layers of the dermis might atrophy as the antler matures, and this process might begin much earlier than the point at which the antler is entirely calcified and cast. Further studies are required to establish when the nerve network to the velvet tissue declines, and if the hardening of the underlying cartilaginous tissue affects this process. It was already documented that the most proximal region of the antler is more calcified compared to the distal tip [41]. Consequently, it could be surmised that the longer and thus, more developed antlers in this cohort would have had increased pain tolerance at the base. Furthermore, it was demonstrated that during the growth phase, male deer have virtually undetectable plasma testosterone (T) hormone levels, which might influence behaviour, making them more docile and less likely to damage the growing tissue [42].

Various authors suggested the use of articaine hydrochloride for local analgesia for human adults [43] and infants [44], and animals such as rats [45], cattle [46,47], and deer [26,27]. Therefore, the increasing demand for this local anaesthetic, its safety and rapid onset, encouraged us to investigate its duration of action on red deer, which is poorly studied. Currently, in New Zealand, the only licensed local anaesthetic used in deer is lignocaine hydrochloride [20,21], which itself is poorly studied in terms of pharmacokinetics and using more objective pain assessments such as MNT. The authors previously, only depended on behavioural analysis [23,48], and electrical stimulation [49] to prove the pain relief effects of lignocaine in deer. Our study in combination with the findings from two studies by Venkatachalam et al. [26,27] suggests that articaine hydrochloride can be a better candidate for pain relief for deer, after velvet antler removal, since it has a rapid onset of action, it is safer than lignocaine and bupivacaine, it has an analgesic duration of up to 6 h, and shows minimal residues

after application. The study was stopped after 6 h, as the recorded force readings reached close to 20 N, which was the boundary of the baseline category (according to our established scaling system in force reading) (see Table 1). As a result of recent studies, which also includes the pharmacokinetic findings, its application in deer now surpasses that of lignocaine hydrochloride, which was used as the only licensed product for deer for two decades.

The primary limitation faced in this study was managing the deer's innate behaviour. Deer are a prey species that display a particular unlearned behaviour known as tonic immobility when faced with a threat. Tonic immobility is a behaviour expressed as a last resort, most typically when a predator is in close proximity or has made contact. This should be distinguished from freezing behaviour that occurs when a prey animal is under pursuit and is attempting to camouflage within its environment [50]. Tonic immobility can occur during periods of physical restraint, and these events might last from seconds to hours and can persist after the animal is physically released. Signs of tonic immobility include bradycardia, bradypnoea, salivation, defecation, and urination, which is a direct result of parasympathetic activity [51]. The mechanism behind this phenomenon is a result of the complex interplay between serotonin, the stress hormone corticotropin-releasing factor (CRF) and dopamine with the amygdala and varies between species [51]. Over the course of this trial, non-resolving tonic immobility was encountered, whilst the deer were physically restrained in the hydraulic crush, occurring in 14 out of 960 measurements. These measurements were recorded as NR, indicating no response, and an algometer reading beyond the predetermined cut-off point (50 N). Habituation to the hydraulic crush, holding pens, and experimental area occurred periodically during the months prior, as the facility was used for other animal husbandry procedures such as anthelmintic drenching and vaccinations. However, animal handlers and the pain assessor could be viewed as a threat by the deer. The removal of antlers without veterinary supervision or anaesthesia is considered a contravention of the Code of Recommendations and Minimum Standards for the Welfare of Deer During the Removal

of Antlers (1992) [52], and Animal Welfare Act 1999 [53], and is accordingly a prosecutable criminal offence in New Zealand. It is for this reason a control group receiving no analgesia was not included in this study to compare against the articaine treatment group.

In this study, a hand-held algometer was used to measure the MNT. This device was successfully used in other species, including humans. The limitations to the use of this device include variability in the rate of application of pressure on the tip and suitability of the tip diameter for the size of the animal. The assessor applied the algometer for approximately 1 s, with the cessation of pressure occurring when the deer moved away from the algometer tip. An additional improvement would be a remotely controlled algometer that can apply pressure at a consistent speed. This study provides a basis for MNT testing of growing deer antlers, and additional investigations and development of standardised testing protocols would allow a comparison between studies. In terms of the articaine study, the current experiment only investigated the effect of this local anaesthetic for a peri-operative pain management. There might be scope to use articaine hydrochloride in a formulation that prides postoperative analgesia with an extended duration of effect.

5. Conclusions

This study validates the use of MNT in red deer, as a method of testing the efficacy of analgesia for antler removal. A number of confounding factors were determined, which could alter the level of tolerance to pain in deer undergoing MNT, such as body weight and antler length. To our knowledge, this is the first study on MNT in red deer antlers and forms the basis for future research. The results from the articaine trial suggests that this local anaesthetic is an ideal alternative for the practice of antler removal due to its rapid onset, suitable duration of pain relief, wide safety margins, and high elimination from the body.

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