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Studies on Renal Safety and Preventive Analgesic Efficacy of Tramadol and Parecoxib in Dogs

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ABSTRACT

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Ovariohysterectomy and castration are common surgical procedures in small animal practice that can result in clinically significant postoperative pain. One way of controlling postoperative pain is administration of a single analgesic or a combination of different classes of analgesics prior to the onset of noxious stimuli. A constraint to the perioperative use of traditional opioids and non-steroidal anti-inflammatory drugs (NSAIDs) is their undesirable side effects. In this series of experiments, the preventive (pre-emptive) analgesic efficacy of two popular human analgesics, tramadol (an 'atypical' opioid) and parecoxib (a NSAID with selective COX-2 inhibition) was evaluated in dogs.

Initially, the efficacy and renal safety of parecoxib, tramadol and a combination of parecoxib, tramadol and pindolol (a β -adrenoceptor blocker and 5-HT_{1A/1B} antagonist) were screened in anaesthetised healthy dogs. These analgesics increased the dogs' nociceptive threshold to mechanical stimuli, without causing significant alterations in the dogs' glomerular filtration rate (GFR) estimated by plasma iohexol clearance. Subsequently, the efficacy of tramadol was compared with morphine, in dogs undergoing ovariohysterectomy or castration. The Glasgow composite measure pain scale-short form score (CMPS-SF) and changes in intra-operative electroencephalogram (EEG) responses were used to assess the efficacy of analgesics. Of the three treatment groups (preoperative morphine, 0.5 mg kg⁻¹; preoperative tramadol, 3 mg kg⁻¹; a 'combination' of preoperative low-dose morphine, 0.1 mg kg⁻¹, and postoperative tramadol 3 mg kg⁻¹), dogs given the 'combination' had significantly lower pain scores after ovariohysterectomy. In castrated dogs, preoperative tramadol (3 mg kg⁻¹) and morphine (0.5 mg kg⁻¹) were tested and no significant difference in the CMPS-SF score were observed between them. Changes in EEG variables were not specific between the treatment groups in ovariohysterectomised dogs.

Finally, the efficacy of test drugs was evaluated against acute noxious electrical stimulation in anaesthetised dogs, using EEG. Median frequency of the EEG, a reliable indicator of nociception, increased significantly in tramadol and parecoxib groups, compared to morphine, after electrical stimulation. These studies demonstrated that tramadol and

parecoxib can produce analgesia in dogs with insignificant side effects. The efficacy of tramadol appears to vary with the type of noxious stimulus. A complete prevention of noxious input by administration of analgesics pre- and post-operatively could have important clinical applications.

Key words: tramadol, parecoxib, morphine, dogs, analgesic efficacy, anaesthesia, renal safety

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the curve
BUN	blood urea nitrogen
CGRP	calcitonin gene related peptide
CL1	clearance calculated by one-compartment model
CMPS-SF	composite measure pain scale-short form
CNS	central nervous system
COX	cyclooxygenase
CRAF	continual rate of application of force
CYP2D6	cytochrome P450 2D6
ECG	electrocardiogram
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
ERPF	effective renal plasma flow
E_T CO ₂	end-tidal CO ₂ tension
$E_{T\text{ HAL}}$	end-tidal halothane tension
F50	median frequency
FFT	fast fourier transformation
GABA	gamma-amino butyric acid
GFR	glomerular filtration rate
GLMM	generalised linear mixed models
HPLC	high performance liquid chromatography
IC50	median inhibition concentration
ICL	iohexol clearance
ICP-AEC	inductively coupled plasma-atomic emission spectroscopy
IV	intravenous
LD50	median lethal dose
LSM	least square means
MAP	mean arterial pressure
mRNA	messenger ribonucleic acid
NA	noradrenaline
NH ₄ OH	ammonium hydroxide
NK	neurokinin
NMDA	N-Methyl D-Aspartate
NSAID	non-steroidal anti-inflammatory drug

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LIST OF ABBREVIATIONS (contd..)

PAG	periaqueductal grey
PG	prostaglandin
PGE2	prostaglandin E2
PPD	pressure of palpation device
PR	pulse rate
Ptot	total EEG power
RBF	renal blood flow
RR	respiratory rate
RVM	rostral ventromedial medulla
SC	subcutaneous
SE	standard error
SEF	spectral edge frequency 95%
SG	strain gauge
SP	substance P
SpO2	oxygen saturation
SRT	spinoreticular tract
STT	spinothalamic tract
T _{max}	time to maximum plasma concentration
UV	ultraviolet
VAS	visual analogue scale
VTH	veterinary teaching hospital
WMWodds	Wilcoxon-Mann-Whitney odds

CHAPTER 1

INTRODUCTION

Universally, pain is a common and distressing phenomenon. The International Association for the Study of Pain has defined pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” (Merskey 1986). The ability to communicate verbally and physically helps identify the pain in humans. In the case of nonverbal animals pain is a sort of behavioural reaction to an aversive sensory experience associated with actual or potential tissue damage (Cambridge et al. 2000).

Pain following surgical procedures, if inadequately treated, results in immediate distress in both people and animals. The noxious stimuli from the surgical site are detected by peripheral afferent nerve fibre terminals and are transmitted to the cerebral cortex through the dorsal horn of the spinal cord. This results in sensitisation of peripheral and central neurones, which in turn leads to hyperalgesia (amplified response to subsequent noxious stimuli), allodynia (previously painless sensations experienced as pain) and intermittent muscle spasm. All of these contribute to chronic pain that can persist for days to weeks beyond surgery (Thurmon et al. 1996). Also, the dose of a given post-operative analgesic may become ineffective once neurones are sensitised. Most of the currently employed anaesthetics provide little or no residual analgesia (Hellyer 1997).

The main objective of preventive (preemptive) analgesia is to block / reduce the development of neuronal hyper responsiveness by administration of analgesics prior to the onset of noxious stimuli that in turn reduces the analgesic requirement postoperatively. This concept is a relatively new approach in treating peri-operative pain, though Crile suggested as early as 1913 the use of regional nerve blocks prior to surgery in addition to general anaesthesia to prevent post-operative pain. Interest in this area was revived in 1988 when Wall suggested that post-operative pain might be reduced by preventing the intraoperative nociceptive impulses from reaching the spinal cord.

Various types of analgesics, such as opioids, N-Methyl D-Aspartate (NMDA) receptor antagonists, α_2 agonists, local anaesthetics, non-steroidal anti-inflammatory drugs (NSAIDs),

etc., have been found to be either experimentally or clinically effective analgesics. Also, these drugs can be administered by different routes – local infiltration, regional nerve blocks, epidural, parenteral or oral administration, etc. Each of these has its own merits and demerits. Opioids and NSAIDs are the main stay analgesics in small animal practice (Slingsby et al. 2006).

Though opioids are the first-rate analgesics, their peri-operative use is often associated with clinically significant side effects in dogs. Tramadol, a centrally acting synthetic analgesic of the aminocyclohexanol group, has synergistic opioid and non-opioid modes of action (Raffa et al. 1992) and is popular as a postoperative analgesic in humans (Lehmann 1994). Respiratory depression and other adverse effects that are characteristic of opioids in humans are insignificant with this drug. However, the use of tramadol in veterinary practice has been very limited. The side effects of NSAIDs include gastro-intestinal irritation and renal dysfunction. However, recent NSAIDs such as parecoxib, which act by selective COX-2 inhibition, are claimed to be free from these side effects in people (Cheer & Goa 2001). In a recent study, it has been shown that pindolol, a non-selective beta-adrenoceptor blocker / 5-hydroxytryptamine antagonist markedly enhanced the analgesic effect of tramadol in experimental mice (Corrales et al. 2000).

Preoperative balanced analgesia involving a combination of two or more classes of analgesics such as an opioid and a NSAID has been demonstrated to be effective in controlling postoperative pain (Slingsby & Waterman-Pearson 2001). These drugs could act synergistically and the resultant low doses of these drugs decrease the likelihood of side effects.

The Research problem

A comprehensive investigation on efficacy and safety of tramadol and parecoxib compared to morphine and saline, administered preventively to dogs, has been carried out in the studies reported in this thesis. The main objective of these studies was to evaluate the suitability of these drugs as pre-and post-operative analgesics in dogs undergoing ovariohysterectomy or castration. To do this in a rational way, drugs and drug combinations were screened in experimental dogs under controlled conditions. All protocols for research involving dogs in this thesis were approved by the Massey University Animal Ethics Committee.

CHAPTER 2

POSTOPERATIVE PAIN: A REVIEW ON NEUROPHYSIOLOGICAL PATHWAYS AND PATHOLOGICAL MECHANISMS, EVALUATION METHODS AND MANAGEMENT STRATEGIES

Clinical pain can be subdivided into acute and chronic. Acute pain results from injury that recedes with wound healing, and tends to be self-limiting (Gayner & Muir 2002). Chronic pain persists beyond the usual course of an acute disease or wound healing, or is associated with a chronic disease process that causes continuous pain or pain recurs at intervals for months or years (Bonica 1990).

Postoperative pain is one of the most prevalent forms of acute pain that threatens the well being of animals. Though acute pain has an adaptive (protective) sensory function as it signals the individual to avoid noxious stimuli, uncontrolled acute pain has a maladaptive role in the sensory activity leading to development of chronic pain states (Scholz & Woolf 2002).

Ovariohysterectomy and castration are common surgical procedures in small animal practice that are carried out under general anaesthesia. Nociceptive input is not abolished under a surgical plane of general anaesthesia though anaesthetics can make the animal unconscious and unable to perceive pain during surgery. This preconscious neural activity causes neuronal hyper responsiveness, which is difficult to control once developed. Effective management of postoperative pain is a major welfare issue in small animal practice. This chapter discusses the neurophysiological pathways and pathological mechanisms involved in generating postoperative pain, different pain evaluation methods and controlling strategies.

2.1 PERIPHERAL NOCICEPTIVE MECHANISMS

During surgery, when the scalpel blade cuts through the skin and deeper tissue, nerve fibres sensitive to mechanical stimuli generate a brief and high intensity afferent flow followed by low intensity afferent input produced by nerve fibres sensitive to various inflammatory chemicals such as K^+ , ATP, H^+ etc. released from the injured cells (Woolf & Bromley 1999).

Nociceptive afferents generated due to the activation of peripheral sensory nerve endings are transmitted to the spinal cord and the cerebral cortex where they are processed resulting in acute nociceptive pain. Moreover, this afferent input is capable of initiating alterations or

“plasticity” in the central nervous system (CNS) processing of subsequent sensory information, which is a chief determinant of post injury pain sensitivity (Woolf & Bromley 1999).

Also, surgery induces a typical inflammatory reaction (some time after tissue injury) resulting in prolonged activation and sensitisation of peripheral afferent nerve fibres (Dray 1995), thereby maintaining sustained afferent input to the higher centres. A variety of inflammatory mediators are released from damaged cells and primary afferent fibres (table 1). They could activate or sensitise the afferent fibres directly or indirectly.

Table 1: Different inflammatory mediators released in response to tissue trauma and their effect on afferent nerve terminals ^a

Substance	Origin	Effect
H ⁺	Damaged cells	Activation
K ⁺	Damaged cells	Activation
Prostaglandins	Damaged cells	Sensitisation
Kinins:		
A.Bradykinin	Plasma cells	Activation
B.Kallidin	Tissue kininogens	Sensitisation
Leukotrienes	Damaged cells	Sensitisation
Adenosine	Adenosine triphosphate (ATP)	Sensitisation
Serotonin	Platelets, Mast cells	Activation
Histamine	Mast cells	Activation
SP & CGRP	Sensory nerve endings (C-fibres)	Sensitisation
Nerve growth factor (NGF)	Sensory nerve endings (C-fibres)	Sensitisation
Catecholamines & Neuropeptide Y	Sympathetic nerve fibres	Potentialiation of local inflammation

^a Modified with information from Dray (1995) & Muir (2002)

All these inflammatory mediators can act synergistically and produce “ inflammatory soup” (Treede et al. 1992; Woolf 1995). Soaking and bathing of peripheral afferent fibres in this algogenic soup reduces their activation threshold or enhances their pain sensitivity, leading to peripheral sensitisation (Cousins & Power 1999). Some inflammatory mediators like bradykinin could cause both whereas others like prostaglandins are primarily associated with afferent fibre sensitisation (Dray 1995).

The sensitised nerve endings at the site of injury show two types of altered response to subsequent mechanical and thermal stimuli; an exaggerated response to normally noxious stimuli, and a noxious response to normally innocuous stimuli, referred to as hyperalgesia and allodynia, respectively (Merskey & Bogduk 1994). This hyperalgesia at the site of injury (inflammation) is termed primary hyperalgesia (Shafford et al. 2001). In addition to primary hyperalgesia, a secondary mechanical hyperalgesia is seen in the uninjured skin surrounding the wound, which is mediated predominantly by central mechanisms (Coderre et al. 1993; Ali et al. 1996; Klede et al. 2003).

In summary, regional injury responses in the periphery are a profound alteration in the threshold of primary afferent sensory neurones, expansion of their receptor fields, activation of dormant afferent peripheral nerve fibres thereby enhancing the number of functional sensory neurones, hyperalgesia and amplified transduction of noxious stimuli (Dray 1995; Kelly et al. 2001).

2.2 CENTRAL NOCICEPTIVE MECHANISMS

2.2.1 The spinal cord

Once a noxious stimulus is transduced into a neural signal, it is then transmitted to the dorsal horn of the spinal cord mainly through A δ and C type afferent sensory fibres, where initial integration and modulation of afferent nociceptive input occurs (Lamont et al. 2000; Kelly et al. 2001; Muir 2002). A brief description of the spinal cord cytoarchitecture is essential when describing the spinal nociceptive mechanisms. The spinal cord is divided into white matter and gray matter. The gray matter in turn is divided into 10 laminae, which have no clearly defined margins (Rexed 1952). Laminae I to VI comprise the dorsal horn, lamina VII comprises the intermediate zone, laminae VIII to IX comprise the ventral horn, and lamina X surrounds the central canal of the spinal cord.

Thinly myelinated A δ fibres terminate in the marginal lamina or lamina I and to some extent in lamina V of the spinal dorsal horn. Unmyelinated C fibres end on lamina I and lamina II, whereas large myelinated A β -type afferent fibres project to deeper layers mostly laminae III through lamina VI (Woolf & Chong 1993; Willis 2005). Further, neurones in the dorsal horn of the spinal cord can be divided into three classes (table 2).

Table 2: Type of dorsal horn neurones and their role in nociceptive transmission^a

Projection neurones	Propriospinal neurones	Interneurones
Relay sensory input to supraspinal centres	Transfer sensory information from one segment of the spinal cord to another (segmental transmission)	Excitatory interneurones relay sensory input to projection neurones
Play significant role in the activation of descending pathways	Negligible contribution to nociception	Inhibitory interneurones control the flow of sensory input to the brain

^a Elaborated from Doubell et al.(1999)

Some of the projection neurones are excited solely by noxious input from both A δ and C nociceptive fibres, known as nociceptive-specific neurones, and the superficial lamina or lamina I possess a high proportion of this class (Woolf 1995; Raffe 1997). Some other projection neurones respond in a graded manner, to both innocuous as well as noxious information from A β , A δ and C fibres and called as wide dynamic range neurones. They are present in high concentrations in lamina V and to some extent in laminae I and II.

In summary laminae I & II are the principal zones for the primary afferent synapse from where the peripheral nociceptive information is projected to higher centres in the brain (Dickenson 1995). As in the periphery, the spinal dorsal horn contains myriad receptors encoding multiple transmitters released in response to peripheral injury from the central terminals of afferent nerve fibres, and the spinal dorsal horn neurones.

Some of the identified as well as putative neurotransmitters implicated in the spinal nociception include several peptides (substance P, SP), calcitonin gene related peptide (CGRP), neurotensin, galanin, cholecystokinin, somatostatin, bombesin and vasoactive intestinal polypeptide); excitatory amino acids (glutamate and aspartate); inhibitory amino acids (gamma-amino butyric acid (GABA) and glycine); nitric oxide and prostanoids; opioid peptides; biogenic amines (serotonin and noradrenaline) and adenosine (Dickenson 1995; Budai 2000).

Of these, the excitatory amino acid glutamate and neuropeptide SP acting at selective and specific receptors on dorsal horn neurone membranes plays a central role in excitatory transmission of nociceptive information and associated 'plasticity' in the CNS (Millan 1999). Receptors for glutamate can be broadly classified as ionotropic. These include AMPA (α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), NMDA and kainate, and seven groups of metabotropic receptors. Receptors for SP in the spinal dorsal horn are primarily neurokinin-1 (NK₁) type, though there are two other receptors (NK₂ and NK₃) in that family (Khasabov et al. 2002).

During surgery, trains of noxious stimuli from the periphery lead to continued release of glutamate from the pre-synaptic end of afferent C-fibres along with (co-release) of the neuropeptide SP. This in turn leads to a synergistic and complementary interaction between the glutamate acting on NMDA and metabotropic receptors and SP acting on NK₁ receptors, generating slow excitatory synaptic potential that lasts tens of seconds (Woolf & Bromley 1999). Electrophysiologically, it has been shown that these slow and long lasting excitatory potentials summate each time with the previous one (frequency of 0.5-2 Hz) resulting in cumulative depolarisation of post synaptic neurones, which is manifested as a substantial and non-linear increase in the number of action potentials produced on each subsequent C- fibre input (Sivilotti et al. 1993; Woolf 1995). This frequency-dependent plasticity of spinal neurones (lasting the duration of the stimulus) in response to a brief and repeated C-fibre afferent input is termed as 'wind-up' (Mendell 1966). At the cellular level, this non-linear rise in action potentials after cumulative depolarisation of post synaptic membranes was explained by a significant alteration in the intracellular calcium levels, a key determinant of neurotransmitter activated signal transduction pathways in dorsal horn neurones.

The intracellular calcium levels could be elevated by changes in three different receptor channels namely voltage-dependent NMDA, voltage-gated calcium and metabotropic glutamate receptor channels. The NMDA receptor is a classic glutamate receptor, the channel which is blocked by a Mg⁺⁺ at normal resting membrane potentials. Glutamate binding to the receptor produces negligible inward currents. However, in the presence of membrane (cumulative) depolarisation, this Mg⁺⁺ block is removed allowing a high calcium influx along with regular Na⁺ currents. Membrane depolarisation also activates voltage-gated calcium channels further increasing the calcium inflow; the net effect of which the neurones are

‘wound up’ (Woolf et al. 2000). As a result, stimulus – response properties of dorsal horn neurones will be altered to a great extent and are manifested by a progressive increase in the magnitude and longevity of responses to brief and repeated C fibre stimulation (Price 1972).

Further, the metabotropic glutamate receptors elevate the calcium influx indirectly through the activation of second messengers (eg: inositol triphosphate) (Miller 1991). Altogether, there is a steep rise in intracellular calcium levels leading to activation of calcium dependent intracellular protein kinases A and C and other enzymes like nitric oxide synthase, resulting in elevation of the level of nociceptive second messengers in the dorsal horn neurones (Budai 2000). Further, calcium activated protein kinases phosphorylate different membrane bound proteins (receptors) on dorsal horn neurones including NMDA receptors. Phosphorylation of the NMDA receptor by protein kinase C results in amplified sensitivity or excitability of the receptor to the actions of glutamate released subsequently, since membrane depolarization is now not necessary to remove the Mg^{++} block (Chen & Huang 1992). Consequently, properties of dorsal horn neurone receptive fields are altered along with recruitment of previously subthreshold inputs (reducing the neurone activation threshold). Thus, phosphorylation, which makes the channel open for longer, of the NMDA receptor, is, at least, in part, responsible for establishing (inducing) sensitisation of central neurones and the resulting hyperalgesia (Dubner 2005). However, there are several other second messenger pathways that are activated by brief and intense C-fibre input contributing to increased excitability of the spinal dorsal horn neurones (Petersen-Zeitz & Basbaum 1999). This activity-dependent central nociceptive facilitation emerges within seconds of an intense nociceptive conditioning stimulus and lasts for several hours longer than the duration of the initiating stimulus (Woolf & Wall 1986; Woolf & Chong 1993).

Once induced, a low level of afferent C-fibre input from the inflamed surgical site would be sufficient to sustain or maintain the central sensitisation (Woolf & Bromley 1999). Further, peripheral inflammation induces some transcriptional changes such as induction of the immediate early genes for C-fos and COX-2 proteins in the dorsal horn neurones (Scholz & Woolf 2002). COX-2 is a major contributor to the spinal prostaglandin E_2 (PGE_2), which in turn contributes to sustaining the central hyperexcitability (Samad et al. 2001; Scholz & Woolf 2002). However, C-fos protein is thought to be involved in the memory of pain, and can be used as a marker of nociception.

Therefore, surgical injury contributes to post operative pain hypersensitivity by two distinct, but collaborative mechanisms: peripheral and central sensitisation (Woolf 1983).

Sensitisation of peripheral and central neurones is clinically demonstrated as a decrease in postoperative mechanical pain threshold and increased pain scores in response to palpation (both at incision site and sites distant from surgery), in dogs undergoing ovariohysterectomy (Lascelles et al. 1995, 1997; Slingsby & Waterman-Pearson 2001).

2.2.2 Ascending transmission from the spinal cord

From the spinal dorsal horn, the nociceptive information is relayed to the supraspinal centres via different nerve tracts ascending from the spinal cord. Simultaneous transfer of dorsal horn output to the ventral horn activates flexor motor neurones generating the withdrawal flexion reflex, causing synchronized occurrence of both the sensation of pain and flexion withdrawal reflex (Willer 1979).

The spinothalamic tract (STT) emanating from the anterolateral quadrant of the spinal cord is the principal (direct) ascending nociceptive pathway (Sherrington 1901). Also, several neurosurgical studies proved that the successful outcome of the anterolateral cordotomy is due to section of the STT (Sosnowski et al. 1992). Lamina I of the spinal dorsal horn contains a relatively high proportion of STT neurones, though there are some in laminae V - VII (Craig 2003). The majority of the STT neurones are nociceptive specific or wide dynamic range type, axons of which cross the midline at the anterior commissure to the contralateral side in the same segment (Sosnowski et al. 1992; Willis 2005). The STT course through the brain stem structures (medulla, pons and mid-brain) and terminate in specific thalamic nuclei relaying nociceptive information to the cerebral cortex (Willis 2005).

The spinothalamic tract is another ascending nociceptive pathway projecting directly to the hypothalamus through the contralateral thalamus. Cells of this bilateral tract arise from the laminae I, V, VII and X and possibly mediate autonomic and emotional aspects of pain (Craig & Dostrovsky 1999).

The other ascending nociceptive projections from the spinal cord include the spinoreticular tract (SRT) and the spinomesencephalic tract (Sosnowski 1992). These tracts originate from the spinal laminae similar to those giving the STT cells and terminate in different brain stem structures including the medulla and the reticular formation, the parabrachial nucleus, and in

the midbrain periaqueductal grey (PAG) region. Also, some SRT fibres project directly to the level of the thalamus. However, nociceptive input from different brain stem areas in turn project to the thalamus and hypothalamus for ascending relay to the cortex. Simultaneously, these brain stem areas send descending (modulatory) projections back to the spinal dorsal horn (Craig & Dostrovsky 1999). Other indirect pathways including the spinocervicothalamic pathway and dorsal column medial lemniscus pathway also transmit somatic nociceptive input to the thalamus (Willis 2005). Thus, the thalamus receives afferent input either directly or indirectly via multiple nociceptive pathways ascending from the spinal cord and functions as the relay centre for afferent input en route to the cerebral cortex (Lamont et al. 2000).

2.2.3 Perception

Processing of afferent input in the cerebral cortex results in conscious pain perception.

However, imaging studies such as positron emission tomography and functional magnetic resonance imaging have shown an increased and consistent activity of different cortical and sub cortical structures including the somatosensory cortex (I & II), anterior cingulate and insular cortices, in response to acute noxious stimulation in humans (Derbyshire et al. 1997). In rats, Morrow et al (1998) first identified the consistent neural activity in multiple forebrain structures and the midbrain PAG during formalin induced noxious stimulation using the regional cerebral blood flow mapping technique. Therefore, noxious stimulation targets not only activation of the 'pain centre' but also processing at large number of cortical and sub cortical regions associated with motor function, attention allocation, emotional processes and sensory localisation (Derbyshire et al. 1997).

2.2.4 Descending modulation

Descending pathways originating in the brain stem and other cerebral structures play an important role in modulation (Millan 1999). Important brain stem structures involved are; the midbrain PAG and the rostral ventromedial medulla (RVM). The PAG receives descending impulses directly from different cortical areas (including the anterior cingulate and other frontal cortices), the hypothalamus and amygdala, and integrates with the ascending input from the spinal dorsal horn. The descending output from the PAG is projected to the spinal dorsal horn indirectly through connections with neurones in the RVM selectively targeting the dorsal horn laminae rich in nociceptive neurones (Fields 2004). The RVM contains the nucleus raphe magnus with which the PAG has direct connection in cats and rats (Pomeroy & Behbehani 1979). The RVM receives spinal input indirectly from the adjacent medullary

nucleus reticularis gigantocellularis, which has projections from the spinoreticular tract neurones (Fields & Bausbam 1999). Other areas that receive modulatory impulses from PAG include the noradrenergic nuclei (A₅ and A₇ cell groups) of the dorsolateral pontomesencephalic tegmentum, which also project to the RVM. Thus, the RVM acts as a major relay in the descending modulation of nociception (Dubner 2005).

Endogenous opioid peptides, serotonin, noradrenaline, GABA and glycine are the major nociceptive modulatory transmitters in the descending modulatory pathway. It has been suggested that endogenous opioids acting through μ -opioid receptors in the RVM neurones (on-cells and off-cells) play a major role in the spinal antinociceptive effect (Roychowdhury & Fields 1996). However, as exemplified by serotonin, a single transmitter may produce divergent actions through expression at multiple receptors, and therefore facilitate and inhibit spinal nociceptive transmission simultaneously (Millan 2002).

It is noteworthy to mention here that electroencephalographic studies in animals under general anaesthesia have shown that the stimulation of the brain stem reticular formation, which has diffuse connections with other brain structures (ascending and descending) including the thalamus, results in desynchronisation of the EEG (Moruzzi & Magoun 1949; Antognini et al. 2000, 2003), a change in EEG activity indicating arousal/awakening. This suggests the indirect contribution of these brain stem structures to the cortical function.

2.3 PAIN ASSESSMENT METHODS

Evaluation of analgesic efficacy needs accurate measurement of pain (Huskisson 1974) which in animals presents problems as they cannot communicate directly about their experience (LeBars et al. 2001). Assessment of pain by monitoring changes in neurohumoral responses (Fox et al. 1994; Mellor et al. 2002), behavioural reactions (Lascelles et al. 1994; Fox et al. 2000) and neurophysiological variables (Short et al. 1992; Greene et al. 1992) are useful to evaluate analgesic drugs.

2.3.1 Neurohumoral indicators

Changes in plasma concentration of adrenal corticosteroids, specifically cortisol and the associated releasing hormones from the anterior pituitary, have been used in animal pain studies (Slingsby 1998; Mellor et al. 2002). Advantages of this method include:

1. minimised observer variability associated with pain related behaviour assessment
2. and more objective method than behavioural assessment

However, there were some significant limitations to the use of this method (Livingston et al. 1994). They were:

1. increase or decrease in plasma levels of hormones indicative of pain were also associated with any stress response,
2. a single sample collected at a specific time period could not correspond with the level of pain an animal was experiencing, and,
3. directly induced changes in hormonal levels by mechanisms other than analgesia greatly restrict the validity of this method in studies evaluating analgesic efficacies.

2.3.2 Behavioural methods

2.3.2.1. Nociceptive threshold testing

Experimentally mechanical, chemical, thermal and electrical stimuli can be used to produce quantifiable nociceptive stimuli (Beecher 1957; Welsh & Nolan 1995) and the level at which these stimuli are painful (pain threshold) is normally associated with various types of behavioural responses or withdrawal reflexes (Livingston et al. 1994). Every nociceptive stimulus may not be harmful. For example, menthol stimulates cold receptors and capsaicin induces a burning sensation, without producing potential tissue damage (Le Bars 2001). Thus, a stimulus that activates the sensory nerve fibres and is algogenic without actual tissue injury can be used experimentally to produce acute pain (Beecher 1957). Beecher proposed certain 'ideals' of producing nociceptive stimuli by testing devices. They were:

1. it can be applied to a body part that has minimum variation (neurohistology) between different individuals
2. it should be sensitive enough to discriminate noxious from innocuous stimuli
3. the intensity of the pain should be related to the intensity of the stimulus
4. the stimulus can be repeated at the same body part without affecting subsequent readings
5. the stimulus can be easily applied with clearly defined end points
6. it is able to produce a reproducible response without any tissue damage at the threshold level
7. it should be sensitive enough to detect the effects of mild analgesics
8. it should be able to show a linear dose-effect relationship with the same analgesic.

The responses elicited by sudden, acute nociceptive stimuli vary from basic reflexes to more integrated behaviours (escape, avoidance). Usually easily identifiable behavioural reactions (terminating the stimulus) will be considered as the endpoint (Flecknell 1999).

Analgesia in response to thermal, electrical, chemical or mechanical stimuli has been studied in humans and laboratory animals as well as large and small domestic animals. However, each method has its own merits and demerits (table 3).

Table 3: Advantages and disadvantages of different types of noxious stimuli used to elicit experimental pain^a

Type of stimulus	Advantages	Disadvantages
Electrical	<ul style="list-style-type: none"> • Noninvasive • Produces synchronized afferent signals • Suitable to study the central pain mechanisms 	<ul style="list-style-type: none"> • Not natural • Intense stimuli may short-circuit the entire peripheral nociceptors (both noxious and innocuous) making study of peripheral nociceptive mechanisms difficult
Thermal	<ul style="list-style-type: none"> • Selective stimulation of specific cutaneous nociceptors 	<ul style="list-style-type: none"> • Asynchronous activation of peripheral and central neurones. Possibility of radiation with conventional radiant heat sources, for which skin is a good reflector.
Chemical	<ul style="list-style-type: none"> • Can be used to produce visceral or peritoneal pain • Pain produced is analogous to clinical pain 	<ul style="list-style-type: none"> • No control over the stimulus • May harm the animal
Mechanical	<ul style="list-style-type: none"> • Most natural type which the animal may experience in its usual life • Responses produced by these stimuli can be graded from reflexes through vocalizations ultimately to complex motor behaviours, in relation to the intensity and duration of the stimulus 	<ul style="list-style-type: none"> • Technical difficulties in application to freely moving animals especially rodents • Probability of producing changes in tissues either transient/permanent

^a Elaborated from Chambers et al. 1994; LeBars et al. 2001).

Application of pressure stimuli using Von Frey hairs to evoke pain in humans (Von Frey 1897) led to the use of different types of nociceptive mechanical stimuli to elicit quantifiable responses to analgesic therapy in animals (Lascelles et al. 1997). For the purpose, various mechanical threshold-testing devices have been developed in small animal studies.

In dogs, quantifiable mechanical nociceptive stimuli were produced by applying force across their toes through a pair of Doyen intestinal clamps (Martin et al. 1963), or by pneumatic pressure through a programmed toe squeezer (Martin et al. 1974; Martin et al. 1976). Flexor (pedal) reflex was the end-point in these experiments. Later, Hamlin et al (1988) designed a device containing aluminium pliers through which force was applied to toes, for assessing the analgesic efficacy of xylazine administered to healthy dogs. With this device they found a clear-cut increase in pain threshold of dogs given xylazine and the dose-response relationship of xylazine given at low and moderate doses.

There were certain limitations to the methods used in these studies. They were:

1. the mechanical stimulus could be applied to a specific body part i.e. toes only, and,
2. there was no control over the rate of stimulus applied, which may lead to operator variability on sensory thresholds (Grindley 1936).

However, these studies demonstrated the use of mechanical nociceptive stimuli in detecting the pain threshold under experimental conditions. In a surgical setting, application of mechanical nociceptive stimuli both at the surgical wound and at sites other than the wound would be very useful to investigate the efficacy analgesics/analgesic strategies (Lascelles et al. 1998).

Lascelles et al. (1997) used a sophisticated handheld device to produce mechanical stimuli in dogs undergoing ovariohysterectomy. The stimulus was applied through a pen-like probe and the rate at which the stimulus was applied was controlled by means of continual rate of application of force (CRAF) meter that was linked to the device. In the device, the force that pressed the probe perpendicular to the skin could be transmitted to a load cell to produce a voltage output. This voltage output was conveyed to a transducer and amplifier to produce a reading. Stimuli were applied both at the site of surgery and at sites other than tissue injury. Thresholds measured at both sites clearly showed the development of post-operative

hyperalgesia and mechanical allodynia respectively, substantiating the pre-emptive value of pethidine in blocking the development of central sensitisation.

Slingsby et al. (2001) developed another mechanical threshold testing device for use in cats. The investigators opined that cats are less tolerant to testing devices used in other species but could tolerate palpation rather than probing to map the degree of hyperalgesia after surgery. They built a ‘pressure of palpation’ (PPD) device based on the design of the palpometers developed by Atkins et al (1992) and Bendtsen et al (1994). There was a good correlation between threshold values obtained by PPD and the CRAF meter devised by Lascelles et al. (1997).

In summary, all these devices and methods developed to test the mechanical nociceptive thresholds of animals let us assess the efficacy of analgesics/ analgesic approaches, level of wound hyperalgesia and development of ‘plasticity’ in the nervous system under clinical or experimental conditions (Slingsby et al. 2001).

2.3.2.2 Subjective evaluation

Changes in normal behaviour are the most commonly observed reactions of animals to painful stimuli (Firth & Haldane 1999). Since an individual’s behaviour is a function of complex relationships between its internal and external environments (Kantor 1958), recognition, interpretation and quantification of complex behaviours associated with pain differ in different individuals of each species. Therefore assessing pain is complicated as these behavioural observations are subjective and an observer must be sensitive, accurate and experienced in subjective assessment and description of various degrees of pain in nonverbal individuals. Moreover, the investigator may have to interpret the animal’s pain behaviour in terms of his own experience (Taylor & Houlton 1984).

2.3.2.2.1 Types of Behavioural responses

Any behaviour that reliably communicates the experience of pain to others is pain behaviour regardless of intent (Keefe & Lefebvre 1994). The Association of Veterinary Teachers and Research Workers (AVTRW 1986) developed some guidelines for the recognition and assessment of pain in animals based on the following three categories of behavioural responses to pain.

1. Automatic (involuntary) reflex responses, which are programmed to protect the animal e.g. reflex withdrawal from the stimulus or removal of the stimulus seen in tail flick test and paw pressure test in rodents and toe squeezing in dogs. However, reflex responses might originate at spinal level that may exclude the supraspinal processing of nociceptive input. More clearly, using spinal reflexes we can measure the sensory but not the emotional component of pain (Flecknell 1999).
2. Learned avoidance responses, which alters the behaviour of the animal. The animal becomes aware of the aversive feeling of pain felt previously and attempts to avoid the repeated situation and,
3. Voluntary, unlearned behavioural responses resulting from supraspinal sensory processing, intended to communicate the aversive/unpleasant feeling to others of the same species or other species e.g. pain specific vocalization, facial expression, posture etc.

Generally, organised, unlearned behaviours are used to assess pain, as they involve a purposeful reaction resulting from supraspinal sensory processing (Dubner 1987).

Hardie et al (1997) conducted a behavioural study in a large number of ovariohysterectomised dogs. They assessed the pain specific dog behaviours in two ways i.e. interactive (single observer) and non-interactive (video recorded). Interactive behaviours included a range of behaviours like tail wagging, lip licking, vocalization and pawing. These were observed when the observer approached and talked to the dogs at specific time intervals in the postoperative period. Signs of aggression like lip lifting or teeth baring were seen when the surgical site was palpated. Non-interactive behaviours were video recorded. Dogs spent more time sleeping in lateral recumbency during the first 12 hours after surgery and thereafter slept more time in sternal recumbency up to 24 hours. Surgery resulted in decreased grooming and frequent wound (abdominal midline) licking.

Conversely, dogs that were anaesthetised without surgery spent one-half to three-quarters of their time awake post-extubation. They spent more time standing next to sternal recumbency. Drinking, eating and grooming were within normal range. The dogs actively interacted with a handler and by six hours after anaesthesia, they tried to escape from the cage when the handler opened the cage door.

These different behaviours in response to anaesthesia alone or that the animals were more deeply anaesthetised for surgery indicate that ovariohysterectomy is a painful surgical procedure that significantly alters the normal behaviour, body posture and activity, movement, orientation towards the handler/owner, facial expressions and appearance, sleeping patterns and response to manipulations.

2.3.2.2.2 Pain scoring systems

The main objective of any pain scoring system is to assess pain as well as analgesic efficacy in patients (Mathews 2000). Pain rating scales used in animals have simply been adopted from scales developed for measurement of pain in humans. The ability to communicate verbally allows reliable estimation of pain intensity using different scales in adult humans (Flecknell 1999). In the case of children who cannot write or communicate verbally objective demonstration of surgical stress coupled with behavioural reactions like crying, facial expression, posture etc. served as basic criteria for developing infant pain scoring systems (McGrath 1987). Scales designed for acute procedural pain in prelingual children might therefore serve as a fundamental template for acute pain studies in veterinary patients (Hansen 2003).

In veterinary practice, most of the commonly used scales measure the intensity of pain unlike humans in which other dimensions like affect, quality and location of pain (multidimensional) are assessed (Holton et al. 2001). Pain intensity is a quantitative estimate of the severity or magnitude of perceived pain (Jensen et al. 1986).

Different scales have been used to measure the pain (acute) intensity in small animals including simple descriptive scale, numerical rating scale and visual analogue scale (VAS) (Lascelles et al. 1994; Holton et al. 1998; Slingsby 1998). Recently, the Glasgow composite measure pain scale-short form (CMPS-SF) has been introduced by Reid et al. (2007) for measuring acute postoperative pain in a routine clinical setting, in dogs.

Simple descriptive scale: This scale usually consists of four or five expressions to describe different levels of pain intensity. These expressions are assigned a number, which represents the pain score for that animal. For example, Holton et al. (1998) looking at dogs after surgery used the following scoring system;

The observer has chosen the most suitable descriptive term, which was then converted into a numerical score like, no pain = 0; mild pain = 1; moderate pain = 2; and severe pain = 3.

Advantages:

1. Simple to use
2. Results will not be affected by visual acuity.

Disadvantages:

1. Lacks sensitivity due to limited number of pain descriptors that lead to over/under assessment of pain and analgesic efficacy.
2. Inter-observer variability.

Numerical rating scale: This scale consists of multiple categories with different descriptive definitions of pain behaviours in each category and numbers are assigned to each descriptive level. The number chosen by an observer represents the pain score (Holton et al. 1998).

Advantages:

1. Thorough evaluation of the patient due to several categories
2. Easy method of tabulating the score.

Disadvantages:

1. Lack of accuracy as categories are scored by whole numbers that implies equal differences between categories which may not be true
2. Lack of specificity with descriptive pain behaviours
3. It encourages inappropriate statistical analysis since the results are not normally distributed.

Visual analogue scale: This technique is widely used in human medicine (McQuay & Moore 1999) and has been used in various veterinary analgesic studies. Typically, visual analogue scale (VAS) is a 100 mm straight horizontal or vertical line anchored with descriptors of pain intensity, such that 0 represents no pain and 100 represents the worst possible pain, on either end of the line (figure 1). The observer draws an intersecting line along the scale that best represents his/her estimated level of animal pain.

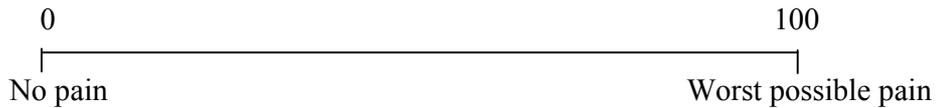


Figure 1: Visual analogue scale for pain scoring

Measuring the length of line from 0 to the mark made in mm i.e. from extreme left to right gives the pain score for the animal. In addition, the VAS can be used to assess the level of dogs' sedation in the peri-operative period. In this system, the level of sedation will be assessed by observing dogs' posture, mental alertness, and its ability to stand and walk. At each assessment, a mark will be made on a 100 mm scale, on which 0 corresponds to 'no sedation' and 100 corresponds to 'fast asleep' (Lascelles et al. 1994).

Advantages:

1. More sensitive in detecting subtle variations as the scale is not limited to some labelled categories of pain
2. Gives a broad sense of whether pain is improving or getting worse in the course of evaluation, and
3. Avoids the use of indefinite descriptive terms and provides many points to select.

Disadvantages:

1. Inter-observer variability when more than one observer evaluates the same animal,
2. Variation due to visual acuity among observers, and
3. VAS scores are not linear, i.e. the difference in pain between each successive increment is not equal.

The Glasgow composite measure pain scale: This pain scale is relatively new and has been derived from the McGill pain questionnaire (Holton et al. 2001). It has been developed based on words and expressions used for describing dogs' pain behaviour by a number of practising veterinary surgeons. These words and expressions were consolidated into seven behaviour categories namely posture, comfort, vocalization, attention to wound, demeanour, mobility and response to touch. Each category includes different descriptions of a dog's pain behaviour. For a quick and reliable assessment of acute pain in a routine clinical setting this composite scale has been shortened as composite measure pain scale- short form (CMPS-SF - appendix). CMPS-SF includes six behavioural categories with 30 pain descriptors. The

descriptors in each category were ranked numerically based on associated pain intensity. Pain score is the sum of the rank scores which was assigned by the observer after picking the best fit descriptor of the dog's pain within each category.

Advantages:

1. Measures more than one aspect of pain.
2. Does not require specialised skills and experience to use since the pain descriptors were taken from practising veterinary surgeons routinely observing acute pain behaviour of dogs.

Disadvantages:

1. Validity of the scale has not been tested in clinical trials after elective surgery.

2.3.3. Neurophysiological technique: EEG

The EEG is a record of the spontaneous electrical activity of the cerebral cortex. It has been used extensively to assess the anaesthetic depth in humans. It is a non-invasive and stress-free technique. However, its use in veterinary practice is still limited owing to the lack of universal standard for the recording technique and the absence of a common restraint method (Pellegrino & Sica 2004).

2.3.3.1 Source of EEG

The underlying mechanism for generation of the EEG activity can be briefly explained as follows (Murrell & Johnson 2006). Changes in the membrane potential of postsynaptic neurones are the chief contributors of EEG. Postsynaptic potentials can be excitatory or inhibitory, and can lead to generation of extracellular fluid currents adjacent to postsynaptic neurones. The characteristic configuration of neurones (particularly pyramidal type) in cortical layers leads to formation of electrical vectors that facilitates transmission of extracellular currents. The EEG is the far-field potential (electrical field created by inherent neuronal activity and recorded away from them) of the vector currents recorded using electrodes on surface of head. Normally, glial cells (located between neurones) do not elicit any action potential and the membrane potential of glial cells equates to extracellular K^+ concentration at equilibrium. Therefore the glial cell contribution to the EEG is minimal and is largely due to transient changes in extracellular K^+ concentration caused by repetitive firing of adjacent cortical neurones (Speckman & Elger 1999).

2.3.3.2 EEG power spectrum

Fast Fourier transformation (FFT), a mathematical procedure, converts the raw EEG signal into its component sine waves of different frequency, characterised by corresponding amplitude. The power spectrum thus generated is simply distribution and derivation of spectral EEG variables from the corresponding frequencies and amplitudes. Quantitation of EEG depends on analysis of the power spectrum. Commonly used quantitative variables of the EEG power spectrum in response to nociception in different species of animals are median frequency (F50), total EEG power (P_{tot}) and spectral edge frequency 95% (SEF) (Miller et al. 1995; Johnson & Taylor 1997, 1998, 1999; Murrell et al. 2000, 2003). F50 is the 50% quantile in the EEG frequency distribution spectrum, P_{tot} is the total area under the EEG frequency spectrum and SEF is the 95% quantile i.e. the frequency below which 95% of total power is located in the EEG power spectrum.

2.3.3.3 EEG and depth of anaesthesia

Gibbs et al (1937) first identified the possible use of EEG for assessing the depth of anaesthesia. Since EEG provides a more direct and quick profile of cortical neuronal activity compared to conventional measures, it has become a monitoring tool of intraoperative patient awareness in human anaesthesia (Mashour 2006). In veterinary species, EEG has been extensively investigated in horses due to high incidence of anaesthesia related perioperative mortality (Johnson & Taylor 1997, 1998; Johnson et al. 1999; Murrell et al. 2003). In dogs, changes in EEG derivatives have been used to assess the depth of anaesthesia with different anaesthetic agents of varying concentration (Scheller et al. 1990; Muthuswamy & Roy 1993; Murrell et al. 2005).

2.3.3.4 EEG changes during nociception and antinociception

Now it is very well established that cerebral cortex participates in the processing of afferent nociceptive input that results in conscious pain perception. It has been demonstrated that changes in EEG activity correlate with verbal reports of human's pain experience (Chen et al. 1989). Correlation of EEG spectral frequency changes with behavioural responses to nociceptive stimulus in conscious sheep (Ong et al. 1997) further supports this.

Changes in EEG power spectrum can be used for objective quantification of nociception in anaesthetised horses, red-deer, lambs, rats and calves (Murrell et al. 2003; Johnson et al. 2005a, 2005 b; Murrell et al. 2007; Gibson et al. 2007). Predominantly, F50 has been shown to be

reflecting alterations in cortical activity in response to nociception in these studies, and also antinociception in anaesthetised horses (Johnson & Taylor 1997, 1999; Johnson et al. 2000; Murrell et al. 2005). In dogs, ovariohysterectomy was shown to cause an increase in mean and median, and a decrease in peak frequency ranges of EEG power spectrum (α and β , and δ rhythms respectively) (Trucchi et al. 2003). This provides a useful tool to demarcate the cerebral cortical processing of analgesic effects from that of anaesthesia.

2.4 POSTOPERATIVE PAIN CONTROLLING STRATEGIES

2.4.1 Preoperative analgesia

Since it is very difficult to abolish central sensitisation once established (Woolf 1983) the conventional method of administering analgesics in response to pain in the postoperative period may not be the most effective strategy for managing acute surgical pain. Therefore ‘timing’ of analgesic administration is one of the major criteria for effective control of surgical pain (Dobromylskyj et al. 2000).

Crile (1913) first formulated the idea of blocking the regional nerves along with general anaesthesia to prevent afferent transmission. This strategy has become popular during late 90’s with the view of preventing central sensitisation by pre-incisional administration of analgesics (Lamont et al. 2000). Administration of analgesics before noxious induction may also diminish or attenuate the possibility of onset of pathological or chronic pain syndrome (Bach et al. 1988).

Kissin (2000) has defined pre-emptive analgesia as ‘an antinociceptive treatment that prevents the establishment of altered central processing of afferent input from injuries’. Preventing the effects of noxious afferent input generated during and after the elective surgery is the fundamental pre-requisite for effective preemptive therapy. Another important factor that may contribute to the success of preemptive analgesia is absence of presurgical pain, which if present would set up central sensitisation well before surgery (Aida & Shimoji 2000).

Several experimental and clinical studies of preventive analgesia both in animals and humans have been conducted in different surgical settings.

2.4.1.1 Animal studies

Two separate experiments in rats conducted by Gonzalez-Darder et al (1986) and Seltzer et al (1991) showed that nerve blockade with lignocaine before neurectomy markedly diminished the degree of autotomy (a pain related behaviour). A more distinct preventive analgesic effect was found with μ -opioid receptor agonists administered before noxious stimulation in rats, in terms of preventing or significantly reducing the development of central hyperexcitability (Woolf & Wall 1986; Dickenson & Sullivan 1987). In contrast, the same analgesic treatment given immediately after initiation of noxious stimuli cannot prevent the development of central sensitisation or wind-up (Woolf & Wall 1986; Katz et al. 1992; Woolf & Chong 1993).

In most of the laboratory studies on rats with acute inflammatory pain produced by injecting formalin or carrageenan, preventive analgesic strategies appeared to be successful in reducing post-injury hyperalgesia at different levels (Goto et al. 1994; Yashpal 1996; Fletcher et al. 1996). However, these studies have many potential limitations. They were;

1. a significant difference in nociceptive responses to injury was observed between control and pretreatment groups, but not between pre and post injury treatment groups as the latter group received opioids as part of the general anaesthetic regime (Yashpal 1996),
2. the pre-emptive effect observed in the formalin induced acute inflammatory pain was dependent on concentration of formalin injected (Yashpal 1996) and time limited (Fletcher et al. 1996).

These results could be extrapolated cautiously in a clinical setting since formalin induced inflammatory pain is basically different from actual surgical pain (Goto et al. 1994; Brennan et al. 1997).

Lascelles et al.(1995) conducted an experiment in rats subjected to ovariectomy which is an accepted model of acute surgical pain (Gonzalez et al. 2000), and showed that intramuscular administration of 15 mg kg⁻¹ pethidine (a μ - receptor opioid agonist) before surgery reduced the development of surgically induced hyperalgesia more effectively than the same opioid given postoperatively. A similar beneficial effect was found with preoperative pethidine in dogs undergoing ovariectomy (Lascelles et al. 1997).

Interestingly, a positive preventive analgesic effect was also shown with carprofen, a NSAID thought to act by inhibition of the enzyme cyclooxygenase peripherally, in two clinical studies in dogs undergoing ovariohysterectomy (Lascelles et al. 1998) and, a variety of soft tissue and orthopaedic surgeries (Woolf 1989; Welsh et al. 1997). Pain scores were lower at all time points in the postoperative period in dogs which received carprofen pre-incisionally than those that received the same drug postoperatively. The investigators opined that the carprofen given prior to surgery would have reduced the amount of noxious information generated peripherally at surgical incision, which in turn resulted in minimal changes centrally.

2.4.1.2 Human studies

McQuay et al (1988) conducted a large retrospective study on postoperative analgesic effects of opioid premedication in people undergoing orthopaedic surgery. They concluded that opioid premedication significantly increased the median time to first analgesic requirement from two to more than five hours after surgery. Richmond et al (1993) demonstrated a positive pre-emptive effect by using a small dose of morphine intravenously in women undergoing abdominal surgery. The investigators emphasised that ‘central sensitisation’ is the key factor in generating postoperative pain and preventing the onset of which could prevent secondary hyperalgesia (Richmond et al. 1993). The beneficial effect of pre-emptive analgesia has been shown by using various pharmacological interventions like epidural administration of local anaesthetics and opioids (Gottschalk et al. 1998; Bach et al. 1988), intravenous opioids (Mansfield et al. 1996), NSAIDs intravenously (Desjardins et al. 2001; Norman et al. 2001), infiltration of the surgical site with local anaesthetics (Jebels et al. 1991), NMDA receptor antagonists given intravenously (Tverskoy et al. 1994).

However, these positive results in terms of diminished pain scores and postoperative analgesic requirement were not consistent at all time points in the postoperative period. Also, some randomised trials could not demonstrate a definitive preventive analgesic effect (Murphy & Medley 1993; Turner & Chalkiadis 1994; Collis et al. 1995). These controversies have been explained by a number of possible reasons. They were:

1. the majority of trials measured the preoperative analgesic effect by comparing the test results between control and treatment groups (Kiss & Kilian 1992). According to Kissin (1996) it does not mean that there is no preventive effect if there is no

significant variation in outcome measures between preoperative and postoperative treatment groups because ‘central hyperexcitability’ can be initiated not only by surgical incision but also by various inflammatory mediators released from injured tissues later in the postoperative period (secondary inflammatory phase),

2. a clear benefit of pre-emptive analgesia cannot be obtained if there were analgesic agents like opioids and nitrous oxide (Goto et al. 1994) as a part of general anaesthetic regimen in the control group (Lascelles et al. 1995; Kissin 1996),
3. type of surgery has a significant bearing on effectiveness of preventive analgesia because organ innervation is dependant on the surgical site (Aida & Shimoji 2000). Moreover, the noxious stimuli generated during surgery should be intense enough to instigate the central sensitisation and the resultant hyperalgesia, otherwise there would be nothing to prevent (Kissin 1996).

Another important factor that contributes to the success of preemptive analgesia is efficacy or potency of the analgesic administered in relation to degree of surgical trauma, and the given analgesic should be able to cover the total surgical period i.e. starting from before the skin incision to last skin suture placed. Hence a single dose of an analgesic agent with limited potency may not prevent the noxious stimuli consistently (Lascelles et al. 1995).

2.4.2 Balanced analgesia

Since postoperative pain results from a series of events at different points in the pathway, involving various mechanisms and transmitter systems, it may not be easy to achieve optimal relief with a single class of analgesic drug or method without potential side effects (Kehlet 1989; Dobromylskyj et al. 2000). Therefore investigations have focussed on achieving optimum analgesia by combining different classes of drugs and techniques, each acting at different steps in the pain pathway (Lascelles 1999). With this approach, administration of analgesics with different mechanisms of action would produce an additive or synergistic interaction resulting in optimal analgesia even with lower doses thereby reducing potential side effects (Kehlet & Dahl 1993; Lascelles 1999; Lamont et al. 2000). A successful example of balanced analgesic therapy is preoperative use of an NSAID and opioid combination. In addition to the primary central mechanism of action, opioids also exert some antinoceptive effects peripherally (Stein et al. 1999).

NSAIDs act peripherally by inhibiting prostaglandin synthesis associated with inflammation (Vane 1971) during and after surgery thereby limiting nociceptive information reaching the CNS. However, some NSAIDs could also act centrally (McCormack 1994). Therefore the mechanisms of interaction of opioid and NSAID analgesia have yet to be elucidated.

Some investigators (Espinet et al. 1996; Campbell & Kendrick 1998) failed to find a positive effect with multimodal strategy compared to single dose regimes. These conflicting results indicate that the choice of analgesic agents, their dose and route of administration are important to achieve success with this strategy.

According to Lascelles (1999) it is also important that the dose and route of administration of a chosen analgesic in the multimodal strategy must be matched to the type of surgery. Also, Kehlet (1994) recommended that the selection of a suitable drug for perioperative use under multimodal strategy should be based on efficacy, convenience, ease of administration, cost, safety and additional advantages associated with outcome variables.

In addition to these factors, choice of analgesic for use also relies on degree of pain intensity expected from surgical trauma. For surgeries involving mild to moderate pain an opioid and NSAID combination appears to be effective under preoperative balanced analgesic strategies (Kehlet & Dahl 1993; Kehlet 1994).

2.4.3 Postoperative analgesics

2.4.3.1 Opioids

Opioids are the most widely used analgesic agents in the management of moderate to severe pain. They produce their effects by binding to specific opioid receptors, mainly μ , δ and κ , located supraspinally, spinally and peripherally (Martin et al. 1976). Opioids have a broad spectrum of action at these receptor sites. Morphine is a well-known potent opioid agonist with greater affinity at μ -receptors than δ and κ receptors (Beckett & Casey 1954). Agents acting predominantly at μ -opioid receptors play an important role in preventive analgesia (Woolf & Bromley 1999).

Activation of μ -opioid receptors located presynaptically on C-fibre terminals and postsynaptically on spinal neurones results in decreased neurotransmitter (substrates for

central hypersensitivity) release and hyperpolarisation of the postsynaptic membrane, respectively. The mechanism through which selective μ -opioid agonist agents act to produce this effect is blockade of Ca^{++} channel while augmenting K^{+} channel activity (Duggan & North 1984). Net effect is reduction in the amplitude and duration of C-fibre evoked slow synaptic potentials and temporal summation (wind-up, Sivilotti et al. 1995), which in turn prevents/reduces the onset of central hyperexcitability.

Peripherally, opioid receptors have been demonstrated on primary afferent sensory nerve terminals in rats and humans (Stein et al. 1990). Peripheral inflammation induces up-regulation of peripheral opioid receptors and activation of endogenous opioid peptides (Stein et al. 1999). There are opioid receptors on activated macrophages too. Inhibition of the release of excitatory proinflammatory compound SP from peripheral sensory nerve terminals has been suggested as the mechanism by which opioids produce local anti-inflammatory effect (Brodin et al. 1983). Clinically, this anti-inflammatory effect was demonstrated by administration of small doses of morphine into inflamed sites (Kalso et al. 1997).

Stimulation of nociceptive modulatory neurones in the brain stem particularly PAG and RVM neurones that exert an inhibitory effect on spinal nociceptive processing is the proposed mechanism of supraspinal analgesia by morphine and other μ -opioid agonists (Heinricher & Morgan 1999). However, opioid activity at other supraspinal sites (solitary tract, and its adjacent areas, locus coeruleus and the ventral tegmental area) is probably related to undesirable potential side effects (table 4; Dickenson 1991).

Table 4: Side effects of opioids acting at different receptors

Receptor type	Side Effect
μ	Euphoria, sedation, respiratory depression, nausea and vomiting, and addiction
δ	Respiratory depression, bradycardia, ileus, Urine retention and hypothermia
κ	Sedation, dizziness, miosis and hallucinations

2.4.3.2 Tramadol

Tramadol is a synthetic, centrally acting atypical opioid analgesic with a low incidence of side effects including minimal respiratory depression and negligible addiction potential

(Radbruch et al. 1996). It is an aminocyclohexanol derivative with clinically effective analgesia in people (Bamigbade & Langford 1998).

2.4.3.2.1 Mechanism of action

Tramadol has two distinct, synergistic mechanisms of action. It showed a weaker binding affinity for μ -opioid receptors than morphine, but had a 10 fold more affinity to μ -receptors than κ - & δ - opioid receptors in rat *in vitro* studies (Hennies et al. 1988). Mean median inhibition concentration (IC_{50}) values for tramadol and morphine in displacing μ -receptor binding of radioactive ligands in rat brain membranes *in vitro* were 1.7×10^{-6} M and 4.6×10^{-9} M, respectively. Further, in animal and human models naloxone, a μ -receptor antagonist only partly reversed or blocked the tramadol induced antinociception even at higher doses (Carlson & Jurna 1987; Collart et al. 1993), indicating that some of the antinociceptive effects of tramadol are not mediated by the opioid receptors alone.

Later, Driessen et al. (1993) investigated the non-opioid component of tramadol analgesia in two different studies using rat brain and demonstrated that tramadol inhibited the synaptosomal re-uptake of noradrenaline (NA) and 5-hydroxytryptamine (5-HT) by central neurones (Driessen & Reimann 1992; Driessen et al. 1993). Both NA and 5-HT are monoamine neurotransmitters involved in the inhibition of nociception by descending inhibitory mechanisms (Millan 2002). Further evidence for monoamine reuptake inhibition by tramadol was provided by Raffa et al (1992). They found that both yohimbine (α_2 -adrenoceptor blocker) and ritanserine (selective antagonist at 5-HT₂ receptors) significantly reduced the antinociceptive action of intrathecally administered tramadol in the rat-tail flick test.

Tramadol appears to be a racemic mixture of (+) and (-) enantiomers and stereospecific as the (-) enantiomer is five- to ten-fold more potent than the (+) enantiomer in inhibiting noradrenaline uptake, whereas the (+) enantiomer is about four fold more potent than the (-) enantiomer for inhibition of serotonin (Raffa et al. 1993; Driessen & Reimann 1992; Driessen et al. 1993). Both enantiomers act synergistically in producing analgesia (Raffa et al. 1993).

In another series of experiments on rat brain *in vitro* (particularly on noradrenergic neurones of locus coeruleus) Sevcik et al. (1993) demonstrated that the analgesic effect of (-) tramadol

was abolished by α_2 -adrenoceptor antagonist rauwolscine only whereas (+) tramadol induced antinociceptive effects were blocked by combination of naloxone and rauwolscine.

In clinically relevant concentrations both racemic tramadol and its (+) - enantiomer blocked the 5-HT uptake by dorsal raphe neurones in rat brain slices (Bamigbade et al. 1997). Further, in human clinical studies, the analgesic effect of a single oral dose (100 mg) tramadol was significantly reduced by a combination of yohimbine and naloxone rather than by yohimbine alone (Desmeules et al. 1996).

Taken together, it appears that tramadol acts by a weak μ -opioid binding and inhibition of 5-HT and NA re-uptake by central neurones, with significant synergism between the two different mechanisms. Also, it is evident that tramadol exists as a racemate with (+) - and (-) - enantiomers, each of which selectively inhibits the re-uptake of 5-HT and NA respectively, by central neurones. It is the (+) - enantiomer, which is responsible for μ -opioid agonism by the racemate.

2.4.3.2.2 Pharmacokinetics of tramadol in dogs

Kukanich & Papich (2004) have investigated the pharmacokinetic properties of tramadol following oral ($11.2 \pm 2 \text{ mg kg}^{-1}$) and intravenous (4.4 mg kg^{-1}) administration to six healthy beagle dogs (table 5).

Table 5: Pharmacokinetics of tramadol following oral and intravenous administration in dogs

Pharmacokinetic property	Route of administration	
	Oral	Intravenous
Bioavailability (%)	65±38	-
Volume of distribution at steady state(L/kg)	-	3.79±0.93
Peak plasma concentration (ng/ml)	1402±695	-
Time to reach peak plasma concentration (hrs)	1.04±0.57	-
Terminal elimination half life ($t_{1/2\beta}$) (hrs)	1.71±0.12	0.8±0.12

An extensive study on tramadol metabolism after oral administration in rats and beagle dogs was conducted by Wu et al (2001). They found:

1. five major metabolites of tramadol,
 - a. O-desmethyl tramadol (M_1),
 - b. N-desmethyl tramadol (M_2),
 - c. N, N-didesmethyl tramadol (M_3),
 - d. N, N, O- tridesmethyl tramadol (M_4), and
 - e. N, O-didesmethyl tramadol (M_5)
2. twenty four other metabolites formed through six pathways. They were O-demethylation, N-demethylation, cyclohexyl oxidation, oxidative N-dealkylation, dehydration and conjugation, and
3. renal excretion was the major route of elimination for tramadol and its metabolites.

Interestingly, from a study on rats Raffa et al (1992) reported that M_1 (O-desmethyl tramadol), the major metabolite of tramadol, showed 6-fold more analgesic efficacy and 200-fold higher affinity for μ -opioid receptors than the parent compound. Also, IV administration of M_1 (1 mg kg^{-1}) to healthy beagle dogs resulted in μ -opioid receptor specific side effects like nausea and sedation (Kukanich & Papich 2004).

Further, in a human experimental pain model Poulsen et al (1996) observed that in poor metabolisers of tramadol, the serum concentrations of (+)- M_1 were below the detection limit after 2-10 hours of tramadol administration and the analgesic levels were also lower than those in extensive metabolisers of the drug. The authors concluded that formation of (+)- M_1 depends on the genetic polymorphic cytochrome P450 2D6 (CYP2D6), which is an isoenzyme of cytochrome P-450 in the human liver, and due to reduced activity of the CYP2D6 in poor metabolisers of tramadol, M_1 concentrations were lower. In dogs, the activity of equivalent enzyme (CYP2D19) has been demonstrated to be 40% of that in people (Chauret et al. 1997). Consequently, formation of M_1 from the parent compound has been reported to be lower in dogs (Wu et al. 2001; Kukanich & Papich 2004).

In summary, it appears that tramadol undergoes an extensive first pass hepatic metabolism and O-desmethylation of tramadol (an important metabolic path way producing M_1) is dependent on the cytochrome P450 enzyme complex in the liver, in humans. The proportion of M_1 formation in dogs appears to be lower than that in people with functional cytochrome P450 enzyme system.

2.4.3.2.3 Postoperative analgesia

In a randomised blinded preoperative clinical trial on dogs undergoing ovariohysterectomy, IV tramadol (2 mg kg⁻¹) provided an equal degree of analgesia with IV morphine (0.2 mg kg⁻¹) and there was no significant difference between the two groups with regard to sedation, arterial oxygen saturation, pH, cortisol levels and cardiovascular variables. The authors concluded that tramadol is as effective as morphine in dogs after ovariohysterectomy (Mastrocinque & Fantoni 2003).

Tramadol has been extensively investigated in humans for postoperative analgesia, compared to animals. Only two studies investigated the efficacy of tramadol in dogs subject to ovariohysterectomy (Mastrocinque & Fantoni 2003; Paolo et al. 2004). Analgesia with negligible side effects would make tramadol attractive in a clinical setting.

2.4.3.2.4 Toxicological studies

In a review on experimental toxicological studies on tramadol in animals, Matthiesen et al (1998) reported the following:

1. LD50 values after single oral administration in dogs were 450 mg kg⁻¹ and from 40 to 100 mg kg⁻¹ after intravenous administration,
2. The main signs of intoxication were restlessness, unsteady gait, exophthalmia, mydriasis, salivation, vomiting, tremors, convulsions, slight cyanosis and dyspnoea,
3. No carcinogenic and teratogenic effects were found on administration of tramadol in rats and rabbits, and
4. Racemic tramadol and both enantiomers were absorbed and cleared equally well in dogs.

2.4.3.3 NSAIDs

2.4.3.3.1 Peripheral anti-inflammatory action

Prostaglandins released in response to tissue trauma and inflammation act synergistically with other algescic inflammatory mediators co-released at the injured site to sensitise the nociceptors and play a predominant role in post injury pain hypersensitivity (Dray 1995). COX is the principal rate-limiting enzyme that catalyses the first step in the conversion of arachidonic acid to prostaglandins (PGs), prostacyclins (PGI₂) and thromboxane (TX) A₂. NSAIDs produce their anti-inflammatory and analgesic effects by inhibiting the activity of

COX enzymes on arachidonic acid (Vane 1971), which in turn reduce the synthesis of eicosanoids.

For many years, COX was thought to be a single enzyme and solely responsible for production of prostaglandins responsible for physiological functions and inflammatory pain (Lawson et al. 1999). Recent studies that demonstrated the gene encoding for COX brought huge modifications in basic concepts about COX (Kujubu et al. 1991; Sirois & Richards 1992; Habib et al. 1993; O'Neill & Ford-Hutchinson 1993; Feng et al. 1993). COX exists in at least three isoforms: COX-1, COX-2 and COX-3 (Jouzeau et al. 1997; Chandrasekharan et al. 2002). It is thought that COX-1 is responsible for generation of physiological PGs that maintain the gastric mucosal integrity, platelet function and auto regulation of renal blood flow (RBF), whereas induction of COX-2 is mainly associated with production of PGs responsible for inflammatory pain.

At therapeutic doses, traditional/non-selective NSAIDs produce anti-inflammation and other clinical benefits by inhibiting COX-2, but due to concurrent COX-1 inhibition side effects like gastric ulceration, platelet dysfunction and renal hypo-perfusion may occur which limit their use in the immediate perioperative period (Kam & Power 2000; Sinatra 2002). Based on this hypothesis selective COX-2 inhibitors (coxibs) were postulated to be safer than non-selective COX inhibitors in people and more suitable to be used before and after surgery (Katz 2002).

However, species differences exist in constitutive expression of COX isoforms in different body tissues. For example, COX-2 is expressed constitutively in the kidney, predominantly by macula densa in dogs and rats, and hence suggests that COX-2 mediated PG production might play a role in renal autoregulation in these species. In primates COX-2 activity in kidneys is limited and hence inhibition of COX-2 may not influence PG mediated renal haemodynamics. Difference in COX isozyme activity (in different body tissues) across species may have clinical importance.

2.4.3.3.2 Central analgesic action

A large body of evidence suggests that NSAIDs, in addition to peripheral effects of anti-inflammation and hypoalgesia, can also exert central antinociceptive effects (Breder et al. 1995; Beiche et al. 1998; Hay et al. 1997; Svensson & Yaksh 2002). Both COX-1 and COX-

2 are constitutively present in the brain and the spinal cord. Intrathecal administration of NSAIDs was shown to block the tail-flick responses induced by substance P or NMDA (Malmberg & Yaksh 1992) which indirectly stimulate the synthesis of PGs in the spinal cord, through action of COX on arachidonic acid. Recently, Chandrasekharan et al (2002) have demonstrated the activity of COX-3 in canine cerebral cortex and proposed that selective inhibition of COX-3 is the primary central mechanism through which some NSAIDs act to produce analgesia. Interestingly, systemically given NSAIDs were also demonstrated to be analgesic in healthy animals (Lizarraga & Chambers 2006). There are several mechanisms other than central PG inhibition through which NSAIDs produce central analgesic effects (Appleton 1997).

Parecoxib sodium is a water-soluble injectable pro-drug of valdecoxib, a potent and highly selective COX-2 inhibitor in human tissues (Talley et al. 2000). Studies in humans have shown that it is safe and effective when given before or after surgery (Desjardins et al. 2001; Barton et al. 2002). However, there are no reports of its use in dogs.

Perioperative use of β -blockers has been shown to reduce post-operative analgesic requirements with better control of intraoperative haemodynamics in humans (Chia et al. 2004). Pindolol is a non-selective beta-adrenoceptor blocker (Frishman 1983) and a 5-HT_{1A/1B} antagonist. Potentiation of tramadol analgesia has been shown with pindolol in mice (Corrales et al. 2000).

Morphine is the archetypal opioid analgesic, which interacts predominantly with μ and κ opioid receptors to exert its analgesic effect. It has been widely used as a gold standard for comparing the efficacy of alternative agents in analgesic studies (Brodbelt et al. 1997).

CHAPTER 3

PLASMA IOHEXOL CLEARANCE TEST, MECHANICAL THRESHOLD TESTING AND EEG MEASUREMENT

This chapter contains a review of different methods of estimating glomerular filtration rate (GFR) as a measure of renal function. The method used for assessing GFR, plasma iohexol clearance test, in dogs of study reported in chapter 4 has been reviewed and described here. Also, the methods used to evaluate effects of treatments (mechanical threshold testing and EEG recording) and drugs used in studies detailed in chapters 4, 5 and 6 are described here.

3.1 GLOMERULAR FILTRATION RATE (GFR)

The most accurate method to assess overall renal function is the determination of GFR, as it is directly proportional to the number of functional nephrons in both kidneys (DiBartola 1995). Since GFR is linearly related (within limits) to the renal blood flow, factors affecting renal blood flow such as changes in the cardiac output and blood pressure, neurohumoral regulatory elements and drugs will in turn alter the GFR (Greene 1996). Therefore an estimate of GFR is considered as a reliable indicator of renal perfusion both in man and animals (Albert et al. 2003).

There are several different methods of estimating GFR. They are described below. They include renal screening tests, scintigraphy methods and clearance techniques.

3.1.1 Screening tests

Tests commonly used for screening the renal function are estimation of blood urea nitrogen and serum creatinine concentration.

3.1.1.1 Blood urea nitrogen (BUN)

Urea is synthesised in the liver as a breakdown product of proteins and is freely filtered through the glomerular basement membrane. Reagent test strips that provide a rapid estimate of BUN concentration are popular in veterinary practice (Berent et al. 2005) The reagent strips contain urease (an enzyme that catalyses the hydrolysis of urea in blood to carbon dioxide and ammonium hydroxide (NH₄OH)) and bromothymol blue. An alkaline shift in pH caused by NH₄OH is detected by bromothymol blue as a change in colour of the strip. The colour of the reagent strip is compared with a colour in the colour chart (provided by

manufacturers with each bottle of colour strips) that has quantitative categories of BUN concentration (mg dL^{-1}) for each colour. Alternatively, serum urea concentration can be detected with automated wet chemistry analysers.

Advantages:

1. Ease of measuring in a routine clinical practice
2. Good reproducibility and low cost

Disadvantages:

1. It not an early diagnostic measure of renal function since BUN concentration only begins to rise when 75% of total nephron population is nonfunctional (DiBartola 2000).
2. Increase or decrease in BUN concentrations are affected by several extrarenal factors like fever, massive muscle trauma or corticosteroid administration (that causes increased protein catabolism) and hepatic insufficiency.

3.1.1.2 Serum creatinine concentration

Creatinine, which is the breakdown product of creatine phosphate and creatin in muscle and food, is eliminated solely through glomerular filtration in the dog and cat (Braun et al. 2003). It provides an indirect measure of GFR. Serum creatinine concentration has traditionally been measured by the alkaline picrate reaction (Jaffe's reaction). Picrate ions cause formation of a yellow-orange chromogen by acting on creatinine at alkaline pH. Colorimetric methods are used to determine creatinine concentration from the chromogens. Alternatively, serum creatinine concentration can be measured by specific enzymatic reactions.

Advantage:

1. Ease of measuring in a routine clinical practice

Disadvantage:

1. Similar to BUN, it not an early diagnostic measure of renal function since serum creatinine concentrations only begin to rise when 75% of total nephron population is nonfunctional (DiBartola 2000).
2. Non-creatinine chromogens (bilirubin, lipids, actoacetate etc.), variation in individual size and muscle mass, food intake and degradation by intestinal bacteria can interfere with serum creatinine concentrations
3. Measurement by the Jaffe reaction can overestimate creatinine measurement by as much as 45% in healthy dogs (Braun et al. 2003).

Therefore, the relationships between changes in BUN and serum creatinine concentrations and GFR are not linear (Ross 1986).

3.1.2 Renal scintigraphy

This method of evaluation of renal function is comparable to standard methods of GFR estimation (Kerl & Cook 2005). Krawiec et al (1986) have used this method in dogs. Before injecting the radio isotope ($^{99\text{M}}$ -Tc-DTPA) to a dog, a precount of the isotope in the syringe is determined with the syringe positioned above the centre of the gamma camera. The dog is positioned in lateral recumbency with the camera positioned dorsally over the cranial abdomen in contact with the spine. The image acquisition starts with the simultaneous intravenous injection of the isotope. Images are acquired for six minutes after isotope injection. After image acquisition, the syringe and injection port are again positioned above the centre of the gamma camera to determine the postcount. The precount minus the postcount gives the injected dose. The GFR is calculated from the net kidney count of the isotope (from the images) and the injected dose.

Advantages:

1. Non-invasive, accurate and reproducible method of GFR estimation
2. Does not require blood or urine collection
3. This method of GFR estimation can evaluate the individual kidney function

Disadvantages:

1. Need for specialised licensing and equipment
2. Following test animal should be isolated for 24 hours (Kerl & Cook 2005).

3.1.3 Clearance techniques

The renal clearance of a substance is that volume of plasma containing the amount of substance that is removed by the renal filtration in unit time (Rang 2003). Therefore the renal clearance of a substance that is neither reabsorbed nor secreted by renal tubules is equal to GFR. The substance can be either naturally occurring (e.g. creatinine), or exogenously administered (e.g. inulin, radioisotopes, radiographic contrast agents). As nearly 50 % of urea nitrogen filtered at the glomerulus is reabsorbed in the renal tubules, clearance of urea nitrogen cannot be used for estimating GFR (Kerl & Cook 2005). The two most commonly used substances to estimate GFR using clearance techniques are inulin and creatinine.

3.1.3.1 Endogenous creatinine clearance

As creatinine is excreted by glomerular filtration without marked tubular reabsorption or secretion, its clearance can be used to estimate GFR (Ross 1986). All urine is removed from the bladder through a catheter and the bladder should be rinsed with sterile saline. Dogs are kept in metabolic cages and all urine produced during a 24-hour period is collected in the cage and through the catheter in the bladder. The volume of urine is measured and recorded. A blood sample is collected nearly 12 hours into the test. Serum and urinary creatinine concentrations can be measured by the Jaffe method. The following formula is used to estimate the endogenous creatinine clearance:

$$C_{\text{creat}} = \frac{U_{\text{creat}} \times V}{SC \times T \times BW}$$

Where, C_{creat} = Creatinine clearance; $\text{mL min}^{-1}\text{kg}^{-1}$

U_{creat} = Urine creatinine concentration; mg dL^{-1}

V = Urine volume; mL SC = Serum creatinine concentration; mg dL^{-1}

T = Time of collection period; minutes

BW = Body weight; kg

Advantages:

1. No need of constant infusion into the animal as it is normally produced in the body.
2. No need for special equipment
3. Easy mathematical calculation
4. A reliable estimate of GFR.

Disadvantages:

1. Difficulty in collecting urine for long periods (24-hours) accurately
2. Metabolic cages used for accurate collection of urine may not be available in most clinical practices
3. Possibility of ascending infection due to indwelling urinary catheters
4. Slightly overestimates GFR of male dogs because of the proximal tubular secretory mechanism, and underestimates GFR in cats
5. Also, slightly overestimates GFR compared to that measured by inulin clearance, as determination of SC by Jaffe method includes non-creatinine chromogens but U_{creat} does not (DiBartola 1995)

To overcome this inaccuracy caused by non-creatinine chromogens, exogenous creatinine clearance test has been developed for GFR estimation.

3.1.3.2 Clearance of exogenous creatinine

In this method, plasma creatinine concentrations are increased by injecting creatinine and high concentrations are maintained during the test procedure to reduce the relative effect of non-creatinine chromogens on GFR estimation (DiBartola 1995). A bolus of sterile creatinine solution is injected intravenously in dogs. During the urinary clearance procedures plasma creatinine concentrations are maintained by injecting creatinine-saline solution at a rate of 0.5 mL/min with a constant infusion pump. The urinary bladder is catheterised and flushed with sterile saline before urine collection at 150 minutes (after bolus injection of creatinine). Urine is collected at 30-minute intervals until 240 minutes after injection of creatinine with the bladder rinsed three times (with saline) just before the end of each collection period. The quantity of urine added with rinses will be measured and recorded. Blood samples will be collected from the jugular vein at the same time points as the urine collection. The concentration of creatinine in urine and blood samples will be determined with the Jaffe reaction (Finco et al. 2001).

The standard formula for urinary clearance of exogenous creatinine is:

$$C = (U_v \times U_c) / P_c, \text{ where } U_v = \text{Urine volume}; U_c = \text{Urine creatinine concentration}; \\ P_c = \text{Plasma creatinine concentration.}$$

Advantages:

1. A sensitive and accurate measure of GFR
2. GFR correlates well with that estimated by inulin clearance, which is considered as 'gold standard' for GFR determination
3. Easy mathematical calculation

Disadvantages:

1. Difficult to obtain sterile creatinine commercially
2. Difficulty of collecting urine for long periods accurately
3. Labour intensive as it requires continuous intravenous infusion of the substance
4. Complicated laboratory analysis

3.1.3.3 Inulin clearance

It is considered as the 'gold standard' for measuring GFR and for comparing the GFR estimated by all other methods (Finco et al. 1991). The method for determining inulin clearance is similar to that described for exogenous creatinine clearance. The concentration of

inulin in urine and plasma is determined by a modified anthrone method (Robinson et al. 1974). Other methods have used radiolabelled inulin (^{14}C -inulin) administered by constant infusion (Finco et al. 1991) or bolus injection (Fettman et al. 1985). The concentration of inulin (^{14}C -inulin) carboxylic acid in plasma and urine is determined by liquid scintillation counting. Though these methods of inulin clearance yield accurate estimate of GFR they are clinically impractical. Recently, plasma clearance of inulin following a single intravenous injection has been used to determine GFR in dogs and cats (Haller et al. 1998). Blood samples are drawn at three, 20, 40, 80 and 120 minutes after inulin injection. Inulin concentration in plasma is determined by acid hydrolysis followed by a modified enzymatic assay commercially available in a test kit (Haller et al. 1998). With this method, inulin concentrations between 5 and 2000 mg L⁻¹ can be measured. Clearance is calculated using dose / area under the curve (AUC) of plasma inulin disappearance. The AUC is calculated using different pharmacokinetic modelling strategies, which are described latter in this chapter.

Advantage:

1. Standard and accurate method of GFR estimation

Disadvantages:

1. Inulin is not available for routine clinical use. Only available as a chemical grade compound in some countries
2. Expensive
3. Difficulty of collecting urine for long periods accurately (with traditional continuous infusion techniques)
3. Labour intensive as one of the methods requires continuous intravenous infusion of the substance
4. Complicated assays and need for specialised equipment for measuring radiolabelled inulin.

3.1.3.4 Plasma clearance of radioactive markers

The plasma disappearance of radiolabelled compounds excreted through renal filtration, following a single intravenous injection yields good estimate of GFR (Moe & Heiene 1995). Radioisotope technetium-diethylenetriaminepentaacetic acid ($^{99\text{M}}$ -Tc-DTPA) is injected intravenously and blood samples are drawn immediately before the injection and five, 15, 30, 60, 120, 180, 240, 300 and 360 minutes after the injection. The plasma radioactivity of the

injected compound (counts mL⁻¹) is determined by gamma counting in a scintillation counter. Plasma clearance of the ^{99m}Tc-DTPA is calculated using dose / AUC of plasma disappearance. The AUC is calculated using different pharmacokinetic modelling strategies.

Advantages:

1. No need of collecting urine for long periods
2. Provide reliable estimate of GFR

Disadvantages:

1. Radioisotopes are expensive
2. Inherent restrictions associated with the handling and processing of radioactive samples
3. Need for a certified nuclear medicine laboratory
4. Clinically impractical method.

3.1.3.5 Plasma iohexol clearance test

More recently, assessment of GFR from plasma clearance of a non-radioactive filtration marker, iohexol, following a single intravenous injection has gained wide acceptance, both in humans and domestic animals with normal to reduced renal function (Krutzen et al. 1984; Nilsson-Ehle 2003; Moe & Heiene 1995; Brown et al. 1996; Nesje et al. 1997; Laroute et al. 1999; Finco et al. 2001). Iohexol is a non-ionic, low-osmolar (780 mOsm/kg water) iodinated radiographic contrast medium frequently used for urography, angiography, myelography, arteriography and in contrast enhancement for computerized tomography. Iohexol satisfies all of the characteristics necessary of a filtration marker in dogs (Mützel & Speck 1980). The characteristics (DiBartola 2000) of an ideal filtration marker are:

1. Freely filtered at the glomerulus
2. Not bound to plasma proteins
3. Not metabolised
4. Non-toxic
5. Excreted only by the kidneys
6. Neither reabsorbed nor secreted by the renal tubules
7. Stable in blood and easily measured

Moreover, iohexol obviates most of the problems associated with standard clearance media (creatinine, inulin, Cr-EDTA etc), while yielding results for GFR similar and comparable to those obtained by the latter (Shihabi et al. 1993). Results from plasma iohexol clearance studies in normal dogs and those with different degrees of renal dysfunction indicate that the technique is quite suitable and valid for estimating GFR and the agent is safe in this species (Moe & Heiene 1995; Gleadhill & Michelle 1996; Heiene & Moe 1999; Finco et al. 2001). The dose of iohexol varied with level of renal function and ranged from 150 to 600 mg I / kg in dogs with reduced to normal renal function respectively (Gleadhill & Michell 1996; Brown et al. 1996; Finco et al. 2001).

In two clearance studies in dogs (n = 18, n =50) with different degrees of renal impairment a dose of iohexol up to 600 mg I / kg did not produce any adverse reaction and was well tolerated, indicating a wide safety margin of the marker (Moe & Heiene 1995; Heiene & Moe 1999). These doses are routinely used in intravenous urography without any adverse reactions. In addition, studies in humans and dogs that evaluated the validity of iohexol method with other standard procedures of estimating GFR showed that intravenous administration of iohexol simultaneously with other standard clearance media ($^{99\text{M}}\text{Tc-DTPA}$, inulin, exogenous creatinine etc.) did not generate any toxic effects (Brown & Reilly 1991; Moe & Heiene 1995; Finco et al. 2001), indicating the compatibility of iohexol with other media in different doses and modes of administration.

In dogs with normal renal function, iohexol (600 mg I kg⁻¹) markedly increased the effective renal plasma flow (ERPF) and GFR assessed by renal clearance of p-aminohippurate and inulin respectively. This renal vasodilation was transient. In dogs with experimentally induced renal insufficiency iohexol caused a rapid decrease in ERPF and GFR at a higher dose (900 mg I kg⁻¹). However, low doses of iohexol (300 mg I kg⁻¹) did not produce marked changes in renal perfusion (Arakawa et al. 1996). Several experiments in domestic animals have been demonstrated that iohexol was a safe and potential marker to assess GFR (Heiene 1995; Gleadhill & Michell 1996; Brown et al.1996; Heiene & Moe 1998; Finco et al.2001). Also, the technique is minimally invasive, simple and inexpensive, when compared to other standard clearance procedures (Rocco et al. 1996).

3.1.3.5.1 Calculation of plasma iohexol clearance (ICL) in dogs

In general, plasma clearance of injected filtration marker can be calculated by using the following formula

$$\text{CL (plasma)} = \frac{\text{Injected dose of the marker (Dose)}}{\text{Area under the plasma concentration versus time curve (AUC)}}$$

The exponential formulae that are specific to different pharmacokinetic models describe the shape of the plasma disappearance curve of the contrast agent. The area under this curve (AUC) is calculated using integration or the trapezoidal rule.

The single injection method to estimate plasma clearance largely depends on distribution and elimination of the marker from extra-cellular fluid (Brown et al. 1996). After intravenous injection, iohexol is rapidly distributed exclusively in the extra-cellular space, followed by an exponential decline (first order kinetics) in the terminal elimination phase in healthy dogs (Mützel & Speck 1980). In a study on 52 dogs with normal to slightly reduced renal function, Heiene & Moe (1999) reported that the marker had reached the terminal mono-exponential slope at 2 hours (range 0.1- 4 hours), indicating the distribution of the marker had been finished within 2 hours, after intravenous injection of the marker and most of the marker was eliminated within 4-6 hours. However, in dogs with severe renal dysfunction, most of the injected marker remained in the body beyond 6 hours.

One-compartment model: Clinically, a one-compartment model for calculation of AUC appears to be simple and time saving. In this model, the body is viewed as a single compartment (plasma) into which the marker is injected and excreted from only that compartment (Frennby & Sterner 2002). Since the model utilises the slope from the terminal elimination phase of the marker, only two or three samples are required, allowing the process to be completed in three or four hours (Gleadhill & Michell 1995; Heiene & Moe 1998; 1999). Sampling before two hrs after intravenous injection of the marker is usually not done in this model as the terminal mono-exponential slope is not reached within two hours both in dogs and humans with normal to slightly reduced renal function (Heiene & Moe 1999; Frennby & Sterner 2002).

Sampling before two hours i.e. before reaching the terminal elimination phase (incomplete distribution) of the marker results in a steeper mono-exponential slope than the original

(Heiene & Moe 1998). When GFR is assumed to be normal, the period of sampling may end four hours after injection of the marker predicting elimination of most of the marker within four to six hours (Frennby & Sterner 2002). However, in patients with severely reduced renal function (when GFR is predicted to be too low) the marker may still be present in the body beyond six hours (slow elimination), necessitating sampling up to 24-48 hours or some times up to 72 hours. In this case, sampling should be started late, may be from three to four hours after injection of the marker owing to incomplete distribution at two hours (Nilsson-Ehle & Grubb 1994; Nilsson- Ehle 2003). In this connection, it is interesting to note that a study (Brändström et al. 1998) on 50 patients with varying degrees of renal impairment showed a strong correlation between Cr-EDTA and iohexol (HPLC) clearance measurements at three-point sample collection (150, 195 and 240 minutes after injection), the former being the reference technique.

The theoretical expression (Heiene & Moe 1998) of mono-exponential function corresponding to a one-compartment model is

$$C_t = C_o \times e^{-kt}, \text{ where}$$

C_t is the plasma concentration of the marker at time t and $-k$ is the terminal elimination rate constant of the marker, given by slope of the straight line. C_o is the plasma concentration of the marker at time zero. Also, AUC in a one-compartmental model is given by:

$$AUC_{1c} = C_o / k$$

In practice, C_o is calculated theoretically by extrapolation of the straight line (line of best fit) plotted C_t against 't' with slope $-k$ (Rang 2003), from time 't' to '0' (Finco et al. 2001). A minimum of two samples is required in this method to obtain a linear regression. However, the clearance value produced by a one-compartment model (CL_1) may overestimate GFR by up to 20-30% because theoretically calculated initial concentration (C_o) may not truly estimate the exact AUC (underestimated AUC), indicating immediate non-mixing of the marker in the distribution phase (Nesje et al. 1997). To correct this clinically relevant error correction formulae have been introduced into both human and animal pharmacokinetics.

From a study on 74 patients with various renal diseases, Brøchner-Mortensen (1972) found that plasma clearance of $^{51}\text{Cr-EDTA}$ estimated by CL_1 always exceeded the total plasma clearance and to correct this error he developed the following equation by least-squares regression analysis.

$$\text{Corrected clearance (CL)} = 0.990778 CL_1 - 0.001218 CL_1^2$$

Clearance studies conducted in humans and animals have demonstrated the feasibility of this correction formula under varied approaches. Plasma iohexol clearance calculated by one-compartment model (corrected with the Brochner- Mortensen formula) showed an excellent correlation with renal clearance of inulin (best indicator of GFR) in 41 patients (Gaspari et al. 1995). Also, in dogs (n=8) and cats (n=10) with normal and reduced renal function Brown et al (1996) found a good correlation between ICL values by one-compartment method (corrected with the Brochner- Mortensen formula) and urinary clearance values obtained by exogenously administered creatinine (widely accepted method for the measurement of GFR in cats and dogs). In dogs (n =24) with known or suspected renal insufficiency Gleadhill & Michell (1996) checked the validity of this correction factor using Tc-DTPA and iohexol as filtration markers and reported that the correction factor was adequate to get absolute clearance values from a one-compartment model that uses the blood samples taken two-four hours after the injection of filtration marker.

Heiene & Moe (1999) established correction formulas for prediction of clearance values by the trapezoidal method (CL_{tr}) from the CL_1 values taking CL_{tr} as the reference method, in dogs. CL_{tr} could be predicted accurately from two samples (at two and three or two and four or two, three, and four hours) after injection of the marker by using the following equation.

$$CL_{tr} = 4.52 + 0.84 CL_1 - 0.00084 (CL_1)^2$$

Two-compartment model: A two-compartment model launches an additional peripheral compartment corresponding to extra vascular space (tissue) in addition to the central plasma compartment. This biphasic model estimates the AUC by resolving the curve into two straight lines, of which the initial steep line represents plasma concentration of the marker in the distribution phase (fast phase) and the second (slow phase) exponential component, the terminal straight line signifies the concentration in the elimination phase (Heiene & Moe 1998; Rang 2003). The bi-exponential equation specific to the model (Saperstein et al. 1955; Nesje et al. 1997) is,

$$C_t = A X e^{-k_1 t} + B X e^{-k_2 t}$$

Also, AUC in 2-compartment model is given as

$$AUC_{2c} = A / k_1 + B / k_2, \text{ where}$$

A = concentration at the y intercept of the fast component at zero time

B = concentration at the y intercept of the slow component at time zero

k_1 and k_2 are the slopes (rate constants) of both components

Though this model directly represents the real kinetics of the marker in the body (Rang 2003), its use has been very limited in clinical practice due to,

1. Need for very early, frequent and late sampling i.e. starting from less than five minutes up to 600 minutes at different time points and collection of multiple samples after injection of the marker (Gaspari et al. 1995; Haller 1998), making the procedure cumbersome.
2. Disorders with body fluid volume some times make separation of the curve difficult (Hall et al. 1977).

Alternatively, clearance by two-compartment model (CL_{2c}) can be estimated from CL_1 using the following regression equation.

$$CL_{2c} = 0.57 + 0.75 \times CL_1 \text{ (Heiene 1995)}$$

Three-compartment model: A three-compartment model calculates AUC by separating the curve into three straight lines according to the tri-exponential equation,

$$C_{pt} = C_1 \times e^{-\lambda_1 t} + C_2 \times e^{-\lambda_2 t} + C_z \times e^{-\lambda_z t}, \text{ where}$$

$$\text{AUC in three-compartment model, } AUC_{3c} = C_1/\lambda_1 + C_2/\lambda_2 + C_z/\lambda_z$$

But a three-compartment model and models that include more than three-compartments (multi-compartment) are not usually used in routine clinical practice because of the need for increased number of blood sampling, though the results from these models are highly accurate (Heiene & Moe 1998).

Non-compartmental models: These calculate AUC by dividing the curve (plotted using actual plasma concentration of the marker at different time points) into different trapezoids and adding the area of each trapezoid (Rowland & Tozer 1995), or by numerical integration of the curve. Within the trapezoidal method AUC from $C_{p_{t(i)}}$ to $C_{p_{t(\text{last})}}$ can be calculated by using simple trapezoidal rule ($\frac{1}{2} (a+b) h$), where $C_{p_{t(i)}}$ and $C_{p_{t(\text{last})}}$ are the plasma concentrations of the marker from initial sampling point to last time segment. After a rapid intravenous injection, C_{p_0} (Plasma concentration at zero time) can be calculated by simple extrapolation. Then the AUC from t_{last} to infinity can be estimated by extrapolation of the line of best fit assuming plasma disappearance of the filtration marker is mono-exponential in the terminal phase. Thus the total AUC can be calculated as,

$$AUC_{0-\infty} = AUC_{0-t_1} + AUC_{t_1-t_{\text{last}}} + AUC_{t_{\text{last}}-\infty}, \text{ where}$$

$$AUC_{0-t_1} = \frac{C_0 + C_{p_{t(i)}}}{2} \times t_1$$

$$AUC_{1-last} = \frac{C_{pt(i)} + C_{pt(2)}}{2} \times (t_2 - t_1) + \frac{C_{pt(2)} + C_{pt(3)}}{2} \times (t_3 - t_2)$$

$$AUC_{last-\alpha} = \frac{\dots\dots\dots + C_{last}}{K_{el}}$$

The non-compartment model appears more comfortable and precise, as it does not split the body in to different compartments, but demands frequent and late sampling for AUC estimation (Heiene 1995).

When compared to other compartment models described to calculate AUC for ICL, a one-compartment model appears simpler, time saving and feasible to use in clinical and research studies, where reducing blood sampling reduces the ethical cost. In addition, correlation of CL_1 values with standard renal clearance techniques makes it more attractive and reliable to use in research trials. Therefore, we decided to use a one-compartment model for calculation of the AUC from two samples collected at two and four hours after single IV injection of iohexol to dogs of the study reported in chapter 4.

3.1.3.5.2 Analysis methods

Several analytical methods have been developed for determination of iohexol iodine concentration in serum or plasma samples. Iohexol is quite stable in plasma or serum samples that can be stored at $-80^{\circ}C$ indefinitely (Braselton et al. 1997; Heiene & Moe 1998; Finco et al. 2001). Iohexol in plasma or serum can be analysed by different methods:

Chemical method (ceric-arsenite reaction): This method of plasma iohexol quantitation was described by Back et al. (1988). Hydrolysis of iohexol by an alkali separates its iodine (I) content and the quantity of liberated iodine was measured by colorimetric method using ceric ammonium sulphate.

Inductively coupled plasma-atomic emission spectroscopy (ICP-AEC): This method measures serum iodine concentration of iohexol at 178.276 nm of the polychromator in plasma-atomic emission spectrometer (Braselton et al. 1997), after deproteinisation of the sample with trichloroacetic acid, hydroxylamine sulphate and hydrochloric acid. The quantification limit for I in the serum sample with this method was $15mg L^{-1}$.

High-performance liquid chromatography (HPLC): With this method, concentration of iohexol I is detected by injection of the diluted serum sample onto a cartridge column of the HPLC system. This column separates iohexol into two isomers in about 4 minutes with ultraviolet detection at 254nm. Peak area from one of the isomers will be used for calculating quantity of iohexol I in serum (Shihabi et al. 1993). The quantification limit for I in the serum sample with this method was 10mg L⁻¹.

Capillary electrophoresis: This is another non-radio-active method that measures iohexol I quantity in the serum sample deproteinised with acetonitrile. The supernatant from the deproteinised serum sample is injected into the capillary and electrophoresed for 2.6 minutes at 12 kV (Jenkins et al. 2000). Peak height or area in capillary electropherogram is used to calculate the amount of iohexol I in serum sample. The limit of detection of iohexol was 10 mg L⁻¹.

X-ray fluorescence: This method uses Americium (²⁴¹Am), a radioisotope which releases high energy photons to bombard the plasma sample. The iodine atoms in iohexol become excited by bombarding photons and emit a characteristic X-radiation. This X-ray fluorescence is proportional to the number of iodine atoms present in the plasma sample that in turn is the measure of iodine concentration (Brändström et al. 1998). The limit of quantification with this method was 0.06 mg iodine per mL. However, each method has its own merits and demerits (table 6).

Of all the methods, HPLC appears to be clinically flexible and highly sensitive and can detect the marker administered at lower doses and from very small samples, which is really useful in small animal clinical trials (Gonda 2002). Detection limit with HPLC was found to be as low as 1 µg mL⁻¹ (Krutzen et al. 1984). The repeatability of a method for GFR estimation reflects its precision in determining changes in renal function (Jenkins et al. 2000). In a study on dogs with normal and reduced renal function Finco et al (2001) demonstrated the repeatability of GFR values by HPLC method with those by ICP-AEC and chemical methods. Also, a large number of trials that used HPLC for quantitation of the marker substance demonstrated the reliability of the method (Krutzen et al. 1984; Gaspari et al. 1995; Brändström et al. 1998; Nilsson- Ehle 2003; Laroute et al. 1999; Meucci et al. 2004).

Table 6: Advantages and disadvantages of different laboratory analytical methods of iohexol in plasma or serum sample^a

Analytical method	Advantages	Disadvantages
HPLC	Smaller samples, high precision, lower detection limits with reduced dose of the marker, excellent linearity and suitable for clinical trials	Labour intensive
Chemical method (Ceric-arsenite)	Accurate determination of concentrations	Nonlinear with the concentrations above 120mg/L, which is in the range seen in renal dysfunction, need for toxic chemicals
ICP-AEC	Smaller samples, accurate with lower detection limits	Expensive
Capillary electrophoresis	Quick and inexpensive with low detection limits	Problem with calibration and analysis of serial samples at very low or very high concentrations
X-ray fluorescence	Simple and convenient with Fast analysis, highly accurate	Expensive, use of radioisotopes, limited diagnostic applications. Needs large dose of iohexol(20 mL) and large sample volume (4-6 ml)

^a Elaborated from Shihabi et al. (1993); Rocco et al. (1996); Braselton et al. (1997); Gonda (2002)

Shihabi et al (1993) developed a simple and rapid method of analysing iohexol based on injecting diluted serum sample directly on to HPLC column without any deproteinisation. They have used an inexpensive cartridge column for chromatographic elution of iohexol. They had compared the new method to that of an acetonitrile deproteinisation technique and found a good correlation between them ($r \sim 1$). The coefficient of variation in this method was 3.1% (n=15) with a detection limit as low as 10 mg I L^{-1} , allowing the use of smaller doses of marker without any risk even in renal insufficiency (Shihabi et al. 1993; Rocco et al. 1996). Direct injection of the sample saves time and labour, which are important in clinical practice. Also, no other drug, except paracetamol interfered with chromatographic peaks of iohexol.

Because of the reported advantages and ease of use of sample without any pretreatment in HPLC method by Shihabi et al (1993), the concentration of iohexol iodine in dog plasma was

detected by HPLC with direct injection of a plasma sample onto the cartridge column, in the study reported in chapter 4.

3.1.3.5.3 Plasma iohexol analysis by HPLC

A plasma iohexol clearance test was conducted twice on each dog, before and 24 hours after analgesia and induction of anaesthesia. Pretreatment iohexol clearance test was conducted following baseline threshold testing of dogs. Dogs were given free access to water until anaesthetic induction. Iohexol (Omnipaque; Amersham Health Pty Ltd, New South Wales, Australia) 5 mL (300 mg I mL⁻¹) was injected as a single dose through a 21 SWG needle into the left cephalic vein over 30 seconds and the completion of the injection was recorded as time zero. An 18 G catheter was placed aseptically in the right cephalic vein and filled with heparinised saline. At 120 and 240 minutes after iohexol injection, 2 mL of blood was collected into a heparin tube after drawing 1-2 mL of blood mixed with heparinised saline into a separate syringe initially. Correct withdrawal times were recorded to the nearest second. Plasma was harvested by centrifugation of the blood sample at 1000 x g for 10 minutes and kept at -80°C until analysed for iohexol I.

Iohexol I concentration in the plasma sample was measured by reverse phase high-performance liquid chromatography (HPLC, Shihabi et al. 1993), using a Shimadzu HPLC system (Shimadzu Corporation; Kyoto, Japan. Photograph appended). This system consisted of a Model LC-10Advp pump, diode array ultraviolet (UV) detector (Model SPDM-10Avp) set at 254 nm and a 100µL loop auto injector (Model SIL-10 ADvp). Two columns were used. A 5 µm Luna C₁₈ 4 x 150 mm gradient column (Phenomenex New Zealand Ltd., Auckland, New Zealand) and a 3 µm C₁₈ 10 X 4.0 mm cartridge column (Mercury (LC/MS) cartridge column; Phenomenex New Zealand Ltd., Auckland, New Zealand). Shimadzu Class VP-7.3 HPLC software (Shimadzu Corporation; Kyoto, Japan) was down loaded onto a personal computer (Microsoft office, windows XP version) and used for operating the HPLC system and obtaining the chromatogram.

Initially, distilled water samples containing a known concentration of iohexol were run on C₁₈ gradient column. The mobile phase was 10 % methanol in phosphate buffer (8 mM, 0.96 gm of sodium dihydrogen phosphate in one litre of water) flowing at 1.3 mL min⁻¹. Buffer pH was 2.2. Initially, buffer was run through flow lines to equilibrate the column, until a

stable baseline appears on chromatogram window (nearly two hours). Sample was then injected through a 100 μ L sample loop by an auto injector. The two peaks of iohexol were eluted in about 4 minutes with UV detection at 254 nm. The outer surface of the autoinjector needle and sample loop line was rinsed before injecting each sample on to the column. After completion of sample running eachday, the flow line was flushed with 50 % methanol (for two hours) and kept in 50 % methanol overnight. With this method, iohexol elution with two isomers was confirmed initially.

Later, dog plasma samples with known concentration of iohexol (standards) were run through the same protocol described above. After 200-fold dilution with the elution solvent, the plasma sample was injected directly onto C₁₈ gradient column. The 4 x 150 mm gradient column caused very high operating pressures and was replaced by a 3 μ m C₁₈ 10 X 4.0 mm cartridge column for further analysis. Standard plasma samples were run with the cartridge column using the same operating procedure. Two peaks corresponding to two isomers of iohexol were eluted on a chromatogram window. Peak area under the second peak was taken. Each sample was run four times and mean \pm SE of the four peak areas of each sample were taken to built the standard graph figure 2.

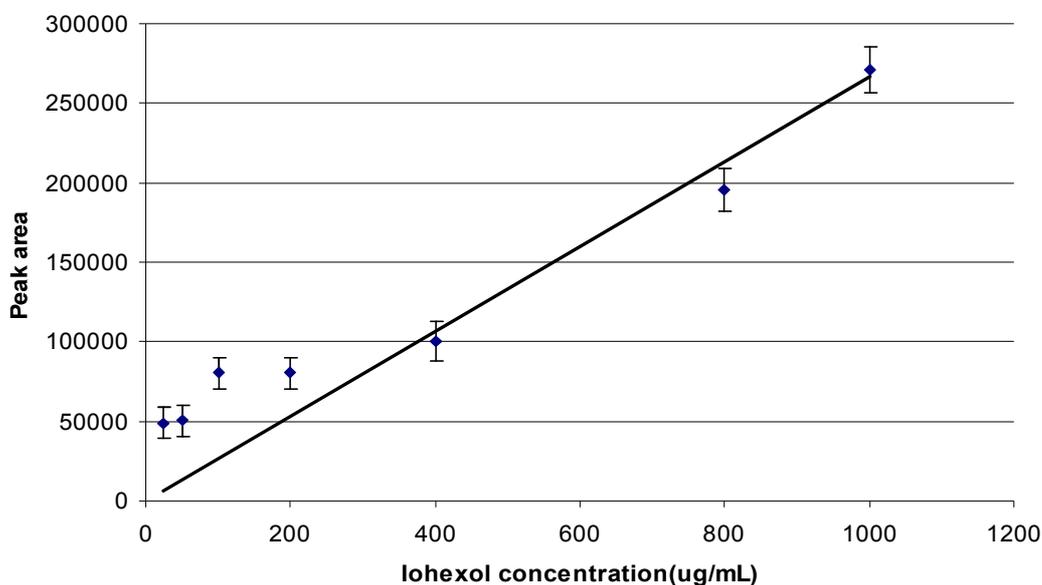


Figure 2: Graph of known concentrations of iohexol (X-axis) in dog plasma plotted against peak area (Y-axis) determined ($y = 267.5x$, $r = 0.8294$)

The concentration of iohexol in a test sample should be calculated from the regression equation of the standard graph, $y = 267.5x$ ($r = 0.8294$). However, the graph obtained is not precisely linear between different concentrations (low concentrations) of iohexol (correlation coefficient r is only 0.83) with this method. Shihabi et al (1993) reported that a 50 rather than 200- fold dilution of the sample yields adequate sensitivity. Therefore it was decided to dilute each plasma sample 50-fold rather than 200-fold with the elution solvent. Also, 8 mM phosphoric acid buffer (pH 2.2) was used instead phosphate buffer with 10 % methanol, as stated by Shihabi et al (1993). The flow rate was changed to 0.6 mL min^{-1} . Two peaks corresponding to isomers of iohexol were eluted in about three minutes with UV detection at 254 nm (figure 3). The protocol used for constructing the standard graph was appended. Area under the second peak was plotted against known concentrations of iohexol for constructing the standard graph (figure 4). The level of detection with this method was $5 \mu\text{g mL}^{-1}$.

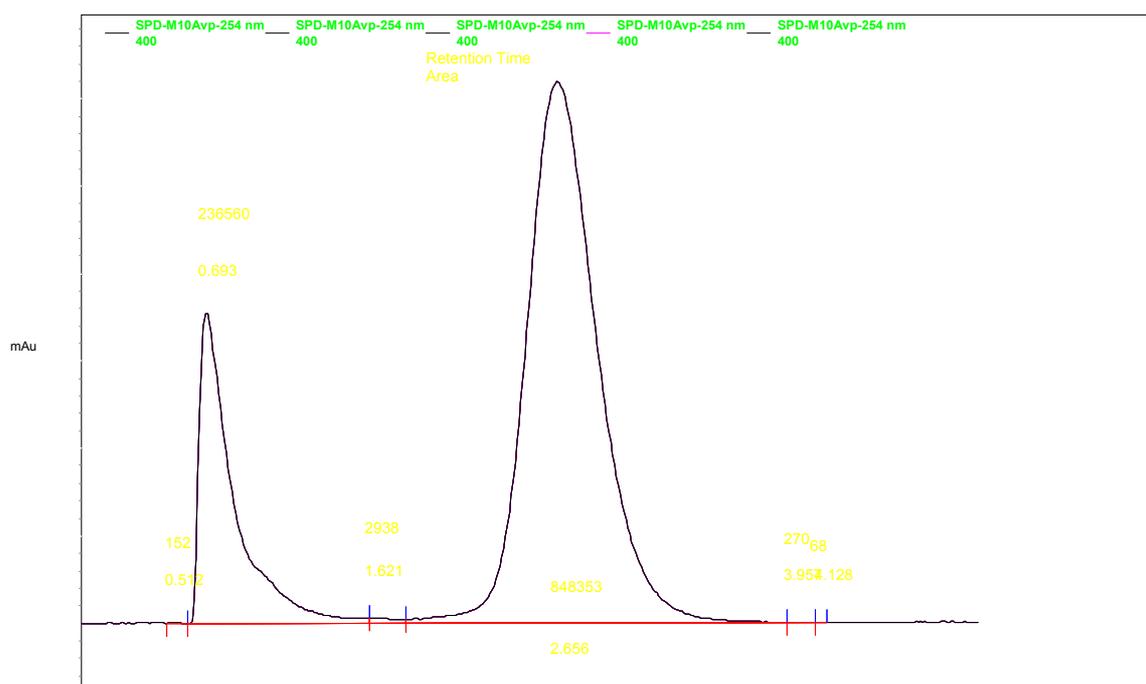


Figure 3: Chromatogram showing two peaks (eluted at 254 nm of UV light) corresponding to two isomers of iohexol

The regression equation obtained from the standard graph (figure 4), $y = 1890.5 x$ ($r = 0.9876$) was used to calculate the concentration of iohexol ($\mu\text{g mL}^{-1}$) in the test sample before and 24 hours after analgesia and induction of anaesthesia. The standard graph is linear between $5 \mu\text{g mL}^{-1}$ and $1200 \mu\text{g mL}^{-1}$ of iohexol I in dog plasma. Each standard sample and

test sample was run on HPLC for four times. Mean±SE of the four peak areas from each sample was used to build the standard graph and to calculate the concentration of iohexol I from the standard equation, respectively.

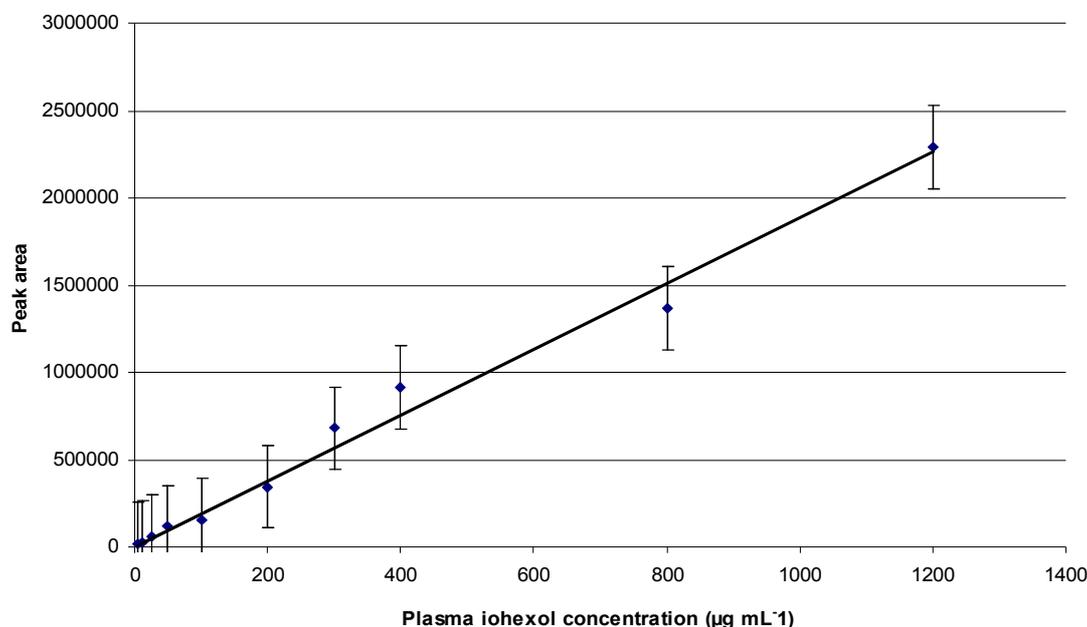


Figure 4: Standard graph of known concentrations of iohexol (X-axis) in dog plasma plotted against peak area (Y-axis) determined by final HPLC method ($y = 1890.5x$, $r = 0.9876$)

ICL was calculated from the plasma levels of iohexol at two and four hours, assuming that iohexol was distributed in a single compartment, with correction for the terminal elimination phase (Brøchner-Mortensen 1972; Heiene & Moe 1998). For example, dogs in one of the treatment groups in the current study had mean plasma iohexol concentrations of 48.05 (120 min) and 17.9 (240 min) before administration of analgesics and anaesthesia. These plasma iohexol concentrations were plotted against time (figure 5) to calculate the AUC as shown below.

$$AUC_{1c} = \text{Concentration intercept } (C_0) / \text{slope (elimination rate constant } -k),$$

$$AUC_{1c} = 311.2$$

$$CL_{1c} = \text{Dose} / AUC_{1c} \quad CL_{1c} = 4.82$$

$$\text{Corrected clearance (CL)} = 0.990778 CL_1 - 0.001218 CL_1^2$$

$$CL = 4.74 \text{ mL min}^{-1} \text{ kg}^{-1}.$$

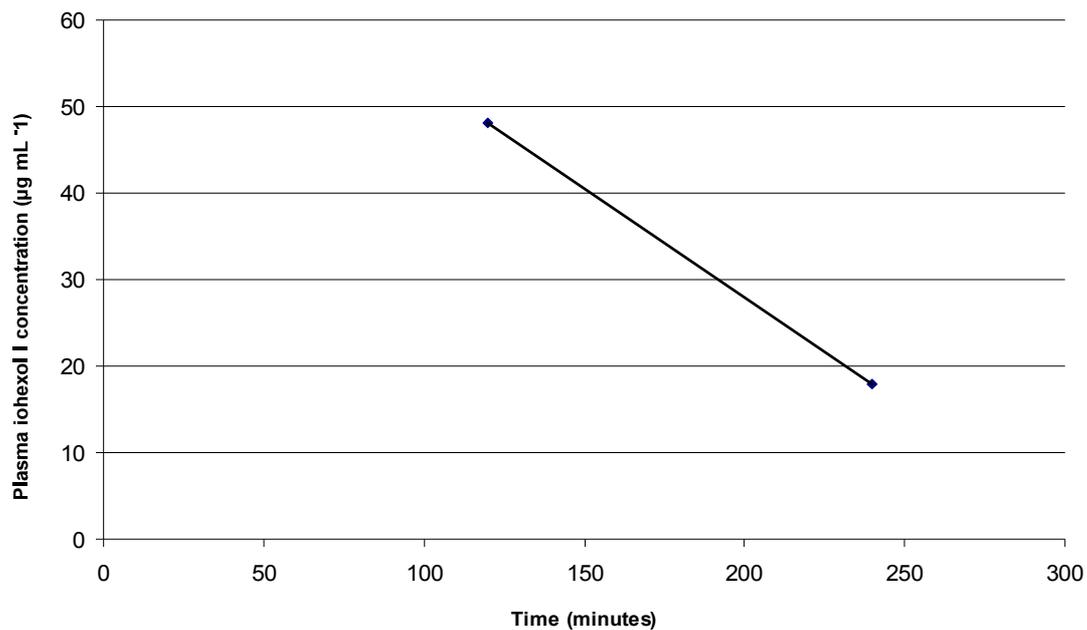


Figure 5: Plasma iohexol concentrations (Y-axis, detected by HPLC technique) plotted against time (X-axis, at 120 min and 240 min). (($y = -0.2513x + 78.2$, $r = 1$))

3.2 MECHANICAL NOCICEPTIVE THRESHOLD TESTING

3.2.1 Threshold testing device

Based on the design of the “pressure of palpation device” developed by Slingsby et al (2001), which has been proven to produce reproducible readings of threshold testing both in dogs and cats, a mechanical nociceptive threshold testing device was built with slight modifications (figure 6). It contained a 2.2mm diameter blunt ended pin with head attached to strain gauge (SG). The SG was connected by a wire to a Wheatstone bridge circuit, which was enclosed in a small fibre box. The box had a digital display, on/off switch and a reset button in the front and the device was battery operated by two nine volt batteries. When switched on an initial reading (- 0.38) appeared each time, which was subtracted from the actual measured value. The device was calibrated using an electronic balance (Model PG 5002-S delta range mercury balance, Watson Victor Ltd. Sydney, Australia). The force applied on the pin head with the thumb, pressed the pin against the plate on the balance and both the weight (g) displayed in the balance and the corresponding reading on the device were recorded. Since there was no means of holding the pressure value, the readings were noted very quickly before a sharp drop, which required help, by another person. Twelve replications were made to keep the standard error as low as possible. 100 g weight was taken as 1Newton (N) and values in Newtons were transformed into their natural log (Ln) for

calibration purpose. A linear graph was built by plotting the log-transformed Newtons against the testing device readings (figure 7). A regression equation obtained from the calibration graph was used to calculate the force from the device reading when dogs were tested.



Figure 6: Mechanical threshold testing device used to assess efficacy of test drugs in dogs

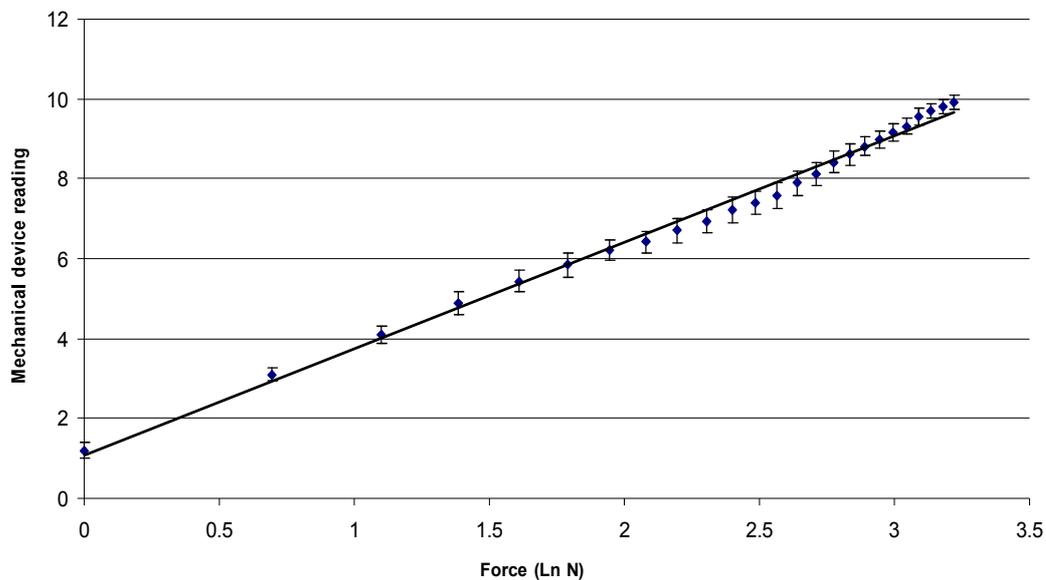


Figure 7: Mechanical nociceptive threshold testing device calibration curve. The Y-axis shows the pressure applied to generate corresponding force in Newtons ($y = 2.6658X + 1.0686$; $r = 0.9927$). The X-axis shows the force in natural log of Newtons (Ln N)

3.2.2 Threshold testing

Dogs were allowed 30 minutes after arrival for initial settlement in the new environment (laboratory). They were tested in pairs. Similar background music was played in low volume throughout the testing period with a minimum number of people around. The dogs were not muzzled during the experiment. Ambient temperature in the laboratory was monitored throughout each experiment, and was between 14⁰C and 22⁰C.

Thresholds were measured at the ventral abdomen (on the midline, half way between umbilicus and pubis) of dogs in either standing or recumbent positions. Stimulus was applied at the same site in all dogs. Baseline threshold measurements were taken four times with five minutes between each pair of recordings. The mean±SE of the four baseline values was used for statistical analysis. The behavioural responses indicating the threshold were aggressive teeth bearing, vocalisation, vigorous turning towards the stimulus or standing suddenly from a lying position (Barnhart et al. 2000). This was followed by a baseline ICL test. Following baseline ICL test, the dogs received pre-anaesthetic medication. Thirty to sixty minutes after pre-anaesthetic medication, they were given the analgesic drugs and anaesthesia was then induced and maintained for 30 minutes. Post-treatment nociceptive thresholds were measured using the same procedure as baseline threshold testing, at 20, 40 and 60 minutes after extubation. A cut-off point of 25 N prevented tissue damage, if any dog did not respond to the stimulus and this value was recorded.

The device was portable, had prompt control over stimulus, easy to operate, and did not require any attachment to the test site. Threshold measurement by single investigator enhanced the repeatability and therefore, all the four baseline readings remained very close to each other in every dog, which acted as its own control. Dogs tolerated the pressure applied through the probe and produced a clear-cut avoidance (behavioural) response indicating the threshold. Initially, the device was tested with co-researchers in the laboratory and was found to produce no residual pain or tissue damage. Visual examination of the testing site in dogs revealed no signs of tissue injury or persistent skin damage.

The box enclosed the measuring circuit was portable and robust. The joint connecting the SG to the first part of the cable wire was reinforced with strong plastic sleeves, top of which was

sealed with an adhesive tape. This provided sufficient strength to handle the SG and avoided variation in readings due to flexion of the joint (Slingsby 1998).

Conversely, there were few drawbacks to the use of the device:

1. Fast fall in readings immediately after ceasing pressure application on the probe,
2. Amount of charge in the batteries influenced the reading. These findings were consistent with those of Slingsby (1998) who originally designed the device.

Nevertheless, some remedies adept to fix these problems were:

1. Positioning the device close to the testing site and careful observation of the reading during pressure increments, and
2. Changing batteries frequently and calibrating the device each time batteries changed.

3.3 EEG RECORDING



Figure 8: Position of the electrodes on an anaesthetised dog for EEG recording

The EEG was recorded during two types of noxious stimulation (surgery and electrical stimulation) of dogs in the studies reported in chapters 5 and 6. The EEG was recorded continuously during anaesthesia and surgery (chapter 5), via an amplifier and analogue to digital converter (Alert System, Medlec, Surrey, UK) and stored on a personal computer

(450/L, Dell Computer Corporation, Bracknell, Berkshire, UK). The EEG was recorded with a sample rate of 1 kHz and a pass band of 0.5-400 Hz using three 27 SWG stainless steel needle electrodes (Medelec, Radiometer, Auckland, New Zealand) placed subcutaneously. The electrodes were positioned with the non-inverting electrode over the zygomatic process of the frontal bone, the inverting electrode over the mastoid process and the ground electrode caudal to the occipital process (figure 8).

The raw EEG was analysed after completion of each experiment, using 'Spectral Analysis Program' (SAP, Chris Jordan, Northwick Park Hospital, Herts, UK). A 30 Hz digital low pass filter was applied and Fast Fourier Transformation (FFT) was carried out on each 2.048 second epoch using a 10% raised cosine window function (Welch 1967). F50, SEF and Ptot were derived from the spectra using standard techniques (figure 9).

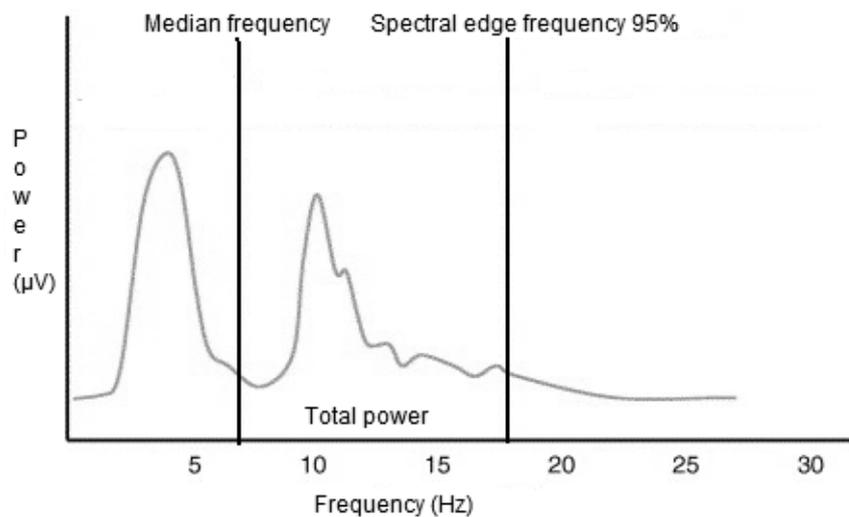


Figure 9: Schematic diagram of an EEG power spectrum

In the study reported in chapter 6, the EEG was recorded using an Apple Macintosh personal computer installed with Chart 5.2.2 recording software and connected to Powerlab 4/20 data recording system (PowerlabTM data acquisition system[®], AD Instruments Ltd, Sydney, Australia). The electrode positioning was similar to that described for dogs undergoing surgery. EEG was recorded at a sampling rate of 1 kHz with low pass filter of 0.5 kHz and high pass filter of 0.1 Hz applied using external amplifier set at a gain of 1000.

Analysis of EEG data was carried out off line after the completion of each experiment. The F50, SEF and Ptot were calculated for consecutive non-overlapping 1-second epochs, using

purpose written software (Spectral Analyser; CB Johnson, Massey University, Palmerston North, NZ, 2002). Data were multiplied using a Welch window. FFT was applied to each epoch, generating sequential power spectra with 1 Hz frequency bins. EEG data contaminated by artifact during electrical stimulus application were prevented by excluding variables derived from five to seven seconds before to five to seven seconds after electrical stimulus from analysis.

Minimal anaesthesia model: In people, it has been suggested that anaesthetics at low doses increase the EEG activity compared to high doses at which the EEG is depressed (Tonner & Bein 2006). Analysis of intraoperatively derived raw EEG signals indicates that a gradual increase in the depth of sedation / hypnosis results in a graded decrease in the EEG activity ranging from slow shift through burst suppression to isoelectric activity (figure 10; Tonner & Bein 2006). However, to date, there has been no single EEG derived parameter that solely indicates the depth of anaesthesia.

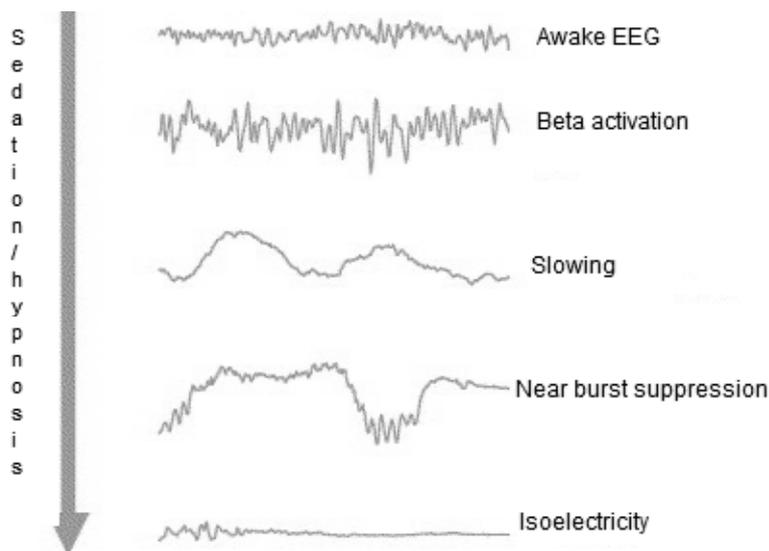


Figure 10: Alterations in the EEG activity with increasing levels of sedation/hypnosis (adapted from Tonner & Bein 2006)

In a review on neurophysiological techniques to assess pain in animals, Murrell & Johnson (2006) suggested that the level of depression of the CNS with different types of general anaesthetics can influence the generation of electrical activity from the cerebral cortex in response to noxious stimuli. Also, the association of the EEG spectral frequencies with nociception varies with the type of inhalation anaesthetic used. General anaesthetics (for example, isoflurane) with considerable antinociceptive activity in their own right would

influence the changes in EEG variables linked to nociception (Haga et al. 2001). The minimal halothane anaesthesia model, using a stable and minimal partial pressure of halothane, would allow generation of consistent EEG responses of nociception in animals (Murrell & Johnson 2006). Halothane has negligible analgesic property compared to other inhalation anaesthetics (England & Jones 1992). This method has been used in EEG studies involving different species of animals, horses, lambs, red deer, pigs, tammar wallaby, rats and calves, with a variety of noxious stimuli (Murrell et al. 2003, 2005; Johnson et al. 2005b, 2005a; Haga & Ranheim 2005; Diesch et al. 2005; McGregor 2005; Murrell et al. 2007; Gibson et al. 2007). The validity of this model has not been tested in the dog EEG studies evaluating nociception. Therefore it was decided to test this model in dogs subjected to noxious electrical stimulation in the study reported in chapter 6.

3.4 DRUGS USED

The following test drugs were used for the methods described in chapters 4, 5 and 6

1. Tramadol (Tramal[®] 100: tramadol hydrochloride 50 mg mL⁻¹. Grunenthal GmbH, Aachen, Germany)
2. Parecoxib (Dynastat: powder for injection. Made up with sodium chloride diluent as 20 mg mL⁻¹: Pharmacia Australia Pty. Ltd, New South Wales, Australia)
3. Pindolol; dissolved in 10% dimethyl sulphoxide in 0.9% saline. Sigma-Aldrich chemicals, Montana, USA.
4. Morphine Sulfate Injection; 10mg mL⁻¹ ; Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia.

Since there were no recommended dose rates available for the use of these test drugs in dogs, their dose rates were chosen based on their experimental or clinical use in human studies.

The following pre-anaesthetic, induction and anaesthetic drugs were used for the anaesthetic methods described in all the studies.

1. Acepromazine Maleate ((Acezine 2; 2 mg mL⁻¹ injection; Delta Veterinary Laboratories, New South Wales, Australia)
2. Propofol ((Propofol Injection 10 mg mL⁻¹; Mayne Pharma Pty Ltd, Mulgrave, Victoria, Australia)
3. Thiopentone Sodium ((Thiobarb 2.5%; Jurox NZ Ltd, Auckland, New Zealand)
4. Halothane (Halothane-vet; Merial NZ Ltd; Manukau; Auckland, New Zealand)
5. Atropine ((Atropine sulphate injection; Phoenix Pharma, Auckland, New Zealand).

CHAPTER 4

ANALGESIC EFFICACY AND EFFECTS ON GLOMERULAR FILTRATION RATE OF PARECOXIB AND TRAMADOL GIVEN ALONE OR TOGETHER WITH PINDOLOL IN ANAESTHETISED DOGS

4.1 INTRODUCTION

Opioids and NSAIDs are the two major classes of drugs commonly used to provide post-operative analgesia in dogs. Tramadol is a centrally acting analgesic with several mechanisms of action. It acts as a weak μ -opioid agonist coupled with inhibition of synaptic re-uptake of serotonin (5-HT) and NA by central neurones (Raffa et al. 1992). In dogs, post-operative analgesic effects of tramadol have been shown to be comparable to those of morphine but with fewer side effects (Mastrocinque & Fantoni 2003). However, there are very few studies that investigated the effects of tramadol in dogs compared to that in humans in which tramadol has been in use for more than two decades (Kukanich & Papich 2004). In normal rats anaesthetised with pentobarbitone sodium a bolus injection of tramadol (2 mg kg^{-1}) was shown to increase serum NA levels that would cause renal hypo perfusion. (Nagaoka et al. 2002). Further, effects of opioids on renal circulation are unclear (Mercadante & Arcuri 2004).

Though NSAIDs have been popular for their potential anti-inflammatory and analgesic effects, the broad range of their side effect profile (due to nonselective COX inhibition) restricts their use in the immediate perioperative period. Attempts to develop NSAIDs with relatively fewer side effects led to development of selective COX-2 inhibitors (coxibs). Parecoxib sodium (an injectable pro-drug of valdecoxib), a potent and highly selective COX-2 inhibitor in human tissues (Talley et al. 2000), is licensed in New Zealand and the European Union for the short-term treatment of acute postoperative pain in adults (2001). Intravenous or intramuscular administration of parecoxib, as a 20 or 40 mg single dose produced analgesia in both pre- and post-operative analgesic trials including a variety of surgical models in humans (Desjardins et al. 2001; Barton et al. 2002; Rasmussen et al. 2002; Ng et al. 2003; Bajaj et al. 2004;). However, there are no reports of its use in dogs.

Anaesthesia and surgery can decrease the circulating volume with significant vasoconstriction resulting in a reduced renal blood flow (RBF) that in turn lowers the GFR. Renal auto regulation by means of endogenous PGs can maintain RBF in response to the

hypotensive effects of general anaesthesia (Lobetti & Lambrechts 2000). Subsequent inhibition of PG production by NSAIDs may lead to acute renal failure after anaesthesia (Palmer 1995). However, intravenous (IV) fluids are usually administered to dogs during general anaesthesia and surgery to maintain effective circulating blood volume (Lobetti & Lambrechts 2000). *In vitro* studies that have explored the basal expression of COX-2 in different anatomical areas of mammalian kidney have questioned the renal safety of selective COX-2 inhibitors (coxibs). Therefore the renal safety of coxibs in dogs needs to be assessed under normotensive anaesthesia before clinical use.

Pindolol is a non-selective beta-adrenoceptor blocker (Frishman 1983) and a 5-HT_{1A/1B} antagonist. Though pindolol is not an analgesic by itself, its use as an adjunct to tramadol analgesia was shown to augment the efficacy of tramadol in mice (Corrales et al. 2000).

GFR is a sensitive and reliable indicator of renal perfusion since it is linearly related to the RBF (Greene 1996). Plasma clearance of iohexol is a validated measure of GFR in dogs (Moe & Heiene 1995; Heiene & Moe 1999; Finco et al. 2001). This method of GFR estimation precludes most of the problems associated with other traditional (standard) techniques.

The purpose of this study was to compare the analgesic efficacy of parecoxib, tramadol and a combination of parecoxib, tramadol and pindolol, to the standard analgesic morphine, and to determine the effect of these drugs on GFR in healthy experimental dogs under normotensive anaesthesia.

4.2 MATERIALS AND METHODS

4.2.1 Animals

Eight adult (ten months to five years old) dogs (males n=4, females n=4) were used for the study. They were obtained from Jennersmead farm, Animal health services centre, Massey University, Bunnythorpe. All dogs were kept under uniform conditions. There was a mix of breeds with Labrador and Huntaway types. Their weights were 29.2 ± 4.2 kg (mean \pm SD). A complete physical examination was performed on each dog on arrival and all were apparently healthy. None of the dogs had any disease history in the farm records. The study was approved by the Massey University Animal Ethics Committee.

4.2.2 Treatment groups and study design

There were five treatments as detailed below.

1. tramadol, 3 mg kg⁻¹ intravenously (IV)
2. parecoxib, 1 mg kg⁻¹ IV
3. combination (parecoxib, 1 mg kg⁻¹ IV plus tramadol, 3 mg kg⁻¹ IV plus pindolol, 5 µg kg⁻¹ subcutaneously, SC)
4. morphine, 0.1 mg kg⁻¹ IV (positive control)
5. saline 0.9%, 2 mL (negative control)

Each dog received every treatment in a cross-over design with a two-week interval between treatments. The sequence of treatment administered was randomised for each dog. The investigator measuring analgesia and GFR was blinded to the treatment.

4.2.3 Drug administration and anaesthesia

Dogs were fasted for 12 hours prior to anaesthesia with free access to water until anaesthetic induction. They were given acepromazine 0.05 mg kg⁻¹ SC. Dogs receiving pindolol were also given it SC at this time by the drug administrator (not the person assessing the effects of treatment). Thirty to sixty minutes after pre-medication, the dogs were given the analgesic drugs IV and then anaesthesia was induced with IV propofol to effect (2.9 ± 0.8 mg kg⁻¹ mean±SE). Once intubated, anaesthesia was maintained with halothane in oxygen using a circle system with a minimum flow rate of 0.5 L min⁻¹. Airway gases were sampled continuously from the end of endotracheal tube connected to the circle system, using an anaesthetic gas analyser (Hewlet Packard M1025B; Hewlet Packard, Hamburg, Germany) and the vaporizer adjusted to maintain end-tidal halothane tension between 0.85% and 0.95%. Arterial blood pressure was recorded every five minutes using a Doppler ultrasound pressure transducer with cuff. If the dogs' systolic blood pressure dropped below 90 mmHg, they were given Hartmann's solution IV and the inspired halothane concentration was reduced. Respiratory rate (RR) pulse rate (PR) and arterial haemoglobin oxygen saturation (SpO₂, measured by using pulse oximeter with probe attached to dog's tongue) were recorded every five minutes together with signs of anaesthetic depth. After 30 minutes the halothane was turned off. As soon as the dogs were able to maintain their own airway, the endotracheal tube was removed. Dogs were monitored carefully for drug induced side effects throughout the study period.

4.2.4 Assessment of analgesia

Each dog's nociceptive thresholds were measured using a mechanical pain threshold testing device and the method was described in chapter 3.

4.2.5 Estimation of GFR

A plasma iohexol clearance test was conducted twice on each dog, before and 24 hours after analgesia and induction of anaesthesia. The method was described in chapter 3.

4.2.6 Statistical analyses

This is a cross-over study with the sequence of drugs administered randomised for each dog. Traditional methods of analysing repeated measures data include the univariate repeated measures analysis of variance and the multivariate repeated measures analysis of variance. PROC GLM in SAS[®] (SAS Institute Inc., Cary, NC, USA) provides both univariate and multivariate tests for repeated measures. Another approach to analyse repeated measures is using generalised linear mixed models (GLMM). Mixed models include the usual fixed effects for the regressors plus the random effects. GLMM has been suggested as an ideal method for analysing repeated measures from cross-over studies (Putt & Chinchilli 1999; Jones & Kenward 2003). PROC MIXED in SAS[®] provides an excellent platform for GLMM analyses. It can handle balanced as well as unbalanced or missing within-subject data, and it offers more options for modeling the within-subject covariance (Littell et al. 1998).

The effects of test drugs on nociceptive thresholds were compared employing PROC MIXED procedure in SAS[®] 9.1 (SAS Institute Inc., Cary, NC, USA). Post-treatment threshold values (at 20, 40 and 60 min) were expressed as percent of pretreatment values and were log-transformed. The employed linear mixed model (Littell et al. 1998) included the fixed effects of treatments, carry-overs, time and treatment- time interaction, and random effects of animals. The covariance error structure for repeated measures over time-points within animals within group was determined using Akaike's information criterion. A first-order autoregressive model was found to be the most appropriate error structure. The effect of carry-overs was found to be non-significant and hence was excluded from the final model. Normality of data was assessed employing PROC CAPABILITY procedure in SAS[®] 9.1. Residuals of data were found to be normally distributed. For graphical presentation, LSM in log-scale were back-transformed to the observed scale.

The effects of test drugs on GFR were also compared employing PROC MIXED procedure in

SAS[®] 9.1. Post-treatment GFR values were expressed as percent of pretreatment values. The employed model included fixed effects of treatment and carry-over, and random effects of animals. Since carry-over effect was non-significant, it was excluded from the final model. Residuals of data were found to be normally distributed. A P -value < 0.05 was considered significant for all statistical analyses.

4.3 RESULTS

Except when given morphine, none of the dogs showed significant side effects after test drug administration, and so none were excluded from the trial. Vomiting, defaecation and prolonged sedation were noticed in four of eight dogs given morphine. There was no significant difference in dogs' RR, PR and SpO₂ during anaesthesia and they were within normal range.

4.3.1 Analgesic efficacy

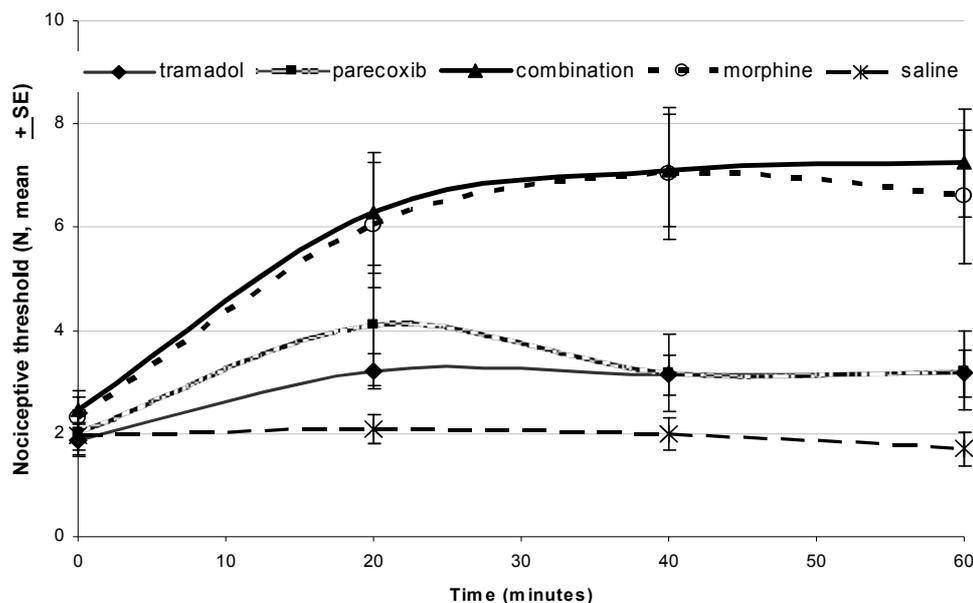


Figure 11: Mean \pm SE nociceptive thresholds of dogs ($n=8$) measured before (values at time 0) and after administration of IV tramadol, IV parecoxib, combination (IV tramadol, IV parecoxib and SC pindolol), IV morphine or saline. Post-treatment thresholds were measured at 20, 40 and 60 minutes after extubation

Trends of nociceptive thresholds (as simple mean \pm SE) over time in dogs of different treatment groups are depicted in figure 11, while the post-treatment least square means (LSM) for nociceptive thresholds (expressed as percent of pre-treatment values) are shown in figure 12. All the four analgesic groups had significantly ($P<0.05$) higher overall (over 60

minutes) post-treatment nociceptive thresholds compared to the saline group (figure 12). Among the treatment groups, tramadol and parecoxib groups had similar nociceptive thresholds that were significantly ($p < 0.001$) lower than those of the combination and morphine groups. There was no significant difference in the overall post-treatment nociceptive thresholds between the combination and morphine groups.

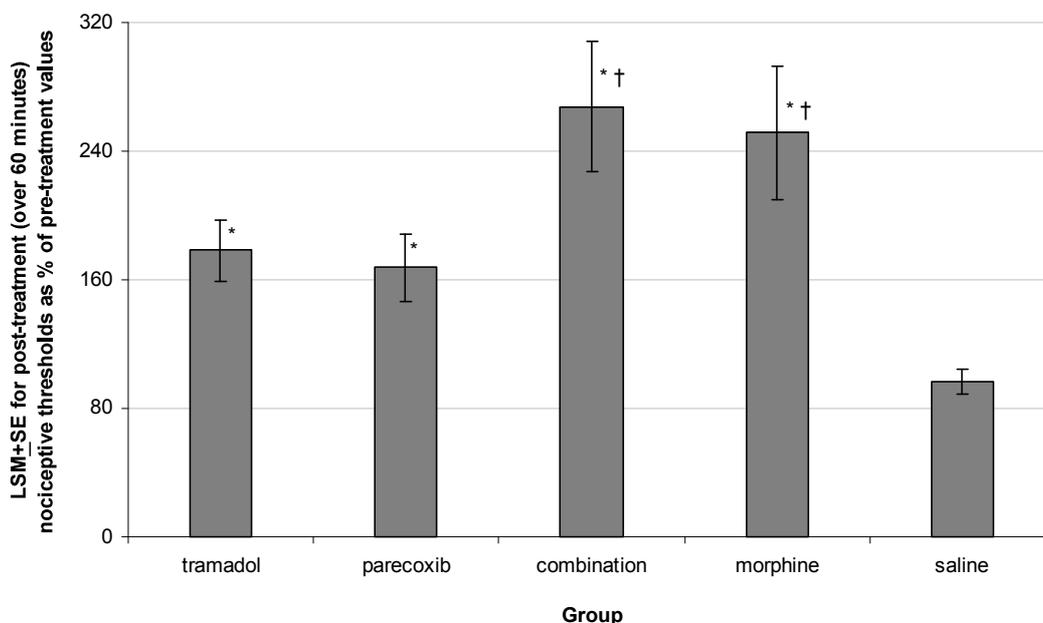


Figure 12: *LSM±SE for post-treatment (over 60 minutes) nociceptive thresholds expressed as percent of pre-treatment values, in dogs (n=8) given either tramadol IV, parecoxib IV, a combination (tramadol IV, parecoxib IV and SC pindolol), morphine IV or saline at anaesthetic induction*

* significantly different from saline ($P < 0.05$)

† significantly different from tramadol and parecoxib ($P < 0.001$).

4.3.2 Renal safety

Plasma iohexol clearance rates (as simple mean±SE) in dogs belonging to the five treatment groups, before and 24 hours after drug administration are presented in table 7. LSM for plasma iohexol clearance rates (expressed as percent of pre-treatment values) 24 hours after administration of analgesics and anaesthesia ranged between 96% (morphine) and 105 % (parecoxib) in the different groups (figure 13). There was no significant difference in clearance rates between the treatment groups. Power of the current study to detect a difference of $1 \text{ mL min}^{-1} \text{ kg}^{-1}$ in clearance rate between groups ($n=8$, determined by PROC POWER in SAS[®] 9.1 using the observed overall variance for clearance rates) was 0.86.

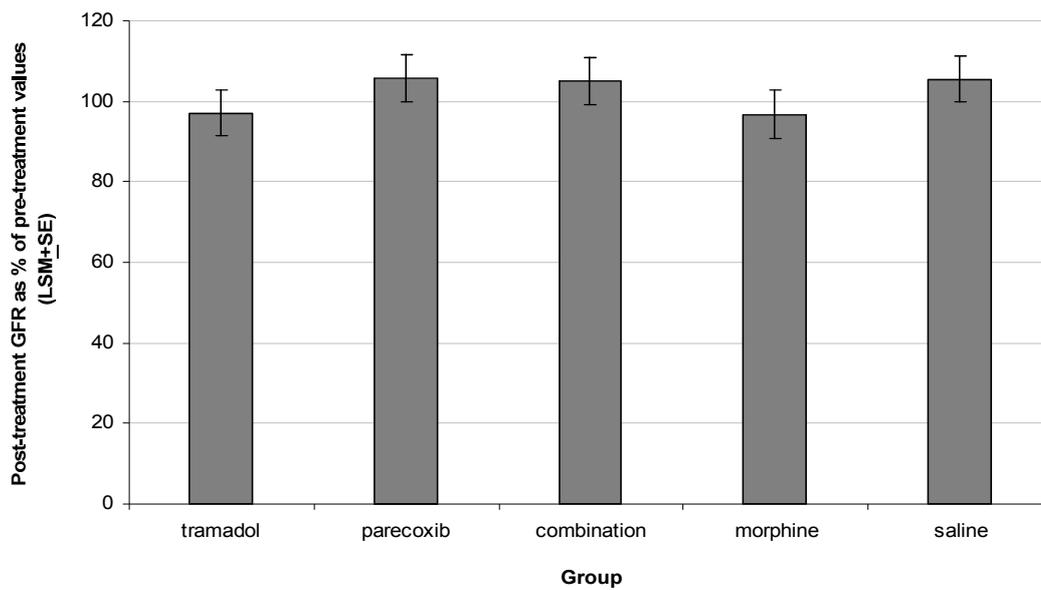


Figure 13: *LSM±SE for plasma iohexol clearance rate (an estimate of GFR) expressed as percent of pre-treatment values, of dogs (n=8) given IV tramadol, IV parecoxib, combination of the two with SC pindolol, IV morphine or saline. Clearance rates were measured before and 24 hours after analgesic administration and anaesthetic induction*

No significant difference in GFR after administration of analgesics was observed in any of the treatment groups.

Table 7: *Simple mean±SE of GFR values estimated from plasma iohexol clearance of dogs (n=8) before and 24 hours after analgesia and anaesthetic induction*

Treatment group	GFR values (mL min ⁻¹ kg ⁻¹ , mean ± SE)	
	Before analgesia and anaesthetic induction	24 hours after analgesia and anaesthetic induction
tramadol	4.61±0.12	4.56±0.18
parecoxib	4.5±0.29	4.56±0.23
combination	4.66±0.17	4.62±0.22
morphine	4.61±0.17	4.54±0.25
saline	4.64±0.24	4.69±0.075

4.4 DISCUSSION

4.4.1 Mechanical nociceptive threshold testing

Assessing pain thresholds to mechanical noxious stimuli is a well established technique in dogs (Lascelles et al. 1997; Barnhart et al. 2000; Slingsby & Waterman-Pearson 2001). The

mechanical threshold testing device built for this study satisfied most of the ideals put down by Beecher (1957) for producing experimental pain using nociceptive stimuli.

Dogs were tested in pairs to minimise separation anxiety and allowed 30 minutes for initial settlement in the new environment (laboratory). This would minimize the stress-induced analgesia in adaptation to environment (Fleetwood & Holtzman 1989; Rubinstein et al. 1996) that otherwise would interfere with baseline nociceptive threshold readings. From previous investigations conducted in humans, monkeys (Miron et al. 1989), and Cows (Whay et al. 1996), it has been suggested that diversion of attention to another stimulus modality may modify the level of perception of pain intensity to noxious stimuli. Therefore, the experiment was conducted with a minimum number of people around. Similar background music was played in low volume throughout the testing period to prevent undue disturbances (if any) from outside the laboratory.

Previous studies in humans and horses showed that the rate of application of pressure influences the threshold responses (Grindley 1936; Chambers 1992). Lascelles et al (1997) could overcome this difficulty in a study on dogs, by devising a CRAF meter that was connected to the algometer. This allowed pressure application at a constant rate. Since there was no means of control over application of pressure in this device, only one investigator (blinded to the treatment given) performed the testing through out the experiment to minimize the operator variability on sensory thresholds.

None of the dogs showed any behavioural responses indicating attainment of threshold level either immediately after placement of the probe without any pressure application or before the stimulus become painful. Barnhart et al (2000) found the similar unlearned, natural responses to repeated noxious stimulus application in dogs with threshold testing. The threshold response of the dogs did not change significantly over time in the saline control group with no analgesia, indicating that the dogs have not been habituated or sensitised to the testing stimuli.

Chambers (1992) observed a significant increase in pressure threshold of sheep at ambient temperature of 0°C - 8°C compared to higher temperatures. To exclude the possibility of ambient temperatures affecting the thresholds, all pieces of this trial were conducted at ambient temperature between 14°C and 22°C . The dogs receiving morphine (0.1 mg kg^{-1}), although sedated, were aware of their surroundings and did respond to the stimulus.

Analgesia can be differentiated from sedation using behavioural responses to a mechanical stimulus in dogs given opioids and sedatives (Barnhart et al. 2000; Slingsby 1998).

4.4.2 Analgesia

In this study, both tramadol and parecoxib significantly increased the mechanical nociceptive thresholds of dogs compared to dogs given saline, indicating that administration of both drugs resulted in analgesia. Specific inhibition of COX-2, which results in inhibition of synthesis of inflammatory PGs that sensitise peripheral nerve endings, is the primary analgesic action ascribed to coxibs. Since there was neither surgical trauma nor peripheral inflammation in dogs in this study, analgesia produced by parecoxib must be related to mechanisms other than inhibition of peripheral inflammation. This is in agreement with a previous study that showed analgesia in the absence of peripheral inflammation with COX-2 specific inhibitor, FR 140423, in normal rats using a tail flick test (Ochi et al. 1999), which was attributed to a central analgesic mechanism. However, the actual site and the exact mechanism of central analgesic effect of NSAIDs are still unclear. Parecoxib would be expected to have a bigger effect in the presence of inflammation.

Parecoxib is rapidly and completely converted to its active metabolite, valdecoxib, in dogs after intravenous injection (Talley et al. 2000). Both human and animal studies have shown that coxibs can cross the blood brain barrier and enter the CNS in concentrations high enough to inhibit COX-2 activity (Buvanendran et al. 2001, 2002; Dembo et al. 2005). It has been suggested that the peripheral inflammatory stimuli cause an increased expression of spinal COX-2 mRNA and increased release of spinal PGE₂, and NSAIDs have a powerful effect upon spinal nociceptive processing evoked by peripheral inflammation (Ebersberger et al. 1999; Malmberg & Yaksh 1992). Since the dogs in the current study had no peripheral inflammation, analgesia produced by parecoxib given alone could indicate tonic spinal PG synthesis or another mechanism of analgesia.

Tramadol is widely used for management of acute post-operative pain and cancer pain in humans (Bamigbade & Langford 1998). It produced significant analgesia in response to acute mechanical stimulation after intravenous administration to healthy dogs in this study. This is consistent with the findings of Raffa et al. (1992) in mice with non-inflammatory pain models. Several studies suggest that tramadol analgesia is mediated by a weak μ -opioid receptor agonistic activity coupled with inhibition of re-uptake of NA and 5-HT, thus

potentiating descending nociceptive inhibitory systems. However, formation of the major metabolite (M_1) responsible for a significant portion of the analgesia in people is minimal in dogs (Wu et al. 2001) so there may be a ceiling for tramadol analgesia in dogs.

Though tramadol ($3 \text{ mg kg}^{-1} \text{ IV}$) and parecoxib ($1 \text{ mg kg}^{-1} \text{ IV}$) had similar analgesic efficacy in the current study, this was significantly ($P < 0.001$) lower than that of morphine ($0.1 \text{ mg kg}^{-1} \text{ IV}$). However, in another study, tramadol ($2 \text{ mg kg}^{-1} \text{ IV}$) showed equal efficacy to morphine ($0.2 \text{ mg kg}^{-1} \text{ IV}$) when given preoperatively to dogs undergoing ovariohysterectomy (Mastrocinque & Fantoni 2003). This discrepancy could be due to the difference in the type and intensity of noxious stimulation activating different pathways in tramadol analgesia.

Dogs that were given a combination of tramadol, parecoxib and pindolol showed pain thresholds that were significantly ($P < 0.001$) higher than those in dogs administered either tramadol ($3 \text{ mg kg}^{-1} \text{ IV}$) or parecoxib ($1 \text{ mg kg}^{-1} \text{ IV}$) alone. Analgesia produced by this combination was comparable to that of morphine ($0.1 \text{ mg kg}^{-1} \text{ IV}$). NSAID analgesia (COX-2 inhibitor, FR 140423, and non specific COX inhibitors, dipyron and flunixin meglumine) was shown to be blocked by naloxone (μ -opioid antagonist,) and atipamezole (α_2 -adrenoceptor blocker) in rats and healthy sheep, respectively (Chambers et al. 1995; Ochi et al. 1999), suggesting the involvement of both opioidergic and adrenergic mechanisms in NSAID analgesia. It is likely that parecoxib could have interacted with tramadol by activating both opioidergic and noradrenergic systems in the present study.

Pindolol was shown to block the putative 5-HT_{1A} autoreceptors present on central serotonergic neurones (Romero & Artigas 1997), which resulted in increased release of 5-HT. 5-HT_{1A} and 5-HT_2 antagonists were shown to augment tramadol analgesia in rats and mice (Pinaridi et al. 1998; Corrales et al. 2000). In the current study, it seems likely that pindolol could have potentiated tramadol analgesia in activating the serotonergic modulatory system. However, since neither parecoxib nor pindolol were given separately with tramadol, it is not possible to attribute the effects of the combination to either parecoxib or pindolol. Due to limited availability of dogs parecoxib and pindolol were not tested separately with tramadol. Pindolol is a β -blocker and could potentially slow the heart rate, but the bradycardic effect is very unlikely to be seen at the low dose used in this trial (the dose used clinically is approximately 15 times the dose used in this trial). None of the dogs in the

present study given pindolol had untoward haemodynamic variables during anaesthesia which might be attributable to pindolol.

There are no reports on the use of parecoxib and pindolol (as an adjunct to analgesics) in dogs. The dose rates of these test drugs were chosen based on human studies. Also, no dose-titration studies are available for tramadol and morphine in dogs. The dose of morphine used in this study (0.1 mg kg^{-1}) was less than that routinely used in our clinic (0.5 mg kg^{-1}). As this is the first study to use these novel drugs in dogs it was thought appropriate to compare their efficacy with low dose morphine initially.

4.4.3 Renal safety

There are several different methods of estimating GFR, which is directly proportional to the number of functional nephrons in both kidneys (DiBartola 1995). Traditional techniques like estimating serum urea and creatinine concentration are not early diagnostic measures of renal dysfunction since their concentrations only begin to rise when 75% of nephrons are non-functional (DiBartola 1995), in addition to being affected by extra renal factors. Plasma clearance following a single intravenous injection of iohexol as a filtration marker has been validated as a measure of GFR in dogs (Moe & Heiene 1995; Heiene & Moe 1999; Finco et al. 2001).

Analysis of iohexol iodine (I) in plasma by HPLC method, though labour intensive, allows detection of lower concentrations which in turn helps reduce the dose of injected marker and possible renal effects of the marker. The dose of iohexol injected to dogs of the current study is 1500 mg I (single dose). The standard graph is linear between $5 \mu\text{g mL}^{-1}$ and $1200 \mu\text{g mL}^{-1}$ of iohexol I. The mean iohexol concentration ($\mu\text{g mL}^{-1}$) in our study dogs ranged from 43.9 (120 min) to 17 (240 min) and from 40.6 (120 min) to 15.2 (240 min) before and 24 hours after analgesia and induction of anaesthesia, respectively. The concentration of iohexol I detected two and four hours after a single injection was within the lower detection range ($5 \mu\text{g mL}^{-1}$). The dose of the injected iohexol (1500 mg I) was well tolerated by all dogs in the present study and it was far less than that used in other studies using ICL to estimate GFR (Moe & Heiene 1995; Finco et al. 2001; Brown et al. 1996).

Calculation of GFR from the area under the iohexol concentration / time curve, assuming a one-compartment model, was simple, time saving and minimally invasive. Only two samples

were required so the procedure took 3 or 4 hours (Heiene & Moe 1998, 1999). However, this method may slightly overestimate GFR due to underestimation of AUC (Finco et al. 2001). Clearance studies in humans and dogs using the Brøchner-Mortensen formula to correct this error found good correlation with clearance values from other standard clearance techniques such as renal clearance of inulin and exogenously administered creatinine (Gaspari et al. 1995; Brown et al. 1996). A slight difference in the range of GFR values from different studies is not surprising due to biological and measurement variations (Heiene 1995).

The dogs in our study had a GFR of $4.61 \pm 0.02 \text{ mL min}^{-1} \text{ kg}^{-1}$ (mean \pm SE) before anaesthesia, which is similar to the reported GFR value for normal dogs ($4.60 \pm 0.15 \text{ mL min}^{-1} \text{ kg}^{-1}$, Izzat & Rosborough 1989), determined by renal clearance of inulin, an accepted reference method for GFR estimation. In the current study, GFR was measured 24 hrs after induction of anaesthesia as a change in GFR should be detected then if there was renal damage (Crandell et al. 2004). Most studies investigating the renal effects of NSAIDs in dogs measured renal function using either creatinine clearance or serum urea and creatinine concentration, 24-48 hrs after anaesthesia and surgery (Forsyth et al. 2000; Lobetti & Joubert 2000; Mathews et al. 2001; Laredo et al. 2004). Since each dog had every treatment in the cross-over design of present study, GFR estimation before each testing episode would detect any compromise to GFR beyond 24 hours of any treatment episode. However, there were no significant differences in pre-treatment GFR values between treatment groups.

The effects of tramadol on renal haemodynamics in normal dogs are not known. Serum NA levels were shown to be elevated after injection of tramadol at clinical doses in rats (Nagaoka et al. 2002), which would further increase the likelihood of anaesthesia affecting RBF. There are no available reports on the renal effects of parecoxib in dogs. This study did not reveal any significant differences in GFR of dogs with parecoxib and tramadol, either singly or together with pindolol (at doses that showed significant analgesia), in comparison with that in dogs given either morphine or normal saline. Morphine is a commonly used pre-operative analgesic in dogs, and is not thought to affect RBF. In the present study, morphine treated dogs had small reductions in GFR that were statistically non-significant.

In anaesthetised subjects renal auto-regulation seems to depend maximally on PG synthesis when mean arterial pressure (MAP) decreases below 65 mm Hg (Herbaczynska-Cedro & Vane 1973). Hence, in dogs given NSAIDs, PG inhibition would be expected to become

critical for RBF when MAP is below 65 mmHg. This would require cardiovascular support such as the administration of fluids. Preventive administration of IV fluids to normal dogs during anaesthesia should be routine practice in anticipation of induced hypo-tension and subsequent renal damage (Lobetti & Lambrechts 2000). None of the dogs in the present study had systolic blood pressures less than 100 mmHg (mean 103 ± 1.6 mmHg) (corresponding to a MAP of approximately 65 mmHg) during anaesthesia. Normotension was maintained with IV fluids, to evaluate the renal effects of test drugs in a routine clinical setting.

The depth of halothane anaesthesia maintained in this study was thought to be sufficient to allow routine elective surgery. Surgery was not conducted in the dogs used in these experiments for ethical reasons, however, surgery can also contribute to perioperative changes in MAP due to insensible fluid losses by evaporation, haemorrhage, and increasing sympathetic activity (Lobetti & Lambrechts 2000). These factors can increase or further decrease MAP. A dose-dependent decrease in MAP has been reported with different general anaesthetics such as halothane, isoflurane and sevoflurane, in dogs (Mutoh et al. 1997). Renal effects of anaesthetic drugs in dogs undergoing extensive surgery at a deep plane of general anaesthesia have yet to be evaluated.

4.5 CONCLUSION

Tramadol and parecoxib administered at induction of anaesthesia to clinically normal dogs produced significant analgesic effects, in response to nociceptive mechanical stimuli. Their combination, together with pindolol produced analgesia comparable to that of a low dose of morphine and was free from discernable side effects within the limitations of this study. Neither tramadol nor parecoxib (alone or in combination) had adverse effects on renal perfusion 24 hours after anaesthesia in healthy dogs with normotensive anaesthesia. The combination requires further evaluation in clinical trials, particularly to assess efficacy against surgical pain.

CHAPTER 5

EFFICACY OF PREOPERATIVE TRAMADOL, PREOPERATIVE MORPHINE OR PREOPERATIVE LOW-DOSE MORPHINE WITH POSTOPERATIVE TRAMADOL IN DOGS UNDERGOING OVARIOHYSTERECTOMY OR CASTRATION

5.1 INTRODUCTION

Opioids are the main stay analgesics for the management of postoperative pain in small animals. Morphine is the gold standard μ -opioid and the most commonly used perioperative analgesic, despite its side effects like vomiting, defaecation, prolonged sedation and respiratory depression (Hall & Clark 1991). Tramadol is a centrally acting analgesic with several mechanisms of action including μ -opioid receptor affinity but devoid of typical μ -receptor specific side effects (Raffa et al. 1992). Timing of analgesic administration plays an important role in optimal control of postoperative pain. Prevention of the entire noxious input of surgical origin is the most effective pain controlling measure (Kissin 2005).

Ovariohysterectomy and castration are common surgical procedures in small animal practice (Dorn & Swist 1977; Gassel et al. 2005). Both surgical procedures can cause significant postoperative pain in dogs (Fox et al. 1994; Lascelles et al. 1998; Gayner & Muir 2002). Since these elective surgeries are performed routinely on healthy and pain free animals the efficacy of test drugs can be more reliably assessed assuming that all postoperative pain resulted from surgery only (Slingsby et al. 2006).

Subjective assessment of the postoperative pain using different pain scales, including the VAS, numerical ratings scale and simple descriptive scale, has been practised in clinical analgesic trials of dogs (Lascelles et al. 1994; 1997; Slingsby 1998; & Waterman-Pearson 2000; 2001; Lemke et al. 2002). Though these scales provide a reliable subjective appraisal of acute pain, lack of linearity, lack of specificity with descriptive pain behaviours and inter-observer variability are claimed as the drawbacks (Holton et al. 1998; Hansen 2003).

Recently, the short form of the Glasgow CMPS-SF has been introduced for assessment of acute pain in a clinical setting (Reid et al. 2007). It includes six behavioural categories with 30 pain descriptors. The descriptors in each category were ranked numerically based on the intensity of pain. Pain score is the sum of the rank scores which will be assigned by the

observer after picking the best fit descriptor of the dog's pain within each category. The maximum score for the six categories is 24 and the recommended analgesic intervention level is six. The authors stated that the scale does not require specialised skills and experience to use as the constructs of the scale were composed and reconfigured from the author's clinical experience, feed back from a large number of veterinary surgeons (more than 500) familiar with postoperative behaviour of dogs in their practice and a validation study (Morton et al. 2005; Reid et al. 2007).

The CMPS-SF appears more attractive as it has been constructed for quick assessment of acute pain in a clinical setting (Reid et al. 2007). VAS has been reported to be satisfactory to measure sedation in dogs undergoing surgery (Reid & Nolan 1991; Welsh et al. 1997; Lascelles et al. 1998; Slingsby & Waterman-Pearson 2001; Slingsby et al. 2006).

Evaluation of anti-nociceptive efficacy of the drugs against noxious surgical stimuli using EEG responses has been studied in horses, red-deer, and calves (Murrell et al. 2005; Johnson et al. 2005a; Gibson et al. 2007). In dogs, ovariohysterectomy has been demonstrated to cause quantitative changes in various frequency bands of EEG power spectrum recorded intraoperatively (Trucchi et al. 2003). There has been no report of studies that used intraoperative EEG to assess the anti-nociceptive efficacy of analgesics in dogs undergoing ovariohysterectomy or castration.

The main aim of this research project was to evaluate the analgesic efficacy of tramadol and parecoxib singly or together (under balanced analgesic strategy) in dogs undergoing ovariohysterectomy or castration in our veterinary teaching hospital (VTH). Though the efficacy and effects of these drugs on renal perfusion were checked in eight anaesthetised dogs in the study reported in chapter 4, our VTH anaesthesia staff were not happy to administer parecoxib preoperatively to dogs since parecoxib has neither been tested in dogs undergoing surgery nor was there an available report of its preoperative use in dogs. Therefore it was decided to compare the analgesic efficacy in dogs of preoperative morphine 0.5 mg kg^{-1} (the standard VTH preoperative analgesic) or tramadol 3 mg kg^{-1} alone, or preoperative low dose morphine 0.1 mg kg^{-1} with postoperative tramadol 3 mg kg^{-1} using intra-operative EEG responses and the Glasgow CMPS-SF score.

5.2 MATERIALS AND METHODS

5.2.1 Ovariohysterectomy study

5.2.1.1 Animals

Twenty four mixed breed dogs undergoing routine ovariohysterectomy were recruited for the trial. The mean (\pm SE) body weight of the dogs was 19.3 (\pm 4.7) kg, with a range from 4.5 to 34.5 kg. Age of the dogs ranged from six months to six years with a mean (\pm SE) of 1.9 ± 0.6 years. All the dogs were clinically normal.

5.2.1.2 Anaesthesia and analgesia

After routine physical examination, each dog was assessed for the presence of pain, using the Glasgow CMPS-SF. The dog was then randomly allocated to one of the three treatment groups described below.

5.2.1.2.1 Pre-anaesthetic medication

Dogs were given pre-anaesthetic medication with acepromazine 0.05 mg kg^{-1} and atropine 0.04 mg kg^{-1} SC, 30-45 minutes before anaesthetic induction. This is our VTH standard pre-anaesthetic medication of dogs.

5.2.1.2.2 Analgesia

Dogs were randomly allocated to one of the three treatment groups (eight dogs per treatment group). Test drugs were given at the same time as the pre-anaesthetic medication. Dogs in control group received morphine 0.5 mg kg^{-1} SC, those in tramadol group received tramadol 3 mg kg^{-1} SC and dogs in low-dose morphine group administered low-dose morphine 0.1 mg kg^{-1} SC. Dogs in this group were given tramadol 3 mg kg^{-1} IV at extubation postoperatively. Each dogs' sedation was assessed using the VAS just prior to induction of anaesthesia (figure 14).



Figure 14: *The visual analogue scale used for assessing dogs' sedation in the perioperative period*

In this system, the level of sedation was assessed by observing dog's posture, mental alertness, and its ability to stand and walk. At each assessment, a mark was made on a 100 mm scale, on which 0 corresponds to 'no sedation' and 100 corresponds to 'fast asleep' (Lascelles et al. 1994). The distance from 0 to the marked point on the scale was later converted to numerical form for statistical analysis.

5.2.1.2.3 Anaesthesia

Anaesthesia was induced with IV thiopentone sodium ($7.1 \pm 0.4 \text{ mg kg}^{-1}$ mean \pm SE) to effect and maintained with halothane delivered in 100% oxygen via a circle system. The concentration of halothane was adjusted to keep the dog at a suitable plane of surgical anaesthesia. As soon as the dog was anaesthetised and its airway, breathing and circulation had been checked, a pulse oximeter and Doppler transducer with cuff was attached so that SpO₂ of the haemoglobin and blood pressure were monitored non-invasively. Dogs were given Hartmann's solution IV to maintain systolic arterial blood pressure above 100 mm Hg throughout the anaesthetic period. Intra-operatively, fentanyl (fentanyl injection; 500 μg in 100 mL; Mayne Pharma Pty Ltd, Victoria, Australia) $1 \mu\text{g kg}^{-1}$ IV was given by the anaesthetist as needed, to control any tachycardia and tachypnoea which occurred in response to surgery. Respiratory rate, end-tidal halothane tension ($E_{T \text{ HAL}}$) and end-tidal CO₂ tension ($E_T \text{ CO}_2$) were also monitored. All these parameters, and the signs of depth of anaesthesia, were recorded every five minutes until the end of surgery.

5.2.1.3 EEG and electrocardiogram (ECG) recording

The EEG was recorded in a three electrode montage as described in chapter 3. The EEG recording (figure 15) was started as soon as the dog was stabilised under halothane anaesthesia (SA). During ovariohysterectomy, EEG data from 100-second blocks were taken at each surgical time point (figure 16). The 100-second blocks immediately preceding the skin incision were taken as the baseline (BL) period and during the skin incision were taken as skin incision period (SI). Data from 100-second blocks following clamping through ligation of the suspensory ligament of each ovary were used for ovary 1 (O₁) and ovary 2 (O₂). The 100-second block data following clamping through ligation of body of uterus were described as the uterine body (UB). The 100-second block data during skin closure was taken skin closure (SC). EEG recording was stopped at the end of anaesthesia (EA).

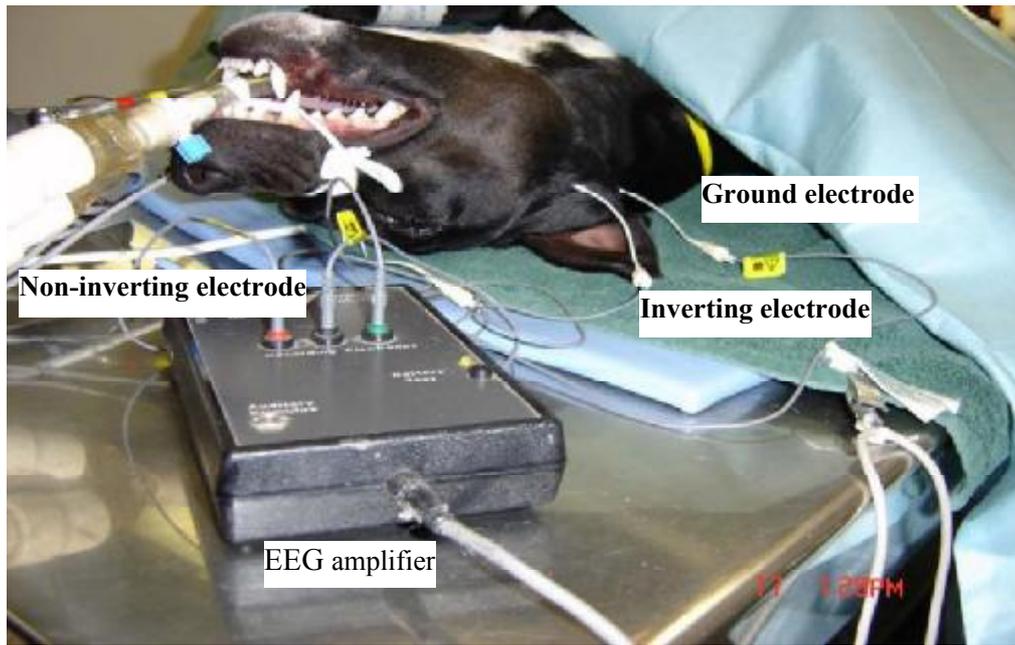


Figure 15: Position of the EEG electrodes on a dog undergoing ovariohysterectomy

For castration, the 100-second blocks immediately preceding the skin incision were taken as the baseline period. Data from 100-second blocks following clamping through ligation of the spermatic cord of each testicle were taken as testicle 1 and testicle 2. Data were averaged over the 100-second blocks at each surgical variable for statistical comparison.

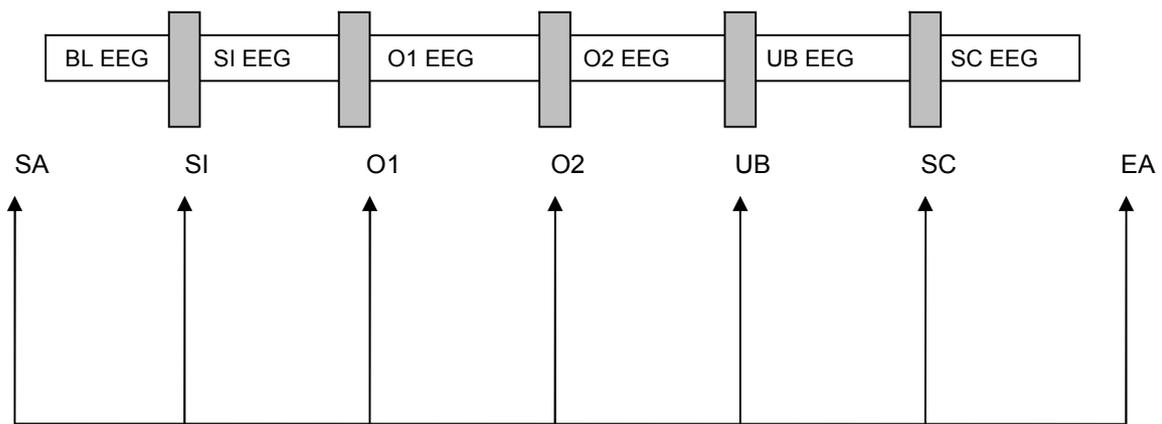


Figure 16: Diagram of the EEG recording pattern for different surgical time points in dogs undergoing ovariohysterectomy

SA = stabilisation of anaesthesia; BL EEG = baseline EEG; SI EEG = EEG recorded during skin incision; O1 EEG = EEG recorded during ligation of ovary 1; O2 EEG = EEG recorded during ovary 2 ligation; UB EEG = EEG recorded during ligation of body of uterus; SC EEG = EEG recorded during skin closure; EA = end of anaesthesia

The raw EEG (figure 17) was analysed using spectral analysis programme as described in chapter 3. The derived spectral variables (F50, Ptot and SEF) were used for statistical comparison.



Figure 17: Raw EEG recorded in an anaesthetised dog undergoing surgery

The ECG was recorded continuously in a lead II electrode configuration, using an Apple Macintosh personal computer installed with Chart 5.2.2 recording software and connected to Powerlab 4/20 data recording system (Powerlab™ data acquisition system®, AD Instruments Ltd, Sydney, Australia). The ECG was recorded at a rate of 1 kHz. The recorded data were analysed off-line using the rate meter function of the Chart 5.2.2 to obtain heart rate.

5.2.1.4 Surgery

Final year veterinary students under the supervision of a veterinary anaesthetist and surgeon performed the ovariohysterectomy with a routine ventral midline approach. The mean±SE surgery time was 87.9±6.7 minutes.

5.2.1.5 Postoperative pain assessment

Before recovery from anaesthesia dogs were moved to a cage in the recovery ward and the endotracheal tube was removed when their laryngeal reflexes were restored. Each dog's pain was assessed by the same person using the Glasgow CMPS-SF, at 1, 3, 6 and 9 hours postoperatively. Sedation was assessed at the same postoperative time points.

If a dog appeared to be in an unacceptable pain (CMPS-SF score ≥ 6 out of 24) rescue analgesia was given with morphine 0.3 mg kg⁻¹ intramuscularly and fentanyl 1 µg kg⁻¹ IV. The dogs were also observed for side effects, if any, of the administered analgesics.

5.2.2 Castration Study

Sixteen mixed breed dogs undergoing castration were recruited for the trial. The mean (±SE) body weight of the dogs was 21.5±5.3 kg, with a range from 8 to 34 kg. The age of the dogs

ranged from 4 months to 15 months, with a mean \pm SE of 9 \pm 0.9 months. Dogs were randomly allocated to one of two treatment groups (n=8). Dogs in control group received morphine 0.5 mg kg⁻¹ SC and those in tramadol group received tramadol 3 mg kg⁻¹ SC. The procedure for premedication, assessment of dogs' sedation, anaesthesia, EEG and ECG recording, haemodynamic variable monitoring and postoperative pain assessment was similar to those in dogs undergoing ovariohysterectomy. Final year veterinary students under the supervision of a veterinary anaesthetist and surgeon performed the castration with a routine prescrotal approach. The mean \pm SE surgery time was 46.75 \pm 4.68 minutes.

5.2.3 Statistical analyses

5.2.3.1 ECG and EEG

Data from heart rate, respiratory rate, end-tidal halothane tension and end-tidal carbon dioxide tension were analysed using analysis of variance (ANOVA- GraphPad Prism v 4.0b for Macintosh, Graphpad Software Inc., CA, USA). The effect of test drugs on EEG spectral frequency during ovariohysterectomy or castration were compared employing PROC MIXED procedure in SAS[®] 9.1 PROC CAPABILITY procedure in SAS[®] 9.1 was used to test the data for normality and residuals of data were found to be normally distributed.

The linear mixed model used for comparing the treatment groups included the fixed effects of time, treatments, and random effects of animals. The covariance error structure for repeated measures over different surgical time-points within animals within group was determined using Akaike's information criterion. A first-order auto-regressive model was found to be the most appropriate error structure. Analysed data from PROC MIXED procedure were presented as least square means (LSM \pm SE). Results were considered significant if $P < 0.05$.

5.2.3.2 Pain and sedation scores - ovariohysterectomy

Preoperative pain scores were all zero and so not considered further for statistical analysis. Postoperative pain scores at each of the recorded time points were categorised into three classes based on intensity of pain, as shown in table 8. Number of dogs under each category at each time point in each treatment group was scored and the numbers over different time points under each category in each group were pooled (Slingsby et al. 2006). The pooled numbers under each pain category were used to compare the between-treatment-group differences in pain scores employing a Wilcoxon-Mann-Whitney odds (WMWodds) macro (O'Brien & Castelloe 2006) in SAS[®] 9.1. The WMWodds statistic is an extension of the

generalized odds ratio (genOR, Agresti 1980), used to summarize the association between two ordinal variables. WMWodds has been considered as an ideal effect-size measure for properly interpreting and reporting results based on the common Wilcoxon-Mann-Whitney two-group test (O'Brien and Casteloe 2006). The three treatment groups were compared in three combinations of two each. The VAS sedation scores were analysed and compared in similar way as that of pain scores, except that they were classified into four classes as detailed in table 9.

Table 8: Categorisation of the Glasgow CMPS-SF pain score into three classes, in ovariohysterectomised dogs, based on intensity of pain

Class number	Description of class
1	pain score 0-2; mild/no pain
2	pain score 3-5; moderate pain
3	pain score \geq 6; severe pain

Table 9: VAS sedation score categorisation into four classes, in ovariohysterectomised dogs, based on level of sedation

Class number	Description of class
1	VAS scores 0-24
2	VAS scores 25-49
3	VAS scores 50-74
4	VAS scores 75-100

5.2.3.3 Pain and sedation scores - castration

Residuals of data were not normally distributed and so non-parametric tests were used to analyse the data. A Mann-Whitney-U test (SPSS 13.0; SPSS Inc. Chicago, Illinois, USA) was used to compare between the two treatment groups and the Kruskal - Wallis ANOVA (Graph pad Prism 4.0b) was used to compare between times within each treatment group. Data were presented as mean \pm SE. and $p < 0.05$ was considered significant.

5.3 RESULTS

5.3.1 Ovariohysterectomy

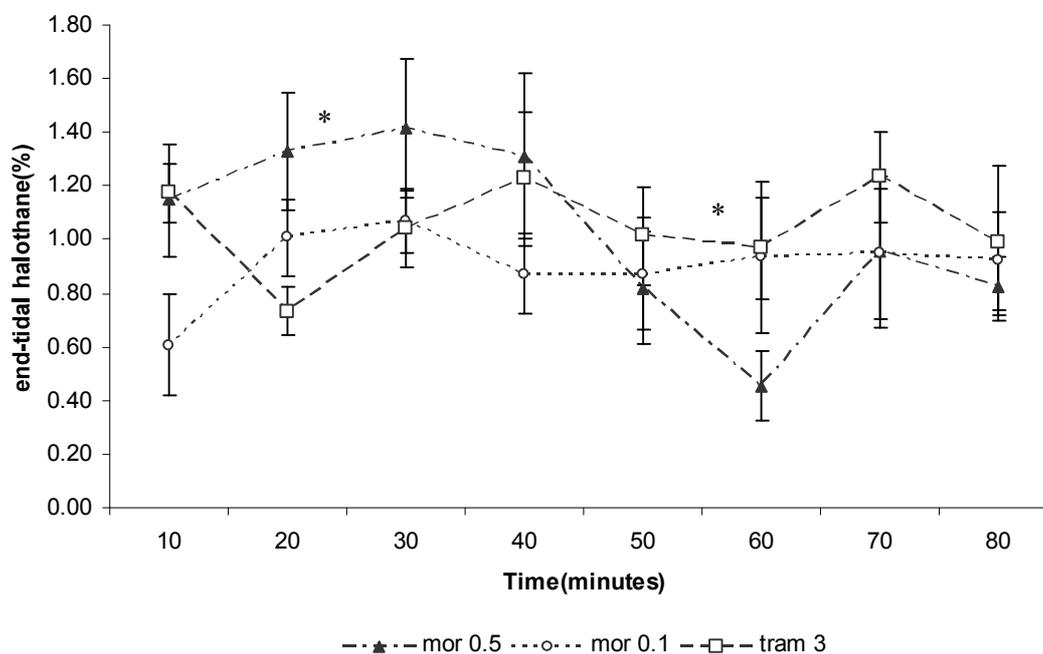


Figure 18: Mean ± SE end-tidal halothane tension (%) in ovariohysterectomised dogs of three treatment groups

*significantly different from morphine (0.5 mg kg⁻¹) (P=0.024; 0.0151).

Table 10: Mean ± SE intra-operative heart rate of ovariohysterectomised dogs given morphine (0.5 mg kg⁻¹, n=8), tramadol (3 mg kg⁻¹, n=8) or morphine (0.1 mg kg⁻¹, n=8) preoperatively, at different surgical time points

Group	Baseline	Skin incision	Ovary 1	Ovary 2	Body of uterus	Skin closure
morphine (control)	122(0.5)	112(0.9)*	109(0.5)*	115(0.7)	114(0.4)	114(0.4)*
tramadol	128(0.9)	116(0.22)*	114(0.5)*	122(0.5)	122(0.9)	121(0.8)
low-dose morphine	126(1.1)	115(0.5)*	115(0.9)	114(0.8)*	114(0.6)*	120(0.8)

*Significantly different from baseline

Preoperatively, vomiting and defaecation were noticed in five out of eight dogs administered morphine (0.5 mg kg⁻¹). None of the dogs given morphine (0.1 mg kg⁻¹) or tramadol (3 mg kg⁻¹) vomited /defaecated. Four out of eight dogs in each of the morphine and tramadol

groups needed rescue analgesia as early as one hour post-surgery, while only one dog in the low-dose morphine group required rescue analgesia by 6 hours post-surgery. The $E_{T_{HAL}}$ differed significantly between the treatment groups (figure 18). Intra-operative RR, systolic blood pressure, end-tidal carbon dioxide tension and SpO_2 did not differ significantly and were within the normal range. Within each treatment group, intra-operative heart rate differed significantly between baseline and different surgical time points (table 10). There was no significant difference in the heart rate between the three treatment groups.

5.3.1.1 EEG responses

Ovariectomy caused statistically non-significant changes in F50 and Ptot between treatment groups (figures 19 and 20). Within each treatment group, there were no significant differences in F50 and Ptot between baseline and different surgical time-points. SEF differed significantly during baseline period, skin incision and ligation of first ovary between treatment groups (figure 21). Dogs of preoperative tramadol (3 mg kg^{-1}) and morphine (0.1 mg kg^{-1}) groups have significantly higher SEF than those received morphine (0.5 mg kg^{-1}) at these surgical time points. There were no significant differences of SEF during ligation of ovary 2 and body of uterus between treatment groups.

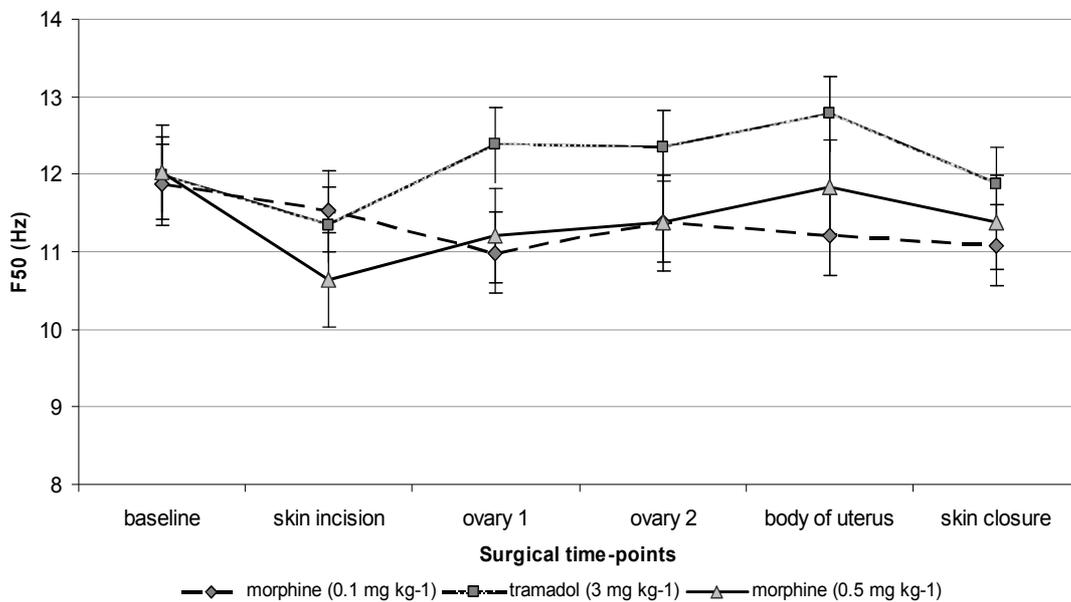


Figure 19: Mean ± SE F50 (Hz) of ovariectomised dogs (n=8) given morphine (0.1 mg kg^{-1}), tramadol (3 mg kg^{-1}) or morphine (0.5 mg kg^{-1}) preoperatively, during baseline and different surgical time-points

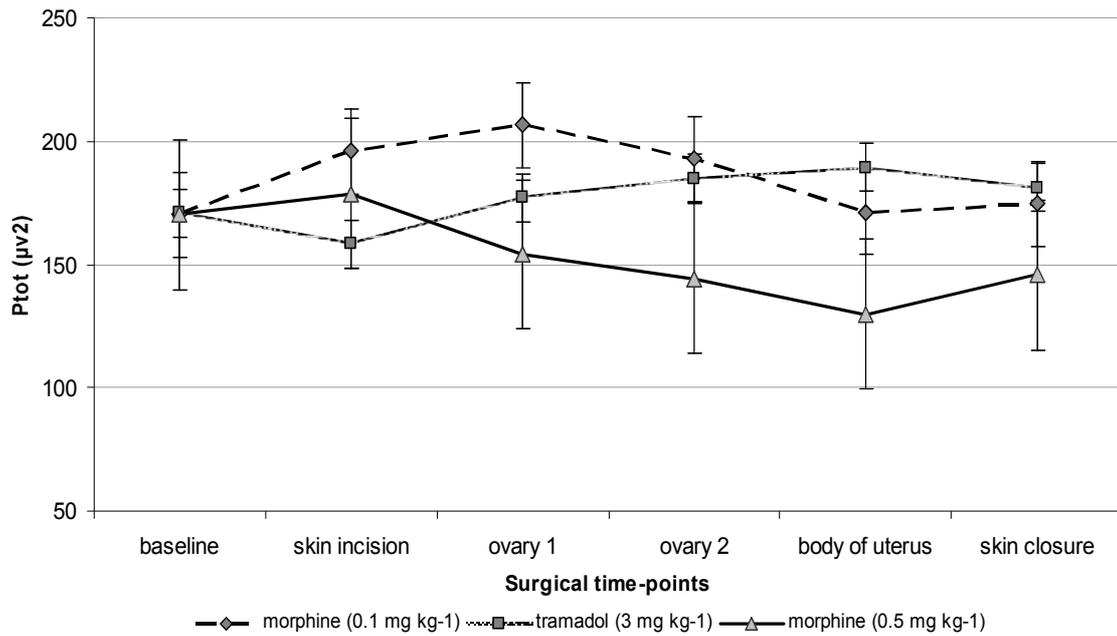


Figure 20: Mean ± SE Ptot (μv^2) of ovariohysterectomised dogs ($n=8$) given morphine (0.1mg kg^{-1}), tramadol (3 mg kg^{-1}) or morphine (0.5 mg kg^{-1}) preoperatively, during baseline and different surgical time-points

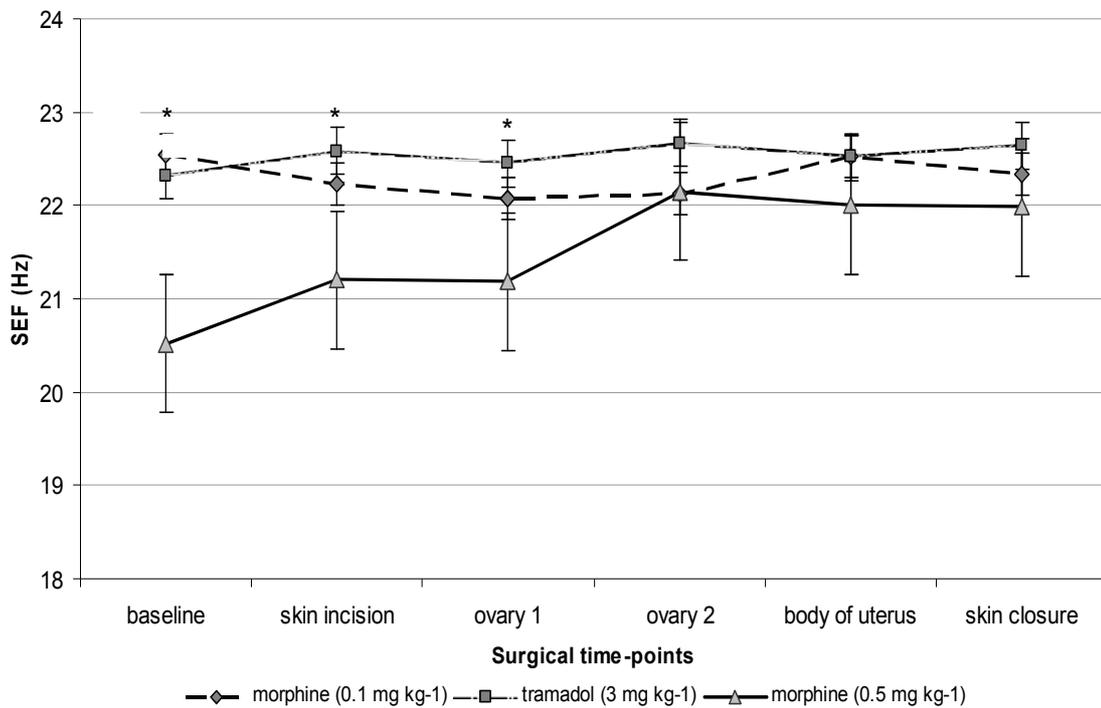


Figure 21: Mean ± SE SEF (Hz) of ovariohysterectomised dogs ($n=8$) given morphine (0.1mg kg^{-1}), tramadol (3 mg kg^{-1}) or morphine (0.5 mg kg^{-1}) preoperatively, during baseline and different surgical time-points

5.3.1.2 Pain and sedation scores

Distribution of dogs in each treatment group, at four different postoperative time points, into three classes of pain intensity (described in table 8) is shown in table 11 below. There was no significant difference in overall mean pain score category between preoperative morphine (0.5 mg kg⁻¹) and tramadol (3 mg kg⁻¹) groups. The overall mean pain score category of dogs administered a combination of preoperative low dose morphine (0.1 mg kg⁻¹) and postoperative tramadol was significantly lower than the other two treatment groups (table 12).

Table 11: Distribution of number of ovariohysterectomised dogs in three treatment groups, into three classes of pain intensity, at different postoperative time points to enable statistical analysis

Group	Class	Number of dogs at postoperative time-points (hrs)			
		1	3	6	9
morphine(control)	1	4	3	4	4
	2	2	1	0	0
	3	2	4	4	4
tramadol	1	2	4	2	4
	2	3	0	2	0
	3	3	4	4	4
low-dose morphine	1	8	8	7	7
	2	0	0	0	0
	3	0	0	1	1

Table 12: Mean and median CMPS-SF pain score categories of ovariohysterectomised dogs of three treatment groups over nine hours postoperatively

Treatment group	Mean pain score category*	Median pain score category
morphine(control)	2.00	2.00
tramadol	2.09	2.00
low-dose morphine	1.12	1.00

* Mean pain score category overall postoperative time points 1= mild, 2=moderate, 3=severe. Statistics (WMWodds) morphine=tramadol ($P=0.655$); low-dose morphine (combination)> morphine ($P=0.001$); low-dose morphine (combination)> tramadol ($P=0.001$)

Distribution of dogs in each treatment group, at four different postoperative time points, into four classes of sedation (described in table 9) is shown in table 13 below. Preoperatively, there was no significant difference in the mean sedation score category between preoperative

low-dose morphine (0.1 mg kg⁻¹) and tramadol (3 mg kg⁻¹) groups. The mean sedation score category of dogs administered morphine (0.5 mg kg⁻¹) was significantly higher than the other two treatment groups, preoperatively (table 14). Postoperatively, morphine (0.5 mg kg⁻¹) produced significantly more sedation than tramadol. There was no significant difference in the level of sedation produced by a combination of low-dose morphine and tramadol (given pre- and post-operatively, respectively) and preoperative tramadol.

Table 13: Distribution of number of ovariohysterectomised dogs in three treatment groups, into four classes of sedation at different postoperative time points to enable statistical analysis

Group	Sedation class	Number of dogs at different time-points				
		Pre-surgery	Post-surgery			
			1 hour	3 hours	6 hours	9 hours
morphine (control)	1	1	1	3	8	8
	2	2	2	3	0	0
	3	1	3	1	0	0
	4	4	2	1	0	0
tramadol	1	6	7	8	8	8
	2	2	1	0	0	0
	3	0	0	0	0	0
	4	0	0	0	0	0
low-dose morphine	1	8	4	7	8	8
	2	0	0	0	0	0
	3	0	1	0	0	0
	4	0	3	1	0	0

Table 14: Mean and median VAS sedation score categories of ovariohysterectomised dogs of three treatment groups during pre- and post-operative periods

Treatment group	Preoperative		Postoperative	
	Mean sedation score category*	Median sedation score category	Mean sedation score category**	Median sedation score category
morphine (control)	3.0	3.5	1.69	1.00
tramadol	1.25	1.00	1.03	1.00
low-dose morphine	1.00	1.00	1.44	1.00

* Mean sedation score category 1= 0-24 (sedation score), 2=25-49, 3=50-74 and 4=75-100. Statistics (WMWodds) preoperatively morphine > tramadol ($P=0.000$); morphine> low dose mmorphine (combination, $P=0.000$); tramadol=low dose morphine (combination, $P=0.404$). Postoperatively morphine > tramadol ($P=0.010$); morphine= low dose morphine (combination, $P=0.223$); tramadol=low dose moorphine (combination, $P=0.449$)

5.3.2 Castration

There were no significant differences between the two treatment groups with regard to dogs' weight, age and duration of surgery. Three dogs vomited after premedication with morphine prior to anaesthesia induction. No such side effects were seen with tramadol. None of the dogs given preoperative morphine (0.5 mg kg^{-1}) or tramadol (3 mg kg^{-1}) required rescue analgesia at any postoperative time point. Intra-operative heart rate, RR, $E_{T \text{ HAL}}$ (figure 22) and $E_T \text{ CO}_2$ did not differ significantly between the two treatment groups and were within the normal range.

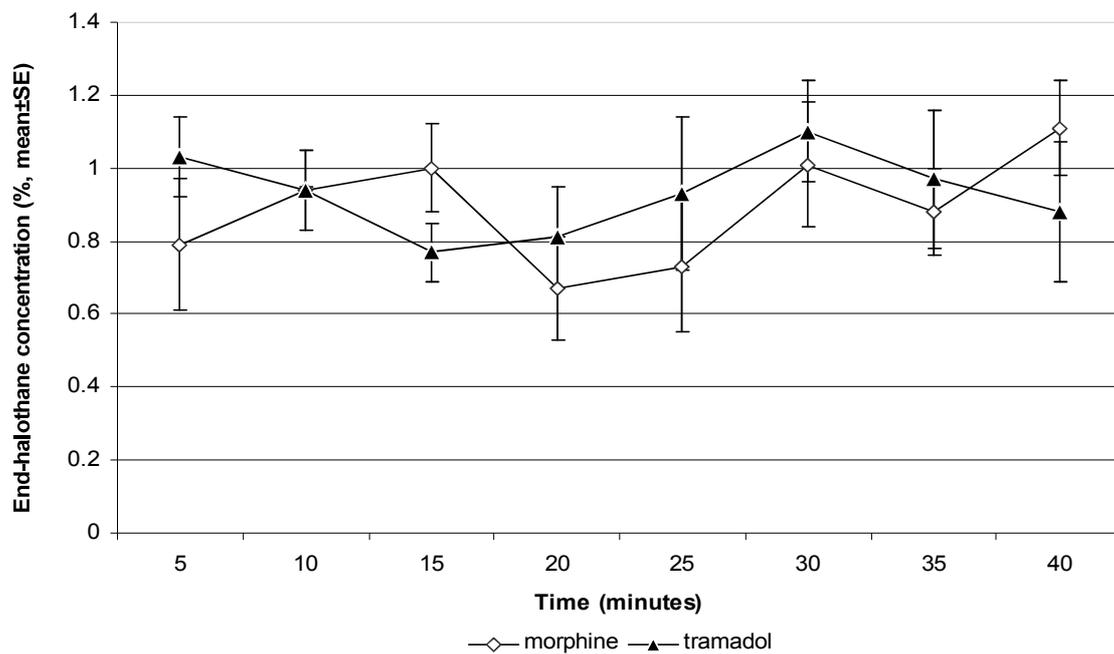


Figure 22: Mean±SE of end-tidal halothane tension (%) in castrated dogs (n=8) given tramadol (3 mg kg^{-1}) or morphine (0.5 mg kg^{-1}) preoperatively

5.3.2.1 EEG responses

In dogs of tramadol group F50 increased significantly during removal of testicle 1 from baseline ($P=0.0025$). There was no significant difference in F50 between baseline and surgical time-points in morphine group. F50 of tramadol (3 mg kg^{-1}) was significantly higher than that of morphine (0.5 mg kg^{-1}) at testicle 1 ($P=0.049$; figure 23). Ptot decreased significantly during testicle 1 from baseline ($P=0.033$) in dogs of tramadol group. No statistically significant difference was found in Ptot between baseline and surgical time-points in morphine group (figure 24). No statistically significant difference in SEF was found within or between the two treatment groups (figure 25).

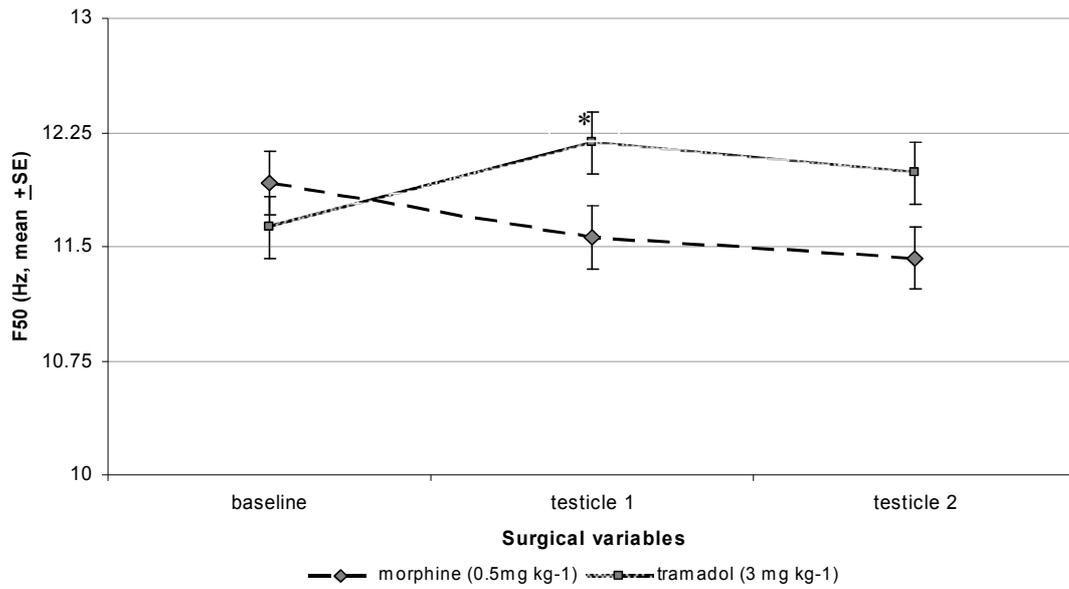


Figure 23: Mean±SE F50 (Hz) of castrated dogs (n=8) given morphine (0.5mg kg⁻¹) or tramadol (3 mg kg⁻¹) preoperatively, during baseline and two different surgical time points

* significantly different from morphine (0.5 mg kg⁻¹) (p=0.049)

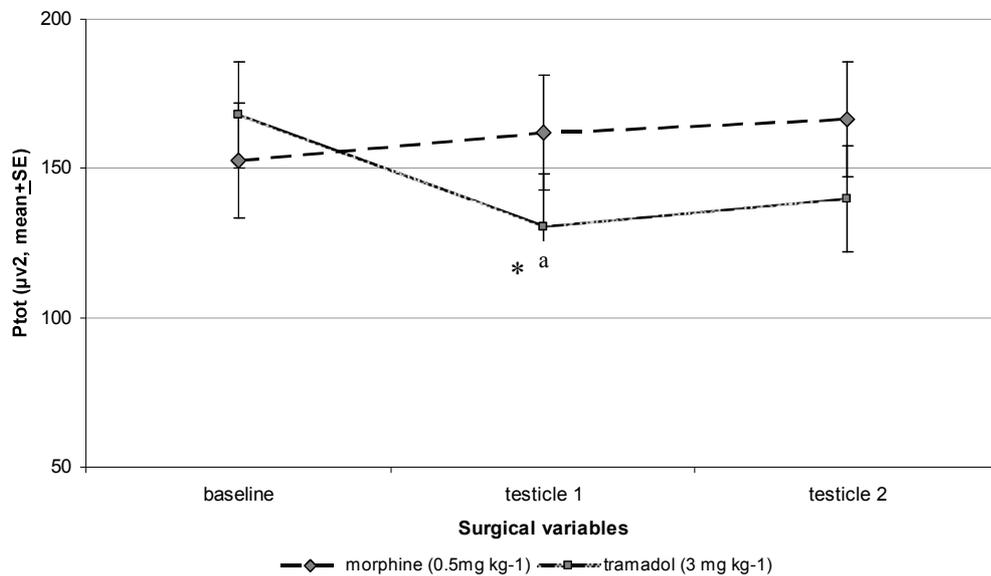


Figure 24: Mean±SE Ptot (µv²) of castrated dogs (n=8) given morphine (0.5mg kg⁻¹) or tramadol (3 mg kg⁻¹) preoperatively, during baseline and two different surgical time points

* tramadol significantly (p=0.042) different from morphine

^a testicle 1 significantly (p=0.033) different from baseline in case of tramadol

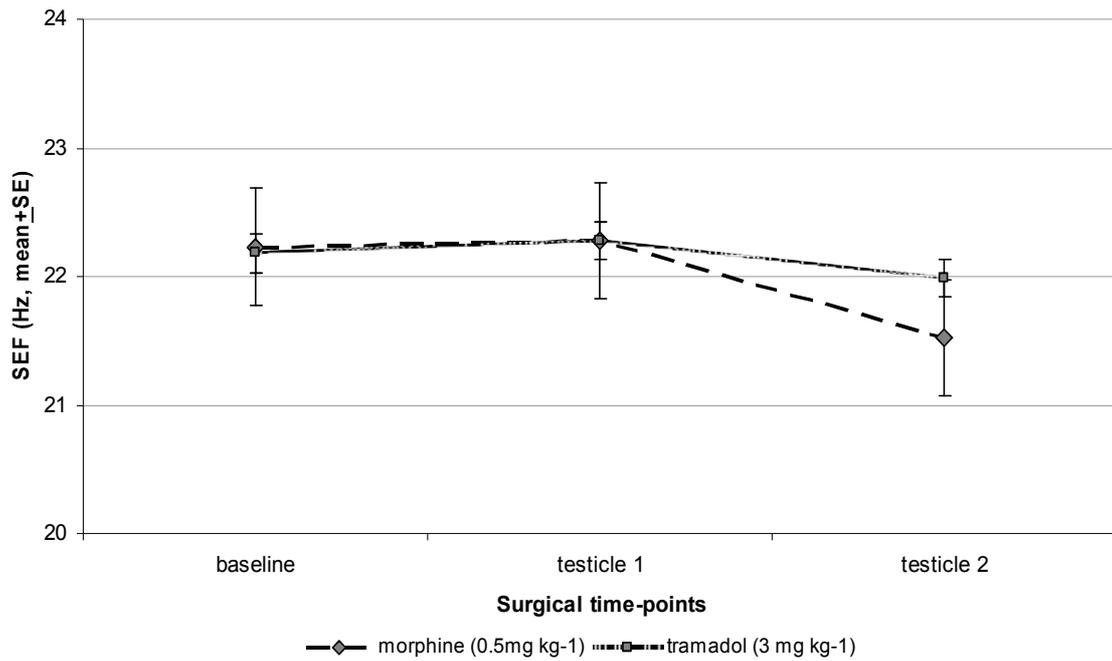


Figure 25: Mean±SE SEF (Hz) of castrated dogs (n=8) given morphine (0.5mg kg⁻¹) or tramadol (3 mg kg⁻¹) preoperatively, during baseline and two different surgical time points

5.3.2.2 Pain and sedation scores

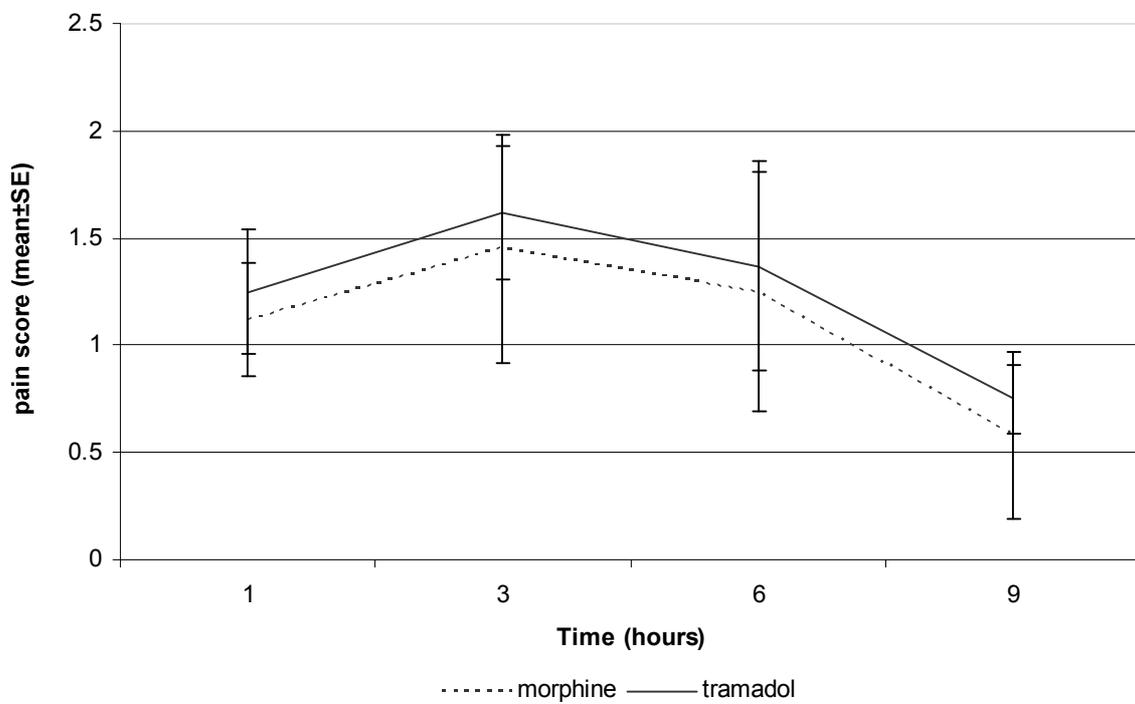


Figure 26: Mean±SE pain score of dogs given preoperative morphine 0.5 mg kg⁻¹ (n=8) or tramadol 3 mg kg⁻¹ (n=8), at 1, 3, 6 and 9 hours after castration

There were no significant differences in the mean CMPS-SF pain score of dogs given preoperative morphine 0.5 mg kg^{-1} or tramadol 3 mg kg^{-1} . The pain scores did not vary significantly with time in the postoperative period, in both treatment groups (figure 26). Preoperative VAS sedation score of dogs given morphine was significantly higher than that of tramadol ($P=0.01$). Postoperative sedation scores were higher at one hour and fell gradually to zero in dogs administered morphine. At this time point (one hour), dogs given tramadol had significantly less sedation score than those given morphine.

5.4 DISCUSSION

5.4.1 Ovariohysterectomy

5.4.1.1 EEG responses

This study did not find significant changes in F50 and Ptot between baseline and noxious surgical events within each treatment group or between the three treatment groups, morphine (0.5 mg kg^{-1}), tramadol (3 mg kg^{-1}) and morphine (0.1 mg kg^{-1}) administered preoperatively to dogs undergoing ovariohysterectomy. Significant changes in intra-operative EEG frequency spectrum have been reported during ovariohysterectomy in dogs (Trucchi et al 2003). Studies in humans, horses, red deer and calves have demonstrated that surgical stimuli produce significant changes in F50 and Ptot (Murrell et al. 2003; Haga & Ranheim 2005; Johnson et al. 2005b; Gibson et al. 2007), and preoperative lignocaine (given IV and ring block) blocked the changes in these two EEG variables. Also, a series of experiments (Johnson 1996; Johnson & Taylor 1997, 1999; Johnson et al. 2000) in halothane anaesthetised, un-stimulated ponies revealed that drugs of well-known analgesic efficacy caused a reduction in F50. Collectively, results from these studies suggest that changes in F50 and Ptot may predominantly be linked to nociception. In contrast, studies in rats, horses and pigs found no significant changes in EEG frequency spectrum in response to noxious surgical and tail clamp stimuli (Miller et al. 1995; Haga & Dolvik 2002 & 2005; Murrell et al. 2008).

Murrell & Johnson (2006) reviewed the studies investigating the EEG indices of nociception in humans and animals, and concluded that the discrepancy in the findings of EEG correlates of nociception was due to variation in experimental conditions that need to be stabilised. They are; 1. depth of anaesthesia, 2. type of anaesthetic agent and 3. severity of noxious stimulation. In addition, use of induction agents with anti-nociceptive/ anti-analgesic efficacy, agents for stabilising intra-operative haemodynamic variables (e.g. MAP and heart

rate) and neuromuscular blockers may influence the EEG responses besides brain activity (Miller et al. 1995; Murrell et al. 2000; Murrell et al. 2002).

There are some possible explanations for not finding the significant differences in EEG indices of nociception/anti-nociception between the three treatment groups in the present study.

1. $E_{T_{HAL}}$: There were statistically significant differences in $E_{T_{HAL}}$ between the treatment groups (figure 18). This finding is consistent with that of Mastrocinque & Fantoni (2003) in dogs undergoing ovariohysterectomy. Change in depth of anaesthesia may influence the response generated by the cerebral cortex. An increase in anaesthetic depth has been shown to increase the latency and decrease the amplitude of somatosensory evoked potentials from the cerebral cortex in halothane anaesthetised sheep (Gibson et al. 2007; personal communication). Experiments conducted under stable alveolar tension of halothane could find significant changes in EEG indices of nociception and drug induced anti-nociception in horses, sheep, red deer, pigs, tamar wallaby, rats and calves with a variety of noxious stimuli (Murrell et al. 2003, 2005; Johnson et al. 2005a, 2005b; Haga & Ranheim 2005; Diesch et al. 2005; McGregor 2005; Murrell et al. 2007; Gibson et al. 2007).
2. Four dogs in morphine (0.5 mg kg^{-1}) group, four dogs in tramadol (3 mg kg^{-1}) group and two dogs in low dose morphine (0.1 mg kg^{-1}) group received 2-3 injections of IV fentanyl ($1 \text{ } \mu\text{g kg}^{-1}$) intraoperatively. Also, one dog in low- dose morphine group received a bolus dose of 2.5 mg kg^{-1} ketamine during surgery. Fentanyl is a potent μ -opioid receptor agonist and has been demonstrated to be altering the amplitude and the frequency of the EEG in un-stimulated dogs (Wauquier et al. 1981). Ketamine is an N-methyl-D-aspartate receptor antagonist (Aida et al. 1994), which has been found to reduce the F50 of the EEG of halothane anaesthetised ponies (Johnson & Taylor 1999).
3. Anaesthesia was induced with IV thiopentone in dogs of all treatment groups. The terminal elimination half life of thiopentone (20 mg kg^{-1}) in dogs after IV administration was said to be 6.9 ± 2.1 hours (mean \pm sd, EMEA 1999). A significant alteration in EEG variables, F50 and P_{tot} , has been demonstrated in ponies given IV

infusion of thiopentone (Murrell et al. 2000). In the present study, EEG was recorded 10-15 minutes after anaesthetic induction with IV thiopentone in all dogs.

An important limiting factor in the current study was the lack of untreated (negative) control group to compare with the effects of three treatments. Though morphine (positive control) has been described as a well-known yard stick for efficacy comparison (Brodbelt et al. 1997), it is unclear whether the observed non-significant difference in the EEG indices of nociception between the treatment groups including low dose morphine (0.1 mg kg^{-1}) is because of the diverse experimental conditions or equal efficacy of tramadol (3 mg kg^{-1}) and morphine (in both 0.5 and 0.1 mg kg^{-1} dose rates). However, a dose-dependent increase in efficacy has been demonstrated with intrathecal morphine in the rat model of neuropathic pain (Hwang et al. 2000). Also, the F50 of the group treated with tramadol was significantly higher than that of the group treated with morphine during castration (this study). A negative control group was not included on ethical grounds as this surgery is likely to cause severe pain.

Ovariohysterectomy induces a distinct viscerosomatic nociception in rats and dogs (Lascelles et al. 1995; 1997; Gonzalez et al. 2000). Procedures like skin incision, body wall incision and skin closure are presumably associated with somatic nociception whereas ligation of ovaries is predicted to be associated with visceral nociception (Gayner & Muir 2002). The ligation of the uterine body proximal to the cervix has been considered to be linked to both types of nociception (McGregor 2005). Stimuli that activate the visceral afferents are distension, ischemia and inflammation (exemplified by hollow organs such as colon which are sensitive to distension and inflammation but are insensitive to cutting and burning, Al-Chaer & Traub 2002). McGregor (2005) from the rat model of ovariohysterectomy performed under controlled experimental conditions found that the Ptot produces more consistent and significant responses to noxious surgical time-points (including skin incision, ligation of ovary and body wall suturing) than F50. In the present study, EEG data were taken during baseline, skin incision, and skin closure, and from clamping through ligation of both ovaries and uterine body with the view of comparing changes in EEG spectral frequencies that may specify the nociception/anti-nociception. However, confounding experimental factors described above may have obtunded the generation of significant EEG responses between the treatment groups.

Spectral edge frequency 95% of the EEG power spectrum showed significant differences between tramadol and morphine groups. This component of the EEG power spectrum reflects more about general CNS depression than nociception (Johnson & Taylor 1999; Murrell et al. 2003, 2005; Chapter 6). Significant differences in overall E_{THAL} (during both anaesthesia and surgery) between treatment groups may be associated with SEF changing. But this study was not designed to study the linear relationship between SEF and overall E_{THAL} changes and no attempt was made to compare these statistically.

The heart rate of dogs in all treatment groups decreased significantly from baseline through different surgical time points (table 10). This finding contrasts with that of dogs in the study reported in chapter 6 where heart rate increased transiently following electrical stimulation. Similar to the present study, dogs in that study received morphine ($0.5 \text{ mg kg}^{-1} \text{ SC}$) and tramadol ($3 \text{ mg kg}^{-1} \text{ SC}$) before anaesthesia and noxious electrical stimulation. The discrepancy in the heart rate changes may be due to difference in the type and intensity of noxious stimulation inducing changes in sympathetically mediated cardiovascular variables.

5.4.1.2 Pain scores

Pain is an ‘abstract’ phenomenon and animals are unable to verbalise their sensation (Reid et al. 2007). Immediate and appropriate assessment of postoperative pain is important for evaluation of analgesic efficacy that also aids in optimal pain control (Lascelles et al. 1994). Significant variation in physiological observations has been demonstrated to be a non-specific indicator of pain (Conzemius et al. 1997; Hansen et al. 1997; Holton et al. 1998). Use of changes in behaviour (non-interactive) of undisturbed animals coupled with responses to handling of the animal and its surgical site (interactive behaviour) has been reported to be the most effective clinical tool for rapid evaluation of postoperative pain (Waterman & Kalthum 1989; Lascelles et al. 1994). The combination of non-interactive and interactive behavioural changes serves as a basic template for constructing different pain scales.

Because of its reported ease of use and quick applicability in the clinical setting, the CMPS-SF has been chosen to score the pain related dog behaviours that indirectly measure the efficacy of test drugs, in the present study. The statistical analyses of pain and sedation scores were complicated by dogs receiving rescue analgesia as early as one hour post-surgery in both preoperative morphine and tramadol groups. The ‘last observation carried forward’ method used by Slingsby and Waterman-Pearson (1998, 2000, 2001) cannot be applied to

this study as dogs received rescue analgesia at the beginning of postoperative pain assessment (one hour). Keeping the sample size in view (8 dogs per treatment group), we used the ‘pain score categorisation’ method suggested by Slingsby et al. (2006) to analyse the treatment effects instead of removing the dogs which received rescue analgesia at one hour postoperatively. Though this kind of statistical analysis lets us assess the overall effect of the treatments, it may not allow for comparison of treatment effects at different postoperative time points.

In the current study, the dogs treated with a combination of preoperative low-dose morphine (0.1 mg kg^{-1}) and postoperative tramadol (3 mg kg^{-1}) had significantly lower overall mean pain score than the dogs treated with either preoperative morphine (0.5 mg kg^{-1}) or tramadol (3 mg kg^{-1}) alone. Clinically, the difference between preoperative analgesia just covering the intra-operative period and the immediate postoperative analgesia covering the early postoperative period has been demonstrated using short acting μ -opioid agonist pethidine in dogs undergoing ovariohysterectomy (Lascelles et al. 1997). The pain scores of dogs given postoperative analgesia only were significantly lower in the early postoperative period (until one hour) whereas those of dogs administered preoperative analgesia only were significantly lower in the later postoperative period (from 8 hours to 20 hours). Interestingly, Gordon et al (2002) in a human dental pain model demonstrated that a combination of pre- and post-operative analgesia with a local anaesthetic produced better analgesia of longer duration than preoperative administration of local anaesthetic alone. At this instance it is noteworthy to mention Kissin’s (2005) review on preoperative analgesia concluding that more complete prevention of nociceptive input generated during surgery and the initial postoperative period has greater clinical value.

Results of this study appear to support the concept of ‘complete prevention’ during and after surgery as the ‘combination’ produced better analgesia. The dogs treated with 0.1 mg kg^{-1} morphine showed no side effects in contrast to those that were given 0.5 mg kg^{-1} morphine. There was no significant difference in the overall mean pain score category between preoperative morphine 0.5 mg kg^{-1} and tramadol 3 mg kg^{-1} . This finding is consistent with that of Mastrocinque & Fantoni (2003) in dogs undergoing ovariohysterectomy. Four dogs in each group (both morphine 0.5 mg kg^{-1} and tramadol 3 mg kg^{-1}) required intervention analgesia. Inclusion of another group of dogs with no treatment (negative control) would

have more clearly demarcated the efficacy of each test drug in its own right (Mastrocinque & Fantoni 2003). It was not possible because of ethical concerns.

The chief characteristics of any pain scale or instrument are sensitivity and responsiveness to changes caused by analgesic administration (Reid et al. 2007). With the pain scores obtained using CMPS-SF in this study we were able to compare the efficacy of test drugs administered at two different time points related to surgery. Both non-interactive and interactive behaviour assessment was accomplished by picking a suitable scoring system to describe a dog's behaviour according to six defined categories under four sections.

Postoperative pain was assessed at 1, 3, 6, 9 hours in this study. It was not possible to accurately determine pain scores at 20 minutes postoperatively as the dogs were deeply sedated at this time point, making identification of pain-related behaviour difficult. The behavioural category ranking the response to pressure 2 inches around the incision site could be scored only. The other behavioural categories with different pain descriptors such as general demeanor (quiet or crying, attention to wound etc.), response to handling (ability of the dog to rise or walk) and the overall behaviour of the dog (happy or non-responsive to surroundings etc.) could not be judged accurately at this time. In the early postoperative period, up to an hour, sedation may mask the overt behaviour of pain (Slingsby & Waterman-Pearson 1998). Dogs administered morphine (0.5 mg kg^{-1}) preoperatively had highest sedation score 20 minutes postoperatively (author's observation).

Tramadol has been shown to have equal efficacy with morphine in ovariohysterectomised dogs (Mastrocinque & Fantoni 2003). As in the current study, the investigators in that study did not have an untreated group because of ethical reasons. To date, there have been no dose titration studies of morphine in dogs (Kukanich et al. 2005). The dosage of morphine ranges from $0.05\text{-}2 \text{ mg kg}^{-1}$ intravenously or intramuscularly or subcutaneously every 2-6 hours (Carroll 1999; Wagner 2002). This range has been recommended based on clinical impressions or subjective visual assessment of analgesia or pain that seems to be significantly variable (Kukanich et al. 2005). Also, no dosage recommendations are available for tramadol in dogs as it has not been investigated extensively in this species. In the present study, inclusion of another treatment group, comprising 'combination' of preoperative morphine and postoperative tramadol, aided in revealing the best pain management strategy for this surgical procedure, though preoperative tramadol and morphine produced similar degree of

analgesia. However, as the surgery times were relatively long, the improved analgesia shown by the combination group could be due to tramadol given at extubation rather than being a reflection of the combination as such. Use of another treatment group that received postoperative treatment only would have more clearly demarcated the benefit of ‘complete prevention’, in this study. Availability of dogs and time were the two factors that restricted the inclusion of postoperative analgesic group.

5.4.2 Castration

5.4.2.1 EEG responses

Castration caused a significant change in EEG variables recorded under stable experimental conditions, between morphine and tramadol groups. In contrast to ovariohysterectomised dogs, none of the dogs castrated received a fentanyl or ketamine bolus intraoperatively and there was no significant difference in $E_{T\text{HAL}}$ between the two treatment groups. In the present study, F50 increased and Ptot decreased significantly from baseline, during removal of a testicle 1 in tramadol group. In contrast, morphine obtunded the F50 and Ptot changing from baseline, during removal of both testicles. Murrell et al. (2003, 2005) found a significant increase in F50 and a decrease in Ptot in horses castrated under stable halothane anaesthesia, which was abolished by preoperative administration of lignocaine. Results from this part of the study seem to be consistent with the findings of Murrell et al. (2003, 2005) in horses.

F50 in the group receiving tramadol was significantly higher than the group receiving morphine during ligation of the spermatic cord of testicle 1. This finding is consistent with the results of the study reported in chapter 6, where in the dogs have been subjected to acute noxious electrical stimulation. Electrophysiological studies suggest that μ -receptor agonist opioids block the afferent passage of nociceptive information by acting pre- and post-synaptically in the spinal dorsal horn (Duggan & North 1984). Clinically, this has been demonstrated by preoperative administration of pethidine (a μ -receptor agonist) blocking the development of central hypersensitivity in terms of a significant decrease in VAS pain scores (Lascelles et al. 1997). During castration, tension on spermatic cord and cremaster muscle may cause visceral nociception (Gaynor & Muir 2002). Because of its weaker binding to μ -receptors (Raffa et al. 1992), tramadol might have failed to prevent the afferent noxious transmission to the same extent as morphine (0.5 mg kg^{-1}). This can be seen as an increase in F50 compared to morphine, during removal of testicle 1 in the present study.

P_{tot} of tramadol (3 mg kg⁻¹ SC) treated dogs has decreased significantly from baseline during removal of testicle 1, and it was significantly less than that of the morphine treated group. Decrease in P_{tot} is linked to nociception (Murrell et al. 2003) and related to a decrease in depth of anaesthesia during noxious stimulation. It represents a different aspect of nociception than F50 (Murrell et al. 2003). There were no significant differences in P_{tot} from baseline during removal of each testicle in the morphine (0.5 mg kg⁻¹ SC) group. This finding is consistent with that of Murrell et al. (2005) and Gibson et al. (2007) in horses and calves administered preoperative lignocaine. However, in the study reported in chapter 6, morphine (0.5 mg kg⁻¹ SC) did not prevent the decrease in P_{tot} after noxious electrical stimulation in dogs. This discrepancy may likely be due to the difference in the type and intensity of noxious stimulation. Changes in SEF might represent the level of general CNS depression. There were no significant differences in SEF between the morphine and tramadol groups. Also, overall ET_{HAL} did not differ significantly between the two groups. These findings further support the concept that SEF is more related to the depth of general anaesthesia.

5.4.2.2 Pain and sedation scores

Inhibition of 5-HT and NA reuptake by central neurones is another mechanism of tramadol analgesia in addition to weak μ -receptor affinity (Raffa et al. 1992). Both 5-HT and NA are the nociceptive modulatory transmitters in the descending inhibitory pathway. The spinal dorsal horn neurones exert a potential nociceptive modulatory effect prior to dispatching the noxious input to supraspinal centres (Millan 2002). There were no significant differences in the CMPS-SF pain score between the tramadol and morphine groups at any postoperative time point. Also, there were no significant differences in F50 between the morphine and tramadol groups during castration of testicle 2. It seems possible that further activation of descending inhibitory mechanism might produce analgesia though tramadol allows for some afferent noxious transmission initially due to weak μ -receptor affinity.

Preoperative tramadol (2 mg kg⁻¹, 3 mg kg⁻¹) showed efficacy equal to morphine (0.2 mg kg⁻¹, 0.5 mg kg⁻¹) and superior to butorphanol (0.2 mg kg⁻¹) in dogs undergoing ovariohysterectomy (Mastrocicque & Fantoni 2003; Paolo et al. 2004; this study). In these three studies tramadol had been given intravenously, intramuscularly and subcutaneously, respectively. It appears that tramadol can produce analgesia in dogs given by any of these routes. However, subcutaneous drug administration along with routine preanaesthetic medication has been noticed to be easier, to conscious dogs in this study.

Morphine caused significantly more sedation in dogs preoperatively than tramadol. Our anaesthetist/anaesthesia technicians were happier to handle morphine administered (more sedated) dogs for IV anaesthetic induction. Postoperatively, it is important to identify and interpret the clinical signs of pain which the animal should be able to show overtly. Masking by sedation makes pain difficult to assess accurately and design appropriate pharmacological interventions. Tramadol caused significantly less sedation postoperatively than morphine. However, as the pain has been assessed starting from one hour postoperatively level of sedation has not influenced the pain assessment with CMPS-SF in dogs given morphine. One important limitation of this study is the small sample size. This was due to the difficulty in recruiting cases, availability of the EEG recording equipment and time.

5.5 CONCLUSION

Both preoperative tramadol and morphine provided an equal degree of postoperative analgesia in ovariohysterectomised or castrated dogs. In ovariohysterectomised dogs, a combination of preoperative low-dose morphine that caused no side effects, and postoperative tramadol produces better postoperative analgesia than either drug given alone preoperatively. Stable intra-operative experimental conditions are required to find a significant difference in EEG indices of nociception / anti-nociception, between different treatment groups. The Glasgow CMPS-SF can be used satisfactorily to evaluate the efficacy of different classes of analgesics in the postoperative period. Further studies are required with a large dog population to compare the efficacy of these two analgesics administered in different dose rates and times of surgery, for better postoperative analgesia.

CHAPTER 6

ELECTROENCEPHALOGRAPHIC RESPONSES OF TRAMADOL, PARECOXIB AND MORPHINE TO ACUTE NOXIOUS ELECTRICAL STIMULATION IN ANAESTHETISED DOGS

6.1 INTRODUCTION

A sensitive and specific way to evaluate pain could deliver significant benefits to the clinical management of patients with painful conditions. This is especially so when dealing with animals because of the absence of verbal communication. EEG is a record of the spontaneous electrical activity from the surface of the cerebral cortex. It has been considered as a reliable representative of the CNS function as it directly indicates the activity of cortical neurones (chiefly bipolar type) that are believed to be its primary generators (Silva 2004). Also, it can indirectly reflect the activity of lower centres of the brain (such as the brain stem reticular formation and the thalamus) as it has been shown that these centres have a potential regulatory influence on cortical neuronal function especially during periods of unconsciousness and general anaesthesia ((Moruzzi & Magoun 1949; Antognini et al. 2000, 2003). In this connection, it should be noted that these brain stem structures possess diffuse connections with the other lower brain areas (such as PAG, RVM etc.) that play a major role in the spinal nociceptive processing through descending modulation (Millan 2002).

It has been demonstrated that changes in the EEG activity correlate with verbal reports of the experience of pain in humans (Chen et al. 1989; Chang et al.2002). Correlation of EEG spectral frequency changes with behavioural responses to nociceptive stimulus in conscious sheep (Ong et al. 1997) further support this. Changes in EEG power spectrum have been used to objectively quantify nociception in anaesthetised rats, horses, lambs, deer and calves (Otto et al.1996; Murrell et al. 2003; Johnson et al. 2005a; 2005b; Haga & Ranheim 2005; Murrell & Johnson 2006; Gibson et al. 2007).

The association of changes in EEG spectral frequencies with nociception seems to be variable. The type and depth of anaesthesia, together with type and intensity of noxious stimulus influence EEG responses (Murrell & Johnson 2006; Murrell et al. 2007; chapter 5). However, the minimal anaesthesia model using halothane at a stable and minimal partial pressure has been shown to produce consistent EEG responses across different animal species with different modalities of noxious stimuli (Murrell et al. 2003; Johnson et al. 2005a; 2005b; Haga & Ranheim 2005; Murrell et al. 2007; Gibson et al. 2007). The validity of this model has yet to be evaluated in dogs.

Quantitative analysis of EEG power spectra has also been used to evaluate the efficacy of opioids and medetomidine in anaesthetised dogs and to correlate EEG changes with the pharmacokinetic properties of these agents (Wauquier et al. 1981; Short et al. 1992; Hoke et al. 1997). However, none of these studies have focussed on the effects of different classes of analgesics on EEG frequency spectrum in response to noxious stimulation in dogs.

Opioids and NSAIDs are commonly used to provide analgesia in dogs. Morphine is the prototype opioid and has been widely used as a gold standard for comparing the efficacy of alternative agents in analgesic studies (Brodbeck et al. 1997). Tramadol is a centrally acting analgesic with several mechanisms of action (Hennies et al. 1988; Raffa et al. 1992; Driessen et al. 1993), acting as a weak μ -opioid agonist and inhibiting synaptic re-uptake of 5-HT and NA into central neurones. Formation of M₁, a major metabolite of tramadol, was shown to be important for the effect of tramadol on experimentally induced pain in people (Poulsen et al. 1996). In dogs, M₁ production from the parent compound has been demonstrated to be low (Wu et al. 2001; Kukanich et al. 2004). Parecoxib sodium is a water-soluble injectable pro-drug of valdecoxib, a recently introduced NSAID which selectively inhibits COX-2 in human tissues (Talley et al. 2000). Parecoxib has been demonstrated to be safe and effective in different human pain models (Daniels et al. 2001; Rasmussen et al. 2002; Barton et al. 2002).

Noxious electrical stimuli can be used to assess the efficacy of centrally acting agents (Le Bars et al. 2001). They produce synchronised afferent signals that are suitable for the study of EEG responses (Murrell et al. 2007). The primary aim of this study was to compare the effects of tramadol and parecoxib on EEG responses to electrical stimulation, with those of morphine (positive control) and saline (negative control) in anaesthetised dogs. The validity of the minimal anaesthesia model in producing reliable EEG responses in dogs was also tested.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design

Eight healthy adult mixed breed dogs (4 male, 4 female) weighing 23.5 ± 2.5 kg (mean \pm SE) were used in this study. The study was approved by the Massey University Animal Ethics Committee. There were four treatment groups: morphine 0.5 mg kg^{-1} , tramadol 3 mg kg^{-1} , parecoxib 1 mg kg^{-1} , or 0.9% saline injected SC. Due to limited availability of dogs for use in

experimentation, the combination of tramadol and parecoxib was not tested. Every dog received each drug in a cross-over design with a two-week interval between treatments. The sequence of drugs administered was randomised for each dog.

6.2.2 Anaesthesia

Dogs were fasted for 12 hours prior to anaesthesia with free access to water. They were given pre-anaesthetic medication with acepromazine (0.05 mg kg^{-1}) SC. Test drugs were given SC, at the time of pre-anaesthetic medication. Forty five to sixty minutes after pre-anaesthetic medication anaesthesia was induced with IV propofol to effect ($3.1 \pm 0.6 \text{ mg kg}^{-1}$, mean \pm SE). After endotracheal intubation anaesthesia was maintained with halothane in oxygen. Dogs were given Hartmann's solution IV to maintain systolic arterial blood pressure above 100 mmHg throughout the anaesthetic period and their systolic blood pressure was recorded every five minutes using a Doppler ultrasound pressure transducer with cuff. Airway gases were sampled continuously from the end of endotracheal tube connected to the circle system, using an anaesthetic gas analyser (Hewlet Packard M1025B; Hewlet Packard, Hamburg, Germany) and the vaporizer adjusted to maintain $E_{T\text{HAL}}$ between 0.85% and 0.95%. All animals breathed spontaneously throughout anaesthesia and $E_{T\text{CO}_2}$ was maintained between 4.60 KPa and 5.92 KPa (35 mmHg and 45 mmHg). Oesophageal temperature was monitored in all animals using a thermistor probe and was maintained between 37°C and 38°C . Arterial haemoglobin SpO_2 was monitored with a pulse oximeter.

6.2.3 EEG and ECG recording

The EEG was recorded (figure 27) as described in chapter 3. A 10 minute EEG baseline was recorded commencing 90 minutes after induction of anaesthesia to allow metabolism of the induction dose of propofol. This was followed by the application of a supramaximal electrical stimulus (50 volts at 50 Hz for 2 s, Valverde et al. 1989), using a Grass Stimulator (S48K square pulse stimulator, Astro-Med Inc., Grass instrument division). Stimulus was applied to the right hindlimb (lateral aspect of the distal metatarsus) through two stainless steel needle electrodes placed subcutaneously two centimeters apart. EEG data were collected for 10 minutes after electrical stimulation. Halothane was discontinued immediately following this and the dogs were extubated as soon as they were able to maintain their own airway. ECG was recorded continuously in a lead II electrode configuration. The analysis of the raw EEG data (figure 28) was carried out off line after the completion of each experiment, as described

in chapter 3. The three EEG variables of interest (F50, SEF and Ptot) were derived from sequential power spectra generated by FFT.

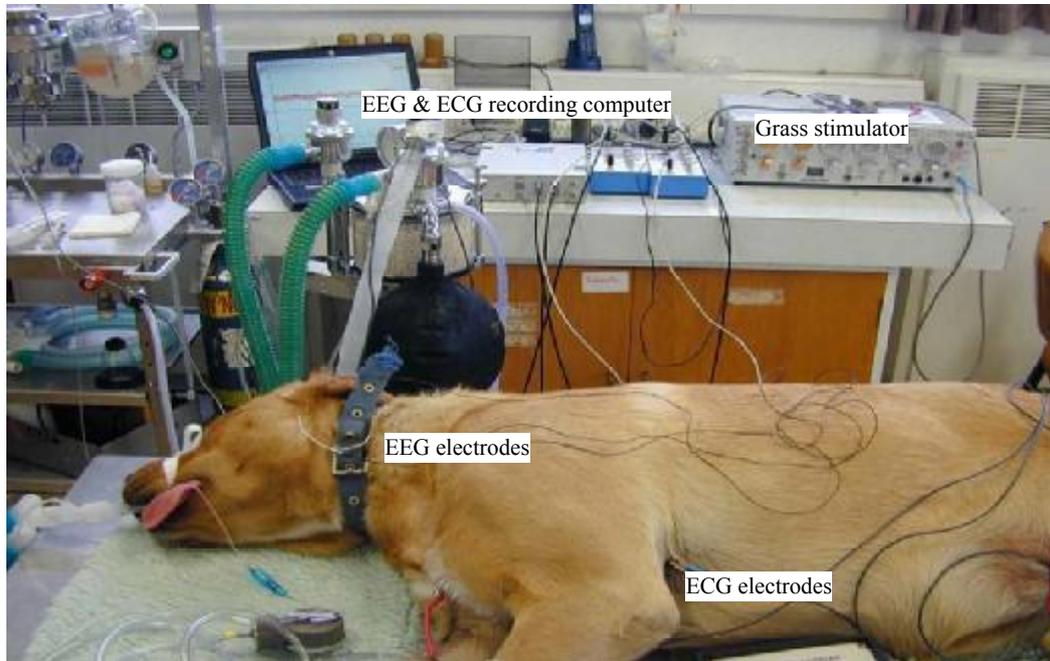


Figure 27: EEG and ECG recording in an anaesthetised dog subjected to electrical stimulation

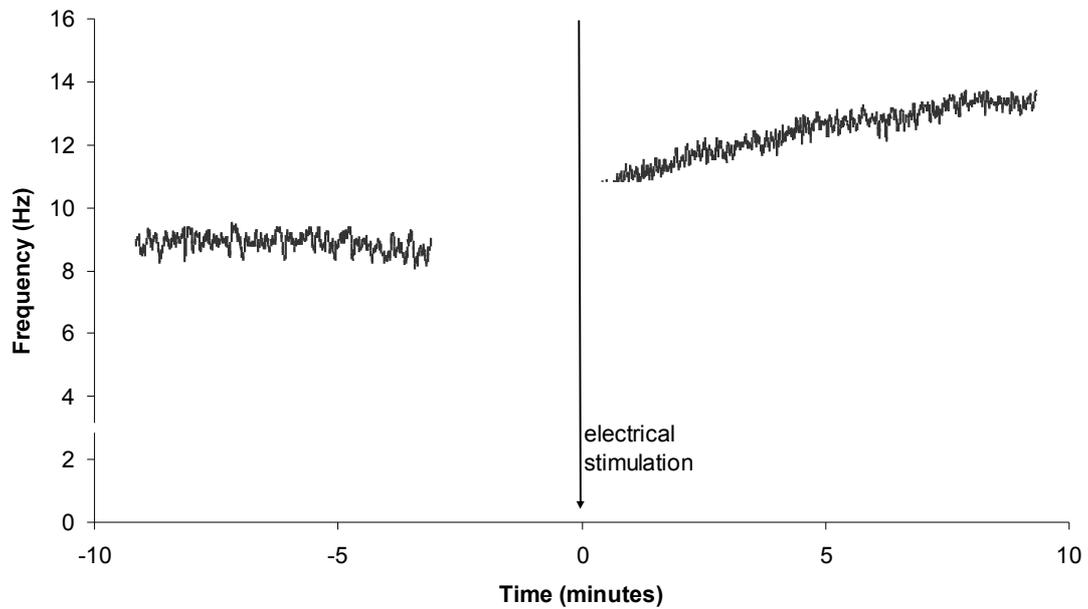


Figure 28: Raw EEG recorded in anaesthetised dogs before and after electrical stimulation

Heart rate was calculated from the ECG data using the rate meter function of Chart 5.2.2 and analysed as follows. Pre-stimulation mean heart rate (pre300) for each dog was calculated from readings during 300 seconds prior to stimulation. Post-stimulation mean heart rate at intervals of 15 seconds (post15, post30....post360) was estimated until six minutes post-stimulation and expressed as percent of pre300. Also, mean heart rates for the immediate 15 seconds prior to stimulation (pre15) were estimated and expressed as percent of pre300. Heart rate during stimulation (for five to seven seconds) was excluded to avoid contaminated data.

6.2.4 Statistical analyses

The effects of test drugs on EEG frequency spectrum were compared employing PROC MIXED procedure in SAS[®] 9.1. PROC CAPABILITY procedure in SAS[®] 9.1 was used to test the data for normality and residuals of data were found to be normally distributed. EEG data from 10-second blocks before to 10-second blocks after the electrical stimulus (after excluding five to seven second blocks immediately before and after the stimulus) were taken for statistical analysis. Post-stimulation data from the three EEG variables of interest were expressed as percent of baseline values. The linear mixed model used for analysis of variance between treatment groups included the fixed effects of treatments, carry-overs, and random effects of animals. Analysed data from PROC MIXED procedure were presented as least square means (LSM±SE).

Between treatment group comparison of pre15 and post-stimulation mean heart rates (post15, post30....post360) was done using one way ANOVA (SPSS 13.0; SPSS Inc. Chicago, Illinois, USA). Also, within each group, each of the post-stimulation mean rates (post15, post30....post360) was compared with pre15 using paired t-test. Results were considered significant if $P < 0.05$ for both EEG and ECG data.

6.3 RESULTS

6.3.1 EEG responses

F50 was significantly increased after electrical stimulation compared to that of baseline in dogs of all treatment groups except morphine (figure 29). There were no significant differences in F50 before and after electrical stimulation in dogs in the morphine group. The percentage change of post-stimulation F50 was significantly lower in the morphine group than that of other treatment groups after electrical stimulation (LSM±SE of F50 as % change

from baseline are 101.68 ± 8.43 , 121.93 ± 8.58 , 134.94 ± 8.37 , 141.56 ± 8.43 for the dogs in the morphine, tramadol, parecoxib and saline groups respectively). There were no significant differences in the magnitude of increase in F50 between the saline, tramadol and parecoxib groups after electrical stimulation ($P = 0.0612$; saline vs. tramadol; $P = 0.4657$; saline vs. parecoxib; $P = 0.1884$; tramadol vs. parecoxib). It should be noted that there was a non-significant difference between the F50 values recorded for dogs between the tramadol and saline treatment groups. P_{tot} decreased in all treatment groups following stimulation (LSM \pm SE of post-stimulation P_{tot} as % change of baseline were 71.72 ± 4.18 ; 73.77 ± 4.27 ; 71.33 ± 4.1 ; 65.61 ± 4.18 for morphine, tramadol, parecoxib and saline respectively). No significant differences in the magnitude of decrease in P_{tot} were found between treatment groups (figure 30). SEF did not change significantly from that of baseline after stimulation in all treatment groups (figure 31).

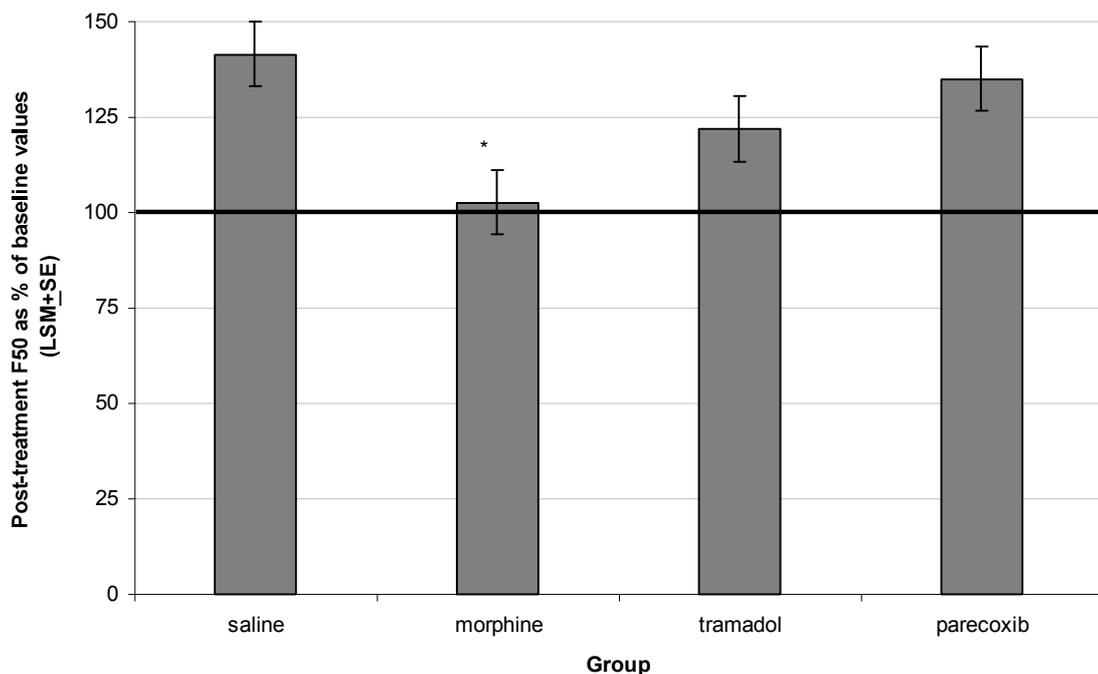


Figure 29: LSM \pm SE for post-electrical stimulation F50 expressed as % of baseline values, of eight dogs given saline, morphine, tramadol or parecoxib subcutaneously in a randomised cross-over design

*Significantly different from saline, tramadol and parecoxib ($P < 0.0002$, 0.0453 and 0.0023 respectively).

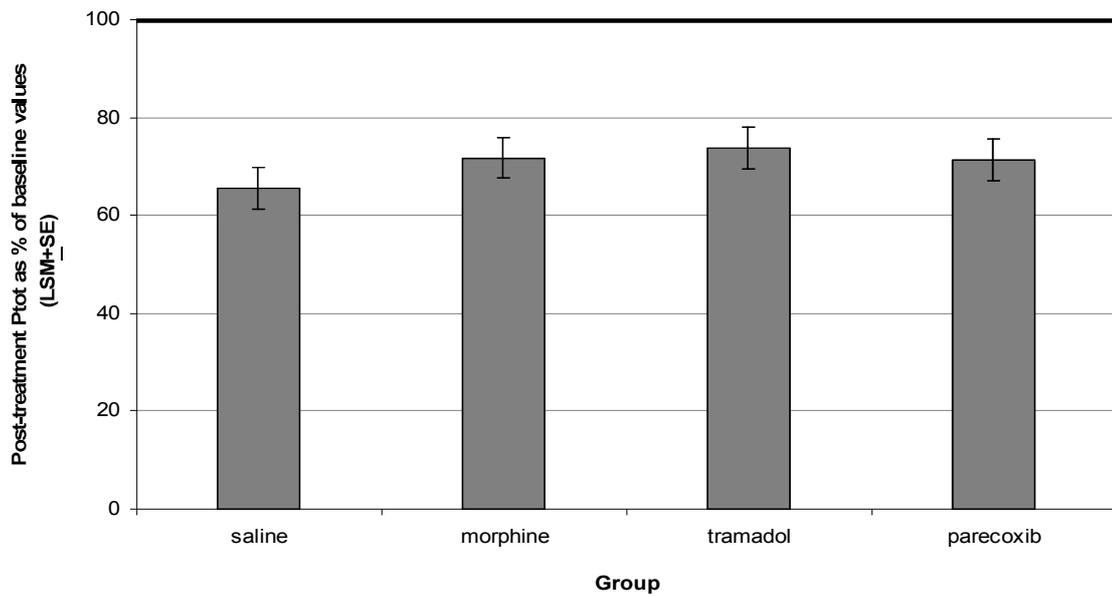


Figure 30: *LSM±SE for post-electrical stimulation Ptot expressed as % of baseline values, of eight dogs given saline, morphine, tramadol or parecoxib subcutaneously in a randomised cross-over design*

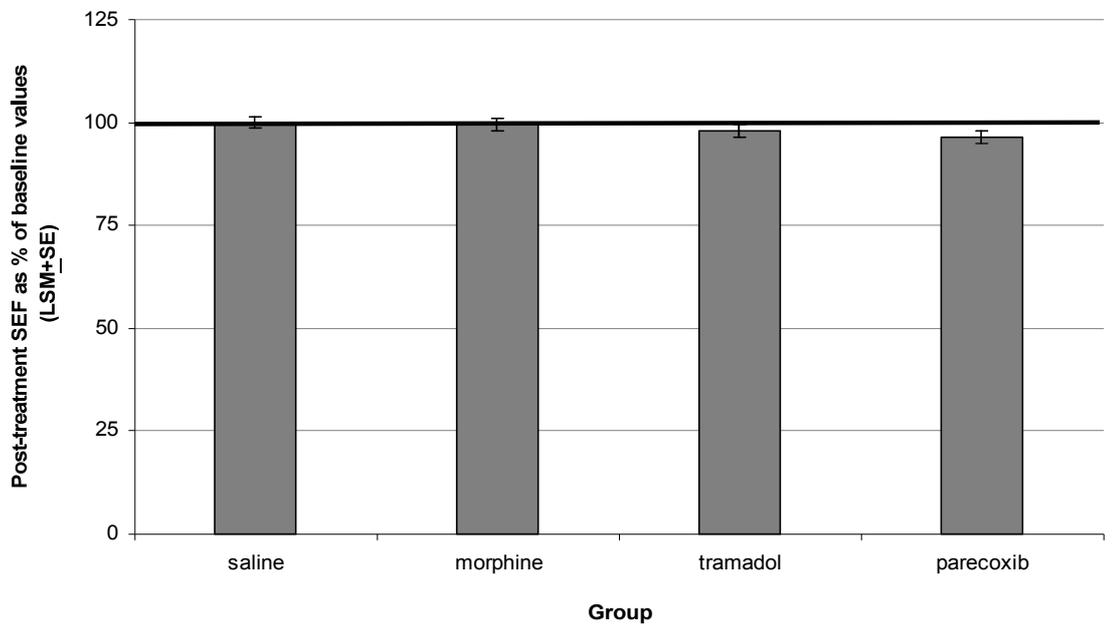


Figure 31: *LSM±SE for post-electrical stimulation SEF expressed as % of baseline values, of eight dogs given saline, morphine, tramadol or parecoxib subcutaneously in a randomised cross-over design*

6.3.2 ECG results

ECG data from three dogs, one each from tramadol, parecoxib and saline groups were not included for heart rate analysis due to a high incidence of movement artifacts. Mean \pm SE of Pre15 heart rate ranged from 100.2 \pm 0.5 (saline) to 102.8 \pm 2.3 (tramadol). Following electrical stimulation, there was a transient increase in heart rate in dogs of all treatment groups. This response was variable between the four treatment groups lasting between one minute (morphine group, 115.97 \pm 6.29, mean \pm SE) and three minutes (parecoxib group, 107.07 \pm 3.39, mean \pm SE). There were no significant differences in mean heart rates between the treatment groups (figure 32).

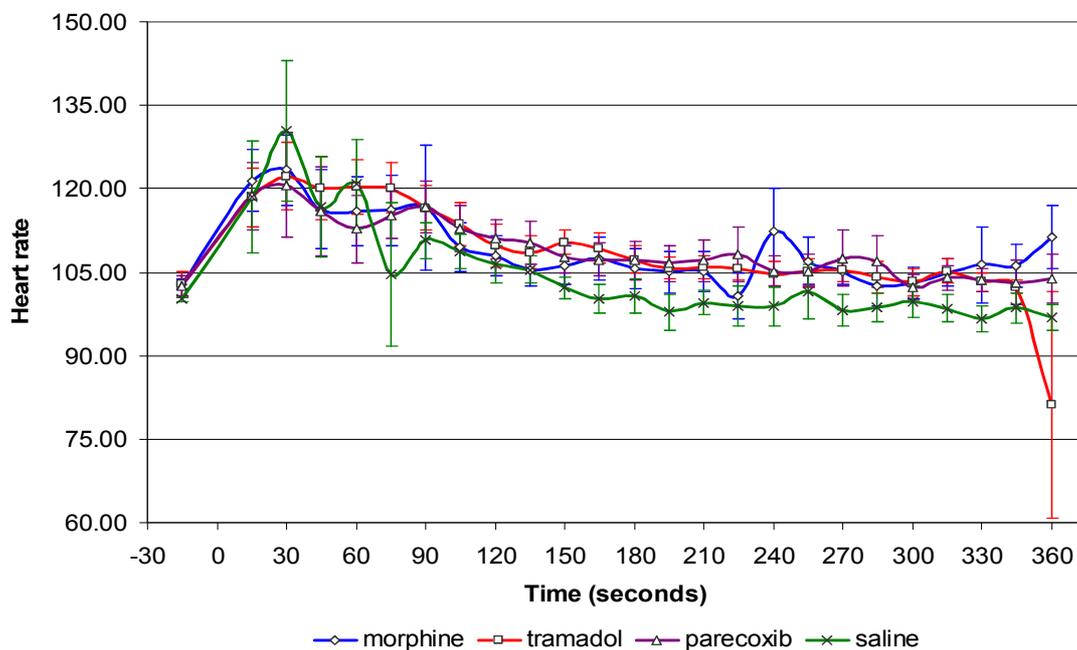


Figure 32: Heart rates post-electrical stimulation in eight dogs administered saline, morphine, tramadol, and parecoxib in a crossover design

Post-stimulation heart rate for every 15 sec, up to 300 sec was compared to pre-stimulation heart rate for 15 sec (expressed as % of heart rate during 300 sec pre-stimulation). The heart rate was significantly different from pre15 ($P < 0.05$) in all treatment groups until 60 seconds post-stimulation.

6.4 DISCUSSION

The minimal anaesthesia model is a sensitive and objective method for the assessment of central responses to noxious stimulation in most species (Murrell & Johnson 2006) and has been utilised in the evaluation of analgesic techniques (Johnson et al. 2005). EEG spectral analysis has been shown to be useful in confirming analgesia in dogs (Short et al. 1992). A significant increase in F50 has been shown to be a typical EEG response marking nociception

in rats, horses, lambs, calves and deer (Otto et al. 1996; Murrell et al. 2003; Johnson et al. 2005a; 2005b; Murrell et al. 2007; Gibson et al. 2007).

In the present study, morphine abolished the rise in F50 in response to noxious stimulation. There were no significant differences in F50 before and after noxious electrical stimulation in the morphine group. This is similar to findings in horses that demonstrated blunting of changes in F50 with intravenous lignocaine given prior to surgical castration (Murrell et al. 2005). The morphine group also had a significantly reduced F50 after noxious stimulation compared to that of the other treatment groups in this study. Other studies have also demonstrated reductions in F50 following the administration of analgesic agents. A pre-operative lignocaine ring block was shown to prevent increases in F50 in deer and calves undergoing velvet antler removal and surgical dehorning respectively (Johnson et al. 2005a; Gibson et al. 2007). Further, in un-stimulated anaesthetised horses drugs of known analgesic efficacy in people such as alfentanil have been shown to reduce F50 (Johnson & Taylor 1997). All these studies were conducted using the minimal anaesthesia model with halothane in oxygen.

In the present study, tramadol (3 mg kg⁻¹ SC) and parecoxib (1 mg kg⁻¹ SC) did not prevent the increase in F50 after noxious stimulation to the same extent as morphine (0.5 mg kg⁻¹ SC, $P < 0.01$). There were no significant differences in the magnitude of increase in post-stimulation F50 of dogs of these two groups and that of saline group. The primary role of μ -opioid agonist agents in pre-emptive analgesia is to prevent pre-synaptic neurotransmitter release and hyperpolarisation of postsynaptic membrane, thereby reducing afferent noxious transmission (Duggan & North 1984). Anti-nociceptive potency of tramadol has been said to be one tenth that of morphine due to weaker binding to μ -opioid receptors. Mean IC₅₀ values for tramadol and morphine in displacing μ -receptor binding of radioactive ligands in rat brain membranes *in vitro* were 1.7x10⁻⁶ mol L⁻¹ and 4.6x10⁻⁹ mol L⁻¹, respectively (Hennies et al. 1988).

There are four possible explanations for the significant rise in post-stimulation F50 in dogs in the tramadol group:

1. Weak μ -opioid receptor affinity may have allowed afferent transmission to the cerebral cortex (Freye et al. 1998), after acute noxious stimulation.
2. Lower production of metabolite M₁ in dogs (Wu et al. 2001), which is the major

metabolite of tramadol with 200-fold higher affinity at μ -receptors than the parent compound (Raffa et al. 1992; Poulsen et al. 1996).

3. Both 5-HT and NA play an antinociceptive role in descending inhibitory pathways from the brain stem. This is an additional proposed mechanism of tramadol analgesia (Raffa et al. 1992).
4. The dose of the tramadol administered (3 mg kg^{-1}) may be too low compared to the intensity of noxious stimulation.
5. In dogs, the time to maximum plasma concentration (T_{max}) following oral tramadol administration (100 mg as a single dose) has been reported to be 1.04 ± 0.51 hours. Also, the T_{max} of O-desmethyltramadol (metabolite (M_1) with higher μ -receptor affinity) following oral tramadol administration (100 mg) was 0.5 ± 0.2 hours (Kukanich & Papich 2004). In the present study, tramadol (3 mg kg^{-1} SC) has been given at the time of pre-anaesthetic medication and anaesthesia was induced 45 to 60 minutes latter. The baseline EEG was recorded 90 minutes after induction with propofol. Therefore the data from the EEG recorded approximately 2.5 hours after tramadol administration has been taken for statistical analysis. Though the pharmacokinetics of subcutaneously administered tramadol are not available in dogs, from the available data following oral tramadol administration, it is likely that the plasma concentration of tramadol/O-desmethyltramadol at the time of noxious electrical stimulation of dogs in this study might not be enough to prevent the afferent transmission fully. Also, it can be seen in the results related to F50 of this study that the P value (0.061) between tramadol and saline treatment groups approached but did not achieve significance.

In the current study, parecoxib was also unable to prevent the rise in F50 after noxious electrical stimulation. The pharmacokinetic profile of parecoxib are not available in dogs. Possible mechanisms of NSAID analgesia in the absence of peripheral inflammation have been shown to be via activation of descending modulatory systems inhibiting excitation of the spinal dorsal horn neurones (Chambers et al. 1995; Vanegas et al. 1997; Lizarraga & Chambers 2006). Tramadol (3 mg kg^{-1} IV) and parecoxib (1 mg kg^{-1} IV) singly and together produced analgesia in response to mechanical nociceptive stimulation in healthy dogs (chapter 4 of this thesis). However, it appears that these analgesics did not completely prevent the afferent flow of noxious signals to the cerebral cortex in response to an acute electrical stimulus.

There were no significant differences in post-stimulation P_{tot} between treatment groups though it decreased from baseline in all treatment groups. Arousal from sedation and anaesthesia due to noxious stimulation causes changes in P_{tot} in horses (Miller et al. 1995) and a significant decrease in P_{tot} may indicate nociception (Murrell et al. 2003). Preoperative lignocaine (given IV and as nerve block) has been demonstrated to prevent changes in P_{tot} as well as changes in F50 due to noxious stimulation in deer, horses and calves under minimal halothane anaesthesia (Johnson et al. 2005a; Murrell et al. 2005; Gibson et al 2007). Based on these reports, it may be hypothesised that drugs that obtund changes in F50 would also obtund or prevent changes in P_{tot}. However, the changes in F50 seen in the present study were not reflected in P_{tot}.

Murrell et al. (2003) showed a direct relationship between changes in E_{T HAL} and P_{tot} in response to surgical castration in horses, and suggested that a reduction in total EEG power reflects a decrease in adequacy of anaesthesia due to nociceptive stimulus. The magnitude of decrease in P_{tot} between individuals varies due to differences in their E_{T HAL}. In contrast, there was no association between alterations in F50 and E_{T HAL} during castration. In the current study morphine (0.5 mg kg⁻¹), an antinociceptive drug of known efficacy in people, did not restrict changes in P_{tot}, in contrast to changes in F50, in response to noxious stimulation. This finding supports the concept of Murrell et al (2003) that changes in P_{tot} and F50 indicate different aspects of nociception.

Changes in SEF did not differ significantly between treatment groups in the current study. These results are consistent with those of Johnson & Taylor (1999) who concluded that alterations in SEF reflect general CNS depression. In the present study, dogs of all treatment groups were maintained under a stable plane of anaesthesia at E_{T HAL} of 0.85% and 0.95%. This was considered sufficient to maintain unconsciousness whilst enabling the generation of EEG responses due to subconscious nociceptive processing (Johnson et al. 2005a). None of the anaesthetised dogs in the present study displayed unexpected awakening either during baseline EEG recording or during stimulus application. However, electrical stimuli produced movement responses (Murrell et al. 2007) in all dogs indicating direct stimulation of neuromuscular junction under minimal anaesthesia.

The results of this study further support the findings of Nickel & Zerrahn (1987) who showed that different classes of analgesics can be separated by their effects on EEG power spectra. 'Desynchronisation', a shift of EEG activity towards high frequency, has been reported as the EEG response to nociception (Murrell et al. 2003). In the current study, desynchronisation was the characteristic finding with tramadol, parecoxib and saline that was not seen with morphine. This is the first study in dogs that has demonstrated clear differentiation of different classes of analgesics based on EEG power spectral analysis using the minimal anaesthesia model.

Electrical stimuli are more suitable in the study of central rather than peripheral anti-nociceptive mechanisms. They excite all peripheral sensory nerve fibres (including A β -type, which are not directly implicated in nociception) and thus bypass the transduction mechanisms in the sensory nerve endings (Handwerker & Kobal 1993; Le Bars et al. 2001). Though all peripheral sensory fibres are activated, stimulation of thin A δ (fast pain) and C type fibres (slow pain) only causes pain (Handwerker & Kobal 1993; Le Bars et al. 2001). However, electrical stimuli are non-invasive and easily-controllable, and therefore useful to study pain mechanisms. A study in rats comparing the EEG effects of three different types of noxious stimuli (mechanical, electrical and thermal) showed that electrical stimuli produced the most consistent EEG responses, with a significant increase in F50, marking nociception (Murrell et al. 2007). Results of the current study further confirm the use of noxious electrical stimuli in generating a reliable EEG response.

Anaesthesia was induced with propofol because of its rapid metabolism and lack of analgesic properties in dogs (Short & Bufalari 1999). The mean elimination half life ($t_{1/2\beta}$) of propofol (6.5 mg kg⁻¹ IV) in dogs anaesthetised with halothane in nitrous oxide and oxygen was reported to be 75 minutes (Nolan et al. 1993). In the present study, baseline EEG was recorded 90 minutes after induction in all dogs to exclude the possibility of any effect of propofol on the EEG at the doses used. Halothane has minimal antinociceptive effects (England & Jones 1992) compared to other inhalation anaesthetics. There were no significant differences between treatment groups with regard to systolic blood pressure, $E_{T\text{CO}_2}$ and core body temperature before and after noxious stimulation.

Heart rate increased transiently in dogs of all treatment groups following electrical stimulation, with variability between post-stimulation time points. Nociceptive stimuli can

induce changes in sympathetically mediated cardiovascular variables in animals under general anaesthesia (Greene et al. 1992; Otto & Gerich 2001; Haga et al. 2001). The association between changes in EEG frequency and haemodynamic variables in response to noxious stimuli appears to vary with species and laboratory. Increases in heart rate and MAP were shown to be associated with an increase or decrease in different frequency bands of the EEG power spectrum in response to surgical and electrical stimulation in sheep and dogs respectively (Greene et al 1992; Otto & Gerich 2001), whereas MAP was increased significantly without significant changes in EEG power spectra in response to surgical castration in horses (Haga & Dolvik 2005). In the present study continuous ECG recording enabled the detection of a transient tachycardia following noxious stimulation in all treatment groups.

6.5 CONCLUSION

In conclusion, this study demonstrated that morphine (0.5mg kg^{-1} sc), a potent μ -opioid agonist, prevented any increase in F50 following noxious electrical stimulation in dogs under minimal halothane anaesthesia. Both tramadol and parecoxib failed to prevent a significant rise in F50 (to the extent of morphine). This is consistent with un-inhibited afferent nociceptive transmission to the cerebral cortex in response to acute noxious stimuli. Changes in Ptot seem to represent a different component of nociception than those of F50. Our findings are consistent with EEG studies in other species of animals using the minimal anaesthesia model (Murrell et al. 2003; Johnson et al. 2005a; 2005b; Murrell et al. 2007; Gibson et al. 2007). This study identified F50 as the most reliable indicator of nociception in dogs. This provides a valuable tool to demarcate the cerebral cortical processing of analgesic effects from those of anaesthesia.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSION

This thesis compares the efficacy and safety of novel analgesics, tramadol and parecoxib to morphine and saline, in dogs. The main objective of these studies was to investigate better analgesic drugs and strategies for effective control of postoperative pain in dogs. Though tramadol and parecoxib have been extensively investigated in human clinical trials their use in small animal practice has not been reported except for a few clinical trials on tramadol in dogs. Since there was a need to assess the efficacy and safety of these drugs in postoperative pain, this project included four experiments: assessment of efficacy against a mechanical noxious stimulus, assessment of efficacy against an electrical noxious stimulus, assessment of efficacy against surgical stimuli and assessment of renal function.

7.1 MECHANICAL NOCICEPTIVE THRESHOLD TESTING

Mechanical nociceptive threshold testing has been in use for many years in analgesic trials in dogs (Martin et al. 1963; Martin et al. 1974; Martin et al. 1976; Hamlin et al 1988; Lascelles et al in 1997; Slingsby 1998). A mechanical nociceptive threshold testing device for use in dogs was built (chapter 4) with slight modifications from the device designed by Slingsby et al. (2001). The stimulus was applied to the ventral abdomen of dogs either in standing or recumbent position. Behaviours indicating threshold were aggressive teeth bearing, vocalisation, vigorous turning towards the stimulus or standing suddenly from a lying position. However, learned avoidance behaviour influences the accuracy of threshold testing. This was avoided by allowing fifteen days interval between each testing episode on every dog. None of the dogs showed 'pain-anticipating' behaviour such as attaining threshold level as soon as probe is placed on the test site, at any testing episode. The device has been effective in discriminating the dogs' threshold level with different analgesic drugs administered preventively. It had prompt control over stimulus and was easy to operate.

But the limitations to the use of device such as fast fall in readings immediately after ceasing pressure application and the variability of pressure application could be fixed by setting a reading holder to the circuit and a CRAF meter (devised by Lascelles et al. 1995), respectively. These modifications could improve the efficiency of the device. It would be

interesting to test the efficacy of a variety of different analgesics/analgesic combinations using the modified threshold testing device in dogs.

There have been no reports of use of pindolol as an adjunct to analgesics in dogs. Based on rat and human studies (Corrales et al. 2000; Chia et al. 2004) it was interesting to know whether pindolol (dose lower than that used for anti-hypertensive effect i.e. 5 µg) would potentiate tramadol analgesia in dogs also. One significant limitation to conclude the effect of pindolol on tramadol analgesia in this study was the lack of a separate treatment group with pindolol and tramadol. This was because of restricted availability of dogs for use in experimentation. However, dogs given a combination of tramadol, pindolol and parecoxib had significantly higher thresholds than those given tramadol alone, though tramadol alone had significantly increased the dogs' thresholds compared to those of saline. The combination produced analgesia equivalent to morphine (0.1 mg kg⁻¹), which is likely to be due to combined potentiation by pindolol and parecoxib of tramadol analgesia. Though inhibition of synthesis of inflammatory prostaglandins is the principal mechanism of action of parecoxib, it produced significant analgesia (when given alone) in the absence of inflammation in dogs of this study. It provides significant insights into central analgesic action of NSAIDs.

It would be interesting to test the efficacy of tramadol and pindolol, tramadol and parecoxib combinations in different doses to assess the effect of each drug on tramadol analgesia in dogs. Also, application of dose-effect analysis methods would be useful to evaluate the efficacy of different combinations given in different doses.

7.2 RENAL FUNCTION UNDER ANAESTHESIA AND ANALGESIA

Veterinary practitioners are often reluctant to use NSAIDs before surgery because of concerns regarding adverse effects of these drugs on renal circulation. Though parecoxib (selective COX-2 inhibitor) is apparently safer for preoperative use in humans (Daniels et al. 2001; Rasmussen et al. 2002; Barton et al. 2002), its effects on renal perfusion in anaesthetised subjects have not been evaluated using a renal function test. There are many renal function tests to detect a change in GFR which indirectly signifies renal perfusion. Plasma clearance of iohexol has been validated for this purpose in dogs (Heine & Moe 1998). Quantification of plasma iohexol by HPLC is a labour intensive technique but can detect as little as 5 µg of iohexol in plasma (Gonda 2002; this study). Also, none of the dogs in this study given iohexol before and 24 hours after anaesthesia showed any untoward side effects.

Clearance calculation by a one-compartmental method (used in this study) simplifies the sampling procedure and time besides good accuracy. This study did not find any significant effect of parecoxib on GFR detected 24 hours after anaesthesia and their administration, measured by plasma iohexol clearance (calculated by a one-compartmental model) in dogs. It would be interesting to compare the accuracy of clearance calculated by ICL with that calculated from the standard renal clearance techniques for assessing the GFR, such as the inulin clearance, in dogs administered NSAID analgesics and anaesthesia.

Though it is not customary to assess the effect of preoperative opioids on renal circulation of dogs undergoing surgery, tramadol has been evaluated along with parecoxib for renal safety in this study. Tramadol has been said to be a weak μ - opioid, and a monoamine (5-HT & NA) reuptake inhibitor (Raffa et al. 1992). It was thought important to know whether increased concentration of NA as a result of tramadol administration further aggravates the effects of anaesthesia on the vascular tone and renal perfusion of the dogs. This study did not find any such effect of tramadol given singly or with parecoxib and pindolol, at the time of anaesthetic induction in dogs.

7.3 EFFICACY AGAINST AN ELECTRICAL NOXIOUS STIMULUS

Chapter 6 compared the efficacy of tramadol and parecoxib to morphine and saline against acute noxious electrical stimulation, using EEG variables recorded under minimal halothane anaesthesia in dogs. $E_{T_{HAL}}$ was stabilised between 0.85% and 0.95% since variation in $E_{T_{HAL}}$ was presumed to obtund generation of significant EEG responses between three treatment groups in the study reported in chapter 5. Though electrical stimuli are brief and phasic, and cannot equate to trains of surgical stimuli, they can be used to evaluate the efficacy of drugs to prevent afferent nociceptive transmission (central mechanisms) using EEG responses. Morphine ($0.5 \text{ mg kg}^{-1} \text{ SC}$) given at the time of pre-anaesthetic medication restricted the changes in F50 of EEG after stimulation where as tramadol ($3 \text{ mg kg}^{-1} \text{ SC}$) and parecoxib ($1 \text{ mg kg}^{-1} \text{ SC}$) did not. There was no significant difference between the latter two treatment groups and saline in post-stimulation F50, which was increased compared to baseline values.

Significant increase in F50 has been demonstrated to be linked to nociception predominantly, in horses, lambs, red-deer, pigs, rats and calves (Murrell et al. 2003; Johnson et al. 2005a; 2005b; Haga & Ranheim 2005; Murrell et al. 2007; Gibson et al. 2007). Since EEG is a

record of electrical activity of the cerebral cortex, the increase in post-stimulation F50 in tramadol, parecoxib and saline groups was attributed to increased cortical activity due to afferent nociceptive transmission. This increase in EEG responses was seen with tramadol in human studies also (Freye et al. 1998). There was no significant difference in baseline F50 between all treatment groups which further supports that the observed difference in F50 between morphine and other treatment groups was due to difference in their antinociceptive efficacy but not due to well-known sedative property of morphine (as tramadol, parecoxib and saline did not produce sedation apparently).

The changes in F50 appear to be more related to nociception and anti-nociception than the other two EEG variables (Ptot and SEF) as there was no significant difference in the SEF and in the magnitude of decrease in Ptot between treatment groups, in this trial. The EEG responses of dogs given a combination of tramadol and parecoxib against a noxious electrical stimulus have not been tested due to limited availability of dogs for use in these experiments, in the present study. Further studies should include evaluation of the EEG changes in dogs given a combination of tramadol and parecoxib.

7.4 INTRA-OPERATIVE NOCICEPTION AND POST-OPERATIVE ANALGESIA

The effects of the drugs on clinical pain during and after surgery were examined using EEG responses during surgery and assessing the animals' behaviour using the Glasgow pain scale during recovery. Apart from its use as a monitor of anaesthetic depth for many years (Gibbs et al. 1937; Mashour 2006) EEG is gaining popularity as a useful tool for measuring nociception in different species of animals (Murrell et al. 2003; Johnson et al. 2005a; 2005b; Haga & Ranheim 2005; Murrell et al. 2007; Gibson et al. 2007). Use of anaesthetics which have an anti-nociceptive property in their own right might interfere with EEG variables (Haga et al. 2001; Haga & Dolvik 2005). Halothane has minimal analgesic efficacy (England & Jones 1992) and has been demonstrated to allow significant EEG responses to nociception and anti-nociception with stable concentrations throughout surgery (Murrell et al. 2003; Murrell et al. 2005; Johnson et al. 2005a; Haga et al. 2005; Gibson et al. 2007). In this study, the intra-operative halothane concentration of dogs (adjusted by the anaesthetist who was guided by clinical signs) varied significantly between treatment groups during ovariohysterectomy. Stabilising intra-operative haemodynamic variables with suitable drugs is imperative. Fentanyl and ketamine, which have been demonstrated to affect EEG variables (Wauquier et al. 1981; Johnson & Taylor 1999), were given as considered necessary by the

anaesthetist during ovariohysterectomy in dogs of three treatment groups. Lack of a conclusive and significant difference in EEG responses between the three treatment groups in ovariohysterectomised dogs appears due to confounding influence of varied end-tidal halothane concentration and intra-operative fentanyl and ketamine administration.

Further studies in dogs being castrated, without a significant difference between groups in end-tidal halothane concentration and intra-operative supporting drug administration, showed a significant difference in F50 and P_{tot} between the morphine and tramadol groups, indicating that morphine was more effective. Changes in these two EEG variables have been shown to be linked to different aspects of nociception across different animal species (Murrell et al. 2003; Johnson et al. 2005a; 2005b; Haga & Ranheim 2005; Murrell et al. 2007; Gibson et al. 2007). Results from this study seem to correlate with those found with electrical stimulation of dogs (study reported in chapter 6 of this thesis), though the type of noxious stimulation is different.

The dose and duration of action of the drugs given before the onset of noxious stimuli should be enough to cover the total nociceptive input generated by surgery for successful prevention of pain (Kissin 1996; 2005). The mean or median pain score of dogs given a combination of preoperative low-dose morphine (0.1 mg kg⁻¹) and post-operative tramadol (3 mg kg⁻¹) was lower after ovariohysterectomy in dogs of this study. Also, only one dog in this treatment group received rescue analgesia at six hours postoperatively compared to the other two treatment groups (0.5 mg kg⁻¹ morphine or 3 mg kg⁻¹ tramadol) where four out of eight dogs from each group received rescue analgesia at one hour after surgery. From these findings it appears questionable whether 0.5 mg kg⁻¹ morphine or 3 mg kg⁻¹ tramadol given singly at the time of premedication can prevent the 'total nociceptive input' in ovariohysterectomised dogs.

However, there are some limitations to this study to answer this question conclusively. Another efficacy evaluation method used in ovariohysterectomy study, intraoperative EEG, did not show any significant differences between the three treatment groups due to confounding experimental conditions. There were neither 'saline control' nor 'postoperative analgesic only', treatment groups for comparison. Lascelles et al. (1997) demonstrated better analgesia with preoperatively given analgesics (short acting) at later postoperative period from 8 hours until 20 hours, with similar pain model in dogs. The method of statistical

analysis used in this study did not permit a comparison of the efficacy of the different treatments at different postoperative time points. Because of intrinsic ethical restrictions of our hospital that dogs undergoing surgery in the morning should receive buprenorphine (a partial μ -agonist) between 8 pm and 9pm, pain assessment was carried out until 9 hours after surgery, only. Dexterity of the surgeon may have affected the intensity of noxious stimulation. This surgery was performed by final year veterinary students under the guidance of a veterinary surgeon and operator variability may have influenced the outcome.

In castrated dogs, there was no significant difference in postoperative pain scores between preoperative morphine (0.5 mg kg^{-1}) and preoperative tramadol (3 mg kg^{-1}), though the EEG responses (F50 & Ptot) differed significantly at removal of testicle 1 ($P=0.049$). However, there was no significant difference between the two groups at removal of testicle 2, in these two EEG variables. The EEG can indirectly reflect the activity of the lower centres of the brain (such as the brain stem reticular formation that has diffuse connections with descending modulatory areas) though it is a record of the cortical electrical activity (Moruzzi & Magoun 1949; Antognini et al. 2000). Both 5-HT and NA are the neurotransmitters in the descending inhibitory pathway, whose concentrations are reported to be increased by tramadol *in vitro*. Collectively, it appears that activation of descending inhibitory mechanisms (Raffa et al. 1992) latter to the initiating stimulus might contribute to tramadol analgesia, with this pain model.

We can assume from EEG responses and postoperative pain scores recorded in the castration study that further activation of descending inhibitory mechanisms might contribute to tramadol analgesia as there were no significant differences in F50 & Ptot between morphine and tramadol at testicle 2, and postoperative analgesic efficacy, respectively. However, EEG responses to acute and brief electrical stimulation (50 Hz, 50 volts for 2 sec were used in this study) may not represent the contribution of descending inhibitory mechanisms in tramadol and parecoxib analgesia. We can differentiate different classes of anti-nociceptive agents using EEG responses, as observed in this study. Morphine prevents afferent noxious transmission (reflected as no change in post-stimulation F50) probably due to potent μ -receptor affinity whereas a weak binding at μ -opioid receptors (Hennies et al. 1988) might cause tramadol fail to prevent entry of noxious input to the extent of morphine. Also, production of M_1 , a major metabolite of tramadol with greater affinity at μ -opioid receptors

than the parent compound, has been reported to be limited in dogs (Wu et al. 2001; Kukanich et al. 2004).

Both tramadol and parecoxib produced analgesia in response to nociceptive mechanical stimuli. In contrast, they did not prevent afferent nociceptive transmission against a standard electrical stimulus. Tramadol showed equal efficacy to morphine against surgical stimuli. Behavioural methods of pain measurement (mechanical threshold testing and pain scale), though can assess analgesia of tramadol, did not

7.5 CONCLUSION

This thesis compared the preventive analgesic efficacy of tramadol and parecoxib to that of morphine (routinely used analgesic in dogs) and saline, using behavioural and EEG responses, in dogs. Tramadol and parecoxib produced analgesia that was significantly less than that of low-dose morphine, against mechanical nociceptive stimuli. Their combination with pindolol produced analgesia comparable to low dose morphine. Neither single nor combined administration of tramadol and parecoxib were found to affect GFR, estimated by plasma iohexol clearance, in dogs under normotensive anaesthesia.

A combination of preoperative low-dose morphine (0.1 mg kg^{-1}) and postoperative tramadol (3 mg kg^{-1}) has been found to yield better postoperative analgesia than preoperative tramadol (3 mg kg^{-1}) or preoperative morphine (0.5 mg kg^{-1}) alone, in ovariohysterectomised dogs. In castrated dogs, preoperative tramadol (3 mg kg^{-1}) can produce analgesia similar to that of morphine with clinically insignificant side effects. The short form of the Glasgow pain scale can differentiate the efficacy of analgesics and analgesic strategies.

Preventive administration of tramadol and parecoxib cannot abolish the afferent nociceptive transmission to the extent of morphine, in response to a standard electrical stimulus. To become a useful tool for measuring anti-nociceptive efficacy, EEG might need a stable and repeatable plane of anaesthesia. Further studies are required with a large dog population to investigate the efficacy of these analgesics administered in different dose rates and times of surgery, for optimal postoperative analgesia.

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APPENDIX

THE GLASGOW CMPS-SF

SHORT FORM OF THE GLASGOW COMPOSITE PAIN SCALE

Dog's name _____
 Hospital Number _____ Date / / Time _____
 Surgery Yes/No (delete as appropriate) _____
 Procedure or Condition _____

In the sections below please circle the appropriate score in each list and sum these to give the total score.

A. Look at dog in Kennel

Is the dog?

(i)		(ii)	
Quiet	0	Ignoring any wound or painful area	0
Crying or whimpering	1	Looking at wound or painful area	1
Groaning	2	Licking wound or painful area	2
Screaming	3	Rubbing wound or painful area	3
		Chewing wound or painful area	4

In the case of spinal, pelvic or multiple limb fractures, or where assistance is required to aid locomotion do not carry out section B and proceed to C
 Please tick if this is the case then proceed to C.

B. Put lead on dog and lead out of the kennel. C. If it has a wound or painful area including abdomen, apply gentle pressure 2 inches round the site.

When the dog rises/walks is it?

(iii)	
Normal	0
Lame	1
Slow or reluctant	2
Stiff	3
It refuses to move	4

Does it?

(iv)	
Do nothing	0
Look round	1
Flinch	2
Growl or guard area	3
Snap	4
Cry	5

D. Overall

Is the dog?

(v)	
Happy and content or happy and bouncy	0
Quiet	1
Indifferent or non-responsive to surroundings	2
Nervous or anxious or fearful	3
Depressed or non-responsive to stimulation	4

Is the dog?

(vi)	
Comfortable	0
Unsettled	1
Restless	2
Hunched or tense	3
Rigid	4