Evaluation of Diphenhydramine as an Antihistamine in Dogs
Anesthetized for Surgical Excision of Mast Cell Tumours

by

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ABSTRACT

EVALUATION OF DIPHENHYDRAMINE AS AN ANTIHISTAMINE IN DOGS ANESTHETIZED FOR SURGICAL EXCISION OF MAST CELL TUMOURS

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University of Guelph, 2015

This thesis determined in phase 1 the pharmacokinetics and cardio-respiratory effects of diphenhydramine (DHP) in conscious research dogs administered 1 mg/kg, IV, or 2 mg/kg, IM. Phase 2 consisted of a blinded clinical trial to investigate the effectiveness of DPH in anesthetized dogs to prevent the negative cardiovascular effects associated with potential histamine release during surgical excision of mast cell tumours (MCT). Dogs were anesthetized with a balanced anesthetic and analgesic technique and then allocated to receive DPH (1 mg/kg, IV) or saline. Plasma DPH and histamine concentrations were measured and correlated with cardio-respiratory parameters.

Phase 1 results provided descriptive pharmacokinetics of DPH administered IV or IM in healthy dogs. Cardio-respiratory parameters remained within normal limits during the experiment and no behavioural changes were associated with DPH administration. The IV protocol was chosen for the clinical phase 2 under anesthesia, due to a shorter time to maximal concentrations ($T_{\text{max}}$) of $6.0 \pm 0.3$ min versus $45 \pm 5.1$ min for the IM group.

During the clinical phase 2, plasma concentrations of DPH remained above concentrations considered therapeutic (25 ng/mL) in humans until the end of
surgery. Despite the lack of statistical differences in histamine concentrations throughout anesthesia between groups, higher histamine concentrations were measured during maximal manipulation of the tumour. Mean arterial and diastolic blood pressures were significantly lower in DPH than in the saline group during surgical excision of the tumour. These results contradict the assumption that hypotension is more likely in dogs undergoing MCT excision that have not received DPH. Values for cardio-respiratory parameters in both groups were considered within acceptable limits for anesthetized dogs.

In dogs, DPH can be administered by either IV or IM routes at 1 mg/kg and 2 mg/kg, respectively, to yield plasma concentrations that exceed therapeutic concentrations in humans without noticeable adverse behavioural or cardio-respiratory side effects. The administration of DPH prior to surgical removal of MCT in dogs did not have clear clinical anesthetic differences related to cardio-respiratory responses compared to dogs receiving a saline placebo.
ACKNOWLEDGEMENTS

I would like to thank for their support and contribution to this thesis to all the members of my committee: Dr. Conny Mosley, Dr. Ameet Singh, Dr. Melissa Sinclair, Dr. Brad Hanna, and Dr. Tony Mutsaers. I would like to specially thank my supervisor; Dr. Alex Valverde, this thesis has become a reality because of his patience and dedication. Hopefully someday I can repay him for all the energy and effort that he put in helping me reach my goals over these years. Thanks for helping me find my way.

Thanks to my family and friends both sides of the Atlantic for being always there even in the distance. I would also like to acknowledge all the anesthesia technicians and residents that not only helped me during the clinical part of this thesis but also filled my path these years with happiness, friendship and fun.

Finally I would like to dedicate this work to the most important men in my life. To my husband, Dr. Luis Gaitero, for surviving your second residency by my side, for keeping me calm and steady when I needed it the most. To my dad, the best person I ever met, because all the work and effort that I put in everything in life is hoping that he is proud of me, wherever he is.
# TABLE OF CONTENTS

**ACKNOWLEDGEMENTS**................................................................................................iv  
**TABLE OF CONTENTS**.......................................................................................................v  
**DECLARATION OF WORK PERFORMED**........................................................................ix  
**LIST OF TABLES**...............................................................................................................x  
**LIST OF FIGURES**.............................................................................................................xii  
**LIST OF ABBREVIATIONS**................................................................................................xv  

## CHAPTER I  
**GENERAL LITERATURE REVIEW**

1.1. Introduction...................................................................................................................1  
1.2. Objectives and hypothesis............................................................................................2  
1.3. Histamine.....................................................................................................................3  
1.4. Histamine receptors......................................................................................................6  
1.5. Biological functions of histamine receptors.................................................................7  
  1.5.a. H₁ receptor.....................................................................................................8  
  1.5.b. H₂ receptor.....................................................................................................9  
  1.5.c. H₃ receptor...................................................................................................10  
  1.5.d. H₄ receptor...................................................................................................12  
1.6. Plasma histamine concentration determinations.......................................................13  
1.7. Cardiovascular effects of histamine release...............................................................15  
1.8 H₁-Antihistamines.......................................................................................................19  
  1.8.a. Side effects of first generation H₁-antihistamines............................................22
CHAPTER II

THE PHARMACOKINETICS OF DIPHENHYDRAMINE AFTER ADMINISTRATION OF A SINGLE INTRAVENOUS OR INTRAMUSCULAR DOSE IN HEALTHY DOGS
CHAPTER II
EVALUATION OF THE ANTIHISTAMINIC EFFECTS OF DIPHENHYDRAMINE IN DOGS UNDERGOING EXCISION OF MAST CELL TUMOURS

3.1. Summary.................................................................................................................. 117
3.2. Introduction...............................................................................................................120
3.3. Materials and methods..............................................................................................123
    3.3.a. Animals...........................................................................................................123
    3.3.b. Experimental design.......................................................................................124
    3.3.c. Study protocol.................................................................................................124
    3.3.d. Monitoring and blood sampling.....................................................................127
DECLARATION OF WORK PERFORMED

I declare that, with the exception of the listed items, all work reported in this thesis was performed by me.

The research proposal providing funding for this study was written and submitted by Dr. Alexander Valverde. Dr. Valverde also performed the statistical and DPH pharmacokinetic analysis. Determinations for DPH plasma concentrations using high performance liquid chromatography were performed by Yu Gu, HPLC Facility coordinator, Department of Biomedical Sciences. Determinations for histamine plasma concentrations using enzymatic immunoassay were performed by Michelle Beaudoin-Kimble, Animal Health technician, Department of Clinical Studies.
LIST OF TABLES

Table 2.1. Physiologic variables after administration of diphenhydramine at 1 mg/kg, IV, and 2 mg/kg, IM in healthy conscious dogs. Data is expressed as mean (SD); n = 6/group............................................................111

Table 2.2 Pharmacokinetic parameters of diphenhydramine using a non-compartmental model after administration of 1 mg/kg, IV, or 2 mg/kg, IM, in healthy conscious dogs (n=6/group). Data expressed as mean (SD)............................................................113

Table 2.3. Plasma concentrations of diphenhydramine after administration of 1 mg/kg, IV, or 2 mg/kg, IM, in healthy conscious dogs (n=6/group). Data expressed as mean (SD)............................................................114

Table 3.1. Pre-operative information of dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group) or the same volume of saline, IV (S group)............................................................152

Table 3.2. Respiratory parameters and temperature in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) after induction............................154
Table 3.3. Cardiovascular parameters in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) after induction………………………………………………..156

Table 3.4. Times of collection of blood samples for DPH determinations (min after DPH administration, mean [SD]; and plasma DPH concentrations [ng/mL], mean [SD]) in dogs receiving 1 mg/kg of DPH, IV (n=8)………………………………………………………………………..158

Table 3.5. Times of collection of blood samples for histamine determinations (mean [SD]) and plasma histamine concentrations (ng/mL; mean [SD]) in dogs receiving 1 mg/kg of DPH, IV (n=8) or saline, IV (n=8). …………………………………………………………………………..160
LIST OF FIGURES

Figure 2.1. Semi-log plot of mean plasma concentrations of diphenhydramine over 24 h after administration of 1 mg/kg, IV, in six healthy dogs…………………………..115

Figure 2.2. Semi-log plot of mean plasma concentrations of diphenhydramine over 24 h after administration of 2 mg/kg, IM, in six healthy dogs……………………………..116

Figure 3.1. Timeline representation of cardiovascular data recording time points (T), and diphenhydramine (D) and histamine (H) blood samples collecting times (min) until end of surgical procedure. Induction of anesthesia and beginning of surgery are indicated with arrows…………………………………………………………………..163

Figure 3.2. Systolic arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement……………………………………………………………………..164

Figure 3.3. Mean arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement……………………………………………………………………..165
Figure 3.4. Diastolic arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement. ..............................................................166

Figure 3.5. Cardiac index (mL/min/kg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement. ..............................................................167

Figure 3.6. Stroke volume index (mL/beat/kg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement. ..............................................................168

Figure 3.7. Heart rate (beats/min) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement. ..............................................................169

Figure A.1. Data for trapezoid calculations of AUC (ng/h/mL) and AUMC (ng-h*h/mL) derived from 6 dogs after 2 mg/kg of diphenhydramine administered IM ..........177
Figure A.2. Data for trapezoid calculations of AUC (ng/h/mL) and AUMC (ng-h*h/mL) derived from 6 dogs after 2 mg/kg of diphenhydramine administered IV..........................178

Figure A.3. Canine blank plasma spiked with diphenhydramine (500 ng/mL) and orphenadrine (100 ng/mL) to determine the retention times observed for diphenhydramine (1.8 min) and for orphenadrine (3.2 min) during the HPLC pharmacokinetic analysis of diphenhydramine performed in Chapter II of this thesis...179

Figure A.4. Canine blank plasma tested for specificity during the pharmacokinetic determinations of diphenhydramine. No interfering peaks were observed in blank plasma samples at retention time corresponding to the drug and internal standard indicating that this clean background of the assay procedure is specific to diphenhydramine............180

Figure E.1. Histamine curve generated from the commercial enzyme immunoassay kit (Enzyme immunoassay Histamine; IM2015; Immunotech, Marseille, France) and then analyzed with online software (MyAssays Ltd., http://www.myassays.com) using a four-parameter logistic curve. This analysis showed a high coefficient of determination (R^2) between kit standard concentrations and measured concentrations..........................185
TABLE OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCT</td>
<td>Mast cell tumour</td>
</tr>
<tr>
<td>DPH</td>
<td>Diphenhydramine</td>
</tr>
<tr>
<td>HNMT</td>
<td>Histamine-N-methyltransferase</td>
</tr>
<tr>
<td>H₁</td>
<td>Histamine receptor 1</td>
</tr>
<tr>
<td>H₂</td>
<td>Histamine receptor 2</td>
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<td>H₃</td>
<td>Histamine receptor 3</td>
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<tr>
<td>H₄</td>
<td>Histamine receptor 4</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
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<td>Prostaglandin D₂</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>AV</td>
<td>Atrioventricular</td>
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<tr>
<td>%F</td>
<td>Bioavailability</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the concentration-time curve</td>
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<tr>
<td>Vd</td>
<td>Volume of distribution</td>
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<tr>
<td>T₁/₂</td>
<td>Terminal half-life</td>
</tr>
<tr>
<td>CI</td>
<td>Plasma clearance</td>
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<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
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<tr>
<td>LOD</td>
<td>Limit of detection</td>
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<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------------</td>
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<tr>
<td>SCF</td>
<td>Stem cell factor</td>
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<tr>
<td>SAP</td>
<td>Systolic arterial pressure</td>
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<tr>
<td>DAP</td>
<td>Diastolic arterial pressure</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>ETCO₂</td>
<td>End-tidal carbon dioxide</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiogram</td>
</tr>
<tr>
<td>RR</td>
<td>Respiratory rate</td>
</tr>
<tr>
<td>CO</td>
<td>Cardiac output</td>
</tr>
<tr>
<td>NICO</td>
<td>Non-invasive cardiac output with partial rebreathing of CO₂</td>
</tr>
<tr>
<td>SVI</td>
<td>Stroke volume index</td>
</tr>
<tr>
<td>IPPV</td>
<td>Intermittent positive pressure ventilation</td>
</tr>
<tr>
<td>SPO₂</td>
<td>Pulse-oximetry</td>
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<td>Temp</td>
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CHAPTER 1: GENERAL LITERATURE REVIEW

1.1. INTRODUCTION

In small animals, mast cell tumour (MCT) is the most frequent malignant skin tumour in dogs and second most frequent in cats (Govier 2003, London & Seguin 2003, Dobson & Scase 2007). Paraneoplastic syndromes in dogs associated with this disease are usually the result of mast cell degranulation that can be precipitated by physical or chemical means, including surgical manipulation or radiation of the tumour (Dobson & Scase 2007),

In dogs, systemic release of histamine may cause non-desired cardiovascular effects such as decrease in systemic vascular resistance (SVR), arterial blood pressure, and cardiac output (CO) (Kapin & Ferguson 1985, Shmuel & Cortes 2013). Patients under general anesthesia are more prone to develop hypotension due to the decrease in vascular resistance and blunted sympathetic response caused by inhalant agents. For this reason, routine pre-operative treatment with antihistamines has been recommended in anesthetized patients with mastocytosis (Klein & Misseldine 2013) and during surgical manipulation of MCT (London & Seguin 2003), although this type of therapy/prevention is not universally instituted.

Diphenhydramine (DPH) is a “first generation” H1-antihistamine used commonly in veterinary medicine for amelioration of motion sickness of vestibular origin, to control mild allergic reactions and chronic pruritus, and as a second line treatment in anaphylactic reactions (Iwasaki & Hasegawa 2006, Dowling 2012, Peters & Kovacic 2009). In addition it is the most widely recommended drug for preventing cardiovascular
effects of endogenous release of histamine from mast cells during surgical excision of MCT (Hofmeister & Egger 2005, Thamm & Vail 2007).

Systematic reviews of the literature and new guidelines in human medicine reveal that antihistamine treatment is empirical and a demonstrated effect for clinical emergencies to treat cardiovascular shock due to histamine release has never been confirmed (Sheikh et al. 2007b, Simons 2010, Shmuel & Cortes 2013). Research has not confirmed or refuted the efficacy of antihistamine treatment for hypotension caused by massive histamine release in veterinary medicine. Evidence is also lacking regarding its ability, when administered prophylactically, to decrease the risks of cardiovascular effects caused by mast cell degranulation.

Despite the lack of evidence, an increase in the number of cancer patients in veterinary medicine, including MCT, is resulting in anesthetists utilizing DPH orally or as an injectable with increased frequency in the pre-operative period. The aim of this therapy is to prevent the potential effects of histamine released from manipulation and excision of the MCT during anesthesia and therefore to decrease the impact of hypotension, a common occurrence during general anesthesia (Ryan et al. 2012). However, the dose, route and time of administration have been empirically recommended and remain unsupported by veterinary or human pharmacokinetic data.

1.2. OBJECTIVES AND HYPOTHESIS

The primary objective of this research thesis is to determine the pharmacokinetics, safety, and general systemic effects of DPH in healthy awake dogs following a single
dose of 1 mg/kg, IV or 2 mg/kg, IM. This will provide the groundwork for the second phase of the research.

The second objective of the research thesis is to develop an appropriate therapeutic protocol for clinical canine cases requiring pre-treatment with DPH under anesthesia prior to MCT excision. To this end, we will evaluate the use of DPH in anesthetized client owned animals undergoing surgical MCT excision. Specific goals of this second phase clinical research will investigate the anesthetic stability and cardiovascular responses during tumour manipulation, and incidence of hypotension in dogs with or without DPH administration, and relate the findings to histamine and DPH concentrations during those events.

We hypothesize that both routes, IM and IV, are well tolerated by healthy dogs and result in detectable DPH plasma concentrations without major side effects. Our second hypothesis is that dogs with MCT have higher histamine levels than healthy dogs and those levels increase with surgical manipulation of the tumour. We also hypothesize that there is a correlation between plasma histamine levels and blood pressure values in dogs under general anesthesia and preoperative administration of DPH minimizes variations in blood pressure associated with changes in histamine levels.

1.3. HISTAMINE

Histamine (2-[4-imidazolyl]ethylamine) is a low-molecular-weight (111.15 g/mol) endogenous biogenic amine isolated for the first time in the early 1900’s (Dale & Laidlaw 1910). Histamine is produced naturally by decarboxylation of histidine by histidine decarboxylase (HDC) in mast cells, basophils, platelets, and enterochromaffin...
cells, where it is stored intracellularly in vesicles and released upon stimulation (Stoelting & Hillier 2006, Maintz & Novak 2007). Mast cells and basophils are the major sources of histamine in normal tissue. Each cell can contain 3-8 pg of histamine or up to 70% of the cell weight (Watt & Ennis 2004). In mammalian species, histamine is also synthetized and stored in neurons known as “histaminergic neurons” with cell bodies in the tuberomamillary nucleus of the posterior hypothalamus, and corresponding axons localized throughout the cerebrum, cerebellum, posterior pituitary and spinal cord, which use histamine as a primary neurotransmitter (Brown et al. 2001, Simons 2004).

Endogenous metabolism of histamine happens within 1-2 min and there is no cumulative effect. When administered exogenously the half-life of pharmacologically active doses of histamine is less than 10 s in the rat and 20-30 s in the dog (Parsons & Ganellin 2006). Histamine inactivation is possible by two different mechanisms: intracellular histamine is usually metabolized by methylation of the imidazole ring by histamine-N-methyltransferase (HNMT) and extracellularly by oxidative deamination of the primary amino group by diamine oxidase (DAO) (Peters & Kovacic 2009).

The first important experiments designed to demonstrate an actual release of histamine in association with anaphylactic reactions in dogs and guinea pigs were performed in the 1930’s (Watanabe 1931, Dragstedt & Mead 1936). Since then, at least 14 non-identical molecular forms of histamine with different electrical charge, conformation, proton-binding, and electron distribution have been identified. Histamine is the most extensively studied chemical compound with physiological activity in human and veterinary medicine since its isoforms enable histamine to participate in more than 20 different physiologic functions (Peters & Kovacic 2009).
Histamine release can cause muscle cell contraction, vasodilation, increased vascular permeability, mucus secretion, tachycardia, arrhythmias, and stimulates gastric acid secretion (Maintz & Novak 2007). Clinically, the most common systemic adverse effects are hypotension and tachycardia, followed by bronchoconstriction, cardiovascular collapse, pruritus and urticaria (Owen et al. 1982). Elevated plasma histamine levels have been documented in dogs and other species in septic and hemorrhagic shock. Histamine seems to play a role in the pathogenesis of the cardiovascular changes in sepsis (Spink et al. 1964, Neugebauer et al. 1996) and appears to be a good marker for insufficient organ perfusion in hemorrhagic shock in veterinary patients (Nagy et al. 1986, Rixen et al. 2002).

Histamine is also involved in the regulation of hematopoiesis, wound healing, cell proliferation, angiogenesis in intestinal ischemia, and tumour models (Kusche et al. 1980, Raithel et al. 1998). It plays an important role in allergic inflammation and immune modulation since histamine controls accessibility to sites of inflammation by modulating vessels permeability and adhesion molecule expression, stimulates the production of cytokines, γ-interferon, and lysosomal enzymes, and is involved in the development of several aspects of antigen-specific and humoral immune responses, including maturation and modulation of dendritic, t helper Th1, and Th2 cells (Simons 2004, Schneider et al. 2010).

Histamine further plays a role in the regulation of the circadian sleep-wake cycle, nociception, vigilance, cognition, learning, memory, and endocrine homeostasis, and it has antiepileptic properties (Brown et al. 2001, Haas et al. 2008).
1.4. HISTAMINE RECEPTORS

Histamine functions are mediated by interaction with different receptors (H₁, H₂, H₃, H₄). The effects of histamine vary depending on which receptor is activated. Histamine itself is not used therapeutically but antihistamine agents are commonly used to block the effects of endogenous histamine (Adams 1995).

Histamine receptors have been classified and defined pharmacologically by the actions of their respective agonists and antagonists. The first compound with antihistamine properties was synthetized by Bovet & Staub in 1937. In 1942, Halpern described the first antihistamine (phenbenzamine) used clinically in humans to treat allergies (Halpern 1942). Later, multiple antihistamines were synthetized in rapid succession, such as pyrilamine, mepyramine, DPH, and chlorpheniramine, all of which are considered “first generation” or “classic” antihistamines; some of them remain in use today (Emanuel 1999).

Early investigations showed that the vasodilatory response to lower doses of histamine in 24 cats under chloralose, initially anesthetized with ether, could be antagonized by the administration of DPH, but the responses obtained by larger doses of histamine were refractory to this treatment, suggesting the existence of more than one histamine receptor involved in vascular responses (Folkow et al. 1948). In addition, histamine has positive inotropic and chronotropic effects in isolated hearts of mammals, which are poorly antagonized by some antihistamines (Trendelenburg 1960, Barlet 1963). The effects easily antagonized by first generation antihistamines were then termed as H₁ and the poorly antagonized ones as non-H₁ (Ash & Schild 1966). An antihistamine drug that is able to antagonize the non-H₁ gastric and cardiovascular effects of histamine in
mammals is known as an H₂-receptor antagonist, for example burimamide (Black et al. 1972).

A third type of histamine receptor, H₃, has been identified in the cerebral cortex of the rat (Arrang et al. 1983) and cloned (Lovenberg et al. 1999). Activation of H₃ receptors has histamine auto-regulatory function by inhibiting the release of histamine and other neurotransmitters such as norepinephrine, dopamine, and acetylcholine in the central nervous system (Parsons & Ganellin 2006).

More recently, a fourth histamine receptor, H₄, has been cloned and pharmacologically characterized in renal and immune system cells (Oda et al. 2000, Morse et al. 2001, Zhu et al. 2001).

1.5. BIOLOGICAL FUNCTIONS OF HISTAMINE RECEPTORS

The known histamine receptors (H₁, H₂, H₃, H₄) are part of the G-protein-coupled receptor family (GPCR) and they transmit the extracellular signals via G-protein (Gₚ, Gₛ, Gᵢₒ, Gᵢₒ, respectively) systems coupled to intracellular second messengers (Hough 2001). Histamine receptors vary in expression, signaling, function, and histamine binding ability; they therefore have different potential therapeutic applications (Peters & Kovacic 2009). There is intra-species variability in the density and location of the different receptors (Peters & Kovacic 2009) and the affinity of the same radioligand for histamine receptors varies widely among species (Leurs et al. 1995). Therefore, some clinical effects of histamine are mediated by different receptors in different species, and even in different parts of the same organ within the same species (McNeil 1984).
1.5.a. H$_1$ receptor

The H$_1$ receptor is a glycoprotein with a molecular weight of 56 kDa, first cloned from bovine tissue (Yamashita et al 1991). It has been isolated in various tissues and is expressed in CNS neurons, smooth muscle cells (vascular, respiratory and gastrointestinal), cardiovascular system, neutrophils, eosinophils, monocytes, macrophages, mast cells, dendritic cells, T and B cells, endothelial cells and epithelial cells (Simons & Simons 2011).

The histamine H$_1$ receptor is coupled to the phospholipase C-enzyme and regulates intracellular Ca$^{2+}$ concentrations. In mammals, activation of phospholipase C results in formation of inositol triphosphate that triggers the release of Ca$^{2+}$ from cytoplasmic stores and also increases the Ca$^{2+}$ influx from the extracellular space (Matsumoto et al. 1986, Pollock et al. 1988). This Ca$^{2+}$ induces smooth muscle contraction, by increasing the frequency of action potential discharge, in the respiratory and gastrointestinal tracts, and it can also induce coronary artery vasoconstriction, vasodilatation in the hepatic arterial bed, vasoconstriction in the hepatic portal vascular bed, catecholamine release from the adrenal chromaffin cells, pruritus, and sneezing by sensory nerve stimulation (Richardson & Withrington 1978, Stoelting & Hillier 2006, Peters & Kovacic 2009).

High levels of intracellular Ca$^{2+}$ trigger multiple secondary signaling pathways and stimulate the production of second messengers such as cyclic guanosine monophosphate (cGMP), cyclic adenosine monophosphate (cAMP), prostaglandin E$_2$ (PGE$_2$), prostacyclin, arachidonic acid metabolites and nuclear factor κB (Leurs et al. 1995). Enzymes such as nitric oxide (NO)-synthetase (Bredt & Synder 1990) are also
activated by Ca$^{2+}$ to convert the amino acid L-arginine into nitric oxide (NO), which upon release from the vascular endothelium stimulates guanylate cyclase to increase levels of cGMP in vascular smooth muscles, causing potent vasodilation (Toda 1990, Stoelting & Hillier 2006).

H$_1$ receptors are also found in conductive tissue of the atrioventricular (AV) node in humans, rodents, and dogs and slow HR by decreasing AV nodal conduction (Hageman et al. 1979, Jochem & Zwiska-Korzala 2004, Matsuda et al. 2004). H$_1$ receptors located on pancreatic duct epithelial cells activate Cl$^-$ and K$^+$ channels with a secretory effect in response to increased intracellular Ca$^{2+}$ (Nguyen et al. 1998).

1.5.b. H$_2$ receptor

This G-protein has an approximate molecular weight of 40 kDa and has been cloned from canine, rat and human cells (Gantz et al. 1991a, Gantz et al. 1991b, Ruat et al. 1991).

The receptor is coupled to adenylate cyclase via the G$_s$ protein pathway, and its activation increases the levels of cAMP in brain, stomach, fat, vascular smooth muscle, heart, and lungs of humans, dogs, monkeys, rats, rabbits, and guinea-pigs (Keller et al. 1981, Newton et al. 1982, Foreman et al. 1985, Hattori et al. 1988, Sarem-Aslani et al. 1991). Accumulation of cAMP activates protein kinase A enzymes that phosphorylate a wide variety of proteins involved in regulatory processes (Peters & Kovacic 2009). High levels of cAMP in gastric parietal cells activate the proton pump to secrete hydrogen ions and increase acid gastric secretion. In cardiac cells, cAMP causes phosphorylation of calcium channels, which increases the number of channels opened during depolarization.
(Levi & Alloati 1988). This has a positive inotropic and chronotropic effect on ventricular myocardium in a similar way to stimulation of β-adrenergic receptors. Consequently, H₂ activation has been linked with worsening of heart failure in dogs, and administration of H₂ blockers has been suggested as a therapy for those cases (Asanuma et al. 2006, Takahama et al. 2010).

Activation of H₂ receptors has been associated with increased intracellular Ca²⁺ through the same G-protein related pathway as H₁ activation, leading to increased capillary permeability and relaxation of vascular smooth muscles (Stoelting & Hillier 2006). Cerebral venodilation in dogs is only associated with activation of H₂ receptors (Monge et al. 1997).

The ability of this receptor to activate multiple G proteins fits into the complex paradigm of cross-talk between signaling pathways (Del Valle & Gantz 1997). This receptor is also involved in other physiological processes and its activation causes inhibition of basophil chemotactic responsiveness (Lichtenstein & Gillespie 1975), inhibition of mitogen mediated immunocyte proliferation via induction of suppressor T cells (Sansoni et al. 1985), differentiation of promyelocytic leukemic cells into mature granulocytes (Seifert et al. 1992), inhibition of PGE₂-stimulated duodenal epithelial bicarbonate secretion (Hogan et al. 1995), and alters the modulation of circadian rhythms (Peters & Kovacic 2009).

1.5.c. H₃ receptor

This histamine receptor was first cloned in 1999 (Lovenberg et al. 1999). Recent molecular studies have shown that a single form of the H₃ gene can give rise to multiple
isoforms in the rat (H3A, H3B, H3C) and guinea pig (H3L, H3S) (Tardivel-Lacombe et al. 2000, Drutel et al. 2001). Part of the H3 signal transduction pathways is due to coupling of G1 or Go proteins, which results in inhibition of adenyl cyclase activity, reduction in Ca²⁺ current, inhibition of the Na⁺/H⁺ exchanger and activation of protein kinase (Bakker et al. 2002). The H3 receptor’s primary function is to act as a presynaptic autoreceptor that inhibits the synthesis and release of histamine from histaminergic neurons in the central nervous system (Parsons & Ganellin 2006). In addition, this receptor modulates the release of other neurotransmitters such as acetylcholine, GABA, dopamine, glutamate, norepinephrine (NE), and serotonin, from the central and peripheral nervous system (Brown et al. 2001).

The H3 receptor is widely distributed throughout the brain, heart, gastric mucosa, kidneys and vascular system in dogs. H3 receptors found in the heart, kidneys, and systemic vasculature are localized within presynaptic membranes of postganglionic sympathetic nervous fibers where they act to inhibit presynaptic norepinephrine release (Yamasaki et al. 2001, Stoelting & Hillier 2006). H3 receptor activation may contribute to cardiovascular dysfunction in anaphylactic reactions by inhibiting adrenergic neural NE, as this catecholamine is released as a part of the adrenergic compensatory response to severe hypotension during anaphylactic shock, causing vasoconstriction (Schellenberg et al. 1991). Treatment with H3 antihistamines during an anaphylactic shock episode also improves left ventricular function in dogs (Chrusch et al. 1999).

In other pathologies such as myocardial ischemia, sympathetic over activity with excessive NE release is a prominent cause of cardiac dysfunction and arrhythmias. Massive release of NE disturbs Ca²⁺ homeostasis in myocytes, pacemaker cells, and
conducting tissue, causing arrhythmias and cardiac dysfunction (Levi & Smith 2000). Consequently, selective H₃ agonists have been proposed as a new therapeutic option in myocardial ischemia (Levi & Smith 2000, Yamasaki et al. 2001).

The role of this receptor in neuropathic pain, myocardial ischemia, osteoarthritis, epilepsy, cognitive impairment, and sleep disorders is currently under investigation (Yawata et al. 2004, Tiligada et al. 2009, Brioni et al. 2011, Cowart et al. 2012).

1.5.d. H₄ receptor

The histamine H₄ receptor is the most recently identified receptor subtype. It was discovered via a genomic approach using the H₃ receptor sequence (Oda et al. 2000). Since then it has been cloned in humans, mice, monkeys, pigs, and dogs (Liu et al. 2001, Oda et al. 2005, Jiang et al. 2008) showing a fairly low homology between species. The highest density of this receptor has been found in bone marrow and leukocytes with moderate levels in spleen and small intestine (Hough 2001). Compared with the other H receptors, the H₄ receptor has a more selective expression pattern being found mainly in cells of hematopoietic origin, including dendritic cells, mast cells, eosinophils, monocytes, basophils, and T cells (Thurmond et al. 2008). In primary cells culture, activation of the H₄ receptor appears to be mainly coupled to Gi/o proteins, thereby inhibiting cAMP formation and increasing intracellular Ca²⁺ levels by mobilization from intracellular stores (Morse et al. 2001, Zhu et al. 2001). One of the most important functions of the H₄ receptor is its role in innate immunity, where its activation mediates NK cell chemotaxis and cytokine release from invariant natural killer T cells (Leite-de-Morales et al. 2009).
This receptor has a major role in allergy and inflammatory diseases. Recently, histamine H4 receptor antagonists with high receptor affinity and specificity have been developed. The potential benefit of a co-administration of H1 and H4 antihistamines for treatment of allergic diseases has been suggested (Thurmond et al. 2008). In addition, the H4 receptor has potential therapeutic applications in asthma (Cowden et al. 2010), atopic dermatitis, autoimmune skin diseases (Thurmond et al. 2008), allergic rhinitis (Takahashi et al. 2009), sepsis (Matsuda et al. 2010), keratoconjunctivitis, allergic conjunctivitis (Nakano et al. 2009, Leonardi et al. 2011), and vestibular disorders (Desmadryl et al. 2012), and may produce antinociceptive effects to reverse thermal hyperalgesia in acute and persistent inflammatory pain, osteoarthritic injury, chronic neuropathic pain, and acute post-operative pain models in animals (Hsieh et al. 2010).

It has been recently shown that H4 receptors are expressed in human melanoma and breast cancer cells, and it is believed that they play an important role in cancer cell proliferation (Massari et al. 2011, Medina et al. 2011). Some H4 agonists have also been shown to partly protect against the hematological toxicity of chemotherapeutic agents (Petit-Bertron et al. 2009) and have therapeutic potential in oncology.

1.6. PLASMA HISTAMINE CONCENTRATION DETERMINATIONS

Basal plasma histamine concentrations of < 1.0 ng/mL are considered normal in humans (Dyer et al. 1982). Similar values (≤ 0.8 ng/mL) have been reported as normal in healthy dogs (Thermann et al. 1975, Robinson et al. 1988, Ishiguro et al. 2003, Guedes et al. 2007). One study reported higher plasma histamine concentrations (2.9 ± 2.2 ng/mL) in dogs with MCT (n= 17 dogs) when compared to normal dogs (0.7 ± 0.4 ng/mL), and
the degree of hyperhistaminemia could not be predicted from initial stage of the disease, histological grade of the tumour or tumour size (Fox et al. 1990). In another study with 11 dogs, higher histamine levels (0.26-2.75 ng/ml) where found in dogs with gross disease when compared with a control group (0.12-0.36 ng/mL), and a correlation between increasing levels of histamine and progression of the disease was found since all dogs that died of MCT (n=7) developed hyperhistaminemia (mean of 14 ng/mL; range of 5.11-30.1 ng/mL) (Ishiguro et al. 2003).

Different methods of measuring plasma histamine levels have been described and validated in different species. The enzyme HNMT has been used in humans and animals as a marker in radioenzymatic essays to determine histamine levels in plasma (Faraj et al. 1984, Fox et al. 1990). A novel acylating agent, succinyl glycinamide N-hydroxysuccinimide ester, was synthesized in 1988 (Morel & Delaage 1988), and monoclonal antibodies were synthetized to react with acylated histamine. Based on this finding, an immunoassay of high selectivity and sensitivity was designed to quantify histamine in any biologic fluid. This assay is used in most of the commercial immunoassays that are currently available for plasma histamine concentration determinations. Acylated histamine is incubated in monoclonal antibody coated wells in presence of alkaline phosphatase acylated histamine conjugate. After incubation, the wells are rinsed in order to remove non-bound components. The bound enzymatic activity is then measured after the addition of a chromogenic substrate. The use of these commercial kits has been validated for their clinical use in dogs (Morel et al. 1990, Ishiguro et al. 2003, Guedes et al. 2006, Guedes et al. 2007).
1.7. CARDIOVASCULAR EFFECTS OF HISTAMINE RELEASE

Histamine has multiple direct and indirect effects on the cardiovascular system, but some of the interactions are complex and still not totally understood due to a high variability. Histamine receptors are present in all parts of the circulatory system and the complex pharmacologic effects of histamine on the cardiovascular system can be explained by selective activation of each receptor subtype (Genovese & Spadaro 1997). Cardiovascular effects of histamine release may also vary depending on the species, since distribution of histamine receptors is different among them (Hirschowitz 1979). Observations also suggest there are individual variations in the physiological response to histamine (Ishiguro et al. 2003).

Direct effects of histamine include regulation of heart function, peripheral vascular resistance, and volume of circulating blood, but histamine also has an indirect influence on nervous and humoral cardiovascular regulation (Peters & Kovacic 2009). In dogs, systemic release of histamine causes dilation of coronary arteries and terminal arterioles that is independent of vascular innervation, and an increase in capillary permeability that leads to a decrease in SVR and consequently in systolic (SAP) and diastolic pressure (DAP) (Adams 1995, Genovese & Spadaro 1997, Peters & Kovacic 2009). It has been demonstrated in dogs and cats that those actions are mediated by H1 and H2 receptors, which are both present in pulmonary, cerebral, and systemic vascular beds (Black et al. 1975, Tucker et al. 1975, Hoffman et al. 1977, Monge et al. 1997). For this reason, simultaneous administration of both and H1 and H2-receptor antihistamines has been recommended to treat vascular depressor responses to histamine in dogs, cats and humans (Black et al. 1975, Owen et al. 1982, Lorenz & Doenicke 1985). Treatment
with the two antihistamines combined has proven to be more effective to prevent the cardiopulmonary responses associated with the intravenous administration of histamine in dogs, but it seems to be less effective for physiological changes associated with systemic anaphylaxis (Silverman et al. 1988). Prostaglandin D$_2$ (PGD$_2$) plays a role in mediating hypotension in humans with mast cell diseases (Roberts et al. 1980); this mediator is also released by canine mast cells and its actions are not prevented by antihistamine therapy (London & Thamm 2013).

The liver is one of the most important targeted organs in dogs with histamine release due to anaphylaxis (Shmuel & Cortes 2013). When histamine is released from the gastrointestinal tract into the portal vein during anaphylaxis, it causes vasodilation of the hepatic arterial and venous vasculature, with a consequent increase in arterial hepatic blood flow and intrahepatic pressure (Lautt & Legare 1987). Those hepatic vascular effects produce massive infiltration of fluid into the liver decreasing venous return, cardiac pre-load and consequently causing a decrease in CO (Richardson & Withrington 1978, Kapin & Ferguson 1985).

Histamine’s direct effects on heart function are complex and mediated by cardiac H$_1$, H$_2$ and H$_3$ receptor activation. The frequency of these effects varies within species due to a different density of histamine receptors in the heart. While in humans the presence of the H$_2$ type predominates, in dogs there is a higher density of H$_1$ receptors. Activation of the H$_1$ receptors may cause a decrease in ventricular contractility (negative inotropic effect) (Zavecz & Levi 1978) and may impair AV conduction (negative dromotropic effect) that can lead to AV dissociation (Levi & Kuye 1974, Hageman et al. 1979). Nevertheless, activation of H$_2$ cardiac receptors appears to increase HR
(chronotropic effect), increase ventricular contractility (positive inotropic effect) (Hirschowitz 1979, Asanuma et al. 2006) and increase AV conduction and the ventricular automaticity. Due to this conduction disturbance, histamine has arrhythmogenic properties. The occurrence of tachyarrhythmias appears to be mediated by H₂ activation causing stimulation of adenylate cyclase and high levels of intracellular cAMP that result in an increment in the slope of spontaneous diastolic depolarization, and restore spontaneity in quiescent tissues (Wolff & Levi 1986). Life-threatening ventricular arrhythmias generally do not occur in humans until plasma histamine concentrations are greater than 12 ng/mL (Lorenz et al. 1982).

Activation of H₃ receptors located on the presynaptic terminals of sympathetic effector nerves in the heart and systemic vasculature inhibits endogenous NE release from the sympathetic nerves. Increased NE release is a physiological compensatory neural adrenergic response that happens during cardiovascular shock. In dogs, inhibition of this compensatory response contributes to cardiovascular collapse during anaphylactic shock (Chrusch et al. 1999).

Histamine also affects the cardiovascular system indirectly through central histaminergic neurons (Peters & Kovacic 2009). As mentioned before, those neurons utilize histamine as a classic neurotransmitter and their bodies are localized in the tuberomamillary nucleus of the posterior hypothalamus. H₁, H₂ and H₃ receptors have been identified that mediate the actions of histamine in the central nervous system. The central histamine system is stimulated by high concentrations of histamine and has two main different indirect mechanisms to control the cardiovascular system (Brown et al. 2001).
- An increase in sympathetic flow by increasing the concentration of plasma catecholamines, especially NE (Akins & Bealer 1991).

- An increase in vasopressin levels (Bealer & Abell 1995).

A recent investigation in anesthetized dogs showed that stimulation of brain mast cells and histamine administration evoked renin and vasopressin release with an elevation of catecholamine levels (Matsumoto et al. 2008). In conscious rats, intracerebroventricular injections of histamine cause hypertension and bradycardia (Gatti & Gertner 1983) whereas hypertension and tachycardia are elicited in anesthetized rats (Finch & Hicks 1976a). In conscious cats, central administration of histamine causes hypertension mediated at least partially by H₁ receptors without changes in HR (Finch & Hicks 1976b).

The relationship between plasma histamine concentrations and adverse cardiovascular effects has not been well established for all species. There are also differences between the cardiovascular effects elicited by endogenous histamine release, exogenous histamine administration, and response to treatment (Silverman et al. 1988). In humans, plasma levels of endogenous histamine of 1 ng/mL are associated with only cutaneous signs, whereas levels of 1-12 ng/mL result in generalized severe cutaneous reactions, mild hypotension, tachycardia, arrhythmias, and difficulty breathing. Plasma histamine levels higher than 12 ng/mL usually require emergency treatment due to cardiovascular collapse (fatal arrhythmias, severe hypotension, cardiac arrest) and severe bronchospasm (Lorenz et al. 1982, Sánchez Palacios et al. 2000).
1.8. H₁-ANTIHISTAMINES

Antihistamines are the largest class of medication used in the treatment of allergic disorders in humans and animals (Parsons & Ganellin 2006).

H₁-antihistamines have been traditionally classified into chemical groups: ethanolamines, athylelediamines, alkylamines, piperazines, piperidines, phenothiazines, and others (Estelle et al. 2009).

Functionally they are usually separated in two groups known as first-generation or second-generation. First-generation drugs easily cross the blood brain barrier acting on H₁ receptors located on postsynaptic membranes of histaminergic neurons (Simons 2004, Simons & Simons 2011). Because of this effect in the CNS, first-generation compounds can be used as sedatives in humans and to treat motion sickness and nausea (Stoelting & Hillier 2006).

Second generation compounds are less fat soluble and compared to the first-generation antihistamines have minimal central nervous system effects (Peters & Kovacic 2009). Another difference between the two groups is that first-generation drugs may also act on muscarinic cholinergic, serotonin and α-adrenergic receptors, increasing the chances of adverse effects, while second-generation compounds have a higher selectivity for H₁ receptors without affecting other receptors (Stoelting & Hillier 2006). The term “third-generation” antihistamine was introduced in 2003 by a consensus group of experts on new-generation antihistamines (CONGA), which defined the characteristics required for a third-generation or multifunctional H₁ antihistamine (Holgate et al. 2003). These characteristics include a drug with demonstrable anti-inflammatory effects, a favorable therapeutic index, and three ‘prerequisites’ that seem necessary as primary components of
a new class of antihistamines: lack of cardio toxicity, lack of drug–drug interactions, and lack of CNS effects (Holgate et al. 2003).

All H₁-antihistamines act as inverse agonists that bind to and stabilize the inactive conformation of the H₁-receptor. Traditionally, these drugs were described as H₁-antagonists or H₁-blockers, but this does not accurately reflect their molecular mechanism of action (Leurs et al. 2002, Simons 2004). In physiological circumstances, the inactive and active states of the H₁ receptor are in equilibrium, and histamine has preferential affinity for the active state. When histamine binds to the receptor it stabilizes the active conformation, shifting the equilibrium towards this state. Conversely, H₁-antihistamines have preferential affinity to the inactive form of the receptor, stabilizing the receptor in this conformation and shifting the equilibrium toward the inactive state, preventing histamine from binding to the receptor (Bakker et al. 2002, Simons & Simons 2011). When histamine interacts with the receptor the equilibrium is already shifted, therefore preventive treatment with H₁-antihistamines is more effective against histamine actions than reversing the effects that have already taken place (Simons & Simons 1983).

Histamine plays an important role in the pathophysiology of IgE mediated conditions such as allergies and pruritus by interacting with H₁ receptors. H₁-antihistamines are primarily administered in allergic pathologies for their ability to block histamine effects on post-capillary venule smooth muscle that results in a decrease in vascular permeability. They also act by inhibiting histamine stimulation of type-C nociceptive nerve fibers, reducing the incidence of itching and sneezing (Thurmond et al. 2008). Anti-inflammatory properties are related to inhibition of inflammatory cell
accumulation by down-regulating the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Leurs et al. 2002).

Patients with systemic mast cell disorders, such as systemic mastocytosis, a hematologic neoplasia characterized by prolonged survival and accumulation of neoplastic mast cells in visceral organs, also benefit from the use of H1-antihistamines (Hadzijusifovic et al. 2010). This pathology resembles the systemic form of MCT in veterinary patients. Second generation H1-antihistamines exert growth-inhibitory effects on canine, feline and human neoplastic mast cells carrying transforming KIT mutations in vitro. In addition, in vitro studies have shown that various H1-antihistamines have the capacity to also inhibit mast cell activation and histamine release in various species (Mota & Da Silva 1960, Church & Gradidge 1980, Weller & Maurer 2009) including the dog (Garcia et al. 1997). The suggested mechanism of action involves a direct inhibitory effect of H1-antihistamines on Ca^{2+} channels present in mast cells and basophils, reducing the intracellular Ca^{2+} store depletion, stabilizing the cellular membrane, and consequently avoiding degranulation (Leurs et al. 2002). However, this hypothesis has not been reproduced in vivo using clinical doses (Perzanowska et al. 1996).

First generation compounds such as DPH, chlorpheniramine, cyproheptadine and hydroxyzine are still the most commonly used antihistamines in veterinary medicine because they are effective and inexpensive (Merchant & Taboada 1989, Peters & Kovacic 2009).
1.8.a. Side effects of first generation H₁-antihistamines

Most of the adverse effects from first-generation H₁-antihistamines result from their ability to cross the blood brain barrier (BBB) and their relatively low specificity for the histamine H₁ receptor. H₁-antihistamines have a high lipophilicity, relatively low molecular weight, and lack of recognition by the P-glycoprotein efflux pump that is expressed on the luminal surfaces of non-fenestrated endothelial cells in the CNS vasculature, all of which facilitate their passage through the BBB (Christy et al. 2001, Chen et al. 2003). First generation antihistamines such as chlorpheniramine and DPH inhibit neurotransmission in histaminergic neurons and potentially impair alertness, cognition, learning, rapid response-waking memory, and attention tasks in humans even at low doses, which may cause drowsiness, somnolence, fatigue, sedation, dizziness, confusion, agitation, headache, dystonia, dyskinesia, and hallucinations (Taglialatela et al. 2000, Pfizer® 2006, Simons & Akdis 2009, Simons & Simons 2011). Because of their poor selectivity for the H₁-receptor, first generation compounds have antimuscarinic, antiserotonergic and anti-α-adrenergic effects, resulting in potential side effects, such as mydriasis, dry eyes, dry mouth, nausea, vomiting, dose-related tachycardia, thickening of bronchial secretions, constipation, urinary retention, increased appetite, dizziness and orthostatic hypotension (Simons & Akdis 2009, Simons & Simons 2011).

Cardiac toxicity associated with H₁-antihistamines treatment is not frequent but it has been reported in humans at doses larger than therapeutic. Some signs of toxicity reported in humans include sinus tachycardia, ventricular arrhythmias, and QT prolongation (Zareba et al. 1997, Khalifa et al. 1999, Taglialatela et al. 2000). There are
no reports of cardiac toxicity in veterinary species but *in vitro* studies have shown that DPH administration may block the repolarizing $K^+$ currents and lengthen cardiac repolarization in guinea pig and feline cardiac cells (Wang et al. 1998, Khalifa et al. 1999).

In dogs, first generation H$_1$-antihistamines abolished autoregulation of renal blood flow (RBF) and glomerular filtration rate (GFR) when used to treat reactive hyperemia and these effects should be considered in dogs with renal pathology or a predisposition to hypotensive events (Banks et al. 1984).

Because of their $\alpha$-adrenergic blockade activity, first-generation antihistamines have the potential to cause peripheral vasodilation, postural hypotension, and dizziness (Simons 2004, Sheikh et al. 2007), often reported after promethazine administration (Estelle et al. 2009, Ellis & Brown 2013). Hypotension and tachycardia are also described as possible adverse reactions in humans with parenteral administration of DPH (Pfizer® 2006). For this reason, some clinicians recommend slow IV administration or prefer the use of the intramuscular route (Plunkett 2000, Fossum et al. 2013). However, there are no reports of hypotensive events after DPH administration in veterinary patients.

In dogs, administration of clinical doses of H$_1$-antihistamines has been associated more commonly with drowsiness, increased pruritus, and vomiting. Ataxia, hypersensitivity, hyperesthesia, diarrhea, seizures, muscle tremors, tachypnea, and anticholinergic effects such as dry mucosa, tachycardia, urinary retention, and hyperthermia have also been described in dogs receiving high doses (> 25 mg/kg, PO) of DPH (Papich 1990, Lagutchik et al. 1997, Clark & Dorfman 2000). Cats are reported to
be more sensitive to antihistamine administration and can exhibit hyperexcitability even at low doses (Gilbert 2009).

1.9. DIPHENHYDRAMINE (DPH)

1.9.a. Pharmacokinetics of DPH

Diphenhydramine hydrochloride or 2-(Diphenylmethoxy)-N,N-dimethylethylamine hydrochloride is a first-generation ethanolamine-derivative antihistamine (Nadendla 2005).

(Imons & Simons 1994)

Diphenhydramine has an oral bioavailability in people of 40-70% and it is the preferred route of administration for therapeutic use. In humans, DPH is metabolized almost completely in the liver via cytochrome P450 2D6, 1A2, 2C9, and 2C19 isoenzymes to nordiphenhydramine (active metabolite), dinordiphenhydramine, diphenylmethoxyacetic acid and DPHM-N-oxide metabolites (Akatsu et al. 2007). Only 2-10 % of unchanged DPH is excreted with in the urine in different species, which reflects the extensive metabolism of the drug (Drach et al. 1970, Albert et al. 1975, Wasfi et al. 2003).
The primary route of metabolism in most species is through two successive demethylations and the resulting primary amine is further oxidized to carboxylic acid (Bilzer et al. 1974, Paton & Webster 1985). Some differences among species have been observed in the final transformation of the third metabolite (diphenylmethoxyacetic acid), which is conjugated with glutamine by rhesus monkeys and rats, or with glycine by dogs (Drach & Howell 1968, Drach et al. 1970). In sheep, DPH metabolism follows other routes since those metabolites represent just 0.5-2% of the dose administered, and hepatic metabolism accounts for only about 32% (Kumar et al. 1998, Kumar et al. 1999a). In horses oxidation to carboxylic acid is less significant than in other species (Wynne et al. 1996).

Studies in pregnant ewes have shown that DPH widely crosses the placenta blood barrier affecting the fetus in a dose-dependent way, and that uptake and metabolism by the fetal liver plays a major role in fetal non-placental elimination of the drug (Rurak et al. 1988, Kumar et al. 1997).

Peak plasma concentrations are achieved in 1.5 to 2.5 h in humans after oral administration of 50 mg (Simons et al. 1990, Scavone et al. 1990), and in 1 to 2 h in dogs administered 1 mg/kg orally (Koyanagi et al. 2014). In people, effective antihistamine concentrations in plasma are greater than 25 ng/mL and are usually achieved after oral therapeutic doses of 25-50 mg in adults (Bilzer et al. 1974, Estelle et al. 2009). Plasma concentrations of 25 to 50 ng/mL were not associated with significant sedation in one study (Carruthers et al. 1978), whereas in another study apparent DPH concentration thresholds to produce drowsiness were found to be 30.4-41.5 ng/mL and concentrations
higher than 60 ng/mL resulted in mental impairment (Carruthers et al. 1978, Gengo et al. 1989).

Recommended doses for DPH in cats and dogs reported in the literature are 1-4 mg/kg, administered orally, IM, or IV (Adams 1995, Papich 2011) but no studies regarding the relationship between therapeutic dose and plasma concentrations have been performed in these species.

Systemic availability

Bioavailability (%F) is generally defined as the fraction of a drug dose reaching the systemic circulation without chemical modification, compared with the same dose given intravenously (IV). Because IV injection of a drug represents complete systemic availability, %F can be calculated from the ratio of the areas under the concentration-time curves (AUC) for an identical bolus dose given by the alternative route (oral, intramuscular, or subcutaneous) and intravenously (Adams 1995, Peck et al. 2003). The %F after intramuscular (IM) or subcutaneous (SQ) administration is determined by the rate and extent of the drug absorption. Drug absorption depends on the physicochemical properties of the substance, drug formulation factors, and blood flow rate to the site of administration (Sams & Muir 2009). First-pass metabolism through the portal circulation and passage through the liver before entering the systemic circulation for delivery to their target tissues also affects the rate of absorption of orally administered drugs and can result in lower %F of the drug ( Boothe 2012, Holford 2012). Plasma concentrations of DPH are relatively low after single oral doses in humans because it undergoes first-pass metabolism in the liver and only 40–70% of the drug reaches the systemic circulation.
Systemic availability after sublingual administration has been reported in humans to be 58% (Scavone et al. 1990). Oral %F in dogs is much lower than in humans with only 2.8-7.3% of the drug contributing to plasma concentrations (Koyanagi et al. 2014). No information is available about the systemic availability of DPH after IM or SQ administration in veterinary species, but in rats and guinea pigs higher concentrations of the drug are attained in tissues when administered by these routes than for oral administration suggesting a higher %F (Dill & Glazco 1949).

**Volume of distribution**

Volume of distribution (Vd) of a drug is a mathematical expression of the sum of the apparent volumes of the compartments that constitute the compartmental model. Values for Vd can vastly exceed any physical volume in the body and because it is theoretical, the Vd is generally referred to as an apparent Vd (Boothe 2012). In quantitative terms, Vd represents the fluid volume that would be required to contain the total amount of absorbed drug in the body at a concentration equivalent to that in the plasma at steady-state (Baca & Golan 2011). It reflects a balance between movement of the drug out of the circulation into other tissues, which decreases plasma concentration and makes the apparent volume larger, and binding to plasma proteins, which increases plasma concentration and makes the apparent volume smaller (Holford 2012). In a simple one-compartmental model it is calculated as the dose of drug administered IV divided by the resulting plasma concentration of drug before elimination begins or when steady-state conditions have been achieved (Initial Vd and Vd$_{ss}$, respectively) (Stoelting...
The Vd is influenced by the physicochemical properties of the drug such as lipid solubility, plasma protein binding, and molecular size. In general, drugs with large molecular size, low lipid solubility, and which are highly bound to plasma proteins have small Vd, close to the extracellular plasma fluid volume. Drugs with small molecular size and very lipophilic properties are found in high concentration in tissues and their plasma concentrations decrease quickly, and therefore have larger volumes of distribution (Riviere 2009, Stoelting & Hillier 2006). The volume of distribution is useful in estimating the dose required to achieve a given plasma concentration, and variation of Vd mainly affects the peak plasma concentration of the drug. Thus, for drugs of equal potency, a drug that is more highly distributed among body tissues generally requires a higher initial dose to establish a therapeutic plasma concentration than does a drug that is less highly distributed (Baca & Golan 2011).

Diphenhydramine is a highly lipophilic drug, with a relatively large Vd that increases with the dose administered (Yoo et al. 1990), and is extensively protein-bound (78.4-98%) in humans, sheep, rhesus monkeys, and dogs (Yoo et al. 1990, Koyanagi et al. 2014). Studies of tissue distribution of DPH performed in rats, guinea pigs and humans showed tissue accumulation in lungs, kidneys, liver, brain, and muscle (Dill & Glazco 1949, Okumura et al. 1978, Hausmann et al. 1983). Mean apparent Vd for adult humans after administration of a total dose of 50 mg of DPH, IV (≈1.4 mg/ kg) has been reported as 4.17 L/kg (Berlinger et al. 1982), 4.54 ± 0.87 L/kg (Blyden et al. 1986), 3.29 L/kg (Carruthers et al. 1978), and 3.56 ± 0.45 L/kg (Scavone et al. 1990). One study in humans demonstrated that the Vd/F of DPH after an oral dose of 1.25 mg/kg decreases with age; 13.6 ± 6.3 L/kg in elderly adults, 17.4 ± 4.8 L/kg in younger adults, and 21.7 ±
6.6 L/kg in children (Simons et al. 1990). Data available in veterinary medicine is more difficult to compare due to differences in methodology of the studies, dose range, and species differences. In one recent study, no difference in Vdss was found when comparing young (10.5 ± 1.0 L/kg) and older (12.1 ± 6.7 L/kg) dogs administered 0.2 mg/kg, IV (Koyanagi et al. 2014). But in sheep, similar to humans, Vdss of DPH after IV administration decreases with age; 13.1 ± 3.1 L/kg for fetal lambs, 4.4 ± 2.4 L/kg for young lambs, and 2.1 ± 1.1 L/kg for adults (Kumar et al. 1999a, Wong et al. 2000). Similar results were found in another study after DPH IV infusion, where Vdss for fetal lambs was significantly higher than in young lambs (Au-Yeung et al. 2007). A decrease in Vdss after birth is expected because the drug cannot distribute to the maternal compartment that is available to the fetus during pregnancy (Kumar et al. 1999b, Au-Yeung et al. 2007). In rabbits administered 2.56 mg/kg of DPH, IV the Vdss was 11.4 L/kg (Simons et al. 1996), and in camels and horses administered 0.625 mg/kg, IV the Vdss was 2.38 L/kg and 5.98 L/kg, respectively (Wasfi et al. 2003).

Elimination Half-Life

Elimination half-life (T1/2) has been defined as the time necessary to eliminate 50% of the drug from the body after IV injection. The recently new introduced term terminal half-life is used now most commonly. More specifically, terminal half-life is defined as the time required for plasma concentrations of the drug to decrease by 50% after pseudo-equilibrium of distribution has been reached (Toutain & Bousquet-Melou 2004a); both terms are interchangeable when the decrease in plasma drug concentration is parallel to its elimination from the body (Stoelting & Hillier 2006).
pharmacokinetic model the terminal $T_{1/2}$ depends on the Vd and clearance of the drug:

$$T_{1/2} = \frac{0.693 \times V_d}{Cl}$$

An exponential process can describe drug elimination; the time taken for a two-fold decrease can be shown to be proportional to the natural logarithm of 2 (approximately 0.693), assuming first-order kinetics (Holford 2012).

The amount of drug remaining in the body is related to the number of elimination half-times that have elapsed, which allows the estimation of frequency of dosing required to maintain the plasma concentration of the drug in the therapeutic range (Baca & Golan 2011). Approximately five half-lives are required for nearly total elimination of the drug from the body after a single bolus. Drug accumulation is expected if rate-dosing intervals are less than this period of time and can lead to toxicosis (Sams & Muir 2006). During a constant rate infusion of a drug 50% of the steady-state concentration is reached after one half-life, 75% after two half-lives, and over 90% after four half-lives. When the rate of elimination equals the rate of administration, the steady-state plasma concentration is achieved. The time necessary for a drug to achieve this state with intermittent dosing is close to five half-lives (Stoelting & Hillier 2006, Holford 2012).

Mean $T_{1/2}$ in people after oral administration of a total dose of 50 mg of DPH ($\approx 1.4$ mg/kg for a 70 kg person) ranges between 3.4 and 9.2 h. The $T_{1/2}$ has been reported in humans as $3.4 \pm 0.2$ and $4.7 \pm 0.5$ h in adults (Carruthers et al. 1978, Scavone et al. 1990), $4.1 \pm 0.4$ h in Caucasian men (Spector et al. 1980), $4.8 \pm 0.33$ h in young adults, $4.9 \pm 0.7$ h in elderly women (Berlinger et al. 1982), $6.0 \pm 0.8$ h in women receiving oral
contraceptives (Luna et al. 1989), and 9.2 ± 1.2 h in adults (Blyden et al. 1986). The T1/2 of 1.25 mg/kg of oral DPH increases with age; 5.4 ± 1.8 h in children, 9.2 ± 2.5 h in young adults, and 13.5 ± 4.2 h in elderly adults (Simons et al. 1990). This prolongation in T1/2 was speculated to be associated with an age-related decrease in hepatic function, associated with reduced activity of the P-450 enzyme system, reduced hepatic blood flow, and diminished number and metabolizing capacity of hepatocytes in geriatric populations (Simons et al. 1990). Higher T1/2 have been reported in studies with sample collection times of 24 h or longer (Blyden et al. 1986, Simons et al. 1990); the differences with other studies may be because shorter times of sampling do not ensure sampling throughout the elimination phase, therefore sampling collection for at least 24 h post-administration has been recommended (Simons et al. 1990). Similar values of T1/2 have been reported after IV administration of equal doses (≈1.4 mg/kg) in adults, young adults, elderly women, Oriental and Caucasian males, and people with chronic hepatic disease (Albert et al. 1975, Carruthers et al. 1978, Spector et al. 1980, Berlinger et al. 1982, Meredith et al. 1984, Blyden et al. 1986, Scavone et al. 1990).

Elimination half-life values after DPH IV administration reported for several species are: 1 h for monkeys (n = 10) after a dose of 10 mg/kg, 2 h for pregnant monkeys (n = 10) after a dose of 10 mg/kg, 1 h for beagle dogs (n = 2) after a dose of 5 mg/kg, 0.3 h in New Zealand white rabbits (n = 2) after a dose of 3 mg/kg, 1 h in guinea pigs (n = 7) after a dose of 5 mg/kg, 0.1 h in mice (n = 15) after a dose of 3 mg/kg, and 1 h in rats (n = 3) after a dose of 7 mg/kg (Drach et al. 1970). More recent studies have reported longer T1/2 of 1.3 ± 0.2 h after IV administration of 3 mg/kg in rabbits of the same breed (Simons et al. 1996) and 2.1-5.9 h after IV administration of 0.2 mg/kg or 4.2-6.6 h after
oral administration of 1 mg/kg in dogs of the same breed (Koyanagi et al. 2014). In horses administered 0.625 mg/kg IV, the T\(_{1/2}\) was 6.11 h, whereas the same dose and route in camels resulted in a T\(_{1/2}\) of 1.58 h (Wasfi et al. 2007). In adult sheep, the T\(_{1/2}\) after an IV dose of 0.25-2.5 mg/kg ranges between 34 and 68 min (Yoo et al. 1990, Kumar et al. 1997, Kumar et al. 1999b, Au-Yeung et al. 2007), between 22 and 70 min in lambs (Wong et al. 2000, Au-Yeung et al. 2007), and between 33 and 53 min in fetal lambs (Kumar et al. 1997, Kumar et al. 1999b, Wong et al. 2000).

**Clearance**

Plasma clearance (Cl) is defined as the plasma volume that is totally cleared of drug by excretion and/or metabolism per unit time (Toutain & Bousquet-Melou 2004b).

\[
\text{Dosing rate} = \frac{Cl \times \text{Therapeutic plasma concentration}}{\%F}
\]

\[
\text{Plasma Cl} = \frac{\text{Total (body) rate of drug elimination}}{\text{Plasma concentration}}
\]

Therefore, more of the drug is cleared from the plasma per unit time immediately after administration, when the plasma concentration is the highest (Baca & Golan 2011). For most drugs administered at therapeutic doses, Cl is constant over the concentration range encountered in clinical settings; therefore a drug is cleared from the circulation at a rate proportional to the amount of drug present in plasma (first-order kinetics; Stoelting & Hillier 2006, Holford 2012). The most frequent technique for estimating total Cl (Cl\(_{\text{tot}}\)) or first-order clearance consists of administering an IV single dose of the drug and
measuring sequentially plasma concentration until drug concentration in plasma is less than the limit of quantification (LOQ) (Toutain & Bousquet-Melou 2004b).

\[ Cl_{tot} = \frac{Dose}{AUC} \]

Because DPH is mainly metabolized in the liver in most species and just a small portion is eliminated unchanged by the kidneys, Cl of the drug is highly dependent on hepatic clearance. Hepatic clearance of a drug is the product of the hepatic blood flow and the extraction ratio of the drug, which compares the drug levels in plasma immediately before entering and just after exiting the organ (Stoelting & Hillier 2006, Sams & Muir 2009, Baca & Golan 2011). In people, a decrease in hepatic clearance is associated with age due to lower hepatic blood flow and a decline in hepatic metabolism (Simons et al. 1990). In another study comparing two populations with and without chronic liver disease, Cl in cirrhotic patients was significantly less than in normal subjects (Meredith et al. 1984) due to a decrease in the hepatic extraction ratio of the drug because of decreased numbers of functional hepatocytes. Plasma Cl reported in healthy adult humans after IV and oral DPH administration ranges between 6.2 and 11.2 mL/min/kg (Carruthers et al. 1978, Spector et al. 1980, Berlinger et al. 1982, Blyden et al. 1986, Simons et al. 1990). In women, Cl was reported to be 21.8 ± 3.2 mL/min/kg, suggesting a higher Cl value in this population (Luna et al. 1989). Clearance values have been reported to be significantly higher in children after oral administration (49.2 ± 22.8 mL/min/kg, Simons et al. 1990); this age-related variation has also been noted in sheep, where the drug is eliminated significantly faster from plasma in lambs than in adults.
(Wong et al. 2000, Au-Yeung et al. 2007), and in 2-year-old versus 10-year-old dogs (51.8 ± 14.6 mL/min/kg versus 30.9 ± 6.1 mL/min/kg) (Koyanagi et al. 2014).

The Cl values in 15-day-old lambs exceeded values for hepatic blood flow, so the presence of an extrahepatic clearance component for young animals has been suggested (Au-Yeung et al. 2007). Studies in pregnant ewes have confirmed that maternal placental Cl and fetal non-placental Cl are greater than clearance values in the mother. It appears that renal excretion is different in the fetus than in the adult: fetal kidney and lungs appear to be responsible for 10% of total non-placental Cl in the fetus, with the fetal liver accounting for the remainder (Yoo et al. 1993, Kumar et al. 1997). Values of total clearance after IV administration in other species are: 23.6 mL/min/kg in camels, 13.16 mL/min/kg in horses (Wasfi et al. 2003) and 128 ± 45 mL/min/kg in rabbits (Simons et al. 1996).

1.9.b. Clinical uses in veterinary medicine

Despite the introduction of newer drugs with fewer side effects, DPH is still one of the most prescribed H₁ antihistamines in veterinary medicine due to its effectiveness and low cost (Peters & Kovacic 2009). In many countries DPH is labeled and authorized for treating canine and feline allergic and pruritic dermatitis (Iwasaki & Hasegawa 2006). The neurological pathways causing pruritus are composed of dedicated C-fibers in the periphery and histaminergic neurons that project their axons into the brain, where they express H₁ receptors (Stander & Steinhoff 2002, Thurmond et al. 2008). Diphenhydramine has moderate efficacy but high individual variability to control angioedema and pruritus associated with allergic reactions and histamine release due to
mast cell malignant proliferation in small animals (Scott & Miller 1999, Gilbert 2009). Although DPH has been used in dogs and cats as a preferred treatment for atopic dermatitis, there is no conclusive evidence of efficacy of topical or oral treatment with this drug and it is no longer recommended for this specific use (Olivry et al. 2010). The use of antihistaminic therapy seems to be unpredictable and especially ineffective in dogs with chronic changes and seems to work better if given consistently and prophylactically rather than after the dog has become moderately pruritic (Peters & Kovacic 2009, Bloom 2013).

Diphenhydramine may cause drowsiness, slow physical reaction times, and sedation in humans (Lines et al. 1992, Kaneko et al. 2000). For this reason it is preferentially prescribed at night for people with sleeping disorders, to decrease anxiety, and for perioperative sedation (Katz et al. 2000, Meltzer et al. 2007). In dogs, drowsiness has been reported after oral administration of DPH at range doses of 10-50 mg/kg (Wauquier et al. 1981, Scott & Buerger 1988), which are higher doses than those prescribed for parenteral administration. In a prospective randomized clinical trial DPH administered IM at 2, 4, and 8 mg/kg doses did not cause any sedation prior to anesthesia in healthy dogs (Hofmeister & Egger 2005).

First generation antihistamines can block both cholinergic and histaminergic nerve signals responsible for transmission of the vestibular stimulus to the vomiting center in the medulla (Simons & Simons 2011), preventing nausea, vomiting, motion sickness and vestibular disorders. The use of DPH in humans is common as an antiemetic during pregnancy, chemotherapy, and opioid administration in the perioperative period (Bleicher et al. 2008, Lu et al. 2010, Niebyl 2010). In dogs, oral
DPH 25 mg (approximately 5.5 mg/kg) administered once a day decreased the incidence and delayed the onset of emesis following exposure to ionizing radiation (Gralla et al. 1979). Oral DPH at 2-4 mg/kg is also commonly prescribed to prevent carsickness in dogs and cats (Papich 2000, Dowling 2012).

Because of its anticholinergic effects, DPH can be used as a coadjuvant treatment in cases of organophosphate and carbamate pesticide poisoning in veterinary patients (Clemmons et al. 1984, Arnot et al. 2011). Preventive administration of DPH in mice and rats has shown protective properties against organophosphate insecticide-induced toxicosis reducing the incidence of toxic manifestations, delaying the onset of death, and decreasing the mortality rate (Faris & Mohammad 1997, Bird et al. 2002). When used as a treatment in rats with signs of organophosphate toxicity, DPH significantly reduced troponin I levels and attenuated myocardial injury (Yavuz et al. 2008). In dogs, DPH corrected the muscle electrical abnormalities caused by organophosphate administration, indicating that it may be effective in treating organophosphate-induced neuromuscular weakness refractory to other forms of therapy (Clemmons et al. 1984).

The use of DPH to treat severe anaphylactic reactions remains controversial; clinically it is better to use DPH as a preventive drug for anaphylaxis. Epinephrine, oxygen, IV fluids, vasopressors, and glucocorticoids are preferred as first line treatment for severe reactions. Studies in anesthetized dogs and cats determined that DPH alone (6-10 mg/kg) was able to abolish the vascular depressor effects of low doses of histamine (Folkow et al. 1948), however, DPH and other H1-anthistamines administered alone, were not able to abolish the severe cardiovascular depression caused by high doses of exogenous histamine (Folkow et al. 1948, Tucker et al. 1975, Black et al. 1975).
Most animal models for anaphylaxis are based on exogenous administration of histamine. Combination of H₁ and H₂ antihistamines in dogs is effective to prevent the cardiopulmonary responses associated with the intravenous administration of histamine but not to prevent physiological changes associated with endogenous systemic anaphylaxis, even though exogenous administration of histamine results in higher histamine plasma levels (Silverman et al. 1988). For this reason, antihistamine treatment remains empirical and a demonstrated benefit for clinical emergencies has not been confirmed (Sheikh et al. 2007, Simons 2010, Shmuel & Cortes 2013).

When administered alone, DPH modestly stimulates ventilation by augmenting the interaction of hypoxic and hypercarbic ventilator drives (Fukuda 2010). It has been shown that in humans, DPH IV (0.7 mg/kg) counteracts the alfentanil-induced decrease in the slope of the ventilator response to carbon dioxide (Babenco et al. 1998).

Diphenhydramine has been shown to be an effective local anesthetic in humans and small mammals for procedures of short duration. The anesthetic properties of DPH have been attributed to its structural similarities with local anesthetics and recent investigations have shown that it is able to block Na⁺ channels of sensory neurons in a similar way (Kim et al. 2000, Kuo et al. 2000). Used as a 0.5-1% solution injected locally with or without epinephrine, DPH appears to be as effective as lidocaine and prilocaine to provide regional anesthesia prior to oral or dermatologic surgery in humans (Gallo & Ellis 1987, Ernst et al. 1994, Uckan et al. 1998, Pavlidakey et al. 2009). In rats, DPH is less potent than bupivacaine at producing cutaneous analgesia but at equipotent doses they have the same duration of action (Chen et al. 2014). Diphenhydramine is not recommended as a substitute for more common local anesthetics since it is an irritant to
the tissues and causes pain at injection, but it appears to be a good alternative in patients with allergy to amides or in cases where the maximum dose recommended of amide anesthetic has been reached (Dire & Hogan 1993, Ernst et al. 1994, Singer & Hollander 1995, Uckan et al. 1998, Pavlidakey et al. 2009). Tissue necrosis has been reported as a possible side effect of DPH injection (Dire & Hogan 1993).

When administered intrathecally in rats, DPH produces spinal anesthesia with sensory and nociceptive blockade more potent than that observed with lidocaine (Hung et al. 2011). It has been also proposed as a good alternative for corneal anesthesia since 5% DPH solution has an anesthetic effect when administered topically to rabbits (Suffridge et al. 2009).

Various authors have reported in the veterinary literature the common use of parenteral DPH (0.5-2 mg/kg, IM) as a premedication agent prior to manipulation of MCTs in an attempt to decrease histamine effects, mainly cardiovascular depression, derived from mast cell degranulation during diagnostic or surgical procedures (Merchant & Taboada 1989, Hofmeister & Egger 2005, Ryan et al. 2012, Fossum et al. 2013). However, no clinical trials or prospective studies have been performed to assess the efficacy, true benefits, or potential detriments under clinical conditions of DPH administration in patients with MCT.

1.10. MAST CELL TUMOURS

The first mast cell tumour (MCT) was described early in the 1900’s (Bashford et al. 1905) and the term “mastocytoma” was first used in 1942 (Bloom 1942). Currently, the terms “mast cell tumour” and “mastocytoma” are equally used to define a malignant
proliferation of mast cells. Cutaneous and subcutaneous mastocytomas are the most common types of MCTs and are thought to arise from mast cells in the dermis and subcutaneous tissue (Bostock 1986), but MCT can potentially arise from any location where mast cells are normally found, including lung, intestinal mucosa and perivascular regions (Maker-Gabb et al. 2003).

1.10.a. Mast cells

Mast cell progenitors are derived from pluripotent CD34 cells located in the bone marrow (Kirshenbaum et al. 1991). In dogs, these precursor cells are known as canine bone marrow-derived mast cells (cBMMCs) (London & Thamm 2013). Most of the progeny of multipotent stem cells leave the hematopoietic tissue after they differentiate, but immature mast cells differentiate into phenotypically identifiable mast cells after they infiltrate connective or mucosal tissue (Kitamura & Fujita 1989). Differentiated mast cells can be found in the lung, gastrointestinal mucosa, and surrounding blood vessels, but in dogs the highest concentration of mast cells can be found in the liver and skin (Tams & Macy 1981). There are multiple mechanisms involved in the regulation of mast-cell differentiation. It is stimulated by contact with fibroblasts and inhibited by the presence of differentiated mast cells (Kitamura 1989), but the mechanism most studied in recent years has been the influence of cytokines (IL-3, IL-4, IL-6, among others) and stem cell factor (SCF), also known as c-kit ligand, in the development and maturation of mast cells (Austen & Boyce 2001). A characteristic feature of mature mast cells is the presence of cytoplasmic granules, which contain many biologically active substances, including heparin, histamine and proteolytic enzymes. It has been demonstrated that
these mature, granulated mast cells have mitotic activity indicating that fully
differentiated mast cells have the potential to proliferate (Curry et al. 1998).

Mature mast cells express high-affinity IgE receptors in their surface. Binding of
antigens to these receptors leads to immunological activation and subsequent release of
the content of granules and production of various mediators such a prostaglandins (PGE₂,
PGD₂), leukotrienes, platelet activating factor, cytokines (TNF-α, IL-3, IL-4, IL-5, and
IL-6) and others (London & Seguin 2003). These mediators lead to several reactions that
can contribute to hypersensitivity reactions. Non-immunological activation might also be
induced by cytotoxic or non-cytotoxic stimulation. Cytotoxic stimulation causes the
rupture of the plasma membrane while non-cytotoxic stimulation induces mediator
release allowing the membrane to remain intact. Substances able to induce non-
immunological activation include cytokines, neuropeptides, anaphylatoxins, free radicals,

Mast cells and basophils are generally thought to be the major sources of
endogenous histamine in normal tissue and can be modulated by histamine as they
express H₁, H₂ and H₄ surface receptors, although how this varies between different mast
cell types is not clear. As a result of those interactions histamine can cause varying and
sometimes counteracting effects in the same cell depending on the concentration of
histamine and the specific histamine receptors that are activated. (Thurmond et al. 2008,
Peters & Kovacic 2009)

Therefore, mast cells have always been primarily defined as effector cells of the
allergic response and activation of mast cells and interaction of certain components with
their various surface receptors plays a critical function in both the initiation of the innate

1.10.b. Canine mast cell tumours

Epidemiology

Cutaneous MCT is the most common malignant cutaneous tumour in the dog and accounts for 11 to 21% of all canine skin tumours (Rothwell et al. 1987, Villamil et al. 2011). They can affect dogs of any breed, but some breeds such as Boxers, English bulldogs, Labrador retrievers, Golden retrievers and Boston terriers, among others, seem to be overrepresented (Bostock 1986, Rothwell et al. 1987). MCT is usually a disease of older dogs with a mean age of approximately 7-9 years (Govier 2003, Mullins et al. 2006), but cases in young animals have also been reported (Conroy 1983, Davis et al. 1992). The visceral form of MCT, often referred as a disseminated or systemic mastocytosis, can also occur. In the dog, the visceral MCT is uncommon and almost always is preceded by an undifferentiated primary cutaneous lesion; bone marrow and peripheral blood involvement is common (Thamm & Vail 2007).

Pathogenesis

The exact etiology of MCT is unknown but is likely multifactorial. Due to the high frequency of appearance in the skin, it has been suggested that chronic cutaneous inflammation or topical carcinogens may play a role in their development (Govier 2003, London & Seguin 2003). A viral etiology was discussed after experimental transmission
using cellular and cell-free material (Lombard et al. 1963, Rickard & Post 1968) but there is no epidemiological evidence to indicate horizontal transmission of the disease.

As mentioned before, SCF is reported to be responsible for the proliferation of mast cell precursors derived from the bone marrow, their recruitment to specific tissue sites and their subsequent development and maturation (Curry et al. 1998). The effects of SCF are mediated by the SCF receptor (SCFR) also known as Kit. This receptor is normally expressed on the mast cell surface and encoded by the proto-oncogene c-kit (Tsai et al. 1991). The fact that the prevalence is higher in some breeds seems to indicate an underlying genetic component. Therefore, recent investigations have been focused on the role of c-kit in the etiology of canine mast cell cancer. Various studies have shown that malignant canine mast cells derived from spontaneous neoplasms express SCFR (Reguera et al. 2000, Webster et al. 2006). Furthermore, several authors have reported a variety of c-kit mutations in canine MCTs with a frequency of 13 to 40%, found more frequently in poorly differentiated tumours (London et al. 1999, Downing et al. 2002, Zemke et al. 2002). It was then suggested that mutations in this gene could be associated with the development or progression of the tumour and c-kit could be used as a potential prognosis factor. A more recent investigation proved that c-kit mRNA is overexpressed in canine MCT and may play a role in the pathophysiology and proliferation of the tumour, but those mutations appear not to be a good indicator of the biological behavior of the tumour (Giantin et al. 2012). Nevertheless, no mutations were found in up to 60% of the dogs and the presence of neoplastic cell growth of mast cells in the absence of mutations in c-kit has been observed, so mutations in other genes are likely to be involved in the pathology of canine MCT (Ohmori et al. 2008, Welle et al. 2008).
**Pathophysiology**

The most common presentation for canine cutaneous MCT is a solitary nodule, although 10-15% of dogs have multiple lesions (Mullins et al. 2006). In human medicine mastocytosis is a term used to define a heterogeneous group of disorders characterized by abnormal growth and accumulation of mast cells in one or more organ systems (Valent et al. 2001). Extended cutaneous mastocytosis is well described in humane medicine (Valent et al. 2007) and is very unusual in dogs, but has been reported (Davis et al. 1992). Cases of canine or feline systemic mastocytosis or visceral MCT are also uncommon and usually the result of systemic spread of an aggressive cutaneous form, although cases of systemic mastocytosis with or without apparent skin involvement have been described in those species (Davies et al. 1981, O’Keefe et al. 1987, Lamm et al. 2009).

The paraneoplastic syndromes associated with MCT in dogs are usually complications related to mast cell degranulation. Clinical signs are caused by extracellular release of the bioactive substances from mast cell granules (Welle et al. 2008). Extracellular release of these mediators following mast cell degranulation may be precipitated by physical or chemical means (Dobson & Scase 2007). A common example of mast cell degranulation by mechanical manipulation is the phenomenon called *Darier’s sign*: the release of histamine and proteases during physical manipulation of the tumour can cause erythema and edema of the adjacent tissues (Govier 2003, London & Seguin 2003). Local delayed wound healing attributed to proteolytic enzymes and vasoactive amines, and coagulation defects caused by heparin are also complications of mast cell degranulation from the tumour (O’Keefe et al. 1987, Thamm & Vail 2007).
Although the local effects are the most commonly described, the most severe paraneoplastic disorders associated with MCT are caused by systemic release of histamine. Plasma histamine concentrations in dogs with MCT are significantly higher than in normal dogs and the severity of the hyperhistaminemia is not related to clinical stage or size or the tumour, so all dogs with MCT are susceptible to histamine-induced paraneoplastic disorders (Fox et al. 1990, Ishiguro et al. 2003). Gastrointestinal signs such as vomiting, anorexia, melena and abdominal pain may appear in dogs with MCT. These gastrointestinal disorders are caused by binding of histamine to H₂ receptors stimulating gastric secretion and causing hyperacidity, increasing gastrointestinal motility, promoting intravascular thrombosis, and subsequent mucosal ulceration (Fox 2002). Gastrointestinal ulceration appears in up to 80% of dogs with MCT (Howard et al. 1969). The effects of histamine release in the respiratory system include airway smooth muscle contraction, vasodilation, an increase in mucous secretion and mucosal edema formation (Peters & Kovacic 2009).

Cardiovascular complications derived from histamine release have to be considered. Acute anaphylactic reactions caused by sudden, massive release of histamine from the neoplastic cells appear to be unusual, but episodes of collapse may be seen in dogs with extensive disease (Silver 1972, Macy 1986, Dobson & Scase 2007).

1.11. ANESTHETIC CONSIDERATIONS AND EFFECTS OF HISTAMINE IN DOGS

Multiple diagnostic, therapeutic, and anesthetic drugs have the potential to cause adverse anaphylactic reactions upon administration in all species. The prevalence of
anaphylactic reactions during the perioperative period has not been determined in veterinary medicine, but it is considered low (Armitage-Chan 2010). The incidence of anaphylaxis during anesthesia in humans has been reported to range from 1 in 4,000 to 1 in 25,000 (Lieberman et al. 2010). Multiple anesthetics and drugs that are administered during the anesthetic period have the potential to cause histamine release with associated negative cardiovascular effects in healthy patients. Some examples of these drugs include muscle relaxants such as succinylcholine, d-Tubocurarine, or atracurium at high doses (Thompson & Walton 1964, Scott et al. 1986), some opioids such as morphine (Guedes et al. 2006), meperidine (Akcasu et al. 2009) or codeine (Thompson & Walton 1964), macromolecular polymers (polyvinypyrrolidone [PVP]) (Thompson & Walton 1964), contrast agents (Girard & Leece 2010), and antibiotics (Mori et al. 2000).

In humans, acute cardiovascular reactions, such as ventricular fibrillation, collapse, acute ischemia, or cardiac arrest are often associated with mast cell-related disorders, including mastocytosis and mast cell activation syndrome. Some of these reactions can be triggered by events commonly linked with increased histamine release, such as the drugs mentioned above, food allergies, or insect bites (Triggiani et al. 2014). Therefore, drugs associated with histamine release should be used with caution during the perioperative period in patients with MCT that have elevated basal levels of histamine and a higher risk of mast cell degranulation during manipulation of the tumour.

Since dogs with MCT have an increased potential for gastrointestinal ulceration due to the H2 receptor activation (Fox 2002), nonsteroidal anti-inflammatory drugs (NSAIDs) should be used with caution or avoided (Ryan et al. 2012, Fossum et al. 2013). Approximately 80% of the dogs with MCT have been shown to have some degree of
gastric ulceration during post-mortem examination (Howard et al. 1969). Enzymes COX-1 and COX-2 are expressed in the canine gastrointestinal tract and produce a variety of homeostatic prostaglandins. Prostaglandin E₂ and PGI₂ have gastroprotective effects by increasing mucosal blood flow, mucus and bicarbonate production, and turnover of gastrointestinal epithelial cells, and by decreasing acid secretion (Kukanich et al. 2012). Therefore, use of NSADs and inhibition of COX-2 and COX-1 can cause gastric ulceration or prevent healing of a pre-existing lesion.

In dogs, there has been no quantification of histamine release or adverse reactions during MCT diagnostic manipulation or surgical resection during general anesthesia of clinical cases, however, extensive research has been performed in research dogs under general anesthesia with canine anaphylactic models or exogenous administration of histamine providing some useful information for veterinary medicine. The definitions, human studies, and canine research findings are summarized below.

In humans, hypersensitivity reactions are classified as mild (skin reactions), moderate (other organ system involvement such as gastrointestinal, respiratory and/or cardiovascular), or severe (involving neurologic compromise, hypoxia, or severe cardiovascular reactions with hypotension (SAP < 90 mmHg). The term anaphylaxis is only used to describe severe hypersensitivity reactions as defined above (Brown 2004, Thomas et al. 2013). Early identification and treatment of anaphylaxis has been shown to improve overall outcomes in people (Lieberman et al. 2010).

In dogs, signs and symptoms indicative of histamine release are divided into cutaneous, respiratory, cardiovascular, and gastrointestinal (Shmuel & Cortes 2013). Cutaneous signs, such as hyperemia and/or edema are observed with mild histamine
release, however these signs can be challenging to detect in dogs due to fur, pigmentation, or inability to visualize during a surgery. Common clinical signs found in dogs with mild reactions to vaccines are facial or periorbital edema (30.8%), and wheals or urticaria (20.8%; Moore et al. 2005). One retrospective study of 96 dogs diagnosed with hypersensitivity reactions of multiple etiologies found that cutaneous manifestations were present in 100% of the dogs with mild reactions; however, cutaneous signs were only recognizable in 57% of the dogs diagnosed with moderate or severe anaphylaxis (Quantz et al. 2009). Unfortunately, in the latter study histamine levels were not measured. In one study in dogs histamine levels were measured after IV administration of morphine as a bolus (0.3 or 0.6 mg/kg) followed by a continuous rate infusion at low (LM, 0.17 mg/kg/h) or high doses (HM, 0.34 mg/kg/h). Flushing of the skin around the lips, ears, and oral mucosa was seen in 60% of the dogs in the LM group during a 30 min period (peak plasma histamine levels of 5.5 ng/mL) and 100% of the dogs in the HM group (peak plasma histamine levels of 10.6 ng/mL) during a 120 min period, indicating a higher incidence and longer duration of flushing with increasing plasma levels of histamine after morphine administration (Guedes et al. 2006). In another study (Guedes et al. 2007) histamine levels were measured after administration of two different doses of morphine or hydromorphone; flushing of the skin and mucous membranes was observed in most dogs that received the higher dose of either morphine or hydromorphone. This phenomenon was more profound and consistent with the higher dose of morphine that resulted in the highest histamine levels (208.6 ng/mL) (Guedes et al. 2007). This latter study concluded that hydromorphone produces minimal histamine release (maximum of 0.9 ng/mL) and the relationship between cutaneous hyperemia and histamine plasma
concentrations was not clearly established. Overall, the presence of mild signs may be the initial sign of a more severe reaction.

In dogs, the most common sign of histamine release and anaphylaxis is hypotension, and may be caused by a combination of factors that include an increment in vascular permeability that allows shifting of fluid out of the intravascular space (distributive shock), a decrease in SVR due to systemic vasodilation, and/or a decreased CO from severe venous hepatic vasodilation and reduced cardiac pre-load (Shmuel & Cortes 2013). Usually, the peripheral vasodilation induced by histamine is dose-related, and in healthy conscious dogs is usually short-lived because of compensatory reflexes and quick inactivation of histamine (Adams 1995). Sick or anesthetized animals that already have hemodynamic depression or a reduced sympathetic tone may not compensate for the histamine-induced vasodilatation, which may lead to dramatic hypotension or even vascular collapse (Guedes et al. 2006).

General anesthesia may exacerbate the effects of histamine release by blunting sympathetic compensation to hypotension. For example, administration of a single dose of morphine (1 mg/kg, IV) in conscious healthy dogs resulted in maximum median histamine plasma concentrations of 208.6 ng/mL without differences in arterial blood pressures. However, one dog had a significantly reduced MAP of 34 mmHg 2 and 5 min after morphine administration corresponding to maximum histamine concentrations of 589 and 351 ng/mL respectively (Guedes et al. 2007). Earlier research in dogs anesthetized with sodium pentobarbital and administered morphine (1 mg/kg, IV), demonstrated similar histamine plasma concentrations (100-300 ng/mL), with a decrement in MAP values to 40-60 mmHg from normal in all dogs (Thompson & Walton
1964). In the same study a correlation between severity of hypotension in dogs and histamine plasma levels was found. Histamine concentrations of 10-1,400 ng/mL correlated with larger reductions in MAP of 10-80 mmHg from normal baseline values (Thompson & Walton 1964). In another study in pentobarbital anesthetized dogs, the administration of 6 μg/kg of histamine IV resulted in plasma histamine concentrations of 17.4-21.4 ng/mL with significant decreases in mean aortic pressure (120 mmHg for median values before histamine administration compared with 63 mmHg after histamine administration) and total SVR (1.09 mmHg/mL/kg/min compared with 0.55 mmHg/mL/kg/min after histamine administration; Thermann et al. 1975).

The most common signs seen in anesthetized dogs after exogenous administration of histamine are a dose dependent decrease in systemic and aortic blood pressures, decreases in hepatic arterial vascular resistance, increases in pulmonary vascular resistance and portal pressures, and an increase in lung airway resistance (Greenway & Oshiro 1973, Black et al. 1975, Richardson & Withrington 1978, Lautt & Legare 1987, Bates et al. 1994). Transient increases in HR and CO during the first 3 min of histamine infusion were also noticed in one study in dogs (Tucker et al. 1975). These increases are likely associated with the positive chronotropic and inotropic effects of histamine (Hirschowitz 1979, Owen et al. 1982 Asanuma et al. 2006).

Models of anaphylaxis using anesthetized dogs in which histamine is released endogenously, have shown marked decreases in MAP, total vascular resistance, CO, stroke volume, left ventricular function, and PaO₂, and an increase in pulmonary vascular resistance (Kapin & Ferguson 1985, Mitsuhata et al. 1995, Mink et al. 1998, Mink et al. 2004). Despite the severe hypotension (MAP < 50 mmHg) seen during anaphylaxis, one
study has shown that anesthetized dogs tend to have a preferential distribution of CO to increase and maintain blood flow to vital organs, such as brain and adrenal glands (Kapin & Ferguson 1985). In these animal anaphylaxis models increases in histamine levels from baseline values were only demonstrated during the initial 15 min after antigen exposure (Mitsuhata et al. 1995, Mink et al. 1998). The transient increase in histamine plasma concentrations coincides with maximum physiological changes. Cardiorespiratory function returns to normal within 30-180 min after antigen challenge in these models.

1.12. CARDIOVASCULAR MONITORING UNDER ANESTHESIA

Monitoring the cardiovascular and respiratory systems for optimal function during general anesthesia is performed to maintain adequate blood flow and oxygen delivery to tissues and to assess anesthetic depth. It is also important to assist in the detection of side effects and complications such as an anaphylactic event from histamine release from MCT manipulation. Monitoring allows for prompt detection of inadequate function that can be addressed accordingly. Commonly monitored parameters of clinical canine cases include: HR, RR, ECG, MAP, SAP, DAP, SPO2, ETCO2, and Temp. Non-invasive CO can also be monitored in clinical cases, and adds a more complete cardiovascular assessment. The following parameters were evaluated and calculated in this thesis: HR, RR, SPO2, ECG, ETCO2, SAP, MAP, DAP, Temp, NICO, and SV.

1.12.a. Arterial blood pressure monitoring and the significance of hypotension

Blood pressure measurement involves the quantification of the distending
pressure, exerted by the volume of blood contained in the distensible vascular space on the wall of blood vessels, and the additional volume ejected by the contracting ventricle (Avolio et al. 2010). Blood pressure reflects left ventricular afterload and in conjunction with HR is considered a fundamental cardiovascular vital sign. Standards for basic anesthetic monitoring in human anesthesia mandate the measurement of arterial blood pressure at least every 5 min in all anesthetized patients to ensure the adequacy of the patient’s circulatory function (American Society of Anesthesiologist, 2010). However, in veterinary medicine the measurement of arterial blood pressure is a guideline. During anesthesia for more critical cases requiring advanced support, such as mast cell tumour removal, arterial blood pressure measurement (direct and indirect) is recommended for identification of potential hypotension and treatment (ACVAA 2009).

Arterial blood pressure is expressed as the product of cardiac output and systemic vascular resistance (BP = CO x SVR). The peak pressure value within the aorta during cardiac ejection is called SAP and is determined by the SV, the velocity of left ventricular ejection, SVR, the distensibility of the aortic and arterial walls, the viscosity of blood, and the left ventricular preload (end-diastolic volume). The minimal pressure reached during diastole and before each new ejection is the DAP and is affected by blood viscosity, arterial distensibility, SVR, and the length of the cardiac cycle (HR). The MAP is the average effective pressure that drives blood through the systemic organs (Wagner et al. 1997). It is not equal to the arithmetic mean of systolic and diastolic pressure because the arterial pressure remains nearer to DAP than the SAP during a larger part of the cardiac cycle under normal circumstances. So MAP is commonly expressed as \((2/3 \times DAP) + (1/3 \times SAP)\) (Lamia et al. 2005, Mohrman & Heller 2014).
Arterial blood pressure can be measured by direct or indirect methods and is one of the most useful measures of cardiovascular function available to the veterinary anesthesiologist (Haskins 2007). Multiple non-invasive monitors are widely available and validated for their use in veterinary patients, but arterial catheterization with direct arterial monitoring is the accepted reference standard for arterial blood pressure monitoring. Direct measurement of blood pressures is considered superior to other methods for early detection of intraoperative hypotension since it offers a beat-to-beat measurement and a visible waveform that allows for waveform analysis and evaluation of pulse pressure variation (Schroeder et al. 2014).

Mean arterial blood pressure in conscious, healthy dogs has been reported as 103 ± 15 mmHg (Haskins et al. 2005). The minimum acceptable MAP, below which organ perfusion is believed to be compromised, is often described as 60-65 mmHg, although there is considerable inter-individual variability (Thooft et al. 2011). A MAP < 80 mmHg in the kidney or < 50 mmHg in the brain (Shipley & Study 1951, Bendo et al. 2006), results in the loss of autoregulation of organ blood flow, so that tissue perfusion becomes directly dependent on the arterial pressure level and further decrements may cause organ hypoperfusion. The main negative consequence of a decrease in arterial blood pressure is reduced perfusion of blood to vital organs (Wagner et al. 1997).

Development of hypotension (defined as a MAP < 60 mmHg or a SAP < 80 mmHg) usually represents a failure of the autonomic nervous system to compensate for reduced CO and impaired oxygen delivery (Cove & Pinsky 2012). Hypotension is a common complication of general anesthesia and also a consequence of systemic histamine release in anaphylaxis (Shmuel & Cortes 2013). Hypotension during general
anesthesia is usually associated with the use of inhalants and injectable anesthetic drugs that cause a decrease in myocardial contractility, HR, CO, SVR, and blunt the sympathetic responses in a dose dependent fashion (Pagel & Farber 2005). In addition to the effects of anesthetic drugs, veterinary patients can also present with clinical conditions that predispose them to hypotension under anesthesia, such as dehydration, blood loss, and septic shock conditions. In anesthetized veterinary patients, it is generally recommended to maintain MAP above 60 mmHg to support individual organ blood flow and tissue delivery (Wagner et al. 1997, Haskins 2007).

1.12.b. Cardiac output monitoring

Monitoring CO is an ideal method to assess organ perfusion, oxygen delivery to the tissues and overall cardiovascular function. However, the measurement of CO may be impractical in a clinical setting due to cost and time. Non-invasive methods of CO determination, such as the NICO, are practical in the clinical setting for case management or clinical research. This measurement indicates the volume (mL or L) of blood pumped or delivered by the heart to the pulmonary or systemic vasculature against the SVR per unit time (min). It is the product of the HR and SV. The SV of the ventricle is determined by the interactions between preload, contractility and afterload (Lavdaniti 2008). One of the determinants of CO is HR, which is easily monitored by different methods, such as the ECG, which can also detect arrhythmias. Multiple non-cardiac factors can also affect HR and prevent the measurement of SV in certain clinical situations, thereby making the interpretation of HR on CO and the overall hemodynamic status variable (Schroeder et al. 2014).
Measurement of CO provides a global assessment of the circulatory system, and in combination with other hemodynamic measurements allows calculation of additional circulatory variables, such as SV, SVR and pulmonary vascular resistance, ventricular stroke work, and oxygen delivery (Schroeder et al. 2014). Normal values for CO in healthy dogs have been reported as 165 ± 43 mL/min/kg (Haskins et al. 2005). Even though CO monitoring is still more commonly used in a research setting, it is possible in some specialty practices in veterinary medicine with non-invasive methods. It is an emergent technique in human medicine for multiple reasons. Organs are dependent on flow as well as pressure to achieve adequate function, and a normal MAP with a decreased CO may still result in organ dysfunction (Linton & Gilon 2002). Cardiac output determination was traditionally reserved in the past for patients with severe circulatory instability or patients undergoing major surgery, because of cumbersome and invasive techniques of CO determination that required right heart catheterization (Botero & Lobato 2001). Currently, other methods for CO determinations that are less invasive are available and used widely for a variety of patients. Methods for CO determination can be classified in 4 categories: indicator methods such as thermodilution and lithium dilution techniques, arterial pulse wave analysis (pulse contour CO), derivation of the Fick principle (partial CO2 rebreathing), and imaging diagnostic techniques such as transesophageal echocardiography, Doppler ultrasound, and thoracic bioimpedance (Shih 2013).

Thermodilution is the accepted clinical gold standard for CO measurement in both human and veterinary medicine. This method is more invasive since a catheter with a thermistor tip (Swanz GanzR) must be inserted into the pulmonary artery via the jugular
vein, right atrium and right ventricle. A cold solution of dextrose 5% or normal saline (temperature controlled at 1-5 °C) is injected into the right atrium from the proximal catheter port. This solution causes a decrease in blood temperature, which is measured by the thermistor placed in the pulmonary artery catheter. The decrease in temperature is inversely proportional to the dilution of the injectate. A special monitor is able to plot a graph of the concentration of the indicator against time to produce a concentration-time curve, and calculates the AUC and extrapolates CO values from a modification of the Stewart-Hamilton equation (Prabhu 2007, Lavdaniti 2008, Shih 2013).

\[
\text{CO} = \frac{\text{amount of indicator}}{(\text{concentration of indicator} \times \text{time})}
\]

Invasive techniques have associated risks such as pneumothorax, dysrhythmias, perforation of a heart chamber, infections, tamponade, or valve damage. These complications and the fact that in human medicine use of these techniques have been associated with a higher morbidity and mortality in critical patients has made less invasive techniques the preferred option in the last 10 – 20 years (Hett & Jonas 2003, Sadham et al. 2003).

The lithium dilution method is a less invasive technique that also uses indicator dilution to derive a concentration-time curve from which CO can be calculated but the indicator used in this case is lithium. A pulmonary catheter is not needed since lithium can be injected into a central or peripheral vein, and its concentration over time is recorded by a sensor consisting of a lithium sensitive electrode in a flow-through cell, which samples blood from a downstream artery. This method is considered minimally invasive and has shown excellent agreement when compared with thermodilution in horses and dogs (Linton et al. 2000, Mason et al. 2001).
Other minimally invasive and non-invasive techniques available are transesophageal echocardiography, Doppler ultrasound, pulse contour cardiac output, thoracic electrical bioimpedance, and partial CO₂ rebreathing (Lavdaniti 2008). The technique of partial CO₂ rebreathing (NICO) estimates CO using the Fick principle and has been validated for use in dogs (Gunkel et al. 2004).

\[
CO = \frac{VCO_2}{CvCO_2-CaCO_2}
\]

The monitor consists of a carbon dioxide sensor, a disposable airflow sensor, a rebreathing loop with a valve, and a pulse oximeter. The VCO₂ is calculated from minute ventilation and its carbon dioxide content and arterial carbon dioxide content (CaCO₂) is estimated from the alveolar partial pressure of CO₂ (PaCO₂) using the following equation:

\[
CaCO_2 = (6.957xHb + 94.864) x \log (1.0+0.1933xPaCO_2)
\]

The technique assumes that due to a high diffusion rate of CO₂, there is no difference in venous CO₂ (CvCO₂) between cycles under normal or rebreathing conditions. Hence, CO may be calculated from the following equation:

\[
CO=\Delta VCO_2/\Delta CaCO_2
\]

This method measures the non-shunted portion of the CO. To make a more accurate determination the monitor uses the shunt equation to correct the value and make also a non-invasive estimation of the intrapulmonary shunt fraction. Therefore, arterial blood gases are necessary to calibrate the monitor including patient’s hemoglobin, PaCO₂, and PaO₂ values (Jaffe 1999).
Limitations of NICO include the assumption of normal pulmonary function and the use of mechanical ventilation to maintain a constant tidal volume and normal CO₂ levels for better accuracy of the monitor. Concentrations of end-tidal CO₂ (ETCO₂) > 65mmHg at baseline or >80 mmHg during rebreathing may interfere with the initiation of rebreathing cycles by the software (Tachibana et al. 2002) therefore, it is recommended to maintain normal ETCO₂ (35-45 mmHg) during measurements. The non-rebreathing mode lasts 60 s, the rebreathing period (valve closed) last 50 s and is followed by a stabilization period of 70 s that allows the ETCO₂ of the patient to go back to baseline values (Jaffe 1999). This means that a new value is obtained every 3 min, which may delay the detection of quick changes in CO. During the rebreathing phase mild increases in CO₂ are observed so the patient has to be able to tolerate changes in PaCO₂ levels.

Although rebreathing loops can be expanded or retracted to an optimized level of rebreathing there is a limitation of size for using NICO. Tidal volumes of more than 200 mL are necessary for the software to initiate rebreathing cycles so the method is not indicated for cats and dogs less than 10 kg of body weight. When compared with other methods partial CO₂ rebreathing has shown good agreement with thermodilution in dogs weighting between 18.2 and 39.5 kg (Capek & Roy 1988, Haryadi et al. 2000), but the limits of agreement were too wide in beagle dogs weighting 9-13 kg (Yamashita et al. 2007). The rebreathing valve accounts for 32 mL of dead space during normal non-rebreathing cycles (Jaffe 1999). This extra volume may be significant for smaller dogs and may explain differences in the accuracy found in these studies. It has been also hypothesized that smaller tidal volumes may lead to lowered VCO₂ and ETCO₂ values.
during mechanical ventilation, leading to underestimations of $\Delta V\text{CO}_2$ (Yamashita et al. 2007).

This system compares well with the lithium dilution method in dogs between 22 and 25.4 kg although it appears to underestimate values when CO is elevated (Gunkel et al. 2004). Despite the size limitations, the NICO system is ideal for use in clinical situations since it is non-invasive, semi-continuous, easy to use, and less expensive than other methods (Kothari et al. 2003). It has been used successfully for CO determination in clinical cases of dogs (>15 kg) undergoing general anesthesia for routine surgical procedures (Rioja et al. 2013).

Overall, for clinical research of client owned animals, both lithium dilution and NICO methods offer advantages, are ethically sound, and scientifically comparable to the standard method of thermodilution.
1.13. REFERENCES


Bloom F (1942) Spontaneous solitary and multiple mast cell tumors (mastocytoma) in dogs. Arch Pathol 33: 661-676.


Cowden JM, Riley JP, Ma JY et al. (2010) Histamine H4 receptor antagonism diminishes existing airway inflammation and dysfunction via modulation of Th2 cytokines. Respir Res 11: 86.


Khalifa M, Drolet B, Deleau P et al. (1999) Block of potassium currents in guinea pig ventricular myocytes and lengthening of cardiac repolarization in man by the histamine H1 receptor antagonist diphenhydramine. J Pharmacol Exp Ther 288: 858-865.


Koyanagi T, Tamaura Y, Yano K et al. (2014) Age-related pharmacokinetic changes of acetaminophen, antipyrine, diazepam, diphenhydramine, and ofloxacin in male cynomolgus monkeys and beagle dogs. Xenobiotica 44: 893-901.


Quantz JE, Miles MS, Reed AL et al. (2009) Elevation of alanine transaminase and
gallbladder wall abnormalities as biomarkers of anaphylaxis in canine

activity. Diamine oxidase as a new biologic marker of colorectal proliferation? Ann
N Y Acad Sci 859: 262-266.

Reguera MJ, Rabanal RM, Puigdemont A et al. (2000) Canine mast cell tumors express

Richardson PD, Withrington PG (1978) Responses of the simultaneously perfused
hepatic arterial and portal venous vascular beds of the dog to histamine and 5-

Rickard CG, Post JE (1968) Cellular and cell-free transmission of a canine mast cell


Iowa. USA. pp: 11-46.

Rixen D, Raum M, Holzgraefe B et al. (2002) Local lactate and histamine changes in
small bowel circulation measured by microdialysis in pig hemorrhagic shock. Shock
18: 355-359.

Roberts LJ II, Sweetman BJ, Lewis RA et al. (1980) Increased production of
prostaglandin D2 in patients with systemic mastocytosis. N Engl J Med 17: 1400-
1484.

induced by morphine and hydromorphone administration in dogs. Am J Vet Res 49:
1699-1701.

Rothwell TL, Howlett CR, Middleton DJ et al. (1987) Skin neoplasms of dogs in


Rurak SW, Yoo SD, Kwan E et al. (1988) Effects of diphenhydramine in the fetal lamb
after maternal or fetal administration. Pharmacol Exp Ther 247: 271-278.


CHAPTER II: THE PHARMACOKINETICS OF DIPHENHYDRAMINE AFTER ADMINISTRATION OF A SINGLE INTRAVENOUS OR INTRAMUSCULAR DOSE IN HEALTHY DOGS

2.1. SUMMARY

Objective- To determine the pharmacokinetics of diphenhydramine (DPH) in healthy awake dogs following a single IV dose of 1 mg/kg or IM dose of 2 mg/kg.

Study Design- Prospective randomized crossover research trial.

Animals- Six purpose-bred hounds, 3 males and 3 females weighting 22.3 ± 2.6 kg.

Methods- Dogs were randomly allocated to two treatment groups. Dogs received DPH at 1 mg/kg, IV, via a cephalic catheter or 2 mg/kg, IM, in the epaxial muscles. Jugular vein blood samples for DPH plasma concentration determination were collected at baseline (time 0) and up to 24 h post-administration. Additional measures included indirect systolic (SAP), mean (MAP) and diastolic pressures (DAP), heart rate (HR), electrocardiogram (ECG), respiratory rate (RR), rectal temperature (Temp), and effects on behaviour.

Results- The DPH clearance (Cl) after IV administration was 20.7 ± 2.9 mL/kg/min and Cl/F after IM administration was 20.8 ± 2.7 mL/kg/min. The Vd was 7.6 ± 0.7 L/kg after IV administration and Vd/F after IM administration was 12.3 ± 1.2 L/kg after IM administration. The T1/2 was 4.2 ± 0.5 h after IV administration and 6.8 ± 0.7 h after IM administration. The bioavailability from IM administration was 88%. No significant differences were found in physiological parameters between groups or within the groups.
and values remained within normal limits. No adverse effects or behavioural changes were observed after administration of DPH.

**Conclusion and clinical relevance:** Both IV and IM administration resulted in rapid DPH plasma concentrations that exceeded therapeutic levels in humans. Complete bioavailability occurred with IM administration when given at twice the dose of IV.
2.2. INTRODUCTION

Diphenhydramine (DPH) is a “first generation” H₁-antihistamine that acts as an inverse agonist, binding to and stabilizing the inactive conformation of the H₁-receptor (Bakker et al. 2000, Simons & Simons 2011). Similar to the older histamine compounds, DPH easily crosses the blood brain barrier acting on central nervous system H₁ receptors. It has lower selectivity for H₁ receptors compared with newer drugs such as loratadine or terfenadine and may also activate muscarinic cholinergic, serotonin or α-adrenergic receptors (Stoelting & Hillier 2006). In veterinary medicine, DPH is used commonly for amelioration of motion sickness of vestibular origin, to control mild allergic reactions and chronic pruritus (Zur et al. 2002, Conder et al. 2008), and as a second line treatment in anaphylactic reactions (Peters & Kovacic 2009, Dowling 2012).

Diphenhydramine has also been recommended to prevent the adverse cardiovascular and local effects of histamine released from mast cells during surgical excision of mast cell tumours (MCT) (Hofmeister & Egger 2005, Thamm & Vail 2007). Doses of 1-5 mg/kg orally, IV and IM route have been recommended (Adams 1995, Govier 2003). The IM route has been described in dogs in clinical manuals in preference to IV administration, due to risk of hypotension and/or cardiovascular instability associated with the latter route (Mathews 2006). Hypotension, SAP < 85 mmHg, has been reported in humans after IV promethazine, 25 mg, which is another first generation H₁-antihistamine (Ellis & Brown 2013).

The pharmacokinetics of DPH are described in humans, rabbits, camels, horses, and sheep (Simons et al. 1990, 1996, Wasfi et al. 2003, Au-Yeung et al. 2007). For effective antagonistic actions at the H₁ receptor, DPH should achieve sufficient plasma
concentrations and for a duration that can block the histamine released from mast cells. In an early paper the results of only two dogs that received DPH at 5 mg/kg, IV, were reported with an estimated half-life \( (T_{1/2}) \) of approximately 1 h (Drach et al. 1970). More recently in dogs, the pharmacokinetics of DPH at 0.2 mg/kg, IV, and 1 mg/kg, orally, were reported in dogs (Koyanagi et al. 2014). The pharmacokinetics of DPH IV or IM at doses used clinically (0.5 - 4 mg/kg) (Papich 2011, Plumb 2011, Fossum et al. 2013) is not known. To determine the potential benefits of DPH administration in clinical cases, it is necessary to establish the pharmacokinetics to optimize the dose, route, time and frequency of administration (pre-emptive), so that effective antagonistic actions at the \( H_1 \) receptor are elicited through sufficient plasma DPH concentrations and for a duration that can block the histamine released from mast cells.

The primary objective of this research is to determine the pharmacokinetics of DPH in healthy dogs following a single IV dose of 1 mg/kg or a single IM dose of 2 mg/kg. A secondary objective is to assess both IV and IM routes for effects on cardiorespiratory parameters (heart rate [HR], respiratory rate [RR]), and oscillometric arterial blood pressures, temperature (Temp), and behavioural effects (alertness, sedation) in healthy research hound dogs. We hypothesized that both routes, IV and IM are well tolerated in healthy conscious dogs and that detectable DPH plasma concentrations would be observed without major cardiorespiratory or behavioural side effects.
2.3. MATERIALS AND METHODS

2.3.a. Animals

Six healthy purpose-bred hounds, three intact males and three intact females, were used in the study. Dogs were considered healthy based on history, physical examination, complete blood count and serum biochemistry analysis. Dogs were 3.0 ± 0.6 years old (2.6 - 4 years) weighing 22.3 ± 2.6 kg (17.6 - 25.4 kg). The study was carried out in accordance with the guidelines of the Canadian Council on Animal Care and was approved by the Institutional Animal Care Committee at the University of Guelph.

2.3.b. Experimental Design

A randomized crossover study design was used to evaluate the pharmacokinetics of DPH in 2 different administration groups, IV and IM. Dogs were assigned to each of the groups equally by a randomization scheme (http://www.randomization.com) with a washout period of one week between group treatments.

2.3.c. Study Protocol

A catheter 19-SWG, 12 inches, (Intracath, Becton Dickinson Infusion Therapy Systems, Sandy, Utah, USA) was placed in the jugular vein, after infiltration of subcutaneous tissues with 1 mL of 2% lidocaine hydrochloride (Alveda Pharmaceutics, Toronto, Ontario, Canada), and secured with a bandage. This catheter was used to sample blood for the pharmacokinetics of DPH. Dogs in the IV group had a cephalic vein catheterized with a 20-GA, 1-inch catheter (BD Insyte-W, BD Infusion Therapy Systems Inc. Sandy, Utah, USA) for DPH (Diphenhydramine hydrochloride injection USP 50
mg/ml, Pharmaceutical partners of Canada Inc. Richmond Hill, Ontario, Canada) administration; the catheter was removed 30 min after DPH administration. Diphenhydramine was administered at doses of 1 mg/kg for the IV group, followed by 3 mL of saline, and 2 mg/kg for the IM group injected in the epaxial musculature with a 22 GA, 1-inch hypodermic needle (Monoject Standard hypodermic needle, Covidien, Mansfield, Massachusetts, USA). Three mLs of blood for determination of DPH plasma concentration was collected in heparinized tubes from the jugular catheter of each dog before DPH administration (baseline, time 0) and at 3, 5, 10, 20, 30, 45, 60 min and 1.5, 2, 4, 6, 8, 12, and 24 h post-administration. The volume of blood removed was replaced through the same catheter with twice the volume of isotonic solution (Plasma-Lyte A® Injection, Baxter Corporation, Mississauga, Ontario, Canada) at each sampling time. Oscillometric arterial blood pressures (SAP, MAP, DAP) (Cardell® Veterinary vital signs monitor, model 9403, Midmark Corporation Versailles, Ohio, USA), ECG, and HR (Datex-Ohmeda S/5 Anesthesia Monitor; GE Healthcare Finland, Helsinki, Finland), and RR (counted over 15 s) were recorded at times 0, 3, 10, 30 min and 2, 4, 8, 12 and 24 h. Three consecutive blood pressure measurements that had no more than 20% variation between them were taken and averaged at each time point by placing a cuff that was 40% of the circumference of the distal radius-ulna area, above the joint. A digital thermometer was used to monitor rectal Temp at times 0, 10, 30 min and 2, 4, 8, 12 and 24 h. Dogs were observed for changes in behaviour, such as sedation or excitement, immediately after DPH administration and for 24 hours.
2.3.d. Plasma DPH analysis

Heparinized blood samples were placed on ice immediately after collection, until centrifugation at 3,000 \( \times \) g for 10 min. Plasma was then collected and stored at -80°C until analysis. Diphenhydramine was quantified in plasma using high-performance liquid chromatography (HPLC) employing fluorescence detection. HPLC Analysis was performed on a Waters Alliance® 2695 HPLC separations system (Mississauga, ON, Canada) coupled with a 2475 Multi-Wavelength Fluorescence Detector. The system was connected to a PC with Empower 2 software (Waters®, Mississauga, ON, Canada) for data collection and processing. Stock solutions of DPH and orphenadrine standards (Sigma-Aldrich, St. Louise, MO, USA) were prepared in methanol at the concentration of 500 \( \mu \)g/mL and also stored at -80°C. Calibration (reference) standards (10, 50, 100, 500 and 1000 ng/mL), and quality controls (QC) (20 and 800 ng/mL) were prepared in blank canine plasma.

Plasma test samples were processed by adding 10 \( \mu \)L of internal standard working solution (1 \( \mu \)g/mL, orphenadrine) to 100 \( \mu \)L aliquots of unknown canine plasma. Acetonitrile (1 mL) and 10 \( \mu \)L of 1N HCl (Fisher Scientific, Ottawa, ON, Canada) (Caledon Laboratories Ltd. Georgetown, ON, Canada) were combined to cause protein precipitation. The mixture was vortex-mixed for 30 s, followed by centrifugation 14,400 g at 4°C for 10 min. The supernatant was then collected and evaporated under a constant flow of nitrogen. An isocratic chromatographic separation was prepared by reconstituting the residue obtained after evaporation with 100 \( \mu \)L of the mobile phase (acetonitrile-water-acetic acid 80:20:1, v/v/v, at a flow rate of 1 mL/min), and 50 \( \mu \)L of it was injected onto the column. The analytical column used was a Sunfire® C18 column.
(50 mm x 4.6 mm I.D., 2.5 µm, Waters®, Ireland), connected with a Security Guard C18 guard column (4 mm x 3.0 mm I.D., Phenomenex®, Torrance, CA, USA). Fluorescence detection was optimized at 229 nm excitation and 268 nm emission wavelengths. The retention times observed were 1.8 min for DPH and 3.2 min for orphenadrine, respectively.

The limit of detection (LOD) for this assay was 5 ng/mL (based on 3 times the signal to noise ratio at the time of elution of the analyte). The technique was optimized to provide a minimum limit of quantification (LOQ) of 10 ng/mL. The limit of quantitation was determined by spiking blank canine plasma at various concentrations of DPH, and the resulting values had a coefficient of variation no greater than 20%. The intra-day and inter-day assay coefficients of variation were 8.9% and 10.7% respectively. The true value for each calibration standard was within 15% of the actual value except at LOQ (10 ng/mL), where it deviated by less than 20%. The recovery rate was determined then by comparing the peak areas of blank plasma spiked with different amounts of drug with the peak areas of the same standards prepared in the mobile phase. Average recovery was 91.7%, with 89.3% at LOQ of 10 ng/mL.

Method validation

Canine plasma samples were quantified using the ratio of the peak area of DPH to that of the internal standard orphenadrine. Peak area ratios were plotted against DPH concentrations. Standard curves, in the form of \( y = A + Bx \), were calculated using weighted \( (1/x^2) \) least squares linear regression. Calibration curves were prepared and assayed on 12 separate days. Five point calibration curves were linear and reproducible.
in the concentration range from 10 ng/mL to 1 µg/mL with a coefficient of determination (R²) > 0.99 for all calibration curves. No interfering peaks were observed in blank plasma samples at retention time corresponding to the drug and internal standard. This clean background showed that the assay procedure was specific to DPH.

2.3.e. Pharmacokinetic Analysis

First order pharmacokinetic analysis of DPH concentration-time data following IV and IM administration and pharmacokinetic parameter analysis were determined using a non-compartmental model with the software PK Solutions 2.0 (Summit Research Services, Montrose, Colorado, USA). Individual drug concentration-time data was subjected to nonlinear least squares regression analysis to determine elimination half-life (T½)

\[ T_{\frac{1}{2}} = \frac{0.693}{\lambda_n} \]

where \( \lambda_n \) is a rate constant (-2.303s)

Descriptive curve parameters calculated included the concentration at time 0 (C₀) for the IV route determined by log-linear regression,

\[ C_0 = \sum C_n \]

The area under the concentration-time curve (AUC) and its first moment (AUMC) to the final concentration time point (Cₜ) were calculated by the log-linear
trapezoidal rule. AUC_{area} was calculated with a trapezoidal model based on AUC_{(0-t)} using the observed data points only (not extrapolated to infinity).

\[ AUC_{(0-t)} = \sum_{i=0}^{n-1} \frac{t_{i+1} - t_i}{2} (C_i + C_{i+1}) \]

where \( n \) is the number of data points

\[ AUC_{(area)} = AUC_{(0-t)} + \frac{C_t}{\lambda_z} \]

The AUMC_{area} was calculated by combining trapezoidal calculation of AUMC_{(0-t)} and extrapolated area with the residual areas to infinity

\[ AUMC_{(0-t)} = \sum_{i=0}^{n-1} \frac{t_{i+1} - t_i}{2} (C_i t_i + C_{i+1} t_{i+1}) + \frac{C_t t_t}{\lambda_z} + \frac{C_{t} t}{\lambda_z^2} \]

From these values the follow parameters were extrapolated:

Ratios of AUC (zero to last time point) values for each route of administration will be compared to determine absolute systemic bioavailability (%F) of DPH following IM dosing.

\[ \%F = \frac{AUC_{IM}}{AUC_{IV}} \times \frac{Dose_{IV}}{Dose_{IM}} \times 100 \]

Mean residence time (MRT) was calculated as:

\[ MRT_{(area)} = \frac{AUMC}{AUC} \]

Volume of distribution area (Vd_{(area)}) was calculated as:
\[ Vd(\text{area}) = \frac{FD}{AUC \lambda_z} \]

The calculated Vd for the IM dose was corrected if F < 100%:

\[ \text{Corrected } Vd(\text{area}) = F \times Vd(\text{area}) \]

Systemic clearance (Cl) based on AUC\(_{(0-t)}\) trapezoidal calculations were:

\[ CL = \frac{FD}{AUC\_{(0-t)}} \]

Clearance\(_{(area)}\) based on trapezoid AUC\(_{(area)}\) calculations:

\[ CL_{(area)} = \frac{FD}{AUC_{(area)}} \]

The calculated Cl for the IM dose was corrected if F < 100%:

\[ \text{Corrected } Cl(\text{area}) = F \times Cl(\text{area}) \]

Terminal T\(_{1/2}\) was calculated from Vd and clearance with the following equation:

\[ T_{1/2} = \frac{0.693 \times Vd}{Cl} \]

The respective Cl and corrected Cl were used for the IV and IM dose, respectively, if F < 100% for the IM dose.
2.3.f. Data analysis

Statistical analysis was performed using standard statistical software (MedCalc Statistical Software version 14.10.2 [MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014]). Normality of data was assessed using D’Agostino-Pearson, Shapiro-Wilk and Kolmogorov-Smirnov tests. Values for physiological parameters are reported as mean ± SD. Statistical comparison of HR, SAP, MAP, DAP, RR, and Temp between the two groups and within groups at the different time points were determined using a repeated measures ANOVA followed by a post-hoc Bonferroni correction. Values of p < 0.05 were considered significant.
2.4. RESULTS

No adverse effects or behavioural changes, such as sedation or excitement were observed after IV or IM administration of DPH. Cardio-respiratory parameters including HR, RR, SAP, MAP, DAP, and Temp were within normal limits in all the dogs at all time points (Table 2.1). No statistically significant differences in HR, SAP, MAP, DAP, RR, or Temp were detected between groups or at different time points within the same group (p > 0.05) after administration of DPH.

Plasma concentrations of DPH (ng/mL) for both routes of administration are represented in Figures 2.1 and 2.2. The DPH plasma concentrations were above the LOQ of the assay (10 ng/mL) 3 min after DPH administration for both groups. Peak plasma concentrations for IV and IM groups were 306 ± 77.0 ng/mL and 188 ± 33.6 ng/mL, respectively. Times to T<sub>max</sub> were 6 ± 0.3 min for the IV group and 45 ± 5.1 min for the IM group. The distribution half-life for the IV dose was 1.1 ± 0.1 h and the absorption half-life for the IM dose was 0.11 ± 0.01 h. Diphenhydramine concentrations were undetectable (<10 ng/mL) 12 h after administration in the IV group, whereas levels of 16 ng/mL were still present at 24 h in the IM group. Pharmacokinetic parameters for DPH for each treatment group are reported in Tables 2.2 and 2.3. Absolute %F in this study after IM administration was 88% assuming a 100% F after IV administration.
2.5. DISCUSSION

This study determined the pharmacokinetics of DPH following IV (1 mg/kg) and IM (2 mg/kg) administration in healthy dogs. Diphenhydramine has been commonly recommended as an ancillary drug in dogs with MCT to counteract the adverse effects of histamine on the peripheral vasculature and on wound healing. Mast cells may degranulate and release histamine in response to surgical manipulation or radiation therapy (Welle et al. 2008, Ryan et al. 2012, Fossum 2013, London & Thamm 2013) and elicit adverse cardiovascular effects that include a decrease in systemic vascular resistance, systemic arterial blood pressures and the development of arrhythmias (Peters & Kovacic 2009). Although these effects may be short-lived due to compensatory reflexes and quick inactivation of histamine, the effects are dose-related and could be adversely significant in sick or anesthetized animals with compromised cardiovascular function (Thermann et al. 1975, Adams 1995, Guedes et al. 2006).

Doses of DPH used in this study are within recommended ranges of those recommended for dogs in clinical practice, 0.5-4 mg/kg (Papich 2011, Plumb 2011, Fossum et al. 2013). The oral route is most commonly used in people and dogs. The IV or IM routes are used for emergency administration of DPH or where oral administration is not feasible. The parenteral formulation of DPH is labeled in humans for both IM and IV administration; however, there is clinical concern of hypotension in dogs after IV DPH (Plunkett 2000, Fossum et al. 2013; Mathews 2006) without scientific investigation. Hypotension is a common complication of general anesthesia and also a consequence of systemic histamine release in anaphylaxis (Shmuel & Cortes, 2013). Hypotension is defined as MAP < 60 mmHg or SAP < 80 mmHg (Haskins, 2007). Administration of
promethazine, 25 mg, IV, another first generation antihistamine, can result in hypotension in humans (Ellis & Brown 2013) but there are no specific reports that describe such hypotensive events after IV DPH in veterinary patients during pharmacokinetic studies of different species.

Dogs in this study had normal cardio-respiratory function with similar values after receiving DPH either IV or IM. Because of DPH’s α-adrenergic blockade and anticholinergic activity, first-generation antihistamines have the potential to cause cardiovascular side effects such as peripheral vasodilation, hypotension, and tachycardia (Simons 2004, Sheikh et al. 2007); however, no changes in any of the cardio-respiratory parameters were detected during this study for both routes of administration.

Signs of sedation or changes in mentation were also not observed after administration by either route. Diphenhydramine and the rest of first-generation antihistamines have the ability to cross the blood brain barrier and can cause drowsiness in humans. Concentrations of 30-40 ng/mL cause drowsiness and concentrations higher than 60-70 ng/mL result in mental impairment in humans (Albert et al. 1975, Gengo et al. 1989). Drowsiness has been reported in dogs administered DPH at 10-50 mg/kg, PO (Wauquier et al. 1981, Scott & Buerger 1988), but in a prospective randomized clinical trial using doses of 2-8 mg/kg, IM, DPH did not cause any sedation prior to anesthesia (Hofmeister & Egger 2005). In our study, plasma concentrations achieved a maximum of 306 ng/mL and 188 ng/mL for the IV and IM group, respectively, which are higher concentrations than those associated with drowsiness and mental impairment in humans (Albert et al. 1975). The dogs in this study, although apparently calm, were research animals that may be more comfortable with handling and exhibit a quiet behavior that
makes sedation less detectable.

Toxic plasma concentrations of DPH in dogs have not been accurately determined, but one case report of non-fatal oral DPH intoxication measured plasma levels of 547 ng/mL on admission. In this case report the dog presented severe ataxia and spastic tremors, dilated pupils, and a decreased level of consciousness (Lagutchik et al. 1997).

The pharmacokinetic profile of DPH has been determined in humans, rabbits, camels, horses and sheep after IV administration (Scavone et al. 1990, Kumar et al. 1999a, Simons et al. 1996, Wasfi et al. 2003). In dogs, the pharmacokinetics of DPH after IV (0.2 and 5 mg/kg) and oral administration (1 and 8.65 mg/kg) are also available (Drach et al. 1970, Wang et al. 2007, Koyanagi et al. 2014) but not after IM administration.

In the present study, IM administration of DPH resulted in high bioavailability (F = 88%) due to rapid absorption with detectable plasma levels at 3 min after administration, and maximum plasma concentrations were achieved 45 min after administration. Bioavailability from oral (40-70%) and sublingual (58%) administration in humans is significantly lower, since DPH administered via these routes undergoes first pass hepatic metabolism through the portal circulation (Albert et al. 1975, Paton & Webster 1985, Blyden et al. 1986, Scavone et al. 1990). In dogs oral administration is often used and recommended, but recent data suggests that this route results in a low bioavailability (2.8-7.3%) (Koyanagi et al. 2014). Similarly, in rats and guinea pigs, higher and more predictable concentrations of DPH were attained in tissues after 5 mg/kg, subcutaneous administration compared with the same dose given orally (Dill &
Glazco 1949), yielding better systemic absorption of DPH after subcutaneous or IM injection (Stoelting & Hillier 2006).

Higher plasma concentrations of DPH were achieved in this study after IV administration of 1 mg/kg compared to the IM administration of 2 mg/kg. For the IV dose, concentrations declined rapidly after reaching maximal concentrations, indicating a fast distribution time to the tissues (1.1 h) and the corresponding large Vd(area) (7.6 L/kg), which exceeds the total body water in the dog (≈ 0.6 L/kg) (Wamberg et al. 2002). Likewise, the Vd(area)/F after the 2 mg/kg IM dose was high (12.3 L/kg). In a recent study, the Vd at steady state after administration of 0.2 mg/kg IV was 10.5-12.1 L/kg (Koyanagi et al. 2014). The high Vd suggests that DPH distributes extensively in the body tissues, probably because of its high lipid solubility (logP_{o/w}=3.27) (Hansch et al. 1995), despite moderate-high plasma protein binding of 76-98% reported in dogs and other species (Albert et al. 1975, Spector et al. 1980, Yoo et al. 1990, Koyanagi et al. 2014). Other studies have shown that DPH distributes extensively in the body tissues with maximum tissue uptake occurring at 1-3 min in rats and a mean initial distribution T_{1/2} of 5-9 min in sheep (Drach et al. 1970, Yoo et al. 1990). High Vd values after IV administration have been reported in adult rabbits (11.4 L/kg), dogs (10.5-12.1 L/kg), camels (5.98 L/kg), humans (3.56-4.54 L/kg), horses (2.38 L/kg), and sheep (2.1 L/kg) (Carruthers et al. 1978, Berlinger et al. 1982, Blyden et al. 1986, Scavone et al. 1990, Yoo et al. 1990, Kumar et al. 1999a, Wong et al. 2000, Koyanagi et al. 2014). In sheep the Vd of DPH increases with higher doses (Yoo et al. 1990), which agrees with the higher Vd values found in the IM group in our study (14.0 L/kg) in such as the dose administered was twice the IV dose and this route showed high %F.
Clearance times were similar for both IM (20.8 mL/kg/min) and IV (20.7 mL/kg/min) DPH doses. Higher values have been reported after IV administration of 0.2 mg/kg in 2 year old dogs (33.8 mL/kg/min) and in 11 year old dogs and in 11 year old dogs (70.1 mL/kg/min), in another study (Koyanagi et al. 2014). In the latter study, differences in clearance between the two age groups were attributed to decreased hepatic function in older individuals from lower hepatic blood flow and hepatic metabolism, and longer T1/2, due to a decrease in hepatic extraction ratio (Meredith et al. 1984, Simons et al. 1990).

Clearance times obtained in this study are higher than those reported after IV DPH in humans (6.2-11.2 mL/kg/min) (Carruthers et al. 1978, Spector et al. 1980, Berlinger et al. 1982, Blyden et al. 1986, Simons et al. 1990) and horses (13.2 mL/min/kg), similar to camels (23.6 mL/kg/min) (Wasfi et al. 2003); but lower than for rabbits (128 mL/kg/min) and adult sheep (31.5-83.3 mL/kg/min) (Yoo et al. 1990, Simons et al. 1996, Au-Yeung et al. 2007). Diphenhydramine is extensively metabolized by the hepatic cytochrome P-450 enzyme system and renal clearance of unchanged DPH seems to be a minor route of elimination since only 2-10 % is excreted in urine in humans, camels, monkeys, dogs, and rats (Drach et al. 1970, Albert et al. 1975, Wasfi et al. 2003). Differences in clearance values among species can be explained by differences in hepatic blood flow amongst them. Humans and horses have similar hepatic blood flow (~20 ml/kg/min) while dogs have higher (31-34 mL/kg/min) (Teranaka & Schenk 1977, Davies & Morris 1993, Dyke et al. 1998). In both, sheep and rabbits, clearance of DPH was found to exceed significantly the normal hepatic blood flow, (Cl of 128 mL/kg/min versus hepatic blood flow of 71 mL/kg/min for rabbits; Cl of 83 mL/kg/min versus
hepatic blood flow of 49 mL/kg/min for sheep) indicating that DPH is likely cleared by
the liver as well as by other organs (Yoo et al. 1990, Davies & Morris 1993, Simons et al.
1996). In fact, gastrointestinal drug uptake from the systemic circulation is responsible
for 50-80% of DPH systemic clearance in adult sheep and the liver accounts for the
remainder (Kumar et al. 1999a).

There are also differences in the hepatic metabolic routes among species that may
explain the variation in Cl and T1/2 values. The major routes of DPH metabolism in dogs
include diphenylmethoxyacetic acid (DPMA) and N-oxide metabolites; the same
metabolites are present in urine of humans, rats, and rhesus monkeys (Drach & Howell
1968, Drach et al. 1970, Chang et al. 1974) but some differences have been found in the
last transformation since DPMA is conjugated with glutamine by rhesus monkeys and
rats, but with glycine by dogs (Drach & Howell 1968, Drach et al. 1970). In horses,
oxidation to DPMA is less significant than in other species (Wynne et al. 1996), whereas
in sheep extrahepatic metabolism of DPH predominates and those metabolites represent
only 0.5-2% of the dose administered (Kumar et al. 1999b).

The T1/2 is a hybrid parameter, dependent on both Cl and Vd. Because Cl
remained unchanged in both the IV and IM groups of this study, while Vd increased with
increasing dose in the IM group, the net effect was an increase in T1/2 of DPH for dogs in
the IM group (6.8 h) compared with the IV group (4.2 h). Similar values of 4.4 ± 0.9 h
were found in dogs after oral administration of 8.65 mg/kg of DPH in combination with
D-amphetamine sulfate and ginger extraction (Wang et al. 2007), and of 4.2-6.6 h after
administration of 1 mg/kg (Koyanagi et al. 2014). Administration of 0.2 mg/kg IV
resulted in a T1/2 of 2.1 ± 0.1 h in 2 year old Beagles and a highly variable 5.9 ± 5.3 h in
11 year old Beagles (Koyanagi et al. 2014). Similar $T_{1/2}$ values have been found in humans after approximately 0.38-0.7 mg/kg of DPH, IV (Carruthers et al. 1978, Berlinger et al. 1982, Blyden et al. 1986, Scavone et al. 1990). In sheep and rabbits, a shorter $T_{1/2}$ of 0.6-1.3 h after IV administration corresponds to similar or lower Vd and faster Cl times (Yoo et al. 1990, Simons et al. 1990, Kumar et al. 1999b, Wong et al. 2000). These results are in contrast with the much shorter $T_{1/2}$ ($\approx$1 h) found in dogs in a prior study (Drach et al. 1970). Variability in these results may be explained by differences in number of dogs, sampling frequencies, and differences in analytic techniques chosen for plasma DPH determinations since HPLC and liquid chromatography/tandem mass spectrometry assays (LC-MS/MS) have shown to have higher sensitivity and selectivity than older techniques such as tritium-labeled assay used by Drach et al (1970). The technique chosen for our study was HPLC employing fluorescence detection, which is precise, accurate, and relatively easy to perform and has been validated in different species for plasma DPH determination (Webb & Eldon 1991).

The MRT reported in this study for the IV route was shorter (3.8 h) than the one for the IM group (9.8 h) or after oral DPH in other study (6.5 h) (Wang et al. 2007). In the latter study, parameters were reported after oral administration of an approximate dose of 8.65 mg/kg of DPH formulated as dimenhydrate, and in combination with D-amphetamine sulfate and ginger extraction, to 6 healthy beagles (Wang et al. 2007). Both, D-amphetamine and DPH, are potent inhibitors of the enzyme (CYP2D6) member of the cytochrome P450 mixed-function oxidase system (Amstrong & Cozza 2003, Krishnan & Moncrief 2007), which may affect the pharmacokinetic parameters determined in that study, due to the competitive inhibition for the same binding site on an
enzyme and the associated reduction in oral clearance (Bibi 2008). The MRT is a measure of the average time a substance spends in the body after an IV bolus dose. When a drug is given by an extravascular route it spends additional time at the site of administration, and MRT is then the sum of the mean times at these sites and in the body and subsequently is expected to be longer than the MRT for the same drug given by IV route (Rowland & Tozer 2010). Mean residence time of DPH has been reported to be shorter in rabbits, sheep, and camels after IV administration but longer in horses (Simons et al. 1996, Kumar et al. 1999a, Wasfi et al. 2003). These differences may be attributed to differences in the metabolism and clearance of the drug among species and differences in sampling time protocols. Since MRT has an inverse relationship with clearance times, is also affected by age in different species and is shorter in lambs and children when compared with adults (Simons et al. 1990, Wong et al. 2000).

Dose-response studies have not been performed in domestic species, but $C_{\text{max}}$ found in this study for both IV and IM groups far exceeded DPH plasma concentrations that are considered therapeutic in humans. In people, effective antihistamine concentrations in plasma, measured by reduction in skin wheal diameter to subcutaneously injected histamine, are considered between 25-50 ng/mL (Carruthers et al. 1978) and are usually achieved after oral therapeutic doses of 25-50 mg in adults (Bilzer et al. 1974, Estelle et al. 2009). Plasma concentrations of DPH considered therapeutic in humans were achieved 3 min after administration in both groups in this study and were maintained above that therapeutic range for up to 4 h in the IV group and up to 8 h in the IM group. The $C_{\text{max}}$ of DPH obtained after 1 mg/kg administered orally was only 2.66–3.34 ng/mL, suggesting that much higher oral doses may be necessary in
dogs to reach plasma concentrations considered therapeutic in humans, due to the low oral F% of DPH (7.3 % in young dogs and 2.8% in aged animals) in this species (Koyanagi et al. 2014). In one study in cats, maximum abolishment of hyperemia caused by low doses of histamine was achieved after DPH administration at doses of 6-10 mg/kg but effective plasma concentrations were not measured (Folkow et al. 1948). Specific studies in dogs are necessary to confirm therapeutic plasma concentrations of DPH in this species for antihistaminic effects.

In conclusion, DPH in dogs by both IV and IM routes of administration resulted in rapid DPH plasma concentrations that exceeded therapeutic levels in humans. The IM administration of DPH resulted in fast absorption and almost complete bioavailability. The lack of side effects in dogs, including alterations in behaviour or cardio-respiratory function, indicate that both routes are appropriate for DPH administration at the doses investigated in this study. Pharmacokinetic parameters reported in this study will assist in the design of clinical protocols for parenteral administration of DPH prior to MCT resection in dogs.
2.6. REFERENCES


Koyanagi T, Tamaura Y, Yano K et al. (2014) Age-related pharmacokinetic changes of acetaminophen, antipyrine, diazepam, diphenhydramine, and ofloxacin in male cynomolgus monkeys and beagle dogs. Xenobiotica 44: 893-901.


Table 2.1. Physiologic variables after administration of diphenhydramine at 1 mg/kg, IV, and 2 mg/kg, IM in healthy conscious dogs. Data is expressed as mean (SD); n = 6/group.

<table>
<thead>
<tr>
<th>Time</th>
<th>0 min</th>
<th>3 min</th>
<th>10 min</th>
<th>30 min</th>
<th>1 h</th>
<th>2 h</th>
<th>4 h</th>
<th>8 h</th>
<th>12 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
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<td>90 (26)</td>
<td>85 (26)</td>
<td>83 (25)</td>
<td>92 (19)</td>
<td>87 (19)</td>
<td>91 (24)</td>
<td>87 (24)</td>
<td>87 (26)</td>
<td>95 (27)</td>
</tr>
<tr>
<td>IM</td>
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<td>18 (2)</td>
<td>19 (2)</td>
<td>18 (2)</td>
<td>19 (2)</td>
<td>17 (3)</td>
<td>18 (2)</td>
<td>18 (2)</td>
<td>20 (3)</td>
<td>18 (2)</td>
</tr>
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<td>144 (22)</td>
<td>155 (13)</td>
<td>148 (13)</td>
<td>148 (20)</td>
<td>151 (19)</td>
<td>142 (23)</td>
<td>142 (12)</td>
<td>143 (13)</td>
<td>155 (20)</td>
</tr>
<tr>
<td>IM</td>
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<td>111 (11)</td>
<td>124 (6)</td>
<td>118 (21)</td>
<td>119 (16)</td>
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<td>110 (20)</td>
<td>114 (13)</td>
<td>111 (13)</td>
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<tr>
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<td>111 (6)</td>
<td>111 (21)</td>
<td>111 (16)</td>
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<tr>
<td></td>
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<tr>
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<td>(11)</td>
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<td>(23)</td>
<td>(17)</td>
<td>(16)</td>
<td>(19)</td>
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<td></td>
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<td>109 (17)</td>
<td>107</td>
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<td>(0.5)</td>
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<td>ND</td>
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<td>ND</td>
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<td>(0.3)</td>
</tr>
</tbody>
</table>

Statistically significant differences (P < 0.05) were not detected for any of the variables within groups or between groups. ND, not determined.
Table 2.2. Pharmacokinetic parameters of diphenhydramine using a non-compartmental model after administration of 1 mg/kg, IV, or 2 mg/kg, IM, in healthy conscious dogs (n=6 /group). Data expressed as mean (SD).

<table>
<thead>
<tr>
<th>Parameter (units)</th>
<th>IV group</th>
<th>IM group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>C(_{\text{max}}) (ng/mL)</td>
<td>306 (77.0)</td>
<td>188 (33.6)</td>
</tr>
<tr>
<td>T(_{\text{max}}) (min)</td>
<td>6 (0.3)</td>
<td>45 (5.1)</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>3.8 (0.3)</td>
<td>9.8 (1.1)</td>
</tr>
<tr>
<td>F (%)</td>
<td>100</td>
<td>88</td>
</tr>
<tr>
<td>Vd(_{\text{area}}) (L/kg)</td>
<td>7.6 (0.7)</td>
<td>14.0 (1.4)</td>
</tr>
<tr>
<td>Vd(_{\text{area}})/F (L/kg)</td>
<td>7.6 (0.7)</td>
<td>12.3 (1.2)</td>
</tr>
<tr>
<td>Cl(_{\text{area}}) (mL/kg/min)</td>
<td>20.7 (2.9)</td>
<td>23.6 (3.1)</td>
</tr>
<tr>
<td>Cl(_{\text{area}})/F (mL/kg/min)</td>
<td>20.7 (2.9)</td>
<td>20.8 (2.7)</td>
</tr>
<tr>
<td>T(_{1/2}) (h)</td>
<td>4.2 (0.5)</td>
<td>6.8 (0.7)</td>
</tr>
<tr>
<td>AUC(_{\text{area}}) (ng-h/mL)</td>
<td>804 (92)</td>
<td>1,413 (156)</td>
</tr>
<tr>
<td>AUC Extrap (%)</td>
<td>0</td>
<td>11.2</td>
</tr>
<tr>
<td>AUMC(_{\text{area}}) (ng-h*h/mL)</td>
<td>3,056 (401)</td>
<td>13,906 (1066)</td>
</tr>
</tbody>
</table>
Table 2.3. Plasma concentrations of diphenhydramine after administration of 1 mg/kg, IV, or 2 mg/kg, IM, in healthy conscious dogs (n=6 /group). Data expressed as mean (SD).

<table>
<thead>
<tr>
<th>Time after administration (h)</th>
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<th>IM group</th>
</tr>
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<tr>
<td>0.05</td>
<td>285 (74.7)</td>
<td>43 (6.7)</td>
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<td>0.08</td>
<td>306 (77.0)</td>
<td>51 (14.6)</td>
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<td>200 (42.4)</td>
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<td>0.75</td>
<td>178 (56.7)</td>
<td>188 (33.6)</td>
</tr>
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<td>1</td>
<td>164 (33.2)</td>
<td>186 (38.9)</td>
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<td>1.5</td>
<td>154 (49.4)</td>
<td>161 (42.0)</td>
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<td>2</td>
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<td>4</td>
<td>50 (8.8)</td>
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<tr>
<td>6</td>
<td>30 (10.5)</td>
<td>75 (18.2)</td>
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<td>8</td>
<td>21 (5.0)</td>
<td>52 (12.8)</td>
</tr>
<tr>
<td>12</td>
<td>13 (1.3)</td>
<td>29 (13.0)</td>
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<tr>
<td>24</td>
<td>ND</td>
<td>16 (4.6)</td>
</tr>
</tbody>
</table>

ND, not detected or value is lower than the limit of quantification of the assay (10 ng/mL).
Figure 2.1. Semi-log plot of mean plasma concentrations of diphenhydramine over 24 h after administration of 1 mg/kg, IV, in six healthy dogs.
Figure 2.2. Semi-log plot of mean plasma concentrations of diphenhydramine over 24 h after administration of 2 mg/kg, IM, in six healthy dogs.
CHAPTER III: EVALUATION OF THE ANTIHISTAMINIC AND CARDIO-RESPIRATORY EFFECTS OF DIPHENHYDRAMINE IN ANESTHETIZED DOGS UNDERGOING EXCISION OF MAST CELL TUMOURS

3.1. SUMMARY

Objective- To evaluate the antihistaminic effects and cardio-respiratory parameters associated with the use of IV diphenhydramine (DPH) in anesthetized client-owned dogs undergoing surgical excision of mast cell tumours (MCT).

Study Design- Prospective, randomized, blinded clinical trial.

Animals- Sixteen client-owned dogs undergoing excision of MCT.

Methods- Dogs were premedicated with hydromorphone (0.1 mg/kg, IM), induced with propofol (2-4 mg/kg, IV to effect), intubated, mechanically ventilated, and maintained with isoflurane (ETIso 1.5%). Dogs were randomly allocated to two treatment groups and received DPH, 1 mg/kg, IV (DPH group) or equivalent volume of saline (S group), 10 min after induction of anesthesia. Heart rate, direct arterial blood pressure, pulse oximetry, end-tidal isoflurane, end-tidal CO₂, cardiac index, stroke volume index, temperature, arterial blood gases, and anesthetic depth were recorded throughout anesthesia every 5 min. Blood samples for determination of DPH and histamine plasma concentrations were collected prior to premedication, during surgical preparation, during excision, and 2 h post-extubation. A general linear mixed model was used for multiple comparisons of cardio-respiratory parameters between and within groups. Sampling times and plasma concentrations for DPH and histamine were analyzed using a repeated measures ANOVA (p < 0.05).

Results- There were no differences in weight (DPH- 25.4 ± 12.2 kg; S- 26.9 ± 9.7; kg),
gender (DPH- 2 males, 6 females; S- 3 males, 5 females), age (DPH- 8.1 ± 3.5; S- 8.0 ± 2.1; years), and propofol dose (DPH- 3.0 ± 0.8; S- 3.5 ± 0.9; mg/kg). Diphenhydramine plasma concentrations of 343 ± 152 ng/mL (5 min post-administration) and 53 ± 40 ng/mL (83 ± 15 min- during surgery) were measured in the DPH group. Mean arterial and diastolic blood pressures were significantly lower in the DPH group than in the S group during the times of surgical dissection of the tumour (73 ± 19 min after DPH or S administration; 52 ± 10 versus 62 ± 9 mmHg diastolic pressure), at the time of complete excision of the tumour (85 ± 19 min; 65 ± 12 versus 78 ± 11 mmHg mean pressure, and 51 ± 10 versus 65 ± 9 mmHg diastolic pressure), and at the time of half-way through closure of the excision site (97 ± 21 min; 67 ± 12 versus 81 ± 11 mmHg mean pressure, and 53 ± 10 versus 67 ± 9 mmHg diastolic pressure). Cardiac index was significantly higher in the DPH group at the time of complete excision of the tumour than in the S group (100 ± 20 versus 82 ± 16 mL/min/kg). No other significant differences in cardio-respiratory parameters were noted between groups. Histamine plasma concentrations before premedication were 1.49 ± 1.71 ng/mL (range from 0.20 to 4.59 ng/mL) in the DPH group in 6/8 dogs and 0.91 ± 0.84 ng/mL (range from 0.06 to 2.11 ng/mL) in the S group in 7/8 dogs. Higher histamine concentrations were measured during maximal surgical manipulation of the tumour in the DPH group at surgical preparation (24 ± 10 min after DPH administration; 6.60 ± 11.54 ng/mL) and at the time of surgical excision (80 min ± 16 min; 4.56 ± 7.81 ng/mL), and at the time of surgical excision in the S group (86 ± 24 min after S administration; 2.48 ± 4.01 ng/mL).

**Conclusion and clinical relevance**- Isoflurane anesthetized dogs administered DPH prior to MCT excision had no clear clinical anesthetic differences related to cardio-respiratory
responses compared to dogs receiving a S placebo. Mean arterial and diastolic blood pressures were higher in the S group during surgical excision of the tumour despite a lower cardiac index than for the DPH group; however cardio-respiratory parameters in both groups were considered within acceptable limits for anesthetized dogs.
3.2. INTRODUCTION

Mast cell tumour (MCT) is the most frequent malignant skin tumour diagnosed in dogs (Govier 2003, Dobson & Scase 2007). Cutaneous mastocytoma is the most common type of MCT and originates from mast cells in the dermis and subcutaneous tissue (Bostock 1986); however, MCT can potentially originate from any location where normal mast cells are found, including lung, intestinal mucosa, and perivascular regions (Maker-Gabb et al. 2003).

Paraneoplastic syndromes in dogs associated with MCT are usually the result of mast cell degranulation. Clinical signs are caused by extracellular release of bioactive substances, such as histamine, heparin, proteolytic enzymes, and vasoactive amines, contained within mast cell granules (Welle et al. 2008). Extracellular release of these mediators following mast cell degranulation may be precipitated by physical or chemical means, including surgical manipulation or radiation therapy (Dobson & Scase 2007).

Dogs with MCT have significantly higher plasma histamine concentrations (hyperhistaminemia) and lower plasma gastrin concentration than normal dogs, which predisposes to hyperacidity and gastric ulceration (Fox et al. 1990). In dogs, systemic release of histamine causes dilatation of coronary arteries and terminal arterioles that are independent of vascular innervation. It also increases capillary permeability that leads to a decrease in systemic vascular resistance and arterial blood pressure (Adams 1995; Genovese & Spadaro 1997; Peters & Kovacic 2009). For this reason, routine pre-operative treatment with diphenhydramine (DPH) has been recommended during manipulation and treatment of MCT (London & Seguin 2003), although this type of therapy/prevention is not instituted universally for excision of MCT. Histamine release is
assumed to occur during surgical manipulation of the tumour but no information is available for changes in histamine levels that may occur in anesthetized dogs undergoing surgical excision of MCT.

Diphenhydramine is a first generation H₁ inverse agonist that binds to and stabilizes the inactive conformation of the H₁-receptor. It also possesses anticholinergic and local anesthetic properties (Stoelting & Hillier 2006). Associated with H₁ antagonism, it can cause drowsiness and sedation in humans; however, these effects do not seem as obvious in dogs (Hofmeister & Egger 2005).

With the increase in caseload of cancer patients seen in our institution, including MCT, the utilization of DPH at home or during diagnostic procedures and prior to anesthesia and surgery has increased with the aim of preventing the potential effects of histamine released from MCT during surgery. Doses of 1-5 mg/kg per os (PO), intramuscular (IM), or intravenous (IV) of DPH have been recommended (Adams 1995; Govier 2003); however, some clinicians anecdotally describe the risk of adverse reactions, including hypotension after IV administration, and prefer PO administration. Since the goal of this therapy is to block the effects of histamine released from mast cells, it is important to time the administration of DPH to obtain peak antagonistic effects on the H₁ receptor during periods of histamine release from the MCT in surgery. Studies in anesthetized dogs and cats determined that DPH was able to abolish the vascular depressor effects of low doses of histamine (Folkow et al. 1948), however, DPH and other H₁-antihistamines administered alone, were not able to abolish the severe cardiovascular depression caused by high doses of exogenous histamine (Folkow et al. 1948, Tucker et al.
1975, Black et al. 1975). Therefore, it is possible than DPH effectiveness will depend on actual histamine concentrations released during surgery.

The main objective of the second phase of this research thesis is to test a therapeutic protocol with IV DPH for clinical canine cases anesthetized undergoing surgical excision of MCT, designed from pharmacokinetic results obtained in the first phase, Chapter II of the thesis. Secondary objectives with anesthesia and MCT manipulation are to investigate, i) anesthetic stability, ii) cardio-respiratory responses, iii) the incidence of hypotension with or without DPH administration, and finally iv) the relationship of anesthesia and cardio-respiratory parameters to histamine and DPH concentrations during those events.

We hypothesize that there is a plasma histamine levels affect negatively arterial blood pressure in dogs under general anesthesia and that pre-operative administration of DPH will minimize significant variations in blood pressures associated with the changes in histamine levels with surgical manipulation of MCT.
3.3. MATERIALS AND METHODS

3.3.a. Animals

Sixteen client-owned dogs (five males and eleven females) admitted to the OVC-HSC requiring general anesthesia for surgical excision of MCT were included in this study. Dogs weighed 10-35 kg and were 4-14 years old. The study was carried out in accordance with the guidelines of the Canadian Council on Animal Care and was approved by the Institutional Animal Care Committee at the University of Guelph. Informed owner consent was obtained prior to anesthesia in all cases.

All dogs underwent physical examination prior to anesthesia, and had complete blood count and serum biochemistry laboratory evaluation. Dogs with significant cardiovascular or renal disease were excluded from the study. Dogs were enrolled in the study if there was confirmed cytologic and/or histopathologic diagnosis of MCT and body weight was > 10 kg. In cases receiving DPH or other antihistamines preoperatively, treatment was stopped at least 12 h prior to the anesthesia. Prior to anesthesia, a pre-operative questionnaire outlining the MCT diagnosis and treatment was completed by the clinician responsible for the case (Appendix B). Additional information gathered included date and method of diagnosis, histological grade, site and number of tumours, history of previous MCT, type of excision to be performed (wide or marginal), presence of local (erythema, wheals, itchiness, hyperemia, mass changing size) or systemic (vomiting, diarrhea, melena, fever, peripheral edema, collapse) signs of disease, and last dose and time of antihistamine drugs administered. In all cases tissue from the surgery was sent for histopathologic confirmation of the diagnosis and grading of the tumour. The World Health Organization Clinical Staging System was used for staging, and the 2-Tier Grading
Criteria (Kiupel et al. 2011) was used to grade every tumour (Appendix C and D), even though there is no available grading system for oral or subcutaneous MCT.

3.3.b. Experimental Design

A randomized blinded study design was used to investigate the anesthetic stability and cardio-respiratory responses during tumour manipulation, incidence of hypotension, and histamine plasma levels in dogs with or without DPH administration. Dogs were equally assigned to each of the groups by a randomization scheme (http://www.randomization.com).

3.3.c. Study Protocol

A physical examination was performed prior to premedication by one anesthesiologist (Andrea Sanchez). Rectal temperature (Temp), heart rate (HR) and respiratory rate (RR) were recorded (PRE). A blood sample (6 mL) was collected by jugular venipuncture for baseline determination of DPH and histamine plasma levels. Immediately after, all dogs were premedicated with hydromorphone (0.1 mg/kg, IM) injected into the epaxial musculature.

Based on the size of the dog and the site of the tumour, a cephalic or saphenous vein was catheterized with a 20 or 22-SWG, 1-inch or 1.5-inch catheter (BD Insyte-W, BD Infusion Therapy Systems Inc. Sandy, Utah, USA). Anesthesia was induced with propofol (2-4 mg/kg, IV) (pms-Propofol 1%, PharmaScience, Montreal, Quebec, Canada) titrated to effect. After endotracheal intubation dogs were maintained with isoflurane (Aerrane Isoflurane, USP, Baxter Corporation, Mississauga, Ontario, Canada) in 100% oxygen
using a coaxial rebreathing system (F-circuit) with oxygen flow rate of 50-100 mL/kg/min. Intermittent positive pressure ventilation was immediately established using an electronically controlled, time-constant pressure-limited ventilator (EMC 2002IE; Hallowell, Massachusetts, USA), with a rate of 8 to 12 breaths/min and a tidal volume (VT) of 10 to 15 mL/kg in order to maintain end-tidal CO₂ values between 30 and 40 mmHg. Dogs were instrumented during the first 5-10 min of anesthesia and the isoflurane vaporizer setting was adjusted to maintain a constant end-tidal concentration (ETI₂o; %) of 1.5%. A balanced electrolyte solution (Plasma-Lyte A® Injection, Baxter Corporation, Mississauga, Ontario, Canada) was administered at a rate of 10 mL/kg/hr throughout anesthesia.

The dorsal pedal artery was catheterized with a 22-SWG, 1-inch catheter (BD Insyte-W, BD Infusion Therapy Systems Inc. Sandy, Utah, USA) for arterial blood pressure monitoring, blood gas analysis, and blood sampling for determination of DPH and histamine plasma concentrations during anesthesia. This arterial catheter was connected by non-compliant tubing to a pressure transducer (Transducer set, Argon Medical Devices, DTXPlus™ DT36, Singapore) from a multi-parametric anesthesia monitor (Carescape B650 monitor, GE Healthcare, Helsinki, Finland) for determination of systolic (SAP), diastolic (DAP) and mean (MAP) arterial blood pressures. The monitor was zeroed prior to blood pressure determinations and the transducer was placed at the level of the manubrium. The same multi-parameter monitor was used to monitor HR, RR, ETIs o, and Temp. A NICO monitor (NICO; Novametrix Medical Systems, Wallingford, Connecticut, USA) was used for cardiac output (CO), pulse oximetry (SpO₂), and end-tidal CO₂ (ETCO₂) measurements. The monitor was zeroed to room air prior to use. The
flow sensor, mainstream capnograph, and rebreathing loop from the NICO monitor were placed between the endotracheal tube and the F-circuit, and the pulse oximeter was placed on the tongue. During the instrumentation phase an arterial blood sample was obtained from the dorsal pedal artery for arterial blood gases analysis (ABL 800Flex Series analyzer; Radiometer, Copenhagen, Denmark). Values of partial pressure of arterial oxygen (PaO₂) and carbon dioxide (PaCO₂) obtained from the arterial blood gas analysis were entered in the NICO monitor as well as values for hemoglobin concentration (Hb) and inspired oxygen fraction. This process was repeated at the beginning of the surgical procedure to ensure accurate CO measurements due to changes in blood gas parameters during anesthesia. Cardiac index (CI) was calculated by dividing the CO value obtained by the patient’s weight (BW), and stroke volume index (SVI) was calculated as SVI = (CO/HR)/BW.

Dogs were randomly divided into two experimental groups. After instrumentation and recording of cardio-respiratory parameters (T0, baseline), treatments were administered. Dogs in the DPH group received DPH at 1 mg/kg, IV (Diphenhydramine hydrochloride injection USP 50 mg/ml, Pharmaceutical partners of Canada Inc. Richmond Hill, Ontario, Canada), and dogs in saline group (S) received an equivalent volume of NaCl 0.9% (0.9% Sodium Chloride Injection USP, Baxter Corporation, Mississauga, Ontario, Canada).

Cefazolin at 22 mg/kg, IV, was administered to all dogs as intraoperative antibiotic after induction and every 90 min until the end of surgery. Once the surgical area was aseptically prepared, dogs were transferred to the surgical suite while continuously instrumented and monitored. In surgery, dogs were suited with a blanket and a delivery
forced warm air system (3M Bair Hugger™ Warming unit, Model 505, Arizant Healthcare Inc. Eden Prairie, Minessota, USA) to maintain body temperature within normal limits and then surgically draped. A board certified surgeon performed complete excision of the tumour (marginal or wide). After the surgical procedure, dogs were transferred to a recovery area and isoflurane was discontinued. Upon extubation, dogs were kept in a cage with the arterial and venous catheters in place and monitored by the investigator (Andrea Sanchez) until fully recovered. At 2 h post-extubation, blood was collected from the arterial catheter for DPH and histamine concentration determinations. Additional doses of hydromorphone (0.025 mg/kg, IV) were administered 2-3 h after the pre-medication dose throughout surgery and thereafter if signs of pain were noticed in the post-extubation period.

3.3.d Monitoring and blood sampling

Cardiovascular parameters (HR, SAP, MAP, DAP, CI, SVI), Temp, ETISO%, VT, RR, ETCO₂, and SpO₂) were monitored continuously after induction and throughout anesthesia and recorded at T0 (baseline), before preparation of the surgical site (T1- 5 min after treatment administration; T2- 10 min after treatment administration), during surgical preparation of the tumour (T3- 20-30 min after treatment administration; T4- 30-40 min after treatment administration), and during surgery (T5- within 10 min of draping the surgical site; T6- half-way through excision of the tumour; T7- immediately before complete excision of the tumour; and T8- half-way through closure of the excision site).

Blood samples of 3 mL were collected for determination of histamine (H) plasma concentrations in tubes with EDTA from the jugular vein before pre-medication (H1 at
PRE), and from the arterial catheter after induction (H2 at T0, baseline after induction), during maximal manipulation of the tumour at the time of surgical preparation (H3 at T3 or T4), intra-operatively just before tumour removal (H4 at T7), and 2 h post-extubation (H5) (Figure 3.1). In addition, blood samples of 3 mL were collected for determination of DPH plasma concentrations in heparinized tubes from the jugular vein before pre-medication (D1 at PRE), and from the arterial catheter 5 min after treatment (DPH or S) administration (D2 at T1), 30 min after treatment administration (D3 at T3 or T4), intra-operatively during surgical dissection of the tumour (D4 at T6), just before tumour removal (D5 at T7), and 2 h post-extubation (D6) (Figure 3.1). The volume of blood removed after every blood sample was replaced through the same catheter with twice the volume of isotonic solution (Plasma-Lyte A® Injection, Baxter Corporation, Mississauga, Ontario, Canada) at each sampling time.

Hypotension during anesthesia was defined as MAP < 60 mmHg and was treated with dopamine (5 μg/kg/min) if it persisted for > 10 min or if deemed detrimental to the patient. The same investigator (Andrea Sanchez) performed all anesthetic procedures, including blood sampling, monitoring of anesthetic depth, and recording of anesthetic parameters.

### 3.3.e. Plasma DPH analysis

Heparinized blood samples were placed on ice immediately after collection, until centrifugation was performed at 3,000 g for 10 min. Plasma was then collected and stored at -80°C until analysis. Diphenhydramine was quantified in plasma using high-performance liquid chromatography (HPLC) employing fluorescence detection.
High-performance liquid chromatography analysis was performed on a Waters Alliance® 2695 HPLC separations system (Mississauga, ON, Canada) coupled with a 2475 Multi-Wavelength Fluorescence Detector. The system was connected to a PC with Empower 2 software (Waters®, Mississauga, ON, Canada) for data collection and processing. The analytical column used was a Sunfire® C18 column (50 mm x 4.6 mm I.D., 2.5 µm, Waters®, Ireland), connected with a Security Guard C18 guard column (4 mm x 3.0 mm I.D., Phenomenex®, Torrance, CA, USA). An isocratic chromatographic separation was performed using a mobile phase containing acetonitrile-water-acetic acid (78:22:1, v/v/v), at a flow rate of 1 mL/min. A 50 µL sample was injected into the column. Fluorescence detection was optimized at 229 nm excitation and 268 nm emission wavelengths. The retention times observed were 1.9 min for DPH and 3.1 min for orphenadrine, respectively.

Stock solutions of DPH and orphenadrine standards were prepared in methanol at the concentration of 1 mg/mL and stored at -80°C. Calibration (reference) standards (10, 50, 100, 500 and 1000 ng/mL), and quality controls (QC) (20 and 800 ng/mL) were prepared in blank canine plasma. To 100 µL aliquots of unknown canine plasma samples, 10 µL of internal standard working solution (1 µg/mL orphenadrine) were added. Proteins were precipitated by adding both 10 µL of 1N HCl and 1 mL acetonitrile. The mixture was vortex-mixed for 30 s, followed by centrifugation for 10 min at 14,400 g at 4°C. The supernatant was then collected and evaporated under a constant flow of nitrogen. The residue was reconstituted with 100 µL of the mobile phase and 50 µL of it was injected onto the column.
Method validation

Canine plasma samples were quantified using the ratio of the peak area of DPH to that of the internal standard orphenadrine. Peak area ratios were plotted against DPH concentrations. Standard curves, in the form of \( y = A + Bx \), were calculated using weighted \( (1/x^2) \) least squares linear regression. Calibration curves were prepared and assayed on 8 separate days. Five point calibration curves were linear and reproducible in the concentration range from 10 ng/mL to 1 \( \mu \)g/mL, with the correlation coefficient \( (r^2) > 0.99 \) for all calibration curves. No interfering peaks were observed in blank plasma samples at retention time corresponding to the drug (1.9 min) and internal standard (3.1 min) (Fig. 2). This clean background shows that the assay procedure is specific to DPH.

The limit of detection for this assay was 5 ng/mL (based on 3 times the signal to noise ratio at the time of elution of the analyte). The limit of quantitation (LOQ) for the assay was 10 ng/mL. The limit of quantitation was determined by spiking blank canine plasma with various concentrations of DPH, resulting values having a coefficient of variation no greater than 20%. The intra-day and inter-day assay coefficients of variation were 6.9% and 13.7% respectively. The true value for each calibration standard was within 15% of the actual value except at LOQ (10 ng/mL), where it deviated by less than 20%.

The recovery rate was determined by comparing the peak areas of blank plasma spiked with different amounts of drug with the peak areas of the same standards prepared in the mobile phase. Average recovery was 93.7%, with 88.6% at LOQ (10 ng/mL).
3.3.f. Plasma Histamine analysis

Venous or arterial blood samples were collected into EDTA tubes and placed on ice immediately after collection, until centrifugation at 3,000 g for 10 min. Plasma was then collected and stored at -80°C until analysis. Analysis of plasma histamine concentrations was performed using previously described methods using a commercial enzyme immunoassay kit (Enzyme immunoassay Histamine; IM2015; Immunotech, Marseille, France) according to the manufacturer’s recommendations. The assay has a LOQ of 0.055 ng/mL (0.5 nM) and has been validated and used for determination of plasma histamine concentrations in dogs (Guedes et al. 2007).

The assay procedure included an acylation step, an immunological step, and an enzymatic step. For the acylation step, 25 µL of acylation buffer were added to 100 µL of the plasma or calibrators (0 nM, 1.1 nM, 2.9 nM, 8.8 nM, 25 nM, 90 nM) or control (4.4-6.6 nM), and to 25 µL of acylation reagent (reconstituted with DMSO), and vortex-mixed immediately. Then in the immunological step, 50 µL of the acylated samples (plasma, calibrators, and control) and 200 µL of a reconstituted lyophilized alkaline phosphatase acylated histamine conjugate were added to each well of a plate with monoclonal antibody coated wells, and then incubated for 2 h at 2-8 °C, while shaking on a titer plate shaker (Model 4625, Thermos Scientific, USA). Finally, in the enzymatic step, the wells were washed with wash solution previously diluted with distilled water, to remove the non-bound components. Then 200 µL of substrate (diethanolmine-HCl solution) were added to the components bound to the coated wells and incubated for 30 min at 18-25°C, while shaking on the titer plate shaker in the dark. Then 50 µL of stop solution (NaOH 1N) was added per well and the bound enzymatic activity read at an absorbance of 405 nm, using a
microplate reader (ELx800 Universal Microplate reader, BioTek Instruments, Inc., Winooski, Vermont, USA). The raw data was then analyzed using online software to generate the plasma concentrations (“Four Parameter Logistic Curve” online data analysis tool, MyAssays Ltd., June 25th 2015, http://www.myassays.com/four-parameter-logistic-curve.assay). The assay has an intra-assay precision of 7.1-9.8% and an inter-assay precision of 6.4-16.8%.

3.3.g. Data analysis

Tests of normality including Shapiro-Wilk, Kolmogorov-Smirnov, Cramer-von Mises and Anderson-Darlin, and UNIVARIATE and PLOT procedures were used to detect unequal variances, outliers and other non-random patterns within the data. The effects on cardio-respiratory variables and the comparisons between the two treatments were analysed with a generalised mixed linear model allowing correlations and non-constant variability for normal distributions (proc MIXED) and NPARIWAY if the assumptions of a normal distribution were not met (SAS Institute, version 9.3, Cary, North Carolina, USA). The error structure over time was selected based on the Akaike Information Criteria (AIC) among structures offered by SAS: ar(1), arh(1), toep(2-5), toeph(2-4), un(2-5); using for each treatment a random effect and a 2 factor (time and treatment)-factorial design with repeated measurements over time. Normal distribution for plasma DPH concentrations was analyzed with Kolmogorov-Smirnov test followed by descriptive statistics to determine symmetry and confidence intervals. Differences in sampling times were analysed using a repeated measures ANOVA followed by a post-hoc Bonferroni correction for comparisons between the two groups. Differences in gender,
age, weight, and propofol dose were determined with a paired samples T-test. Values of p < 0.05 were considered significant.

Plasma DPH within the DPH group and histamine concentrations within and between groups were compared using a repeated measures ANOVA followed by a post-hoc Bonferroni correction (MedCalc Statistical Software version 15.6 [MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014]). Normality of data was assessed using Kolmogorov-Smirnov test. Values are reported as mean ± SD for normally distributed data or as median and confidence intervals for non-normally distributed data. Values of p < 0.05 were considered significant.
3.4. RESULTS

Sixteen client-owned dogs were included in this study and were equally distributed between the two groups. Breeds represented in this population were: Pug (n=3), Mixed breed (n=3), Boxer (n=2), Labrador Retriever (n=2), Golden Retriever (n=2), Boston Terrier (n=1), Rottweiler (n=1), Weimaraner (n=1), and Beagle (n=1). Pre-operative information is summarized in Table 3.1. As part of the staging process, pre-operative thoracic radiographs were performed in all the dogs, abdominal ultrasound was available in 13 of 16 dogs and 3 dogs underwent computerized tomography (CT) of the MCT mass prior to surgery. During surgery, wide excision with 30 mm free margins of the circumference of the tumour was possible in 5 of 11 dogs. Histopathologic results showed that in 8 of the cases tumours were subcutaneous while 8 were cutaneous; of these 16 tumours 4 were classified as high grade. Seven dogs were in stage I of the disease (3 in DPH group and 4 in S group), 4 dogs in stage II (2 in DPH and 2 in S group), 4 dogs in stage III (2 in DPH group and 2 in S group), and one dog in the DPH group had systemic disease with spleen involvement and was considered to be in clinical stage IV.

No differences in weight (DPH- 25.4 ± 12.2 kg, S- 26.9 ± 9.7 kg), gender (DPH- 2 males and 6 females, S- 3 males and 5 females; p = 1.0), age (DPH- 8.1 ± 3.5 years, S- 8.0 ± 2.1 years), or propofol dose required for induction (DPH- 3.0 ± 0.8 mg/kg, S- 3.5 ± 0.9 mg/kg) were found between the two groups. Induction of anesthesia was 42 ± 23 min after premedication and treatment with DPH or saline was administered 11 ± 7 min after induction. No differences between groups were found for blood sampling time points for DPH or histamine plasma determinations.
Respiratory parameters and Temp are included in Table 3.2. No significant differences were observed between groups for Temp (DPH- 36.6 ± 0.8 °C, S- 36.9 ± 0.7 °C), ETIso% (DPH- 1.5 ± 0.1%, S- 1.4 ± 0.1 %), SpO2 (DPH- 100 ± 0.7 %, S- 99 ± 0.7 %), PaO2 (DPH- 408 ± 89 mmHg, S- 473 ± 80 mmHg), or PaCO2 (DPH- 41 ± 5 mmHg, S- 40 ± 5 mmHg) at any time point. Temperature decreased significantly at T2, T3, T4, T5, T6, T7, and T8, with respect to T0 (p < 0.05) in both groups. Hemoglobin concentrations decreased (p < 0.0001) between T0 and T5. An increase in PaCO2 was noted between T0 and T5, although not significant; however ETCO2 also increased during surgical manipulation of the tumour and it was significantly elevated with respect to T0 during T6 (p = 0.0171), T7 (p = 0.0246) and T8 (p = 0171) in the S group.

Cardiovascular parameters are reported in Table 3.3. No significant differences were found between groups in SVI (DPH- 1.5 ± 0.5 mL/beat/kg, S- 1.2 ± 0.3 mL/beat/kg), HR (DPH- 82 ± 19 beats/min, S- 73 ± 19 beats/min), and SAP (DPH- 110 ± 11 mmHg, S- 100 ± 10 mmHg) (Figures 3.2, 3.6, and 3.7). Heart rate decreased after premedication with hydromorphone in both groups; in the DPH group HR was significantly lower at T0 compared to PRE values (p < 0.0003) and in the S group the decrease in HR was significant after induction (T1) when compared to T0 (p = 0.0464). No significant changes in any cardiovascular parameters were detected within the DPH group between baseline (T0) values and 5 min (T1) after administration of DPH. Systolic arterial blood pressure was significantly lower than T0 at T2 (p = 0.0291), T3 (p = 0.0319), T4 (p = 0.0168), and T5 (p = 0.0081) in the S group, whereas it was lower at T3 (p = 0.0378), T4 (0.0156), T7 (p = 0.0197), and T8 (p = 0.0421) than for T0 in the DPH group (Figure 3.2). Diastolic and mean arterial pressures were lower than T0 at T4 in the DPH group (p =
0.0461 for DAP and p = 0.0216 for MAP). Mean and DAP increased significantly at the
time of surgery (T6, T7 and T8) with respect to T0 in the S group (p = 0.0374 for T6, p =
0.0037 for T7, and p = 0.0004 for T8 for MAP; p = 0.0066 for T6, p = 0.0003 for T7, and
p < 0.0001 for T8 for DAP). At times T7 (p = 0.0209) and T8 (p = 0.0139) MAP was
significantly higher in the S group compared to the DPH group, and at times T6 (p =
0.0490), T7 (p = 0.0072), and T8 (p = 0.0072) DAP was also higher in the S group than in
the DPH group (Figures 3.3 and 3.4).

Cardiac index values were significantly lower from T0 at T3 (p = 0.0269) in the S
group (Figure 3.5). A clinical increase in CI was observed in both groups (Figure 3.5) and
an increase in SVI in the DPH group (Figure 3.6) during surgery with respect to previous
values, although these changes were not statistically significant. Cardiac index was
significantly higher in the DPH group at the time of complete excision of the tumour (T7)
than in the S group (p = 0.0469). Surgical stimulation also resulted in significant increases
in HR (p = 0.0022), DAP (p = 0.0079), and MAP (p = 0.0049) between T5 and T6 in dogs
from the S group but not in the DPH group (Figures 3.3, 3.4, and 3.7).

Four dogs in each group developed transient (> 10 min) hypotension (MAP range
of 44 to 59 mmHg) between T1-T5 but dopamine administration was only required in 2/4
dogs; 1 dog was from the S group (administration time of 7 min) and 1 dog in from the
DPH group (administration time of 75 min).

Results for DPH plasma concentrations from dogs receiving DPH and sampling
times are included in Table 3.4. Only one dog in the DPH group had low, but detectable,
concentrations of DPH in plasma (14 ng/mL) prior to premedication. This dog received a
dose of DPH of 2.4 mg/kg, PO, the morning of the surgery, approximately 5 h before the
first blood sample was taken. Two dogs in the S group received DPH within the 12 h prior to premedication and obtaining the first blood sample; however DPH plasma concentrations were not detected. One of these dogs received DPH at 0.73 mg/kg of DPH PO 1 h prior and the other dog received 1.5 mg/kg 4 h prior to blood collection. The other 13 dogs did not receive any antihistamine treatment or treatment was stopped for more than 24 h prior to the experiment.

Maximal DPH plasma concentrations (343 ± 152 ng/mL) were found at T1 and were 53 ± 40 ng/mL at T7 in the DPH group (Table 3.4). In 5 of 8 dogs DPH concentrations were detectable 2 h post-extubation, 27 ± 26 ng/mL (263 ± 29 min after administration). In the S group, DPH plasma concentrations were below LOQ at all times.

Results for histamine plasma concentrations and sampling times are included in Table 3.5. No statistically significant differences were found in sampling times between the two groups (p < 0.05). Baseline histamine plasma concentrations (H1) before premedication were 1.49 ± 1.71 ng/mL (range from 0.20 to 4.59 ng/mL) in the DPH group in 6/8 dogs and 0.91 ± 0.84 ng/mL (range from 0.06 to 2.11 ng/mL) in the S group in 7/8 dogs (Table 3.6). The remaining three dogs, had histamine plasma concentrations below LOQ (< 0.055 ng/mL) at H1. No statistical significant differences were found in plasma histamine levels between or within groups (p < 0.05). Despite the lack of statistical differences, higher histamine concentrations were measured at H3 (24 ± 10 min after DPH administration; 6.60 ± 11.54 ng/mL) and at H4 (80 min ± 16 min; 4.56 ± 7.81 ng/mL) in the DPH group, and at H4 (86 ± 24 min after S administration; 2.48 ± 4.01 ng/mL) in the S group.
3.5. DISCUSSION

This study evaluated the cardio-respiratory responses during isoflurane anesthesia in dogs undergoing surgical excision of MCT for the occurrence of hypotension, and other adverse events, with or without prior administration of DPH (1 mg/kg, IV).

Endogenous release or exogenous administration of histamine may elicit adverse cardiovascular responses. In dogs, actions of histamine on H₁ and H₂ receptors elicit a decrease in systemic vascular resistance, which often results in hypotension (Peters & Kovacic 2009). Histamine’s direct effects on heart function in dogs include predominant activation of H₁ receptors, which may cause a decrease in ventricular contractility (negative inotropic effect) (Zavec & Levi 1978) and may impair AV conduction (negative dromotropic effect) that can lead to AV dissociation (Levi & Kuye 1974, Hageman et al. 1979). Although these adverse cardiovascular effects may be short-lived due to compensatory reflexes and quick inactivation of histamine (Thermann et al. 1975, Adams 1995, Guedes et al. 2006), they are dose-related and it is likely that cardiovascular function of the anesthetized patient is more affected due to impairment of cardiovascular pressor responses from the effects of anesthetic drugs, especially from drugs with vasodilatory effects (Pagel & Farber 2005). This has been part of the reason of why antihistamines are often recommended in patients undergoing excision of MCT, due to the risk of degranulation of mast cells and histamine release during surgical manipulation of the tumour, and the higher risk of developing hypotension in anesthetized patients (Welle et al. 2008, Ryan et al. 2012, Fossum 2013, London & Thamm 2013).

Histamine release from mast cells is rapid and measurable concentrations are detected in plasma within 1 minute of anaphylactic or anaphylactoid episodes (Peters &
Under physiological conditions endogenous histamine is metabolized within 1-2 min and there is no cumulative effect; however, the plasma half-life of histamine during anaphylactic or anaphylactoid episodes has been estimated to be 15-20 min in humans due to saturation of enzymatic metabolism from persistent immunological or non-immunological mast cell activation and continuous release of histamine to the systemic circulation (Laroche et al. 1995, Shmuel & Cortes 2013). Conversely, when administered exogenously the half-life of a single pharmacologically active dose of histamine is 20-30 s in the dog (Parsons & Ganellin 2006). It is likely that histamine release in dogs with MCT is intermittent and depends on stimulation and handling of the tumour, which may expose the dog to the effects of the histamine repeatedly.

The dose of DPH used in this study (1 mg/kg, IV) was effective in achieving plasma concentrations for a duration that should counteract the effects of histamine throughout surgery. However, plasma concentrations of histamine necessary to cause cardiovascular responses are larger than concentrations that elicit cutaneous responses (Lorenz et al. 1982). Studies in anesthetized dogs and cats determined that DPH alone (6-10 mg/kg) was able to abolish the vascular depressor effects of low doses of exogenous histamine; however, these doses did not abolish the severe cardiovascular depression caused by higher histamine doses (Folkow et al. 1948).

In this study, maximum DPH plasma concentrations of 343 ± 152 ng/mL were achieved 5 min after DPH administration at 1 mg/kg, IV. These values are very similar to the peak plasma concentrations of 306 ng/mL obtained at 3 min post-administration in Chapter II for the pharmacokinetics of DPH using research hounds. Dose-response studies in humans have considered plasma concentrations of at least 25 ng/mL of DPH to be
therapeutic on reducing the skin wheal diameter to subcutaneously injected histamine (Carruthers et al. 1978); however, similar studies are not available in dogs. At the time of surgical excision of the tumour (83 ± 15 min), plasma concentrations were still higher than 25 ng/mL in six of the eight dogs that received DPH in this study.

Diphenhydramine administration may not be effective or necessary in dogs undergoing surgery for MCT excision for two reasons; first, DPH at commonly used recommended doses may not counteract the cardiovascular effects of histamine, and second, histamine plasma concentrations in dogs with MCT are higher than for normal healthy dogs, but not necessarily associated with cardiovascular adverse effects. Histamine plasma concentrations in healthy dogs are usually ≤ 0.8 ng/mL (Thermann et al. 1975, Robinson et al. 1988, Ishiguro et al. 2003, Guedes et al. 2007), although one study reported plasma concentrations of 1.72 ± 2.69 ng/mL (Smith et al. 2001). Higher histamine plasma concentrations have been reported in dogs with MCT (2.9 ± 2.2 ng/mL) versus healthy dogs (0.7 ± 0.4 ng/mL) (Fox et al. 1990). Another study found no differences in histamine levels between healthy dogs (0.19 ng/mL, range 0.12-0.36 ng/mL) and dogs with MCT (0.39 ng/mL, range 0.11-2.75 ng/mL) but when histamine concentrations from dogs with microscopic disease were compared to dogs with gross disease, the latter group showed significantly higher histamine plasma concentrations (0.73 ng/mL, range 0.26-2.75 ng/mL) than healthy dogs (Ishiguro et al. 2003).

Histamine plasma concentrations determined for both groups in this study were similar throughout the anesthesia time. Mean values range between 0.73-6.60 ng/mL in the DPH group and 0.39-2.48 ng/mL in the S group. The higher concentrations were usually present during surgical manipulation of the tumour (times T6-8). However these
concentrations tend to be lower than those associated with hypotension in other studies (Thompson & Walton 1964; Thermann et al. 1975). In one study, dogs anesthetized with sodium pentobarbital and administered morphine (1 mg/kg, IV) had histamine plasma concentrations that increased to 100-300 ng/mL and were associated with a decrease in MAP values of 40-60 mmHg from baseline in all dogs (Thompson & Walton 1964). Morphine is known for its histamine releasing properties in dogs due to mast cell degranulation (Guedes et al. 2006). In another study, dogs under pentobarbital anesthesia and administered exogenous histamine had measured plasma histamine concentrations of 17.4-21.4 ng/mL, which were associated with a significant decrease in MAP (median dropped to 63 mmHg from 120 mmHg) and total SVR (dropped to 0.55 mmHg/mL/kg/min from 1.09 mmHg/mL/kg/min) (Thermann et al. 1975). The differences in histamine concentrations in these studies compared to ours, likely account for the lack of significant hypotension observed in our study.

In humans, plasma concentrations of endogenous histamine of 1 ng/mL are associated with only cutaneous signs, whereas concentrations of 1-12 ng/mL result in generalized severe cutaneous reactions, mild hypotension, tachycardia, arrhythmias, and difficulty breathing. Plasma histamine concentrations higher than 12 ng/mL usually require emergency treatment due to cardiovascular collapse (fatal arrhythmias, severe hypotension, cardiac arrest) and severe bronchospasm (Lorenz et al. 1982, Sánchez Palacios et al. 2000). Cutaneous signs, such as hyperemia and/or edema are commonly observed in dogs with mild anaphylactic reactions but histamine levels have not been measured (Moore et al. 2005). In one study, flushing of the skin around the lips, ears, and oral mucosa that lasted up to 120 minutes was seen in 100 % of conscious dogs
administered a bolus of morphine at 0.6 mg/kg, IV, followed by a 0.34 mg/kg/h infusion and had median peak histamine plasma concentrations of 10.6 ng/mL (Guedes et al. 2006). Despite higher histamine plasma concentrations measured in some of the dogs in our study, no signs of cutaneous symptoms were observed. The effects of isoflurane on systemic vascular resistance could interfere with localized effects of histamine on cutaneous signs. Vasoactive substances such as histamine increase the intracellular Ca$^{2+}$ of endothelial cells, which leads to the activation of nitric oxide synthase (NOS). Isoflurane and halothane interfere with this NOS histamine-induced Ca$^{2+}$ entry by depressing influx and preventing the production and action of nitric oxide (NO) on relaxation of the underlying smooth muscle and mediated vasodilation (Simoneau et al. 1996, Tas et al. 2003, Tas et al. 2008); therefore, isoflurane may actually protect against histamine’s actions.

In this study, dogs in the S group showed significantly higher MAP and DAP during surgical manipulation of the tumour (times T6-T8). These results contradict the premise that the administration of DPH will help to prevent the adverse effects of histamine and improve cardiovascular function in dogs with MCT undergoing surgery. Despite similar values in SVI and CI in both groups, except for a higher CI at T7 in the DPH group, the increases in HR, MAP, and DAP observed in the S group in association with surgical stimulation were not seen in dogs receiving DPH. Interestingly, the one dog in the S group that had a MAP below 60 mmHg for > 10 min, required the dopamine administration for only 7 min, as MAP improved as soon as surgery started; whereas the one hypotensive dog in the DPH group required dopamine for the duration of anesthesia despite surgical stimulation. Hence, these results of our study, despite small numbers, do
not demonstrate protection of DPH from hypotension during MCT surgical excision.

Histamine is released pre- and post-synaptically in peripheral sympathetic terminals, where it may act as a primary neurotransmitter or modulate norepinephrine release in dogs, mice, and guinea pigs (Li et al. 2006, Hu et al. 2007, He et al. 2008). Studies performed in mice and guinea pigs indicate that H₁ receptors play a major role in the regulation of sympathetic nerve activity and that histamine potentiates synaptic transmission in sympathetic ganglia through H₁ activation (Christian et al. 1992, Murakami et al. 2015). Baroreflex heart rate response is attenuated in H₁R-null mice and in mice treated with H₁ antihistamines indicating that sympathetic nerve activity is reduced when H₁ receptor is blocked or not present (Murakami et al. 2015). It is possible that dogs that received DPH in our study had less sympathetic responses to surgical stimulation compared to control group due to inverse agonism of the H₁ receptor and the consequent decrease in sympathetic transmission.

The hypotension that occurred during anesthesia in some of the dogs in this study may also be the result of dose-dependent vasodilatory effects of isoflurane (Mutoh et al. 1997). Isoflurane end-tidal concentrations of 1.5% exceed the 1.0 MAC value for isoflurane (1.28 %) (Steffey & Howland 1977), and in combination with the premedication and induction anesthetic drugs, may exacerbate cardiovascular depression from synergistic effects and MAC sparing effects. Hydromorphone (0.1 mg/kg, IV) reduces the MAC of isoflurane in dogs by 50% at 1.5 h and by 33% at 4.5 h after administration (Machado et al. 2006), it causes anti-nociception for 120 min and minimum histamine release in conscious healthy dogs (Guedes et al. 2007, Guedes et al. 2008), and is associated with minimal cardiovascular effects, including no change in blood pressure (Guedes et al.
2007). Similarly, 0.22 mg/kg, IM, of hydromorphone causes no histamine release in healthy dogs (Smith et al. 2001). Propofol used in high doses of 15 mg/kg, IV, for induction does not cause histamine release (Mitsuhata & Shimizu 1993).

Mast cells of different species express H1 receptors in their cellular membrane. In vitro studies have shown that various H1-antihistamines have the capacity to inhibit human and canine mast cell activation and histamine release by interacting with these H1-membrane receptors (Church & Gradidge 1980, Garcia et al. 1997, Weller & Maurer 2009). Histamine levels were similar in DPH treated dogs and S treated dogs in this study, suggesting that DPH administered to anesthetized dogs with MCT does not prevent mast cell degranulation. These results are in agreement with another in vivo study performed in humans were neither cetirizine nor loratadine administered at clinical doses inhibited codeine-induced histamine release (Perzanowska et al. 1996).

Histamine’s vascular actions are mediated by H1 and H2 receptors, which are both present in pulmonary, cerebral, and systemic vascular beds (Black et al. 1975, Tucker et al. 1975, Monge et al. 1997). For this reason, simultaneous administration of both and H1 and H2-receptor antihistamines has been recommended to treat vascular depressor responses to histamine (Black et al. 1975, Owen et al. 1982, Lorenz & Doenicke 1985). Receptor H3 activation also appears to contribute to cardiovascular dysfunction in anaphylaxis (Schellenberg et al. 1991, Chrusch et al. 1999). Prostaglandin D2 (PGD2) plays a role in mediating hypotension in humans with mast cell diseases (Roberts et al. 1980), and this mediator is also released by canine mast cells and its actions are not prevented by antihistamine therapy (London & Thamm 2013). Because of DPH specific actions on H1 receptors, its protection against histamine effects is limited in dogs with
Histamine plasma concentrations have prognostic survival value. Histamine concentrations were followed during progression of the disease for 600 days in dogs with MCT and a correlation was established between increased levels of histamine and progression of the disease since all dogs that died of MCT (n=7) developed hyperhistaminemia (mean of 14 ng/mL; range of 5.11-30.1 ng/mL) (Ishiguro et al. 2003). However, single histamine plasma concentration determinations are not correlated with clinical stage of the disease, histological grade of the tumour, or tumour size (Fox et al. 1990). In our study histamine levels were only measured throughout the anesthetic period and manipulation of the tumour. The only dog that was diagnosed with clinical stage IV MCT and had confirmation of systemic spread of the disease developed the most severe hyperhistaminemia during manipulation of the tumour (31.68 ng/mL) and had the lowest MAP values that did not improve with surgical stimulation and use of dopamine.

Changes observed for the decrease in hemoglobin concentration during anesthesia were expected and described for dogs receiving fluid replacement rate (Valverde et al. 2008). No significant bleeding occurred during surgery in any of the dogs, so this was not a contributing factor to the drop in hemoglobin concentration. The increase in arterial and end tidal CO₂ observed in both groups during anesthesia, although statistically significant, was not deemed clinically relevant. In this study CO was determined using NICO, a non-invasive technique that uses partial re-breathing of CO₂ through a re-breathing loop and temporarily adds a portion of the expired CO₂ to the breathing circuit, resulting in small increases in CO₂. Other clinical studies using this method have also detected increases in PaCO₂ over time (Rioja et al. 2013).
The sample size (n = 8 dogs/group) used in the clinical study could contribute to a low power that prevented significant differences in other of the measured cardiovascular parameters. However, the estimated that the sample size required to detect a difference of 15 mmHg in mean arterial blood pressure with a standard deviation of 10-15 mmHg between groups with at least 95% confidence for a Type I error (α value) at 0.05 and 80% power was 8-16 animals in each group based. Therefore, we still consider that our results are valid. In any instance, the results were somehow unexpected since higher mean and diastolic arterial pressures were determined in the S group. Increasing the sample size could result in no difference between the groups or a corroboration of the already obtained results, but unlikely that it will shift our findings to higher pressures in the DPH group.

In conclusion, this study showed that dogs that received DPH pre-emptively had similar cardio-respiratory stability to dogs receiving S and for some parameters (MAP and DAP) cardiovascular function was better in the S group during maximal surgical excision, despite a lower CI. Despite these differences, cardio-respiratory parameters in both groups were considered within acceptable limits for anesthetized dogs and no clear benefit of DPH administration could be demonstrated in dogs with MCT since histamine plasma concentrations and possible related adverse effects were similar in both DPH and S groups during MCT manipulation and excision in dogs anesthetized with isoflurane.
3.6. REFERENCES


Table 3.1. Pre-operative information of dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group) or the same volume of saline, IV (S group).

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<th>S (n=8)</th>
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<td></td>
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<tr>
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Systemic signs of histamine release

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Table 3.2. Respiratory parameters and temperature in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) after induction.

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<th>T4</th>
<th>T5</th>
<th>T6</th>
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<td>37.0 (0.9)</td>
<td>36.9 (0.9)$^a$</td>
<td>36.7 (0.9)$^a$</td>
<td>36.2 (0.9)$^a$</td>
<td>35.8 (0.9)$^a$</td>
<td>35.9 (0.9)$^a$</td>
<td>36.0 (0.9)$^a$</td>
</tr>
<tr>
<td></td>
<td>38.2 (0.8)</td>
<td>38.2 (0.8)</td>
<td>38.0 (0.8)</td>
<td>37.4 (0.8)</td>
<td>37.0 (0.8)$^a$</td>
<td>36.9 (0.8)$^a$</td>
<td>36.7 (0.8)$^a$</td>
<td>36.3 (0.8)$^a$</td>
<td>36.2 (0.8)$^a$</td>
<td>36.0 (0.8)$^a$</td>
</tr>
</tbody>
</table>

Expressed as mean (SD). $^a$ Indicates value significantly different from T0. Times; T0 baseline, after induction and immediately before treatment administration, T1 at 5 min after treatment administration; T2 at 10 min after treatment administration, T3 and T4 during surgical preparation of the tumour, T5 during surgical draping, T6 half-way through maximal tumour excision, T7 immediately before complete tumour excision, and T8 half-way through closure of the excision site.
Table 3.3. Cardiovascular parameters in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) after induction.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>PRE</th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T4</th>
<th>T5</th>
<th>T6</th>
<th>T7</th>
<th>T8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CI (mL/min/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(DPH)</td>
<td>74 (39)</td>
<td>11 (7)</td>
<td>5 (0)</td>
<td>10 (0)</td>
<td>24 (5)</td>
<td>35 (8)</td>
<td>51 (12)</td>
<td>73 (19)</td>
<td>85 (19)</td>
<td>97 (21)</td>
</tr>
<tr>
<td>(S)</td>
<td>85 (21)</td>
<td>84 (19)</td>
<td>80 (17)</td>
<td>74 (16) (a)</td>
<td>72 (17)</td>
<td>77 (17)</td>
<td>86 (18)</td>
<td>82 (16)</td>
<td>85 (18)</td>
<td></td>
</tr>
</tbody>
</table>

| SVI (mL/beat/kg) | | | | | | | | | | |
| \(DPH\) | 1.5 (0.6) | 1.4 (0.6) | 1.4 (0.6) | 1.4 (0.6) | 1.4 (0.6) | 1.3 (0.6) | 1.7 (0.6) | 1.7 (0.6) | 1.7 (0.6) | |
| \(S\) | 1.2 (0.4) | 1.4 (0.4) | 1.4 (0.4) | 1.2 (0.4) | 1.1 (0.4) | 1.2 (0.4) | 1.2 (0.4) | 1.2 (0.4) | 1.2 (0.4) | |

<p>| HR (bpm) | | | | | | | | | | |
| (DPH) | 111 (42) (a) | 80 (25) | 82 (20) | 83 (22) | 80 (23) | 74 (25) | 77 (18) | 76 (20) | 77 (16) | 79 (18) |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S</strong></td>
<td>88 (41)</td>
<td>72 (25)</td>
<td>63 (19) (^a)</td>
<td>63 (22)</td>
<td>65 (23)</td>
<td>73 (24)</td>
<td>68 (18)</td>
<td>78 (19)</td>
</tr>
<tr>
<td>SAP (mmHg)</td>
<td>DPH</td>
<td>-</td>
<td>122 (18)</td>
<td>122 (18)</td>
<td>111 (18)</td>
<td>107 (18) (^a)</td>
<td>104 (18) (^a)</td>
<td>109 (18)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>-</td>
<td>109 (18)</td>
<td>102 (16)</td>
<td>94 (16) (^a)</td>
<td>94 (16) (^a)</td>
<td>92 (16) (^a)</td>
<td>90 (16) (^a)</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>DPH</td>
<td>-</td>
<td>72 (12)</td>
<td>73 (12)</td>
<td>68 (12)</td>
<td>65 (12)</td>
<td>63 (12) (^a)</td>
<td>67 (12)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>-</td>
<td>67 (11)</td>
<td>64 (11)</td>
<td>63 (11)</td>
<td>64 (11)</td>
<td>65 (11)</td>
<td>65 (11)</td>
</tr>
<tr>
<td>DAP (mg/dL)</td>
<td>DPH</td>
<td>-</td>
<td>57 (10)</td>
<td>57 (10)</td>
<td>53 (10)</td>
<td>53 (10)</td>
<td>50 (10) (^a)</td>
<td>52 (10)</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>-</td>
<td>52 (9)</td>
<td>54 (9)</td>
<td>51 (9)</td>
<td>53 (9)</td>
<td>52 (9)</td>
<td>53 (9)</td>
</tr>
</tbody>
</table>

Expressed as mean (SD).  \(^a\) Indicates value significantly different from T0.  \(^§\) Significant difference between groups. Times; T0 baseline, after induction and immediately before treatment administration, T1 at 5 min after treatment administration; T2 at 10 min after treatment administration, T3 and T4 during surgical preparation of the tumour, T5 during surgical draping, T6 half-way through maximal tumour excision, T7 immediately before complete tumour excision, and T8 half-way through closure of the excision site.
Table 3.4. Times of collection of blood samples for DPH determinations (min after DPH administration, mean [SD]; and plasma DPH concentrations [ng/mL], mean [SD]) in dogs receiving 1 mg/kg of DPH, IV (n=8).

<table>
<thead>
<tr>
<th>DPH sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of collection (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>-89 (50)</td>
<td>5 (0)</td>
<td>30 (0)</td>
<td>76 (18)</td>
<td>83 (15)</td>
<td>263 (29)</td>
</tr>
<tr>
<td>Plasma DPH concentration (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dog 3</td>
<td>14</td>
<td>292</td>
<td>124</td>
<td>101</td>
<td>84</td>
<td>ND</td>
</tr>
<tr>
<td>Dog 4</td>
<td>ND</td>
<td>277</td>
<td>79</td>
<td>48</td>
<td>25</td>
<td>13</td>
</tr>
<tr>
<td>Dog 6</td>
<td>ND</td>
<td>698</td>
<td>257</td>
<td>132</td>
<td>124</td>
<td>72</td>
</tr>
<tr>
<td>Dog 8</td>
<td>ND</td>
<td>233</td>
<td>126</td>
<td>102</td>
<td>63</td>
<td>12</td>
</tr>
<tr>
<td>Dog 10</td>
<td>ND</td>
<td>382</td>
<td>102</td>
<td>82</td>
<td>74</td>
<td>25</td>
</tr>
<tr>
<td>Dog 11</td>
<td>ND</td>
<td>350</td>
<td>174</td>
<td>61</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>Dog 13</td>
<td>ND</td>
<td>270</td>
<td>93</td>
<td>34</td>
<td>16</td>
<td>ND</td>
</tr>
<tr>
<td>Dog 15</td>
<td>ND</td>
<td>244</td>
<td>69</td>
<td>30</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>Mean</td>
<td>14</td>
<td>343</td>
<td>128</td>
<td>74</td>
<td>53</td>
<td>27</td>
</tr>
<tr>
<td>(SD)</td>
<td>(0)</td>
<td>(152)</td>
<td>(62)</td>
<td>(37)</td>
<td>(40)</td>
<td>(26)</td>
</tr>
</tbody>
</table>
ND, not detected or value is lower than limit of quantification of the assay (10 ng/mL). D1 was collected before pre-medication, D2 5 min after treatment administration, D3 30 min after treatment administration, D4 at maximal tumour excision, D5 just before complete tumour excision, and D6 at 2 h post-extubation.
Table 3.5. Times of collection of blood samples for histamine determinations (mean [SD]) and plasma histamine concentrations (ng/mL; mean [SD]) in dogs receiving 1 mg/kg of DPH, IV (n=8) or saline, IV (n=8).

<table>
<thead>
<tr>
<th>Histamine (H)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of collection (min) in DPH group</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>-89 (50)</td>
<td>-12 (8)</td>
<td>24 (10)</td>
<td>80 (16)</td>
<td>263 (29)</td>
</tr>
<tr>
<td>Confidence Intervals</td>
<td>-130 – -47</td>
<td>-17 – -5</td>
<td>16 – 33</td>
<td>76 – 93</td>
<td>238 – 287</td>
</tr>
</tbody>
</table>

Plasma Histamine concentration (ng/mL) in DPH group

<table>
<thead>
<tr>
<th>Dog</th>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
<th>Sample 4</th>
<th>Sample 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog 3</td>
<td>1.04</td>
<td>1.46</td>
<td>1.07</td>
<td>3.64</td>
<td>1.21</td>
</tr>
<tr>
<td>Dog 4</td>
<td>0.35</td>
<td>0.27</td>
<td>0.62</td>
<td>0.17</td>
<td>ND</td>
</tr>
<tr>
<td>Dog 6</td>
<td>0.43</td>
<td>0.07</td>
<td>0.75</td>
<td>0.44</td>
<td>0.25</td>
</tr>
<tr>
<td>Dog 8</td>
<td>ND</td>
<td>0.14</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Dog 10</td>
<td>4.59</td>
<td>2.26</td>
<td>31.68</td>
<td>20.30</td>
<td>0.89</td>
</tr>
<tr>
<td>Dog 11</td>
<td>2.31</td>
<td>0.76</td>
<td>9.65</td>
<td>1.20</td>
<td>0.15</td>
</tr>
<tr>
<td>Dog 13</td>
<td>0.20</td>
<td>0.73</td>
<td>1.14</td>
<td>1.60</td>
<td>0.11</td>
</tr>
<tr>
<td>Dog</td>
<td>Plasma Histamine concentration (ng/mL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>---------------------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dog 1</td>
<td>0.66</td>
<td>0.58</td>
<td>0.77</td>
<td>3.43</td>
</tr>
<tr>
<td></td>
<td>Dog 2</td>
<td>0.80</td>
<td>0.28</td>
<td>ND</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Dog 5</td>
<td>2.07</td>
<td>0.28</td>
<td>0.44</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Dog 7</td>
<td>0.31</td>
<td>0.22</td>
<td>0.06</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Dog 9</td>
<td>0.06</td>
<td>0.14</td>
<td>ND</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>Dog 12</td>
<td>0.33</td>
<td>0.21</td>
<td>0.15</td>
<td>11.92</td>
</tr>
<tr>
<td></td>
<td>Dog 14</td>
<td>ND</td>
<td>0.93</td>
<td>1.29</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Dog 16</td>
<td>2.11</td>
<td>0.49</td>
<td>2.53</td>
<td>2.59</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dog 15</th>
<th>ND</th>
<th>0.12</th>
<th>1.13</th>
<th>ND</th>
<th>0.08</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD)</td>
<td>1.49 (1.71)</td>
<td>0.73 (0.78)</td>
<td>6.60 (11.54)</td>
<td>4.56 (7.81)</td>
<td>0.45 (0.48)</td>
</tr>
<tr>
<td>Median</td>
<td>0.74</td>
<td>0.5</td>
<td>1.13</td>
<td>1.40</td>
<td>0.20</td>
</tr>
<tr>
<td>Confidence</td>
<td>0.23 – 4.15</td>
<td>0.11 – 1.61</td>
<td>0.68 – 20.77</td>
<td>0.22 – 17.07</td>
<td>0.09 – 1.15</td>
</tr>
<tr>
<td>Intervals</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of collection (min) in S group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean (SD) (min)</td>
</tr>
<tr>
<td>Confidence</td>
</tr>
<tr>
<td>Intervals</td>
</tr>
<tr>
<td>Mean (SD)</td>
</tr>
<tr>
<td>-------------</td>
</tr>
<tr>
<td>Median</td>
</tr>
<tr>
<td>Confidence</td>
</tr>
</tbody>
</table>

ND, not detected or value is lower than limit of quantification of the assay (0.055 ng/mL).

H1 was collected before pre-medication, H2 was collected after induction at T0, H3 at maximal manipulation of the tumour during surgical preparation, H4 just before complete tumour excision, and H5 at 2 h post-extubation.
**Figure 3.1.** Timeline representation of cardiovascular data recording time points (T), and diphenhydramine (D) and histamine (H) blood samples collecting times (min) until end of surgical procedure. Induction of anesthesia and beginning of surgery are indicated with arrows.

Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through excision of the tumour, T7 at 85 (19) min immediately before complete excision of the tumour, and T8 at 97 (21) half-way through closure of the excision site.
Figure 3.2. Systolic arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement.

*a Indicates value significantly different from T0 within the group. Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
**Figure 3.3.** Mean arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement.

\[ a \] Indicates value significantly different from T0 within the group. \[ \delta \] Significant difference between groups. Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
**Figure 3.4.** Diastolic arterial pressure (mmHg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement. 

*a* Indicates value significantly different from T0 within the group. *δ* Significant difference between groups. Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
Figure 3.5. Cardiac index (mL/min/kg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement.

\[ a \text{ Indicates value significantly different from T0 within the group.} \]
\[ \delta \text{ Significant difference between groups.} \]

Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
Figure 3.6. Stroke volume index (mL/beat/kg) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement.

Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
**Figure 3.7.** Heart rate (beats/min) in dogs anesthetized with isoflurane undergoing surgical excision of MCT that received 1 mg/kg of DPH, IV (DPH group, n=8) or the same volume of saline, IV (S group, n=8) immediately after T0 measurement.

\[ \text{HR} \]

\( a \) Indicates value significantly different from T0 within the group. Times as mean (SD); T0 (baseline, after induction and immediately before treatment administration), T1 at 5 (0) min after treatment administration; T2 at 10 (0) min, T3 at 24 (5) min and T4 at 34 (8) min during surgical preparation of the tumour, T5 at 51 (12) min during surgical draping, T6 at 73 (19) min half-way through maximal tumour excision, T7 at 85 (19) min immediately before complete tumour excision, and T8 at 97 (21) half-way through closure of the excision site.
CHAPTER IV
GENERAL DISCUSSION AND CONCLUSIONS

Significant advances in diagnostic tools and development of new treatment options in oncology has led to more specialized veterinary care in recent years. This care often involves advanced surgical procedures carried out under general anesthesia. Mast cell tumours are the most commonly diagnosed skin tumours in dogs. Mast cell degranulation may be triggered by mechanical stimulation such as surgery or radiation therapy (Dobson & Scase 2007), resulting in local and systemic release of histamine and other bioactive substances from mast cell granules (Welle et al. 2008). Systemic histamine release in dogs may cause systemic vasodilation, increment in vascular permeability, and a decreased CO (Shmuel & Cortes 2013).

Dogs with MCT undergoing general anesthesia for excision of the tumour are potentially at higher risk of hypotension because of the combined adverse cardiovascular effects of anesthetic drugs and possible histamine release from mast cells during tumour handling. Preemptive administration of antihistamines such as DPH to patients with MCT undergoing surgical excision of the MCT has been recommended to minimize the risk of adverse cardiovascular effects during anesthesia. We investigated if DPH administration under this premise is of any benefit in dogs with MCT undergoing surgical excision under isoflurane anesthesia. In this study, we first determined the pharmacokinetics of DPH in conscious dogs administered 1 mg/kg, IV, and 2 mg/kg, IM in a crossover fashion. This study is the first one to determine the complete pharmacokinetics and safety of clinical doses of DPH used in dogs. Both doses investigated in this study did not cause any
adverse cardio-respiratory effects or behavioural changes and were effective in providing DPH plasma concentrations that are considered therapeutic, based on studies of DPH reducing the skin wheal diameter to subcutaneously injected histamine in humans (Carruthers et al. 1978).

In the second phase of this study, the 1 mg/kg, IV, dose of DPH was selected as preemptive treatment for clinical cases with MCT that underwent surgical excision of the MCT. The purpose of this study was to test the validity of using DPH to prevent or improve cardiovascular function in dogs that have MCT and may be at higher risk of adverse cardiovascular effects from histamine release during anesthesia. The IV dose was selected due to its effectiveness in providing rapid and high DPH plasma concentrations for a duration that could exceed the surgery time for these cases and therefore counteract histamine’s actions if released from mast cells during tumour handling. Dogs were randomized in a blinded study to receive either DPH or saline after induction of anesthesia and were monitored for cardiovascular effects, as well as measurement of DPH and histamine plasma concentrations throughout the perioperative period, while maintained at the same anesthetic depth and surgical conditions. Results from this study demonstrated that cardiovascular parameters were similar between groups in dogs receiving DPH and in dogs receiving saline, and contrary to expectations, mean and diastolic blood pressures were lower in the DPH group despite similar histamine plasma concentrations in both groups and presence of DPH plasma concentrations in the DPH group.

Therapeutic DPH plasma concentrations to treat or prevent cardiovascular effects of histamine have not been determined in dogs; however, evidence suggest that high doses of DPH are needed to achieve plasma concentrations that are effective against histamine’s
cardiovascular adverse effects. Plasma concentrations of DPH that are considered therapeutic against histamine’s cutaneous effects may not be effective against the higher histamine concentrations that have been associated with adverse systemic cardiovascular effects such as hypotension (Folkow et al. 1948, Thompson & Walton 1964, Thermann et al. 1975, Lorenz et al. 1982). Diphenhydramine may not be effective against histamine’s cardiovascular effects because these are mediated through H₁, H₂ and H₃ receptors (Black et al. 1975, Tucker et al. 1975, Schellenberg et al. 1991, Monge et al. 1997, Chrusch et al. 1999). Histamine concentrations measured in dogs in this study tended to be lower than those associated with cardiovascular impairment (Lorenz et al. 1982) and although these concentrations have been associated with cutaneous signs (Guedes et al. 2006), the latter signs were not observed in this study, probably due to a protective action of isoflurane on inhibiting nitric oxide synthase (NOS) and nitric oxide production, which is the mechanism by which histamine mediates vasodilation (Simoneau et al. 1996, Tas et al. 2003, Tas et al. 2008).

The sample size (n = 8 dogs/group) used in the clinical study could contribute to a low power that prevented significant differences in other of the measured cardiovascular parameters. However, the estimated sample size required to detect a difference of 15 mmHg in mean arterial blood pressure with a standard deviation of 10-15 mmHg between groups with at least 95% confidence for a Type I error (α value) at 0.05 and 80% power was 8-16 animals in each group. Therefore, we still consider that our results are likely to be valid. In any instance, the results were somehow unexpected when higher mean and diastolic arterial pressures were determined in the saline group at the time of maximal surgical stimulation than in the DPH group. Increasing the sample size could result in no
difference between the groups or a corroboration of the already obtained results, but unlikely that it will shift our findings to higher arterial pressure in the DPH group. There are also plausible explanations for higher blood pressures in the saline group. Histamine released from pre- and post-synaptic peripheral sympathetic terminals potentiates sympathetic actions through H₁ receptor activation (Christian et al. 1992, Li et al. 2006, Hu et al. 2007, He et al. 2008, Murakami et al. 2015) and blockade of this receptor by DPH can potentially result in exacerbation of the hypotensive effects of general anesthesia.

In conclusion, this investigation demonstrated the following results:

1. Plasma pharmacokinetics values were determined for DPH at doses used clinically in dogs via IV and IM administration.

2. The doses used in this research resulted in DPH concentrations that are therapeutic against cutaneous signs of histamine’s actions in other species.

3. These DPH plasma concentrations did not cause any adverse cardio-respiratory or behavioural effects in conscious dogs.

4. Administration of DPH preemptively to dogs undergoing surgical excision of MCT under isoflurane anesthesia did not cause any clinical benefits to cardio-respiratory function despite effective plasma concentrations of DPH, compared to dogs receiving saline.

5. Histamine plasma concentrations were similar in dogs receiving DPH and dogs receiving saline during anesthesia and surgical excision of the MCT.
FUTURE AREA OF RESEARCH

Our study demonstrated that histamine’s presumed adverse cardiovascular effects were limited in dogs undergoing excision of MCT under isoflurane anesthesia. For this reason, a direct correlation between histamine plasma concentrations that affect negatively the cardiovascular system needs to be established in dogs with MCT and other conditions that enhance the release of endogenous histamine.

Since histamine’s adverse cardiovascular effects are the result of agonistic actions on H₁, H₂ and H₃ receptors, it would be important to establish if an antihistamine drug with actions on more than one histamine receptor is better than a drug like DPH, which is only a specific antagonist to the H₁ receptor, in protecting against histamine’s potential adverse cardiovascular effects.
References


APPENDIX A

Figure A.1. Data for trapezoid calculations of AUC (ng/h/mL) and AUMC (ng-h*h/mL) derived from 6 dogs after 2 mg/kg of diphenhydramine administered IM.

<table>
<thead>
<tr>
<th>Description:</th>
<th>Diphenhydramine IM</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Trapezoid Calculation of AUC (ng-h/mL)</th>
<th>Trapezoid Calculation of AUMC (ng-h*h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (hr)</td>
<td>Conc (ng/mL)</td>
</tr>
<tr>
<td>0.0</td>
<td>285.4</td>
</tr>
<tr>
<td>0.1</td>
<td>43.0</td>
</tr>
<tr>
<td>0.1</td>
<td>51.0</td>
</tr>
<tr>
<td>0.2</td>
<td>83.0</td>
</tr>
<tr>
<td>0.3</td>
<td>121.0</td>
</tr>
<tr>
<td>0.5</td>
<td>142.0</td>
</tr>
<tr>
<td>0.8</td>
<td>158.0</td>
</tr>
<tr>
<td>1.0</td>
<td>165.0</td>
</tr>
<tr>
<td>1.5</td>
<td>161.0</td>
</tr>
<tr>
<td>2.0</td>
<td>133.0</td>
</tr>
<tr>
<td>4.0</td>
<td>92.0</td>
</tr>
<tr>
<td>6.0</td>
<td>75.0</td>
</tr>
<tr>
<td>8.0</td>
<td>52.0</td>
</tr>
<tr>
<td>12.0</td>
<td>29.0</td>
</tr>
<tr>
<td>24.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

**AUC Summary**

<table>
<thead>
<tr>
<th>Last Conc Value</th>
<th>16.0 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative Observed AUC(0→t)</td>
<td>1755.0 ng-h/mL</td>
</tr>
<tr>
<td>Remaining AUC(0→t)</td>
<td>158 ng-h/mL</td>
</tr>
<tr>
<td>AUMC(0→t)</td>
<td>1413 ng-h*h/mL</td>
</tr>
</tbody>
</table>

**AUMC Summary**

<table>
<thead>
<tr>
<th>Last Conc Time Value</th>
<th>584.0 ng-h/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cumulative Observed AUMC(0→t)</td>
<td>5554 ng-h*h/mL</td>
</tr>
<tr>
<td>Remaining AUMC(0→t)</td>
<td>5352 ng-h*h/mL</td>
</tr>
<tr>
<td>AUMC(0→t)</td>
<td>13938 ng-h<em>h</em>h/mL</td>
</tr>
</tbody>
</table>

**Cumulative AUC**

**Cumulative AUMC**

**Curve Parameters**:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax</td>
<td>285.4 ng/mL</td>
</tr>
<tr>
<td>tmax</td>
<td>0.0 h</td>
</tr>
</tbody>
</table>
Figure A.2. Data for trapezoid calculations of AUC (ng/h/mL) and AUMC (ng·h·h/mL) derived from 6 dogs after 1 mg/kg of diphenhydramine administered IV.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Conc (ng/mL)</th>
<th>Partial</th>
<th>Cumulative</th>
<th>Time (hr)</th>
<th>Time · Conc</th>
<th>Partial</th>
<th>Cumulative</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>277.1</td>
<td>0.00</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>0.1</td>
<td>283.0</td>
<td>14.05</td>
<td>14.05</td>
<td>0.1</td>
<td>14.3</td>
<td>0.36</td>
<td>0.36</td>
</tr>
<tr>
<td>0.2</td>
<td>252.0</td>
<td>9.65</td>
<td>23.90</td>
<td>0.2</td>
<td>25.5</td>
<td>0.66</td>
<td>1.02</td>
</tr>
<tr>
<td>0.3</td>
<td>225.0</td>
<td>23.25</td>
<td>47.15</td>
<td>0.3</td>
<td>42.0</td>
<td>2.81</td>
<td>3.83</td>
</tr>
<tr>
<td>0.5</td>
<td>208.0</td>
<td>35.42</td>
<td>122.32</td>
<td>0.5</td>
<td>100.0</td>
<td>14.58</td>
<td>28.16</td>
</tr>
<tr>
<td>0.6</td>
<td>178.0</td>
<td>47.25</td>
<td>169.57</td>
<td>0.6</td>
<td>133.5</td>
<td>29.19</td>
<td>57.35</td>
</tr>
<tr>
<td>1.0</td>
<td>164.0</td>
<td>42.75</td>
<td>212.32</td>
<td>1.0</td>
<td>164.0</td>
<td>37.19</td>
<td>94.54</td>
</tr>
<tr>
<td>1.5</td>
<td>154.0</td>
<td>79.50</td>
<td>291.82</td>
<td>1.5</td>
<td>231.0</td>
<td>98.75</td>
<td>193.29</td>
</tr>
<tr>
<td>2.0</td>
<td>117.0</td>
<td>67.75</td>
<td>359.57</td>
<td>2.0</td>
<td>234.0</td>
<td>116.25</td>
<td>399.54</td>
</tr>
<tr>
<td>4.0</td>
<td>50.0</td>
<td>107.00</td>
<td>526.57</td>
<td>4.0</td>
<td>200.0</td>
<td>434.00</td>
<td>745.54</td>
</tr>
<tr>
<td>6.0</td>
<td>30.0</td>
<td>60.00</td>
<td>606.57</td>
<td>6.0</td>
<td>180.0</td>
<td>380.00</td>
<td>1123.29</td>
</tr>
<tr>
<td>8.0</td>
<td>21.0</td>
<td>51.00</td>
<td>657.57</td>
<td>8.0</td>
<td>168.0</td>
<td>348.00</td>
<td>1471.54</td>
</tr>
<tr>
<td>12.0</td>
<td>13.0</td>
<td>68.00</td>
<td>725.57</td>
<td>12.0</td>
<td>156.0</td>
<td>648.00</td>
<td>2119.54</td>
</tr>
<tr>
<td>24.0</td>
<td>7.00</td>
<td>78.00</td>
<td>803.57</td>
<td>24.0</td>
<td>0.0</td>
<td>936.00</td>
<td>3055.54</td>
</tr>
</tbody>
</table>

**Curve Areas**

<table>
<thead>
<tr>
<th>Description</th>
<th>Diphenhydramine IV</th>
</tr>
</thead>
</table>

**AUC Summary**

<table>
<thead>
<tr>
<th>Last conc. value</th>
<th>AUC(0-1) (ng·h/mL)</th>
<th>Cumulative observed AUC(0-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>804 ng·h/mL</td>
<td>3056 ng·h·h/mL</td>
</tr>
</tbody>
</table>

**AUMC Summary**

<table>
<thead>
<tr>
<th>Last conc. time value</th>
<th>Remaining AUMC(0-1) (ng·h·h/mL)</th>
<th>AUMC(0-1) (ng·h·h/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>6 ng·h·h/mL</td>
<td>3056 ng·h·h/mL</td>
</tr>
</tbody>
</table>

**Cumulative AUC**

<table>
<thead>
<tr>
<th>Curve Parameters</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax</td>
<td>804 ng/mL</td>
</tr>
<tr>
<td>Tmax</td>
<td>0.1 hr</td>
</tr>
</tbody>
</table>
Figure A.3. Canine blank plasma spiked with diphenhydramine (500 ng/mL) and orphenadrine (100 ng/mL) to determine the retention times observed for diphenhydramine (1.8 min) and for orphenadrine (3.2 min) during the HPLC pharmacokinetic analysis of diphenhydramine performed in Chapter II of this thesis.
**Figure A.4.** Canine blank plasma tested for specificity during the pharmacokinetic determinations of diphenhydramine. No interfering peaks were observed in blank plasma samples at retention time corresponding to the drug and internal standard indicating that this clean background of the assay procedure is specific to diphenhydramine.
## Evaluation of the Antihistaminic Effects of Diphenhydramine in Dogs Undergoing Excision of Mast Cell Tumors

### Pre-operatively questionnaire:

<table>
<thead>
<tr>
<th>VTH #:</th>
<th>Name:</th>
<th>Weight (kg):</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age:</td>
<td>Breed:</td>
<td>Date:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Date of diagnosis</th>
<th>Diagnosis confirmation?</th>
<th>Histological grade?</th>
<th>Is the tumor confined to the dermis?</th>
<th>Single or multiple tumors?</th>
<th>Site of the tumor/s</th>
<th>Size of the tumor/s</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NO Cytology Histopathology</td>
<td>I II III</td>
<td>YES NO If no, where?</td>
<td>#</td>
<td>Head Neck Flank Extremities</td>
<td>_____ cm x _____cm x _____ cm</td>
</tr>
<tr>
<td>History of previous MCTs?</td>
<td>YES</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td>-----</td>
<td>----</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clinical staging?</td>
<td>Thoracic Rx</td>
<td>U/S</td>
<td>CT of the mass</td>
<td>NO</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type of excision?</td>
<td>Marginal</td>
<td>Wide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local signs?</td>
<td>Erythema</td>
<td>Wheals</td>
<td>Itchiness</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hyperemia</td>
<td>Mass changing size</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Systemic signs? (Vomiting, diarrhea, melena, fever, peripheral edema, collapse...)</td>
<td>Vomiting</td>
<td>Diarrhea</td>
<td>Melena</td>
<td>Fever</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peripheral edema</td>
<td>Collapse</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment with antihistamines? (Drug, dose, rate) Last dose?</td>
<td>Drug:</td>
<td>Dose:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Route:</td>
<td>Frequency:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Last dose:</td>
<td>NO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**APPENDIX C**

**Table 3.1.** World Health Organization clinical staging system for MCT

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>One tumour incompletely excised from the dermis, identified histologically, without regional lymph node involvement</td>
</tr>
<tr>
<td></td>
<td>1. Without systemic signs</td>
</tr>
<tr>
<td></td>
<td>2. With systemic signs</td>
</tr>
<tr>
<td>I</td>
<td>One tumour confined to the dermis, without regional lymph node involvement</td>
</tr>
<tr>
<td></td>
<td>1. Without systemic signs</td>
</tr>
<tr>
<td></td>
<td>2. With systemic signs</td>
</tr>
<tr>
<td>II</td>
<td>One tumour confined to the dermis, with regional lymph node involvement</td>
</tr>
<tr>
<td></td>
<td>1. Without systemic signs</td>
</tr>
<tr>
<td></td>
<td>2. With systemic signs</td>
</tr>
<tr>
<td>III</td>
<td>Multiple dermal tumours; large, infiltrating tumours with or without regional lymph node involvement</td>
</tr>
<tr>
<td></td>
<td>1. Without systemic signs</td>
</tr>
<tr>
<td></td>
<td>2. With systemic signs</td>
</tr>
<tr>
<td>IV</td>
<td>Any tumour with distant metastasis, including blood or bone marrow involvement</td>
</tr>
</tbody>
</table>
APPENDIX D

2-Tier Grading Criteria for MCT

High grade MCT are characterized any of the following criteria:

- At least 7 mitotic figures every 10 HPF (evaluated in regions with higher mitotic activity).
- At least 3 multinucleated cells every 10 HPF (where more than 3 nuclei constitutes a multinucleated cell).
- At least 3 bizarre nuclei every 10 HPF (Highly atypical with marked indentations, segmentation, and irregular shape).
- Kariomegaly (where at least 10% of neoplastic cells vary by 2 fold).

Tumours not following these criteria would be considered as low grade MCT

(Adapted from Kiupel et al. 2011)
APPENDIX E

Figure E.1. Histamine curve generated from the commercial enzyme immunoassay kit (Enzyme immunoassay Histamine; IM2015; Immunotech, Marseille, France) and then analyzed with online software (MyAssays Ltd., http://www.myassays.com) using a four-parameter logistic curve. This analysis showed a high correlation factor ($R^2$) between kit standard concentrations and measured concentrations. Concentration expressed as nM.