Efficacy of Sustained-Release Novel Bupivacaine Formulations in Sheep

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Abstract:

The objective of this thesis was to prepare and assess several formulations of the local anaesthetic bupivacaine to achieve a longer duration of action. Intralipid® emulsion (a soybean oil emulsion) and collagen combined with titanium oxide nanoparticles were used to develop slow release bupivacaine formulation. These formulations were tested both *in vitro* as a pilot study and *in vivo in sheep*.

Collagen was extracted from bovine limed split hide (a by-product of the leather industry). The collagen as a 1% solution was mixed with bupivacaine hydrochloride 0.5% aqueous solution (Marcain® 0.5%, AstraZeneca, New Zealand) giving a final concentration of 0.25% bupivacaine. Intralipid® (20%, Fresenius Kabi Australia) and bupivacaine 0.5% were mixed resulting in a 0.25% bupivacaine lipid emulsion. Both formulations were tested in vitro pilot study for the release of bupivacaine through a dialysis membrane. The concentration of bupivacaine in the dialysate was measured using High-Performance Liquid Chromatography (HPLC). In the animal studies, 18 sheep were used to compare bupivacaine (control) and bupivacaine-Intralipid®, and another 18 sheep for commercial bupivacaine (control) and collagen- bupivacaine. Each sheep received a nerve block using the control or test formulation in each forelimb. The nerve block was placed at the level of the accessory digits with three injections totalling 4 mL using a 22G needle. The efficacy was tested by manually applying a mechanical noxious stimulus with a blunt instrument below the level of the block. This test was performed first after 15 min and then at one-hour intervals. The time at which a response was observed was considered as the end-point for that formulation.

In the *in vitro* pilot study, both collagen and Intralipid®-based formulations showed slightly more sustained release compared to the control group. However, collagen-based formulation of bupivacaine had the most sustained-release among all.

In the sheep study, the Intralipid®-based formulation significantly extended the duration of the nerve block compared to the control group ($P<0.05$). On the contrary, the collagen-based

formulation of bupivacaine shortened the duration of action significantly compared to control group ($P<0.05$).

In conclusion, an Intralipid®-based formulation provided a more sustained action after nerve blocks in the sheep metacarpal region compared to aqueous bupivacaine or the collagen based formulation. Further research on structure and activity of collagen and its interactions with bupivacaine is required to develop a longer acting formulation.

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Abbreviations:

Col	Collagen
HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
IVRT	*In vitro* drug release test
LLQ	lower limit of quantification
MNT	Mechanical nociceptive testing
NaCl	Sodium Chloride
NaOH	Sodium hydroxide
NH4Cl	Ammonium chloride
NP	Nanoparticle
PAA	Poly (acrylic acid)
PVP	Poly (vinylpyrrolidone)
UV	Ultra Violet
TiO_2	Titanium oxide
ZnO	Zinc oxide

Chapter 1

Introduction

1.1. Background

Sheep welfare is critical from both of ethical and economical aspects in New Zealand. One of the five freedoms included in the Animal Welfare Act 2006 is “freedom from pain, injury and disease”. Pain management in sheep is still a controversial issue due to the difficulty in recognition of the pain, lack of knowledge of the use of analgesic and local anaesthetic agents, the relatively high cost of the treatments and the possibility of residues in meat or milk. High doses could also cause side effects. Moreover, there are very few analgesic and local anaesthetics registered for sheep, which exacerbates the situation (Lizarraga and Chambers, 2012, Martinsen et al., 2011, Chambers et al., 2002). Overall, local anaesthetics, particularly lignocaine, are the most common pre-operative analgesic agents used in food animal practice such as sheep medicine (Goldberg, 2014, Muir, 2013).

For these reasons, it is essential to discover new ways to provide long lasting analgesia in sheep. One approach is sustained-release formulations of local anaesthetics. Our study aimed to develop and test the efficacy of two different formulations of bupivacaine in sheep to prolong the duration of action and thus reduce the total dose used, and so the chances of side effects and residues (Coetzee, 2013).

1.2. Bupivacaine

Bupivacaine is a typical local anaesthetic which blocks nerve conduction. It has a longer duration of action than the most commonly used drug, lignocaine. Local anaesthetics are usually injected around the nerve or neurones intended to be blocked, penetrate the neurones and block the voltage gated sodium channels. Their action is ended when they are absorbed and distributed away from the site of action.

Bupivacaine is an amide (aminoacyl aniline) local anaesthetic commonly used in both human and veterinary clinical practice (Mather et al., 1994). For peripheral nerve blocks in humans, the usual dose is 12.5 mg (5 mL, when the solution is 0.25%) or 25 mg (5 mL, when the solution is 0.5%) (Sweetman, 2009). The onset of action of bupivacaine is dose dependent and can vary from 3 to 6 min after administration, and duration of action varied from 80 to 97 min (McMorland et al.,

1986). The onset of action of bupivacaine is pH-related; at higher pH, onset is shorter compared to lower pH (McMorland et al., 1986). In sheep, the onset of action was within five min after a metacarpal block and lasted for 110 min (Lizarraga et al., 2013). The maximum effect of standard bupivacaine after epidural injection is reported to be around 16.5 min which can be decreased to 14 min by increasing the pH of the bupivacaine injection solution (McMorland et al., 1986). There are numerous reports of the pharmacokinetics of bupivacaine in animals. Feldman *et al.* reported that epidural injection of bupivacaine lasted for 81 ± 42 min in twelve male beagles and it involved both motor and sensory nerves (Feldman et al., 1996). They stated that bupivacaine reached its peak in the serum within 8 min after epidural administration in sheep and the terminal elimination half-life in serum was 6 hr. They also reported that there was no systemic toxicity from bupivacaine in sheep at the dose given (37.5mg)(Feldman et al., 1997). A slow release formulation of bupivacaine has also been shown to produce longer blockade with lower plasma levels than the aqueous solution in sheep brachial plexus block (Estebe et al., 2001).

The plasma protein binding of bupivacaine in both humans and animals is 95% (Heavner, 2007, Sweetman, 2009). In the blood, the two main binding proteins for bupivacaine are α1-acid glycoprotein (predominantly at low concentrations of bupivacaine), and albumin (which plays the major role at high concentrations of bupivacaine) (Sweetman, 2009). Reduction in pH from 7.4 to 7.0 reduces the affinity of the α1-acid glycoprotein for bupivacaine but has no effect on the affinity to albumin (Sweetman, 2009).

The final metabolism of bupivacaine occurs in the liver via conjugation with glucuronic acid, and its phase one metabolite is 2,6-pipecoloxylidine (Sweetman, 2009). This metabolite is produced by cytochrome P450 3A4 and excreted in the urine (Sweetman, 2009). The hepatic extraction of bupivacaine is higher in sheep (Mather, 1991, Rutten et al., 1991, Rutten et al., 1993) compared to humans (Tucker et al., 1977, Wiklund, 1977). In humans, the plasma half-life varies from 1.5 - 5.5 hr in adults and 8 hr in neonates (Sweetman, 2009).

Local analgesia means that the nociceptive signals transmitted by afferent nerve fibres are efficiently blocked (Butterworth and Strichartz, 1990, Hartrick, 2004). In both humans and animals, bupivacaine acts by changing with the conformation of proteins in voltage-gated sodium

channels which are located in nerve cell membranes (Butterworth and Strichartz, 1990, Hartrick, 2004, Goldberg, 2014). The sodium channels become less permeable to sodium ion influx, thereby inhibiting depolarization and generation of action potentials along the axons. A small dose of bupivacaine is sufficient to block action potential in small C fibres and Aδ-fibres responsible for pain transmission. The large Aβ fibres responsible for motor function are not affected at low doses (Butterworth and Strichartz, 1990, Hartrick, 2004, Goldberg, 2014).

In comparison to other local anaesthetics, bupivacaine has a longer duration of action. It is also cardiotoxic when given in higher doses to prolong its duration of action up to 20 hr (Rutten et al., 1991, Sweetman, 2009). Since the side effects are related to plasma concentration but nerve blockade is related to the concentration at the neuron, one strategy to increase the duration of action and reduce side effects is to reduce absorption form the site of injection. Multiple small doses of bupivacaine can also be used but, multiple injections are associated with other problems in animals, such as increased stress and potential injuries to animals due to repeated handling. It can also be challenging for veterinarians to administer repeated injections. Since postoperative pain is likely to last for several days, long duration pain management techniques are required. Thus, there is a need to develop a sustained-release delivery method for bupivacaine in which it is released gradually over an extended period, which should produce fewer side-effects. It may be possible to provide suitable local anaesthesia or analgesia using single dose, thereby further minimising the adverse effects. Synergy in combination with another drug or entrapping the molecules in some specific structures are possible methods which could lead to a greater analgesic effect and fewer side effects rather than multiple injections of the same components (Hartrick, 2004).

Consequently, this project studied two different formulations to test their sustained-release characteristics and their effect on prolonging the action of bupivacaine. The formulations were an Intralipid®-based formulation of bupivacaine and a collagen-based formulation of bupivacaine.

1.3. Collagen

Collagen is one of the most abundant proteins, and more than 30 percent of the structural proteins in animals are made from collagen (Friess, 1998). It is present in tissues including skin, bone, tendon, cartilage and cornea. The various functions of collagen in these tissues are related to the different collagen types and structures. In vertebrate species, there are more than 27 types of collagen found in the tissues and all of them are made of three alpha chains in triple helical molecules (Bhattacharjee and Bansal, 2005, Shoulders and Raines, 2009b).

1.3.1. Collagen structure at the molecular level

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

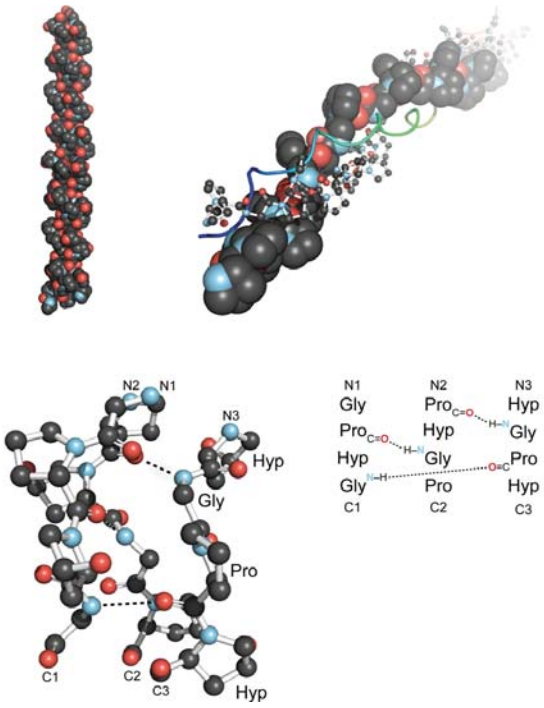

Figure 1.1 *– The collagen triple helix formed from (ProHypGly)4–(ProHypAla)–(ProHypGly)5.*

(Photo:(Shoulders and Raines, 2009a))

The polypeptide has a repeating amino acid sequence Gly-Xaa-Yaa (Shoulders and Raines, 2009b). Glycine is usually present in every third position (Figures 1.1 and 1.2); while, Xaa and Yaa can be any amino acid (Fratzl, 2008). With the smallest side group in all amino acids, glycine is the only amino acid that can efficiently approach the central region of the triple helix (Bhattacharjee and Bansal, 2005). The various types of collagen are distinguished by the alpha chains (Shoulders and Raines, 2009b). For example, fibrillar collagens which contain collagen type I, II, III, V, etc., have continuous triple helical domains at the centre of the alpha chains (Fratzl, 2008) whereas the non-fibrillar collagens (fibril-associated collagens) have intermittent triple helical domains linked by one or more non-helical domains (Fratzl, 2008). The alpha chains are joined primarily by intermolecular H-bonding. Also, every three-peptide unit makes one direct H-bond between the N-H of glycine and C=O of Xaa in the adjacent peptide. Meanwhile, water molecules support the formation of indirect H-bonds between every two peptides (Bhattacharjee and Bansal, 2005).

The three alpha chains can be either identical or different in a collagen molecule, depending on the collagen type (Fratzl, 2008). A type I collagen molecule has two equal α1 chains and one α2 chain (Fratzl, 2008). The Xaa and Yaa amino acids are mainly proline and 4-hydroxyproline, respectively (Friess, 1998).

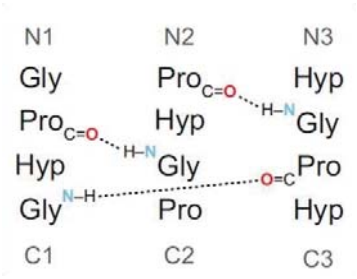

***Figure 1.2**- Schematic description of direct inter H-bonding formed in a segment of collagen triple helix (Shoulders and Raines, 2009b).*

1.3.2. Clinical applications of collagen

Collagen has been widely used in biomedical research for drug delivery and tissue engineering (Glowacki and Mizuno, 2008). Abundance in raw form, simple extraction process, biocompatibility and biodegradation (Lee et al., 2001) makes collagen an excellent agent for tissue engineering. It can be easily extracted from tendons, fish skin (Singh et al., 2011), bovine hide, chicken skin and bovine limed split (Li et al., 2008, Zhang et al., 2006). Most of these are the by-products of leather or food industries and do not usually have a high value themselves. As a natural material, collagen is an essential protein in human extracellular matrix and tissues like muscles, skin and bones. Collagen is very bio-compatible, and the use of collagen for implants is unlikely to evoke immunogenic responses. Furthermore, enzymes inside a mammal's body can break down collagen into its smaller components which makes it biodegradable, thus reducing concerns about side effects after application (O'brien, 2011).

The idea of tissue engineering is to provide a template with a blend of cells to support the re-growing process in damaged tissue (O'brien, 2011). Collagen for skin replacement was primarily prepared in the sponge form (Lee et al., 2001). The encapsulated fibroblasts inside the lyophilized collagen sponge supported the transplantation of artificial skin and epithelialisation of the defected area (Lee et al., 2001). Collagen is also significantly osteoinductive, and it aids in bone regeneration (Lee et al., 2001). Altered collagen biomaterials have also been invented to make artificial blood vessels, heart valves, etc. (Lee et al., 2001). Collagen biomaterials for drug, gene or protein delivery can be manufactured in sponge-like forms to facilitate wound healings, or as film to protect the eye in ophthalmology (Lee et al., 2001). Another use of collagen is its ability to entrap drug molecules in its matrix by electrostatic interactions, covalent interactions or H-bonding (Lee et al., 2001). Thus, collagen's biocompatibility, simple extraction procedure, abundance and drug entrapping abilities, should make it an excellent vehicle for sustained-delivery of bupivacaine.

Efficacy or safety of collagen and bupivacaine formulations has not been reported in sheep. The only studies on this drug combination's effectiveness in reducing postoperative pain were performed on humans after embedding collagen sponges under the skin following surgical

operations (for correcting inguinal hernias and gynaecological surgeries in patients). This formulation was considered effective because it produced longer action with minor side effects (Cusack et al., 2012a, Cusack et al., 2013). Since implantation is only possible in open surgeries, we investigated the use of an injectable combination in our study on sheep.

1.4. Collagen nanocomposite

Friess (1998) stated that fast degradation, low thermal stability and the weak mechanical structure of extracted collagen restricts the applications of collagen in some areas (Friess, 1998) , although these are not necessarily disadvantages in drug delivery. Structural variations in collagen matrix are required for drug entrapment and its controlled release (Lee et al., 2001). Collagen matrix can be strengthened by polymers, organic or inorganic cross-linkers. (Lee et al., 2001).

Nanocomposites are a new class of materials which contain multi-phases with at least one phase having a size smaller than 100 nm in one or more dimensions (Aimé and Coradin, 2012). The nano-materials are inorganic, are implanted in other phases like polymers, and can tune the mechanical, conductive or even magnetic properties of the polymers to give functions that are not possible with organic polymers alone (Aimé and Coradin, 2012). Zinc oxide nanoparticles are added to rubber to increase its thermal conductivity while preserving its high electrical resistance (Kołodziejczak-Radzimska and Jesionowski, 2014). Similarly, other inorganic materials are used as cross-linkers to improve the thermal and mechanical functions of collagen biomaterials. These collagen nanocomposites can be made-up by mechanically combining the nanoparticle suspension with collagen matrix (Aimé and Coradin, 2012).

Numerous studies have been conducted on collagen nanocomposite hydrogels containing gold, iron oxide and chromium oxide nanoparticles. Carboxylic acid functionalized gold nanoparticles are used to cross-link collagen to form hydrogels with enhanced thermal stability and viscoelasticity (Castaneda et al., 2008, Schuetz et al., 2013). A study conducted by Wilson *et al*. (2009) on gold nanoparticle collagen composites demonstrated that polyanionic terminated gold nanoparticles made the fibrillogenic process faster and also improved the stiffness and elasticity of the collagen composite (Wilson et al., 2009). Nidhin *et al.* (2014) cross-linked collagen with

starch-coated iron oxide, and showed that this compound could significantly reduce iron oxide toxicity. This compound made the composite a fascinating material for magnetic resonance and fluorescent imaging (Nidhin et al., 2014). Collagen fibrils generated with the fibrillogenic procedure were cross-linked via H-bonding with OH groups of the starch molecule which improved the mechanical strength and was more appropriate for tissue engineering compared to the collagen without the nanoparticles.

In this project, the focus was on preparing collagen nanocomposite hydrogels with TiO_2 or ZnO nanoparticles. Both titanium oxide (TiO_2) and zinc oxide (ZnO) nanoparticles are inorganic metal oxide nanoparticles, and they have many similar properties. Both are broadly used as components or additives in many areas due to their diverse properties (Yin et al., 2013, Ludi and Niederberger, 2013, Cargnello et al., 2014, Kołodziejczak-Radzimska and Jesionowski, 2014).

TiO_2 and ZnO nanoparticles also have self-cleaning and anti-bacterial effects on different surfaces because of their photocatalytic properties (Kołodziejczak-Radzimska and Jesionowski, 2014, Qi and Xin, 2010). They have been used in biomedical areas including bio-sensing, phototherapy and bio-imaging (Yin et al., 2013, Xu et al., 2007).

TiO_2 and ZnO have been used to improve the mechanical properties of polymeric materials in three different ways. The integration of TiO_2 nanoparticle can play a role as a filler to enhance the viscosity of a polymer solution (Harzallah and Dupuis, 2003). The degree of viscosity improvement is affected by how well the nanoparticles are spread through the polymer matrix (Nasu and Otsubo, 2006). TiO_2 and ZnO nanoparticles can also enhance the mechanical stability (higher tensile strength) of the polymer materials by playing roles as cross-linkers. Also, TiO_2 and ZnO nanoparticles can work as photo cross-linkers since they are photo-active and can produce superoxide radicals (Kołodziejczak-Radzimska and Jesionowski, 2014, Liao et al., 2014).

1.5. Intralipid®

Intralipid® (Fresenius Kabi, New Zealand Limited) is a sterile emulsion containing soybean oil, egg lecithin and glycerol and 10%, 20% and 30% solutions are available. It is commonly used as a source of calories and essential fatty acids (West et al., 2010, Lee, 1977) for parenteral nutrition (Fischer et al., 1980). It is also used for treating toxicity due to lipophilic or cardiotoxic agents such as local anaesthetics (especially bupivacaine as it is cardiotoxic) and macrocyclic lactones (Weinberg et al., 1998, Weinberg et al., 2003, McCutchen and Gerancher, 2008). Intralipid® intravenous infusion was proven to be useful in the treatment of bupivacaine toxicity in dogs and rats (Weinberg et al., 1998, Weinberg et al., 2003), probably due to the strong interaction of lipids with bupivacaine. Intralipid® significantly reduces the plasma concentration of available bupivacaine, thus reduces the toxicity after overdose (Laine et al., 2010).

There are quite a few articles regarding injectable emulsions and drug targeting especially in parenteral form (Hörmann and Zimmer, 2016). Long chain triglycerides (LCTs) which are lipid emulsions have been approved for medical use, and soybean oil is a good example of these lipids (Hippalgaonkar et al., 2010). Intralipid® is a commercial injectable emulsion which contains soybean oil and is more affordable than previous methods such as liposomes and strongly interacts with bupivacaine (Laine et al., 2010). There is evidence of pain reduction during injection of anaesthetic drugs specifically Propofol with a combination of LCTs in humans which also could be very suitable while injecting bupivacaine mixed with a lipid-based emulsion (Doenicke et al., 1996). However, there is no evidence so far of its efficacy in the delivery of bupivacaine and prolongation of local anaesthesia in animals such as sheep. Therefore, due to its reported interactions with bupivacaine, it could be an excellent candidate for its prolonging the duration of action of local anaesthesia.

1.6. Pain in sheep

Pain is a universal sensation, and all mammals can experience it in a similar way although the intensity of the feeling might be different in every individual (Beecher, 1957). The International Association for the Study of Pain (IASP) describes the 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). Healthy adult humans can report their pain accurately, but in non-speaking humans' and animals' behaviour must be used to express their feeling of experiencing pain (Anand and Craig, 1996).

Pain is a very complicated process, and it includes both physical (sensory) and emotional categories (Hellebrekers, 2000). For maximally effective pain management, understanding the precise mechanism and pathways in which pain is perceived is of great importance (Figure 1.3); since, analgesic and local anaesthetic agents affect these pathways in different ways and animals do not have the ability to speak and express their feelings of pain (Farquhar-Smith, 2008). Pain sensation from the time the noxious stimulus touches the skin until it reaches the brain involves transduction, transmission, modulation, projection and finally perception (Goldberg, 2014).

Pain can be clinically classified as acute or chronic depending on its duration; it can also be considered as musculoskeletal (somatic), neuropathic and visceral regarding its location (Hsu, 2013, Lorenz et al., 2010). Livingston and Chambers (2000) and Woolf and Chong (1993) categorised two types of pain for easier understanding as physiological and pathological pain (Woolf and Chong, 1993, Livingston and Chambers, 2000).

The term, "physiological pain" is the feeling of a noxious stimulus which is acute and often defensive to protect from tissue damage. Perception of this pain depends on the intensity of the noxious stimulus (Woolf and Chong, 1993). On the other hand, "pathological pain" is perceived as greater than justified by the noxious stimulus. It is usually associated with an inflammatory response and possibly tissue damage and this pain can be either acute or chronic (Woolf and Chong, 1993). However, some cases without an obvious inflammatory response are still called pathological pain. They usually involve nerve damage and impairment (Loeser and Treede, 2008, Livingston and Chambers, 2000).

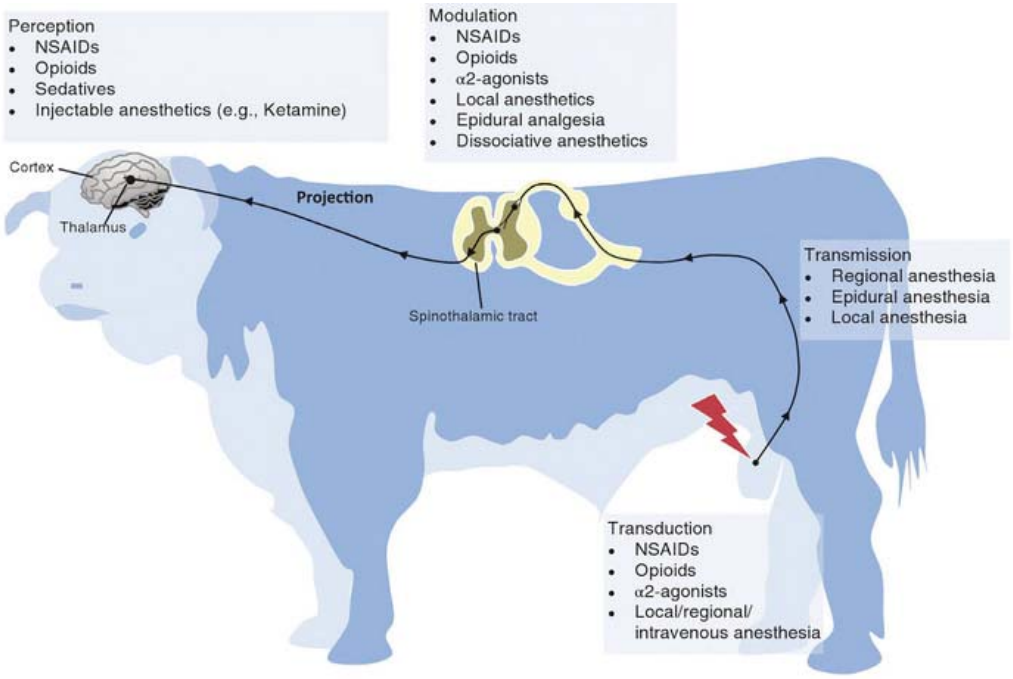

Figure 1.3 *– The nociception pathway in ruminants such as sheep. This picture also shows the target receptors for various local anaesthetic and analgesic agents.*

("Pain Management for Veterinary Nurses and Technicians" by Goldberg, ME (Goldberg, 2014).)

Some surveys suggest that 81% of consumers believe that food animals and humans have the same ability to feel pain (Coetzee, 2013). However, it should not be denied that pain recognition is very challenging in stoic species such as sheep (Coetzee, 2013, Goldberg, 2014). Human infants and juvenile animals might experience pain more intensely compared to older animals (Moss et al., 2007, Johnson et al., 2005, Mellor and Gregory, 2003).

Pain signs in sheep are generally like those in cattle and other ruminants. They often tolerate severe injury without noticeable signs, and they usually act normally even when they are exposed to painful stimuli (Fitzpatrick et al., 2006). The reason behind this could be because animals showing signs of injury are more likely to be chosen by predators in nature and sheep may regard people as predators (Beausoleil et al., 2005).

There are various signs which might occur when the sheep is in pain:

Guarding: The animal keep changes his posture as it feels irritation and discomfort and tries to move in a way to avoid body contact with the stimulant.

Abnormal appearance: The animal stops grooming behaviour and in general shows visible changes comparing to a healthy animal.

Altered behaviour: The animal may stop moving or become hesitant to move its body. It also may show restlessness with signs of circling or weight shifting and even changed sleep patterns. Sheep usually grind their teeth or move their tail or sometimes they might curl their lips as a sign of discomfort.

Vocalisation: The animals might make sounds and vocalise while being handled specially in some parts of the body.

Mutilation: For example, licking, biting, scratching, shaking, or rubbing behaviour when a part of the body is painful.

(Institute for Laboratory Animal Research (U.S.). Committee on Recognition and Alleviation of Pain in Laboratory Animals, 2009, Molony et al., 2002).

Pain can also be assessed by applying a noxious stimulus and noting the animal's response (with suitable controls). Mechanical nociceptive testing (MNT) usually includes the application of pressure externally to produce a noxious stimulus. The stimulus is typically measurable, and the responses often include lifting a leg (when it is applied to the leg or foot) or skin flicking, vocalisation, ear or tail flicking, gait and posture changing or even standing still etc. depending on the animal species or how the stimulus is located (Chambers et al., 1990, Le Bars et al., 2001, Ley et al., 1989, Vivancos et al., 2004). The most frequently used equipment for MNT, particularly in farm animals is a blunt object like a haemostat in a specific area of the animal's body, usually to compress the highly innervated skin against a bone (Chambers et al., 1990, Ley et al., 1989).

1.7. Objective

The objective of this study was to investigate a longer acting bupivacaine formulation compared to the one currently being used in practice. Two different formulations of bupivacaine were developed and compared to aqueous bupivacaine hydrochloride using *in vitro* pilot study, followed by *in vivo* techniques. Nanocomposite collagen and titanium oxide formulations and the commercially available Intralipid® emulsion were used to develop sustained-released bupivacaine formulations.

1.8. Hypotheses

1) The collagen-based formulation of bupivacaine can entrap bupivacaine and slow its release thus increasing its duration of action compared to aqueous bupivacaine hydrochloride.
2) The Intralipid®-based formulation of bupivacaine can slow the release and increase the duration of action of bupivacaine compared to aqueous bupivacaine hydrochloride.

Chapter 2

Materials and Methods

2.1. *In vitro* pilot study

This experiment was a pilot study conducted to determine the rate of release of bupivacaine from different formulations.

2.1.1. Collagen extraction procedure

The collagen was extracted from a waste product of the leather industry called limed split. Bovine limed split is the layer shaved off from the inner side of the hide after it has been treated with lime during leather processing (Albu et al., 2011, Ocak, 2012). Zhang et al. (2006) proposed a methodology for neutralising limed split, which was followed in this study. The pH of the limed split was neutralised by adding 2% NH4Cl and 0.5% HCl. At the end of the procedure, the pH of the solution increased to higher levels and was neutralised by 0.5% HCL to reach around pH 8 at the end. The limed splits were freeze-dried and kept at 4°C before collagen extraction (Zhang et al., 2006). Then, the pieces of the dried limed split were soaked in 0.5 M acetic acid 30:1 (v/w). Pepsin (20 kU/g dry split) was used, and the mixture was incubated and stirred for 48 hr at 4˚C. The final steps of the procedure were based on the methods of Singh et al. (Singh et al., 2011). The supernatant or floating pieces of collagen was collected and combined with the first extract. A saturated NaCl solution (359 g/L) was added to the supernatant until the collagen precipitated out and then it was collected and re-dispersed in the smallest volume of 0.5 M acetic acid solution. Then it was dialysed against 0.005 M acetic acid solution for about three days, with the dialysing solvent (was changed every 24h). Eventually, the pH of the collagen was around 4.

2.1.2 Collagen-based formulation of bupivacaine crosslinked with zinc oxide

Collagen solution 1% (2mL) was mixed with 2 mL of commercial bupivacaine (Marcain® 0.5%, AstraZeneca, New Zealand) and vortex-mixed for a few minutes. Then, in a Petri dish, 300 microliters of ZnO-PVP (polyvinylpyrrolidone) were added to the mixture with a pipette to make the structure and texture stiffer (Figure 2.1). Finally, this gelatinous combination was put in different syringes and was used in both laboratory tests and injections in sheep. Figure 2.1 shows how stiff the collagen solution structure became after addition of zinc oxide-PVP.

Figure 2.1 *– Collagen formed a harder and more stable structure after addition of ZnO-PVP. pH was around 7 which is neutral.*

2.1.3 Collagen-based formulation of bupivacaine crosslinked with titanium oxide

2-mL of commercial bupivacaine (Marcain® 0.5%, AstraZeneca, New Zealand) was vortex-mixed with 1-mL of collagen followed by 1mL of TiO_2 in a 50mL sterile conical test tube. This mixture was vortex mixed for 10 min. The pH of the resulting solution was adjusted between 7 and 7.8 using sodium hydroxide. The volume of the solution was made up to 4 mL using sterile Milli-Q water.

2.1.4. Intralipid®-based formulation of bupivacaine

Intralipid® (Fresenius Kabi, Australia) was a mixture of 10% soybean oil, 1.2% egg yolk phospholipids, 2.25% glycerine, and water for injection. To prepare the bupivacaine formulation, 2mL of this emulsion was vortex-mixed with 2mL of bupivacaine (Marcain® 0.5%, AstraZeneca, New Zealand).

2.1.5. Spectrophotometry

Varian Cary® 50 UV-visible spectrophotometer (USA) was used for analysis of bupivacaine concentration. The specifications of this spectrophotometer were dual beam, Czerny-Turner monochromator, 190–1100 nm wavelength range, approximately 1.5 nm fixed spectral bandwidth, full spectrum Xe pulse lamp single source, dual Si diode detectors, quartz overcoated optics, scan rates up to 24000 nm/min, 80 data points per maximum measurement rate, non-measurement phase stepping wavelength drive, room light immunity. The central control was operated by PC with Microsoft® Windows® system. Data were recorded and graphs produced by Varian Cary Win UV software program. For serial dilution, Eppendorf Research® plus pipettes (Germany) were used.

2.1.6. Calibration of the UV spectrophotometer

The UV spectrophotometer was calibrated by solutions of 0.01, 0.03, 0.06, 0.1 and 0.2 W/V% bupivacaine. The linear model based on these standard calibration solutions were calculated and shown automatically by Varian Cary Win UV software program.

2.1.7. *In vitro* drug release test (IVRT)

This study was conducted to assess the release of bupivacaine from its collagen matrix (Figures 7.6 and 7.7).

2.1.7.1. Equipment used in *in vitro* drug release test (IVRT)

A glass beaker (100 mL) was filled with normal saline 0.9% (80- 100 mL), and a dialysis membrane made from cellulose (Visking tubing, The Scientific Instrument Centre Ltd, England) was used. The temperature of normal saline was maintained at 40˚C. The solution was continuously stirred on a magnetic stirrer (200 rpm).

2.1.7.2. The procedure of *in vitro* drug release test and the calculations

Control bupivacaine formulation was prepared by diluting it with normal saline at 1:1 proportion. The final total amount of bupivacaine in the solution was 12.5 mg (in total of 4 mL) for this test. A glass beaker filled with normal saline was placed on the hot plate which was set to give the

final temperature of 40±3 ˚C (which mimics sheep body temperature). The dialysis membrane was cut and tied at one end, and the bupivacaine solution was injected through the other end of the membrane to fill it up after which it was placed in the beaker. After closing the open end of the membrane, it was pushed into the beaker and was placed in the centre of the solution. The first sample was collected after 15 min, and 3mL of the dialysate solution was removed and poured into a cuvette. 3 mL of normal saline solution was returned to the beaker to maintain the volume. The same process was followed for other samples which were collected 30, 45 min, 1 and 2 hr after the dialysis membrane was immersed in the beaker. The absorption spectrum was recorded, and the concentration of the drug which was leaked through the dialysis membrane was measured using the following formula:

The *calculation was based on this formula after spectrophotometry:*

*Calibration curve: Absorption = 1.36339 * Concentration + 0.00591*

The percentage of drug leaked through the dialysis membrane to the normal saline solution in the beaker was calculated using the following formula:

% of drug in leaked through the membrane = (x/12.5) *100

X = amount of drug leaked through the dialysis membrane. (group 1 – bupivacaine and normal saline; group 2 – bupivacaine and Intralipid®; group 3 – bupivacaine and collagen)

2.1.8. HPLC (High-Performance Liquid Chromatography)

2.1.8.1. Chromatographic procedure

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 DGU-20A3 degasser (Shimadzu, Japan). All chromatograms were analysed by LC solution 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, 5 µm particle size). The mobile phase consisted of 70% 30mM dihydrogen phosphate buffer and 30% acetonitrile with a flow rate of 1 mL/min. The separation was achieved under isocratic/binary

conditions at 40°C. The DA detector was set at 202 nm wavelength throughout the experimental procedure.

2.1.9. Validation of HPLC assay

2.1.9.1. Lower limit of quantification

The lower limit of quantification (LLQ) in mobile phase was measured by running a series of low concentrations of bupivacaine standard (1000, 500, 250 ng/mL) diluted in the mobile phase. The LLQ was set at the lowest concentration showing a signal to noise ratio of 10.

2.1.9.2. Intra-day and inter-day accuracy and precision

The inter-day accuracy and precision were determined using bupivacaine in mobile phase three different concentrations (1000, 500 and 250 ng/mL) every day for three consecutive days. The intra-day accuracy and precision were calculated using these concentrations on each day for three days.

2.1.9.3. Linearity

The linearity of the measurements was checked by running three different concentrations (1000 to 500 and 250 ng/mL) three times in mobile phase. These data were analysed by linear regression in GraphPad Prism® software.

2.1.9.4. Standard Calibration

Standard solutions with different concentrations of commercial bupivacaine were made in the laboratory (1000, 500, 250ng) and were measured by HPLC. The linear model was used to determine the amount in nanograms in the experiment samples which were collagen (TiO_2) treatment group, Intralipid® treatment group and commercial bupivacaine formulations.

After plotting a graph in Microsoft Excel (version 2016), a linear model was extracted to calculate the concentration of the samples afterwards.

$$Y = 114.16X$$

This formula was used to calculate all the concentrations in all three groups:

Control group (bupivacaine and normal saline) at time points of 15, 30, 45, 60 and 120 min; first treatment group (Intralipid® (20%) combined with bupivacaine) at the time points of 15, 30, 45, 60 and 120 min; second treatment group (collagen crosslinked with titanium oxide nanoparticles and combined with bupivacaine) at time points of 15, 30, 45, 60 and 120 min.

2.2. *In vivo* Study:

2.2.1. Safety assessment for collagen-based formulation of bupivacaine

This study was approved by Massey University Animal Ethics Committee (protocol 16/68). A basic target animal safety test was performed on four sheep before progressing to the efficacy experiments. The solutions tested were a normal saline solution, the mixture of normal saline and bupivacaine, normal saline and collagen solution and finally the collagen-based formulation of bupivacaine. They were all injected subcutaneously at four different sites in the neck regions. The skin thickness, redness and any signs of inflammatory reaction were monitored hourly for the first six hr, then once a day for a week. Figures 2.2 and 2.3 show the location of the injection sites, and each injection site was marked for further monitoring.

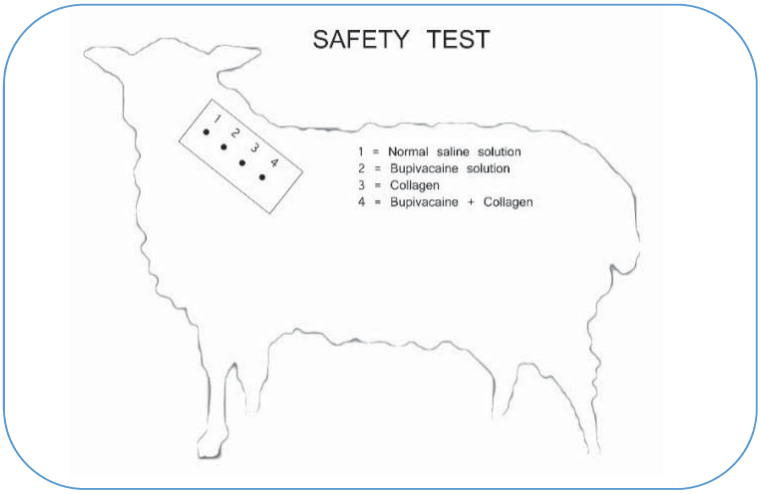

Figure 2.2 *– Safety assessment method which was performed on the neck region of the sheep to compare four different groups.*

Figure 2.3 *– The picture on the right shows the neck of the sheep which was clipped and divided into four parts for different injections. The picture on the left shows the same sheep without any redness and swelling in the following day.*

2.2.2. Efficacy study design

These studies received prior approval from Massey University Animal Ethics Committee (protocol 16/68). All sheep used for the experiments were 30 sourced from the teaching and research herd maintained by Massey University at the Large Animal Teaching Unit.

After calculation of statistical power with G-power software and defining the power above 0.8. 36 (Mix of males and females) Romney-cross mature sheep (30 ± 10kg) were used in this study: divided into two different groups of 18 sheep.

Sheep were kept under typical extensive husbandry conditions including housing on grass pasture in a small flock. Sheep had free access to grass grazing and water, with supplemental feeding providing as seasonally required. Sheep were observed by animal care personnel at least once per day. Sheep were acclimatised to handling as they were frequently used for teaching and low-impact research. A veterinarian clinically examined all the sheep before enrolment in the experiment.

Sheep were manually restrained and clipped over the metacarpal region above the coronary band. The clipped area was washed and scrubbed. Then, 18 sheep received commercial bupivacaine (control) and bupivacaine-Intralipid®, and another 18 sheep received commercial bupivacaine (control) and collagen- bupivacaine formulation. Each sheep received bupivacaine commercial formulation (0.5%) and collagen or Intralipid® formulations randomly in either right or left forelimb. Forelimb nerve block was placed at the level of the accessory digits with three subcutaneous injections of 1-2 mL of solution (for both groups) using a 22G needle by an operator who was not later involved in pain assessment (Figures 7.20, 7.21, 7.22, 7.27 and 7.28). This operator recorded which solution was injected in each foot and informed the pain assessor at the end of the experiment (Figures 7.24 and 7.25).

2.2.2.1. Nerve block technique

The medial and lateral proper palmar digital nerves and medial and lateral proper dorsal digital nerves were blocked by injecting the bupivacaine solutions at four different sites. As shown in Figure 2.4 the injection sites were in a horizontal line at the level of dew claw, i.e. the level of P1

bone, and the nerves which were targeted for injection were proper dorsal digital nerves (medial and lateral proper palmar digital nerve and medial and lateral proper dorsal digital nerve).

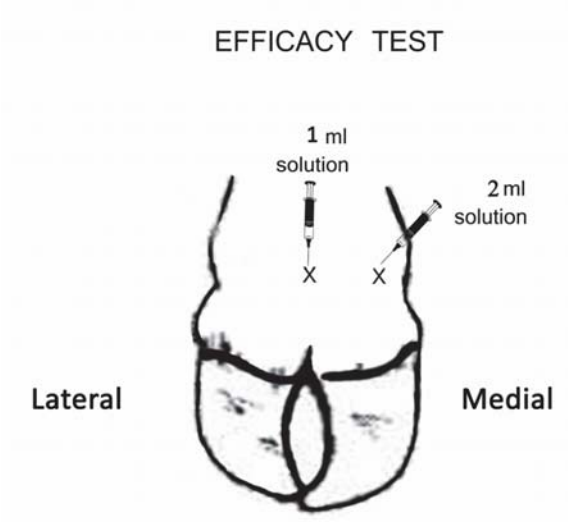

Figure 2.4 *– The schematic image of injection sites in anterior and medial aspects (posterior aspect is not shown in this picture). The lateral side of the forefeet was considered as a control for pain assessment. Only the medial aspect of the forefeet was anaesthetised.*

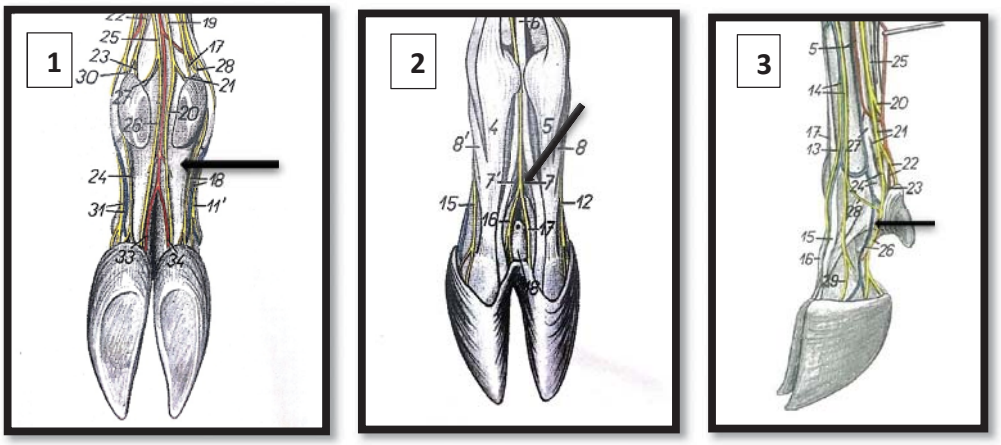

Figure 2.5 *– Distal part of the thoracic limb from three different aspects of medial, dorsal and palmar. The sites of injection are shown by black arrows in each picture. The nerves which pass are proper medial, dorsal and palmar and dorsal nerves. Number 1 shows the proper palmar nerve. Number 2 shows the proper dorsal nerve, and picture number 3 shows medial proper nerve.*

(photo: Peter Popesko, Atlas of Topographical Anatomy of the Domestic Animals, 1978, W B Saunders Co; 2 edition, Figure 71, Page 74, 76, 77.)

2.2.2.2. Pain assessment and data collection

This was a blinded study. The person assessing the pain was blinded to what was in the injections. Fifteen min after the injection, a mechanical nociceptive technique was used to assess the pain by pressing the blunt object (a haemostat) against the skin over the bone in the craniomedial aspect of the forefeet. No response to this noxious stimulus was considered as adequate local anaesthesia. This process was repeated every hour until the sheep responded by withdrawing its foot and that was recorded as the cut-off point for duration of the local anaesthesia.

2.2.3. Statistical analysis

Normality of the distribution between groups was assessed with Kolmogorov-Smirnoff (KS) test, and it was normal. Then, parametric test (Student's *t*-test) was used with SPSS statistical analysis program (version 2017), and the *p-value* was measured among the study groups: Intralipid®-based formulation, collagen-based formulation and the control group (bupivacaine and normal saline). After the analysis, the *p-value* of <0.05 was considered as significant. Finally, diagrams and graphs were designed in Prism® Graph Pad software (version 2017).

Chapter 3

Results

3.1. *In vitro* pilot study results

3.1.1. Spectrophotometry results of collagen/bupivacaine vs. collagen/bupivacaine/zinc oxide vs. commercial bupivacaine formulations

Table 3.1 shows results of the control group expressed in concentration (mg/mL) and percentages of bupivacaine in each time point. Samples were drawn from the beaker in which bupivacaine crossed/passed through the dialysis membrane. Figure 3.1 shows the trend of bupivacaine crossed the dialysis membrane in the control group. The concentration of bupivacaine increased with time and 78% of bupivacaine was released within 2 hr of the initial dosage through the dialysis membrane.

Group 1: Spectrophotometry of Plain Bupivacaine solution (12.5 mg of Bupivacaine)			
Sample	Time (min)	Concentration	Percentage of the drug which leaks out
1	15	0.0361	**22%**
2	30	0.0632	**38%**
3	45	0.0834	**50%**
4	60	0.104	**62%**
5	120	0.130	**78%**

Table 3.1 *– Results of the drug release test for the control group which was the mixture of bupivacaine and normal saline. The percentage at the right side of the table shows what percentage was leaked from the injected solution into the dialysis membrane. (n=1, for each time point)*

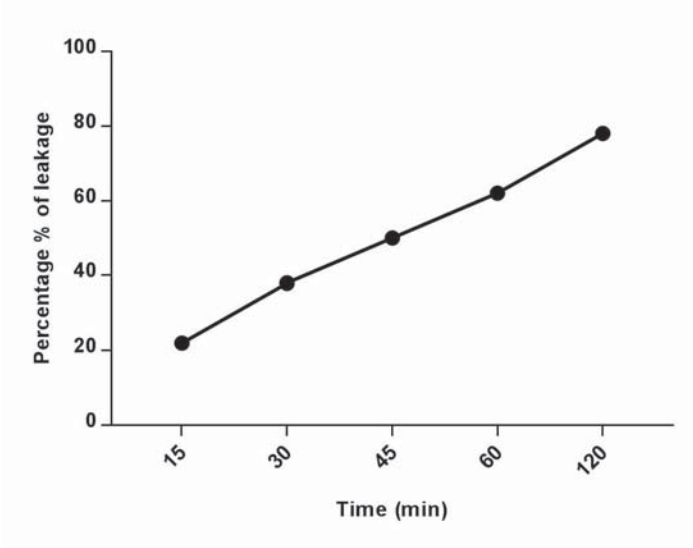

Figure 3.1 – *The trend of bupivacaine leakage in the first group which was commercial bupivacaine and normal saline. The Y axis is the amount of the bupivacaine in percentage, and the X axis is the time in which the samples were drawn from the beaker. (n=1, for each time point)*

A similar technique was performed in the collagen group in which a combination of collagen and bupivacaine at 1:1 was used. Table 3.2 shows the results of the *in vitro* drug release test (IVRT) for the collagen group. The leakage of the drug is shown in concentration (mg/mL) and percentage. Figure 3.2 shows the increasing trend of the bupivacaine leakage although not very consistent. After 45 min, only 44% of drug was released as compared to 50% in the case of control group. Only 65% drug was released in collagen group compared to 78% in the control group at 120 min.

Group 2: Spectrophotometry of bupivacaine + collagen (12.5 mg of bupivacaine)			
Sample	Time (min)	Concentration (mg/mL)	Percentage of the drug which leaks out
1	15	0.0283	**19%**
2	30	0.0419	**28%**
3	45	0.0598	**41%**
4	60	0.0648	**44%**
5	120	0.0951	**65%**

Table 3.2 *– Results of the drug release test for the first group which was the mixture of bupivacaine and collagen. The percentage at the right side of the table shows what percentage was leaked from the injected solution into the dialysis membrane. (n=1, for each time point)*

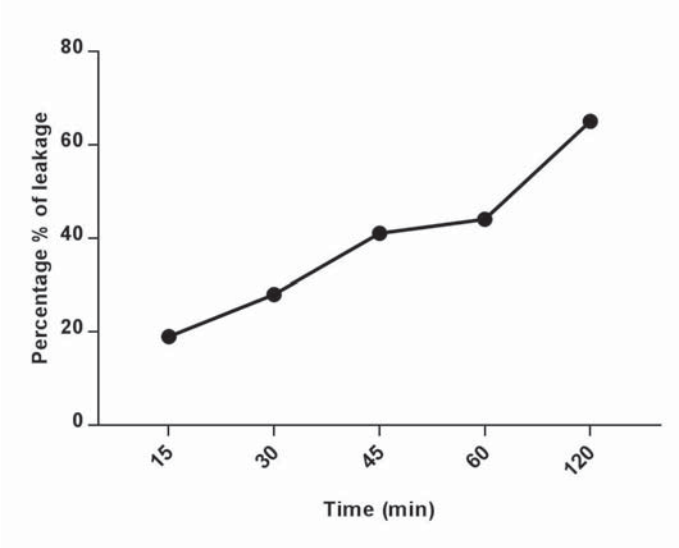

Figure 3.2 *- The trend of bupivacaine leakage in the second group which was commercial bupivacaine and collagen 1% solution. The Y axis is the amount of the bupivacaine in percentage, and the X axis is the time in which the samples were drawn from the beaker. (n=1, for each time point)*

In the third experimental group, 2mL of zinc oxide-PVP (polyvinylpyrrolidone) was added to the collagen and bupivacaine mixture, the results are shown in Table 3.3. The increasing trend of the drug release through the dialysis membrane is illustrated in Figure 3.3 in percentage. After 2 hr only 40% of the drug was released which was less than the collagen-bupivacaine and the control group.

Group 3: Spectrophotometry of bupivacaine (12.5 mg) + collagen + 2mL zinc oxide-PVP			
Sample	Time (min)	Concentration	Percentage of the drug which leaks out
1	15	0.0160	**9.6%**
2	30	0.0276	**17%**
3	45	0.0401	**23.7%**
4	60	0.0496	**27.7%**
5	120	0.0840	**40%**

Table 3.3 *– Results of the drug release test for the third group which was the mixture of bupivacaine and collagen and final addition of 2mL of zinc oxide-PVP. The percentage at the right side of the table shows what percentage was leaked from the injected solution into the dialysis membrane. (n=1, for each time point)*

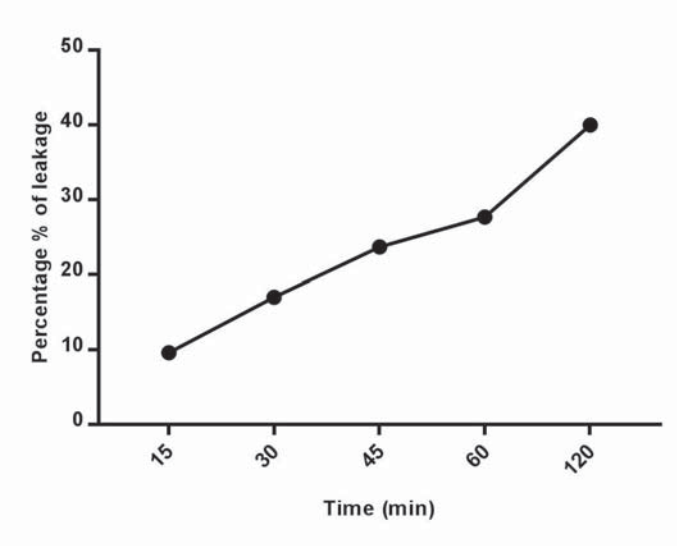

Figure 3.3 – *The trend of bupivacaine leakage in the second group which was commercial bupivacaine, collagen 1% solution and the addition of 2mL zinc oxide-PVP. The Y axis is the amount of the bupivacaine in percentage, and the X axis is the time in which the samples were drawn from the beaker. (n=1, for each time point)*

In another experimental group, 4mL of zinc oxide-PVP was added to the collagen and bupivacaine mixture, the results are shown in Table 3.4. Both concentration (mg/mL) and the percentages of the bupivacaine leakage are shown in this table. The increasing trend of this release is illustrated in Figure 3.4, and the units are in percentage. In the end, after 2 hr, only 30% of bupivacaine was released.

Group 4: Spectrophotometry of bupivacaine (12.5 mg) + collagen + 4mL zinc oxide-PVP			
Sample	Time (min)	Concentration	Percentage of the drug which leaks out
1	15	0.0191	**12%**
2	30	0.0222	**13%**
3	45	0.0328	**18.3%**
4	60	0.0448	**23%**
5	120	0.0840	**30%**

Table 3.4 *– Results of the drug release test for the fourth study group which was the mixture of bupivacaine and collagen and the addition of 4mL of zinc oxide. The percentage at the right side of the table shows what percentage was leaked from the dialysis membrane in different time points. (n=1, for each time point)*

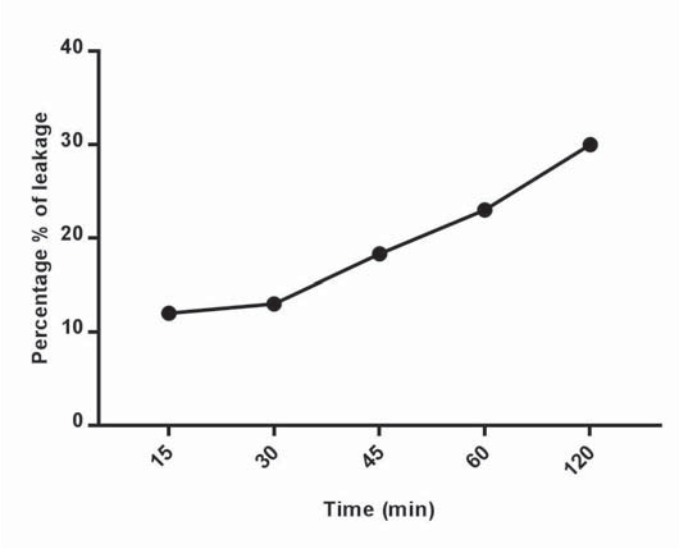

Figure 3.4 - *The trend of bupivacaine leakage in the second group which was commercial bupivacaine, collagen 1% solution and the addition of 4mL zinc oxide-PVP. The Y axis is the amount of the bupivacaine in percentage, and the X axis is the time in which the samples were drawn from the beaker. (n=1, for each time point)*

Finally, in the last group, 5 mL of zinc oxide-PVP solution was first poured in the combination of collagen and bupivacaine mixture, and after the gelatinous structure was formed, the remaining zinc oxide-PVP solution was removed by aspiration with a syringe. Table 3.5 shows the concentrations (mg/mL) and the percentages of the leakage of bupivacaine through the dialysis membrane in different times. Besides, the increasing trend of the drug release is illustrated in Figure 3.5, and the units are all in percentage. This group showed 44% of drug release after 2 hr.

Group 5: Spectrophotometry of bupivacaine + collagen + zinc oxide-PVP (5mL Bpv + Coll and 5mL ZnO-PVP to form a gel) (12.5 mg of bupivacaine)			
Sample	Time	Concentration	Percentage of the drug leaked out
1	15 min	0.0228	**14%**
2	30 min	0.0404	**25%**
3	45 min	0.0514	**30%**
4	60 min	0.0616	**34%**
5	120 min	0.0910	**44%**

Table 3.5 *– Results of the drug release test for the last study group which was the mixture of bupivacaine and collagen also of 5mL zinc oxide-PVP. Zinc oxide-PVP was removed after the gel was formed. The percentage at the right side of the table shows what percentage was leaked from the dialysis membrane in different time points. (n=1, for each time point)*

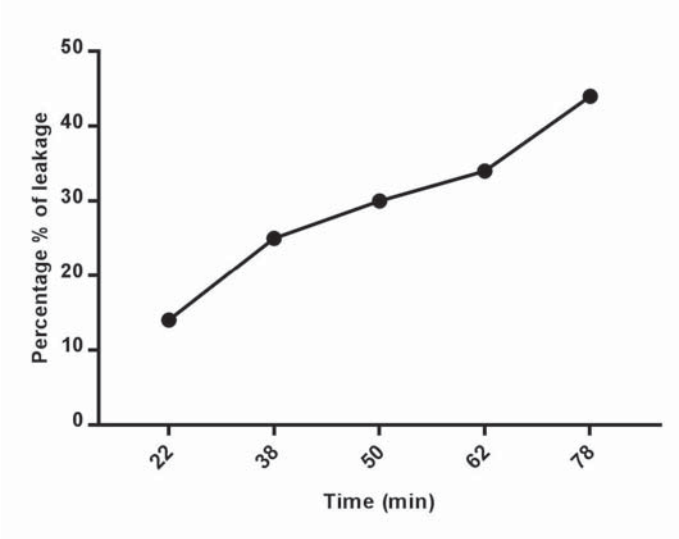

Figure 3.5 - *The trend of bupivacaine leakage in the second group which was commercial bupivacaine, collagen 1% solution and the addition of 5mL zinc oxide-PVP. In this group, the ZnO-PVP solution was removed after gel formation. The Y axis is the amount of the bupivacaine in percentage, and the X axis is the time in which the samples were drawn from the beaker. (n=1, for each time point)*

Finally, the trends in drug leakage of all groups containing the control groups which were bupivacaine/normal saline and bupivacaine/collagen and treatment groups which were collagen/ZnO-PVP (2mL)/bupivacaine, collagen/ZnO-PVP (4mL)/bupivacaine and collagen/ZnO-PVP (5mL) are shown in Figure 3.6. All the numbers show the percentages of the bupivacaine which were leaked into the beaker (contained normal saline) at different times starting from 15 min to 2 hr.

Overall, there is increasing trend in all groups which means in all of them bupivacaine leaked through the dialysis membrane into the beaker. There is an inconsistency in bupivacaine/collagen group at 60 min, but the rest of the groups are consistent in releasing the drug. In the end, bupivacaine mixed with collagen and 4mL of ZnO-PVP showed the least release (only 30%) compared to the other groups, and the control group showed the most release of bupivacaine through the membrane (78%).

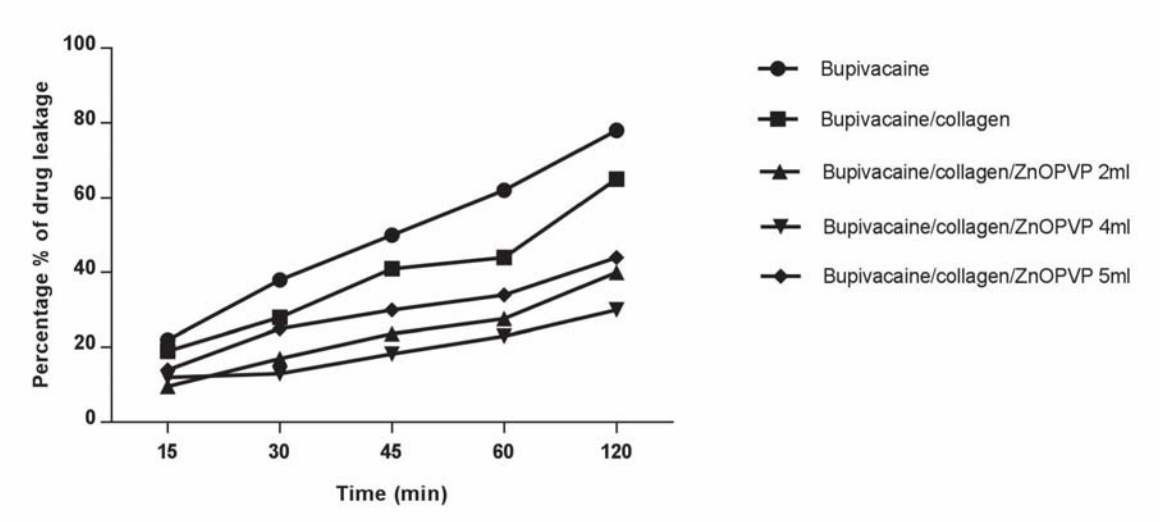

Figure 3.6 – *The bupivacaine leakage from the dialysis membrane in percentage and in different times starting from 15 min to 2 hr (15, 30, 45, 60, 120 min).*

3.1.2. Collagen (TiO_2) vs. Intralipid® vs. bupivacaine commercial formulation

Table 3.6 shows the results expressed as percentage of the bupivacaine leaked out of the injected solution inside the dialysis membrane in different groups of the study. The samples were obtained from the normal saline inside the beaker. After 2 hr, 91% of the bupivacaine in the control group (commercial bupivacaine and normal saline), 84% of first treatment group which was Intralipid® and bupivacaine and 78% of collagen/TiO_2 was released into the beaker.

Sample	Time (min)	Percentage of bupivacaine leaked out in different groups		
		Control	Intralipid®	Collagen/TiO_2
1	15	**26%**	**26%**	**22%**
2	30	**44%**	**43%**	**33%**
3	45	**62%**	**53%**	**41%**
4	60	**71%**	**63%**	**50%**
5	120	**91%**	**84%**	**78%**

Table 3.6 *– The percentages of bupivacaine which leaked out the dialysis membrane in the drug release test in three study groups. (n=1, for each time point)*

As it is illustrated in Figure 3.7, it is evident that the more bupivacaine was leaked through the beaker in the control group compared to the Intralipid®/bupivacaine group. The collagen/TiO_2 group mixed with bupivacaine shows the least leakage of the drug into the beaker.

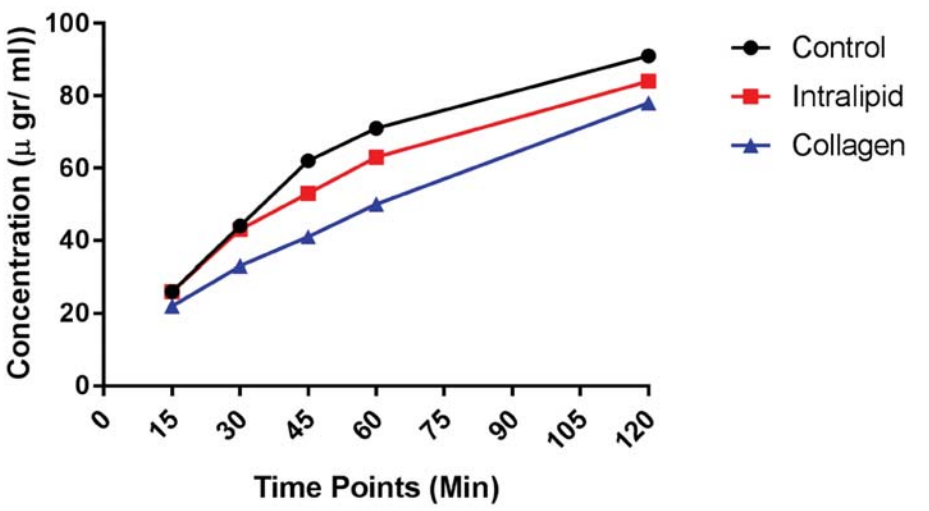

Figure 3.7 *– The study groups - control group (bupivacaine and normal saline solution), Intralipid® combined with bupivacaine and collagen and bupivacaine mixture cross-linked with TiO_2. (n=1, for each time point)*

3.1.3. HPLC method validation

The chromatogram for bupivacaine standard solution is given in Figure 3.8, and it illustrates the concentrations of the bupivacaine in the standard samples. The retention time for bupivacaine was 5:40 min. The linearity of the standard curve was 0.9951. The inter day and intraday variations are given in table 3.7.

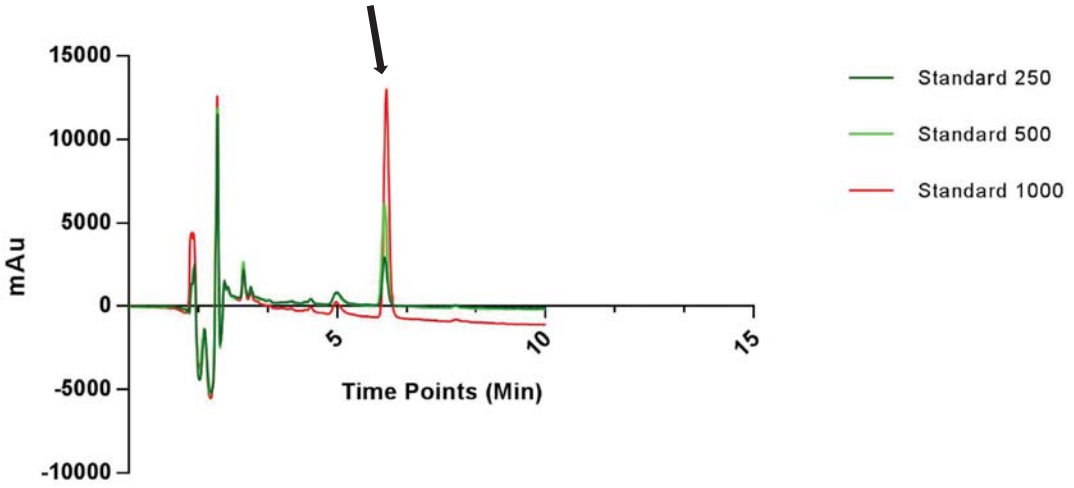

Figure 3.8 *– HPLC histogram for the standard solutions of bupivacaine. Different colours show different concentrations (ng/mL).*

Concentration	Intraday	Inter-day
1000	4.5	5.4
500	6	3.6
250	5	5.5

Table 3.7 *– RSD or relative standard deviation in both intraday and interday study of standard samples. The units of RSD are expressed in percent and was obtained by multiplying the standard deviation by 100 and dividing it by the average. (n=1)*

3.2. *In vivo* Results

3.2.1. Safety study

In the safety assessment, nothing abnormal was found after the subcutaneous injections in the neck region. There were no visible reactions, swellings or inflammation in the area. Skin thickness measurements were not different.

3.2.2. First Part: effects of Intralipid®-based formulation of bupivacaine

There was a significant increase of the duration of local anaesthesia with the Intralipid®-based formulation of bupivacaine. The mean of analgesic period in the control legs was 4.23±1.8 hr compared to 5.81±1.78 hr in the legs which were anaesthetised by Intralipid®-based formulation of bupivacaine, p=0.013 (Table 3.8). Thus Intralipid®/bupivacaine was successful in prolonging the action of local anaesthesia compared to the control group of bupivacaine and normal saline.

Groups	Intralipid® + Bupivacaine	Control
Sheep #1	6	3.45
Sheep #2	3.45	1.45
Sheep #3	8.85	3.85
Sheep #4	5.85	3.85
Sheep #5	4.85	2.85
Sheep #6	6.85	6.85
Sheep #7	8.15	7.15
Sheep #8	8.15	7.15
Sheep #9	5.15	2.15
Sheep #10	7.15	5.15
Sheep #11	8.15	7.15
Sheep #12	5	3.15
Sheep #13	5.15	3.15
Sheep #14	4.15	2.15
Sheep #15	6.15	3.15
Sheep #16	3.15	4.15
Sheep #17	3.15	5.15
Sheep #18	5.15	4.15
Mean	5.80*	4.22
St. deviation	1.78	1.83

Table 3.8 *- All the numbers show the period of local anaesthesia in hr. the time difference between the Intralipid®-based formulation versus control was 1.58 ± 1.5 hr which means the period of local anaesthesia was longer in the treatment group compared to the control group (p-value = 0.013) *.*

3.2.2. Second Part: effects of collagen/TiO_2-based formulation of bupivacaine

The results of the second study in which collagen/TiO_2-based formulation of bupivacaine was compared with the control group in sheep, showed a significant difference between the results of these two groups with the *p-value* of 0.014. The mean of local anaesthesia period in the control group was 2.87±0.96 hr, and in the leg which was anaesthetised by the collagen-based formulation of bupivacaine, it was 2.15±0.68 hr (Table 3.9). It means the collagen-based formulation of bupivacaine mixed with titanium not even had no effect on prolonging the action of local anaesthesia but made it even shorter than the control group which was bupivacaine and normal saline.

Groups	Collagen + TiO_2 + Bupivacaine	Control
Sheep #1	2.15	4.15
Sheep #2	2.15	3.15
Sheep #3	2.15	2.15
Sheep #4	1.15	2.15
Sheep #5	1.15	2.15
Sheep #6	2.15	3.15
Sheep #7	3.15	4.15
Sheep #8	2.15	3.15
Sheep #9	2.15	3.15
Sheep #10	2.15	2.15
Sheep #11	2.15	4.15
Sheep #12	4.15	4.15
Sheep #13	2.15	2.15
Sheep #14	2.15	2.15
Sheep #15	2.15	1.15
Sheep #16	2.15	2.15
Sheep #17	2.15	4.15
Sheep #18	1.15	2.15
Mean	2.15*	2.87
St. deviation	0.68	0.96

Table 3.9 *– All the numbers show the period of local anaesthesia in hr. The collagen-based formulation produced analgesia which lasted 0.72 ± 0.83 hr less than the control group (p-value = 0.014) *.*

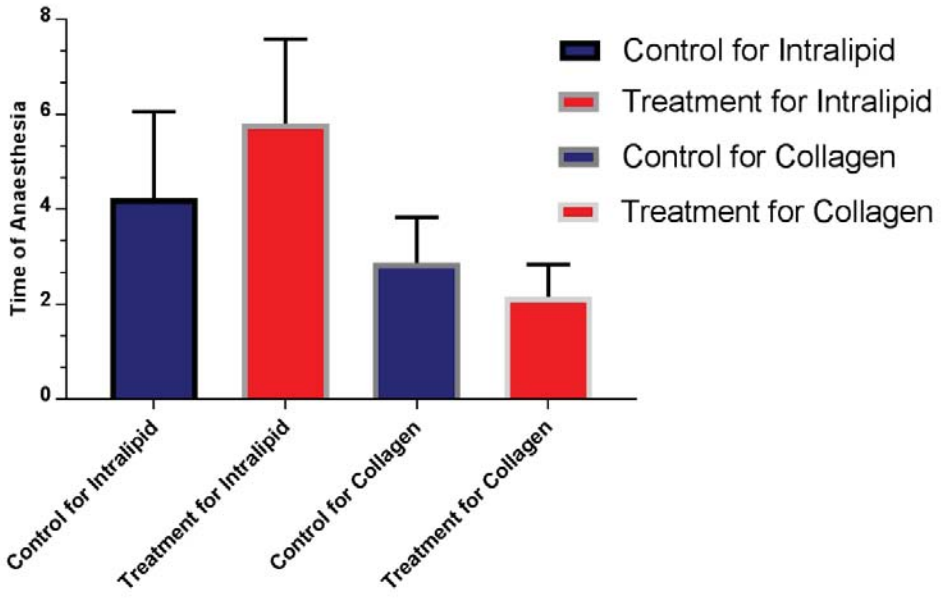

Figure 3.9 *– Duration of analgesia in different groups of the in vivo study. The blue-coloured bars are the control groups of each study, and the red-coloured bars shows each treatment groups. The Y axis is the period of local anaesthesia, and its unit is hr. (n=18 in each experiment group)*

Chapter 4

Discussion

4.1. Discussion

The objective of this study was to develop a sustained-released bupivacaine formulation which can provide local anaesthesia for a longer duration than the commercially available formulation. The short duration and side effects of local anaesthetics have always been a challenge for veterinarians (Coetzee, 2013). Therefore, in this study, we investigated two different ways to reduce systemic absorption of bupivacaine and extend the action by ensuring that the drug stayed around the nerve.

Intralipid® is able to entrap bupivacaine molecules, presumably by dissolution in the soya bean oil, resulting in their slower release. The duration of local anaesthesia provided by Intralipid® bupivacaine was significantly longer than bupivacaine hydrochloride aqueous solution. Intralipid® interactions with bupivacaine are already known (Laine et al., 2010), and it is commonly used intravenously to treat bupivacaine toxicity. Intralipid® has a high affinity to lipophilic substances, and it entraps these molecules in circulation by creating a significant lipid phase in plasma "lipid sink theory" (Weinberg et al., 2010). In this theory, it is believed that Intralipid® separates the bupivacaine molecules from the aqueous tissue phase. Since bupivacaine is highly lipophilic (compared to most commonly used local anaesthetics), the Intralipid® interacts with its molecules strongly (Muhonen et al., 2009); therefore, the availability of free molecules decreases (around 88%) (Laine et al., 2010). Provided the Intralipid stays around the nerve, the bupivacaine should be slowly released from it, resulting in longer duration of action. The results of the *in vitro* pilot study also indicated a sustained-release pattern of bupivacaine from the Intralipid® formulation which confirms the results obtained in the *in vivo* study.

In the *in vitro* pilot study, where the drug release tests were conducted, the collagen-based formulation of bupivacaine showed a better sustained-release of the drug than the Intralipid®-based formulation of bupivacaine, and they were both more successful comparing to the control group of bupivacaine and normal saline. On the contrary, in the *in vivo* study, the Intralipid®-based formulation showed a significant prolongation of local anaesthesia of 1.58 ± 1.5 hr more than the control group.

As illustrated in Figure 3.9, Intralipid®- based formulation of bupivacaine extended the period of local anaesthesia significantly compared to its control group. This result makes Intralipid® a good candidate for sustained-release of bupivacaine in clinics. On the other hand, as it is shown in Figure 3.9, collagen-based formulation of bupivacaine cross-linked with TiO_2 nanoparticles was not successful in prolonging the period of local anaesthesia compared to its control group.

The results in the *in vivo* study and their comparison with the *in vitro* pilot study casts doubt on whether sustained-release of the drug in a dialysis test necessarily means prolonging the action of local anaesthesia in an animal. One hypothesis is that collagen fibres were not efficient enough to keep the bupivacaine molecules inside the tissue for an extended period, but they somehow protected sodium channels from the bupivacaine and that is why bupivacaine did not provide prolonged analgesia in the injected region. It is possible that titanium oxide nanoparticles, after functioning as cross-linkers, made stacks of collagen microfibrils which entrapped a considerable amount of bupivacaine and not enough was released to provide prolonged analgesia. This effect could possibly be overcome by adding more bupivacaine to the formulation.

Four pilot studies were performed prior to the actual project to find the most effective collagen-based formulation of bupivacaine. They were all tested in the *in vitro* pilot study with the drug release tests, and the passage of bupivacaine through the dialysis membrane was measured by UV spectrophotometry. However, in the *in vivo* study, none of them showed suitable results. In two out of four pilot studies, different concentrations of bupivacaine were used. In one solution collagen was mixed with a proportion of (1:4) with commercial bupivacaine and in the other the proportion of (1:1). In other two parts, zinc oxide PVP solution was added to the collagen. Zinc oxide nanoparticles can cross-link the collagen fibres and form a harder and stronger structure when they are added to the collagen matrix. It was hypothesised that when the collagen matrix forms a gelatinous structure, it could potentially hold and restrain more drug molecules within itself. Thus, it could make the release slower inside the tissue after being injected. However, an immediate gelatinous formation of the collagen matrix made the combination impossible to inject through a needle. Therefore, this formulation was not considered as a suitable formulation for the *in vivo* study.

The collagen which was used in these studies was extracted and refined from bovine limed split which was obtained from the leather industry. Although this by-product is not valuable, is toxic for the environment and harmful for nature due to the refining procedures which involve salts and tanning agents (Kanagaraj et al., 2006), its main ingredient is collagen type one. Collagen type one is the most abundant collagen through the body, and many bio-medical products can be produced from it. Consequently, developing ways to use this waste has potential economical and environmental benefits.

This study has a number of limitations. The time for assessment of the drug release in the *in vitro* pilot study was only two hours, which may have been too short. This was because the only purpose of the *in vitro* pilot studies was to assess the sustained-release of bupivacaine to aid the design of the *in vivo* study. It would have been useful to extend the time of obtaining samples from the *in vitro* pilot studies for several hours until 100% of the bupivacaine passed through the dialysis membrane. Repeating the drug release test studies would be advantageous for obtaining more reliable and concrete results. In fact, we mostly focused on the *in vivo* study since sheep were available with inconsiderable cost compared to expensive laboratory consumables. Besides, the time was limited in master's degree to cover all different kinds of experiments, and we are hopeful to continue the procedure in the PhD program.

The other challenge in this study was the pain assessment technique. The sheep did not always produce a clear-cut pain reaction. The only reaction which was considered a true response was quick limb withdrawal after applying pressure just above the coronary band with a blunt-tip haemostat clamp as a noxious stimulus. The other problem was the conditioning process in sheep after several pain stimulations. It was almost impossible to work on the same sheep which was used previously since they were easily agitated by a simple touch.

In further studies, it would be useful to use a mechanical threshold testing device which can automatically detect the force which causes a reaction in the animal. This technique could be more reliable than the conventional techniques which do not always have high levels of certainty in pain assessment. However, neither test will tell if an animal is analgesic enough for surgery.

In future studies, because of the abundance of collagen, it would be useful to investigate different techniques and methods of modification of the collagen structure to solve the problem of its effectiveness after being combined with various anaesthetic and analgesic agents, possibly at higher doses. Other possible routes of administration such as dermal patches to assess the pharmacokinetics and pharmacodynamics of the local anaesthetics should be considered in the future.

Various oil based compounds and excipients with different concentrations should be investigated to compare their results with soybean oil which was assumed to be the active excipient in Intralipid®. More information is needed to understand the interactions of bupivacaine (and other local anaesthetics) with various different lipids.

Chapter 5

Conclusion

5.1. Conclusion

In conclusion, this study proved that both collagen-based formulation and Intralipid®-based formulation of bupivacaine prolonged the release of the drug more than the control group in the *in vitro* pilot study. However, only Intralipid® was successful in significantly prolonging the action of local anaesthesia (by around 1.58 ± 1.5 hr) after perineural injections in the metacarpophalangeal (forefeet) regions of the sheep, compared to the control group. The collagen-based formulation crosslinked with titanium oxide nanoparticles significantly decreased the time of local anaesthesia as compared to the control group. These results showed that Intralipid® could be considered as a useful vehicle for bupivacaine to make local anaesthesia longer and safer in sheep. Collagen still needs more investigation as a vehicle for slow release formulations of bupivacaine.

No side effects were seen with any formulation.

Chapter 6

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Chapter 7

Appendix

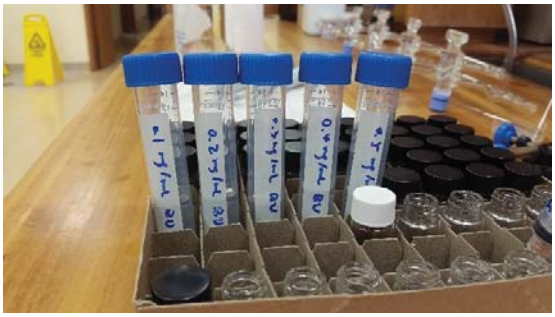

Figure 7.1 *– Plastic test tubes for preparing calibration solution of bupivacaine for the UV-spectrophotometer.*

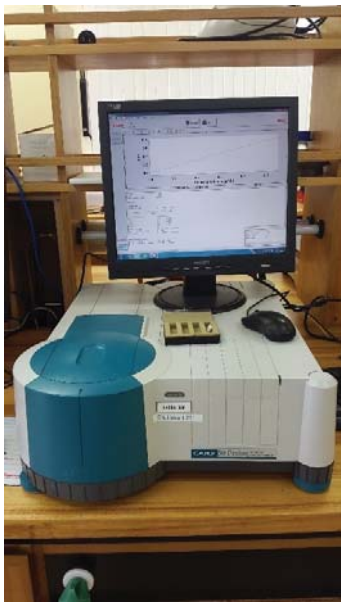

Figure 7.2 *– UV Spectrophotometer device used in this experiment.*

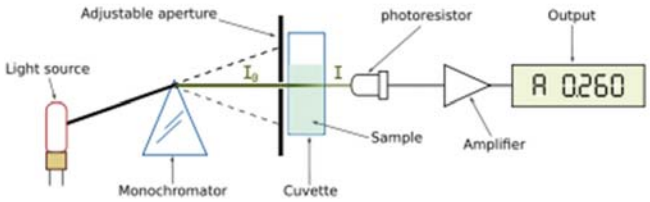

Figure 7.3 *– Schematic mechanism of UV spectrophotometer.*

"Photo: https://en.wikipedia.org/wiki/Spectrophotometry"

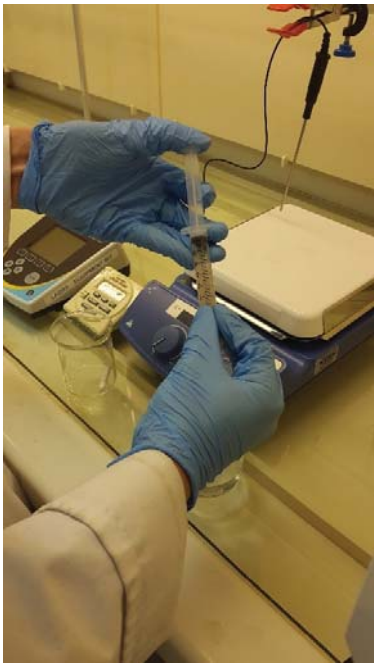

Figure 7.4 *– Injection of the sample into the dialysis membrane.*

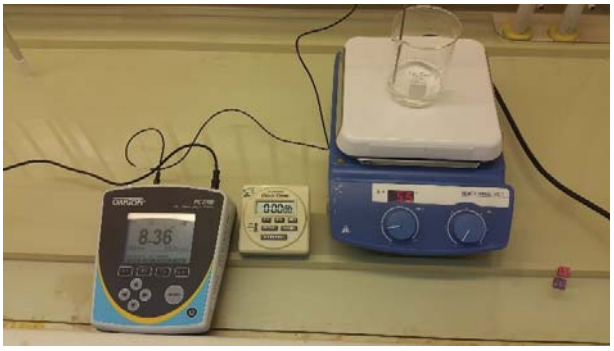

Figure 7.5 *– pH and temperature meter on the left, the timer in the middle and the hot plate in the right corner. The beaker is on the hot plate, and the detector is inside the beaker for detecting the temperature of the solution.*

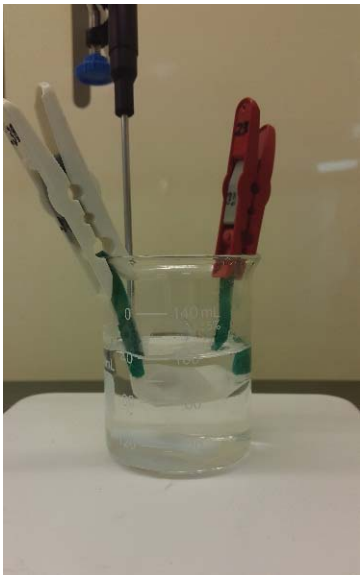

Figure 7.6 *– The beaker which contained normal saline and the dialysis membrane floating inside the solution.*

Figure 7.7 *– Parafilm used to seal the container and prevent evaporation of the water in the container.*

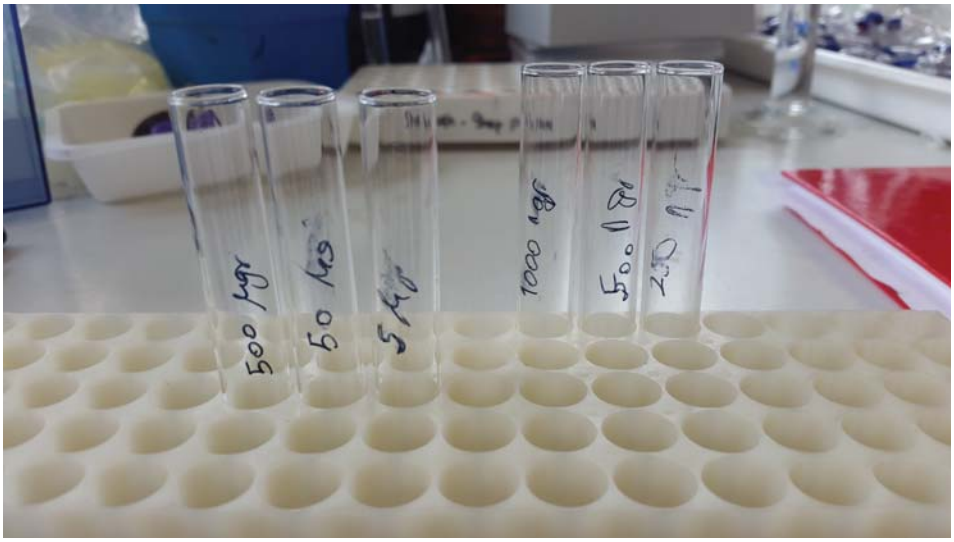

Figure 7.8 *– Serial dilution and preparation of lower concentrations of bupivacaine. The concentrations 500 micro-grams, 50 micro-grams, five micrograms, 1000 Nano-grams, 500 Nano-grams and 250 Nano-grams were prepared for standard calibration.*

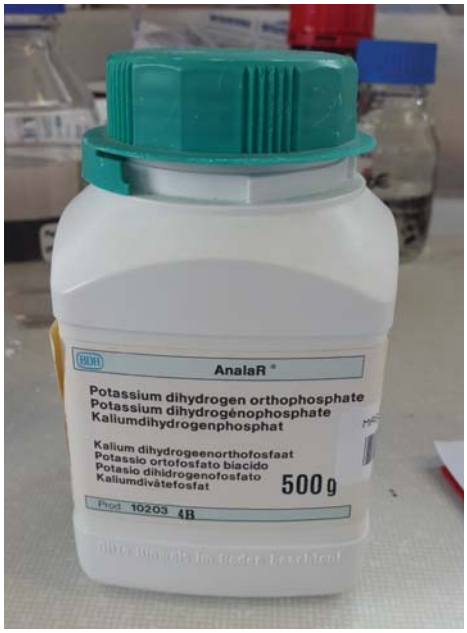

Figure 7.9 *– Potassium dihydrogen orthophosphate used to make the mobile phase.*

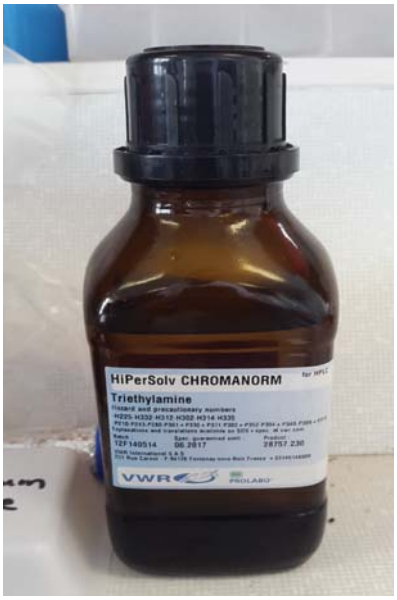

Figure 7.10 *– Triethylamine used to prepare the mobile phase.*

Figure 7.11 *– Acetonitrile used to inject in HPLC device with 30% of volume proportion.*

Figure 7.12 *– The pH meter used to adjust the pH on 4.9 for mobile phase preparation.*

Figure 7.13 *– Shimadzu® HPLC device.*

	Concentration 1		Concentration 2		Concentration 3	
	Concentration	***Area***	***Concentration***	***Area***	***Concentration***	***Area***
	1000	118094.67	500	55741	250	26897
	1000	122445.222	500	52254	250	24412.1111
	1000	110061.333	500	52632.3333	250	24587.3333
MEAN	**1000**	**116867.07**	**500**	**53542.4444**	**250**	**25298.8148**
SD	0	**6282.54834**	0	**1913.37893**	0	**1386.83908**
RSD	0	**5.375807**	0	**3.5735741**	0	**5.48183417**

Table 7.1 *- Inter-Day Variations in Standard Group*

	Concentration 1		**Concentration 2**		**Concentration 3**	
	1000	123214	500	62609	250	23191
	1000	124751	500	61717	250	23423
	1000	116625	500	54143	250	24541
	1000	116800	500	54422	250	24315
	1000	129009	500	57009	250	25712
	1000	128793	500	58069	250	26154
	1000	129780	500	54458	250	26120
	1000	130257	500	54214	250	26130
Mean	1000	**124903.63**	500	**57080.13**	250	**24948.25**
SD	0	**5625.2174**	0	**3460.688**	0	**1241.419**
RSD	0	**4.5036462**	0	**6.06286**	0	**4.975977**

Table 7.2 *– Intraday Variations in Standard Group*

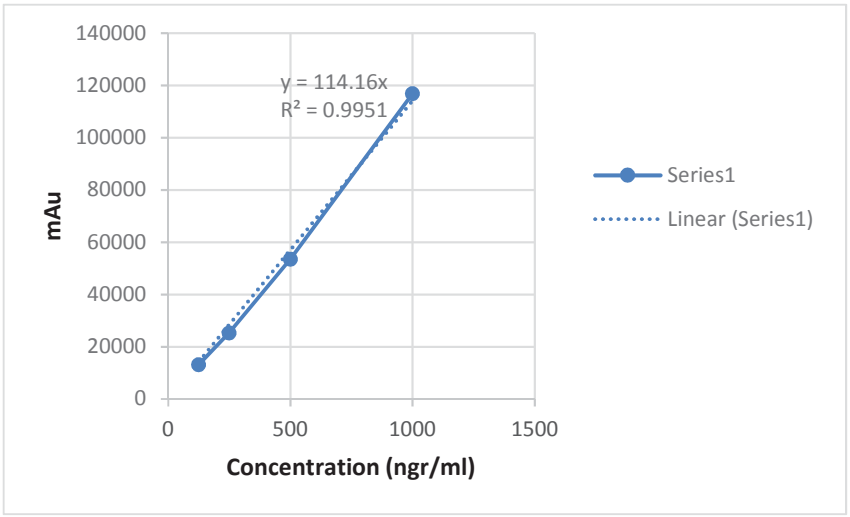

Figure 7.14 *– Graph of linearity in standard group. The Y axis shows the milli absorbance unit (mAU) and the X axis shows the concentration of the standard solutions in nanograms.*

Figure 7.15 *– Bupivacaine hydrochloride (Marcain® 0.5%).*

Input and output of G-power software

Analysis: A priori: Compute required sample size

Input: Tail(s) = Two

Effect size d = 1.018631

α err prob = 0.05

Power (1-β err prob) = 0.8

Allocation ratio N2/N1 = 1

Actual power = 0.821122

Output: Noncentrality parameter δ = 2.969794

Critical t = 2.036933

Df = 32

Sample size group 1 = 17

Sample size group 2 = 17

Total sample size = 34

Figure 7.16 *– The input and output numbers in G-power software program. The number at the bottom left side shows the calculated statistical power.*

Figure 7.17 *– The injection area was washed, scrubbed and clipped.*

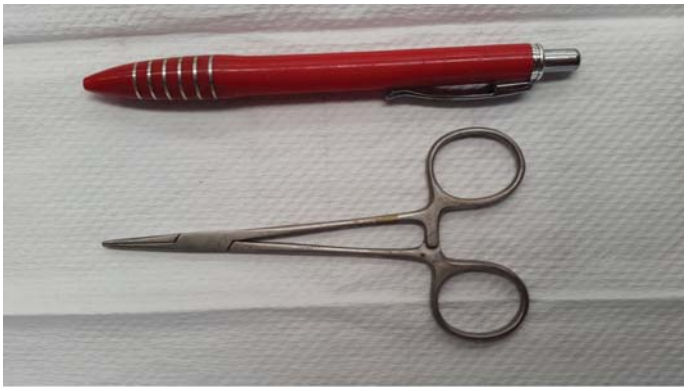

Figure 7.18 *– The tip of a haemostat used as a blunt object for pressing on the nerve as a mechanical noxious stimulus.*

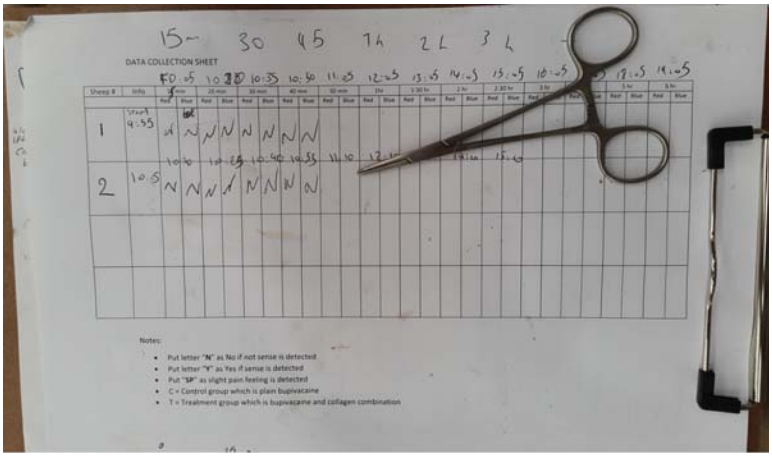

Figure 7.19 *– Designed table for data collection. Letter "N" means No pain; letter "Y" means pain and "SP" means Slight pain.*

Figure 7.20 *– Medial digital nerve block in the right forefoot.*

Figure 7.21 *– Cranial digital nerve block in the right forefoot.*

Figure 7.22 – Palmar digital nerve block in the right forefoot.

***Figure 7.23** – Two different colours used to the make the experiment blinded.*

Figure 7.24 *– The lateral nerves tested as a control for pain assessment.*

Figure 7.25 *– Applying pressure on the medial side of the right forefoot for pain assessment in sheep.*

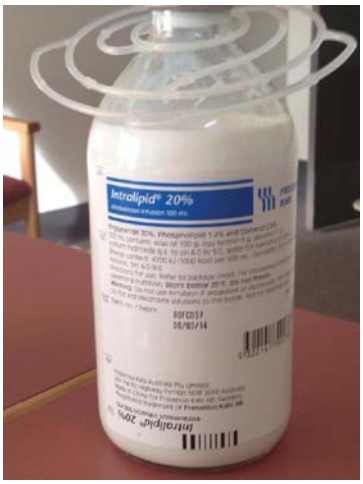

Figure 7.26 *– Intralipid® 20%.*
"Picture extracted from the website: https://ofrjosemi.wordpress.com/"

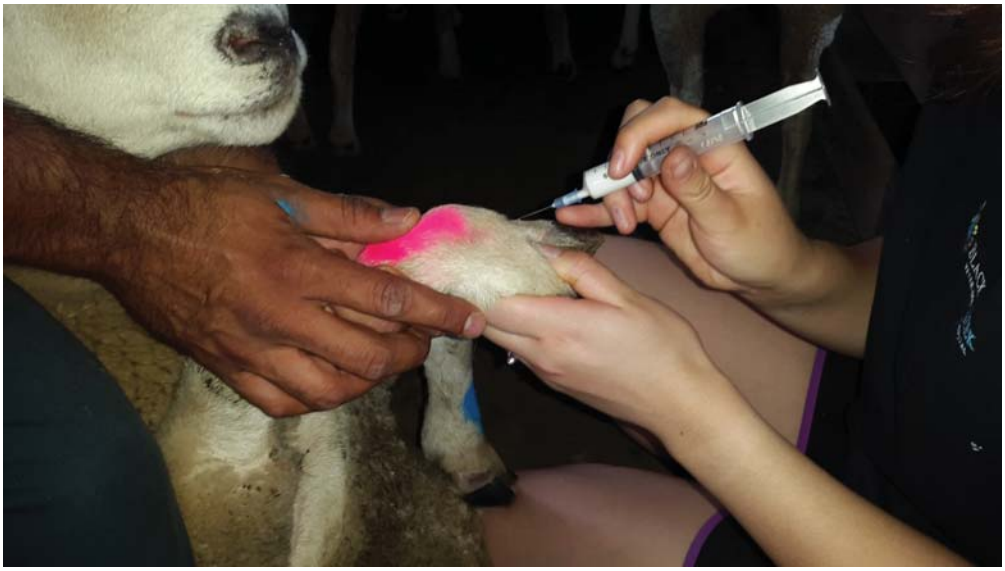

Figure 7.27 *– Subcutaneous injection of Intralipid®-based formulation of bupivacaine in sheep as the treatment group.*

Figure 7.28 *– Subcutaneous injection of the mixture of bupivacaine and normal saline in the carpophalangeal region of the sheep as a control group.*