The Pennsylvania State University

The Graduate School

Department of Comparative Medicine

MIDAZOLAM ENHANCES THE ANALGESIC PROPERTIES OF DEXMEDETOMIDINE IN THE RAT

A Thesis in

Laboratory Animal Medicine

by

Christine A. Boehm

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

May 2009

The thesis of Christine A. Boehm was reviewed and approved* by the following:

Ronald P. Wilson
Professor of Comparative Medicine
Chair, Department of Comparative Medicine
Thesis Advisor

Elizabeth L. Carney
Assistant Professor, Department of Comparative Medicine

Victor Ruiz-Velasco
Assistant Professor of Anesthesiology

*Signatures are on file in the Graduate School

ABSTRACT

Objective Investigate the antinociceptive properties of intraperitoneal-administered dose combinations of midazolam and dexmedetomidine in the rat.

Animals Seventy adult male Sprague Dawley rats (250-300 g) were used in this study.

Methods Dexmedetomidine (D) 0.03, 0.06, 0.09, 0.12, 0.15, 0.18, 0.21 mg kg^{-1} and midazolam (M) 5, 10, 25, 50 mg kg^{-1} were administered intraperitoneally (i.p.), first alone then in combination (D:M) ranging from 0.03 D:5 M to 0.18 D:30 M mg kg^{-1}. Antinociception was evaluated using the tail-flick test at time 0 (before injection), 15, 30, 45, 60 and 75 minutes. To correlate antinociceptive properties with surgical analgesia, a small pilot study was conducted on four rats anesthetized with 0.06 mg kg^{-1} D + 10 mg kg^{-1} M, 0.09 mg kg^{-1} D + 15 mg kg^{-1} M, or 0.12 mg kg^{-1} D + 20 mg kg^{-1} M administered i.p. Time to loss of righting reflex, presence or absence of pedal reflex, and response to surgical incision were measured.

Results Midazolam at all doses administered alone (5 – 50 mg kg^{-1}) did not significantly change tail-flick latencies from baseline values whereas D showed clear dose-dependent increases in tail-flick latency for doses administered in the range of 0.03 – 0.18 mg kg^{-1}. Tail-flick latencies in rats administered combinations of D and M were significantly greater than D alone ($p<0.05$). Further analysis of tail-flick latencies at 60 minutes post-administration (presumed steady state) indicated the drug-drug interaction with respect to tail-flick latency was synergistic.

Conclusions A dose-related antinociceptive effect was demonstrated for D in the rat, which was enhanced by co-administration of M.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

I would first like to thank the members of my committee for their support and encouragement over the course of this project.

I would also like to thank Dr. Wilson and Dr. Tallarida for their assistance with the statistical analysis.

My final thanks go to my husband, Eric, for his tolerance of my stress levels, late nights, loving support, and the cold winters he tolerated in order for me to reach my goal.

Chapter 1

Introduction

Dexmedetomidine is the active isomer of the analgesic medetomidine that binds to α_2–adrenergic receptors (α_2-AR) in an agonist fashion with high specificity. Medetomidine is a racemic mixture of dexmedetomidine and levomedetomidine. The latter is the inactive form (Murrell 2005). Recently, dexmedetomidine became available to the US market as a sedative–analgesic for cats and dogs.

The α_2-AR agonists are known for reliable sedation, analgesia, muscle relaxation, anxiolysis, and a decrease in the anesthetic requirements of injectable and inhalant agents (dose sparing) (Sinclair 2003). Dexmedetomidine is ten times more selective for the α_2-AR than the analgesic and sedative, xylazine (receptor selectivity ratio of 1620 vs. 160 respectively; Scheinin 1988). The onset of action is variable, from 5 minutes when administered intravenously, to 10-15 minutes for intramuscular administration. The most common side effects of dexmedetomidine are bradycardia, decreased respiration, and hypothermia. Dexmedetomidine is easily reversed with atipamezole, an α_2-AR antagonist.

The sedative action of dexmedetomidine is generally ascribed to inhibition of the locus coeruleus in the pons and lower brainstem, which contains pathways involved in the maintenance of vigilance, mediated through presynaptic α_2-AR (Nishimura et al. 1993). In the brain, dexmedetomidine has been reported to bind to α_2-AR (Murrell 2005). Alpha$_2$–agonists bind to the membrane receptors and prevent the release of norepinephrine (Sinclair 2003). The inhibition of norepinephrine release is mediated by a decrease in calcium entry to the cell resulting from the direct inhibition of voltage-gated calcium ion channels (Murrell 2005). Norepinephrine is

necessary for centrally-mediated arousal. When this arousal is decreased, sedation ensues (Sinclair 2003). Muscle relaxation is another effect of dexmedetomidine, and is due to the inhibition of α_2-AR at the interneuronal level of the spinal cord (Sinclair 2003).

Dexmedetomidine produces analgesia by stimulating receptors at various sites in the pain pathways throughout the brain and spinal cord. High expression levels of α_2– AR binding sites are found in the dorsal horn of the spinal cord, where nociceptive fibers synapse, as well as in the brainstem, where nociceptive signals are modulated (Murrell 2005). The binding of α_2-agonists to their receptors results in activation of a membrane-associated G protein that eventually opens potassium channels leading to hyperpolarization of the cell, that renders the neurons less responsive to excitatory input, thus stopping the pain pathway. This method of action is similar to that seen with μ-opioid agonists (Sinclair 2003).

Midazolam is a benzodiazepine that is valued for its sedative-hypnotic, anxiolytic, and muscle relaxant properties. It has a rapid onset of action with a very high affinity for the ionotropic gamma–aminobutyric acid ($GABA_A$) receptor subtypes, up to twice the affinity of diazepam. Once midazolam binds to $GABA_A$ receptors, chloride ions enter the cells via the receptors, which results in hyperpolarization of the neurons, making them more refractory to other stimuli (Nishimura et al. 1993).

Because the affinity of midazolam for its receptor is high, midazolam's potency is nearly three times that of diazepam. The rapid onset of action is attributed to its high lipophilicity at pH values greater than 4.0 (Thurmon 1997). It is water-soluble, highly protein-bound (94-97%), and rapidly crosses the blood-brain barrier. While midazolam is similar to diazepam, it has a shorter duration of action, and a more rapid elimination half-life and total body clearance than diazepam. Midazolam is water soluble in its injectable form, but becomes lipid soluble at body temperature, whereas the carrier for diazepam is 40% propylene glycol, 10% ethanol, 5% sodium benzoate, and 1.5% benzyl alcohol. Midazolam is easily injected intramuscularly or intravascularly, while

diazepam is incompletely absorbed if given intramuscularly, and intravenous injections must be given carefully and slowly to prevent thrombophlebitis or cardiotoxicity (secondary to the propylene glycol carrier) (Plumb 2007a).

Midazolam is metabolized in the liver by the microsomal oxidation system. Elimination half-lives range from 77 minutes in dogs to approximately 120 minutes in humans. The most common side effect is respiratory depression. Midazolam can be antagonized by flumazenil if necessary (Plumb 2007a).

Midazolam has been shown to have a hyperalgesic effect when given with opiates (Pakulska 2001) (Rosland 1990), barbiturates (Tatsuo et al. 1997), dissociative agents (Okulicz-Kozaryn et al. 2000), and when given alone (Tatsuo et al. 1999). Midazolam has not been reported to induce a hyperalgesic state when given with an α_2– AR agonist. This investigation is the first to explore the analgesic state of midazolam combined with dexmedetomidine in commonly used dose combinations.

The combination of medetomidine-midazolam to produce sedation and anesthesia has been explored for canines (Hayashi et al. 1994, Kojima et al. 1999, Itamoto et al. 2000), sheep (Raekallio 1998,), swine (Nishimura et al. 1993, Nishimura et al. 1994), and Japanese macaques (Kimura et al. 2007). Salonen et al (1992b) first demonstrated the synergistic interaction between dexmedetomidine and midazolam, in which the combination of the two drugs, when used for sedation, reduced the individual dose of both drugs and a faster loss of righting reflex than either drug alone. This study, however, did not evaluate antinociceptive properties of either drug or the combination. The synergistic effect was further explored by Salonen et al (1992a), in which they demonstrated the synergistic effect transferred to other behaviors, namely the elevated plus-maze test in rats. They found that while flumazenil blocked the hypnotic response to midazolam, it was not effective against dexmedetomidine-induced loss of righting reflex. Conversely, atipamezole attenuated the hypnotic response to dexmedetomidine, but not to midazolam. They also noted no

"cross-displacement" by the agonists for the alternative receptor in a radiolabeled ligand binding study. Their data strongly support a pharmocodynamic mechanism for the synergistic interaction between midazolam and dexmedetomidine that is exerted at either pre- or post receptor locus (Salonen et al. 1992b).

The purpose of the present study was to investigate the sedative and analgesic properties of the combination of dexmedetomidine and midazolam in the rat and to explore the nature of the drug:drug interactions with respect to antinociception.

Chapter 2

Materials and Methods

Animals

Seventy male 250-300g Sprague Dawley rats (Harlan, Indianapolis, IN) were utilized in this study. Rats that were used in the midazolam study were given a two-week washout period and re-used in the dexmedetomidine study. Rats were pair-housed in a temperature (21 ± 0.5°C) and light (12 light:12 dark with no twilight) controlled environment. Food (2018 Global Rodent Diet, Harlan Teklad, Indianapolis, IN) and water were provided *ad lib* throughout the study. This work was approved by the Institutional Animal Care and Use Committee of the Pennsylvania State University College of Medicine.

Drugs

The drugs utilized in this investigation were dexmedetomidine HCl (Dexdomitor®, Pfizer Animal Health, New York, NY), midazolam HCl (Midazolam HCl Injectable, USP, Hospira, Inc. Lake Forest, IL), and atipamezole HCl (Antisedan®, Pfizer Animal Health, New York, NY). Dexmedetomidine was diluted with sterile water for injection to the appropriate concentrations so manageable volumes were administered. All drugs were administered by i.p. injection into the right caudal abdominal quadrant.

Assessment of Antinociception

Antinociception was assessed by the tail-flick test using an analgesia meter (Ugo Basile, Model 37360, Schwenksville, PA). An intensity setting of 50 on a scale of 1-50 and a cut-off time of 10 seconds were used throughout the study. These settings were determined from the results of preliminary studies to yield baseline tail-flick latencies between 3-5 s. This intensity was chosen because it did not produce immediate or latent burns of the tail in rats exposed to the maximal 10 seconds stimulus.

Antinociceptive tests were conducted in a room separate from the animal colony with racks containing cages transported into the room on the day of testing. During the test period, rats were kept in the same groups and cages as they were housed. Rats were restrained by hand with a small hand towel. Each rat was acclimated to this method of restraint and the tail flick device on three different days before the experiment commenced. During training periods, rats were restrained for 20-30 seconds with no stimulation.

Rats were weighed, and using a permanent marker, their tails were marked with a line in 1 cm increments starting at the tip, for a total of 6 increments. This ensured that the same area of the tail was not reused, and the tissue did not become sensitized to the stimulus (Wilson et al. 1992). Baseline tail-flick latencies were measured at the first position on the tail (1 cm).

Rats were injected with drug as described above 30 minutes after the baseline measurement. Tail-flick latencies were measured 15, 30, 45, 60, and 75 minutes after injection; each measurement was conducted at a different position on the tail, commencing with a position 2 cm from the tip. The 75 minute time intervals permitted evaluation of antinociception in a period of time considered suitable for surgical procedures. Rats that lost their righting reflex were maintained in right lateral recumbency on a 37°C circulating water blanket (Micro-Temp Pump, Cincinnati Sub-Zero, Cincinnati, OH).

Surgical Castration

A pilot study was conducted to determine if antinociceptive data correlated with a surgical plane of anesthesia. Four rats were administered three dexmedetomidine and midazolam (D + M) dose combinations determined from the antinociceptive study to produce maximal analgesia. The time at which the animal lost its righting reflex (LORR), defined as lateral recumbency with no purposeful movements to right itself, was measured. An additional 5 to 10 minutes (depending on the drug dose) was allowed to elapse, during which time the animal was prepped for surgery. A standard castration procedure as described by Waynforth (1992) was performed. Before surgical incision, the pedal withdrawal reflex was assessed and recorded. Response to the initial surgical incision was also noted. Following surgery, animals were reversed with a half-dose of atipamezole 60 minutes after LORR. The rats were allowed to recover fully and monitored for a week for any adverse effects.

Data Analysis

Antinociceptive data are expressed as the mean maximum possible effect (% MPE) using the following formula:

$$\%\,\mathrm{MPE} = \frac{(\text{post - drug latency} - \text{baseline latency})}{(10.0\,\mathrm{s} - \text{baseline latency})} \mathrm{X}\,100$$

Differences among treatments on tail-flick latencies at 15, 30, 45, 60 and 75 minutes after drug administration were analyzed by two-way analysis of variance (ANOVA) for repeated measures with time and treatment as the main factors using Graph Pad Prism version 5.0 (Graph Pad Software Inc., San Diego, CA). When overall treatment effect was detected, subsequent

planned comparisons were analyzed using Bonferroni multiple comparison tests to determine what treatments at which time points differed. Statistical significance was considered at $p < 0.05$.

The analysis of drug-drug interactions was made at the time of presumed steady state, 60 minutes post-injection (Fig. 4). The effect metric (in seconds) was the calculated difference between the test score and the baseline score. Analysis of the effect of dexmedetomidine alone compared to dexmedetomidine combined with midazolam was performed using a two sample t-test. Statistical significance was considered at $p <0.05$.

Chapter 3

Results

Antinociception

The baseline tail-flick latency was 3.9 ± 0.2 seconds (mean ± SEM) (n=68; two animals were discarded as their baselines were greater than the mean plus two standard deviations). Tail-flick scores in sedated or anesthetized rats were analyzed at specified times following varying doses of either midazolam (M), dexmedetomidine (D), or a 167:1 ratio of D:M on a mg kg^{-1} basis, administered i.p. No significant differences in tail-flick latency for rats administered 5-50 mg kg^{-1} M over the 15 – 75 minutes following administration were detected, $p = 0.06$ (Fig. 1).

In contrast, D showed a clear dose-dependent increase in tail-flick latency for doses 0.03 – 0.18 mg kg^{-1} $p = 0.04$; however, drug-time interactions were not statistically reliable, $p = 0.05$ (Fig. 2). Significance was achieved at the following comparisons: for T=45 minutes, 0.03 v. 0.15, 0.03 v 0.18; for T=60 minutes, 0.03 v. 0.15, 0.03 v. 0.18, 0.06 v. 0.15, 0.06 v. 0.18; and for T=75 minutes 0.03 v. 0.18. Groups without error bars all timed out; i.e., all the animals had the same reading, so there was no error. As well, the antinociceptive effect at the 0.03 and 0.06 doses peaked at fifteen minutes and then showed a continuing decline over time.

The combination of D and M increased tail-flick latencies 2–4 fold over D alone (Fig. 3). The highest statistical significance ($p<0.001$) was obtained at T=75 minutes, between the 0.12:20 v. 0.15:25 and the 0.12:20 v. 0.18:30 groups. A statistical significance of $p<0.01$ was found for the following pair-wise comparisons: at T=75 minutes: 0.03:5 v 0.15:25, 0.03:5 v 0.18:30, 0.06:10 v 0.15:25, 0.06:10 v 0.18:30, and 0.09:15 v 0.12:20. Statistical significance of $p<0.05$ was found for the following pairwise comparisons: T=45 minutes: 0.03:5 v 0.12:20, 0.03:5 v 0.15:25, 0.03:5

v 0.18:30, 0.06:10 v 0.12:20, 0.06:10 v 0.15:25, 0.06:10 v 0.18:30; T=60 minutes: 0.03:5 v 0.12:20, 0.03:5 v 0.18:30, 0.06:10 v 0.12:20, 0.06:10 v 0.15:25, 0.06:10 v 0.18:30, T=75 minutes: 0.03:5 v 0.09:15, 0.06:10 v 0.09:15. One animal was discarded from both the 0.15:25 and the 0.18:30 group as their baselines was greater than the mean plus two standard deviations. One other animal from the 0.15:25 group was also discarded as the animal never lost its righting reflex. It is possible that the drugs were injected into an inappropriate location and were never distributed throughout the body as intended. As with the D alone, the antinociceptive effect of the lower dose combinations (0.03:5 and 0.06:10) peaked at 15 minutes and then showed a continual decline over the 75 minutes.

To investigate the effects of the dexmedetomidine-midazolam interaction, we further analyzed data at 60 minutes after dosing, a time that appears sufficient for the establishment of a steady-state of anesthesia. Because M showed no efficacy in the tail-flick test, a simple additive interaction of the combination should produce effects that do not differ from the effect of D alone. The tail-flick latencies resulting from administration of D + M were significantly ($p = 0.021$) greater than those of D alone, thereby indicating a synergistic interaction between the two drugs with respect to antinociception. The four dose combinations compared in Figure 4 all show synergism; the 0.06:10 combination showed the greatest difference in tail-flick latencies when compared to D alone. However, the 0.12:20 combination showed the longest tail-flick latencies overall.

In contrast to the marked enhancements produced by the four combination doses shown above, two very high combination doses, 0.15:25 and 0.18:30, showed reduced effects when compared to D alone. This indicates the synergism is dependent on the concentration of M in the combination.

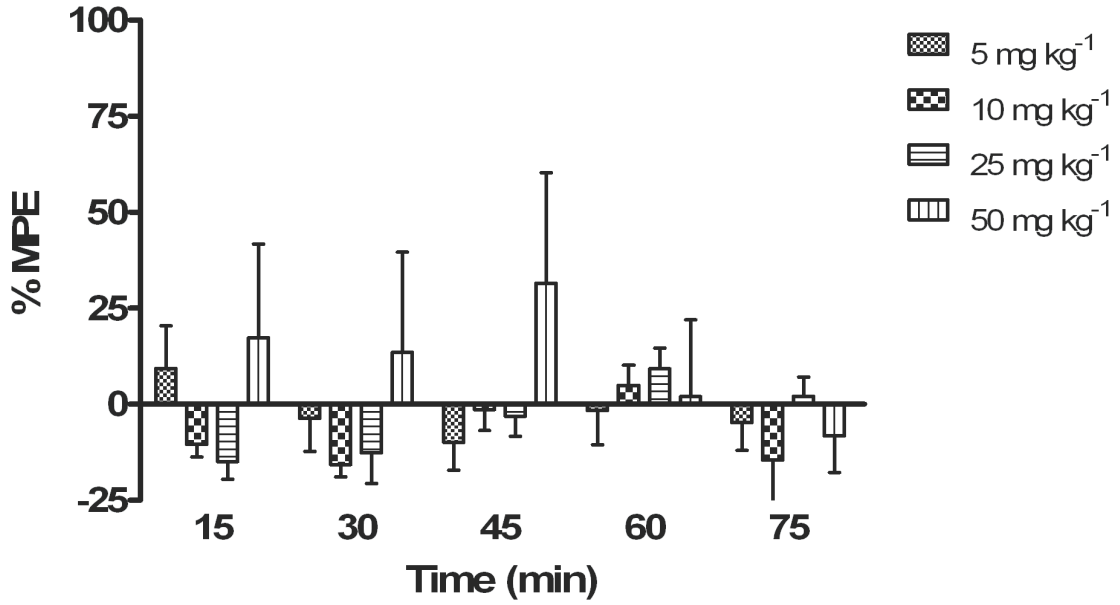

Figure 1: Midazolam alone

Effects of i.p. administration of midazolam (mg kg^{-1}) on tail flick-latency in rats. Each bar represents the mean ± SE of group (n = 5) tail-flick latency expressed as percent maximum possible effect (MPE%).

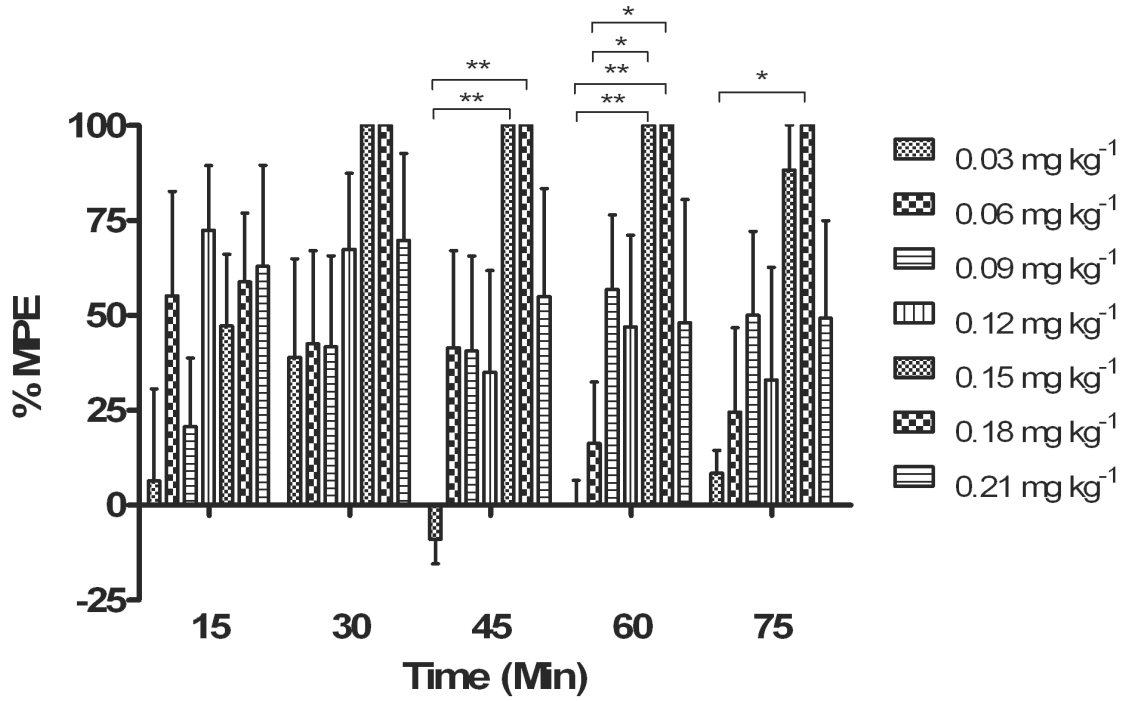

Figure 2: Dexmedetomidine alone

Effects of i.p. administration of dexmedetomidine (mg kg^{-1}) on tail-flick latency in rats. Each bar represents the mean ± SE of group (n = 5) tail-flick latency expressed as percent maximum possible effect (MPE%).

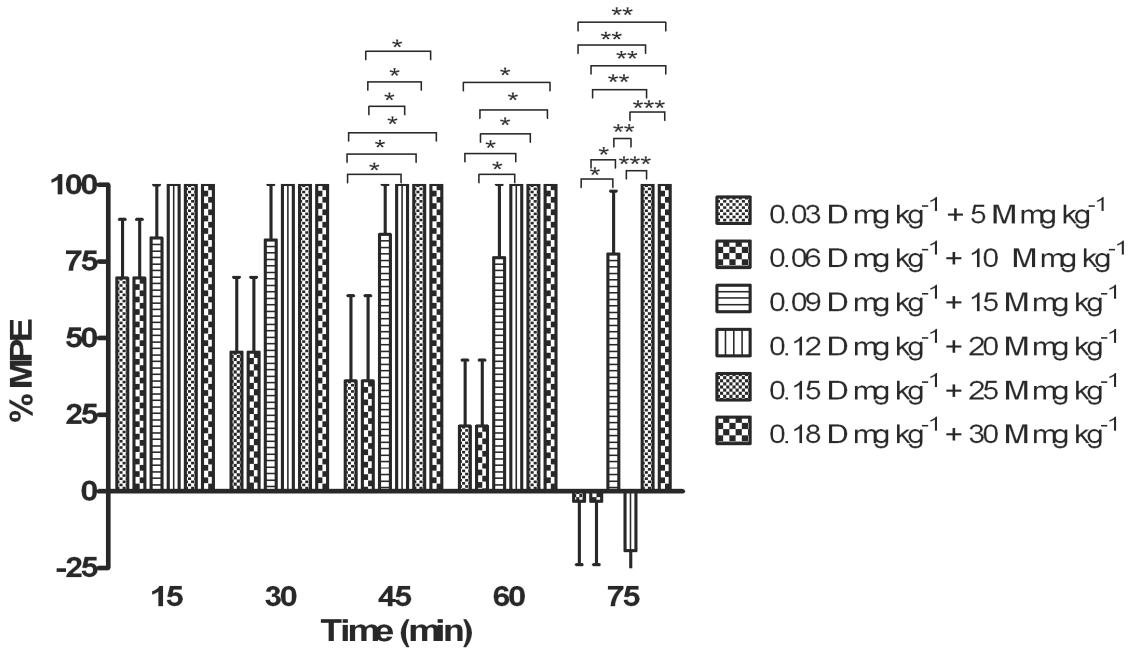

Figure 3: Dexmedetomidine plus midazolam

Effects of i.p. co-administration of dexmedetomidine (D) and midazolam (M, mg kg^{-1}) on tail-flick latency in rats. Significantly different pairwise comparisons; *p < 0.05, **p < 0.01, ***p < 0.001. Each bar represents the mean ± SE of group (*n* = 5 for all groups except 0.15:25 [n=3] and 0.18:30 [n=4]) tail-flick latency expressed as percent maximum possible effect (MPE%).

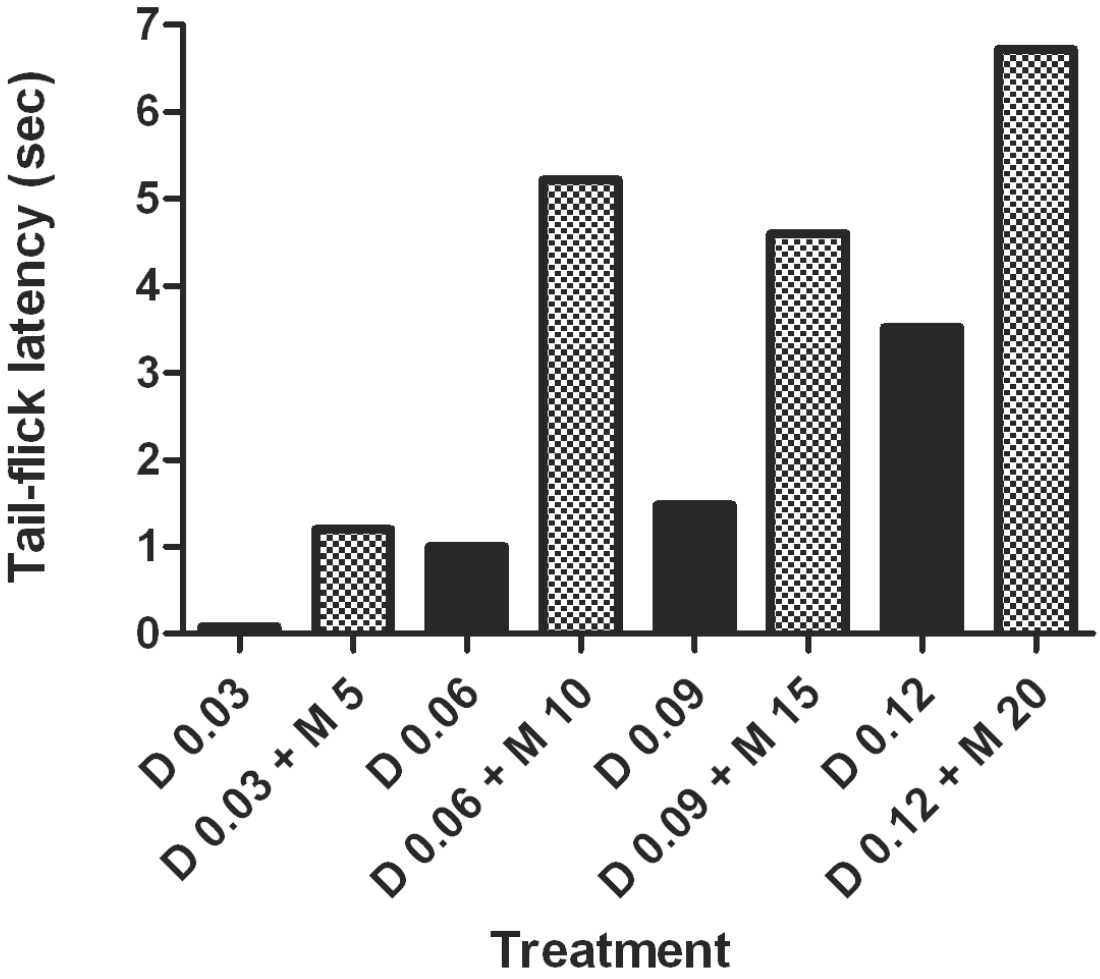

Figure 4: Combinations vs. dexmedetomidine alone at 60 minutes

Effect of dexmedetomidine (D) versus dexmedetomindine (D):midazolam (M) combinations, dose expressed as mg kg^{-1}, on tail-flick latency at 60 minutes post-administration in rats. Each bar represents the change in tail-flick latency from baseline.

Surgical Castration

A small study was conducted to see if the results obtained in the laboratory would translate into surgical anesthesia for a scrotal castration. Based on the results presented in Figure 3, the doses of 0.09:15 and 0.12:20 were chosen because of the depth and duration of anesthesia provided at T=60. Pedal withdrawal reflex, a subjective but commonly used indicator of the depth of anesthesia and analgesia, was assessed every five minutes for sixty minutes after the loss of righting reflex by pinching the rat's toe. Loss of righting reflex was between 84-240 seconds. Table 1 shows the effects of the D:M combination, administered i.p., the time between administration and incision, the presence or absence of the pedal withdrawal reflex during the 60 minutes, and whether the surgeon felt that an appropriate plane of analgesia and anesthesia had been met. The pedal withdrawal reflex was never present. If, at the time of the first incision, it was determined that a surgical plane was not reached, the rat was given additional time to reach that plane, rather than re-dosing it. The rats recovered fully and showed no ill effects for the week after the castration.

Table 1: Surgical Castration Pilot Study

Pilot study of Spraque-Dawley rats anesthetized with three different doses of the D:M (mg kg^{-1}) combinations undergoing surgical castration. The time to the loss of righting reflex, the presence or absence of the pedal withdrawal reflex every 5 minutes measured for 60 minutes, and the depth of anesthesia are indicated.

Rat	Dose (mg kg^{-1})	Time to LORR (sec)	Time after LORR to Incision (min)	Pedal Withdrawal Present?	Anesthetic Depth Sufficient?
1	0.12 D + 20 M	90	5	No	Yes
2	0.09 D + 15 M	84	5	No	No
3	0.09 D + 15 M	240	10	No	Yes
4	0.06 D + 10 M	150	10	No	No

Chapter 4

Discussion

Pakulska, 2001, reported that midazolam, when given with morphine, metamizol, or indomethacin in mice, decreased the antinociceptive effect of all three drugs. In contrast, Rosland, 1990, reported that midazolam induced a dose-dependent attenuation of the effect of morphine, fentanyl, and buprenorphine in the hot-plate and tail-flick tests in mice. Similar results were found when midazolam was used with low doses of ketamine (3.0 mg kg^{-1}) in rats, but not with higher doses (5.0 mg kg^{-1}) (Okulicz-Kozaryn et al. 2000). It has also been shown that midazolam given alone can induce hyperalgesia in rats (Tatsuo et al. 1997). In the present investigation, we observed tail-flick latencies suggestive of hyperalgesia in rats administered M alone at doses of 5 – 25 mg kg^{-1}, but not 50 mg kg^{-1}, consistent with Tatsuo's report (1997). Because the tail-flick test is a reflex-based test requiring some level of muscle function, we attribute the increased tail-flick latency in the 50 mg kg^{-1} M group to minimal or abolished muscle tone. Despite the hyperalgesic effect of M, when combined with D, it actually potentiated the antinociceptive effect of the α_2-AR agonist.

This potentiation, rather than attenuation, of the antinociceptive effect may be attributed to the difference in receptors. Benzodiazepines, ethanol, and barbiturates all potentiate the actions of the neurotransmitter GABA by prolonging the open state of the chloride-associated channels (Tatsuo et al. 1997). Dexmedetomidine is a specific and selective α_2-AR agonist. By binding to the presynaptic α_2-AR, it inhibits the release of norepinephrine, and thereby decreases the propagation of pain signals. The combination of two drugs that act on different receptors showed an obvious synergism in the present study.

Bol et al (2000) measured the synergism of the dexmedetomidine-midazolam combination in rats. They found that the interaction was synergistic for all stimulus-response measures, which included the whisker reflex, startle reflex to noise, tail clamp response, and corneal reflex, similar to the synergism we found with the tail-flick test. They also found the degree of synergy increased with deeper levels of CNS depression. However, each parameter was only measured at two time points per target concentration, and they measured the drug concentrations as plasma concentrations, rather than dose concentrations. Also, the tail clamp test was a modified clipboard clamp test; latencies between 0-15 seconds were defined as positive values and latencies between 15-30 seconds were denoted as negative values, resulting in quantitative data points rather than qualitative data points. They did not report any hyperalgesic effect with the midazolam. Our study also found a synergism for stimulus-response measures.

Dexmedetomidine at the highest dose, 0.21 mg kg^{-1} showed less antinociception than the next three lower doses (Fig. 2). A possible explanation for the attenuation of antinociceptive effect of D at the highest dose could be abolishment of all inhibitory pain modulating pathways, permitting an 'over-expression' of another α-adrenergic insensitive pain pathway (Mense 2000).

In our study, we observed that rats given higher doses of dexmedetomidine (0.12 – 0.21 mg kg^{-1}) produced large amounts of urine. Central stimulation of α2-AR in the hypothalamus decrease the secretion and or production of anti-diuretic hormone from the pituitary (Sinclair 2003). $Alpha_2$-AR agonists also antagonize the renal tubular effects of antidiuretic hormone (Sinclair 2003). A decrease in sympathetic tone may also contribute to the increased urine volumes. The large amounts of urine are noteworthy when performing abdominal surgery so as not to inadvertantly nick a full urinary bladder. We also observed that the higher doses of midazolam (25-50 mg kg^{-1}) caused many of the rats to begin shredding paper towels after the midazolam injection. Both of these phenomena occurred to a lesser degree with the lower doses and dose combinations.

Our study looked at the antinociceptive effect of D and M alone, and in combination with each other. We confirmed that M alone has no antinociceptive effect; D has a dose-dependent effect, and a combination of D + M had the most statistically significant effect on antinociception. The mechanisms by which M may enhance the action of D could include the facilitation of the release and/or binding of the inhibitory neurotransmitter GABA to its receptor. Also, α_2-AR agonists are able to inhibit adenylate cyclase, thereby changing the activity of cAMP-dependent protein kinase, which may alter the conductance properties of the chloride channel (Salonen et al. 1992b).

The results clearly show that D, in combination with M, can provide sedation and analgesia at the appropriate doses; 0.12 mg kg^{-1} D + 20 mg kg^{-1} M and 0.09 mg kg^{-1} D + 15 mg kg^{-1} M provided appropriate levels of anesthesia sufficient to perform surgical castration of the rat, provided 5-10 minutes were allowed to elapse between the LORR and the initial skin incision. This observation indicates that the synergism of the combination is dependent on the addition of M, as the D alone at those doses showed only a partial loss of antinociception, while the addition of M showed a total loss of antinociception. Salonen 1992b found a faster loss of righting reflex (less than 30 seconds) with the M:D combination than our study did. This could be attributed to the higher doses of dexmedetomidine they used (0.07, 0.14, 0.28, 0.35, 0.50 mg kg^{-1}). It may seem, after looking at Figure 3, that the 0.15 and 0.18 doses of D alone would produce surgical anesthesia and M may not be needed. However, the addition of M, noted for its muscle relaxant properties, allows us to decrease the dose of D and provide a more balanced anesthesia, as well as possibly avoid the marked bradycardia and decreased respiration that is sometime seen with high doses of dexmedetomidine. The results of our pilot study correlate well with the data presented in Figure 3. Based on personal preference, I would recommend the 0.12:20 combination, as the LORR was quicker and a surgical plane of anesthesia was reached quicker and with more confidence.

Future studies (more combinations in various ratios) will further clarify the drug-drug interaction with respect to antinociception and anesthesia. It would be interesting to see the D doses we used for surgical castration (0.09 and 0.12 mg kg $^{-1}$) in combinations with different doses of midazolam to further optimize the combinations.

Chapter 5

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Appendix

Literature Search

The purpose of this study was to investigate the effects of a dexmedetomidine-midazolam anesthesia combination on the depth of sedation in the rat.

- Onset, duration, and depth of anesthesia
- Nocioception (tail flick)

Minor procedures for which the suitability of dexmedetomidine-midazolam may be appropriate include: catheter implantation, tail clipping, ear notching, superficial tumor implantation, orbital sinus venotomy, superficial lymphadenectomy, ocular procedures, multiple IV antigen injections, intracerebral electrode implantation, vasectomy, castration, and vascular access port implantation (Kohn et al. 2007). The ability to reverse the effects of dexmedetomidine with atipamezole is an added advantage for minor, short procedures.

What is pain?

The International Association for the Study of Pain defines pain as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage or described in terms of such damage. It is always subjective.” (Kohn et al. 1997). Laboratory animal veterinarians are frequently called upon to evaluate animal protocols in which an animal is to be subjected to a painful procedure, and frequently help provide guidance with anesthetic protocols to alleviate any pain associated with such procedures. Objective measurements of pain and the relief provided by anesthesia and analgesia are necessary for all species.

What is anesthesia?

Anesthesia is a general loss of consciousness that results in the animal not consciously perceiving pain. However, noxious stimuli are still transmitted to the CNS, resulting in central hypersensitivity and heightened perception of post-operative pain (Flecknell 1999).

Anesthesia is commonly needed to restrain laboratory rodents for minor procedures (Kohn et al 2007). Both inhalent anesthesia and injectable anesthesia are commonly used with laboratory rodents. However, inhalent anesthesia requires a precision vaporizer, which can be expensive, and it requires the researcher to either intubate the rodent, which is done infrequently; use a nose cone to maintain a continuous plane of anesthesia, which can be cumbersome; or used for very short procedures when an induction chamber is the sole method of anesthesia.

Injectable anesthetics are favored because they are easy to administer with proper training and have a predictable depth and length of anesthesia. Many injectable anesthetics also confer analgesic properties. Injectables only require needles and syringes, which are inexpensive.

There is no ‘perfect’ anesthesic agent. Rather, the optimum agent depends on the level of anesthesia and analgesia provided, the type of procedure being done (minor or major), the need for muscle relaxation, and the effect of the agent on any research being done.

What is analgesia?

Analgesia is the prevention of noxious stimuli from reaching the central nervous system; thus it’s a lack of perception of certain pain. Peripheral inflammation increases input to the CNS, thus it is also important to minimize peripheral inflammation (Flecknell 1999)

Those that object to using analgesics offer the following arguments:

- The need for repeated injections can be time consuming and may in and of itself cause distress (Flecknell 1999). However time consuming it may be to give injections, it is not justification to withhold anesthesia. It is incumbent on the investigator to prove that injections would cause more distress than that alleviated by analgesics. As well, Buprenorphine Jell-O has been used in many situations to dose laboratory animals after a proper acclimation period (Pekow 1992).
- Alleviation of post-operative pain will result in self-injury. It is rare that animals will remove sutures because the pain has been relieved. (Flecknell 1999) In fact, many animals will remove sutures because of tissue reactions or boredom (personal experience).
- Possible side effects such as respiratory depression can occur. This type of side effect is most common when opioids are used. Respiratory depression in animals is less than that in humans. As well, a respiratory pressor such as doxapram can be administered, or the opioid can be partially reversed with a mixed agonist/antagonist such as butorphanol or buprenorphine, or a pure antagonist such as naltrexone (Flecknell 1999).
- Dose rates and regimens are not established. This is, especially in the digital age, untrue, as there are many different ways to find doses and dose regimens. Every drug has been tested on animals before being manufactured and marketed. It may be difficult to extrapolate from species to species, but reasonable protocols are available, especially from veterinarians involved with a research institution's Animal Care and Use program (Flecknell 1999).
- Pain relieving drugs may interfere with the results of an experiment. There are many different classes of drugs available (Flecknell 1999). For example, when

> doing gastric surgery in a swine, it would be prudent to not use an NSAID that may have a reputation for producing gastric ulcers, but an opiate such as buprenorphine would certainly be appropriate and should not cause undesirable side effects.

Researchers have an ethical responsibility to show that pain will be managed. The USDA requires an investigator to justify when pain medications will not be used; these exemptions are highly scrutinized by Institutional Animal Care and Use Committees. The reduction of pain in research animals also falls within Russell and Burch's principle of Refinement – "to reduce to an absolute minimum the pain and distress experienced by those animals that are used (in research procedures)" (Flecknell 1999). The three 'R's' are general guiding principles embraced by the research community; the other two R's are Reduce and Replace, i.e., use an appropriate number of animals (not too many that lives are wasted, and not so few that the results have no statistical power), and use computer modeling, in vivo testing, and lower species whenever possible (Russell 1992).

Choice of tail flick test

There are many different assays available for measuring analgesia, all of which rely on a change in reaction time to a given stimulus. The most common assays are those that measure a spinal reflex as part of the observed response (Van Pelt 1976). The tail flick and tail withdrawal tests involve a reflex that is localized to the spinal level. Responses to these tests may be due to an alteration in motor and/or sensory pathways. The tail flick test allows the selective stimulation of thermal nociceptors; the routine use of this test in pharmacology studies; and the availability of commercial equipment that permits the use of an adjustable quantifiable stimulus (D'Amour 1941, Dewey 1975). The tail flick also has the advantage of minimal risk for tissue damage when

standard cut-off temperatures and cut-off times are used, as well as the ease of which the investigator could try the stimulus on herself. The tail flick incorporates a photocell that permits the rat to terminate the stimulus when it flicks its tail.

The hot plate test relies on the animal having some level of consciousness. It would be inappropriate in this study as we were attempting to find a surgical plane of anesthesia, which in and of itself does not allow the animal any consciousness. The hot plate test is a less appropriate test because while the animal can react to the stimulus, it cannot terminate the stimulus (Fennessy 1975). The tail withdrawal test is time consuming. Thermal stimulation tests stimulate only thermonociceptors and spinal reflexes, except for the hot plate test which, since the stimulus is directed to a body part and an action is directed at the body part, a supraspinal reflex is also activated (Fennessy 1975).

Mechanical stimulation tests commonly activate low-threshold mechanoreceptors as well as mechanonociceptors (Ward 1974). Varying concentrations of receptors in different physical locations on the body may also produce variability in results (Wixson 1987). Many mechanical stimulation tests also cannot quantify the amount of pressure being applied, which will confound results. As well, many people will pinch the paw to assess anesthesia, and for a study of this length, paws need to be alternated, and there is the possibility that the paw could become desensitized to the stimulus.

Common injectable anesthetic protocols

Pentobarbital is a barbiturate commonly used for anesthesia and analgesia at low doses, and for euthanasia at higher doses. The most common side effect seen with pentobarbital is respiratory depression (Plumb 2007g). Pentobarbital is a poor antinociceptive agent, especially

when compared to other easily obtainable injectables such as ketamine/xylazine, so it is only appropriate for procedures requiring minimal manipulation of the animals (Wixson 1987).

Ketamine is a dissociative general anesthetic agent. It is commonly used in veterinary medicine as a sole agent or in combination with a muscle relaxant such as diazepam. It is being explored as a therapeutic for chronic pain. Common side effects include respiratory depression, emesis, hypertension, muscle spasticity, and vocalization. It is painful upon injection (Plumb 2007d). Ketamine is classified as a Schedule III drug in the US.

Xylazine is an α_2 adrenergic agonist valued for its sedative, analgesic, CNS-depressive, and muscle relaxant properties. Skeletal muscle relaxation is mediated through centrally mediated pathways. Side effects include emesis, hypoventilation (at high doses), muscle tremors, and bradycardia (Plumb 2007h).

Dexmedetomidine

Dexmedetomidine is the active isomer of the analgesic medetomidine that binds to α_2–adrenergic receptors (α_2-AR) in an agonist fashion with high specificity. Medetomidine is a racemic mixture of dexmedetomidine and levomedetomidine. The latter is the inactive form (Murrell 2005). Recently, dexmedetomidine became available to the US market as a sedative–analgesic for cats and dogs.

The α_2-AR agonists are known for reliable sedation, analgesia, muscle relaxation, anxiolysis, and a decrease in the anesthetic requirements of injectable and inhalant agents (dose sparing) (Sinclair 2003). Dexmedetomidine is ten times more selective for the α_2-AR than the analgesic and sedative, xylazine (receptor selectivity ratio of 1620 vs. 160 respectively; Scheinin 1988). The onset of action is variable, from 5 minutes when administered intravenously, to 10-15 minutes for intramuscular administration. Dexmedetomidine is frequently dosed on body surface

area (mcg/m^2), but those values can be converted to body weight (mcg/kg). It is important to note that a smaller dose is used when given IV, and larger doses do not increase the degree of sedation, but rather prolong the length of sedation (Sinclair 2003). The most common side effects of dexmedetomidine are bradycardia, decreased respiration, and hypothermia (Plumb 2007 e). Dexmedetomidine is easily reversed with atipamezole, an α_2-AR antagonist.

The sedative action of dexmedetomidine is generally ascribed to inhibition of the locus coeruleus in the pons and lower brainstem, which contains pathways involved in the maintenance of vigilance, mediated through presynaptic α_2-AR (Nishimura et al. 1993). In the brain, dexmedetomidine has been reported to bind to α_2-AR (Murrell 2005). Alpha$_2$–agonists bind to the membrane receptors and prevent the release of norepinephrine (Sinclair 2003). The inhibition of norepinephrine release is mediated by a decrease in calcium entry to the cell resulting from the direct inhibition of voltage-gated calcium ion channels (Murrell 2005). Norepinephrine is necessary for centrally-mediated arousal. When this arousal is decreased, sedation ensues (Sinclair 2003). Muscle relaxation is another effect of dexmedetomidine, and is due to the inhibition of α_2-AR at the interneuronal level of the spinal cord (Sinclair 2003).

Dexmedetomidine produces analgesia by stimulating receptors at various sites in the pain pathways throughout the brain and spinal cord. High expression levels of α_2– AR binding sites are found in the dorsal horn of the spinal cord, where nociceptive fibers synapse, as well as in the brainstem, where nociceptive signals are modulated (Murrell 2005). The binding of α_2-agonists to their receptors results in activation of a membrane-associated G protein that eventually opens potassium channels, which leads to hyperpolarization of the cell, rendering the neurons less responsive to excitatory input, thus stopping the pain pathway. This method of action is similar to that seen with μ-opioid agonists (Sinclair 2003).

The specific method of action by which the cardiovascular effects of dexmedetomidine are seen are two-fold: the bradycardia is due to reduced sympathetic tone (which also decreases

the heart rate) and increased systemic venous return. The increase in systemic venous return is due to the action of the α_2 agonist on the peripheral α_2 adrenoceptors. This manifests as an increase in arterial blood pressure (Sinclair 2003). A benefit to the increased peripheral resistance may be seen as a slower decline in body temperature (Sinclair 2003).

The respiratory depression seen with dexmedetomidine administration is related to the CNS depression produced by α_2 adrenoceptor stimulation. It should be noted that dexmedetomidine produces a less pronounced degree of respiratory depression than other anesthetic agents, such as propofol, ketamine, and opioids (Sinclair 2003).

Other minor effects of dexmedetomidine include (Sinclair 2003):

- Hypothermia – which is mild compared to that induced by other sedatives.
- Muscle twitching – an occasional occurrence that also happens with xylazine and romifidine.
- Endocrine – α_2 agonists increase serum glucose levels, thus its use in diabetic animals is contraindicated.
- Diuretic effect – animals sedated with α_2 agonists frequently have large volumes of dilute urine upon recovery. This is due to a decrease in the secretion of anti-diuretic hormone and effects on the renin-angiotension system.
- Vomiting – vomiting is induced in 8-20% of dogs and up to 90% of cats.
- Gastrointestinal motility – the motility of the colon in dogs is dramatically inhibited, but it is a temporary effect.
- Intraocular pressure – there are conflicting reports of the effect of dexmedetomidine in different animals: in cats and rabbits, mydriasis and decreased intraocular pressure occur. In the dog, miosis and normal intraocular pressure were noted. However, because vomiting can be a side effect, the use of

dexmedetomidine in animals with an increased intraocular pressure should be limited.

- Intracranial pressure – cerebral blood flow is decreased in dogs sedated with dexmedetomidine. However, because vomiting can be a side effect, the use of dexmedetomidine in animals with a possible increase in intracranial pressure should be avoided.

Midazolam

Midazolam is a benzodiazepine that is valued for its sedative-hypnotic, anxiolytic, and muscle relaxant properties. It has a rapid onset of action with a very high affinity for the ionotropic gamma–aminobutyric acid ($GABA_A$) receptor subtypes, up to twice the affinity of diazepam. Once midazolam binds to $GABA_A$ receptors, chloride ions enter the cells via the receptors, which results in hyperpolarization of the neurons, making them more refractory to other stimuli (Nishimura et al. 1993).

Because the affinity is high, midazolam's potency is nearly three times that of diazepam. The rapid onset of action is attributed to its high lipophilicity at pH values greater than 4.0 (Thurmon 1996). It is water-soluble, highly protein-bound (94-97%), and rapidly crosses the blood-brain barrier. While midazolam is similar to diazepam, it has a shorter duration of action, and a more rapid elimination half-life and total body clearance than diazepam. Midazolam is water soluble in its injectable form, but becomes lipid soluble at body temperature, whereas the carrier for diazepam is 40% propylene glycol, 10% ethanol, 5% sodium benzoate, and 1.5% benzyl alcohol. Midazolam is easily injected intramuscularly or intravascularly, while diazepam is incompletely absorbed if given intramuscularly, and intravenous injections must be given

carefully and slowly to prevent thrombophlebitis or cardiotoxicity (secondary to the propylene glycol carrier) (Plumb 2007c).

Midazolam is metabolized in the liver by the microsomal oxidation system. Elimination half-lives range from 77 minutes in dogs to approximately 120 minutes in humans. The most common side effect is respiratory depression. Midazolam can be antagonized by flumazenil if necessary (Plumb 2007a).

Midazolam has been shown to have a hyperalgesic effect when given with opiates (Pakulska 2001) (Rosland 1990), barbiturates (Tatsuo et al. 1997), dissociative agents (Okulicz-Kozaryn et al. 2000), and when given alone (Tatsuo et al. 1999). Midazolam has not been reported to induce a hyperalgesic state when given with an α_2– AR agonist.

Pakulska, 2001, reported that midazolam, when given with morphine, metamizol, or indomethacin in mice, decreased the antinociceptive effect of all three drugs. In contrast, Rosland, 1990, reported that midazolam induced a dose-dependent attenuation of the effect of morphine, fentanyl, and buprenorphine in the hot-plate and tail-flick tests in mice. Similar results were found when midazolam was used with low doses of ketamine (3.0 mg kg^{-1}) in rats, but not with higher doses (5.0 mg kg^{-1}) (Okulicz-Kozaryn et al. 2000). It has also been shown that midazolam given alone can induce hyperalgesia in rats (Tatsuo et al. 1997).

Combination

The combination of medetomidine-midazolam to produce sedation and anesthesia has been explored for canines (Hayashi et al. 1994, Kojima et al. 1999, Itamoto et al. 2000), sheep (Raekallio 1998), swine (Nishimura et al. 1993, Nishimura et al. 1994), and Japanese macaques (Kimura et al. 2007).

In dogs, ME-MI has been found to produce predictable depth and duration of sedation with a moderate reflex depression, analgesia, muscle relaxation, regular respiration and immobilization (Hayashi 1994). The same group further explored the ME-MI combination in 1995 and found it caused bradycardia (<50-70 bpm) and a transient mild pressor response characterized by a mild but rapid increase in arterial pressure and a systemic vascular resistance above baseline values, probably due to the vasoconstrictive effects of medetomidine.

Kojimo et al also reported on the sedative effects of ME-MI in dogs in 1999. They found the ME-MI combination produced deep sedation, characterized by immobilization with moderate reflex depression, muscle relaxation, and analgesia. However, they report variation in the time to sedation; it has been seen that with medetomidine, one must administer the drug and then not continually stimulate the animal to obtain maximum and quick sedation (personal experience). They were concerned by the amount of bradycardia and decreased respiration accompanying the sedation, thus leading to a second study in 2002.

Kojima et al (2002) explored the cardiopulmonary effects of ME-MI in canines. They confirmed the large cardiovascular changes such as bradycardia, hypertension, decreased cardiac output, and vasoconstriction. They did not find that the respiratory parameters changed markedly. They postulate that the bradycardia was caused by the medetomidine, and is brought about by its vasoconstrictive effects, which leads to activation of baroreceptors, and the decreased cardiac output is induced by this bradycardia and an increase in afterload by the vasoconstriction.

Itamoto et al (2000) confirmed the bradycardia (average = 60 bpm) and the increase in mean arterial pressure 5-15 minutes after injection of ME-MI. They also looked at the acid-base status of dogs anesthetized with ME-MI, and found a slight increase in $PaCO_2$; however, PaO_2 and HCO_3^- did not change significantly.

Raekallio et al. (1998) found that in sheep, the ME-MI combination produced bradycardia to the extent that one sheep suffered cardiac arrest. (The dose was 15 mcg kg^{-1}

medetomidine and 0.1 mg kg^{-1} midazolam). They also found a marked hypoxemia and hypercapnea. Arterial pH stayed within normal range. Systolic and mean arterial pressures decreased, in contrast to the dogs, in which pressure increased.

In Japanese Macaques (*Macaca fuscata*), ME-MI induced a deep sedation accompanied by analgesia, muscle relaxation, and depressed reactions to external stimuli. Kimura et al (2007) noted mild bradycardia and hypothermia, but did not give levels because it does not seem they collected data regarding these parameters. They also looked at hematological and serum biochemical findings, and concluded there was no significant difference between ME-MI and monkeys anesthetized with ketamine. Thus, they found ME-MI to be suitable for immobilization of monkeys for minor procedures.

Nishimura et al. (1993) investigated the sedative effects of ME-MI in swine. They found that despite stimulation during the induction phase, the swine became sedated quickly and smoothly, enough that the swine could be placed in dorsal recumbency easily. They also noted moderate analgesic effects and muscle relaxation, along with depressed swallowing and laryngeal reflexes. They found hypothermia to be dependent on the dose of medetomidine.

The same group further characterized ME-MI sedation in swine by looking at cardiopulmonary effects (Nishimura et al. 1994). They found a pressor response similar to that seen in canines (a rapid increase in arterial and pulmonary arterial pressure with systemic and pulmonary vasoconstriction followed by a decrease 5-10 minutes post-injection). However, the swine did not evidence bradycardia, subsequent hypotension, or a significant decrease in cardiac output. The respiratory response showed minimal change in PaO_2, $PaCO_2$, pH, and base excess.

The physiologic effect and level of sedation of the medetomidine-midazolam (ME-MI) combination has been thoroughly characterized in dogs and swine, with differences (bradycardia, hypoxemia, hypercapnea) appearing to be species-related.

Salonen et al (1992b) first demonstrated the synergistic interaction between dexmedetomidine and midazolam, in which the combination of the two drugs, when used for sedation, reduced the individual dose of both drugs and a faster loss of righting reflex than either drug alone. This study, however, did not evaluate antinociceptive properties of either drug or the combination. The synergistic effect was further explored by Salonen et al (1992a), in which they demonstrated the synergistic effect transferred to other behaviors, namely the elevated plus-maze test in rats. They found that while flumazenil blocked the hypnotic response to midazolam, it was not effective against dexmedetomidine-induced loss of righting reflex. Conversely, atipamezole attenuated the hypnotic response to dexmedetomidine, but not to midazolam. They also noted no "cross-displacement" by the agonists for the alternative receptor in a radiolabeled ligand binding study. Their data strongly support a pharmocodynamic mechanism for the synergistic interaction between midazolam and dexmedetomidine that is exerted at either pre- or post receptor locus (Salonen et al. 1992b).

Bol et al (2000) measured the synergism of the dexmedetomidine-midazolam combination in rats. They found that the interaction was synergistic for all stimulus-response measures, which included the whisker reflex, startle reflex to noise, tail clamp response, and corneal reflex. However, each parameter was only measured at two time points per target concentration, and they measured the drug concentrations as plasma concentrations, rather than dose concentrations. Also, the tail clamp test was a modified clipboard clamp test; latencies between 0-15 seconds were defined as positive values and latencies between 15-30 seconds were denoted as negative values, resulting in quantitative data points rather than qualitative data points.

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