

**PHARMACOKINETICS AND *IN VITRO* EFFECTS OF
IMIPRAMINE HYDROCHLORIDE
ON THE VAS DEFERENS IN CATTLE**

by

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Declaration

I, Claudia Cordel, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this university or any other university.

This dissertation is presented in partial fulfillment of the requirements for the degree MMedVet (Gyn).

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Signed
Claudia Cordel

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List of Key Terms

Imipramine hydrochloride

Domestic bull

Intravenous pharmacokinetics

In vitro effects on vas deferens smooth muscle

Organ bath

Noradrenaline

α_1 -receptors

Optimal *in vitro* imipramine dose

Abstract

This project was divided into two studies. The first investigated the pharmacokinetics of imipramine hydrochloride (IMI) in bulls. IMI was administered intravenously to three bulls (600 - 705.5 kg) at a dose of 2mg/kg body weight (BW). Intravenous plasma concentrations of IMI over time were determined by fluorescence polarization immunoassay (FPIA). IMI plasma concentration versus time profile was best described by a two compartmental open model with first-order rate constants. IMI distributed rapidly, ($t_{1/2\alpha}$) at 7.2 ± 4.2 min, exhibited a very large apparent steady state volume of distribution ($V_{d_{ss}}$) of 4.2 ± 0.9 l/kg BW, had a very short terminal elimination half-life ($t_{1/2\beta}$) of 140 ± 15 min and showed a rapid total body clearance (Cl) of 22.7 ± 7 ml/min/kg. Both IMI and the pharmacologically active metabolite, desipramine was negligible in serum at 24 hours. All three bulls treated with IMI showed pronounced central nervous system signs immediately post injection. Signs of generalised weakness and ataxia were evident. All CNS signs dissipated 15–20 minutes post injection and should therefore not influence the treatment interval. An interval of at least 23 hours between repeat treatments of IMI, representing a period of at least 10 half-lives, is recommended. The dose of 2 mg/kg BW used in this study was similar to that routinely used in stallions without fatal side effects. One of the three bulls exhibited spontaneous emission and ejaculation with this dose.

The second study investigated the effects of IMI on ampullar strips of bulls in organ baths. Vasa deferentia were collected from 16 freshly slaughtered post-puberal bulls of various breeds. Longitudinal ampullar strips were prepared and placed into 20 ml modified Krebs bicarbonate solution, aerated with a mixture of O₂ (95 %) and CO₂ (5 %) in water-jacketed organ baths. The effect on the smooth muscle tissue of noradrenaline (NA) alone, NA in combination with IMI and IMI alone was evaluated. NA alone consistently produced dose-dependant smooth muscle tissue contractions. IMI doses equivalent to ≤ 1 mg/kg BW (body weight equivalent; bwe) had NA potentiating effects. Doses of <0.1 mg/kg bwe were consistently potentiating while doses of >0.1 mg to ≤ 1 mg/kg bwe partially blocked NA stimulating effects. Amplitude of rhythmic

contractions increased while contraction frequency decreased at this level. This study supports the adrenergic potentiating effects of IMI at doses of 0.05 - 0.2 mg/kg bwe with higher doses having paradoxical effects. Doses of IMI ≥ 2 mg/kg bwe completely blocked NA effects. Tissue response to NA, after IMI blockade, started to recover 146-186 minutes after application of IMI at ≥ 2 mg/kg bwe. In the absence of NA, IMI had no effect on smooth muscle activity. The time to an IMI effect on NA initiated smooth muscle activity was 8 minutes. On the basis of the results of this *in vitro* study, we propose that IMI can be used to enhance semen collection by means of electro-stimulation in domestic bulls and immobilised wildlife species such as buffalo, provided that the correct dose is used.

Chapter 1

Introduction

The economic value of ecotourism in Africa, to both governments and communities, is becoming increasingly transparent and is reported to be some three quarters of the value of the whole tourism sector with contributions of trophy hunting at least 18% of this (Humavindu and Barnes, 2003). The bulk of the ecotourism industry comprises non-consumptive activities i.e. game viewing. The African buffalo (*Syncerus caffer*), as one of Africa's "Big Five", is potentially a valuable earner of foreign currency for developing countries from hunting activities (consumptive use), as well as non-consumptive use.

Buffalo in South Africa can be divided into two populations: those that are so-called disease-free buffalo and those that are carriers of specific diseases. Animals of the latter group are carriers of foot and mouth disease virus (FMDV) (Kruger National Park (KNP) only), Corridor/buffalo disease (*Theileria parva lawrenci*) and bovine tuberculosis (*Mycobacterium bovis*). The implications are that buffalo carrying these diseases -may not be moved from the areas where they occur, because of the threat they would pose for the commercial livestock industry. Foot and mouth disease virus provides the biggest threat, considering the disastrous implications this disease could have on the agricultural industry. Strict laws govern the movement of buffalo in South Africa and buffalo that carry diseases may only be moved within the areas where the diseases occur.

A genetic study of four buffalo populations of differing size in South Africa showed a significant positive correlation between the degree of genetic diversity and both population size and park area (O'Ryan, C M; Harley, E. H.; Bruford, M. W.; Beaumont: Wayne, R.K. and Cherry, M. I., 1998). The greatest genetic diversity was found in the KNP, a relatively large population in a large park area, compared with the other three populations sampled (St. Lucia, Umfolozi Complex and Addo Elephant National Park). The Addo Elephant National Park with the smallest area and smallest population size also has the lowest genetic diversity. Buffalo of the Addo Elephant National Park are currently

disease-free, but considered to be substantially substandard with regard to trophy quality, presumably due to a lack of genetic diversity. Buffalo of the KNP meet international trophy hunting standards but are not accessible because of the diseases they carry. The economic benefits derived from using KNP buffalo to genetically upgrade disease-free buffalo would be enormous. Under strictly controlled conditions such as those governed by the Organisation International des Epizooties (OIE), assisted reproductive techniques such as artificial insemination (AI) and embryo transfer (ET), would allow the introduction of new genetics, locally and internationally without disease introduction. However, in comparison to their routine use in domestic livestock species (ruminants and equidae), little is known about the application of these techniques in wild bovidae. Initial problems included the collection of semen samples of good quality. Compared to cattle, electro-ejaculation of chemically immobilised African buffaloes produce poor semen quality. This initial work, attempting to collect semen samples from African buffalo, formed the origins of this work, looking into alternative methods of semen collection in bovidae. Our own experience has shown that the processing, freezing and storage of buffalo semen is similar to that in cattle, with similar post-thaw viability provided that the initial sample is of good quality. Thus, collection of a semen sample of good quality is an important prerequisite in a successful AI or ET programme in non-domestic bovidae. Concurrently it may provide alternative semen collection methods for domestic bovidae.

The use of pharmacological stimulants to enhance seminal emission and ejaculation in both man and stallions is well documented (Brookes, M. E.; Berezin, M. and Braf, Z., 1980; Mitchell, J.E. and Popkin, M. K., 1983; Rowley, D. D.; Lock, T. F. and Shipley, C. F., 1999; Johnston, P. F. and DeLuca, J. L., 1998; Nijman, J. M.; Schraffordt Koops, H.; Oldhoff, J.; Kremer, J. and Jager, S., 1987; McDonnell, S. M., 1992; McDonnell, S. M. and Oristaglio Turner, R. M., 1994; McDonnell, S. M. and Love, C. C., 1991; Gilja, L.; Parazajder, J.; Tadej, M.; Cvitkovic, P. and Kovacic, M., 1994; Oristaglio Turner, R. M., Love, C. C., McDonnell, S. M., Sweeney, R. W., Twitchell, E. D., Habecker, P. L., Reilly, L. K., Pozor, M. A. and Kenney, R. M., 1995; Brinsko, S. P., 2001; Card, C. E., Manning, S. T.,

Bowman, P. and Leibel, T., 1997; McDonnell, S. M., 2001; McDonnell, S. M., Garcia, M. C. and Kenney, R. M., 1987; McDonnell, S. M. and Odian, M. J., 1994). Their use in cattle and non-domesticated terrestrial species, to aid seminal emission and collection of semen samples suitable for cryopreservation for dissemination of genetic material locally and internationally would be a great advantage.

The use of isolated tissue preparations *in vitro*, to establish the effects of pharmacological agents on selected tissues is well established (Livingstone, E. & S., 1970; McLeod, D. G., Reynolds, D. G. and Demaree, G. E., 1973; Hukovic, S., 1961). Human (McLeod, D. G., Reynolds, D. G. and Demaree, G. E., 1973) and guinea pig (Livingstone, E. & S., 1970) vas deferens preparations are known to contract in a dose-dependant excitatory response to adrenalin and noradrenaline. The receptors associated with this response are classified as α -adrenergic (McLeod, D. G., Reynolds, D. G. and Demaree, G. E., 1973; Livingstone, E. & S., 1970). In the guinea pig potentiation of vas deferens response to NA is reportedly produced in most specimens with the addition of cocaine. Imipramine is known to have cocaine-like effects and should similarly potentiate the NA effect.

The pharmacokinetics of imipramine and desipramine have been extensively investigated in humans (Sallee, F. R. and Pollock, B. G., 1990) and a single study reports on pharmacokinetics in narcoleptic horses (Peck, K. E., Hines, M. T., Mealey, K. L. and Mealey, R. H., 2001).

This study was divided into two phases: pharmacokinetics of imipramine hydrochloride in cattle, which has not been done before, and the *in vitro* effects of imipramine hydrochloride on isolated cattle vas deferens tissue.

The aims of the pharmacokinetic study (Chapter 3) were to:

- establish safety of IMI in cattle
- establish time to optimal effect post administration on emission/ejaculation

- establish a dosing interval

The aim of the *in vitro* study using isolated cattle vasa deferentia (Chapter 4) were to:

- quantify and qualify IMI effects at the level of the vas deferens
- establish an optimal dose

Chapter 2

Review of related literature

2.1. Gross anatomical organization of the autonomic nervous system

A brief outline of the anatomical features of the autonomic nervous system (ANS) is necessary for an understanding of the actions of autonomic drugs.

The autonomic nervous system is divided into sympathetic and parasympathetic divisions on the grounds of physiological and anatomical differences. Classically, the two divisions of the autonomic nervous system are often functionally antagonistic, with one stimulating and the other inhibiting a particular response. Anatomically, the autonomic nervous system differs considerably from the rest of the nervous system in that final innervation of a target tissue involves two neurons and not just one. The cell body (soma) of the first neuron is situated inside the central nervous system (CNS), while the soma of the second neuron lies in an autonomic ganglion outside the CNS. The axon of the first (presynaptic) neuron synapses with the short dendrites of the second (postsynaptic) neuron in the autonomic ganglion. The axon of the postsynaptic neuron ends on the target tissue; these axons are generally long in the case of the sympathetic and short in the parasympathetic system. However, in the case of the male genital system, there are many autonomic ganglia situated close to the pelvic organs that contain so-called short noradrenergic neurons whose axons project to the urogenital organs (Kolbeck, S. C. and Steers, W. D., 1993). The presynaptic neuron has a myelinated axon known as the preganglionic fibre; the postsynaptic neuron has a non-myelinated axon that is known as the postganglionic fibre. This applies to both autonomic systems.

Preganglionic sympathetic fibres that innervate the pelvic viscera originate in the lower thoracic and upper lumbar spinal cord segments, T10/11-L2/3 (Gosling, J. A., Dixon, J. S. and Jen, P. Y. P., 1999). Nerve fibres leave the cord through the ventral roots of the spinal nerves. Some of these sympathetic preganglionic fibres synapse with the ganglia

of the paravertebral sympathetic chain, while others pass through the sympathetic trunk to synapse in peripheral ganglia (Fig. 1).

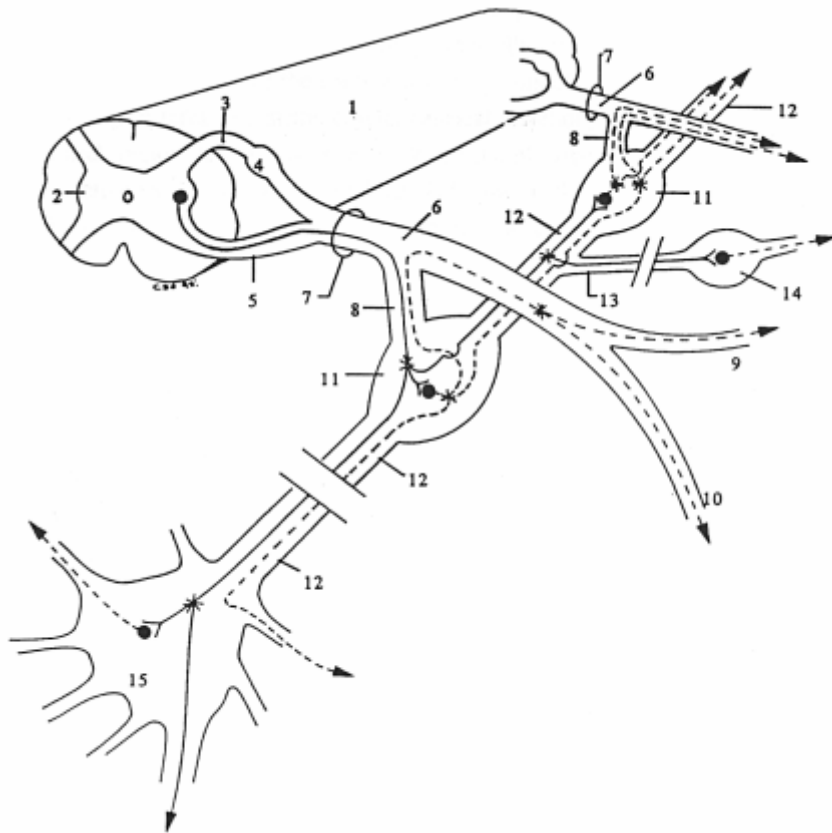


Figure 1: Schematic representation of sympathetic tracts from the central nervous system.

1. spinal cord; 2. Cornu laterale); 3. Radix dorsale; 4. spinal ganglion; 5. Radix ventrale; 6. N. spinalis; 7. intervertebral foramen; 8. R. communicans; 9. R. dorsalis of 6; 10. R. ventralis of 6; 11. Ggl. thoracicum; 12. sympathetic trunk; 13. N. splanchnicus; 14. collateral ganglion; 15. Ggl. cervicothoracicum.

*alternative paths; •>synapse; solid lines indicate pre-ganglionic fibres; dashed lines indicate post-ganglionic fibres.

(From: Bezuidenhout, A. J.; Groenewald, H. B.; Hornsveld, M.; Soley, J. T. and Turner, P. H. Veterinary Anatomy, 2000).

Preganglionic sympathetic fibres that innervate the urogenital organs synapse in the caudal mesenteric ganglion. The postganglionic sympathetic fibres form the hypogastric nerve, (*N. hypogastricus*), which proceeds caudally and enters the pelvic cavity (Fig.2).

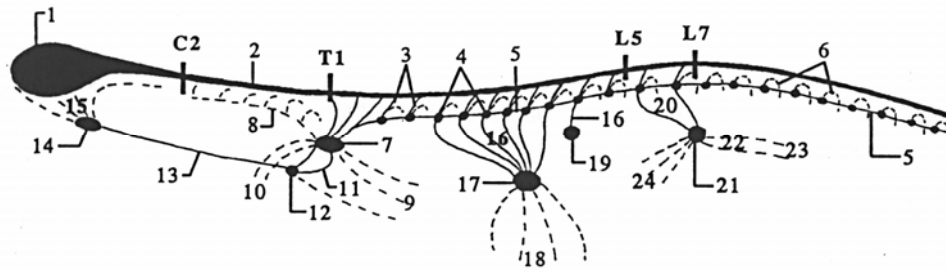


Figure 2: Schematic representation of the sympathetic division of the autonomic nervous system.

1. Brain; 2. Spinal cord; 3. *R. communicans*; 4. *Ggl. thoracicum*; 5. sympathetic trunk; 6. spinal nerves; 7. *Ggl. cervicothoracicum*; 8. *N. vertebralis*; 9. to thoracic viscera; 10. to thoracic limb; 11. *Ansa subclavia*; 12. *Ggl. cervicale medium*; 13. *Tr. vago-sympathicus*; 14. *Ggl. cervicale craniale*; 15. to head/cranial neck; 16. *Nn. splanchn. maj./min.*; 17. *Ggl. Celiacomesenteric*; 18. to abdominal viscera; 19. *Gl. Suprarenalis*; 20. *Nn. splanchn. lumbales*; 21. *Ggl. mesenteric caudalis*; 22. *Nn. hypogastrici*; 23. to pelvic viscera; 24. to pelvic limb
(From: Bezuidenhout, A. J.; Groenewald, H. B.; Hornsvelt, M.; Soley, J. T. and Turner, P. H., 2000.)

Together with the parasympathetic pelvic nerves, (*Nn. pelvini*), the hypogastric nerves form the pelvic plexus, from where nerves accompany blood vessels of the pelvis directly to the pelvic viscera (Gosling, J. A., Dixon, J. S. and Jen, P. Y. P., 1999). These nerves also convey afferent (sensory) fibres to the spinal cord. The role of afferents supplying the vas deferens is not known but they may relay nociceptive (pain) or mechanoreceptive (mechanical pressures/distortions) input. The efferent input from peripheral ganglia contributes to contractility of the vas deferens.

Somas of the preganglionic neurons of the parasympathetic system are located in the second to the fourth sacral spinal cord segments S2-S4 (Fig. 3). Preganglionic fibres leave the spinal cord via the ventral roots of the sacral spinal nerves; the fibres separate from the spinal nerves and then join together forming the pelvic nerve, *N. pelvinus*. The pelvic nerve divides to form an extensive plexus, *Plexus pelvinus* together with fibres from the hypogastric nerves (sympathetic), on the lateral wall of the rectum (Gosling, J. A., Dixon, J. S. and Jen, P. Y. P., 1999). Ganglia are present in the plexus as well as in the walls of the viscera supplied. Postganglionic parasympathetic fibres innervate the descending colon, urogenital organs and pelvic viscera.

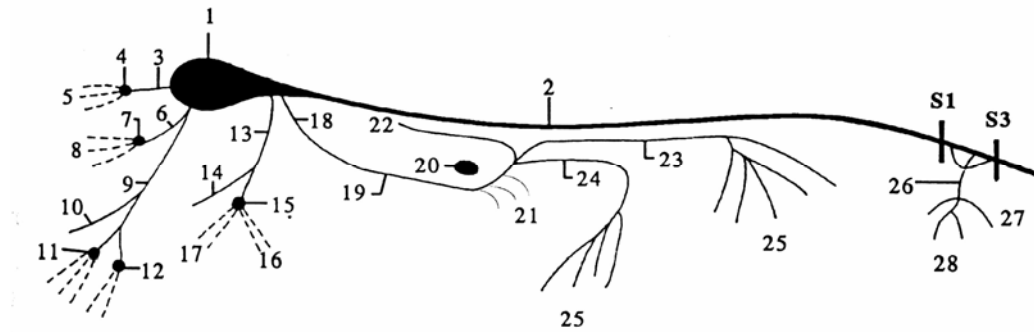


Figure 3: Schematic representation of the parasympathetic division of the autonomic nervous system.

1. brain; 2. spinal cord; 3. *N. oculomotorius*; 4. *Ggl. ciliare*; 5. *Nn. ciliares breves*; 6. *N. petrosus major VII*; 7. *Ggl. pterygopalatinum*; 8. to nasal and lacrimal gland; 9. *Chorda tympani VII*; 10. to rostral 2/3 tongue; 11. *Ggl. sublingualis*; 12. *Ggl. mandibularis*; 13. *N. petrosus minor IX*; 14. to caudal 1/3 tongue; 15. *Ggl. oticum*; 16. to parotid gland; 17. to zygomatic/buccal gland; 18. *N. vagus*; 19. *Tr. vagosympathicus*; 20. *Ggl. cervicale medium*; 21. to thoracic viscera; 22. *N. larynx recur*; 23. *Tr. vagalis dorsalis*; 24. *Tr. vagalis ventralis*; 25. to abdominal viscera; 26. *Nn. pelvini*; 27. *Plexus pelvini*; 28. to pelvic viscera

(From: Bezuidenhout, A. J.; Groenewald, H. B.; Hornsveld, M.; Soley, J. T. and Turner, P. H., 2000).

More recently, it has been determined that the vast majority of all vas deferens projecting autonomic neurons are contained in the paired dorsal root ganglia of L2, L3 and S2, S3 (Gosling, J. A., Dixon, J. S. and Jen, P. Y. P., 1999; Kaleczyc, J., Scheuermann, D. W., Pidsudko, Z., Majewski, M, Lakomy, M. and Timmermans, J. P., 2002) and show a clear ipsilaterally organized projection pattern. Denervation experiments showed that the neurons located within the lumbar dorsal root ganglia send their processes through the ipsilateral hypogastric nerve, whereas those found within the sacral dorsal root ganglia send their processes through the ipsilateral and contralateral pelvic nerve. Furthermore, the pelvic plexus is an undivided structure consisting of interconnected left and right parts in most mammalian species. This study suggests that the processes of the neurons located in the contralateral sacral ganglia probably run first through the contralateral pelvic nerve, cross at the level of the pelvic plexus and finally reach the ipsilateral vas deferens. The crossing point of fibres of neurons found in the lumbar dorsal root ganglia is located above the hypogastric nerve, probably at the level of the caudal mesenteric ganglion. This ganglion consists of two interconnected ganglia located to the left and right of the common trunk of the caudal mesenteric. Such an arrangement with interconnecting branches makes this

prevertebral ganglion ideally suited to act as a crossing point. In a similar vein, a study by Kihara, K and de Groat, W. C. (1997), showed that the lumbar sympathetic pathway to the vas deferens in the rat runs bilaterally and exhibits two crossing points, one at the level of the cranial mesenteric ganglion and one at the level of the pelvic accessory ganglia.

2.2 Neurotransmitters of the autonomic nervous system

Neurotransmitters are the chemical mediators of nerve impulses within the nervous system. Knowledge pertaining to neurotransmitters affects our knowledge of the mechanism of action of drugs at these sites.

The preganglionic neurotransmitter of the autonomic nervous system is acetylcholine (ACH). Postganglionic neurotransmitters, however, differ and the characteristic response of the target tissue depends on the specific neurotransmitter released. The postganglionic parasympathetic neurotransmitter is ACH and the response is described as cholinergic, whereas the postganglionic sympathetic neurotransmitter is noradrenaline (NA) and the response is described as adrenergic.

NA, adrenaline and dopamine are endogenous catecholamines and are the sympathetic neural and humoral transmitter substances in most mammalian species. NA, as the common neurotransmitter at most peripheral sympathetic neuroeffector junctions, is stored in minute granular vesicles in nerve endings. During the resting state, there is a continual slow release of isolated quanta of the transmitter, associated with maintenance of physiological responsiveness of the effector organ. Following stimulation of the adrenergic nerves NA and adrenaline are released in several hundred quanta of neurotransmitter and produce their characteristic responses on receptive tissue. The termination of action of NA and adrenaline occurs by a combination of simple diffusion and reuptake by the axonal terminals into the sympathetic nerve endings, as well as the surrounding tissues (Axelrod, J., Weil-Malherbe, H. and Tomchick, R. 1959). Extra and intraneuronal metabolism of catecholamines also occurs through the enzymatic

degradation by the enzymes catechol-O-methyl transferase (COMT) and monoamineoxidase (MAO), respectively. (Baldessarini, R. J., 2001).

Adrenergic receptors are generally divided in α , β and dopaminergic receptors. The α and β receptors are further subdivided into α_1 , α_2 and β_1 , β_2 receptors, respectively. α_1 -receptors occur on postsynaptic effector cells, especially of smooth muscle. The physiological reaction that results from stimulation includes contraction of all smooth muscle. α_2 -receptors occur both pre- and postsynaptically. Physiological effects include inhibition of noradrenaline release from both central and peripheral adrenergic nerves. Generally, central adrenergic nerves possess postsynaptic α_2 receptors and peripheral adrenergic nerves presynaptic α_2 -receptors. β_1 -receptors occur on postsynaptic effector cells, particularly in the atria, ventricles and conductive tissue of the heart. Stimulation of these receptors causes positive inotropic, chronotropic and dromotropic effects on the heart. β_2 -receptors occur postsynaptically on effector cells of especially the smooth muscle of the bronchi, skeletal muscle arterioles and uterus. Stimulation of these receptors causes smooth muscle relaxation. (Reference: Pharmacology Final Year Class notes, Faculty of Veterinary Science, University of Pretoria)

ACH is the neurohumoral transmitter of all preganglionic autonomic, all postganglionic parasympathetic, a few postganglionic sympathetic fibres and at the neuromuscular junction. ACH is synthesized within cholinergic nerves and stored within axonal vesicular structures. ACH is released from these stores on the arrival of a nerve impulse and causes depolarisation and propagation of the nerve impulse in the second neuron. Once ACH has been liberated it is rapidly metabolized by the enzyme acetylcholine esterase (ACH-E) to acetic acid and choline in the junctional space. ACH-E is present in cholinergic nerves, autonomic ganglia and neuromuscular and neuroeffector junctions. There are two basic types of cholinergic receptors within the peripheral efferent autonomic nerve tracts namely, muscarinic and nicotinic. Muscarinic receptors occur on all postsynaptic effector cells including smooth muscle, heart muscle and

conductive tissue, exocrine glands and central nervous cells. Nicotinic receptor sites are present in autonomic nervous ganglia, adrenal medullary chromaffin tissue as well as the neuromuscular endplates of the somatic nervous system (striated muscle). A nicotinic response usually denotes an excitatory response whereas muscarinic receptor activation may cause an excitatory (GIT) or inhibitory (heart muscle) response.

From the responses of the various effector organs to autonomic nerve impulses and the knowledge of the intrinsic autonomic tone, one can predict the actions of drugs that mimic or inhibit the action of these nerves. In most instances, the sympathetic and parasympathetic neurotransmitters can be viewed as physiologically or functionally antagonistic. If one neurotransmitter inhibits a certain function, the other usually augments that function. Both divisions of the ANS innervate most viscera, including the male genital tract, and the level of activity at any one moment represents the integration of influences of both components. Despite the conventional concept of antagonism between the two portions of the ANS, their activities on specific structures may be either discrete and independent or integrated and inter-dependant. Their actions on male sexual organs are complementary and integrated to promote sexual function. Cholinergic impulses are responsible for erection, whilst adrenergic impulses result in ejaculation.

2.3 Other autonomic neurotransmitters

Although the anatomical separation of the parasympathetic and sympathetic components of the ANS and the actions of the primary neurotransmitters ACH and NA still provide the essential framework of autonomic function, a host of other chemical messengers, the so-called non-adrenergic, non-cholinergic co-transmitters have been identified (Darlison, M.G. and Richter, D., 1999, Lundberg, J.M., 1996; Hokfelt, T., Broberger, C., Xu, Z.Q., Sergeev, V., Ubink, R. and Diez, M., 2000.), demonstrating the possibility of additional peptidergic co-transmission in the autonomic nervous system. These neuropeptides include enkephalins, substance P, somatostatin, cholecystokinin, calcitonin gene-related peptide, galanin (Kaleczyc, J., Scheuermann, D. W., Pidsudko, Z.,

Majewski, M, Lakomy, M. and Timmermanns J-P., 2002), VIP, neuropeptide Y (NPY) (Stjarne, L. and Lundberg, J. M 1986) and nitric oxide (NO) (Jen, P. Y.P., Dixon J. S., Gearhart J. P. and Gosling J. A., 1996). Numerous cell bodies containing colocalised nitric oxide synthetase (NOS) and tyrosine hydroxylase (TH) (a marker for noradrenergic sympathetic neurons) and either NO or TH alone have been identified (Jen, P. Y.P., Dixon J. S., Gearhart J. P. and Gosling J. A., 1996). These additional components play a role in the local and nervous control of the smooth muscle activity in the vas deferens and other genitourinary organs. Many of these peptides are found in the ANS together with ACH or NA and possibly act as neurotransmitters, neuromodulators or neurotrophic factors. It has also been suggested that the presence of SP and/or CGRP and/or GAL in fibres supplying the vas deferens are sensory in nature (Kaleczyc, J., Scheuermann, D. W., Pidsudko, Z., Majewski, M, Lakomy, M. and Timmermanns J-P., 2002). In light of the fact that these neuropeptides are thought to occur in nociceptive nerve cells, it is tempting to assume that the peptidergic neurons transmit nociceptive information from the vas deferens. It is now generally accepted that peripheral processes of sensory neurons provide synaptic collaterals to principal neurons in autonomic ganglia, forming the basis for "axon reflexes" permitting sensory modulation of autonomic neuron activity. Thus, these non-adrenergic, non-cholinergic containing nerve fibres are able to influence the function of the organ by affecting the activity of vas deferens projecting pelvic neurons (Kaleczyc, J., Scheuermann, D. W., Pidsudko, Z., Majewski, M, Lakomy, M. and Timmermanns J-P., 2002). The physiology of peptidergic nervous control and its integration with autonomic control of smooth muscle activity in the genital organs is, however, still poorly understood. Nevertheless, mechanoreceptive and chemoreceptive input that could influence secretory or motor reflex pathways should be considered. Afferents transmitting biochemical or mechanical information from the vas deferens could also influence emission, erection or ejaculation by means of a reflex mechanism. These findings support a dominant adrenergic innervation of the musculature of the vas deferens. (Gosling J. A., Dixon J. S. and Jen P. Y.P., 1999; Jen, P. Y. P., Dixon J.S., Gearhart J. P. and Gosling J. A., 1996) in combination with non-adrenergic, non-cholinergic peptidergic substances which may

have neurotransmitter, neuromodulatory or neurotrophic roles on the predominantly adrenergic innervation.

2.4 Physiology of male sexual function

Three nervous systems are of physiological and clinical significance for normal sexual function in the male. The pudendal nerve (somatic nerve from the sacral CNS), provides motor innervation to the perineal striated muscles as well as sensory innervation of the penis and scrotum. The hypogastric (sympathetic) nerves, supply motor innervation to the vas deferens, the seminal vesicles, the prostate and the bladder neck. The motor innervation operates through α -adrenergic receptors, as discussed for the sympathetic ANS previously. The pelvic (parasympathetic) nerves, supply motor innervation to the corpora cavernosa and corpus spongiosum. The pelvic nerves constitute the major motor input to the penis; however, sympathetic fibres from the thoracolumbar spinal cord also supply the penile vasculature, particularly of the corpus spongiosum. Penile erection is a parasympathetically mediated event, but there is evidence that penile erection may be partially controlled by the sympathetic nervous system, and that postsynaptic α -receptor-mediated smooth muscle contraction participates in the erectile process.

Ejaculation is a complex process consisting of emission into the posterior urethra, bladder neck closure and antegrade ejaculation. Emission is the release of sperm and accessory gland fluid into the pelvic urethra. Ejaculation is the forceful expulsion of these combined fluids from the urethra. The final step in this process, ejaculation, depends on a neural reflex. In this reflex afferent sensory stimuli are initially relayed from the genitalia through the dorsal nerve of the penis. These afferents are carried in the spinothalamic tracts and relay in the thalamus and sensory cortex. The efferent neural fibres travel through the anterolateral column to the thoracolumbar sympathetic outflow, emerging through the sympathetic ganglia. This sympathetic neural output process produces (1) smooth muscle contraction of the tail of the epididymis and the vas deferens, stimulating peristalsis with propulsion of spermatozoa from the cauda epididymis to the ampulla of the vas deferens, and (2) smooth muscle contraction in the

ampulla, prostate, and seminal vesicles with partial closure of the bladder neck, resulting in emission of fluid into the posterior urethra. Further efferent neural control is mediated through the parasympathetic (pelvic nerve) and somatic efferents (pudendal nerve), which causes contraction of the striated bulbocavernosus and ischiocavernosus muscles and associated movements of the pelvic floor muscles. These responses, together with complete closure of the bladder neck, result in ejaculation through the external urethral orifice. Emission is predominantly controlled by the hypogastric (sympathetic) nerve (McDonnell, S. M., 1992).

The unequivocal determination of adrenergic innervation dominance in the male genital system, including the vas deferens, in animals and man has been extensively recorded (Baldessarini, R. J., 2001; Gosling, J. A., Dixon, J. S., Jen, P. Y. P., 1999; Jen, P. Y. P., Dixon, J. S., Gearhart, J. P. and Gosling, J. A., 1996; Kolbeck, S. C. and Steers, W. D., 1993; Wakade, A. R. and Kirpekar, S. M., 1971; Dixon, J. S., Gilpin, C. J., Gilpin, S. A., Gosling, J. A. and Grant, J. F. B., 1987; Arver, S. and Sjostrand, N. O., 1982). Analysis of NA content of various parts of the normal vas deferens reveals a uniform distribution of NA content from prostatic to epididymal end of the tissue, which suggests a uniform distribution of sympathetic fibres all along the vas deferens mediating transport of spermatozoa from epididymis to ejaculatory duct (Wakade, A. R., and Kirpekar, S. M., 1971). Motor responses of isolated human vasa deferentia to selected pharmacological agonists demonstrated dose-dependant excitatory responses only to noradrenaline and adrenaline (McLeod, D. G., Reynolds, D. G. and Demaree, G. E., 1973; Ventura, W. P., Freund, M., Davis, J. and Pannuti, C., 1973). The data suggest that motor innervation is adrenergic in nature and operates through α -adrenergic receptors and that the neurotransmitter in the human vas deferens is noradrenaline.

2.5 Effect of imipramine hydrochloride on the sympathetic nervous system

Iminodibenzyl, the precursor to present day tricyclic antidepressant (TCA) drugs, was developed during the late 1940's (Baldessarini, R. J., 2001). A derivative of iminodibenzyl, imipramine, the first TCA drug developed, was initially classified as a

dibenzazepine and is chemically similar to phenothiazine tranquillisers (Johnson, L. R., 1990). Imipramine was produced in the mid-1950's and was first tested as a medication to control psychoses. Imipramine proved to be ineffective in the treatment of psychotic disorders; however, it demonstrated an ability to correct endogenous depression in human patients. The ability of imipramine to significantly improve clinical depression was documented as early as 1958, and its use for this purpose has continued ever since. Tricyclic antidepressant medications are principally employed in the treatment of endogenous depression. In companion animals, TCA have been used, albeit infrequently, to treat canine narcoleptic hypersomnia syndrome, canine narcoleptic cataplexy syndrome and canine separation anxiety.

Neurotransmission within the ANS has been briefly described above and depicted in Fig. 5, in order to understand the mechanism of action of drugs that act at the level of the neurotransmitter. Each step involved in neurotransmission (Fig 4) represents a possible point of therapeutic intervention.

Imipramine is a dibenzazepine compound that is commonly referred to as a synthetic tricyclic antidepressant (TCA). The proposed mechanism of action is based on inhibition of the re-uptake of biogenic amines such as NA, dopamine and serotonin in the CNS and peripherally (Fig 5). Both central and peripheral adrenergic potentiating effects of IMI are assumed to play a role in enhancement of the emission process. The precise site of action of IMI on emission, however, is not known.

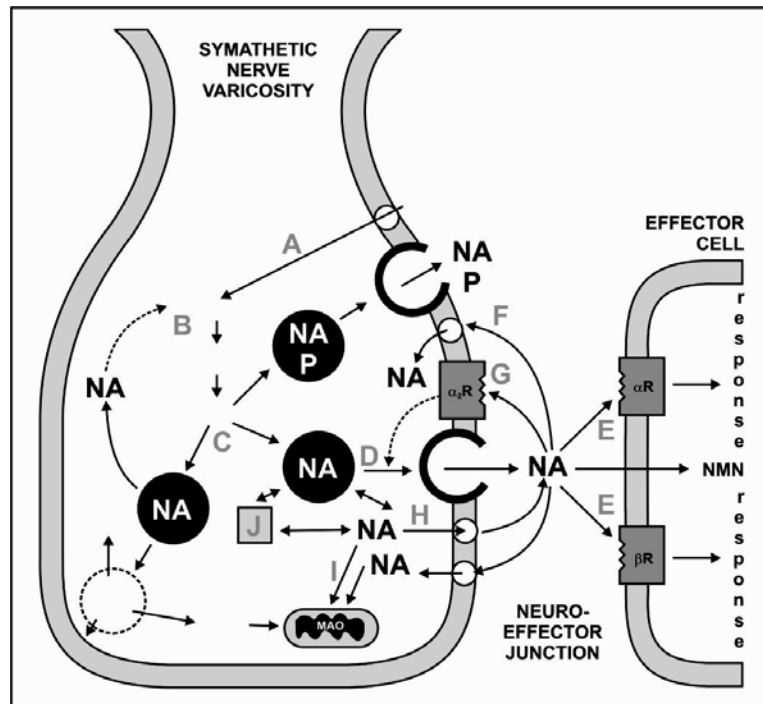


Figure 4: Model of the physiology of neurotransmission at sympathetic neuroeffector junction

Tyrosine is transported actively into the axoplasm (A) and is converted to DOPA and then to dopamine by cytoplasmic enzymes (B). Dopamine is transported into the vesicles of the varicosity, where the synthesis and the storage of noradrenaline (NA) take place (C). An action potential causes the influx of Ca^{2+} into the nerve terminal, fusion of the vesicle with the plasma membrane and exocytosis of NA (D). The transmitter then activates α and β adrenergic receptors of the postsynaptic cell (E). NA that enters postsynaptic cells is rapidly inactivated by catechol-O-methyltransferase to normetanephrine (NMN). The most important mechanism of NA inactivation is the active reuptake into the presynaptic nerve and storage vesicles (F). NA in the synaptic cleft can also activate presynaptic α_2 -adrenergic receptors (G), which has a negative feedback on exocytotic release of NA (dashed line). Other potential neurotransmitters (e.g. ATP and peptides (P)) may be stored in the same or a different population of vesicles.

(modified according to Baldessarini, 2001).

In addition to their transport inhibiting effects, TCAs have variable interactions with adrenergic receptors. The presence or absence of such receptor interactions appears to be critical for subsequent responses to increased availability of extracellular NA in or near synapses. Most TCAs have at least moderate and selective affinity for α_1 -adrenergic receptors, much less for α_2 and virtually none for β receptors.

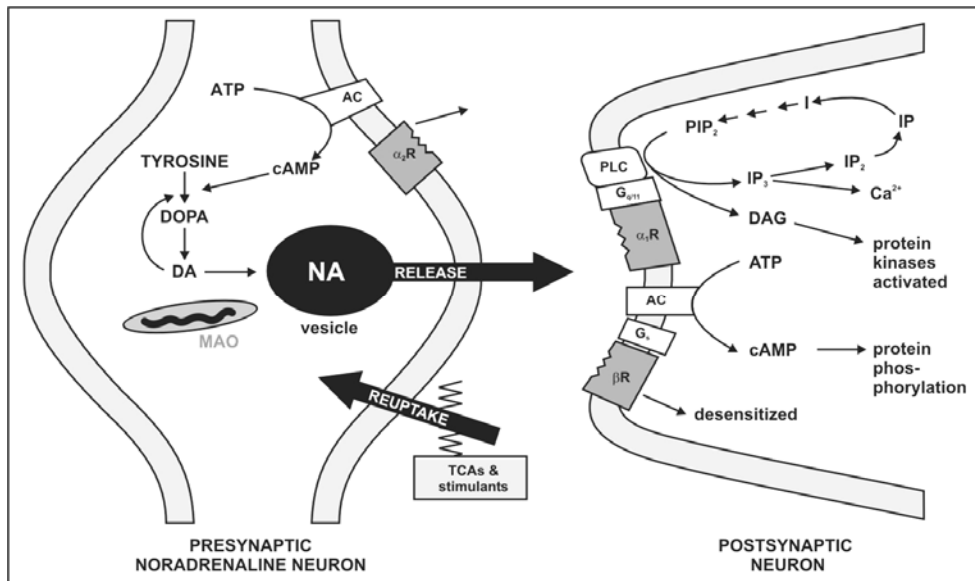


Figure 5: Sites of action of antidepressants

In varicosities, tyrosine is oxidized to dihydrophenylalanine (DOPA) by tyrosine hydroxylase (TH), then decarboxylated to dopamine (DA) by aromatic L-amino acid decarboxylase and stored in vesicles, where side-chain oxidation by dopamine β -hydroxylase ($D\beta H$) converts DA to NA. Following exocytotic release by depolarization in the presence of Ca^{2+} , NA interacts with postsynaptic α - and β -adrenergic receptor subtypes as well as presynaptic α_2 autoreceptors. Inactivation of transsynaptic communication occurs primarily by active reuptake into presynaptic terminals (inhibited by most TCA's and stimulants), with secondary deamination by MAO. Blockade of inactivation of NA by TCA's initially leads to α_2 -receptor-mediated inhibition of firing rates, metabolic activity, and transmitter release from NA neurons, gradually, however, α_2 -autoreceptor response diminishes and presynaptic activity returns. Postsynaptically, β -adrenergic receptors activate adenylylase (AC) through G proteins to convert ATP to cyclic AMP. Adrenergic α_1 receptors activate phospholipase C (PLC) via additional G proteins, converting phosphatidylinositol biphosphate (PIP_2) to inositol triphosphate (IP_3) and diacylglycerol (DAG), with secondary modulation of intracellular Ca^{2+} and protein kinases. Postsynaptic β -receptors also desensitise, whereas α_1 -receptors do not. (modified according to Baldessarini, 2001).

2.6 Pharmacokinetics of imipramine in species other than cattle

(Sallee, F. R. and Pollock, B. G., 1990 (humans); Baldessarini, R. J., 2001 (humans); Peck, K. E., Hines, M. T., Mealey, K. L. and Mealey, R. H., 2001 (horses)).

2.6.1 Absorption and distribution

Absorption after oral administration takes place in the small intestine, with little or no absorption in the stomach due to ionisation. Absorption is rapid and complete (>95%) with peak plasma concentrations occurring 2 to 6 hrs after dosing.

Due to its highly lipophilic nature, IMI and its biologically active metabolite in humans, desipramine (DMI), distributes widely to various tissues. A high degree of peripheral tissue binding results in an apparent volume of distribution (V_d) in the range of 10-20 L/kg for IMI and 10-50L/kg for DMI. This volume of distribution far exceeds plasma volume. The brain accumulates IMI to concentrations 30 to 40 times that in plasma. At steady-state tissue concentrations after long term dosing of IMI, the following ratios of 96:12:3:1 are observed for lung: brain: adipose: plasma (Suhara, T., Sudo, Y., Yoshida, K., Okubo, Y., Fukuda, H., Obata, T., Yoshikawa, K., Suzuki, K., and Sasaki, Y., 1998).

DMI, the major metabolite of IMI in people, is not detected in horses. The $V_{d_{ss}}$ in horses is 0.5 L/kg. The clearance rate of 0.5 L/h/kg is similar for that reported for humans (0.5 - 1L/h/kg). MRT or $t_{1/2}$ of 1.8 hrs is much faster than that reported in people (9.5-20hrs).

2.6.2 Metabolism

IMI and DMI are metabolised almost exclusively in the liver (the first-pass effect in case of oral administration). IMI is mainly eliminated by demethylation to the active metabolite DMI and to a lesser extent by aromatic 2-hydroxylation to 2-hydroxyimipramine. DMI is metabolised by aromatic 2-hydroxylation to 2-hydroxydesipramine. Hydroxylation is the most important intermediate metabolic pathway facilitating clearance of IMI and DMI and it is the rate-limiting step for the elimination of these compounds. The demethylation of IMI and the aromatic 2-hydroxylation of IMI and DMI are carried out by at least 2 different isoenzymes of the cytochrome P450 mono-oxygenase. Metabolism and subsequent glucuronide conjugation are required for significant elimination of IMI and DMI via the urine. Competitive inhibition of hydroxylation of IMI and DMI may occur with concurrent use of drugs utilizing the same metabolic pathways or drugs inhibiting the cytochrome P450 mono-oxygenase system (eg. neuroleptics and cimetidine). This may lead to significant rise in plasma concentration of IMI and DMI, resulting in increased incidence of side effects or toxic conditions. Drugs inducing the cytochrome P450 system increase

clearance rates of IMI and DMI. The extent of the first-pass effect (liver metabolism in case of orally administered drugs) is determined by genetic differences/phenotype between individuals/subjects. Thus, genetic and environmental factors may influence plasma drug levels of IMI and DMI and occurrence of side effects/toxic conditions. In human subjects many environmental factors (smoking, alcohol, age) significantly alter IMI and DMI pharmacokinetics.

2.7 Clinical experience using imipramine

Clinical use of imipramine to treat ejaculatory dysfunction in man and stallions is based on the knowledge that ejaculation is mediated by combined autonomic and somatic innervation, originating at the sacral and thoracolumbar spinal cord levels (McDonnell, S. M., 1992) and that seminal emission is controlled by α -adrenergic mechanisms (Lipshultz, L.I., McConnell, J.A. and Benson, G.S., 1981; Ventura, W.P., Freund, M. Davis, J. and Pannuti, C., 1973; McLeod, D. G., Reynolds, D. G and Demaree, G. E., 1973).

Most recent animal studies utilizing pharmacological manipulation of sexual function using imipramine, has been done in the stallion (Oristaglio Turner, R. M., Love, C. C., McDonnell, S. M., Sweeney, R. W., Twitchell, E. D., Habecker, P. L., Reilly, L. K., Pozor, M. A. and Kenney, R. M., 1995; Brinsko, S. P., 2001; Card, C. E., Manning, S. T., Bowman, P. and Leibel, T., 1997; Brookes, M. E., Berezin, M and Braf, Z., 1980; Johnston, P. F. and DeLuca, J. L., 1998; McDonnell, S. M., Garcia, M. C. and Kenney, R. M., 1987; McDonnell, S. M. and Odian, M. J., 1994; McDonnell, S. M., 1992). Imipramine induces spontaneous erection and ejaculation in the absence of a sexual stimulus in stallions. Spontaneous ejaculates induced with IMI have lower total volumes, with higher concentration and total numbers of sperm (McDonnell, S.M. and Odian, M.J., 1994; McDonnell S.M and Oristaglio Turner, R.M., 1994; McDonnell, S.M., 2001), suggesting increased emission of the sperm rich fraction and reduced emission of accessory gland fluids. Pregnancies in mares have resulted from IMI and xylazine induced ex-copula ejaculates (Card, C.E., Manning, S.T., Bowman, P. and Leibel, T. 1997).

Return to normal sexual function in human male patients using imipramine, has been extensively reported (Gilja,I., Parazajder, J., Radej, M., Cvitkovic, P. and Kovacic, M., 1994). Retroperitoneal lymphadenectomy, used in the treatment of testicular cancer, historically resulted in extensive sympathetic nervous system damage or injury. Many patients suffered from retrograde ejaculation or loss of emission post surgery. Treatment with imipramine converted patients with emission impairment to anterograde ejaculation (Gilja,I., Parazajder, J., Radej, M., Cvitkovic, P. and Kovacic, M., 1994; Nijman, J. M.; Schraffordt Koops, H.; Oldhoff, J., Kremer, J. and Jager, S., 1987).

Chapter 3

Pharmacokinetics of intravenous imipramine hydrochloride in cattle

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3.1 Introduction

There is no reported pharmacokinetic data for imipramine hydrochloride in cattle or the use of imipramine in cattle. This study was developed to determine pharmacokinetics of imipramine in cattle, preceding the determination of *in vitro* effects of imipramine on the vas deferens in cattle. Observation for spontaneous ejaculation at the initial dose used for the pharmacokinetic study would allow an early subjective assessment of the use of imipramine for semen collection purposes. The dose of IMI used in this pharmacokinetic study was extrapolated directly from doses reportedly used in stallions for purposes of semen collection.

3.2 Material and methods

Imipramine hydrochloride (IMI) 200mg/ml (Centre for Pharmaceutical Services, Technikon Pretoria, South Africa) was administered as a bolus injection intravenously to three bulls (600 - 705.5 kg) at a dose of 2mg/kg body BW. Blood samples were collected over time from the contralateral jugular vein. Plasma was harvested by centrifugation, transferred into polypropylene vials and frozen at -20 °C. Stored, frozen samples were thawed immediately prior to analysis.

Plasma concentrations of imipramine were determined over time by fluorescence polarization immunoassay (FPIA). The method was validated for cattle by spiking plasma collected from healthy, non-treated bulls with imipramine of known concentration. The TCA bioassay technique (Abbott Laboratories, Diagnostic Division, Abbott Park, IL,

USA) used, gave satisfactory limits of sensitivity. The lower and higher limits of sensitivity were 20 and 1000 ng/mL, respectively. The test is specific for IMI, with an 84.7 - 90 % cross-reactivity for desipramine (DMI), the biologically active metabolite of imipramine. Additional compounds, except DMI, having a marked cross-reactivity with IMI were not present in plasma at the time of the study.

Using statistical moment analysis, area under the plasma concentration vs. time curve (AUC , zero moment) and the first non-normalized moment ($AUMC$) were calculated according to the trapezoidal method from time zero to the last sampling time and both extrapolated to infinity using the slope (β) of the terminal phase. Mean residence time (MRT , first moment) was derived from $AUMC_{(\infty)} / AUC_{(\infty)}$. The biological half-life ($t_{1/2\beta}$) was calculated by $t_{1/2\beta} = 0.693/\beta$, where β was the slope of the terminal portion of the curve determined by linear regression of the last 4-5 points of the curve (Gibaldi, M. & Perrier, D., 1982; Yamaoka, K., Nakagawa, T., Uno, T., 1978).

3.3 Results

Following intravenous administration, IMI distributed rapidly (Fig 6) from the central compartment with a mean distribution half-life ($t_{1/2\alpha}$) of 7.2 ± 4.2 min, IMI exhibited a very large apparent steady-state volume of distribution ($V_{d_{ss}}$) of 4.2 ± 0.9 L/kg BW, had a very short terminal elimination half-life ($t_{1/2\beta}$) of 140 ± 15 min and showed a rapid total body clearance (CL) of 22.7 ± 7 ml/min/kg.

The first-order rate constants of elimination indicate that elimination of IMI was linear at the dose of 2 mg/kg administered to the cattle.

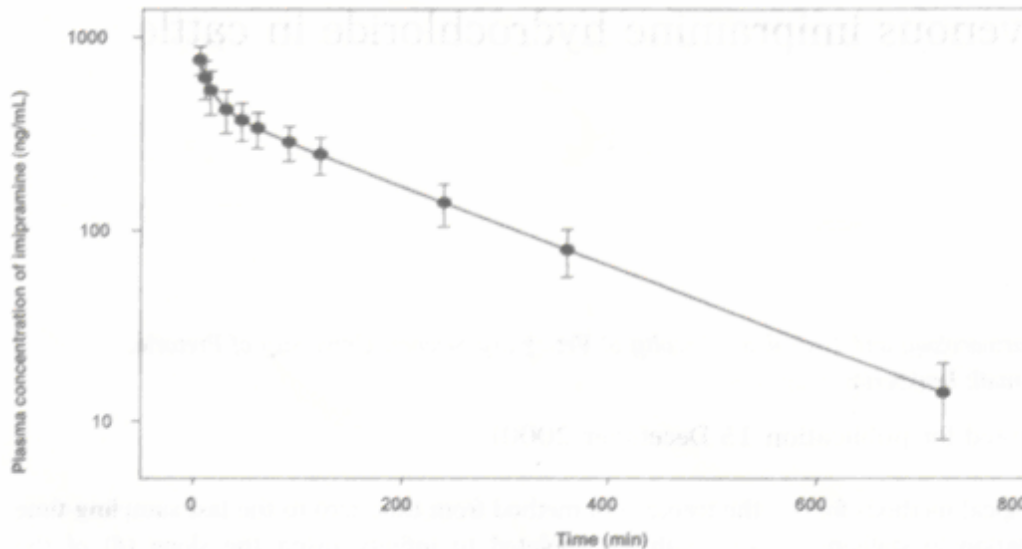


Figure 6: *Mean plasma concentration of imipramine following iv administration (2mg/kg) in cattle*

3.4 Discussion

The mean volume of distribution of IMI (4.2 ± 0.9 L/kg) suggested an extensive peripheral distribution of IMI. However, this was considerably less than the 10-20 L/kg for IMI and 10-50 L/kg for DMI reported in humans (Sallee, F. R. and Pollock, B. G., 1990; Baldessarini, R. J., 2001). Such extensive peripheral distribution is attributed to high lipid solubility and low plasma binding. Furthermore, the low k_{21}/k_{12} (mean = 0.67) ratio suggests that the drug moves freely from the central compartment and is retained for longer periods in the peripheral compartment. Although TCAs (including imipramine) are widely bound in all peripheral tissues, they preferentially bind to myocardial, hepatic, pulmonary and brain tissue (Johnson, L. R., 1990; Besret, L., Debruyne, D., Rioux, P., Bonvalot, T., Moulin, T., Zarifian, M. and Baron, J-C., 1996).

The mean biological half-life ($t_{1/2\beta} = 140 \pm 15$ min) suggests that IMI was rapidly eliminated in cattle. This is similar to that reported in rats (Besret, L., Debruyne, D., Rioux, P., Bonvalot, T., Moulin, T., Zarifian, M. and Baron, J-C., 1996), but much shorter than that reported in humans (Baldessarini, R. J., 2001). The mean clearance value of 22.67 mL/min/kg attests to the fast rate of elimination of IMI in cattle and was

slightly faster than that reported for humans (15 ± 4 mL/min/kg) ((Sallee, F. R. and Pollock, B. G., 1990).

As a result of rapid distribution and high affinity of IMI to central nervous system and other peripheral tissue compartments (including the vas deferens), elimination half-life of IMI from these tissue sites must be considered when establishing parameters for the purposes of clinical uses/trials involving repeat treatments, where it is important that no residual drug effect remains at the peripheral tissue compartment. From studies in the rat, elimination half-lives of IMI from brain tissue is similar to those for serum (Besret, L., Debruyne, D., Rioux, P., Bonvalot, T., Moulin, T., Zarifian, M. and Baron, J-C., 1996). As available data from IMI studies in rats is more closely correlated to the data found in this study than data relating to IMI kinetics in humans, we will assume that as for the rat, elimination half-life of IMI from brain and other peripheral tissues is similar to that from serum in bulls. Thus, elimination of IMI from plasma can be equated to peripheral tissue elimination.

As imipramine is rapidly metabolised to DMI *in vivo* in humans (Sallee, F. R. and Pollock, B. G., 1990) and rats (Besret, L., Debruyne, D., Rioux, P., Bonvalot, T., Moulin, T., Zarifian, M. and Baron, J-C., 1996) and because DMI is the biologically and pharmacologically active metabolite of IMI, with similarly high volumes of distribution and high peripheral tissue concentrations, DMI $t_{1/2\beta}$ can influence the pharmacological action of IMI. Desipramine is formed by demethylation of imipramine and is a pharmacologically active metabolite and is believed to contribute substantially to the therapeutic efficacy of imipramine in humans (Sallee, F. R. and Pollock, B. G., 1990). Other routes of metabolism for imipramine include aromatic 2-hydroxylation and dealkylation reactions. Interestingly, desipramine, the major metabolite of imipramine in humans, was not detected in any samples obtained from horses (Peck, K. E., Hines, M. T., Mealey, K. L. and Mealey, R. H., 2001). Desipramine was not directly measured in this study in cattle and may not even be present, as is the case in horses. Should cattle similarly metabolize IMI to DMI *in vivo*, or the elimination half-life of DMI exceed that of IMI, it would be

important to consider this in establishment of a treatment interval for IMI. The elimination half-life of DMI is reported to be longer than that for IMI in rats (Besret, L., Debruyne, D., Rioux, P., Bonvalot, T., Moulin, T., Zarifian, M. and Baron, J-C., 1996), but does not exceed 27 h in any peripheral tissue examined. In addition, the high cross-reactivity for DMI in the assay used in this study shows that both IMI and its metabolite are negligible in serum by 24 h. Based on this, a treatment interval of at least 23 h between repeat treatments of IMI, representing a period of at least 10 half-lives, can be recommended.

Mean Cl of imipramine in cattle is much greater than values reported for horses (8.7 ml/kg/min) (Peck, K. E., Hines, M. T., Mealey, K. L. and Mealey, R. H., 2001) and humans (15ml/kg/min) (Sallee and Polloc). Although the route of elimination of imipramine in cattle and horses is unknown, it is speculated that the slower rate in horses reflects a slower rate of hepatic metabolism (Peck, K. E., Hines, M. T., Mealey, K. L. and Mealey, R. H., 2001). Furthermore, the fact that desipramine is not detected in serum samples in horses suggests that another metabolic pathway exists in horses, compared with that in people. The presence or absence of desipramine in serum samples in cattle has not been determined and the metabolic pathway of imipramine in cattle can thus only be speculated. Further studies are needed to elucidate the route of elimination of imipramine in horses, as well as in cattle. A genetic polymorphism for the oxidative metabolism of imipramine is believed to exist in humans (Sallee, F. R. and Pollock, B. G., 1990). The limited variability in the clearance values of imipramine in horses (8.7 ± 1.7 ml/kg/min) suggests that no such polymorphism exists within the species. Clearance variability of imipramine in cattle (22.7 ± 7 ml/kg/min) approximate those of people (15 ± 4 ml/kg/min) more closely, suggesting the existence of polymorphism and hence interindividual variability with regard to imipramine clearance in cattle. Polymorphism or individual biological variation among animals is another important factor that may influence half-life of imipramine. A range of plasma elimination times from 10 -21 hours have been reported in human patients (Baldessarini, R. J., 2001).

The most significant clinical effect seen in cattle immediately post administration of 2mg/kg bw IMI were pronounced CNS signs, including generalized weakness and ataxia. Signs are presumably related to the rapid distribution of IMI to peripheral tissues particularly, amongst others, the brain. All central nervous system signs dissipated 15-20 min post injection and should therefore not influence the treatment interval. Due to rapid and extensive distribution of IMI to peripheral tissues, with preferential binding to brain tissue, amongst others, these clinical signs should be expected. Notably and probably due to the rapid clearance rate of IMI in cattle and other species, CNS signs dissipate within 15-20 min post injection. This clinical phenomenon supports the reported rapid clearance rate of IMI from serum and peripheral tissue alike. CNS signs are expected to be dose dependant, becoming negligible or non existent at doses lower than 2mg/kg BW. High serum concentrations of imipramine are associated with a number of adverse effects in humans, including cardiac dysrhythmias, seizures, tremors, hypotension and blurred vision (Sallee, F. R. and Pollock, B. G., 1990). Similar adverse effects of ataxia and drowsiness are reported in horses (Peck, K. E, et al., 2001, McDonnell, S. M., et al., 1987; McDonnell, S. M. and Odian, M. J., 1994, Johnson, L. R.,1990).

Plasma discoloration of the blood samples was also observed in this trial. Macroscopic observation of the discoloured plasma was suggestive of haemolysis. Initial high performance liquid chromatography studies, however, are suggestive of myoglobin as being the cause of plasma discoloration. Further analysis is required to substantiate this finding. At high concentrations, imipramine causes lysis of erythrocytes and hepatocytes in rats by altering membrane fluidity (Yasuhara, H., Tonooka., and Kamei, K., 1985). A similar mechanism could result in lysis of erythrocytes in cattle.

Spontaneous emission and ejaculation was not observed in cattle at the 2 mg/kg BW dose used in this study. We, therefore, conclude from this study that doses of 2 mg/kg BW, although not fatal, have moderately severe CNS side effects, without enhancing emission and lower doses should be used when emission of a concentrated semen sample

is the objective. Studies supporting fascilitatory effects of imipramine, report such effects at doses of ≤ 1 mg/kg BW, with higher doses having paradoxical (inhibitory) effects (Sigg, 1959).

From the pharmacokinetic parameters determined in this study, the time to optimal effect (emission and ejaculation) post treatment of IMI given intravenously in cattle, based on the distribution half-life, is 7.2 ± 4.2 min and the treatment (dosing) interval, based on the elimination half-life of intravenously administered imipramine in cattle is 23 hours, or for practical purposes, once daily (oid). Additionally, a dose of 2 mg/kg BW is paradoxical to enhancement of emission and ejaculation and in the light of other studies reporting similar inhibitory effects of IMI at higher doses, while doses < 1 mg/kg are more likely to have fascilitatory effects, it seems logical to reduce the dose for clinical use to ≤ 1 mg/kg BW.

Chapter 4

In vitro effects of imipramine hydrochloride on the vas deferens of cattle

4.1 Introduction

The major pharmacological action of imipramine is to potentiate the actions of biogenic amines such as norepinephrine, dopamine and serotonin in the CNS, and peripherally it blocks the reuptake of these amines at nerve terminals (Baldessarini, R.J., 2001). Consequently, availability of these biogenic amines increases at nerve terminals, resulting in greater postsynaptic excitation. Imipramine has a narrow therapeutic index, with the recommended therapeutic serum concentrations for humans ranging from 50 to 300 ng/ml (Baldessarini, R. J., 2001).

Imipramine is one of a number of pharmacological agents that is thought to affect genital smooth muscle activity (McDonnell, S. M., Garcia, M. C. and Kenney, R. M., 1987). Imipramine has been used as an alternative to conventional semen collection techniques to collect semen from stallions that as a result of physical impairment or psychological sexual behaviour dysfunction, are unable to ejaculate *in copula* (McDonnell, S. M. 1992; McDonnell, S. M. and Oristaglio Turner, R. M., 1994), and to treat retrograde (Brookes, M.E., Berezin, M. and Braf, Z., 1980; Brinsko, S.P., 2001; Gilja, I., Parazajder, J., Radej, M., Cvitkovic, P. and Kovacic, M., 1994) and premature ejaculation (Mitchell, J.E. and Popkin, M. K., 1983) in man. Human patients suffering from nonseminomatous testicular tumours (NSTT) used to be treated largely by bilateral retroperitoneal lymph node dissection (RLND). Postoperatively, most of these patients suffered loss of antegrade ejaculation, due to removal of or damage to the sympathetic ganglia and hypogastric plexus during RLND. This resulted in retrograde ejaculation or loss of fluid emission into the urethra (Nijman, J. M., Schraffordt Koops, H., Oldhoff, J., Kremer, J. and Jager, S., 1987). These patients could be treated with α -sympathomimetic drugs such as imipramine HCl and thus be offered the chance of fatherhood by coitus (Nijman, J. M., Schraffordt Koops, H., Oldhoff, J., Kremer, J. and Jager, S., 1987).

Treatment with imipramine or detomidine appear to enhance contraction of the ampullae, inhibit contractions of the accessory sex glands, both resulting in ejaculates of lower volume, higher sperm concentration, and higher total numbers of sperm, and lower pH compared to *in copula* ejaculates (McDonnell, S. M. and Odian, M. J., 1994; McDonnell, S. M. and Oristaglio Turner, R. M., 1994; Rowley, D. D., Lock, R. F. and Shipley, C. F., 1999). In stallions good results have been reported with the oral use of imipramine to induce ejaculation (Johnston, P. R. and DeLuca, J. L., 1998).

Pharmacological experiments on isolated tissue preparations to understand and record effects of drugs on specific tissues or organs have been used for more than four decades. Preparations of the guinea-pig vas deferens to test the effects *in vitro* of compounds in organ baths have been used since the 1960's (Hukovic, S 1961; Livingstone, E & S., 1970). Single and multiple combinations of drugs have been tested with or without endogenous substances. These experiments showed that the sensitivity of the Guinea pig vas deferens in response to adrenaline or noradrenaline varies considerably with age, with younger animals being up to 10 times more sensitive. The receptors associated with this response are classified as being of the α -type. Concentrations of adrenaline and noradrenaline required to produce contractions are known from these experiments (Livingstone, E & S., 1970). Actual studies to quantify and qualify the *in vitro* effects of drugs like imipramine HCL on the vas deferens have not been reported.

The aim of this study was to test the *in vitro* effects of imipramine on vas deferens tissue of bulls.

4.2 Materials and methods

4.2.1 Collection and preparation of ampullar strips

The vasa deferentia of 16 post puberal domestic bulls of various breeds were collected over a period of 4 months at a local slaughterhouse. After slaughter and evisceration, the vasa deferentia were dissected from the ampullae to the cauda epididymides. The vas deferens with surrounding connective tissue were placed in a zip-lock bag, covered

with crushed ice in a styrofoam cooler box and transported 75 km to the School of Physiology, School of Health Sciences, University of the Witwatersrand.

On arrival, the vas deferens was dissected free from the surrounding connective tissue. A 4 mm glass rod was introduced into the lumen of the ampulla and the tissue was cut longitudinally into two equal strips. Each strip half was again cut in half to provide 4 equal longitudinal strips of the ampulla, 5-6 cm in length. Cotton threads were tied to each end of two of the strips. One was used as a control while the other one was used to test the effects of imipramine. Only one ampulla from a single bull was used on any particular day.

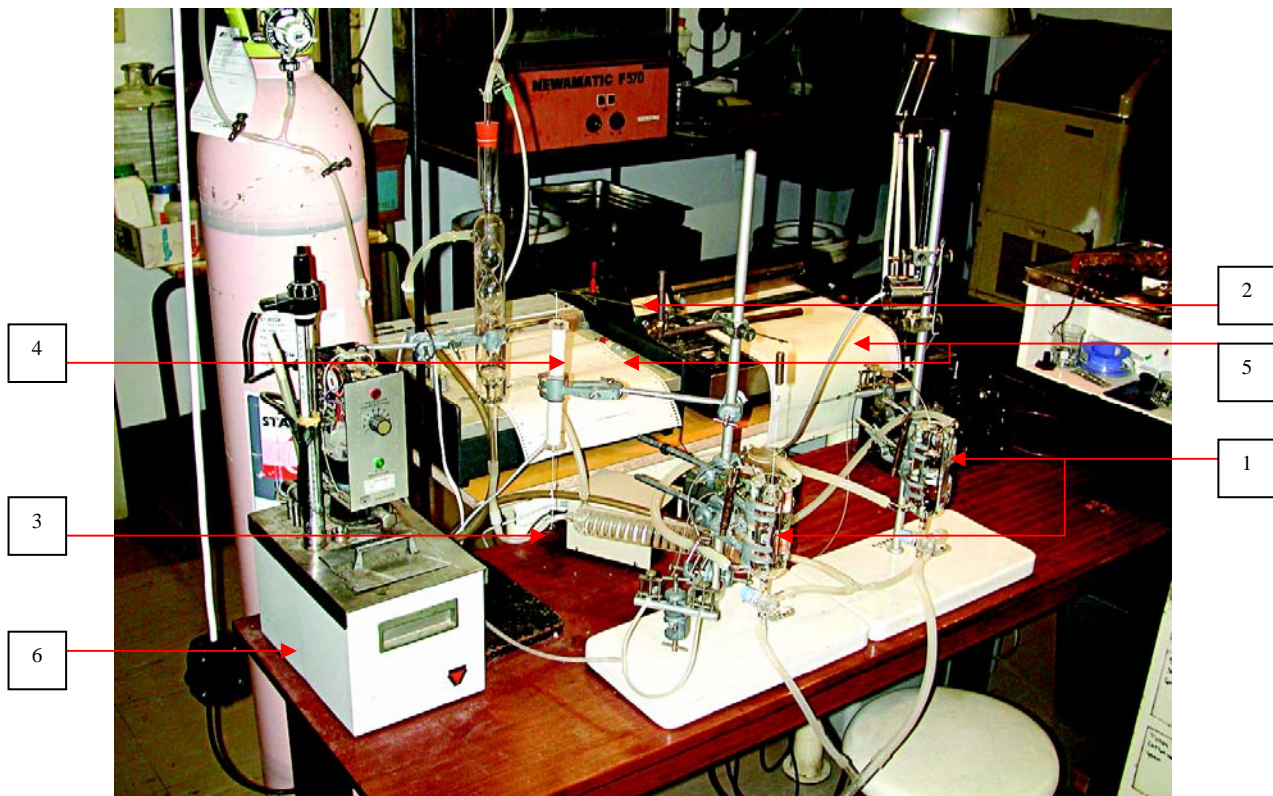


Figure 7: *Overview of in vitro laboratory set up.*

1. Water-jacketed organ baths, 2. lever arm, 3. 1,5 - 3 g weight attached to the lever, applying tension to tissue, 4. Electronic recording device measuring tissue contraction/relaxation and connected to 5. Potentiometers recording an electronic signal, 6. Thermostat.

4.2.2 Organ bath procedure for ampullar strips

Each strip was mounted in a separate water bath. These were jacketed glass baths and water temperature was thermostatically maintained at 37 °C. Baths contained 20 ml modified Kreb's bicarbonate solution, which was aerated continuously with a mixture of oxygen (95 %) and carbon dioxide (5 %). The one thread of the strip was tied to a fixed pin at the bottom of the bath, while the top thread was tied to a lever with loads of between 1,5 - 3g attached, applying tension on the strips. The strips were then allowed to equilibrate under the tension of the weights for 30-40 min until a constant length was recorded on the graph paper. Movement of the lever was measured electronically and the signal recorded on two flat bed potentiometer recorders (JJ Lloyd Instruments Ltd, Brook Ave, Warsash, Southampton SO3 6HP, England) one for each bath (Fig 7-8).

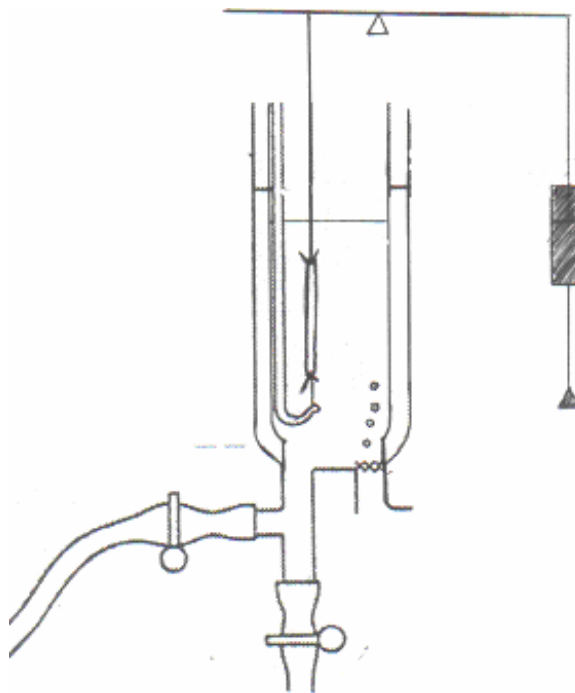


Figure 8: *Schematic representation of organ bath*

Ampulla strips are mounted in Kreb's solution aerated with a mixture of oxygen (95%) and carbon dioxide (5%). The thread attached to one end of the ampulla is tied to a fixed pin and the thread attached to the other end is tied to lever with a load of 1,5 - 3g. Temperature water in the jacket is maintained at 37 °C. The volume of the bath is 20 ml.

4.2.3 Addition of noradrenaline and imipramine

A routine procedure was used to stimulate the strips in the organ baths on each particular experimental day. The required dose and concentration of NA varied between strips from different animals. To establish the required concentration and dose of NA for each set of strips, NA was added to both the control and experimental strips at different doses (0.05 ml, 0.1 ml, 0.2 ml, 0.4 ml, 0.8 ml) and concentrations (10^{-6} , 10^{-5} , 10^{-3}). Depending on the response elicited to initial NA addition, concentration and dose of NA was adjusted. After 5 min of recording, the bath was drained and new warm Krebs's solution added. Once strips had returned to pre-stimulation length the next NA concentration and dose was added, until a dose-response curve to NA was established. The dose halfway between the minimum and maximum response was used then for the remaining part of the experiment. IMI was only added once consistent results after NA application had been achieved. In order to simulate the clinical application of IMI, amounts added have been converted to mg/kg body weight equivalent (bwe).

A further NA stimulation at the determined dose was applied to provide control contractions. After replacing the buffer and equilibration, increasing volumes of a 1:10 000 IMI (0,1ml equivalent to 0,1mg/kg in a 1000kg bull) solution was added to the experimental bath only. Ten minutes later NA was added to both the baths without buffer replacement and the contractions recorded. Paper speed was 5 mm/min.

4.3 Results

Fig 9 shows the typical pattern observed when ampullar strips are stimulated with increasing amounts of NA. An increased muscle tone occurred with superimposed rhythmic contractions. The response of the specimens varied in sensitivity from day to

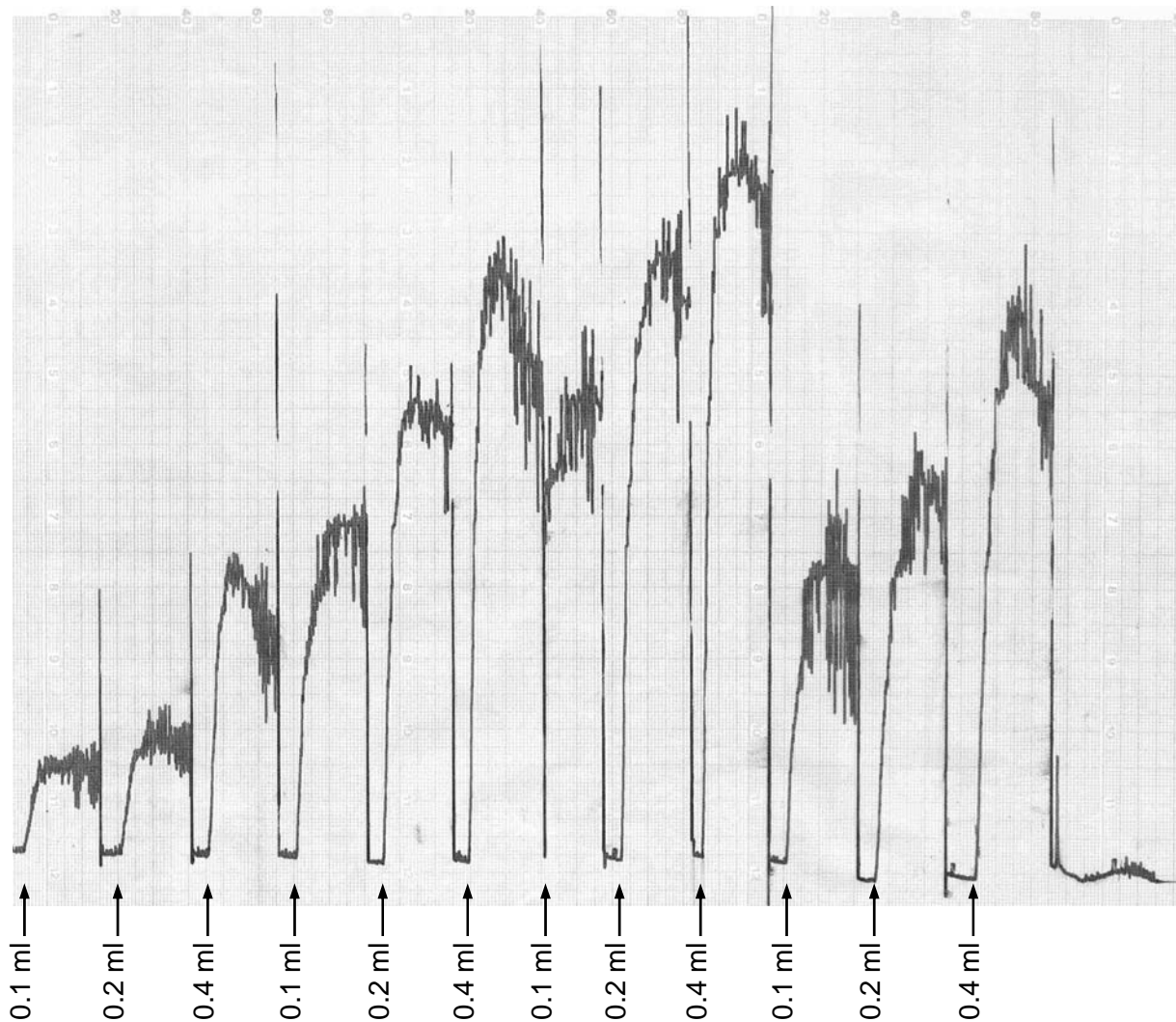


Figure 9: *Repeatable, dose dependant, ampullar responses to NA*

Affects of increasing volumes of a $10^{-6}M$ of NA on a bull ampullar strip.

day with some responding to 0,001 molar while others already responded to 0,000001 molar NA solutions. As can be seen from Fig 10 there was clearly also cumulative effect from previous stimulations when the volume of NA solution was reduced from 0,4 to 0,1 ml. Once stabilized, however, a constant dose of NA produced a consistent result. Virtually no spontaneous motility was observed.

Fig 10 shows the effect of a low dose (0,05 mg/kg bwe) IMI on an ampullar preparation. Immediately after application (provide time on the graph Claudia) IMI induced no effect. The tissue response to IMI was only observed after the second application of NA reaching maximum effect after the 3rd to 4th NA application. The change was an increase in muscle tone and especially amplitude of rhythmic contractions. Application

of 0.1 mg/kg bwe during a NA-induced contraction period produced an even greater effect on amplitude of rhythmic contractions.

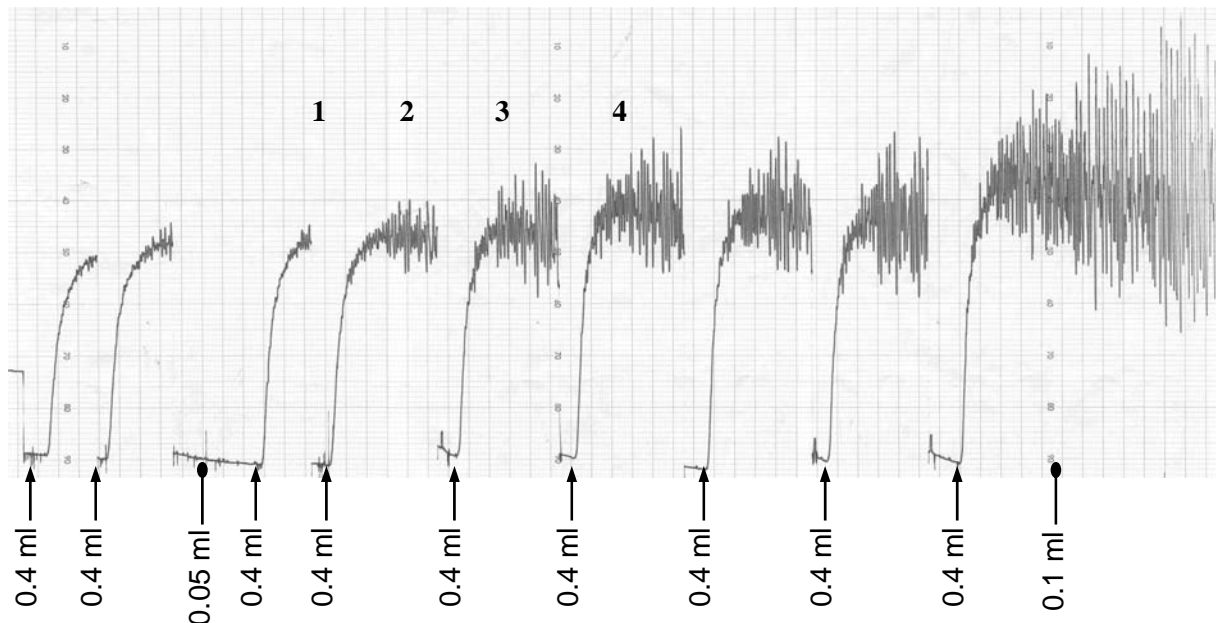


Figure 10: *NA and low dose IMI effects in vitro on ampullar strips*

NA ($10^{-5}M$) potentiating effects of a low dose of IMI ($0.05mg/kg$ bwe; $0.05ml$) and the effect of $0.1mg/kg$ bwe ($0.1ml$) application during a NA-induced contraction wave.

The tracing in Fig 11 was made after a single addition of IMI ($0,1mg/kg$ bwe) and sequential additions of $0,4ml$ $0,00001mol$ NA. The effect of IMI is only evident after the 3rd NA addition reaching maximum effect after the 4th. After the 9th addition of NA the tissue was not flushed. This resulted in a sustained contraction, a gradual decrease in baseline muscle tone but ever increasing amplitudes of rhythmic contractions.

In Fig 12 the effects of $0,5$ and $1mg/kg$ bwe IMI can be seen on NA-stimulated ampullar tissue. This particular specimen had a lower muscle tone to start with when stimulated by NA alone. Addition of $0,5mg/kg$ bwe slightly decreased muscle tone and increased contraction amplitude. At the same time contraction frequency decreased. Thereafter, from NA applications 1-5, contraction frequency slowly recovered. The addition of $1mg/kg$ bwe partially blocked the effect of NA with only a single contraction after the first application and a few more after the second.

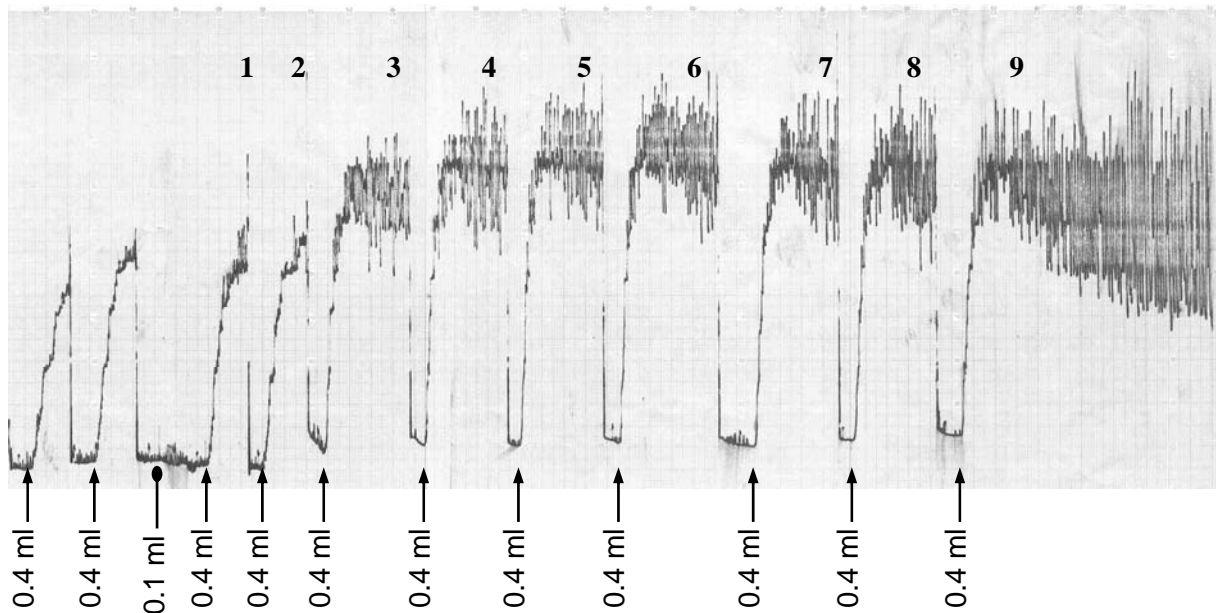


Figure 11: NA and IMI (0.1 mg/kg bwe) effect in vitro on ampullar strips

Low dose IMI (↑) (0.1mg/kg bwe; 0.1 ml) on NA ($10^{-5}M$ (↑)) potentiating effects. An increase in muscle tone and amplitude of rhythmic contractions is seen.

Increasing the dose of IMI sequentially from 0,1 to 4 mg/kg bwe on another ampullar preparation produced similar effects (Fig 13). A dose of 0,5 mg/kg bwe IMI reduced

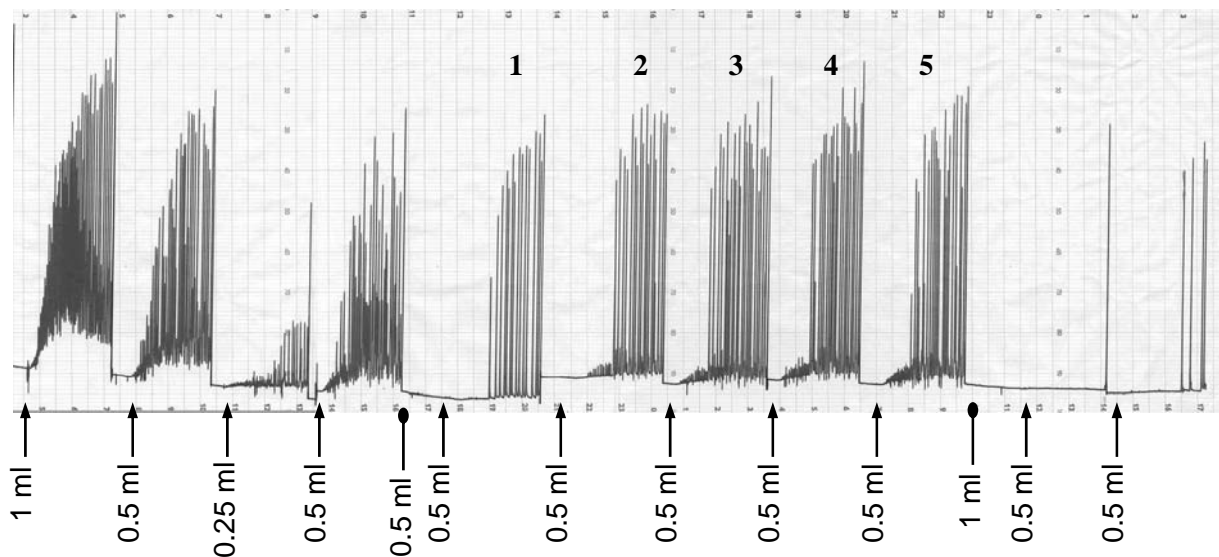


Figure 12: NA and IMI (0.5 mg/kg bwe) effects in vitro on ampullar strips

Addition of 0,5ml IMI (↑) (0.5 mg/kg bwe) decreases overall muscle tone and frequency of contractions while increasing amplitude of rhythmic contractions. At 1 mg/kg bwe (1 ml) the tissue is partially blocked to NA stimulation (↑).

muscle tone as shown in Fig 14 to a consistent level after the 3rd NA addition. Amplitudes of rhythmic contracts were initially increased but gradually returned to pre-IMI addition. A further addition 1 mg and then 2 mg/kg bwe stepwise reduced muscle tone and contraction frequency until the tissue was finally unresponsive to NA stimulation.

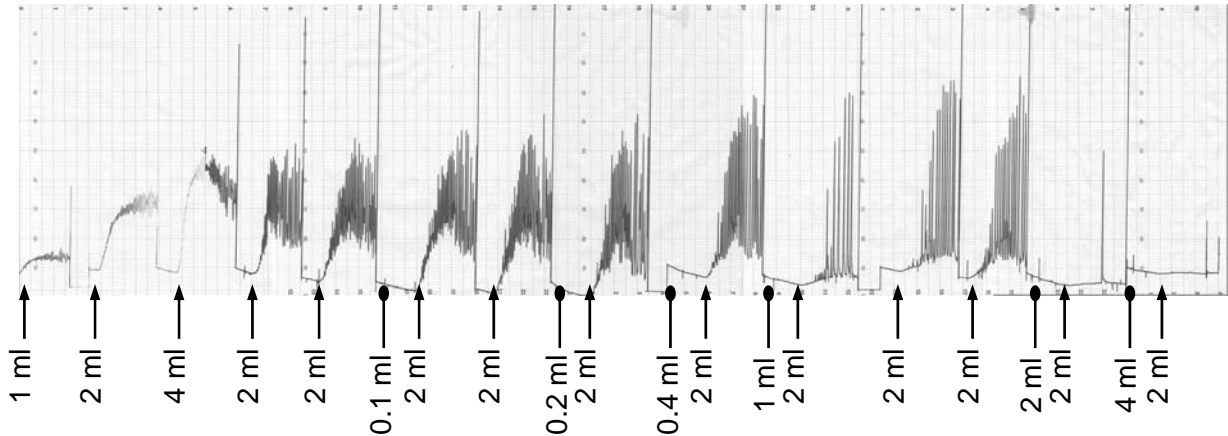


Figure 13: *NA and high dose IMI effects in vitro on ampullar strips*
Effects of increasing doses of 0.1 to 4mg/kg bwe (0.1 to 4 ml) of IMI (↓) on response to NA 10⁻⁵M(↑)

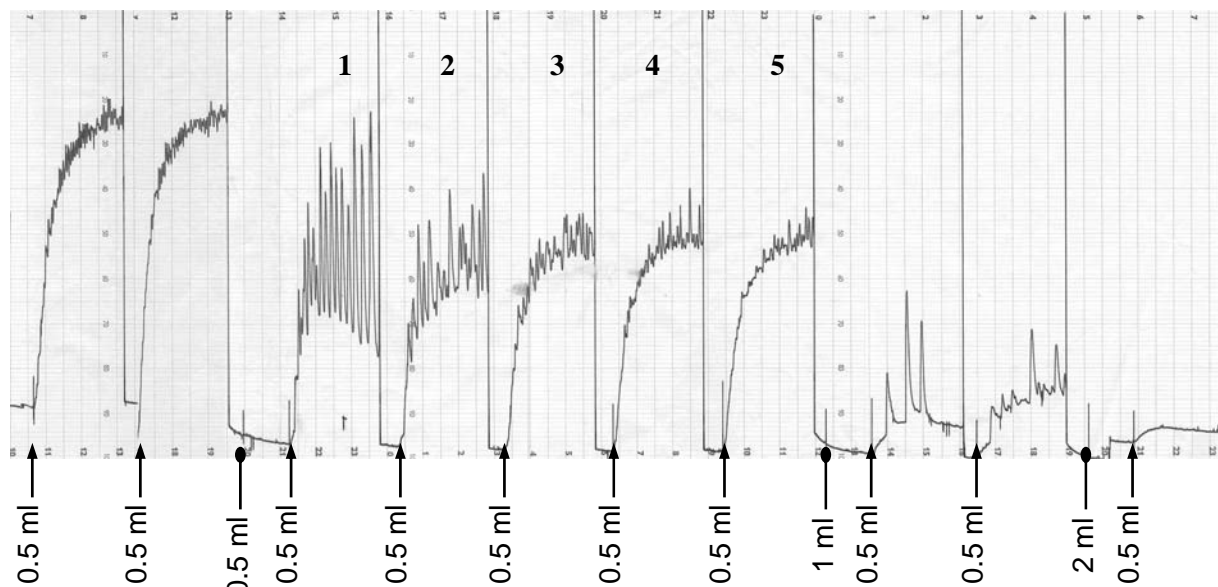


Figure 14: *NA and IMI (0.5, 1.0 and 2.0 mg/kg bwe) effects in vitro on ampullar strips*
Effect of 0.5 mg/kg bwe (0.5 ml) on ampullar response to 0.5 ml NA 10⁻³M(↑) stimulation. Increased doses lead to a partial suppression of contractions (1 ml; 1 mg/kg bwe) and finally the tissue becomes totally unresponsive at 2 mg/kg bwe (2 ml)

Time taken to recover from the blocking effect of IMI after repeated buffer changes and NA stimulation is shown in Figs 15 and 16. In both cases a dose of 2 mg/kg bwe was used and recovery period was 146 and 186 mm, respectively, when the tissue became responsive to NA again.

4.4 Discussion

This study clearly supports previous findings, which showed that the excitatory receptors of the vas deferens are adrenergic (Lipshultz *et al.*, 1981). However, this was the first study reported on the ampullae of bulls. From human studies, it is largely accepted that emission is the result of a powerful series of coordinated contractions of the vas deferens from the cauda epididymides to the proximal part of the vas where it enters the urethra (Ventura *et al.*, 1973). Moreover, these contractions are initiated in response to the administration of NA. The response was found to be dose related. There were marked differences in sensitivity of different

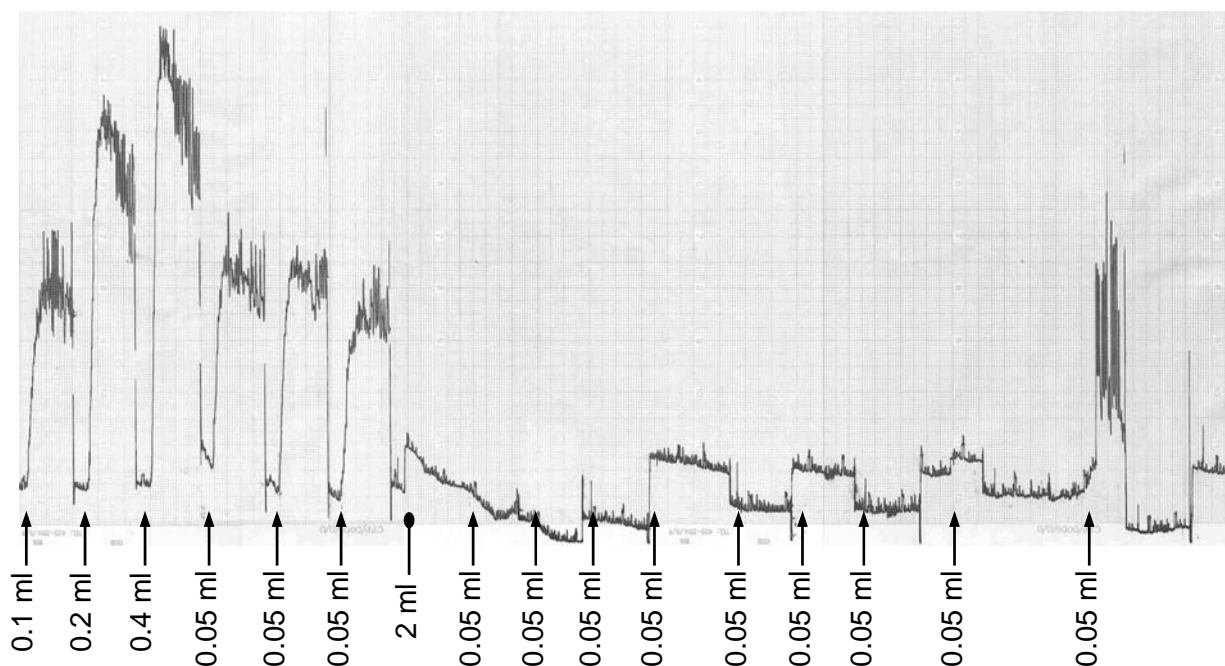


Figure 15. Recovery from high dose IMI effects of ampullar strips in vitro
Time required for an ampullar preparation to recover from a single dose of 2mg/kg bwe (2 ml) IMI until contractions in response to repeated, doses of NA $10^{-6}M$ (\uparrow). The IMI block is reversed after 146 minutes of repeated buffer replacement.

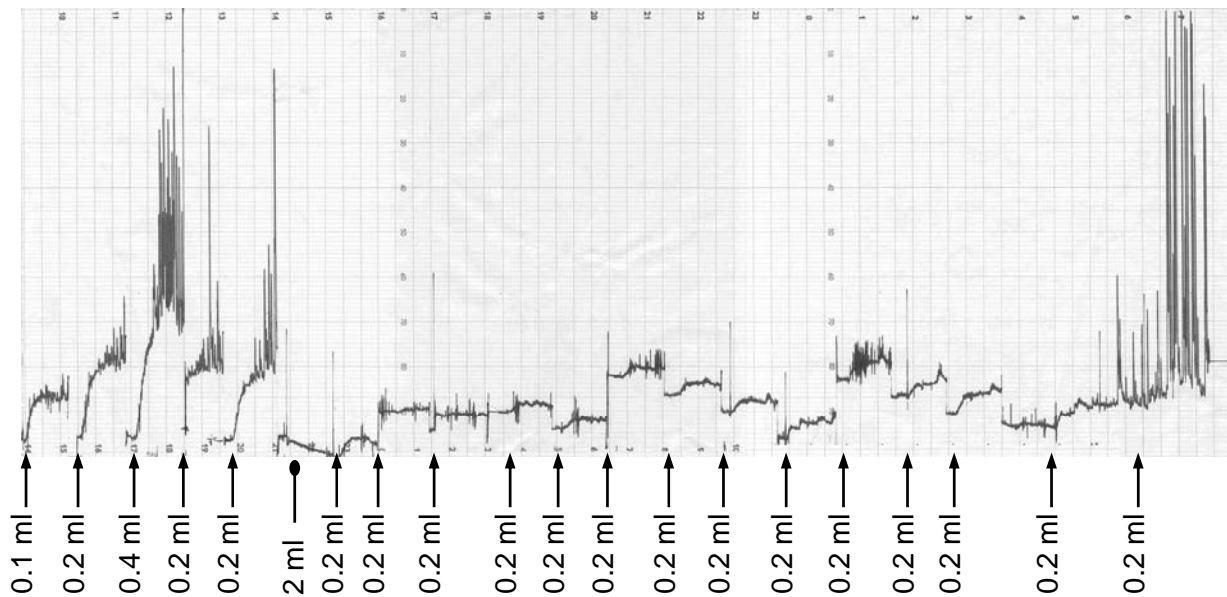


Figure 16: *Recovery from high dose IMI effects of ampullar strips in vitro*
 Time required for an ampullar preparation to recover from a single dose of 2mg/kg bwe (2 ml) IMI until contractions in response to repeated, doses of NA $10^{-6}M$ (\uparrow). The IMI block is reversed after 186 minutes of repeated buffer replacement.

bulls to NA stimulation. Considerable differences in sensitivity between vas deferens tissues have been reported in dogs (Arver and Sjostrand, 1982) and Guinea pigs. In Guinea pigs the differences were found to be due to age (Livingstone, 1970). Tissues from younger Guinea pigs were as much as ten times more sensitive to NA than older ones. It is possible that the differences in sensitivity in our study were also age related. Due to the fact that ages of bulls from which ampullae were collected, were not recorded, we cannot confirm this. It does, however, raise the possibility that age may affect the sensitivity of bulls to IMI and thus the optimal dose of the drug at a particular age.

Despite variation between bulls in response to NA, the use of IMI produced distinct patterns. IMI alone had no effect. Used prior to or during a NA-induced contraction it facilitated contractions. At low IMI doses (0,05 and 0,1 mg/kg bwe) it increased muscle tone and amplitude of rhythmic contractions. The effects are delayed somewhat (Figs 10 and 11). Step-wise increases in the dose of IMI from 0,1 to 0,5 mg/kg bwe decreases muscle tone and contraction frequency, while contraction

amplitude increases. Further increases from 0,5 to 2 mg reduce tone, amplitude and frequency of contractions and finally render the tissues refractory to NA effects. The facilitatory effects of IMI are thus dose dependent and if overdosed, paradoxical blocking of NA stimulatory effects occurs.

Our results suggest that clinical emission enhancing properties of IMI occur at doses \leq 1 mg/kg bwe, with definite paradoxical effects at doses \geq 2 mg/kg bwe. High doses should block NA effects on emission and ejaculation and therefore be entirely useless in the management of ejaculatory dysfunction or for collection of semen samples. Our findings are supported by clinical reports in which doses of IMI resulting in enhanced erection, emission and ejaculation in stallions, does so at relatively low doses, between 0,2 - 1,5 mg/kg (McDonnell, 1992). *Ex copula* ejaculates obtained using IMI at 0,5 mg/kg resulted in pregnancies after artificial insemination of fresh semen (Card *et al.*, 1997). Higher IMI doses (\geq 1 mg/kg) produced heavy sedation in this study and were thus not repeated. Oral treatment with imipramine at doses ranging from 0,2 - 1 mg/kg led to frequent erection and masturbation in sexually unstimulated stallions, while intravenous treatment over a range of doses (50 - 1000 mg total dose) similarly induced erection and masturbation in all animals (McDonnell, *et al.*, 1987). Doses of 2 mg/kg iv have also been used with success in stallions in other studies (McDonnell and Oristaglio Turner, 1994; McDonnell and Odian, 1994), with an ejaculation rate of 27 - 33 %. It is possible that the success rate may have been higher if slightly lower doses had been used. At the dose of 2 mg/kg used during the pharmacokinetic trial (Chapter 3), no spontaneous ejaculation or emission was observed in our bulls.

Studies in human patients with sexual dysfunction attributable to various causes, had improved sexual function with respect to normograde ejaculation, emission and ejaculation after treatment with IMI at doses of 0,3 - 0,7 mg/kg (Nijman, *et al.*, 1987) and 1 mg/kg (Gilja *et al.*, 1994). Doses \leq 1 mg/kg restored loss of seminal emission in 42, 8 % of human patients with emission problems.

Concluding, this work showed that *in vitro* studies on ampullar tissue could provide valuable insight into the nervous control of emission in bulls. It also proved to be useful in determining the mechanism by which IMI facilitates emission. Although it is wrong to extrapolate from *in vitro* results, we recommend a dose of 0.1 to 0.2 mg /kg for clinical application in bulls. Probably it would be wise to test a range of doses from 0.1 to 0.5 mg/kg in bulls. Because there is some delay until the effects are seen it seems logical to wait a while after administration of IMI before electro-stimulation is applied. The same would apply to immobilised wildlife species such as the buffalo.

Chapter 5

General conclusions

Electro-stimulation as a means of semen collection is common practice in large animal practice. It is envisaged that the technique will become increasingly useful in non-domesticated terrestrial species to obtain specimens for cryopreservation of genetic material and determine breeding potential of individuals. Semen emission is controlled by α -adrenergic mechanisms, mediated by biogenic amines (NA, serotonin and dopamine). Pharmacological agents, such as TCAs potentiate the actions of biogenic amines centrally and peripherally, by blocking the re-uptake of amines at nerve terminals. As such, they have an adrenergic potentiating effect. Imipramine hydrochloride is a synthetically manufactured TCA, which has been used successfully to stimulate emission of semen in stallions and humans.

This is the first report of imipramine pharmacokinetics in cattle. IMI was administered intravenously to three bulls (600 - 705.5 kg) at a dose of 2mg/kg BW. Intravenous plasma concentrations of IMI over time were determined by fluorescence polarization immunoassay (FPIA). IMI plasma concentration versus time profile was best described by a two compartmental open model with first-order rate constants. IMI distributed rapidly, ($t_{1/2\alpha}$) at 7.2 ± 4.2 min, exhibited a very large apparent steady state volume of distribution ($V_{d_{ss}}$) of 4.2 ± 0.9 l/kg body weight, had a very short terminal elimination half-life ($t_{1/2\beta}$) of 140 ± 15 min and showed a rapid total body clearance (Cl) of 22.7 ± 7 ml/min/kg. Both IMI and the pharmacologically active metabolite, desipramine was negligible in serum at 24 hours. In all three bulls, treatment with IMI at 2 mg/kg resulted in pronounced central nervous system signs immediately post injection. Signs of generalised weakness and ataxia were evident. All CNS signs dissipated 15–20 minutes post injection and should therefore not influence the treatment interval. An interval of at least 23 hours between repeat treatments of IMI, representing a period of at least 10 half-lives, is recommended. The dose of 2 mg/kg BW used in this study was similar to that routinely used in stallions without fatal side effects. Spontaneous emission and ejaculation was not observed in the three bulls treated with this dose.

The second study on the use of ampullar strips for testing the *in vitro* effects of NA and IMI produced valuable results, which can be applied to the clinical situation in bulls and perhaps wildlife species. NA alone consistently produced dose-dependant smooth muscle contractions of the ampullae confirming that the vas deferens has α_1 -receptors. IMI doses equivalent to ≤ 1 mg/kg BW had NA potentiating effects. Doses of < 0.1 mg/kg bwe were consistently potentiating while doses of > 0.1 mg to ≤ 1 mg/kg bwe partially blocked NA stimulating effects. Amplitude of rhythmic contractions increased while contraction frequency decreased at this level. This study supports the adrenergic potentiating effects of IMI at doses of 0.05 - 0.2 mg/kg bwe with higher doses having paradoxical effects. Doses of IMI ≥ 2 mg/kg bwe completely block NA effects. Tissue response to NA, after IMI blockade, start to recover 146-186 minutes after application of IMI at ≥ 2 mg/kg bwe. In the absence of NA, IMI has no effect on smooth muscle activity.

The time to an IMI effect on NA initiated smooth muscle activity was 8 minutes. If IMI is administered to bulls for semen collection one should consider two time factors. The first is the distribution half-life of 7.2 ± 4.2 min. The second is the refractory period from application of the drug to the effect observed. This was approximately 8 minutes in our *in vitro* study. Taking these two factors into account it would seem sensible to wait at least 20 minutes from administration of IMI until electro-stimulation commences. Indeed, a delay of a few hours from IMI administration until electro-stimulation would probably not significantly reduce response. Extrapolating from the *in vitro* study, we suggest that a dose of 0.1 to 0.5 mg/kg be tested in bulls. This is far below the dose of 2 mg/kg used in the pharmacokinetics trial, which, although produced side effects of nervous symptoms, proved to be safe. It did not, however, result in ejaculation.

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