THE EFFECT OF GONADOTROPIN-RELEASING HORMONES (GnRH) I & II ON SPERM MOTILITY AND ACROSOME STATUS OF THE VERVET MONKEY (*Chlorocebus aethiops) in vitro*

by

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Declaration

I, Charon de Villiers, declare that **"THE EFFECT OF GONADOTROPIN-RELEASING HORMONES (GnRH) I & II ON SPERM MOTILITY AND ACROSOME STATUS OF THE VERVET MONKEY *(Chlorocebus aethiops) in vitro*"** is my own work and that all sources I have used or quoted have been indicated and acknowledged by means of complete references.

Signature: ……. **Date: ……..**

Dedicated to my son Rhyno

TABLE OF CONTENTS

		Page
LIST OF TABLES		**vii**
LIST OF FIGURES		**viii**
Abstract		**1**
CHAPTER 1		**4**
Introduction		**4**
1.	**General introduction and background to GnRH**	**4**
1.1	**Classic role of GnRH**	**4**
1.2	**Novel locations and functions of GnRH**	**6**
1.2.1	*Tissue distribution*	**6**
1.2.2	*Function in the central nervous system*	**9**
1.2.3	*Functions in the reproductive system*	**13**
1.2.3.1	*Mammalian GnRH (GnRH I)*	**13**
1.2.3.2	*Chicken GnRH (GnRH II)*	**16**
1.3	**GnRH and its seven structural forms**	**18**
1.4	**Conservation of GnRH structures in vertebrates**	**19**
1.5	**Molecular biology of GnRH**	**20**
1.5.1	*GnRH gene and GnRH-associated peptide (GAP)*	**20**
1.5.2	*Regulation of the GnRH gene*	**22**
1.5.3	*GnRH Agonists and Antagonists*	**24**
1.6	**GnRH Receptors**	**29**
1.7	**Evolution of the GnRH gene**	**38**
1.8	**General primate spermatogenesis and GnRH**	**40**
1.9	**Spermatology of the Vervet monkey**	**45**

		Page
1.10	**Purpose of the study**	**47**
CHAPTER 2		**50**
Materials and Methods		**50**
2.	**The non-human primate model – Vervet monkey**	**50**
2.1	**The choice of subjects for this study**	**50**
2.2	**Spermatology and medium**	**51**
2.2.1	*Semen collection*	**51**
2.2.2	*Extenders for semen*	**52**
2.2.3	*Medium for acrosome staining*	**53**
2.2.4	*GnRH analogues*	**53**
2.3	***In vitro incubation***	**53**
2.3.1	*Swim-up incubation*	**53**
2.3.2	*Staining for acrosomal integrity*	**55**
2.3.3	*Evaluation for acrosomal integrity*	**55**
2.4	**Motility**	**56**
2.4.1	*Subjective motility*	**56**
2.4.2	*Computer Aided Sperm Motility Analysis (CASMA)*	**57**
2.5	**Statistical analysis**	**61**
CHAPTER 3		**62**
Results		**62**
3.	**Electro ejaculation**	**62**
3.1	**Hams F10 extender**	**62**
3.2	**Swim-up incubation**	**62**
3.3	**Acrosomal integrity**	**63**

Page

3.3.1 *Intact acrosome (Pattern I)* **63**

3.3.2 *Equatorial band (Pattern III)* **64**

3.3.3 *Absent acrosome (Pattern IV)* **65**

3.4 Sperm motility **76**

3.4.1 *Computer Aided Sperm Motility Analysis (CASMA)* **76**

3.4.2 *Effect on CASMA parameters observed during one hour* **76**

3.4.2.1 *Immediate effect (0 – 5 minutes)* **76**

3.4.2.2 *Effect at 15 minutes* **77**

3.4.2.3 *Effect at 30 minutes* **77**

3.4.2.4 *Effect at 60 minutes* **77**

3.5 Percentage sperm motility and sperm motility tracks **86**

3.5.1 *Percentage sperm motility* **86**

3.5.2 *Sperm motility tracks* **88**

CHAPTER 4 **93**

Discussion **93**

4. Electro ejaculation **95**

4.1 Hams F10 extender **95**

4.2 Swim-up incubation **97**

4.3 Acrosomal integrity **98**

4.4 Sperm motility **100**

4.4.1 Total percentage sperm motility **100**

4.4.2 Computer Aided Sperm Motility Analysis (CASMA) **101**

4.4.3 Sperm track analysis **102**

Page

4.4.4 Hypothesis on GnRH I and II in relation to sperm motility development/inhibition and conclusion 103

References 107

Acknowledgements 138

LIST OF TABLES

		Page
Table 2.1	**Sperm motility quantification variables**	**59**
Table 2.2	**Experimental layout for acrosome status and sperm motility evaluations for GnRH I & II at different concentrations for one hour from a total of 84 ejaculates**	**60**
Table 3.1	**Percentage (means ± SD) of intact acrosomes for GnRH I & II (concentrations 10^{-5}M, 10^{-6}M, 10^{-8}M) Propylene glycol 0,2% and controls**	**64**
Table 3.2	**Percentage (means ± SD) of equatorial band staining for GnRH I & II (concentrations 10^{-5}M, 10^{-6}M, 10^{-8}M) Propylene glycol 0,2% and controls**	**65**
Table 3.3	**Percentage (means ± SD) of absent acrosomes for GnRH I & II (concentrations 10^{-5}M, 10^{-6}M, 10^{-8}M) Propylene glycol 0,2% and controls**	**66**
Table 3.4	**Means and standard deviations (mean ± SD) for Computer Aided Sperm Motility Analysis (CASMA) results**	**78**
Table 3.5	**Statistically significant differences when experimental groups were compared to control for CASMA parameters. GnRH peptides I and II indicated as I, II, and concentrations of peptides in Molars. *P* values of control *versus* peptides at significance level of $p < 0.05$ used as a cut off point.**	**79**
Table 3.6	**Percentage sperm motility (means ± SD) after exposure to GnRH I & II and controls**	**86**
Table 4.1	**Comparison of acrosome status reported for Vervet monkey sperm**	**99**

LIST OF FIGURES

		Page
Figure 2.1	**Electro ejaculation equipment**	**52**
Figure 2.2	**PNA labelling patterns of Vervet monkey spermatozoa**	**56**
Figure 2.3	**Sperm Motility Quantifier system and equipment for recording sperm motility**	**58**
Figure 3.1	**Staining of Vervet monkey spermatozoa after labelling with FITC-PNA**	**63**
Figure 3.2	**FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-5}M for one hour**	**67**
Figure 3.3	**FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-6}M for one hour**	**68**
Figure 3.4	**FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-8}M for one hour**	**69**
Figure 3.5	**FITC-PNA staining for equatorial band with GnRH I & II treatment at concentration 10^{-5}M for one hour**	**70**
Figure 3.6	**FITC-PNA staining for with equatorial band GnRH I & II treatment at concentration 10^{-6}M for one hour**	**71**
Figure 3.7	**FITC-PNA staining for with equatorial band GnRH I & II treatment at concentration 10^{-8}M for one hour**	**72**
Figure 3.8	**FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-5}M for one hour**	**73**
Figure 3.9	**FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-6}M for one hour**	**74**
Figure 3.10	**FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-8}M for one hour**	**75**
Figure 3.11	**Curvilinear velocity (VCL) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour**	**80**
Figure 3.12	**Straight line velocity (VSL) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour**	**81**

Page

Figure 3.13 Linearity (LIN) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour **82**

Figure 3.14 Average path velocity (VAP) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour **83**

Figure 3.15 Amplitude of lateral head displacement (ALH) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour **84**

Figure 3.16 Mean angular displacement (MAD) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour **85**

Figure 3.17 Percentage motility (+SD) for sperm treated with GnRH I & II at different concentrations for one hour **87**

Figure 3.18 Representative sperm motion tracks for control, GnRH I and II at 10^{-5}M one hour after sperm exposure **90**

Figure 3.19 Representative sperm motion tracks for control, GnRH I and II at 10^{-6}M one hour after sperm exposure **91**

Figure 3.20 Representative sperm motion tracks for control, GnRH I and II at 10^{-8}M one hour after sperm exposure **92**

Figure 4.1 GnRH sites (*) in the male and female reproductive systems. Sperm travelling through male and female genital ducts could be exposed to GnRH **106**

Abstract

Introduction: Gonadotropin Releasing Hormone (GnRH) is a hypothalamic decapeptide, which regulates mammalian gonadotrophin secretions by binding to specific, high affinity receptors in the pituitary. Two forms of GnRH (GnRH I and GnRH II) are expressed in the brain of humans and some primates. GnRH I play a key role in the process of reproduction. After binding to its cognate receptor (GnRH I receptor), it stimulates the secretion of LH and FSH, which in turn regulate gonadal steroidogenesis and gametogenesis in both sexes. Clinically, some GnRH I analogues have been used as an effective treatment for a variety of reproductive disorders.

The second form of GnRH (GnRH II) differs from GnRH I in that it is expressed at higher levels outside the brain. It seems to play an important role in reproductive functions since GnRH-like material has been observed in various reproductive cells and tissues such as human seminal plasma, follicular fluid, testis and prostate gland. *In vivo* primate studies have shown the contraceptive capabilities of GnRH II analogues and have identified different possible sites of action for fertilization disruption in the reproductive tract. In the male, one site could be the fertilizing sperm since a receptor was found in the acrosomal region. However, GnRH exposure to human sperm *in vitro* has been shown to enhance the ability of the sperm to bind to the zona pellucida of the oocyte.

Even though primates have been used extensively in a variety of investigations in relation to the role of GnRH in reproduction, there is no evidence of any research to investigate the direct effect of GnRH on primate sperm.

Materials and methods: Semen from ten sexually mature Vervet monkeys was obtained by electro-stimulation applied per rectum. Motile sperm were selected through the swim-up technique in Hams F10 medium. Sperm samples were exposed to three different concentrations (10^{-5}M, 10^{-6}M and 10^{-8}M) of GnRH I and GnRH II for one hour. Slides were stained with FITC/PNA for acrosomal integrity evaluation. The proportions of spermatozoa with fluorescent patterns were recorded for intact acrosome, equatorial staining and no acrosome. The results were expressed as percentages after evaluating 200 spermatozoa.

Sperm motility was evaluated by computer-aided sperm motion analysis (CASMA), using the Sperm Motility Quantifier [version 1.01: Wirsam Scientific & Precision Equipment (Pty) Ltd, South Africa]. The parameters considered in this study were straight-line velocity (VSL), curvilinear velocity (VCL), linearity (LIN), average path velocity (VAP), mean angular displacement (MAD) and amplitude of lateral head displacement (ALH).

Results: Neither GnRH I nor II affected acrosomal integrity in terms of any of the staining patterns for all concentrations and time intervals (immediate exposure to one hour). The percentage sperm motility was significantly decreased after one-hour exposure to both GnRH I and II at 10^{-8}M.

The changes in the CASMA variables revealed that immediate exposure (0-5 minutes), showed a statistically significant decline in VCL ($p<0.05$) and VSL ($p<0.05$) in groups treated with GnRH I & II 10^{-8}M when compared to the control and Propylene Glycol 0,2%

(vehicle) groups. After fifteen minutes of GnRH I & II exposure at 10^{-8}M, a statistically significant decline ($p<0.05$) was found in VCL, VSL, LIN, and ALH.

After thirty minutes of GnRH exposure, VCL demonstrated a statistically significant decrease ($p<0.05$) in GnRH I & II 10^{-8}M. VSL and VAP were also significantly decreased ($p<0.05$) but only for GnRH I at 10^{-8}M.

After sixty minutes of GnRH exposure, the statistically significant decline in groups treated with GnRH I & II 10^{-8}M compared to the control was evident for all variables. However, MAD continued to increase, and was significantly different when GnRH II at 10^{-8}M was compared to the control.

Discussion: The results of this study showed that both GnRH I & II at the lowest concentration (10^{-8}M) had an effect on the sperm motility *in vitro*. However, the high prevalence of intact acrosomes in all GnRH treated groups suggests that GnRH I & II have little or no effect on the functional changes in sperm plasma membrane composition.

Conclusion: GnRH I and II inhibit motility of Vervet monkey sperm *in vitro*. It is postulated that sperm can be affected by GnRH in various parts of the female reproductive tract during transport to fertilize the egg. The results of this study suggest that GnRH could play a role in sperm motility modulation and selection.

CHAPTER 1

Introduction

1. General introduction and background to GnRH

1.1 Classic role of GnRH

GnRH is the central regulator of the reproductive hormonal cascade and was first isolated from mammalian hypothalamus. It is also known as Luteinizing Hormone Releasing Hormone (LHRH) or Mammalian GnRH I and controls the reproductive system through its stimulation of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion from the pituitary [Hazum and Conn 1988, Conn and Crowley 1994]. GnRH is one of the families of hormones and neurotransmitters, which require Ca^{2+} for their biological actions [Conn *et al*. 1986, Denef 1988]. The latter plays an important role in the pulsatile way GnRH is released into the systemic circulation. GnRH is synthesized in the hypothalamus and then transported in a pulsatile manner to the anterior pituitary through the hypothalamo-hypophyseal portal system to bring about the release of the gonadotropins, LH and FSH, which, in turn, affect gonodal function [Huckle and Conn 1988, Clayton 1989]. This action of GnRH is achieved by binding to and activation of its high affinity receptors on the pituitary [Johnson and Everitt 1990, Clayton 1989, Stojilkovic *et al.* 1994]. The pulsatile-timed and concentration levels of GnRH are critical for the maintenance of gonodal steroidogenesis and for normal reproductive function [Huckle and Conn 1988].

The C-terminal portion of the GnRH molecule is necessary for attaching to the target receptor, and the first three amino acids are necessary for activating LH and FSH release

[Reeves 1987]. LH stimulates ovulation and corpus luteum formation in females [Moss and McCann 1973] and testosterone secretion in males. FSH stimulates the growth and maturation of ovarian follicles in females and spermatogenesis in males [Conn *et al.* 1986, Hazum and Conn 1988, Ganong 1999]. In addition to the orchestration of gonadal activity through the pulsatile release of LH and FSH from the anterior pituitary, GnRH may play an important role in the regulation of extra-pituitary physiological functions [Stojilkovic *et al.* 1994]. The existence of GnRH and GnRH receptor mRNAs in normal human non-reproductive tissues, suggests that GnRH may also be involved in the regulation of cellular function in an autocrine or paracrine manner [Emons *et al.* 1998, Kakar and Jennes 1995]. There is also strong evidence from several *in vitro* physiological studies that GnRH acts as a meiosis-stimulating factor in oocytes [Hillensjö and LeMaire 1980, Nabissi *et al.* 1997]. In mature granulosa cells, GnRH I is associated with stimulatory effects on oocyte cleavage, ovulation and luteinization [Von Schalburg *et al.* 1999, Nabissi *et al.* 1997] and evidence of enhancing sperm-zona binding, which is an essential step in the fertilization process [Morales 1998].

A second form of GnRH (GnRH II) conserved in all higher vertebrates, including humans, is present in extra-hypothalamic brain and many reproductive tissues. GnRH II is thought to have an important regulatory role in gonadotropin secretion because it potently stimulates gonadotropin discharge in monkeys [Lescheid *et al.* 1997, Okada *et al.* 2003, Densmore and Urbanski 2003] and in the bullfrog [Wang *et al.* 2001]. The wide distribution of GnRH II in the brain, suggests important neuromodulatory functions [Millar *et al.* 2001] such as the contribution to regulating the primate reproductive system, which may also be under

neuroendocrine control from GnRH I [Lescheid *et al.* 1997]. GnRH II might be involved in the suppression of cell growth in tumour cell lines with an auto regulatory role on the rate of cell regulation and proliferation [Peng *et al.* 1994, Emons *et al.* 1989, Emons *et al.* 2000, Quyam *et al.* 1990, Azad *et al.* 1993], and yet may act as a growth or transforming factor that contributes to the promotion of prostate cancer in humans [Fekete *et al.* 1989]. Later studies also proved that GnRH II stimulates gonadotroph secretions, even though it is less effective than GnRH I [Okada *et al.* 2003, Densmore and Urbanski 2003].

In many vertebrate species, a third form of GnRH occurs which is localized to the forebrain in fish and is designated GnRH III. Its localization in the midbrain does support a central role in the regulation of gonadotropin secretion. The role of this third peptide in the hypothalamic-pituitary-gonodal axis may be conveyed by modulation of GnRH I activity, or by the regulation of GnRH I expression or release. It could also play a role as the physiological regulator of reproductive behaviour [Yahalom *et al.* 1999].

1.2 Novel locations and functions of GnRH

1.2.1 *Tissue distribution*

The pituitary is the gonatroph that expresses high affinity GnRH receptors and secretes gonatrophic hormones. GnRH functions not only as a releasing hormone and is distributed throughout the nervous system and other non-reproductive tissues [Rama and Rao 2001]. Due to the lack of a homogeneous *in vitro* model of gonadotropes, the cell line LβT2 was identified as a useful tool to investigate the cellular and molecular events during gonadotropin synthesis [Turgeon *et al.* 1996]. Using reverse transcriptase polymerase chain

reaction (RT-PCR) techniques, PCR products from GnRH were also obtained from human liver, heart, skeletal muscle, kidney, placenta and pituitary. The highest levels of GnRH II were reported in the kidney, prostate, and bone marrow. Compared to the brain, the levels in the kidney are approximately 30-fold higher and four-fold greater in bone marrow and the prostate [White *et al.* 1998].

Despite the apparent role of GnRH I in regulating reproduction, GnRH II is reported to be expressed in humans, and is 70% identical to GnRH I. It is expressed ubiquitously in human tissue with high levels outside the brain. GnRH I have not been observed at a high level outside the brain. GnRH II was also isolated in the brain of various mammals and in the mesencephalic cells of non-placental animals [Dong *et al.* 1996, Urbanski *et al.* 1999, Lescheid *et al.* 1997, Kasten *et al.* 1996 and Montaner *et al.* 1998]. Chicken GnRH (cGnRH) and GnRH I was shown to be expressed in the median eminence of the hypothalamus, a strategic location for its secretion into the circulation of the pituitary gland [Urbanski *et al.* 1999]. GnRH neurons were present at the medial preoptic area (MPOA) and the medio basal hypothalamus (MBH) of the lemur *(Microcebus murinus)* [Aujard *et al.* 2005]. A third isoform of GnRH (GnRH III) is localized in the telencephalon of several fish and in the hypothalamus and midbrain of humans and calves [White *et al.* 1998].

The GnRH gene is expressed in reproductive tissues such as the ovary, placenta, and mammary gland [Dong *et al.* 1993, Peng *et al.* 1994, Nabissi *et al.* 1997 and Von Schalburg *et al.* 1999]. The cDNA sequences of this peptide were shown to be identical in the placenta, the hypothalamus form [Seeburg and Adelman 1984], and the pituitary GnRH [Kakar *et al.*

1992]. Two types of GnRH-like factors are found in human seminal plasma [Sokol *et al.* 1985] and in human testis, which differ in immunoreactivity from hypothalamic GnRH [DiMeglio *et al.* 1998]. It also acts on multiple extra-pituitary sites to regulate various reproductive functions and it has been identified in granulosa and theca cells of the rat ovary, and the Leydig cells of the rat testis. GnRH II was also localized in the testis, epididymis, and seminal vesicle and fallopian tube of the baboon [Siler-Khodr *et al.* 2003]. The peptide is also present in human granulosa-luteal cells [Peng *et al.* 1994] and a GnRH-like protein found in human ovaries showed similarities to the GnRH found in the rat ovary [Hsueh and Schaeffer 1985, Aten *et al.* 1987]. Compounds with GnRH-like activity were found in the ovary of the African catfish and sea bream [Habibi *et al*. 1994, Nabissi *et al*. 1997] and GnRH II was reported in the baboon ovary [Siler-Khodr *et al.* 2003].

Recent studies have shown that about 50% of breast and approximately 80% of ovarian and endometrial cancers express GnRH [Emons *et al.* 1998, Emons *et al.* 2000]. The presence of GnRH in rat and human experimental prostatic cancer, prostatic cancer cells in culture and biopsy specimens has also been reported. It was found that 68% of benign and 27% of malignant human prostate tissues contained a GnRH-like peptide [Quyam *et al.* 1990, Azad *et al.* 1993]. In 30% of biopsies from breast cancer patients, various hypothalamic hormones and GnRH were seen in the cytoplasm or in the nuclei of the tumour cells. The fact that these hormones were consistently found in premalignant and malignant lesions, but not in normal breast tissue, suggests the possibility that the hormones could be produced locally during the process of malignant transformation [Ciocca *et al.* 1990].

The findings that the tissue distribution patterns of GnRH I and GnRH II are dissimilar and that their expressions are differentially regulated, indicate a distinct role for these decapeptides in the body [Cheng and Leung 2005].

1.2.2 *Function in the central nervous system*

GnRH I is secreted from hypothalamic neurosecretory neurons centered in the arcuate nucleus with axons projecting to the median eminence. Its release occurs in response to diverse neural inputs operating through neurotransmitters [Samuel and Yen 1975, Fritz and Speroff 1982]. GnRH, like many neuropeptides, may act as a neurotransmitter, neuromodulator, or local hormone [Hsueh and Schaeffer 1985]. GnRH is assumed to be the most important final common mediator of all influences on reproduction conveyed through the central nervous system (CNS) [Silverman *et al.* 1994]. Any abnormalities in GnRH synthesis, storage, release or action will result in partial or complete failure of gonadal function. Destruction of the GnRH producing neurons in the hypothalamus, or immunization against the peptide, prevents gondotrophin function and results in gonadal atrophy [Johnson and Everitt 1990].

The distinguishing feature of GnRH II and its receptors is its wide distribution in extra-hypothalamic regions of the brain such as the discrete regions of the central and peripheral nervous system in areas associated with sexual behaviour. GnRH II is dispersed in brain areas where lesions and/or electrical stimulation of these areas have effects on reproductive behaviours, such as sexual interest, erection, intromission, thrusting and ejaculation in rats, dogs, cats, monkeys and humans [White *et al.* 1998, Urbanski *et al.* 1999, Millar 2003]. It is

also much more effective than mammalian GnRH in stimulating reproductive behaviour in the ringdove [Millar *et al.* 2001, Millar 2003].

Molecular variants of GnRH similar to GnRH I, salmon GnRH (sGnRH) and GnRH II, were also found in the olfactory bulbs and preoptic-hypothalamic region of the water hog (*Hydrochaeris hydrochaeris*) [Montaner *et al.* 1998]. Studies in rats showed that GnRH cell bodies located in the septal-preoptic-hypothalmic region send only 50-70% of their axons to the median eminence. The remaining axons are widely distributed in the brain and some of these axons can terminate on other GnRH cell bodies that can be involved in the pulsatile release of GnRH. The GnRH neurons migrate from the olfactory placode outside the brain to the forebrain during early development. These GnRH neurons are part of the terminal nerve and may be the source of the GnRH neurons in the septal-preoptic-hypothalmic area [Pfaff *et al.* 1994, Sherwood *et al.* 1993].

Reproductive behaviour is affected by olfactory stimuli being transmitted by the terminal nerve and the medial olfactory tract in fish and other species that could mediate responses to sex pheromones [Pfaff 1973, Moss and McCann 1973]. Sexual behaviour could then be directly regulated by GnRH due to GnRH bodies in the nucleus of the midbrain, hindbrain and spinal cord of birds, fish, reptiles and amphibians. GnRH also acts on the neurons since it promotes mating behaviour in hypophysectomized rats [Pfaff 1973, Moss and McCann 1973]. The neurons in fish receive input from cells that show GnRH-like immunoreactivity, which shifts from the midbrain cell bodies to the terminal regions of the brain to initiate courtship [Sherwood *et al.* 1993].

Chemical signals play an important role in social communication and these signals have been shown to act on physiological mechanisms and behaviour [Schilling *et al.* 1984]. Chemosensory cues stimulate male sexual arousal. The main olfactory system has an important role in attracting males to oestrous females, and the vomeronasal receptors are important for activating olfactory pathways that engage mating behaviour in a sexually dimorphic manner. The GnRH neurons like the vomeronasal organ neurons (VNO) take their origin in the olfactory placode and migrate to the basal forebrain along pathfinder axons that take their origin in the developing VNO [Keverne 2004]. The vomeronasal organ plays an important role in the GnRH system because it transmits chemical signals that activate GnRH coordination. The latter induces changes in the olfactory sensitivity to pheromones and, in some primates there is a possible link between the vomeronasal function and the number of GnRH neurons in the anterior part of the hypothalamus. Lesions of hypothalamic preoptic areas in marmoset monkeys and mouse lemurs caused a profound suppression of sexual arousal associated with both reduced inter male aggressive interactions [Aujard 1997]. Further, the removal of the VNO in the male mouse lemur led to an increase in the number of immunoreactive GnRH neurons in the medial preoptic area [Aujard *et al.* 2005]. The importance of the medial preoptic system in sexual behaviour and reproduction was also demonstrated in a number of studies on male hamsters' response to female hamster odours and the restoration of mating behaviour after VNO lesions via intra-cerebral GnRH injections [Pfeiffer and Johnston 1994, Westberry and Meredith 2003a, Westberry and Meredith 2003b].

The biological function of GnRH II is unknown and only limited attempts have been made to describe its regional expression in the primate brain. Previous studies have shown that GnRH II is expressed in the mesencephalic cells of non-placental mammalian species. In the brain of the tree shrew (*Tupaia glis belangeri),* a novel mammalian, GnRH II is expressed in the neurons of the mesencephalon and was the first nonhypothalamic GnRH isolated from a placental animal [Kasten *et al.* 1996]. It was also shown to be present in the median eminence of the hypothalamus of the adult stump tail monkey (*Macaca speciosa*) and adult, as well as foetal Rhesus monkeys (*Macaca mulatta*). Immunocytochemistry showed that there are some cGnRH-like immunopositive cells in the basal hypothalamus and these results suggested that GnRH might function as the primary gonadotropin releaser.

GnRH III fibres are expressed in the rat median eminence. GnRH is possibly transported to the pituitary by way of the portal system and may interact with pituitary cells. This interaction could indicate a role other than the putative function as a neurohormone at the hypothalamic-pituitary axis, such as a physiological regulator of reproductive behaviour [Yahalom *et al.* 1999]. However, it is still unclear whether these pituitary cells represent a source of GnRH II synthesis or whether they represent a site of GnRH II uptake. It appeared that a synthetic GnRH II is a stimulator of gonadotropin release and the investigators postulated that an anterior and posterior GnRH system may exist within the brains of recently diverged vertebrates, and possibly even in humans. They are: a) the anterior, terminal nerve-septo-preoptic system, which is the main regulator of gonatropin (LH and FSH) release, and b) a posterior system of neurons that express cGnRH II, with fibres projecting throughout the brain, including the hindbrain, posterior pituitary, and spinal cord [Lescheid *et al.* 1997,

Montaner *et al.* 1998]. The later cloning of GnRH cDNA from the Rhesus macaque confirmed that both peptides exist in the Rhesus hypothalamus. The high levels of GnRH II in the supraoptic nucleus raise the possibility that some of the GnRH II may reach the pituitary and peripheral circulation by a different route than GnRH I, which reaches the pituitary gland via the hypothalamic portal vessels. Even though GnRH II is expressed in a concentrated fashion in at least three distinct regions of the Rhesus hypothalamus, it is not co expressed with GnRH I, suggesting unique regulation and function [Urbanski *et al.* 1999].

An established function of GnRH II is the inhibition of M currents (K^+ channels) through the GnRH II receptor in the amphibian sympathetic ganglion. The wide distribution of GnRH II in the central and peripheral nervous system suggests that it might act through this mechanism as a neuromodulator in the central nervous system [Troskie *et al.* 1997, Millar *et al.* 2001, Pawson *et al*. 2003, Millar 2003 and Millar *et al.* 2004].

1.2.3 *Functions in the reproductive system*

1.2.3.1 *Mammalian GnRH (GnRH I)*

All vertebrates, from cyclostomes to primates, rely on GnRH for the development and maintenance of reproductive functions. GnRH I, synthesized within the cells of the hypothalamus, regulates the release of pituitary gonadotrophins, which, in turn, stimulate the release of steroid hormones from the gonads [Conn *et al.* 1986, Kasten *et al.* 1996, Rama and Rao 2001].

Even though it was speculated that two GnRHs in the chicken hypothalamus might assist the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in avian species, GnRH I is suggested to be solely responsible for the secretion of gonadotropic hormones in mammals, humans, amphibians and avian species [Miyamoto *et al*. 1984, Wang *et al.* 2001]. It also plays a role in a variety of other functions such as the direct effects on reproductive behaviour and sexual arousal in rodents. Administration of GnRH into the midbrain central grey matter facilitates sexual behaviour in rats. Given that the ovarian cycle is regulated by gonadotropins, there is evidence that LH secretion facilitates lordosis and mating behaviour in ovariectomized female rats [Pfaff 1973, Pfaff *et al*. 1994, Moss and McCann 1973]. This is an indication that mammalian GnRH has behavioural effects not mediated by the pituitary. Other stimulants of sexual behaviour have been observed in species of fish, amphibians, reptiles and mammals.

Isolation of GnRH I has been reported in various reproductive tissues such as the ovary and testis of humans and monkeys, as well as rat ovary [Aten *et al.* 1987]. Compounds with GnRH-like activity were found in the ovaries of the African catfish (*Clarias gariepinus*) [Habibi *et al.* 1994] and the sea bream (*Sparus aurata*) [Nabissi *et al.* 1997]. The addition of sGnRH to cultured sea bream oocytes directly stimulated oocyte meiosis and binding sites for GnRH in the ovaries, suggesting that GnRH, or compounds with GnRH-like activity, plays an autocrine/paracrine role in the regulation of ovarian function in sea bream and catfish. These findings were first shown in a study where GnRH and GnRH agonists were used in rat oocytes *in vitro* [Hillensjö and LeMaire 1980]. If there is a requirement for GnRH at stages just before ovulation, as in the trout [Von Schalburg *et al.* 1999], then caution

should be taken in interpreting the results from rats. A number of effects have been reported when gonodal cells were exposed to GnRH *in vitro*. However, these effects may not be physiological if the rat gonad does not express GnRH peptide *in vivo* at that specific stage of development.

The expression of GnRH in reproductive tissues may modulate various processes or mechanisms during the fertilization process. The presence of GnRH and GnRH receptors has been shown to play a role during spermatogenesis, sperm maturation, and fertilization [Morales and Llanos 1996] such as the increase of sperm binding to the ovum, an effect that is inhibited by GnRH antagonists [Morales *et al.* 1994, Morales 1998, Morales *et al*. 1999]. Immunoreactive GnRH in human reproductive tissue, such as ovary, granulosa-luteal cells, mammary gland and testis, along with the low expression of the gene, suggests that the peptide may be synthesized for functioning locally as an autocrine or paracrine factor [Dong *et al.* 1993, Peng *et al.* 1994]. In cultured rat granulosa cells, GnRH induces apoptosis and can thus be involved in the process of follicular atresia. GnRH may also play a role in follicle rupture and oocyte maturation during the pre ovulatory period in the rat. In human granulosa luteal cells, GnRH I and II, may play a role in regulating luteolysis during the luteal phase of the menstrual cycle [Samuel and Yen 1975, Fritz and Speroff 1982] and might promote apoptosis as has been shown for rats [Khosravi and Leung 2003]. In the human placenta, GnRH functions as a local regulator, where GnRH or GnRH-like peptides are synthesized by cytotrophoblasts and syncytiotrophoblasts during embryogenesis. It also initiates the secretion of human chorionic gonadotropin (hCG), which in turn stimulates the synthesis and secretion of progesterone from the corpus luteum [Rama and Rao 2001]. This suggests that

the action of GnRH is mediated through its typical high affinity receptor [Kakar and Jennes 1995] and that the placenta might possess its own GnRH system, analogues to the hypothalamus-pituitary system [Cheng *et al*. 2001].

Pubertal development in mammals results from a complex cascade and progressive maturational events involving the entire gonodal axis. The appropriate expression of the receptor in gonadotrophs is critical for GnRH signalling and hence for gonadotropin secretion and sexual development [Samuel and Yen 1975]. The importance of GnRH for sexual development was shown by the strong induction of the gene and its receptor during the infantile period followed by a weaker persistent activation during puberty in the female rat [Zapatero-Caballero *et al.* 2004].

1.2.3.2 *Chicken GnRH (GnRH II)*

As with GnRH I, GnRH II is found in reproductive tissue such as in baboon ovary where it has been proposed to act as a potent regulator of ovarian function and hormone regulation during pregnancy. Like type I, GnRH II is also capable of inducing apoptosis in the human ovary [Siler-Khodr *et al.* 2003, Khosravi and Leung 2003].

Previous studies have shown that synthetic GnRH II is a potent stimulator of gonadotropin release in Rhesus macaques *in vivo* [Lescheid *et al.* 1997]. It is likely that GnRH I and GnRH II receive a distinct set of inputs since they use two different routes to reach the pituitary and thus together may control steroidogenesis, sperm production, follicular development and ovulation [Urbanski *et al.* 1999]. The expression of GnRH II in the testis, epididymis,

seminal vesicle and baboon fallopian tubes further suggests that this peptide may play a role in sperm development and in tubal function [Siler-Khodr *et al*. 2003].

GnRH II has less than one-tenth or one-third the LH-releasing activity of GnRH I in rat and sheep pituitaries *in vitro* [Miyamoto *et al.* 1984] and no effect of a GnRH analogue could be observed on baboon pituitary LH release [Siler-Khodr *et al.* 2003]. On the other hand, there have been reports of an increase in LH concentration after GnRH II was administered systemically during the luteal phase to Rhesus monkeys, but with little effect on LH concentration in the follicular phase [Lescheid *et al.* 1997, Densmore and Urbanski 2003]. Results of pituitary cell culture studies from male Rhesus monkeys supported previous findings that GnRH II stimulates LH and FSH release from the pituitary cells by activating GnRH I receptors [Okada *et al.* 2003]. Recently, some specific FSH-releasing factors, such as sGnRH II and lamprey GnRH III, have been reported. Thus, GnRH II possibly functions as a specific stimulator for FSH [Millar *et al*. 2004].

GnRH II has a potent effect on the inhibition of progesterone production in the ovary and hCG release in the placenta. The high affinity binding sites for GnRH II in baboon ovary and human placenta suggest a possible role of paracrine activity in extra-hypothalamic tissues. The specific binding sites and action of GnRH II and its analogues in reproductive tissues and reproductive cells such as the ovary, ovum, male reproductive tract, sperm, fallopian tube, endometrium, and/or trophoblast, support the possibility of GnRH II being a regulator of the reproductive system [Siler-Khodr *et al.* 2004].

1.3 GnRH and its seven structural forms

Seven forms of non-mammalian GnRH distinct from mammalian GnRH (GnRH I) have been identified. The different forms of GnRH belong to a family of peptides that are structurally related.

All the GnRH structures are decapeptides with at least 50% to 90% sequence identity. The length of the GnRH and five of the amino acids have been conserved. The seven GnRHs, with their distinct primary structures, are named after the animals from which they were first isolated by peptide chemistry. In addition, the primary structure of GnRH for at least one representative species for each vertebrate class has been determined for Agnatha or jawless fish (Lamprey), Chondrichthyes or cartilaginous fish (shark), ratfish, Osteichthyes or bony fish (salmon), catfish (Ngamvongchon), Amphibia (frog), Reptilia (Alligator), Aves (chicken) and Mammalia (pig and sheep) [Sherwood *et al.* 1993].

The GnRH family does not appear to be limited to only seven non-mammalian members and it is generally accepted that different forms of GnRH have arisen through gene duplication from a single ancestral GnRH form whose origin pre-dates vertebrates [Sherwood *et al.* 1993]. Data on the appearance of different forms in one species, such as the three different forms expressed in teleost fish and the basal salmonid, are crucial in understanding the evolution of multiple forms of GnRH within individuals [White *et al.* 1995, Adams *et al*. 2002].

Various studies have shown other GnRHs with distinct structures and to date, 23 GnRH forms or GnRH-like sequences have been identified in various non-mammalian vertebrates. In most vertebrate species, GnRH I or GnRH II is present suggesting early gene duplication. Both peptides are distributed in a wide range of tissues in vertebrates [Sealfon *et al.* 1997, Sherwood *et al.* 1993] and apparently have diverse functions. These include neuroendocrine, paracrine and autocrine neuro- modulatory roles in the central and peripheral nervous system [Millar *et al.* 2001, Millar 2003, Millar *et al.* 2004, Cheng and Leung 2005].

1.4 Conservation of GnRH structures in vertebrates

The mammalian gonadotropin-releasing hormone (GnRH I) was the first isoform of GnRH that was identified in mammals with the linear sequence of (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH^2). It is found in humans and has a wide distribution in vertebrate species [Seeburg and Adelman 1984, White *et al.* 1998]. All forms of GnRH identified to date have 10 amino acids with a pyroglutamic acid N terminus and an amidated glycine C terminus. Amino acids 1 through 4 are tightly conserved except for position 2 in guinea pig (gp) GnRH and position 3 in lamprey (l) GnRH I; the conservation may reflect the importance of residues 1 through 3 for the functional release of LH and FSH. Variations in GnRH peptide structure are typically the result of different amino acids in positions 5 to 8. The structure of the peptide remained unchanged. The conservation of this structure reflects the importance of the peptide for reproductive success [Adams *et al.* 2002, Millar *et al.* 2004].

Of all the structural variants of gonadotropin-releasing hormone, GnRH II (cGnRH) is remarkably conserved without any sequence substitutions among vertebrates. It is regarded

as the most ancient and conserved member of the GnRH family, because it has been found in representative members of every vertebrate class [Sherwood *et al.* 1993]. GnRH II is present in all jawed vertebrates, except jawless fish, and is conserved from bony fish to humans indicating that this is probably the earliest evolved form of GnRH that remained unchanged for at least 500 million years. It was first cloned and sequenced from the chicken hypothalamus (cGnRH II) with an amino acid sequence of pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2 [Kasten *et al.* 1996, White *et al.* 1998 and Urbanski *et al.* 1999]. It differs from GnRH I by having His^5, Trp^7 and Tyr^8 residues [Miyamoto *et al.* 1984, Sealfon *et al.* 1997, Densmore and Urbanski 2003, Ikemoto and Park 2003, Cheng and Leung 2005].

In many vertebrate species, a third conserved form of GnRH (salmon GnRH) was found in teleost fish and is designated GnRH III. Even though the origin of GnRH-III is less clear, it seems that the gene duplication only occurred recently and originated after the divergence of teleosts from the vertebrate lineage [Sherwood *et al.* 1993, White *et al.* 1998, Yahalom *et al.* 1999 and Millar *et al.* 2004].

1.5 Molecular biology of GnRH

1.5.1 *GnRH gene and GnRH-associated peptide (GAP)*

It is widely recognized that the structure of all known vertebrate GnRH genes is conserved and is composed of four exons and three introns. The first exon encodes the 5'-untranslated region (UTR) and consists of 61 bp in mRNA expressed in the hypothalamus. The second exon encodes the signal sequence, the GnRH decapeptide, the GKR processing signal and the first 11 GAP residues. The third exon encodes GAP amino acids 12-43 and the fourth exon

encodes the remaining GAP residues and contains the translation termination codon, as well as the entire 3'UTR [Sherwood *et al.* 1993, Sealfon *et al*. 1997].

The GnRH I gene is derived from a larger, precursor molecule called pre-pro-GnRH. This molecule comprises the decapeptide GnRH preceded by a signal sequence of 23 amino acids and is followed by a Gly-Lys-Arg sequence necessary for enzymatic processing and C-terminal amidation of GnRH. The C-terminal region of the precursor is occupied by a further 56 amino acids that constitute the so-called GnRH associated peptide or GAP. GAP has been shown to release LH and FSH equipotentially from the anterior pituitary. In all species examined, immunocytochemical techniques have located the GnRH precursor, together with GAP and GnRH itself, in a major group of neurons within the medial preoptic area and adjacent anterior hypothalamus [Johnson and Everitt 1990, Dong *et al.* 1996].

The length of GnRH remained at 10 amino acids, the amino (pGlu) and carboxyl (Gly-amide) residues are unchanged, and five of the 10 residues are identical from lamprey to human. The most stable region of the peptide, residues 1-3, is responsible for releasing gonadotropins, while the region with the most changes, 5-8, is thought to mediate receptor binding [Sealfon *et al.* 1997].

Two forms of GnRH, termed GnRH I and GnRH II, encoded by separate genes have been identified in humans. Although they share comparable cDNA and genomic structures, their tissue distribution and regulation of gene expression are significantly dissimilar. Humans may be unusual with respect to GnRH control of reproductive endocrinology in that they

possesses another gene encoding GnRH-II located on chromosome 8p11-p21 and 20p13 , distinct from the GnRH I precursor gene that is encoded within the genome, on chromosome 4 [White *et al.* 1998, Pawson *et al*. 2003]. The pre-pro-GnRH II gene consists of a 5'-untranslated exon and three coding exons, with the mature peptide encoded within coding exon 1 [White *et al.* 1998]. It has also recently been found in the reptilian species, the leopard gecko (*Eublepharis macularis)*. The genomic structure consists of four exons and three introns [Ikemoto and Park 2003].

However, the GnRH II gene (2.1kb) is shorter than the GnRH I gene (5 kb) because introns 2 and 3 of GnRH I are much larger. Although the corresponding precursor proteins are quite similar in length, the GAP is 50% longer in the GnRH II precursor, suggesting that a relatively larger GAP may be a common characteristic among mammalian GnRH II precursors [Cheng and Leung 2005].

1.5.2 *Regulation of the GnRH gene*

The mammalian GnRH gene appears to have different transcriptional start sites depending on the species. Changes in the organization of the 5'-flanking region and in the regulation of gene expression by specific transcription factors should elucidate functional changes in the regulation of reproduction during evolution [Sherwood *et al.* 1993]. In humans, transcription of the gonadotropin-releasing hormone (GnRH) gene can be initiated at two transcription start sites to produce different GnRH mRNAs. The upstream transcription start site is used only in non-hypothalamic reproductive tissues and tumours. In the hypothalamus, the transcription start site utilized is located 61 base pairs upstream of the first exon/intron

junction. However, in many reproductive tissues a second start site 579 bases upstream of the hypothalamic start site is also utilized. RT-PCR analysis of the first strand cDNA derived from RNA isolated from human placenta, mammary gland, testis and ovary showed that the upstream pro-GnRH gene transcription site was preferentially used in these tissues. GnRH transcripts were detected in the rat ovary and the major transcription start site is located downstream of primer 11 [Goubau *et al.* 1992]. The rat and mouse pro-GnRH gene promoter regions are very different from those in the human in the 579 region, and they are lacking the upstream transcription start site [Dong *et al.* 1993]. To determine if a similar pattern of GnRH gene expression exists in non-human primates, a GnRH cDNA was cloned from Rhesus monkey hypothalamic RNA by using PCR to show a 96% similarity between monkey and human, with 94% similarity in the upstream promoter region. An upstream transcription site was also identified in Cynomolgus monkey testicular mRNA, which was different from the human GnRH gene. However, this proved that the GnRH gene expression pattern in this monkey is similar to that seen in the human and differs markedly from that in rodents. A reproductive tissue-specific transcriptional start site was located 504 bases upstream from the hypothalamic start site in the monkey testis, placenta, ovary and mammary gland. As with the human GnRH gene, two transcription start sites were used in transcribing the monkey GnRH gene. Because of the multiple transcription sites for the GnRH gene found in Rhesus and Cynomolgus monkeys, non-human primates make excellent models for studying GnRH gene regulation [Dong *et al.* 1996].

Some studies have shown that both estradiol and cortisol may be involved in regulation of GnRH gene expression in the human placenta. Based on some results, it is suggested that the

paracrine and autocrine regulation of hCG secretion by placental GnRH is mediated through the regulation of gonadotropin-releasing-hormone receptor (GnRHR) message. GnRHR gene expression was shown to be up regulated by GnRH, but down regulated by hCG [Peng *et al.* 1994, Khosravi and Leung 2003]. It is hypothesized that gonadal steroids may regulate GnRH I and II. The presence of the progesterone response element (PRE) in the 5'flanking region of the human GnRH receptor, suggests the possible regulation of this gene by progesterone at the placental expression [Cheng *et al.* 2001, An *et al.* 2005].

1.5.3 *GnRH Agonists and Antagonists*

Recent advances in biochemical techniques have made it possible to synthesize a new peptide once the structure is known. Forms that are structurally altered are called analogues and some synthetic analogues are many times more potent than the naturally occurring hormone and prove very useful in both experimental and clinical work. Inhibitory analogues block actions of the natural hormone by binding to the same receptor but not effecting the anticipated actions [Norris 1985].

Studies in receptor binding have shown that certain GnRH analogues that are antagonists of mammalian receptors behave as agonists in *Xenopus laevis* and chicken receptors, and since new generation peptide and non-peptide antagonists are being sought, this phenomenon provides the opportunity to elucidate interactions and the mechanism underlying receptor activation [Ott *et al.* 2002].

GnRH and its analogues have led to exciting new avenues of therapy in virtually every subspecialty of internal medicine, as well as in gynaecology, paediatrics, urology and oncology. The hypothalamic-pituitary-gonodal axis can be influenced in two distinct classes of therapeutic applications. The first application provides a natural sequence of GnRH in a pulsatile fashion via a portable infusion pump to mimic the normal physiology of hypothalamic secretion, and the second mode uses long-acting GnRH agonists administered in a depot delivery to produce a paradoxical desensitization of pituitary gonadotropin secretion.

This biochemical castration induced by GnRH agonist administration is a safe, effective, complete and reversible method of removing the overlay of gonadal steroids from a variety of diseases that they are known to exacerbate. This is also an ideal management tool to control population size in wild or captive animals without any negative side effects [Conn and Crowley 1994]. When the GnRH receptor is exposed to high concentrations of agonist, it becomes down regulated and results in the suppression of LH and FSH secretion. This led to the widespread use of GnRH analogues for the treatment of a number of endocrine-related disorders or hormone dependent diseases such as endometriosis and prostatic cancers [Eidne *et al.* 1992]. The presence of GnRH-like peptide in prostatic cancer cells may contribute to the appearance or promotion of cancer [Fekete *et al.* 1989] and at the same time lead to the possible role of direct anti-proliferative effect of GnRH analogues on prostatic tissue [Quyam *et al.* 1990, Azad *et al.* 1993]. Triptorelin, a GnRH agonist, has been shown to exert a double inhibitory-stimulatory action on cell growth when tested on two prostatic cell lines depending on the dose and environmental conditions [Ravenna *et al.* 2000].

In the rat and mouse, mammary tumours regress after administration of GnRH. Human patients with breast, prostate and pancreatic tumours have also shown clinical improvement after GnRH analogue treatment [Bützow *et al.* 1987]. An exogenous GnRH analogue addition to culture medium has been shown to inhibit growth of cultured mammary carcinoma cells (MCF-7) [Bützow *et al.* 1987] and the proliferation of cancer cells [Emons *et al.* 1989, Kakar and Jennes 1995].

Another study supported the previous findings that the GnRH agonist Triptorelin inhibits the proliferation of endometrial cancer cells, epithelial ovarian cancer cells and estrogen-stimulated breast cancer cells [Ravenna *et al.* 2000, Ciocca *et al.* 1990 and Emons *et al.* 2000]. The incorporation of [^{3}H] thymidine to tumour cells in culture suggested the tumour suppressing effect of GnRH agonists and antagonists may be a direct effect of GnRH on cell growth mediated through its high affinity receptors [Kakar *et al.* 1994, Kakar and Jennes 1995].

The effects of GnRH agonists on various reproductive functions have been investigated. Administration of GnRH or its potent agonist results in sustained increases in serum gonadotropins and treatment with high doses has been seen as a potential means of enhancing fertility. In female rats, long-term administration of pharmacological doses of GnRH or agonist inhibits ovarian steroidogenesis, ovulation, ovum transport, ovum implantation, pregnancy and uterine growth. It also exerts an extra pituitary action, as shown by their ability to inhibit ovarian function by decreasing ovarian oestrogen production [Hsueh and

Erickson 1979]. GnRH and its analogues have also been used extensively to induce ovulation and spawning in farmed fish [Pagelson and Zohar 1992].

The GnRH I analogue, Buserelin, caused suppression of progesterone production from Baboon granulosa cells *in vitro* [Siler-Khodr *et al.* 2003]. The analogue had no effect on pituitary release after long-term exposure, which may indicate pituitary specificity for mammalian GnRH. Specific GnRH II analogues may be useful for the site-specific regulation of ovarian function and may have a limited effect on pituitary function. In another study using Buserelin, the dependency of Ca^{2+} -dependent CI channels by *Xenopus* oocytes injected with rat pituitary mRNA was shown by measuring the responses of the oocytes to this analogue [Yoshida *et al.* 1989].

GnRH II analogues also demonstrated their role as a contraceptive agent when administered chronically around the time of ovulation or early luteal development in the Rhesus monkey. These GnRH II analogues may serve as an effective, non-steroidal, postcoital contraceptive [Siler-Khodr *et al.* 2004].

Continuous GnRH agonist treatment suppresses the pituitary-testicular axis in a wide range of species. This suppression is characterized by a decline in the peripheral concentrations of LH and testosterone, and regression of the testis, with eventual induction of azoospermia. In male rats, the agonists inhibit testicular steroidogenesis, spermatogenesis and male accessory sex organ growth [Hsueh and Erickson 1979, Hsueh and Jones 1983, Hsueh and Schaeffer 1985]. In monkeys, the acute and chronic administration of gondotropin-releasing hormone

antagonists and agonists can result in a decrease in testicular volumes, ejaculate volumes and sperm production [Bint Akhtar *et al.* 1985, Weinbauer *et al.* 1987a, Weinbauer *et al.* 1987b, Mann *et al.* 1987, Rao *et al.* 1990]

Antide, a highly selective GnRH I antagonist that blocks gonadotropin secretion, was used to show that GnRH II stimulates LH and FSH secretion *in vivo* (Rhesus monkey), as well as *in vitro* (monkey pituitary cells), and that this action occurs through the GnRH I receptor [Densmore and Urbanski 2003, Okada *et al.* 2003]. Antide also disrupted the normal oestrous cycle in an application of the antagonist in the preoptic area of female rats. This antagonist was shown to be a potential contraceptive in the marmoset, without affecting the postnatal rise in testosterone in the male infants [Fraser *et al.* 1994] as well as in Cynomolgus monkeys by suppressing gonadotrophins for 40-50 days [Conn and Crowley 1994].

In a number of species, however, males appear to be insensitive to the inhibitory effects of GnRH agonists. A male marsupial, *Macropus eugenii,* maintained a tonic LH secretion and appears to be resistant to the contraceptive effects of the chronic GnRH agonist, Deslorelin, treatment [Herbert *et al.* 2004].

In two separate studies on sexual development, the GnRH antagonist, Cetrorelix, was used to block the effect of endogenous GnRH action. These results showed that the sexual development of male and female rats is accompanied by the expression of the GnRH receptor and gonadotropin subunit genes during pubertal development [Zapatero-Caballero *et al.* 2003, Zapatero-Caballero *et al.* 2004]. Cetrorelix has also been shown to block GnRH I but

not GnRH II. These effects might be mediated via a GnRH II-specific receptor since antagonists bind to receptors at sites that are distinct from those occupied by agonists [Pawson *et al.* 2003].

The increased biologic activity of GnRH agonists is due to their ability to stay bound to the pituitary receptor longer than the natural hormone and to their capacity to resist enzyme degradation. The half-life of the GnRH analogues in circulation is identical to that of the natural hormone and therefore does not contribute to their increased biologic activity [Densmore and Urbanski 2003].

1.6 GnRH Receptors

The interaction between GnRH and its receptor is a critical event in the endocrine regulation of reproduction and therefore, understanding the regulation of the receptor is important for the study of normal reproductive physiology. The pituitary GnRH receptor is primarily located in the plasma membrane of gonatrophs and it is evident that almost all neurotransmitters and neuropeptides have more than one receptor. It is thus hard to conceive that three GnRH isoforms that are 70%-80% homologous to each other would share a common, single GnRH receptor. However, cross-activity of these isoforms with the GnRH I receptor has been suggested [Yahalom *et al.* 1999]. Amphibia, like most vertebrates, have two forms of GnRH and a receptor for chicken GnRH II has been identified in the sympathetic ganglion of the bullfrog (*Rana catesbeiana)* and platana *(Xenopus laevis)* [Troskie *et al.* 1997]. The presence of at least two GnRHs in a particular species led to the concept of the existence of more than two types of receptors, suggesting that the duplication

of the GnRH gene was accompanied by a coordinated structural evolution of the cognate receptors. The cloning of two subtypes of receptors with differential distribution in the brain and pituitary of the goldfish *(Carassisius auratus)* [Illing *et al.* 1999] and three subtype receptors in the pituitary and hindbrain of the bullfrog, confirmed this theory [Wang *et al.* 2001].

The GnRH I receptor is an established member of the Ca^{2+}-dependant family that is expressed on the surface of pituitary gonadotrope cells [Conn 1986, Conn *et al.* 1986, Stojilkovic *et al.* 1994]. The predicted amino acid sequence for the receptor has more than 80% overall arrangement identity in mammals and is highly conserved with the putative TM domains. It belongs to the group of the rhodopsin-like G protein-coupled receptor (GPCR) family that is characterized by seven-transmembrane (7TM) domains connected by alternating intra cellular and extra cellular loop (EL) domains [Kakar *et al.* 1992, Huckle and Conn 1988, Sealfon *et al.* 1997, Illing *et al.* 1999, Cui *et al.* 2000]. The extra cellular loop 2 of the receptor has been identified as the determinant for agonist activity [Ott *et al.* 2002]. A unique feature of the mammalian receptor I is the absence of the cytoplasmic carboxyl-terminal tail [Eidne *et al.* 1992, Kakar *et al.* 1992, Sealfon *et al.* 1997].

The GnRH I receptor was first cloned from mouse and several other mammalian species [Eidne *et al.* 1992] and homologous receptors were soon identified in the human [Kakar *et al.* 1992, Chi *et al.* 1993, Fan *et al*. 1995, Millar *et al.* 1999].

There is a high degree of homology between cloned GnRHR, particularly between mice and rats, in which 93% of the amino acid sequences are conserved, while the receptor from the pituitary of the rat shows the same homology to that of human receptors [Eidne *et al.* 1992]. The sequencing of receptors from a number of fish such as the GnRH receptors found in the pituitary of the Gilthead Sea bream *(Sparus aurata)* enabled researchers to study the contribution of receptor affinity in the determination of relative biopotencies of GnRH analogues [Pagelson and Zohar 1992].

The GnRH II ligand structure is conserved from bony fish to humans, indicating that this is probably the earliest evolved form of GnRH and thus has critical and specific functions. The cow, sheep and human receptors are 328 amino acids long, while the mouse and rat receptors are 327 amino acids, due to the absence of a residue in the second extra cellular domain. In type II receptors, the N-terminal domain is two residues longer and more negatively charged than that of the type I receptors. Extra cellular loops two and three are also shorter in the type II receptor [Pawson *et al.* 2003]. The catfish receptor is 370 amino acids in length and together with the GnRH II receptor and those found in the goldfish, retained a 56-residue cytoplasmic tail domain at the carboxyl-terminus that is phosphorylated in response to GnRH II binding. This leads to receptor internalization and desensitization along with the coupling of both receptors to the G coupled-protein to mediate the intracellular production of inositol phosphates [Kakar *et al.* 1992, Cui *et al.* 2000].

The tail sequence is not conserved between mammalian and amphibian homologues [Illing *et al.* 1999, Neill 2002, Stojilkovic *et al.* 1994 and Pawson *et al.* 2003]. The importance of the

tail for rapid agonist-induced internalization has been demonstrated in studies for the marmoset type II and the chicken GnRH receptor, while the carboxyl-terminal tail of the catfish GnRH receptor proved to be important for cell surface expression, ligand binding, and receptor phosphorylation and internalization [Millar *et al.* 2001, Pawson *et al.* 2003, Millar *et al.* 2004].

GnRH regulates reproduction via the well-characterized mammalian pituitary GnRH I receptor. This action was confirmed in an *in vitro* and *in vivo* study where results showed that GnRH II stimulated LH and FSH release by activating GnRH I receptors [Okada *et al.* 2003, Densmore and Urbanski 2003]. Three GnRH receptors or receptor-like genes were identified in the human genome:

1) the well established GnRH I receptor gene located on chromosome 4

2) an apparent GnRH II receptor gene located on chromosome 1, and

3) a sterile GnRH II receptor-like homolog gene on chromosome 14 [Kakar *et al.* 1992, Neill 2002, van Biljon *et al.* 2002].

The chromosome 1 gene is comprised of exons 1, 2 and 3, whereas the chromosome 14 gene is comprised of only exons 2 and 3 of the putative receptor. The transcripts for chromosome 1 are either not expressed or only expressed in low levels, but exon 2-3 amplicons for chromosome 14 are abundant at high levels [Neill *et al.* 2001, Neill 2002, van Biljon *et al.* 2002]. A study using an exon 1-specific probe for chromosome 14 was found with relatively strong signals in putamen, occipital lobe, cerebellum, caudate nucleus, heart and testis [Millar *et al.* 2001, van Biljon *et al.* 2002]. To facilitate the identification and

characterization of selective non-peptide GnRH antagonists, the GnRH I receptor in the dog was cloned and is 92% identical to the human GnRH receptor [Cui *et al.* 2000, Millar *et al*. 2004].

An immunohistochemical assay confirmed the presence of a GnRH receptor in the human pituitary that was localized on the surface of the anterior pituitary cells co-localizing with FSH [Lee *et al.* 2000] and a highly selective type II receptor has been cloned and identified in the monkey brain. The receptor was cloned from the pituitary of the Bonnet monkey [Santra *et al.* 2000], a receptor specific for GnRH II from the Rhesus monkey pituitary, a kidney-derived cell line from African green monkeys, [Neill *et al.* 2001] and from the pituitary of the marmoset [Millar *et al.* 2001], but its physiological function remains unknown. All three receptors revealed that they code for a typical seven TM domain GPCR. The primary amino acid sequence of the Marmoset type II GnRH receptor has 39% identity with the type I receptor. The mRNAs of all mammalian GnRH receptors cloned to date are encoded by three exons, and the gene structures are conserved. Type II receptors are expressed in the anterior pituitary of several mammalian species and throughout the brain, as shown in Marmoset and human brains. The type II receptor is more widely distributed than type I and this includes areas in the brain associated with sexual arousal. It might even have a unique function in the monkey brain and pituitary other than the regulation of gondadotropin secretion. The pharmacological characterization revealed that the Marmoset type II receptor is highly selective for GnRH II and less so for GnRH I. Two other GnRHs reportedly expressed in mammals are also active with the type II receptor [Sealfon *et al.* 1997, Neill *et al.* 2001, Neill 2002 and Millar *et al.* 2001]. Furthermore, the marmoset type II receptor can

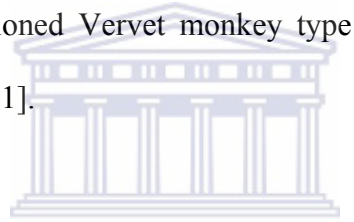
be activated by a GnRH I antagonist, which is a full antagonist of the human type I receptor. This observation may help explain why certain GnRH I antagonist exhibit agonistic activities in some gynaecological cancer cells expressing the type II receptor [Millar *et al.* 2001].

Using reverse transcript polymerase chain reaction and Southern blot analyses, expression of GnRH I receptor mRNA indistinguishable from its pituitary counterpart has been demonstrated in various ovarian compartments such as the human ovaries and human granulosa-luteal (GL) cells [Kakar *et al.* 1992, Peng *et al.* 1994]. In human GL cells, treatment with hCG for 24 hours induces a dose-dependent inhibition of GnRH receptor mRNA levels [Kakar and Jennes 1995]. Even though binding sites for GnRH were detected in the human placenta, no receptor could be found. The failure to amplify the placental GnRH receptor mRNA consistently at all stages of gestation, using the same set of primers, suggests that there might be different receptors expressed at different stages of gestation [Rama and Rao 2001]. Human GnRH receptor gene expression is regulated by progesterone at pituitary and placental levels [Cheng *et al.* 2001]. This effect of progesterone was also shown *in vitro*, using a human neuronal medulloblastoma cell line (TE671) [An *et al.* 2005].

GnRH receptors are expressed in Leydig cells but not the Sertoli cells [Kakar *et al.* 1992] and are also found in both mouse and rat testicular germ cells, even though there was not conclusive evidence for the presence of a receptor in mature sperm cells. These results demonstrated that the receptor may interact in testicular germ cells with GnRH or GnRH-like peptides produced in the testis and may be part of a paracrine system [Bull *et al.* 2000]. An immunohistochemical assay using antibodies revealed the presence of a GnRH receptor

in human sperm that is mainly localized in the acrosomal region [Lee *et al.* 2000]. This could explain the responsiveness of human sperm to GnRH in enhancing sperm-zona binding shown in a previous study [Morales 1998].

The confirmed presence of a type II GnRH receptor immunoreactivity in human tissue led to the search for evidence that the homologous gene for receptor II located on chromosome 1 in humans might be functional. These investigators cloned a potentially full-length type II GnRH receptor transcript from the gene on chromosome I in human sperm. It showed the presence of a UGA translation stop codon within exon 2, as well as a frame shift within exon 1 compared to the recently cloned Vervet monkey type II receptor that contains a CGA arginine codon [Neill *et al.* 2001].



The *in situ* localization of type II GnRH receptor transcripts to the ad luminal region of the seminiferous epithelium is consistent with the distribution of other haploid-specific mRNAs and suggests that this receptor is post-meiotically expressed in round and elongated spermatids [Wykes *et al.* 1997]. These transcripts are distributed throughout the entire sperm head. Differentiating spermatids might require a functional type II GnRH receptor at specific stages during spermatogenesis [Willson and Ashworth 1987]. Sperm have also been shown to express receptors for other hormones or signalling molecules such as the oestrogen receptor and A1 adenosine receptor [Wassarman 1990, Durkee *et al.* 1998 and Minelli *et al.* 2000]. Whether a full length GnRH II receptor protein is expressed in mature sperm and whether the transcripts found are functional, still need to be clarified [van Biljon *et al.* 2002].

A full-length human type II receptor from testis was reported with the presence of a frame shift and a stop codon [Neill *et al.* 2001, Neill 2002].

mRNA contains a stop codon and it seems likely that the gene is a transcribed pseudo gene although it seems functional in primates. This may reflect the involvement of the type II GnRH receptor in the induction of mating behaviour of other primates that are seasonal breeders, unlike humans [Neill *et al.* 2001].

Some observations seemed to indicate that GnRH receptor expression is significantly elevated when human tissue becomes cancerous or is in an abnormal or diseased state. Several studies support the concept that about 50% of breast and approximately 80% of ovarian and endometrial cancers express GnRH I receptors. They are found in other tumorous tissues and cancer cell lines [Emons *et al.* 1989, Kakar and Jennes 1995, Emons *et al.* 1998], are absent in normal breast and liver cells, and are present in low levels in normal heart and brain tissue [Lee *et al.* 2000]. The type I receptor is effective in inhibiting the proliferation of tumour cells due to its resistance to densitization, a common and rapid event of the GPCR super family and undergoes very slow internalization compared to the type II receptor. The GnRH I receptor remains active at the cell surface for longer than most other GPCRs [Pawson *et al.* 2003]. Another study suggests that the inhibitory and stimulatory effects of GnRH on cell proliferation, using four cell lines, exhibit distinct different patterns of ligand sensitivity and that these effects occur via different types of GnRH receptors. For the first time these results proved that the human type II receptor could be functional [Enomoto *et al.* 2004].

Recent cloning of the gonodotropin-releasing hormone (GnRH) receptor from the human breast tumour cell line (MCF-7), and from an ovarian tumour, and its expression in various other human tumours, tumour cell lines and reproductive organs, have been reported [Bützow *et al.* 1987]. An ovarian cancer cell line shown to be type II receptor mRNA positive, but type I receptor mRNA negative, responded only to GnRH II but not GnRH I.

Results have also shown that the GnRH receptors in human extra-hypophysial tissues, such as breast and ovarian tumours, have the same high binding affinities commonly associated with the GnRH receptor of the pituitary gland and that the nucleotide sequences are the same [Kakar *et al.* 1994, Emons *et al.* 2000]. In rats, high affinity and low capacity receptors have been demonstrated in the ovaries comparable to pituitary GnRH receptors [Hsueh and Schaeffer 1985] but reports for GnRH I receptors in human prostate cancer and prostatic cell lines have shown that the affinity of these sites is generally lower than that of the pituitary receptor [Ravenna *et al.* 2000]. The receptors for LHRH (GnRH), Somatostatin and Prolactin in human and rat prostate cancers suggest that LHRH (GnRH), along with other hormones, may act as a growth or transforming factor that contributes to the promotion of prostate cancer in humans [Fekete *et al.* 1989].

Several sources indicate that the expression of extra-pituitary GnRH receptor is not limited to reproductive tissues only. It has been demonstrated by RT-PCR and Southern blot hybridization that the type I receptor is also expressed in the liver, heart and kidney [Kakar and Jennes 1995], whereas the type II receptor mRNA is expressed in human heart, pancreas and skeletal muscle [Millar *et al.* 2001].

The sexual development of female and male rats is accompanied by a progressive and concerted expression of the GnRH receptor but gonadotropin subunit genes in the anterior pituitary reach maximal rates during infancy and are kept at a minimum during juvenile prepubertal development [Zapatero-Caballero *et al.* 2003, Zapatero-Caballero *et al.* 2004].

In humans, it seems as if the type II receptor has become non-functional and that its function is now served by the type I receptor as the target for GnRH II. The type II receptor also appears to be silenced in the mouse, chimp, cow and rat genomes. GnRH II receptor immunoreactivity in the human pituitary and brain [Miller *et al.* 2001] and the wide expression of the type II GnRH, suggest that a functional GnRH II receptor exists and that the gene on chromosome 1 is not a pseudo gene [Neill 2002, Millar 2003, Neill *et al*. 2004]. However, the reports on the disruption of the human type II receptor gene by a frame shift and premature codon in tumours, is an indication that a conventional type II GnRH system is absent in humans [Cheng and Leung 2005, Pawson *et al*. 2005].

1.7 Evolution of the GnRH gene

The origin of the GnRH molecule may precede the evolution of the vertebrates or even the invertebrates. GnRH is an example of a peptide whose gene has apparently undergone duplication to produce a second form of peptide. Duplicated genes may relocate to another portion of the genome [Sherwood *et al.* 1993].

The regulation of reproduction by GnRH has been highly conserved during 500 million years of vertebrate evolution despite the fact its amino acid sequence varies by 50%. The

constraints that appear to have limited the GnRH molecule to substitutions that are exclusively in positions 5, 7 and 8, may be related to receptor binding [Sherwood *et al.* 1993]. In order to study the evolutionary relationship among GnRH forms, a molecular phylogenetic reconstruction technique (the phylogenetic tree) [White *et al.* 1998] was used to show the existence of three evolutionary distinct GnRH groups. These are "releasing" forms localized to the hypothalamus (GnRH I), forms previously localized solely to the midbrain nuclei (GnRH II), and forms localized to the telencephalon in several fish species (GnRH III). The structure of the tree suggests that multiple forms of GnRH exist in many different species because of their ancient duplications of a gene encoding GnRH. GnRH I and GnRH II include representatives from fish and mammals. The origin of GnRH III is less clear because there are only representatives from fish in this group [White *et al.* 1995].

The type II GnRH seems to have been conserved longer than the mammalian GnRH type I. This peptide appears in all classes of vertebrates through primitive placental mammals, revealing that it has remained unchanged for decades, also indicating an important conserved role and a discriminating receptor (or receptors) that have been selected against any structural change in the ligand. This points to essential functions, that have yet to be identified [Millar *et al.* 2001, Millar *et al.* 2004].

Recent studies provided evidence for the presence of a functional type II GnRH receptor in primates and pigs, and a type III receptor for fish and amphibians. Besides the wide tissue distribution of GnRH II, it is becoming evident that a conventional type II receptor system,

specific for GnRH II, does not exist in humans [Pawson *et al.* 2003, Cheng and Leung 2005, Pawson *et al.* 2005].

1.8 General primate spermatogenesis and GnRH

Various factors are important regarding the maintenance of spermatogenesis, e.g. the role of sex steroids and other hormones during this process have been reported. Gonadotropin-Releasing Hormone (GnRH) is well known for its function in the reproductive processes of mammalian and non-mammalian species. The treatment with high doses of GnRH and its analogues was predicted as a potential means for enhancing fertility. Moreover, a decrease in reproductive function has also been shown with long-term administration of pharmacological doses GnRH or its agonists [Hsueh and Jones 1983, Clayton 1989].

Two types of GnRHs in primates [Lescheid *et al.* 1997] and the cloning of the type I and II receptors have been reported [Santra *et al*. 2000, Millar *et al.* 2001]. GnRH has an indirect function on spermatogenesis since LH and FSH are mainly involved in the maintenance of spermatogenesis and GnRH regulates the synthesis and secretion of these gonadotropins, which in turn regulate the hormonal and gametogenic functions of the gonads. It seems that GnRH II and the mammalian type II GnRH receptor specifically regulate FSH secretion, a peptide hormone involved in the development of meiotic spermatocytes and post meiotic spermatids [Conn *et al.* 1986, Glander and Krantzsch 1997]. In an *in vivo* study, a synthetic GnRH II was administered to the Rhesus macaque and the results confirmed that GnRH II is indeed the potent stimulator of gonodal release. A difference in the mechanism of the two peptides also became clear in the involvement of two different routes by which each peptide

could reach the pituitary to coordinate control of gonadosteroidogenesis and sperm production [Lescheid *et al.* 1997]. The results of an *in vitro* study, using monkey pituitary cultures, confirmed that GnRH II stimulates FSH and LH secretion, but that this action occurs via the GnRH I receptor and that the GnRH II receptor found in the primate brain may have a unique function other than regulation of gonadotropin secretion [Densmore and Urbanski 2003, Okada *et al.* 2003].

The production of spermatozoa and the secretion of testosterone by the testis are both dependent on stimulation by the pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinising hormone (LH). Testosterone, which is essential for the initiation and maintenance of spermatogenesis, is secreted by the Sertoli, Leydig and peritubular cells. Sertoli cell number is an important determinant of the maximum achievable sperm output, but another key factor is the efficiency of spermatogenesis. Large differences between species were found when daily sperm productions were compared in terms of per gram of testis and spermatid *versus* Sertoli cell ratio. Of the nonhuman primates that have been investigated, the Rhesus monkey shows vastly superior efficiency of spermatogenesis compared to man, whereas the orang-utan and Cynomolgus monkey (*Macaca fascicularis*) are somewhat intermediate [Sharpe 1994]. The most notable differences between non-human primates and man are the number of generations of differentiated B spermatogonia (four in the monkey and one in man). Other differences include the duration of spermatogenesis, which in the monkey is 48 days, whereas that for man has been reported to vary around 74 days [Plant and Marshall 2001]. The only resemblance in humans and Vervets (*Chlorocebus*

aethiops) is the multiple stages of spermatogenesis observed in a transverse section of a seminiferous tubule [van der Horst 2005].

The FSH receptor is expressed in the testes of the Cynomolgus monkey (*Macaca fascicularis*) and various studies of non-primate species have demonstrated that FSH binding in the testis is restricted to the Sertoli cells. Recently, a study provided compelling evidence for the view that gonadotropin control of testicular inhibin B secretion by the primate testis involves opposing stimulatory and inhibitory actions of FSH and LH, respectively. FSH is posited to act directly on the Sertoli cell and LH appears to be mediated by a paracrine action of testosterone from the Leydig cell to regulate inhibin-B gene expression by Sertoli cells [Ramaswamy *et al.* 2003].

Many *in vitro* and *in vivo* systems have been used to study regulation of spermatogenesis by FSH and testosterone. FSH has a key role in the development of the immature testis, particularly by controlling Sertoli cell proliferation. To support this action of FSH, a study in foetal hypophysectomy in the Rhesus monkey resulted at term in a marked reduction in testicular size, suggesting that gonadotropin secretion by the foetal pituitary plays an important role in regulating Sertoli cell proliferation *in utero.*

The role of FSH in the initiation and maintenance of testicular function in higher primates also confirmed marked species differences. Most notably, although hypophysectomy of adult primates leads to regression of the seminiferous epithelium, as it does in rats, only Sertoli cells and stem spermatogonia remain after removal of the pituitary. In the rat, these cells are

arrested during spermiogenesis [Plant and Marshall 2001]. Thus, in primates, the gonadotropic hormones are obligatory for development to differentiated spermatogonia, spermatocytes and spermatids; whereas in rats it appears that a limited number of spermatids may be produced in the absence of a gonadotropin drive [Plant and Marshall 2001].

Based on relatively selective withdrawal and replacement studies, it was shown that FSH is important in the progression of type A to B spermatogonia and, in synergy with testosterone, in regulating germ cell viability. Acute suppression models, such as hypophysectomy and GnRH antagonist treatment, have been used to demonstrate the role of FSH and testosterone in the maintenance of germ cell viability. Acute gonadotropin suppression resulted in germ cell death and exogenous testosterone suppressed both FSH and LH in primates (Macaque monkeys), profoundly impairing spermatogenesis. During this action, testosterone acts directly at both the hypothalamus and anterior pituitary. Even though FSH has an important role in spermatogenesis, the abolition of FSH secretion alone will not prevent either the initiation or maintenance of spermatogenesis and therefore will not lead to azoospermia [Plant and Marshall 2001, Anderson and Baird 2002, McLachlan *et al.* 2002].

Since primates share very similar hormonal dependencies and structural patterns of spermatogenesis compared to humans, methods for the development of safe and reversible male hormonal contraception are being investigated. Various studies have shown the effect of acute and chronic effect of GnRH antagonist treatment in male primates. Chronic antagonist treatment resulted in decrease in testicular volumes and ejaculate [Bint Akhtar *et al.* 1985] and the induction of azoospermia within nine weeks in male Cynomologus

monkeys (*Macaca fascicularis*) [Weinbauer *et al.* 1987a, Weinbauer *et al.* 1987b]. In the male Bonnet monkey (*Macaca radiata*), no ejaculate could be obtained by week six of antagonist treatment [Rao *et al.* 1990].

Although azoospermia can be achieved by daily GnRH antagonist treatment, the continuous infusion of large doses of antagonist can cause undesirable side effects. A significant loss of body weight was reported for treated male Rhesus monkeys (*Macaca mulatta*) with a high dose, but a low dose GnRH infusion by means of using osmotic mini pumps did not alter the bodyweight [Mann *et al.* 1987]. Similar results on body weights have been reported for the male Bonnet monkey on a low dose mini pump infusion. In all the studies reported, the inhibitory effects of GnRH antagonists were reversible and serum hormones, testis size and sperm counts returned to pre-treatment values.

Seminal tubular fluid (STF) has always been viewed primarily as a means of transporting spermatozoa, inhibin and other substances out of the testis, but it was hypothesized as perhaps the single most important factor in the regulation and maintenance of spermatogenesis. It is the secretion of STF that changes first, in virtually every situation in which spermatogenesis modifies, whether during puberty, seasonal regression of the testis or in the failure of spermatogenesis after the withdrawal of testosterone. Nearly every messenger protein, nutrient and energy source has to be transported to the germ cells from the Sertoli cells, and STF is the major route via which this can occur. Since GnRH II is found in tissues such as in the baboon testis, epididymis and seminal vesicle [Siler-Khodr *et al*.

2003], STF and GnRH II regulation might play an important role in the maintenance of primate spermatogenesis.

1.9 Spermatology of the Vervet monkey

Most studies on Vervet semen were confined to investigating sperm concentrations and motility. The percentage motility reported for Vervet monkey sperm seems to be in the range of 34% to 58%. Semen samples from five non-human primate species were reported; a mean motility of 53% was described for three Vervet monkeys [Roussel and Austin 1967]. Another study involving Vervets, reported sperm motility ranging from 34% to 38% [Ackerman and Roussel 1968]. Other parameters such as sperm concentrations and ejaculate volumes were also investigated. Ejaculate volumes ranging from 0.9ml to 1.24ml and sperm concentration from 57 million/ml to 440 million/ml were reported [Valerio and Dalgard 1975, Hendrickx *et al*. 1978].

Results of semen analysis were reported from a closed breeding colony of Vervet monkeys where breeding males were compared to singly caged males [Seier *et al*. 1989]. The average sperm motility for 11 breeding males and 58 singly caged males was 43% and 55%, respectively. A total semen volume of 0.45 – 0.86ml and a mean concentration per ml ranging from 117 to 185 ($x10^6$) was reported. The values obtained were much lower than described for Macaques, but were relatively the same as previous findings for Vervet monkeys. However, large variations in semen characteristics were found within individuals, such as sperm concentration and motility that differed by 100% and more in at least 2

consecutive ejaculates from 21 males. Considerable differences in a consecutive ejaculate from the same individual were also reported [Seier 1995].

Abnormalities of the sperm were also investigated. The most common morphological abnormality in the same colony of Vervet monkeys (as above), were detached tails. Other abnormalities were microcephalus, macrocephalus, narrow tapered heads, cytoplasmic droplets, thick midpieces and bent or coiled tails [Seier *et al.* 1989]. The same author later reported that tail abnormalities predominate in the sperm of Vervet monkeys and that males with the highest number of folded or coiled tails were successful breeders [Seier 1995]. A total of 28 different sperm abnormalities were found amongst the ejaculates of 28 Vervet monkeys. From a total of 13 head abnormalities, three types were found in colony-bred individuals. In a total of seven midpiece abnormalities, only three types were found in wild-caught individuals, while end piece abnormalities occurred in both groups.

Fertility potential of cryopreserved semen can be poor due to damage to the acrosome and the Vervet is a good model for studies on cryopreservation. The effects on post-thawed sperm and acrosomal status of the Vervet monkey have been reported. A mean recovery of 63.6% and progressive motility of 43% for Vervet monkey sperm was found with a well-established cryopreservation method. Acrosome damage was found to be higher for post-thawed sperm compared to damage before cryopreservation. Intact acrosome levels were 61.2% at pre-freeze *versus* 31.6% at post-thaw and for severely damaged acrosome (equatorial staining) 16.8% compared to 29% for pre-freeze and post-thawed sperm.

Acrosome losses were found to be higher for cryopreserved sperm [Conradie *et al*. 1994, Seier 1995].

The maturation of Vervet sperm motility was also explored using sperm kinematics. A computer-aided sperm motility analysis system revealed clear trends in the development of sperm motility in the epididymis and vas deferens. The motion of spermatozoa from the caput epididymis was sluggish and irregular but the motility increased sharply as it moved through the corpus epididymis and continued to improve through the cauda epididymis and vas deferens. The results proved that the full maturation of motion capabilities was only completed once spermatozoa reached the vas deferens with final emergence in the ejaculate [van der Horst *et al*. 1999].

1.10 Purpose of the study

Gonadotropin-Releasing Hormone (GnRH) and its receptors have been cloned and its structure and functions have been studied extensively in both mammalian and non-mammalian vertebrates. GnRH I and GnRH II are found in humans and primates along with a functional type II receptor that has been located in primates but seems to be non-functional in humans.

In addition to the established paracrine role, it is possible that this peptide has autocrine functions in several reproductive and non-reproductive tissues. In male reproduction, these peptides play an important role in the maintenance of spermatogenesis, sperm maturation and fertilization. *In vitro* expression and immunoidentification of a GnRH receptor in mouse and

rat testicular germ cells and in the acrosomal region of human sperm have been reported. A GnRH II receptor transcript has also been located in human sperm, but it contains a stop codon, and the function is unknown. In addition, GnRH-like substances have been located in human and primate testis, epididymis and seminal fluid with receptors that seem to be situated in the Leydig cells.

In vitro studies on human sperm have confirmed that GnRH plays a role in sperm binding during fertilization and that GnRH and its antagonists did not affect the motility or percentage of acrosome-reacted spermatozoa. These findings raise a challenging question as to how GnRH will affect primate sperm. Primates do have a functional type II receptor and the fact that GnRH II is found ubiquitously in primate reproductive tissue suggests that this peptide has a biological function in sperm. Further, the type II receptor has been confirmed to specifically regulate FSH secretion in primates. However, the investigation of the function of GnRH and its receptors in primate male reproduction has been mainly limited to either sex hormone control studies or understanding the mechanism of the contraceptive activity of GnRH. A literature search provided no evidence of any *in vitro* or *in vivo* work on the direct effect of GnRH on primate sperm. The possible role of GnRH in regulating sperm motility and on acrosome reaction, possibly via one or both GnRH receptors, needs consideration in the above context.

Specific aims are:

1. To investigate the effect of GnRH I and GnRH II at different concentrations on sperm acrosome status.
2. To study the effect of GnRH I and GnRH II at different concentrations on the motility of the Vervet monkey sperm by computer aided sperm analysis.

The results should improve our understanding of the function of both gonadotrophin releasing hormones and their direct effect on Vervet monkey spermatozoa *in vitro*.

CHAPTER 2

Materials and Methods

2. The non-human primate model – Vervet monkey

The Vervet monkey (C*hlorocebus aethiops*), also called the African green monkey, is one of four primate species indigenous to Southern Africa. They adapt well to captivity and breed successfully within a closed environment. It is widely accepted that defined captive-bred primates are preferable to wild-caught individuals as an ideal model for biomedical research [Seier 1986].

2.1 The choice of subjects for this study

Adult males were chosen randomly but availability at the time of sampling determined, and often limited, the choice. The criteria for inclusion were sexual maturity and a 50% progressive motility rate for spermatozoa, due to the rapid decline in semen motility. Ten males who consistently offered reliable sperm concentrations, motility and normal morphology, were retained for this study.

All animals were from a captive-bred stock of Vervet monkeys with a history of successful reproductive performance for two generations. All males used were successful breeders and were housed singly for the duration of the study. The conditions in the closed indoor environment were maintained at 25 - 27°C, a humidity of 45%, about 15-air changes/hour and a photoperiod of 12 hours [Seier 1986]. All individuals had regular access to exercise cages and environmental enrichment (foraging and food puzzles).

2.2 Spermatology and medium

2.2.1 *Semen collection*

Semen was obtained by peri-prostatic electro stimulation applied per rectum. The electro ejaculator consisted of a 175mm long homemade rectal probe with a diameter of 15.4mm and two brass bands embedded in the tip. Both bands were wired internally to contacts at the base of the probe. The two probe contacts were connected to a transformer with five taps, which controlled voltage output from 2.5 – 5.5V by 1.0V increments and had one additional 8.0V output [Seier *et al.* 1989, Cseh *et al.* 2000] (Fig. 2.1).

To achieve ejaculation, a male was placed in dorsal recumbency after anaesthesia with ketamine hydrochloride (Anaket V, Cape Medical Supplies, Cape Town), at 10mg/kg bodyweight intramuscularly. The probe was lubricated with a gel containing disinfectant (Betadine, Lakato Vet., Cape Town) and inserted into the rectum with the brass bands at the level of the prostate gland.

Electro stimulation was started at 2.5V and the full current was applied by switching on the transformer. The probe was held steady at a point until erection was achieved. The current was applied for 1-2 minutes followed by a 15-20 second rest period. Voltages were increased by connecting the leads of the probe into the tap of the transformer for the next higher voltage, until ejaculation was achieved. All ejaculates were collected in a 10ml pre- warmed graduated glass tube. A total of 84 ejaculates were collected for this study.

Figure 2.1 Electro ejaculation equipment

2.2.2 *Extenders for semen*

Because sperm motility of the Vervet monkey declines rapidly if spermatozoa are maintained in seminal fluid [Seier *et al.* 1989], semen was diluted with Hams F10 extender. A volume of 25ml Hams F10 was used containing 0.247g Hams F10 nutrient mixture, 0.03g $NaHCO_3$ (Sodium Bicarbonate) (Sigma, Cape Town), 0.025g Bovine Serum Albumin and 25ml distilled water. The pH was adjusted to 7.5 with Na_2HPO_4 (dibasic) or NaH_2PO_4 (monobasic) (Sigma, Cape Town) and a 744 Metrohm pH Meter (Metrohm Ltd., Switzerland). The extender was freshly prepared for every experiment one hour prior to semen collection. The

medium was kept in a water bath at 37°C throughout the course of the experiment and discarded at the end.

2.2.3 *Medium for acrosome staining*

Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) was used for acrosome staining. A stock solution was prepared by dissolving 5mg of FITC-PNA in 5ml phosphate-buffered saline (PBS), and it was stored in 600µl aliquots at -20°C. For the final working solution, 15.5ml PBS were added to a 600µl aliquot of FITC-PNA.

All buffers and chemicals were purchased from Sigma Chemicals (Cape Town, South Africa).

2.2.4 *GnRH analogues*

The 2 peptides with a molecular weight of 1411 for GnRH I and 1463 for GnRH II were prepared as a stock solution of 50µl aliquots at a concentration of 10^{-3}M, with 20% propylene glycol as the vehicle medium. Analogues were stored in 1ml cryotubes at -20°C. Analogues and propylene glycol were supplied by Prof. A Katz from the Receptor Biology Research Group, UCT Medical School, Cape Town, South Africa.

2.3 *In vitro* incubation

2.3.1 *Swim-up incubation*

Due to the coagulation of semen, the standard swim-up [Vijayakumar *et al.* 1987, Oliva *et al.* 1991 and Esteves *et al.* 2000] had to be adjusted according to the sample to get the most motile sperm. In samples with fluid semen, a volume of 0.5ml seminal plasma was slowly

expelled at the bottom of a 5ml plastic tube containing pre-warmed (37°C) 1ml Hams F10. If the ejaculate contained only coagulum, 1ml of the extender was layered over the sample to allow spermatozoa to swim out of the coagulant mass. The tubes were placed at a 45° angle and incubated for 10 minutes in a water bath at 35°C. During this period, motile spermatozoa migrated from the under layered sperm suspension to the upper layer.

After 10 minutes, a drop of the swim-up was collected with a glass Pasteur pipette from the upper layer of the extender and observed under a microscope at 100 and 400x magnifications. If the swim-up was estimated to yield 50% motile sperm, the top 550µl of the supernatant was gently aspirated with a Gilson pipette and the specimen was divided into three equal aliquots of 180µl. The first aliquot received no treatment (control) and 20µl Hams F10 extender was added with a positive displacement pipette. The second and the third aliquot sperm samples were treated by adding 20µl of GnRH I and 20µl of GnRH II, respectively. All three aliquots were kept in loosely capped 1ml cryotubes (LASEC, Cape Town) and placed in a water bath at 37°C. The effect of concentrations 10^{-5}M, 10^{-6}M and 10^{-8}M for GnRH I and II on motile sperm was evaluated for one hour. The time factor only allowed one peptide and one concentration to be evaluated at a time. The effect of the GnRH vehicle medium, propylene glycol (0.2%), was analyzed separately in the same manner as for the GnRH exposure tests (refer to table 2.2).

To prevent "cold shock" to motile sperm [Faulkner 1971], all tubes, pipette tips, extender and microscope slides used during each experiment, were pre-warmed in either the water bath

(Forma Scientific, Scientific Associates, Tokai, Cape Town) or with temperature controlled heated stages (Photax, Protea Holdings Cape, Cape Town) and kept at 37°C.

2.3.2 *Staining for acrosomal integrity*

For the analysis of motile sperm acrosomal integrity [Mortimer *et al.* 1990], two slides were stained for every time point (refer to table 2.2) for control, GnRH treatment and propylene glycol. A 20µl drop of each sperm specimen was removed and spread over one end of a microscope slide and left to air-dry in the dark. Air dried slides were fixed for five minutes by immersion in 95% ethanol, followed by more air-drying. Slides were stained at room temperature for 15-20 minutes in a foil covered Coplin jar containing 100µg/ml FITC/PNA solution. The slides were washed by gently dipping (x3) in PBS and fixed by immersion in 4% para-formaldehyde fixative for 15 minutes. After rinsing gently with PBS, the slides were allowed to air dry, which was followed by mounting with non-fluorescing media containing anti-fading n-Propyl Gallate (Sigma, Cape Town) and 22 x 22 cover slips. Scoring was completed within 24 hours of staining.

2.3.3 *Evaluation for acrosomal integrity*

Slides were examined with an Olympus (BX 50) microscope (Wirsam Scientific, Cape Town, South Africa) with a 100-x objective (Uplan FI Oil Ph3) and appropriate filters (Olympus U-MWB with BP 450-480) for the FITC-PNA labels. The proportions of spermatozoa with fluorescent patterns were recorded for labels I= intact acrosome,
III= equatorial staining and IV= no acrosome (Fig. 2.2). Type II pattern was not observed in this study. The results were expressed as percentages of 200 counted spermatozoa.

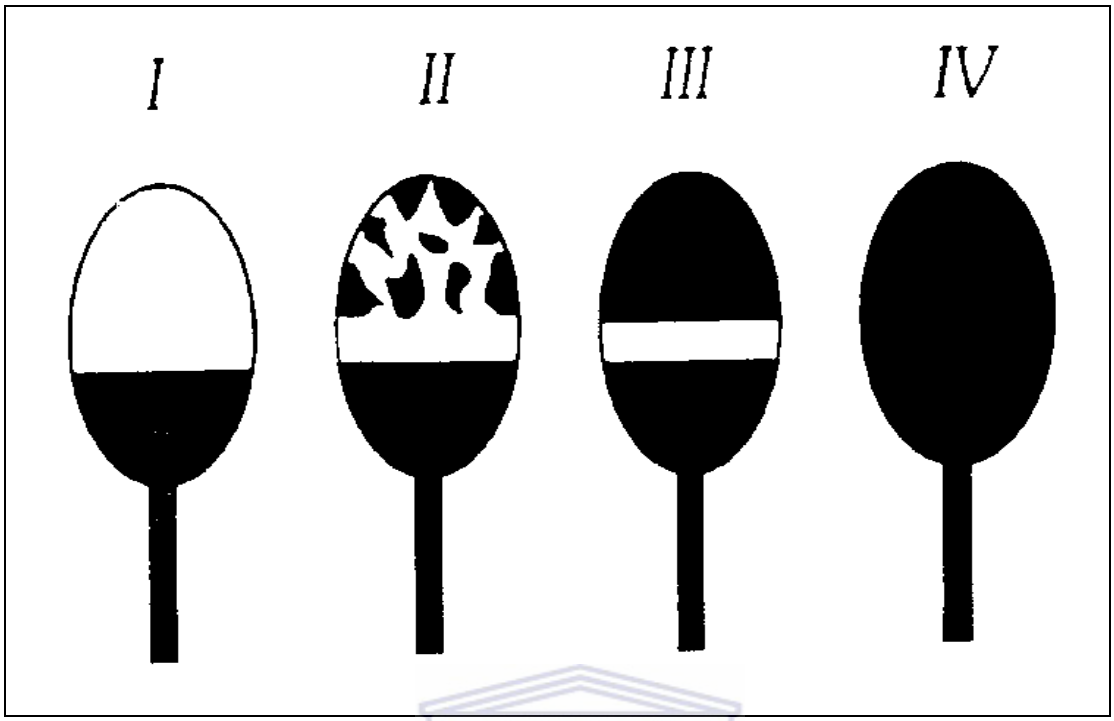


Figure 2.2 PNA labelling patterns of Vervet monkey spermatozoa

FITC-PNA lectin (According to Mortimer *et al.* 1990)**.**
Fluorescence pattern I showing intensely fluorescent region of acrosome intact sperm and the dark postacrosomal region. Fluorescence pattern II displaying irregular fluorescence of the acrosomal region. Note this type of fluorescence is also limited to the acrosomal region of the head. Fluorescence pattern III showing bright fluorescence at the equatorial region of the sperm. Note the lack of fluorescence at both the acrosomal and post-acrosomal regions. Fluorescence pattern IV, showing lack of fluorescence on the sperm head due to absence of the acrosome.

2.4 Motility

2.4.1 *Subjective motility*

Immediately after ejaculation, a drop of semen was placed under a cover slip on a pre-warmed (37°C) microscope slide, and motility and speed of forward progression (FP) were evaluated under a microscope with bright field illumination at 100 and 400x magnifications. Motility was estimated in 10% units of progressively motile spermatozoa and the FP rated on a scale of 0 – 4 [Seier *et al.* 1989]. Zero represented no progressive motility and four

maximum speed. Only samples with more than 50% progressive motility were used for the experimental protocols.

2.4.2 *Computer Aided Sperm Motility Analysis (CASMA)*

A 10µl drop of the sample was pipetted onto a microscope slide and subsequently mounted with a cover slip, then placed on the pre-warmed (37°C) stage of an Olympus CH2 microscope. Electrical coils built into the microscope stage ensured that the temperature was accurately controlled within 0.1^{o}C by means of an external temperature control device developed by Wirsam Scientific (Cape Town, South Africa) for the Olympus CH2 (see Fig.2.3).

Sperm motion characteristics of the three samples of each experiment including control, GnRH I and GnRH II, were recorded at 0 minutes, 15 minutes, 30 minutes and 60 minutes. After the GnRH treatment, 2 – 5 minutes were allowed before analyzing the sample.

Sperm motility for each treatment and each experiment was recorded onto a video tape using a Phillips VHS recorder connected to a Panasonic monitor. Sperm motility was evaluated by the computer-aided sperm motility analysis (CASMA) system, Sperm Motility Quantifier (version 1.01: Wirsam Scientific & Precision Equipment (Pty) Ltd, South Africa), (Fig.2.3).

The recordings of sperm motion were captured at an analysis rate of 50 Hz. The minimum and maximum sperm size was preset at 6 and 47 pixels and spermatozoa were tracked for 0.7 seconds [Mdhluli and van der Horst 2002]. Ten fields from each slide were recorded with a maximum of a 100 - 200 motile spermatozoa. Randomly representative sperm trajectories

were saved as *.tiff files to facilitate qualitative comparison of patterns of sperm motility for each time point of GnRH treatment and the controls. Seven sperm motion characteristics, viz. curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement (ALH), linearity (LIN), mean angular displacement (MAD) and percent motility, were measured [van der Horst *et al.* 1999] (Table 2.1).

Figure 2.3 Sperm Motility Quantifier system and equipment for recording sperm motility

Table 2.1 Sperm motility quantification variables

Variable	Definition	Abbreviations	Units
Straight line velocity	Time-averaged velocity of sperm head projected along straight line between its first & last detected positions	VSL	µm/s
Curvilinear velocity	Time-averaged velocity of sperm head along its actual path	VCL	µm/s
Average path velocity	Time-averaged velocity of sperm head projecting along its spatial average trajectory	VAP	µm/s
Linearity	Ratio of projected length to total length of curvilinear trajectory; LIN = VSL/VCL	LIN	%
Mean Angular Displacement	Measured in Radian = Radius/3.14 x 180	MAD	Radians
Amplitude of lateral head displacement	Magnitude of lateral displacement of a sperm head about its average path.	ALH	µm
Total motility	Percentage motility of spermatozoa swimming with a VCL>20µm.sec and VSL>9µm.sec, the rest were considered immotile	-	%

Table 2.2 Experimental layout for acrosome status and sperm motility evaluations for GnRH I & II at different concentrations for one hour from a total of 84 ejaculates.

	TIME POINTS (minutes)							
	0 Minutes	n	15 Minutes	n	30 Minutes	n	60 Minutes	n
GnRH I								
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-5}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC	-	FITC-PNA	6	FITC-PNA	6
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-6}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-8}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
GnRH II								
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-5}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-6}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
10^{-8}M	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
Peptide vehicle								
Control	SMQ	6	SMQ	6	SMQ	6	SMQ	6
	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC-PNA	6
Propylene	SMQ	6	SMQ	6	SMQ	6	SMQ	6
glycol 0.2%	FITC-PNA	6	FITC-PNA	-	FITC-PNA	6	FITC	6

2.5 Statistical analysis

Descriptive statistics were used to analyze data and ANOVA was used to compare more than two sets of data at a given time interval. Student-Newman-Keuls tests for all pair wise comparisons were subsequently performed. In instances where data did not have a normal distribution, Kruskal-Wallis analysis combined with unpaired Wilcoxon analysis were applied. In all instances the statistical package, MedCalc for Windows, version 4.16 (MedCalc Software, Mariakerke, Belgium), was used.

Graphic presentations of data were done using Excel spreadsheets and concomitant histogram presentations.

CHAPTER 3

Results

3 Electro ejaculation

The method of electro ejaculation consistently produced ejaculates from all male Vervet monkeys. During electro stimulation, the Vervets responded with strong contractions of the thigh muscles, testicular and tail movements, erection with enlargement of the glans penis and ejaculation. Weakening of these responses and cessation of erection indicated the need to change to the next higher current. There were inter-individual differences in the stimulation period required to achieve ejaculation and some males occasionally ejaculated before erection or during rest periods. Urination frequently occurred if the males were stimulated after ejaculation and at 5.5 – 8.0V of electro stimulation.

3.1 Hams F10 extender

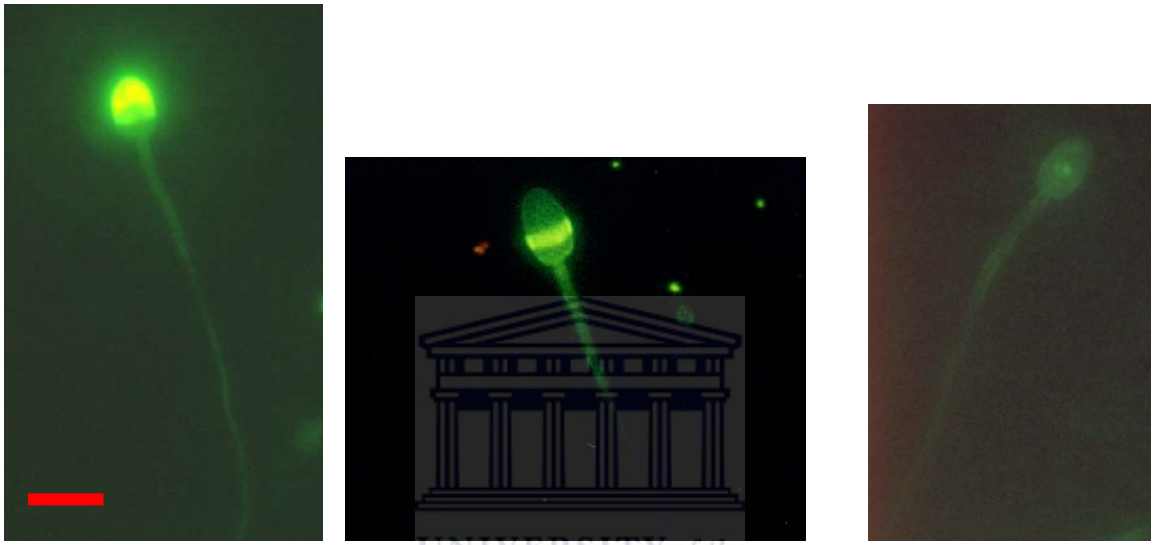
In this study, Hams F10 that consisted of all amino acids, fatty acids and mineral salts, as well as protein (BSA), was used as the physiological extender in all experiments and consistently supported sperm motility for the duration of the experiment.

3.2 Swim-up incubation

The swim-up method provided sufficient motile sperm, even though the method had to be adjusted from time to time to accommodate coagulated ejaculates. Injecting semen at the bottom of the extender or layering Hams F10 on top of the coagulum continuously resulted in a sample containing a high quality of motile sperm, clear of dead sperm and cellular debris.

3.3 Acrosomal integrity

There were three distinct staining patterns of FITC-PNA in the acrosomal and equatorial regions of the Vervet monkey spermatozoa.



Pattern I
Intact Acrosome

Pattern III
Equatorial band

Pattern IV
Absent acrosome

Figure 3.1 Staining of Vervet monkey spermatozoa after labelling with FITC-PNA
The bar at the bottom of pattern I photomicrograph represents 6.3µm **(**From Mdhluli 2003).

3.3.1 *Intact acrosome (Pattern I)*

There were no statistically significant differences in intact acrosomes (Figure 3.1) of the treated groups (GnRH I & II at concentrations of 10^{-5}M, 10^{-6}M and 10^{-8}M) compared to the control. There were also no statistically significant differences between the vehicle (propylene glycol 0.2%), the control or any treatment groups. The means and standard deviations for all groups at the three different time points are summarized in Table 3.1, as well as graphically represented in Figures 3.2 to 3.4.

Table 3.1 Percentage (means ± SD) of intact acrosomes for GnRH I & II (concentrations 10^{-5} M, 10^{-6}M, 10^{-8}M), propylene glycol 0.2% and controls.

Treatment	0 min	30 min	60 min
Propylene glycol 0.2% control	94.42 ± 7.61	91.75 ± 11.72	92.08 ± 6.05
Propylene glycol 0.2%	93.42 ± 6.05	94.75 ± 2.70	95.02 ± 4.65
GnRH control	97.11 ± 4.80	95.78 ± 4.87	96.61 ± 5.42
GnRH I 10^{-5}M	97.30 ± 2.54	98.00 ± 0.97	98.30 ± 1.83
GnRH I 10^{-6}M	96.40 ± 5.83	96.30 ± 7.05	96.30 ± 4.55
GnRH I 10^{-8}M	90.10 ± 7.47	90.60 ± 7.80	92.30 ± 8.10
GnRH II 10^{-5}M	97.60 ± 2.96	96.70 ± 1.44	96.40 ± 3.20
GnRH II 10^{-6}M	95.40 ± 6.41	97.30 ± 5.36	96.00 ± 6.62
GnRH II 10^{-8}M	92.30 ± 5.26	93.10 ± 5.58	94.50 ± 3.87

3.3.2 *Equatorial band (Pattern III)*

The percentage of equatorial band staining (Figure 3.1) showed a larger variation within groups than between groups when the control was compared to all treatment groups for all time intervals. The large variation in each group is also exemplified as relatively large standard deviations (Table 3.2).

Consequently, no statistically significant differences were found between the control and GnRH I & II at concentrations of 10^{-5}M to 10^{-8}M as well as the vehicle, propylene glycol (Figure 3.5 to 3.7). The means and standard deviations for all groups at three different time points are summarized in Table 3.2.

Table 3.2 Percentage (means ± SD) of equatorial band staining for GnRH I & II (concentrations 10^{-5}M, 10^{-6}M, 10^{-8}M), propylene glycol 0.2%, and controls.

Treatment	0 min	30 min	60 min
Propylene glycol 0.2% control	1.80 ± 2.90	4.30 ± 6.50	4.30 ± 4.40
Propylene glycol 0.2%	3.40 ± 1.90	2.50 ± 1.00	3.50 ± 2.20
GnRH control	1.31 ± 3.37	1.89 ± 2.76	1.56 ± 3.43
GnRH I 10^{-5}M	0.58 ± 0.80	0.83 ± 0.68	0.50 ± 0.58
GnRH I 10^{-6}M	0.20 ± 0.41	0.40 ± 0.66	1.70 ± 1.51
GnRH I 10^{-8}M	4.60 ± 7.18	5.30 ± 5.18	2.60 ± 7.01
GnRH II 10^{-5}M	0.41 ± 0.66	0.58 ± 0.80	0.83 ± 0.99
GnRH II 10^{-6}M	0.70 ± 1.40	1.70 ± 1.97	1.70 ± 1.74
GnRH II 10^{-8}M	3.60 ± 4.31	3.40 ± 3.34	3.40 ± 4.02

3.3.3 *Absent acrosome (Pattern IV)*

The results for all groups showing the percentage of absent acrosomes (Figure 3.1) at three different time points are summarized in Table 3.3 and shown in Figures 3.8 to 3.10. The results clearly show that a small percentage of acrosomes are absent in all groups. Furthermore, no statistically significant differences were evident between the controls and any of the experimental groups. The results for absent acrosomes in controls and treatment groups accordingly follow the same trend as for the previous two staining patterns (bright and equatorial).

Table 3.3 Percentage (means ± SD) of absent acrosomes for GnRH I & II concentrations (10^{-5}M, 10^{-6}M, 10^{-8}M), propylene glycol 0.2% and controls.

Treatment	0 min	30 min	60 min
Propylene glycol 0.2% control	3.80 ± 4.80	3.70 ± 5.50	2.20 ± 1.90
Propylene glycol 0.2%	3.20 ± 4.20	2.20 ± 2.50	1.80 ± 2.40
GnRH control	1.58 ± 3.22	2.22 ± 3.47	2.19 ± 4.85
GnRH I 10^{-5}M	2.08 ± 2.35	1.33 ± 0.82	1.00 ± 0.71
GnRH I 10^{-6}M	3.20 ± 5.91	3.30 ± 6.77	1.90 ± 3.50
GnRH I 10^{-8}M	5.20 ± 4.97	4.10 ± 2.96	2.40 ± 1.83
GnRH II 10^{-5}M	1.91 ± 2.89	1.33 ± 1.47	2.58 ± 3.54
GnRH II 10^{-6}M	3.10 ± 5.32	2.90 ± 6.90	2.30 ± 4.92
GnRH II 10^{-8}M	4.30 ± 3.61	3.50 ± 2.95	2.30 ± 1.70

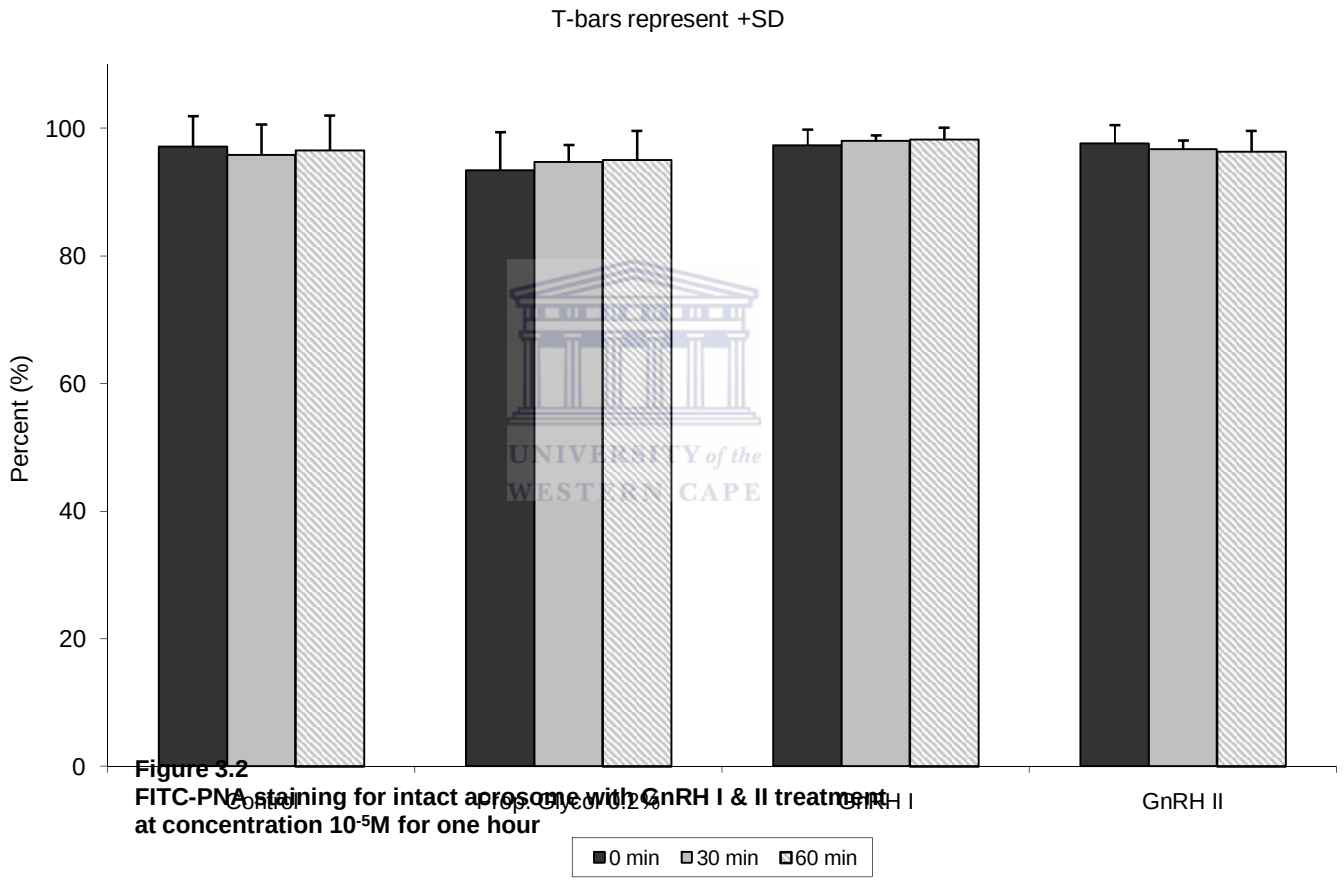


Figure 3.2
FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-5}M for one hour

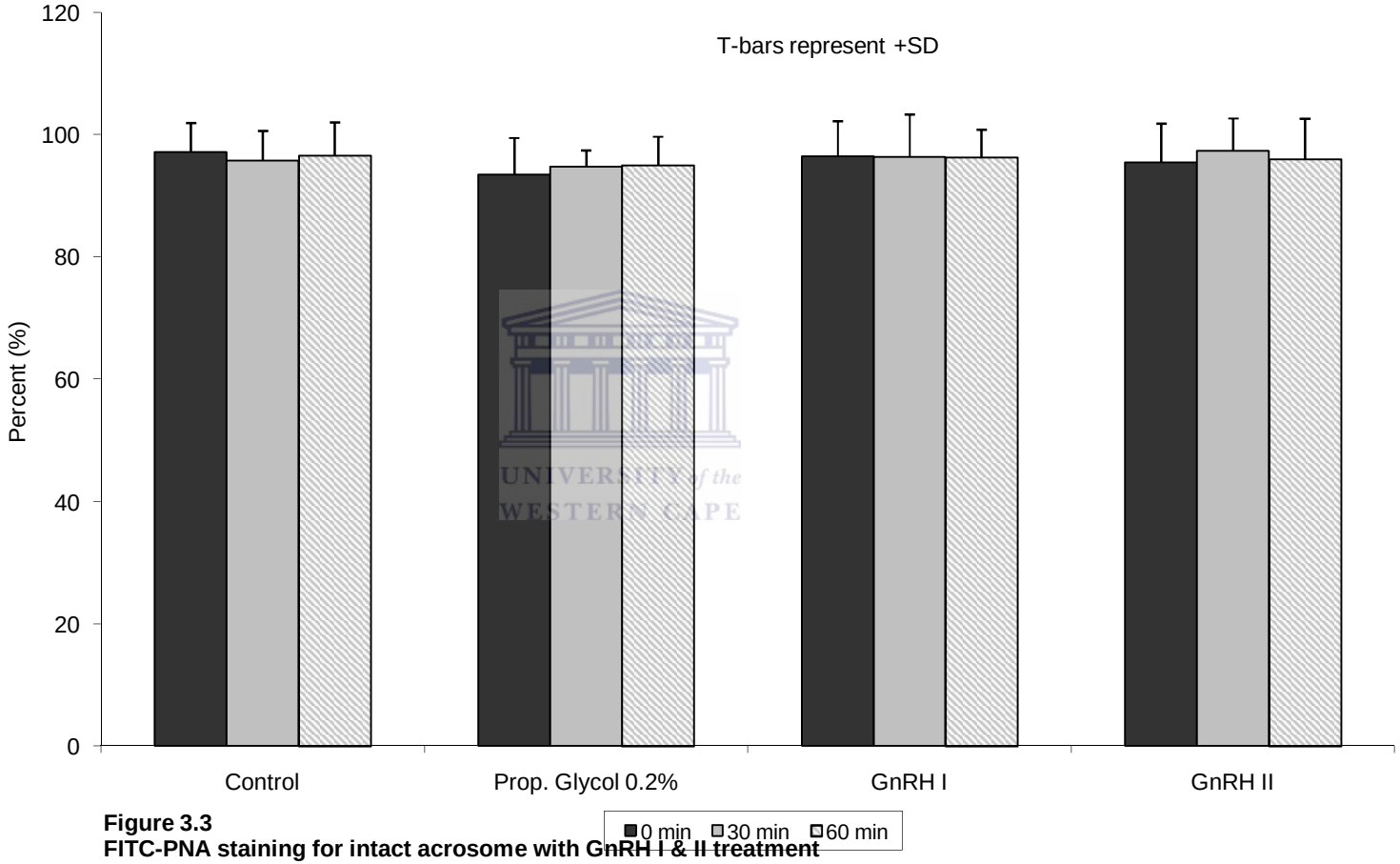


Figure 3.3
FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-6}M for one hour

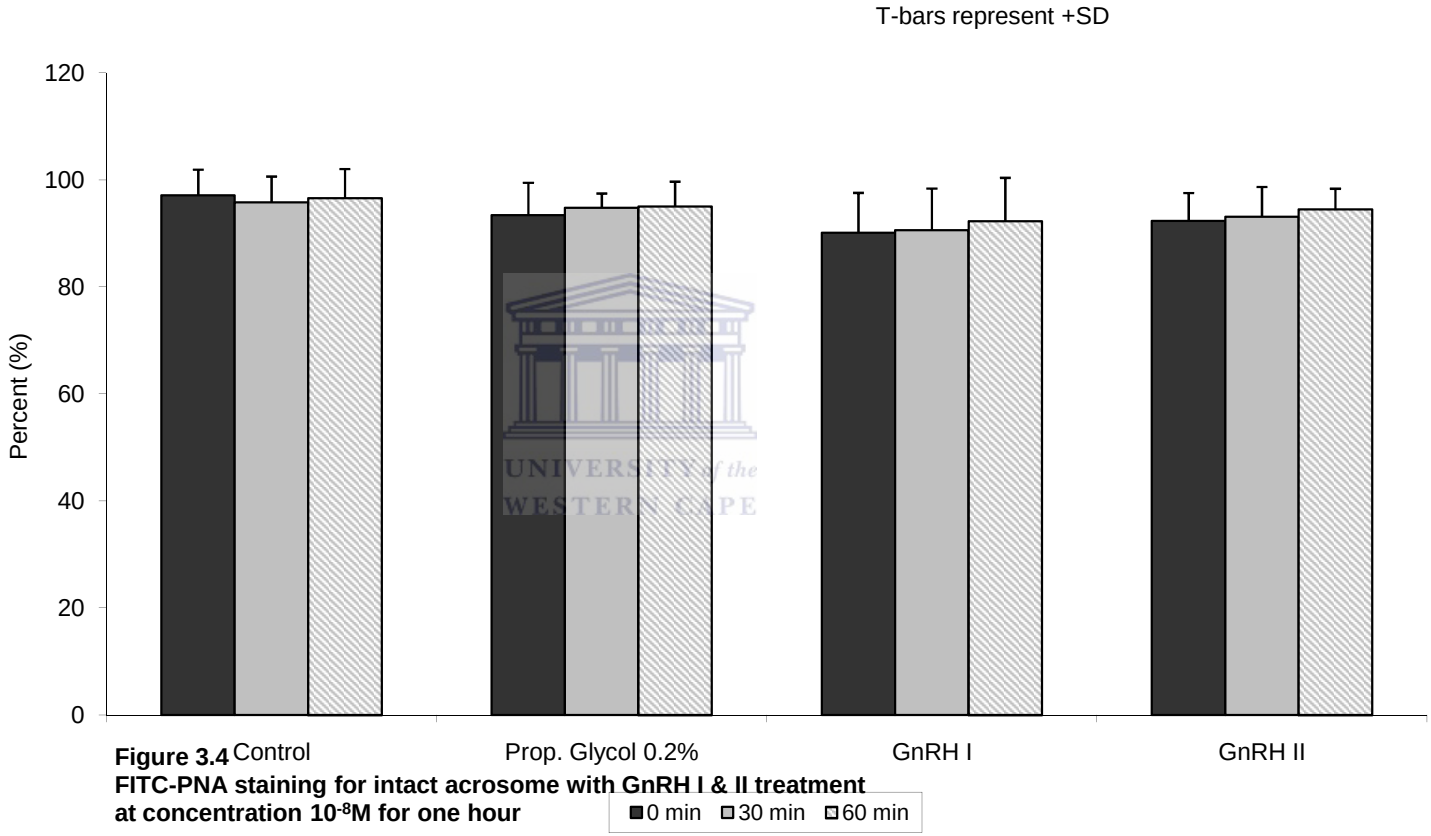


Figure 3.4 **FITC-PNA staining for intact acrosome with GnRH I & II treatment at concentration 10^{-8}M for one hour**

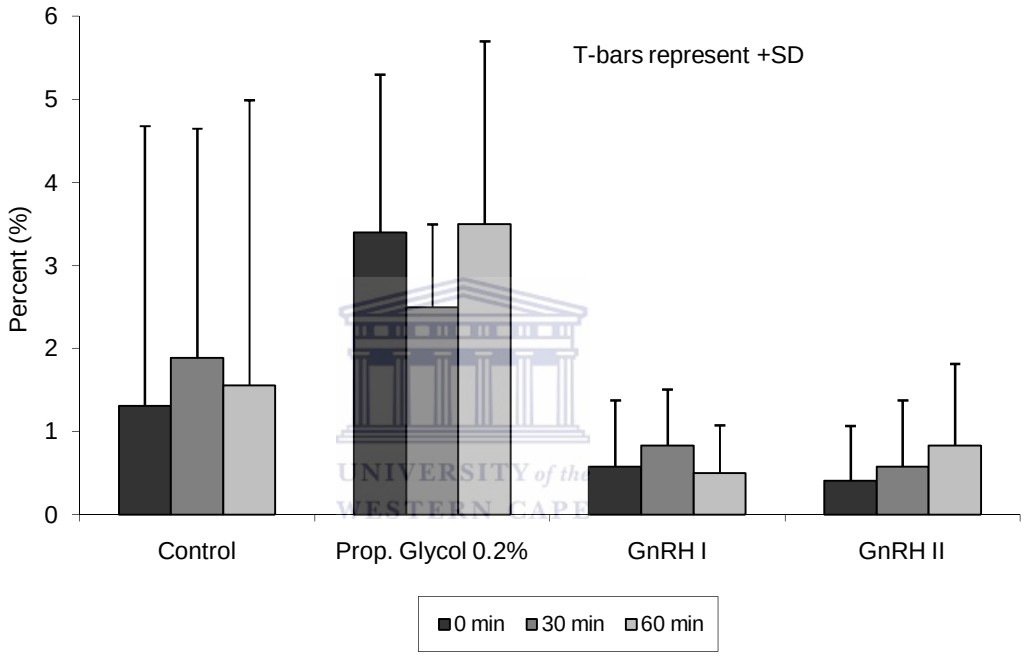
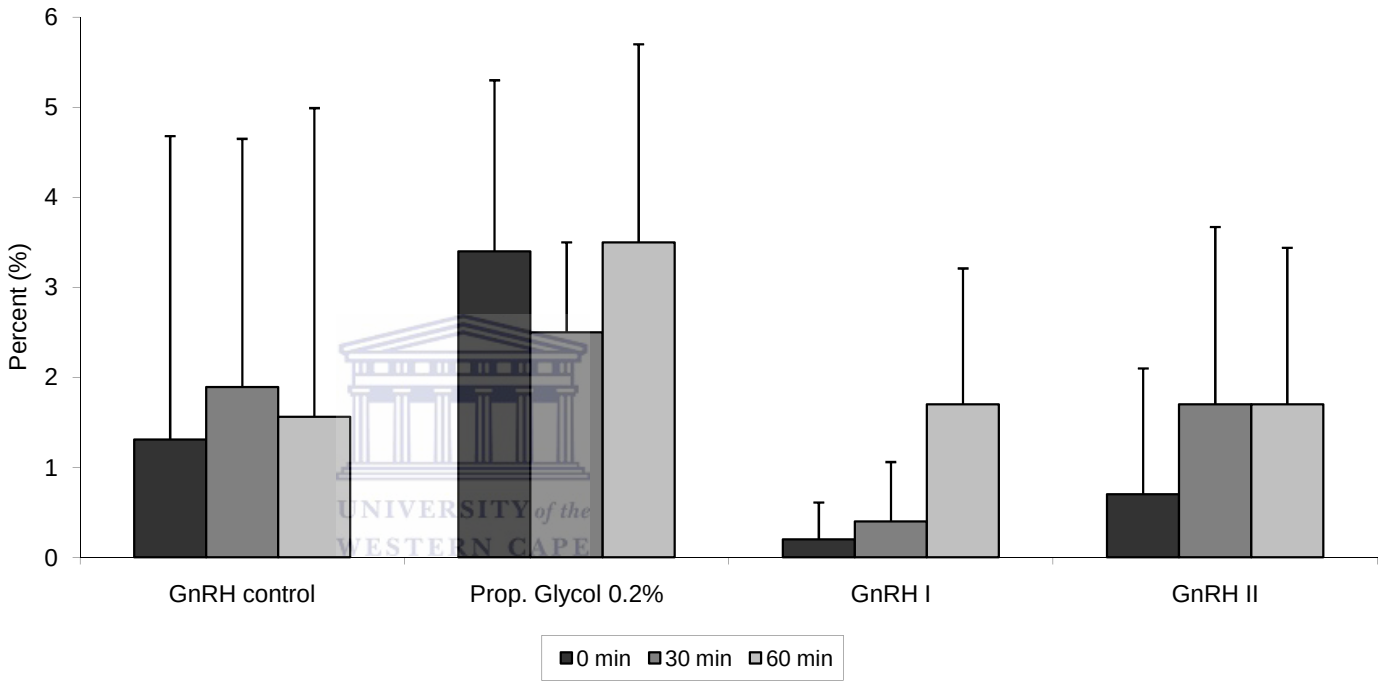


Figure 3.5
FITC-PNA staining for equatorial band with GnRH I & II treatment at concentration 10^{-5}M for one hour



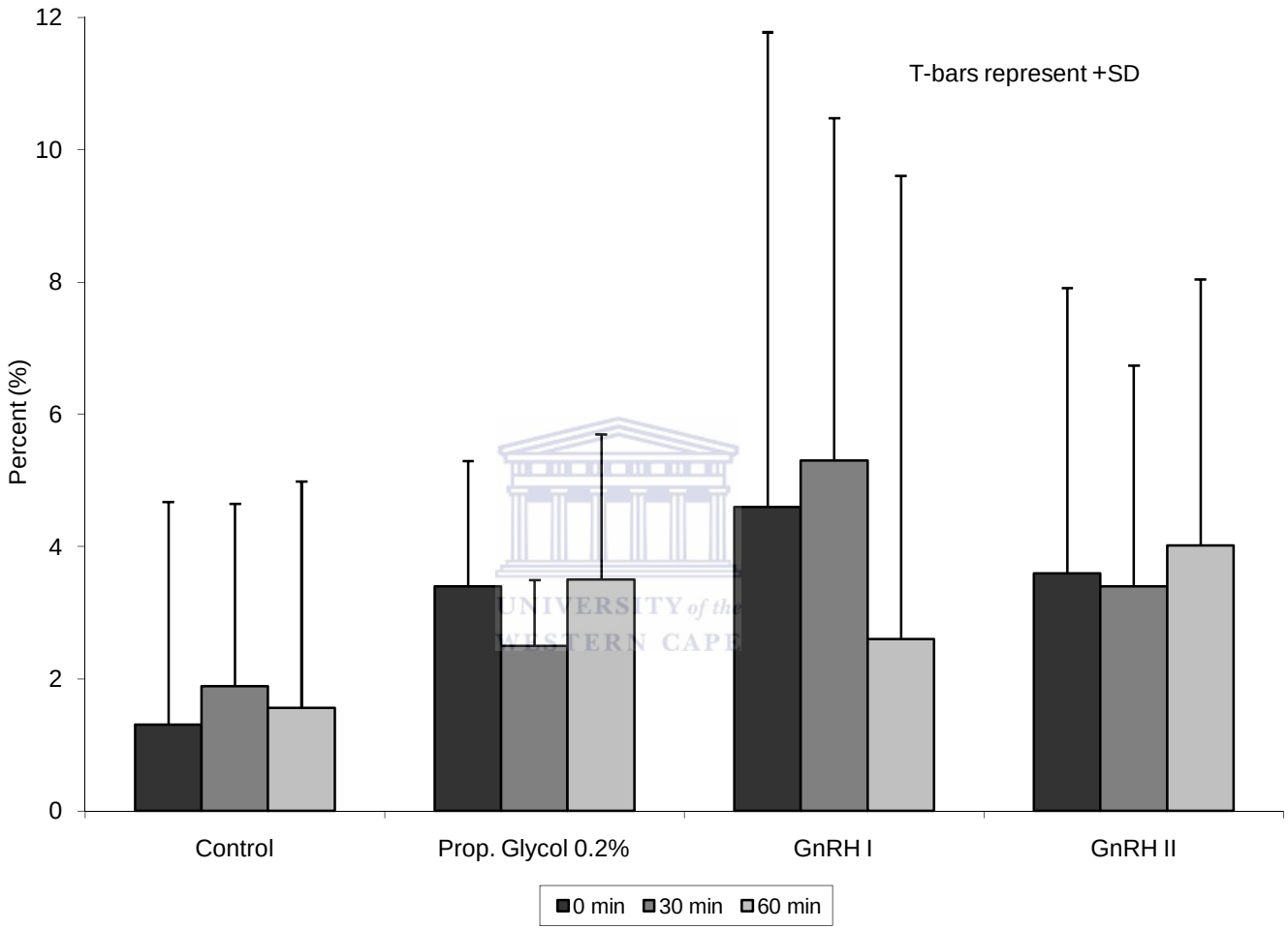


Figure 3.7
FITC-PNA staining for equatorial band with GnRH I & II treatment at the concentration 10^{-8}M for one hour

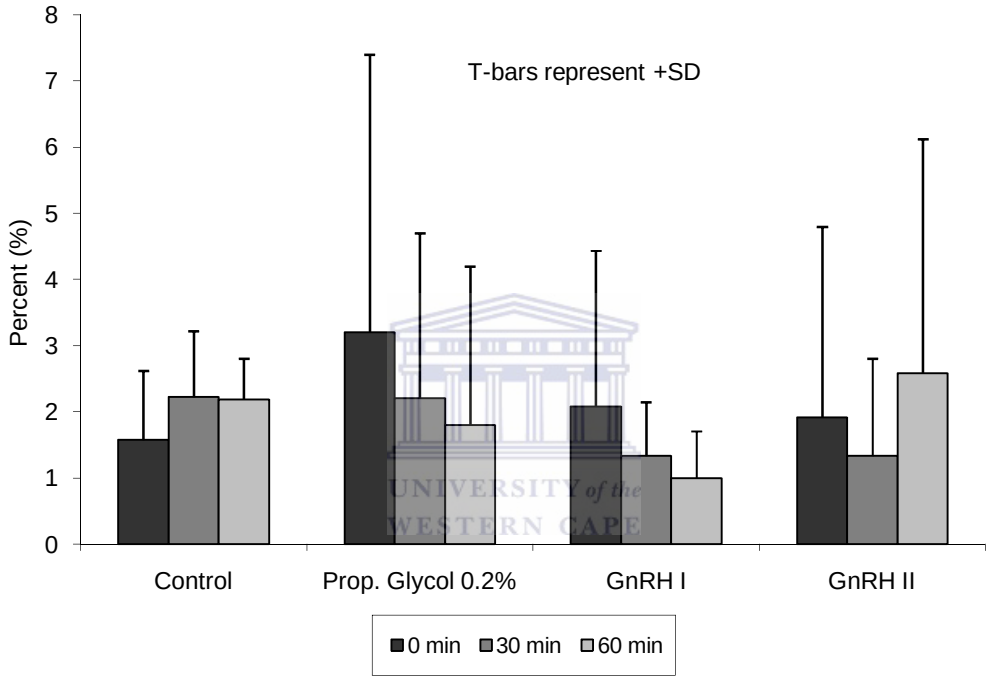


Figure 3.8
FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-5}M for one hour

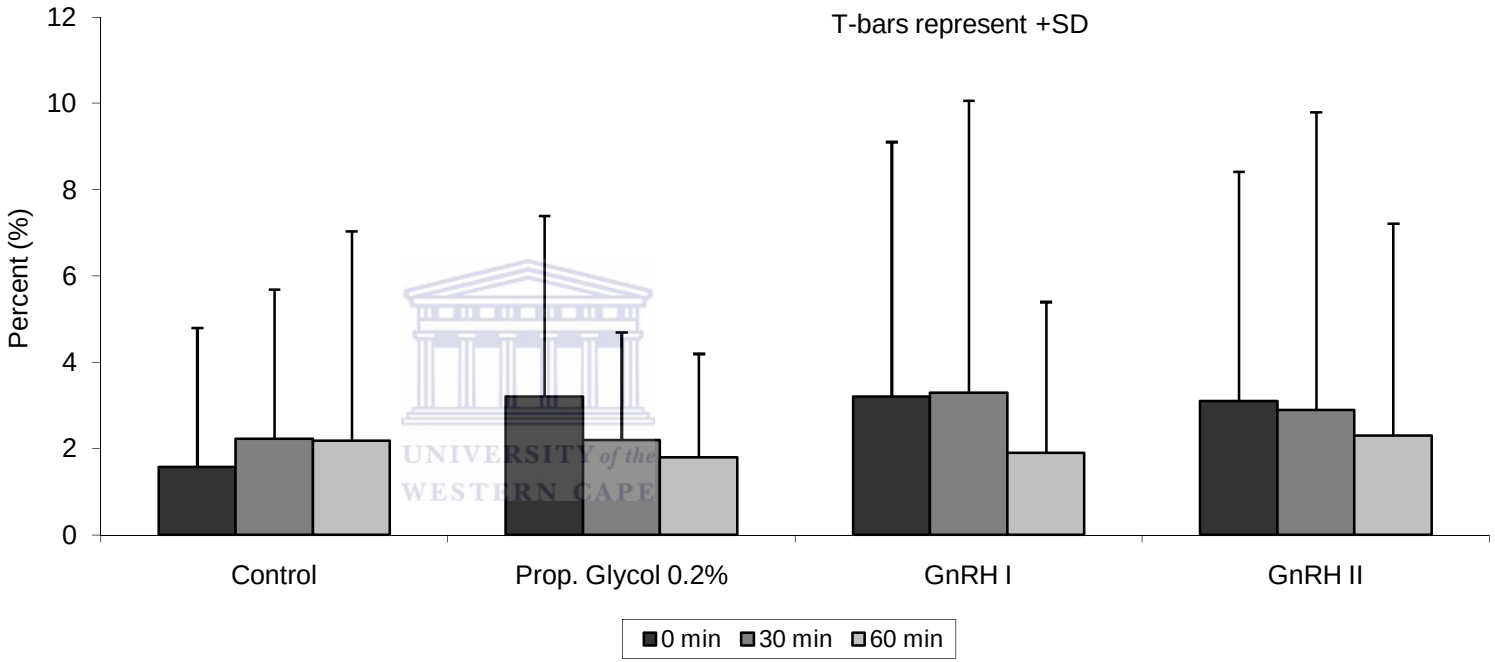


Figure 3.9
FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-6}M for one hour

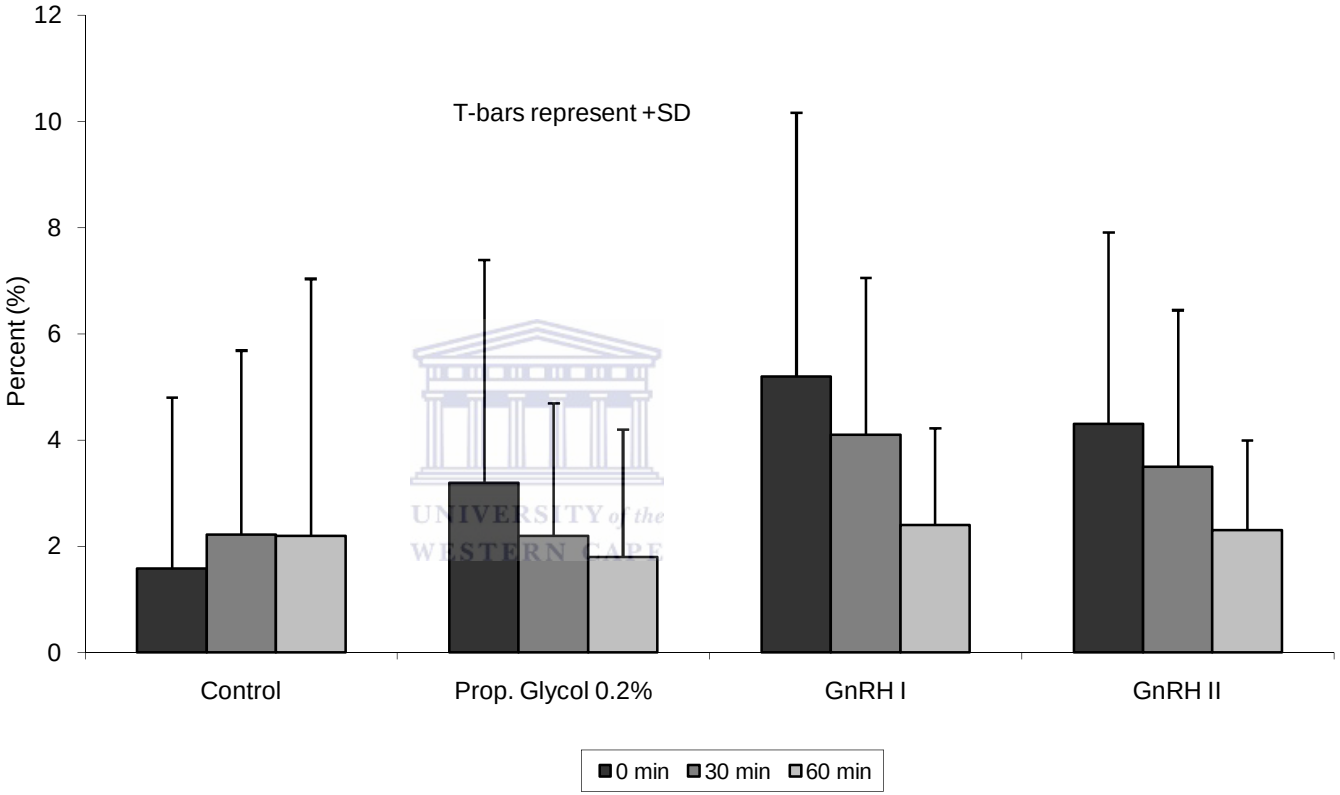


Figure 3.10
FITC-PNA staining for absent acrosome with GnRH I & II treatment at concentration 10^{-8}M for one hour

3.4 Sperm motility

3.4.1 *Computer Aided Sperm Motility Analysis (CASMA)*

There were noticeable changes in CASMA variables in the treated groups compared to the control and vehicle groups over a period of one hour. The most significant differences appeared to be a steady decline from 0 minutes to 60 minutes in all variables for samples treated with GnRH I & II at the concentration 10^{-8}M. Table 3.4 demonstrates the quantitative (± SD) sperm motility results.

3.4.2 *Effect on CASMA parameters observed during one hour*

GnRH treatment was associated with statistically significant changes on CASMA parameters at different time points and different concentrations of GnRH I & II (for *P*- values see Table 3.5) and subsequently described under 3.4.2.1 to 3.4.2.4.

3.4.2.1 *Immediate effect (0 - 5 minutes)*

A statistically significant decline ($p< 0.05$) in VCL (Figure 3.11), VSL (Figure 3.12), LIN (Figure 3.13) and VAP (Figure 3.14) was evident in groups treated with GnRH I & II at 10^{-8}M when compared to the control and Propylene Glycol (vehicle) group. There was a statistically significant increase in MAD (Figure 3.46) ($p<0.05$) for GnRH II at 10^{-8}M when compared to the control. However, no statistically significant differences were observed for ALH in the control *versus* treated groups (Figure 3.15). There were no significant differences between the GnRH control and the Propylene Glycol (PG) control for any parameter, which indicates no PG effect.

3.4.2.2 *Effect at 15 minutes*

There were further statistically significant decreases in VCL, VSL, and LIN, at 15 minutes when GnRH I and II at 10^{-8}M were compared to the control (Tables 3.4 and 3.5). In addition to the immediate affect there was a decline in VAP and ALH for both GnRH peptides at 10^{-8}M at 15 minutes, which was statistically significant when compared to the control ($p<0.05$) (Figures 3.11 – 3.15). MAD was still significantly enhanced for GnRH II at 10^{-8}M when compared to the control (Figure 3.16).

3.4.2.3 *Effect at 30 minutes*

In comparison to the control and all other treated groups, groups GnRH I & II at 10^{-8}M continued to show a significant decline in VCL, VSL, LIN, VAP, and ALH and an increase in MAD (Figure 3.11 – Figure 3.16).

3.4.2.4 *Effect at 60 minutes*

A statistically significant decline in groups treated with GnRH I & II at 10^{-8}M compared to the control, was evident for VCL, VSL, LIN, VAP and ALH (Figure 3.11 – Figure 3.15). Furthermore, MAD continued to increase statistically significantly at a concentration of GnRH II 10^{-8}M compared to the control (Figure 3.16).

Table 3.4 Means and standard deviations (means ± SD) for Computer Aided Sperm Motility Analysis (CASMA) results

Treatment	Parameter	0 min	15 min	30 min	60 min
GnRH control	VCL	212.52 ± 54.77	202.15 ± 53.04	197.26 ± 66.91	175.80 ± 51.73
	VSL	150.22 ± 39.97	136.63 ± 37.20	120.29 ± 54.45	107.44 ± 34.75
	LIN	66.41 ± 9.75	62.86 ± 8.33	55.67 ± 14.66	56.35 ± 9.96
	ALH	6.22 ± 2.59	6.38 ± 2.29	6.74 ± 2.92	6.11 ± 2.29
	VAP	166.45 ± 38.78	152.89 ± 38.28	139.33 ± 54.00	123.69 ± 36.80
	MAD	1.69 ± 0.38	1.86 ± 0.36	2.07 ± 0.52	2.02 ± 0.40
Propylene glycol 0.2%	VCL	241.42 ± 42.26	218.28 ± 32.34	174.71 ± 22.22	151.40 ± 59.66
	VSL	161.31 ± 41.35	142.61 ± 44.92	107.27 ± 34.66	75.81 ± 41.70
	LIN	61.16 ± 12.33	59.38 ± 14.10	52.03 ± 12.19	43.99 ± 11.34
	ALH	7.55 ± 1.96	6.85 ± 1.04	5.58 ± 0.71	5.78 ± 2.74
	VAP	181.54 ± 34.00	163.31 ± 39.77	128.25 ± 29.03	98.05 ± 40.12
	MAD	1.9 ± 0.36	2.03 ± 0.46	2.27 ± 0.35	2.57 ± 0.39
GnRH I 10^{-5}M	VCL	196.63 ± 30.27	193.76 ± 46.60	171.94 ± 49.98	126.62 ± 48.14
	VSL	142.09 ± 21.98	119.73 ± 27.97	111.57 ± 48.30	76.67 ± 53.42
	LIN	69.54 ± 8.12	58.74 ± 4.02	60.31 ± 18.22	50.93 ± 20.91
	ALH	6.38 ± 1.93	7.17 ± 2.40	5.52 ± 2.61	4.35 ± 1.02
	VAP	153.69 ± 20.64	136.49 ± 32.40	127.76 ± 45.01	90.84 ± 49.97
	MAD	1.65 ± 0.34	1.83 ± 0.30	1.91 ± 0.60	2.04 ± 0.51
GnRH I 10^{-6}M	VCL	199.39 ± 66.74	201.42 ± 59.85	186.65 ± 90.85	160.20 ± 42.47
	VSL	135.90 ± 48.38	111.48 ± 37.48	104.83 ± 51.30	91.84 ± 32.35
	LIN	63.34 ± 12.93	47.39 ± 8.71	49.49 ± 12.70	52.07 ± 10.79
	ALH	6.46 ± 3.22	6.93 ± 2.68	5.99 ± 4.36	6.14 ± 2.38
	VAP	150.04 ± 51.54	137.51 ± 37.20	126.82 ± 48.40	106.72 ± 33.88
	MAD	1.89 ± 0.58	2.30 ± 0.35	2.39 ± 0.42	2.27 ± 0.40
GnRH I 10^{-8}M	VCL	157.04 ± 37.85	124.08 ± 14.05	114.64 ± 28.05	101.35 ± 27.05
	VSL	103.29 ± 25.21	73.56 ± 14.87	61.96 ± 36.81	51.36 ± 27.66
	LIN	56.45 ± 12.77	47.17 ± 8.08	42.05 ± 17.54	39.83 ± 11.93
	ALH	5.52 ± 1.48	3.97 ± 0.84	3.80 ± 1.30	4.32 ± 0.78
	VAP	118.78 ± 27.51	90.24 ± 14.16	83.12 ± 27.60	65.91 ± 25.26
	MAD	1.84 ± 0.47	2.15 ± 0.43	2.30 ± 0.58	2.11 ± 0.53
GnRH II 10^{-5}M	VCL	179.48 ± 28.43	178.73 ± 34.70	176.20 ± 41.87	151.81 ± 56.19
	VSL	130.96 ± 20.22	108.03 ± 32.44	102.14 ± 29.07	91.49 ± 51.22
	LIN	68.32 ± 7.07	54.73 ± 11.62	54.35 ± 13.44	54.34 ± 16.00
	ALH	5.82 ± 1.58	6.71 ± 1.98	6.64 ± 2.79	5.66 ± 2.79
	VAP	143.29 ± 21.55	125.97 ± 30.28	120.25 ± 25.99	108.73 ± 49.05
	MAD	1.59 ±0.24	2.07 ± 0.57	2.16 ± 0.57	2.05 ± 0.60
GnRH II 10^{-6}M	VCL	191.73 ± 54.75	162.27 ± 61.76	167.55 ± 54.41	172.82 ± 74.50
	VSL	122.95 ± 32.71	93.75 ± 53.96	100.67 ± 45.89	90.96 ± 36.87
	LIN	58.85 ± 8.79	47.87 ± 14.77	50.71 ± 13.51	48.68 ± 8.64
	ALH	6.11 ± 2.83	4.72 ± 1.97	4.77 ± 2.18	6.65 ± 3.94
	VAP	126.98 ± 56.67	119.66 ± 48.04	123.78 ± 39.69	113.81 ± 42.67
	MAD	2.12 ± 0.36	2.41 ± 0.56	2.29 ± 0.45	2.40 ± 0.29
GnRH II 10^{-8}M	VCL	137.20 ± 21.36	124.08 ± 18.81	109.38 ± 30.69	95.61 ± 21.26
	VSL	82.14 ± 24.94	61.95 ± 21.18	56.96 ± 38.27	37.06 ± 14.55
	LIN	50.28 ± 13.20	42.65 ± 12.53	43.15 ± 17.00	34.57 ± 8.21
	ALH	4.12 ± 0.95	4.14 ± 0.86	3.63 ± 1.19	3.88 ± 1.48
	VAP	102.01 ± 21.45	83.16 ± 19.32	75.78 ± 30.22	58.46 ± 15.91
	MAD	2.21 ± 0.42	2.45 ± 0.47	2.41 ± 0.60	2.55 ± 0.26

Table 3.5 Statistically significant differences when experimental groups were compared to control for CASMA parameters. GnRH peptides I and II indicated as I, II, and concentrations of peptides in Molar. *P*-values of control *versus* peptides at significance level of $p<0.05$ used as a cut off point

Variable	Control *versus* ($p<0.05$)	Time	Increase (▲) Decrease (↓)
VCL	I 10^{-8} & II 10^{-8}	Immediate effect	↓
VSL	I 10^{-8} & II 10^{-8}	Immediate effect	↓
VAP	I 10^{-8} & II 10^{-8}	Immediate effect	↓
LIN	I 10^{-8} & II 10^{-8}	Immediate effect	↓
MAD	II 10^{-8}	Immediate effect	▲
VCL	I 10^{-8} & II 10^{-8}	15 minutes	↓
VSL	I 10^{-8} & II 10^{-8}	15 minutes	↓
LIN	I 10^{-8} & II 10^{-8}	15 minutes	↓
VAP	I 10^{-8} & II 10^{-8}	15 minutes	▲
ALH	I 10^{-8} & II 10^{-8}	15 minutes	▲
MAD	II 10^{-8}	15 minutes	▲
VCL	I 10^{-8} & II 10^{-8}	30 minutes	↓
VSL	II 10^{-8}	30 minutes	↓
LIN	I 10^{-8} & II 10^{-8}	30 minutes	↓
VAP	II 10^{-8}	30 minutes	↓
ALH	I 10^{-8} & II 10^{-8}	30 minutes	↓
MAD	I 10^{-8} & II 10^{-8}	30 minutes	▲
VCL	I 10^{-8} & II 10^{-8}	60 minutes	↓
VSL	I 10^{-8} & II 10^{-8}	60 minutes	↓
LIN	I 10^{-8}, II 10^{-8} & II 10^{-5}	60 minutes	↓
VAP	I 10^{-8} & II 10^{-8}	60 minutes	↓
ALH	I 10^{-8} & II 10^{-8}	60 minutes	↓
MAD	II 10^{-8}	60 minutes	▲

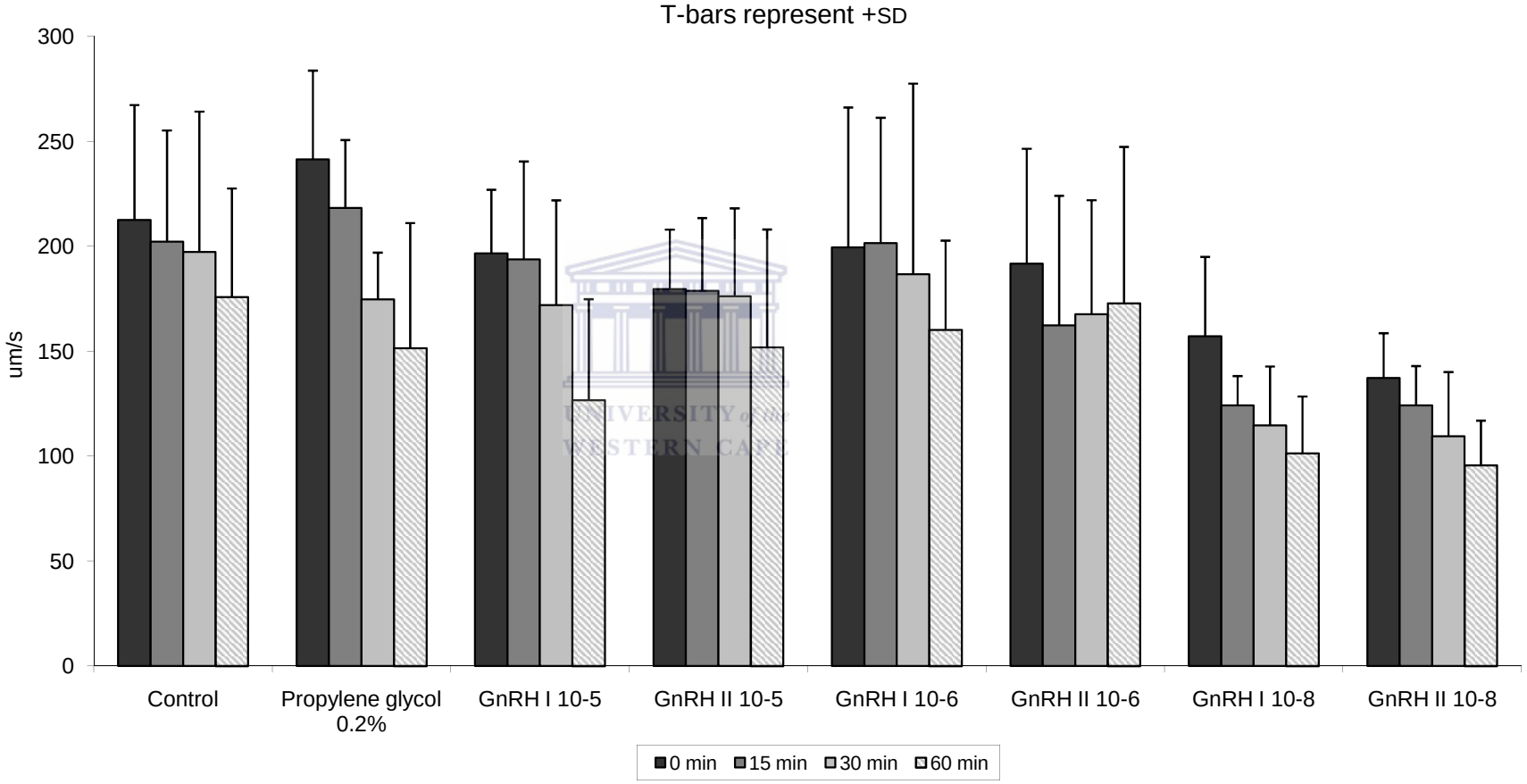


Figure 3.11
Curvilinear velocity (VCL) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

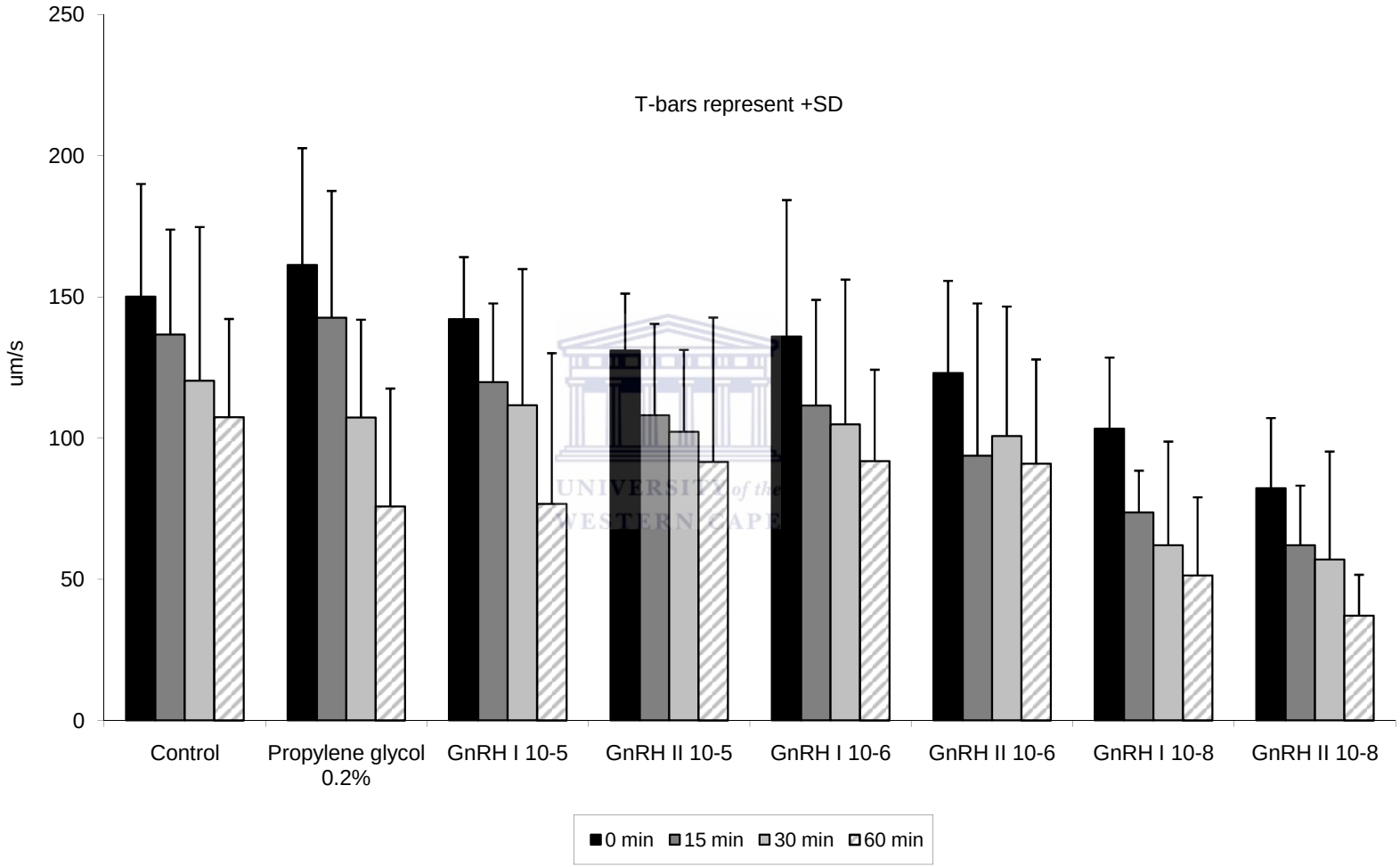


Figure 3.12
Straight line velocity (VSL) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

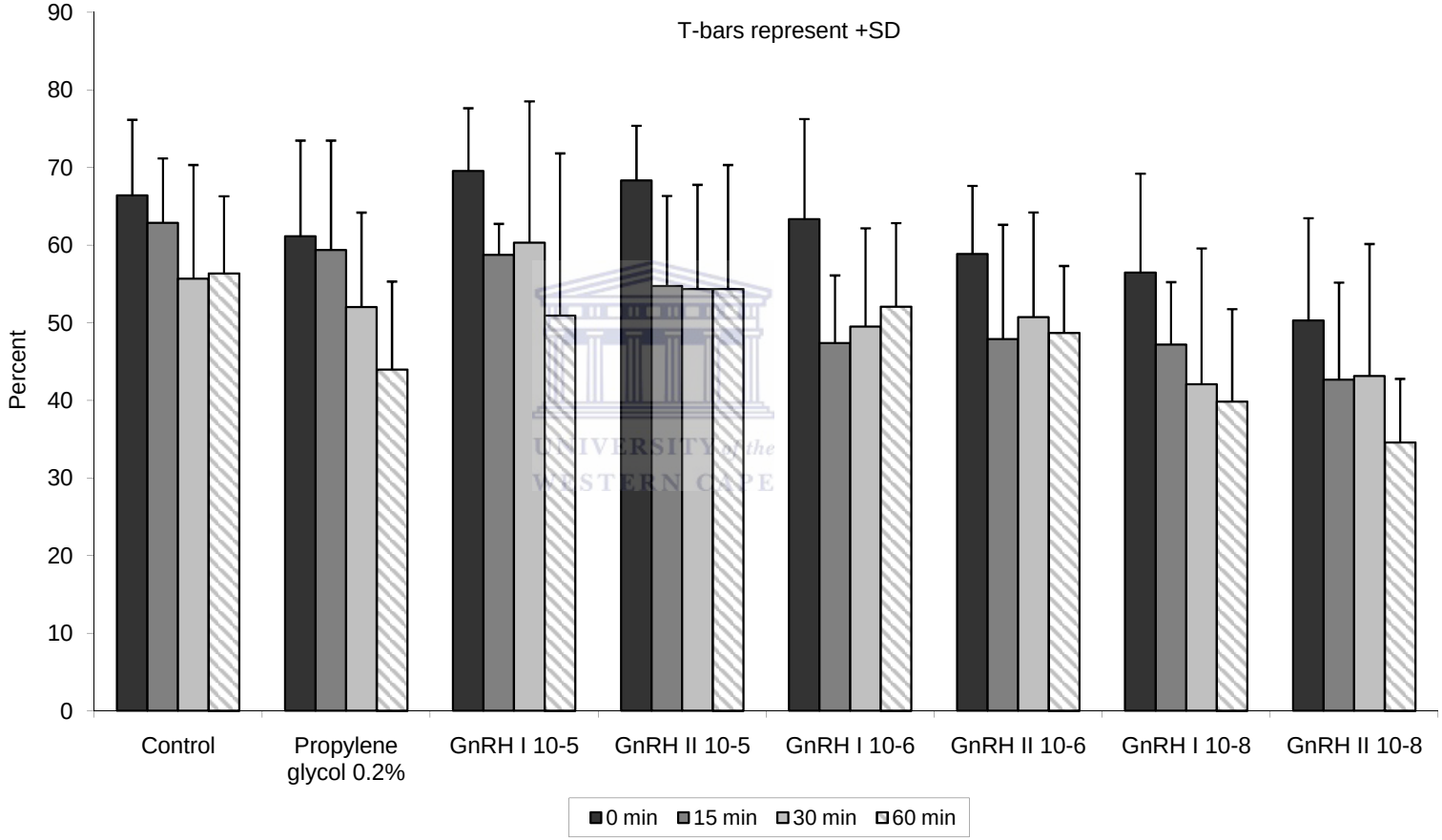


Figure 3.13
Linearity (LIN) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

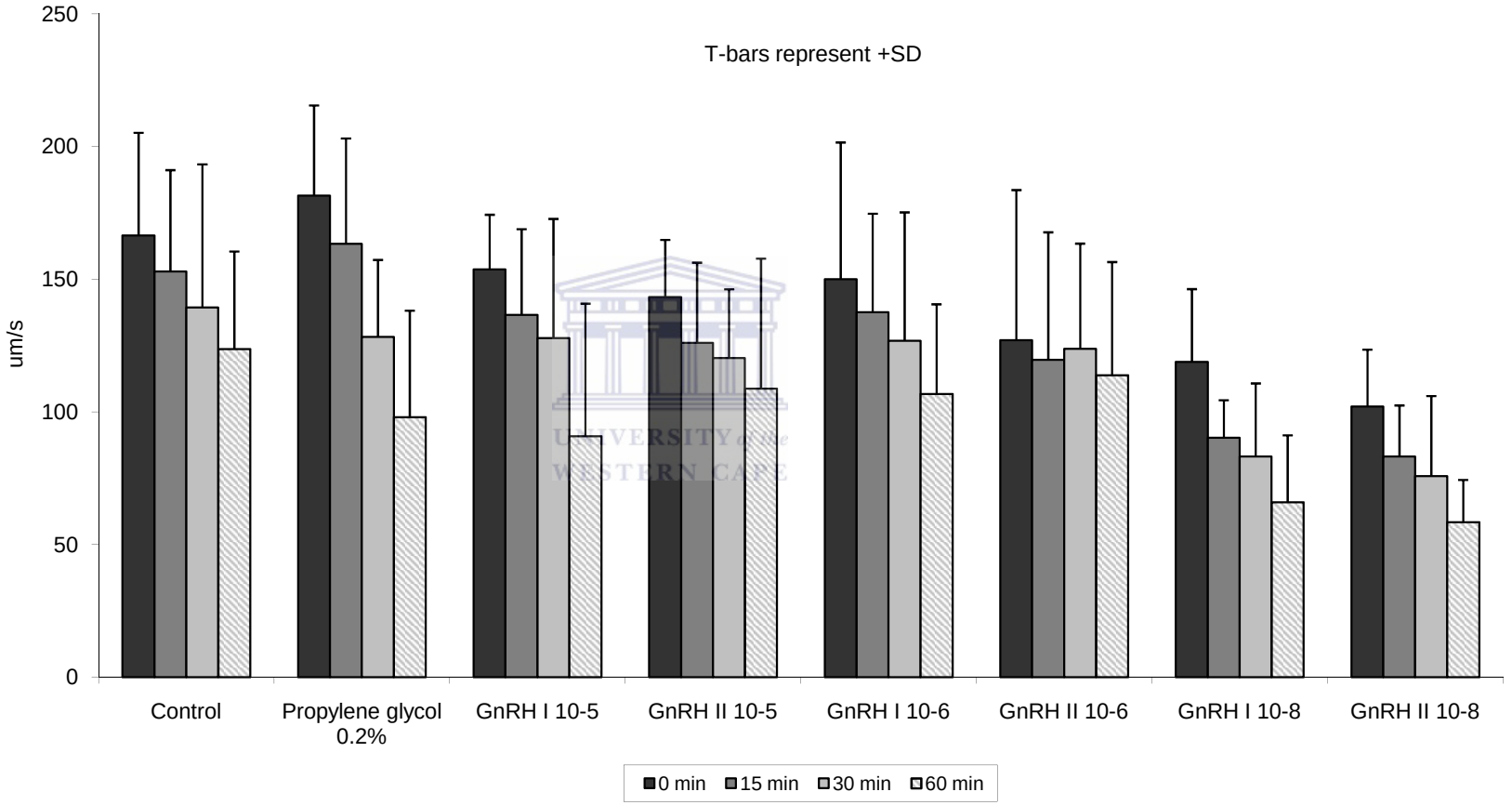


Figure 3.14
Average path velocity (VAP) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

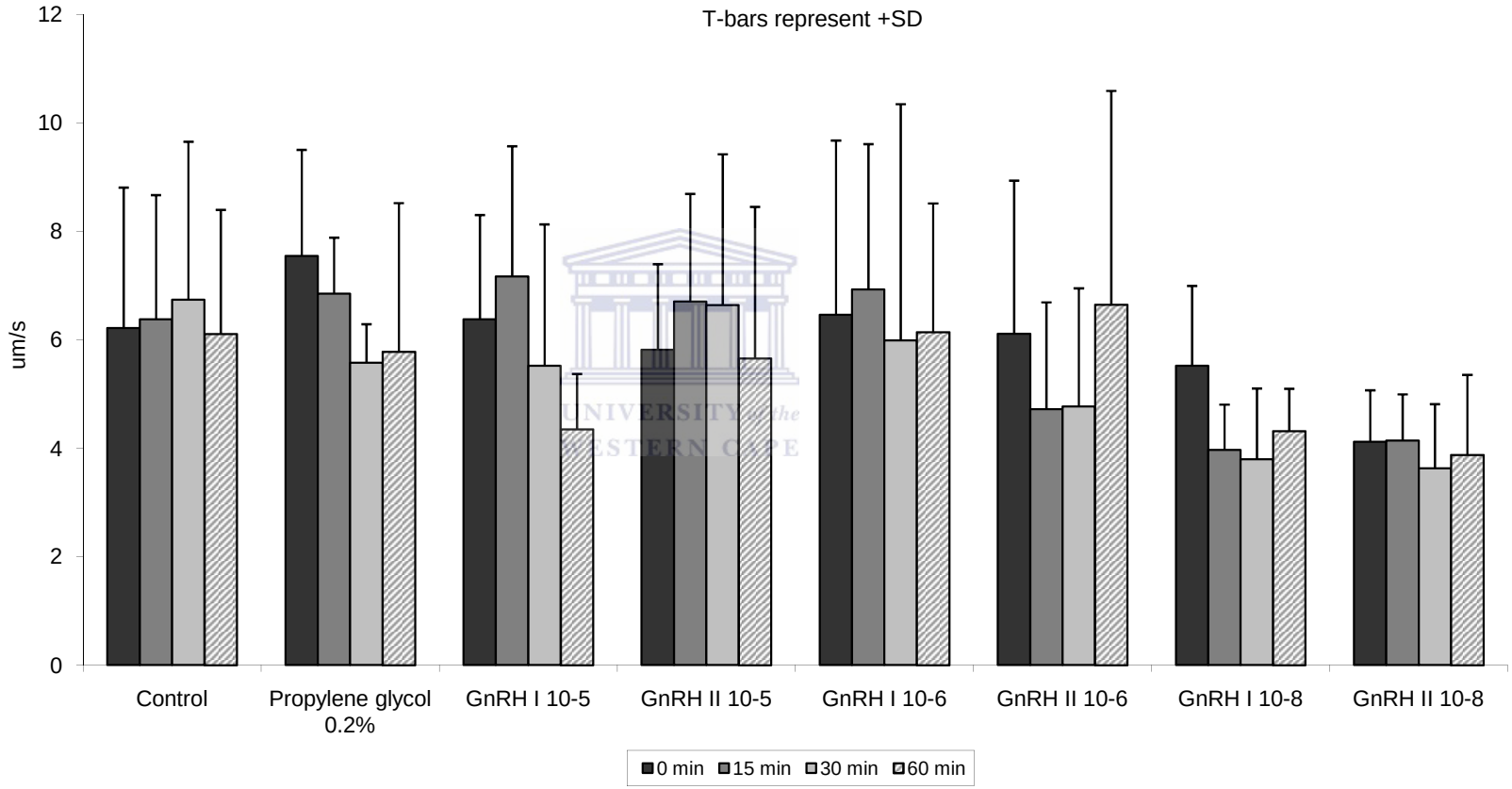


Figure 3.15
Amplitude of lateral head displacement (ALH) (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

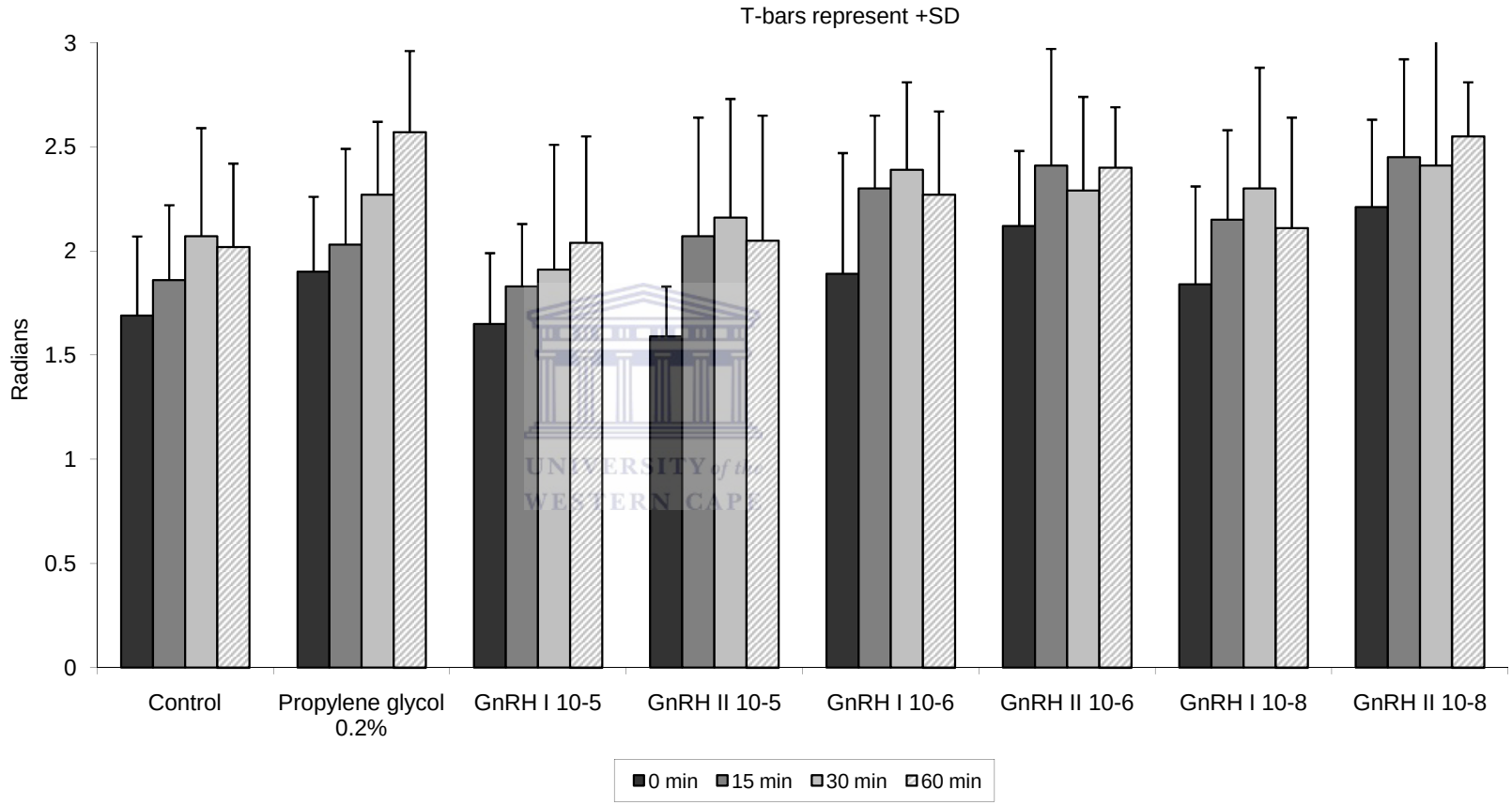


Figure 3.16
Mean angular displacement (MAD) (+SD) for sperm treated with GnRH I & II at various concentrations for one hour

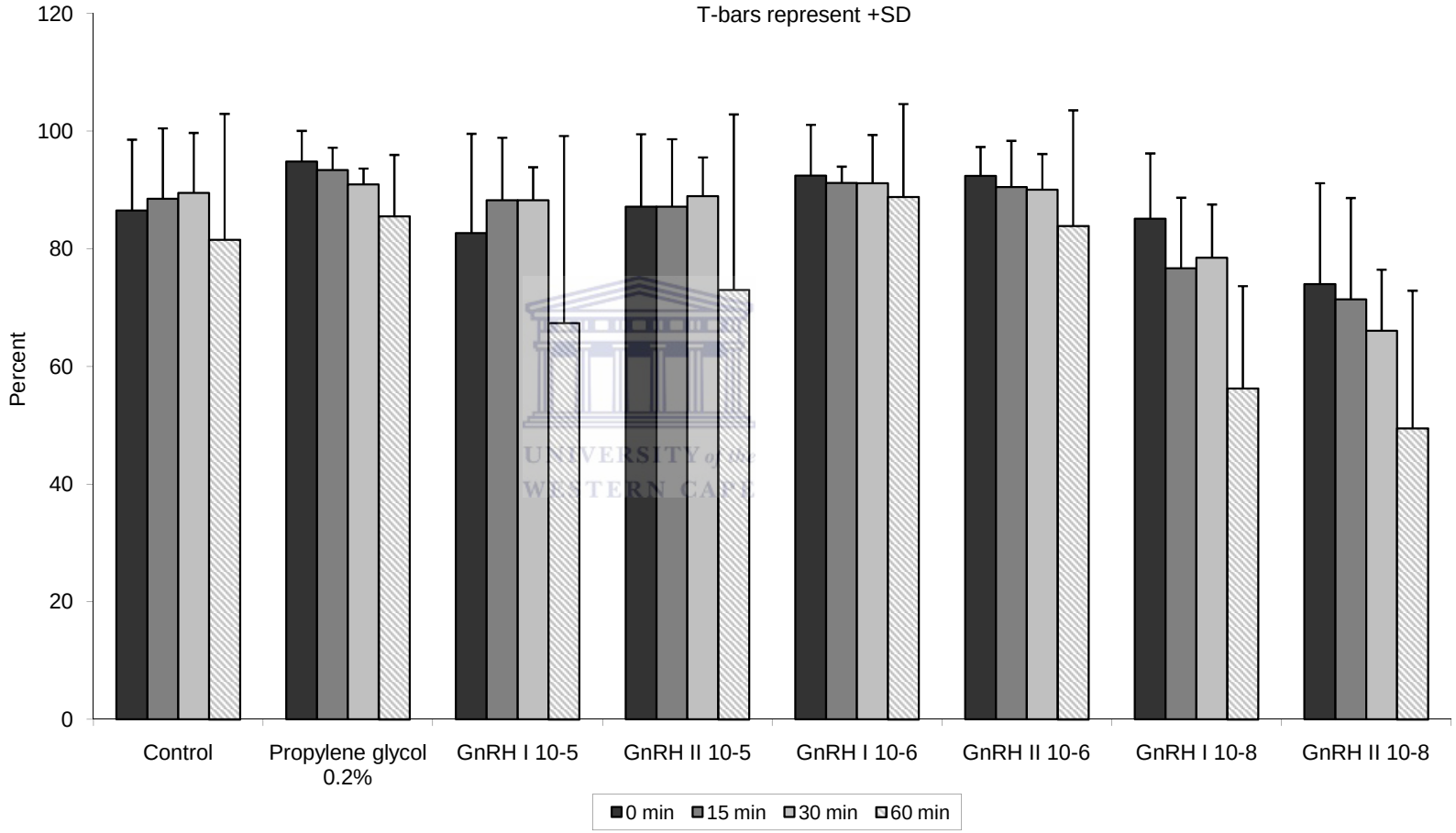


Figure 3.17
Percentage motility (+SD) for sperm treated with GnRH I & II at different concentrations for one hour

3.5.2 *Sperm motility tracks*

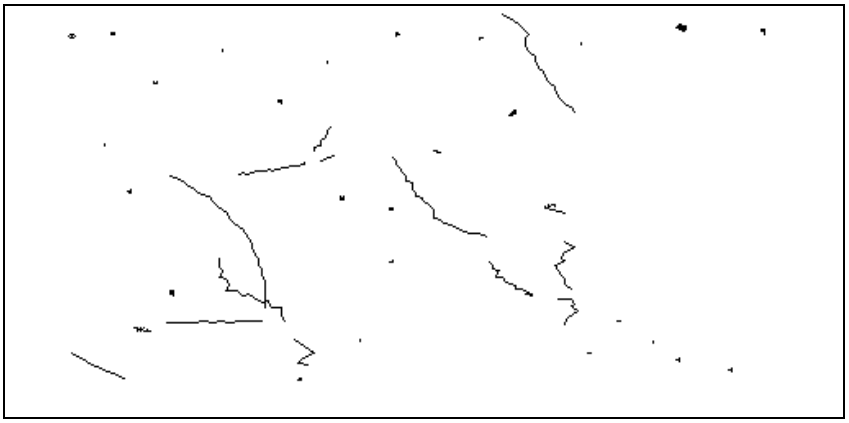
Figures 3.18, 3.19 and 3.20 depict representative sperm motility tracks after SMQ analysis. The purpose of presenting these tracks is to show that in most cases it is possible to recognize patterns of sperm motility for different treatments. While these evaluations are subjective, it is useful in conjunction with the quantitative analysis in section 3.4.

Figures 3.18A, B and C show sperm motility tracks of the control (A), GnRH I at 10^{-5}M (B) and GnRH II 10^{-5}M (C) at 60 minutes. For Figures A and B, the patterns appear quite similar. Both have fast swimming sperm (long tracks) as well as slow swimming sperm (short tracks) with a few immotile sperm (black dots) in Figure A. Shorter tracks are evident for Figure C and some immotile sperm for Figure A, but the patterns of movement seem to be similar. In this instance, the sperm motility pattern largely reflects the quantitative CASMA results as depicted in Table 3.4 and support the validity of the quantitative analysis (no statistical differences observed).

Figure 3.19 A (control at 60 minutes), B (GnRH I at10^{-6}M) and figure C (GnRH II 10^{-8}M) look quite similar. Even though Figure C demonstrate more immotile sperm and shorter tracks, the CASMA results for these tracks showed no statistically significant differences for A, B and C (Table 3.4). However, on closer visual examination the tracks of Figure B are more irregular than those of Figure A. It visually emphasizes more "serrated" or irregular sperm motility tracks usually indicative of a higher ALH and lower LIN. These irregular tracks (Fig. 3.19B) show a resemblance to the early phases of hyper activation.

In contrast to the above observations, the sperm motility tracks in Figure 3.20 A (control at 60 minutes), B (GnRH I at 10^{-8}M) and Figure C (GnRH II at 10^{-8}M) show distinctly different sperm motility patterns. These visual observations are in agreement with the CASMA analysis for this comparison (Table 3.4) which showed statistically significant differences for most parameters between control, GnRH I and GnRH II at 10^{-8}M. Furthermore, these subjective sperm motility track analysis, support the observations that low concentrations of GnRH I and II seem to inhibit sperm motility while higher concentrations have no effect on sperm motility.

A Control 60 at minutes



B GnRH I 10^{-5}M at 60 minutes

C GnRH II 10^{-5}M at 60 minutes

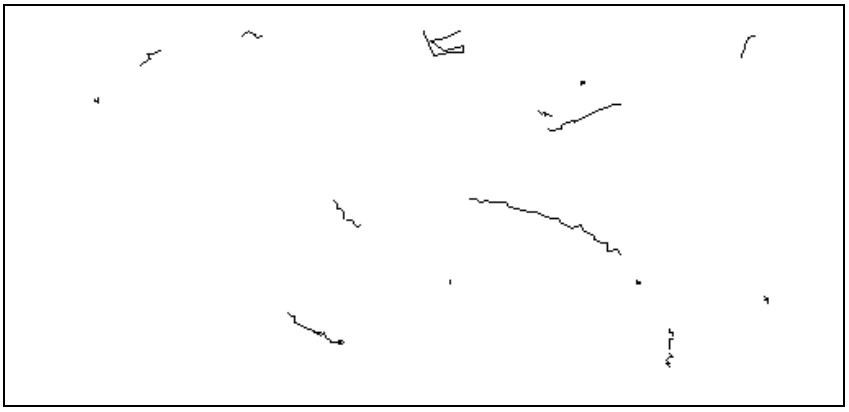
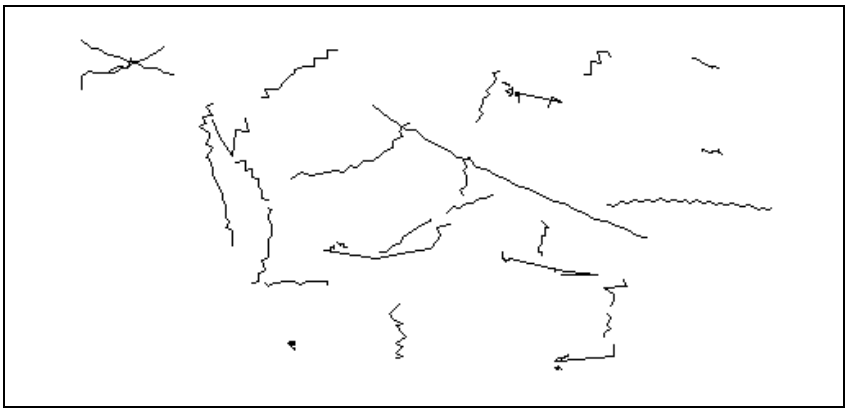


Figure 3.18 Representative sperm motility tracks for control, GnRH I and II at 10^{-5}M for one hour after sperm exposure.

A Control at 60 minutes



B GnRH I 10^{-6}M at 60 minutes

C GnRH II 10^{-6}M at 60 minutes

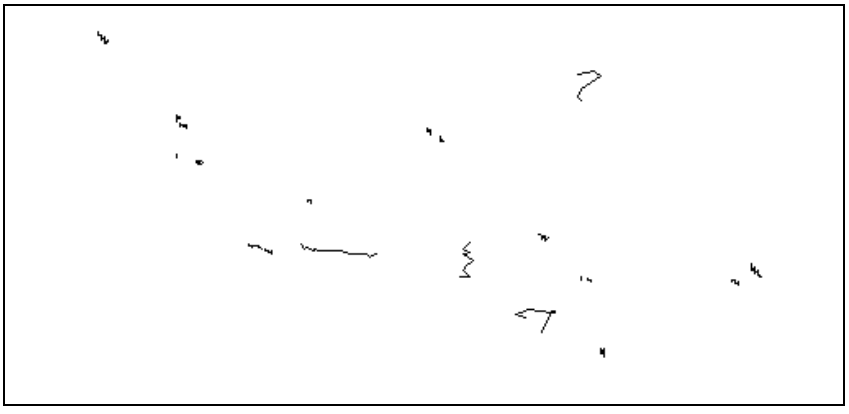
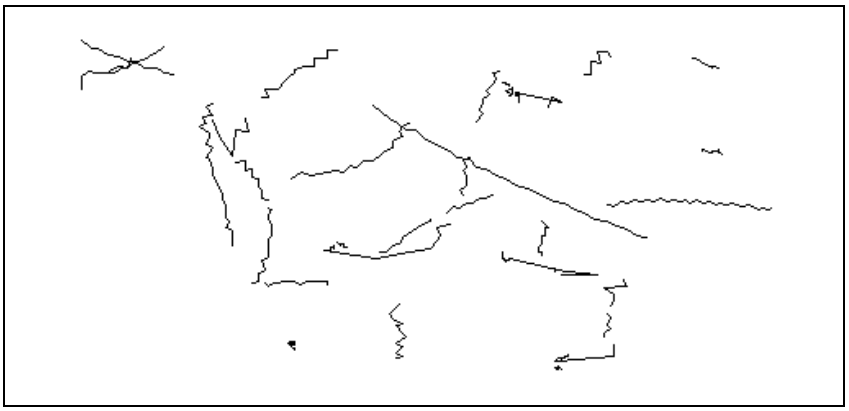


Figure 3.19 Representative sperm motility tracks for control, GnRH I and II at 10^{-6}M for one hour after sperm exposure.

A Control at 60 minutes



B GnRH I 10^{-8}M at 60 minutes

C GnRH II 10^{-8}M at 60 minutes

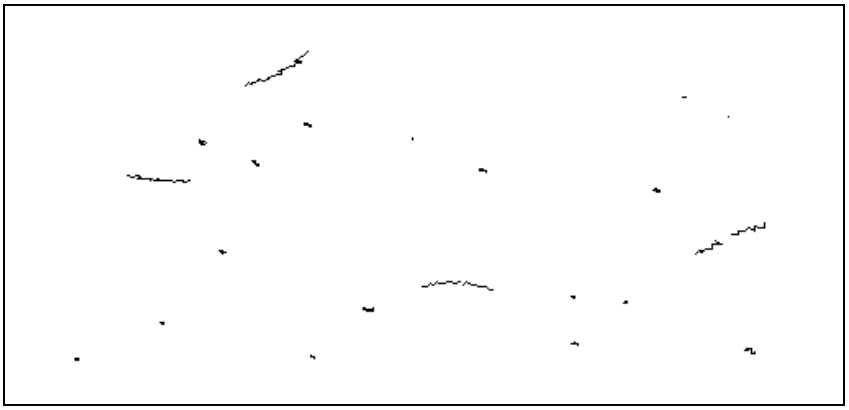


Figure 3.20 Representative sperm motility tracks for control, GnRH I and II at 10^{-8}M for one hour after sperm exposure.

CHAPTER 4

Discussion

Although numerous reports have confirmed the well-established role of GnRH in spermatogenesis, the only data available on its direct effect in mature sperm are from the research done by Morales [Morales *et al.* 1999, Morales 1998 and Morales *et al.* 1994]. There appears to be no comparable published information on the *in vitro* effect of GnRH on non-human primate sperm, despite the significant use of this order as a model for human reproduction. Therefore, this is the first *in vitro* study to present data on the effect of GnRH in relation to sperm motility and acrosome status of the adult Vervet monkey.

The wide tissue distribution and different functions of Gonadotropin-releasing hormone (GnRH) are well documented. There are numerous reports on the essential role of this peptide in the regulation of reproduction, viz. that it binds to a specific receptor located on the pituitary gland to cause the release of LH and FSH [Stojilkovic *et al*. 1994]. Furthermore, the reports of the inhibiting effect of GnRH antagonists and their possible use as a contraceptive agent, has paved the way for answering questions as to the specific role of GnRH in the male reproductive system.

The local production of GnRH was reported in the testis and "GnRH-like" molecules were detected in human seminal plasma [Sokol *et al*. 1995]. The obvious investigations to follow were the effects of GnRH on mature sperm. *In vitro* studies have shown the expression of the GnRH receptor in rat and mouse testicular germ cells [Bull *et al*. 2000]. Even though a type II GnRH system is absent in humans, a receptor transcript in human sperm was found [Lee *et*

al. 2000, van Biljon *et al*. 2002]. Previous reports have described various other peptide receptors found on the acrosome region of the sperm and that these peptide molecules play a role in the acrosome reaction. The latter is a crucial event for the success of fertilization and takes place after the fertilizing spermatozoon binds to the surface of the zona pellucida (ZP). Thus, molecules that modify the ability of spermatozoa to bind to the ZP are physiologically important, as they could interfere with the fertilization process.

Additional *in vitro* studies revealed that GnRH increased sperm binding to the human ZP by a direct effect on the sperm cells [Morales 1998] that is mediated by calcium influx into the sperm. Furthermore, it has been shown that GnRH antagonists inhibit sperm binding in a dose dependent manner [Morales *et al*. 1999] and that GnRH increased sperm-zona binding 300% when Ca^{2+} and progesterone (P) were present in the culture medium, progesterone as a stimulator of acrosome reaction [Morales *et al*. 2000, Morales *et al*. 2002]. However, neither GnRH nor any antagonists were reported to have any effect on the pattern of sperm movement and acrosome-reacted sperm.

GnRH-like molecules have been reported in the ovary [Aten *et al*. 1987] and human follicular fluid [Ying *et al*. 1981]. It is postulated that spermatozoa might interact with GnRH (or with GnRH-like molecules) during their journey through the male and female genital tracts and this interaction might provide an increase or decrease in the zona-binding capabilities of the spermatozoa. The bulk of evidence indicates that fluids secreted in the upper female genital tract play a role in the fertilizing ability of sperm and follicular fluid has been reported to inhibit sperm binding [Munuce *et al*. 2004].

4. Electro ejaculation

Rectal probe electro ejaculation is an accepted non-invasive procedure for the collection of non-human primate sperm [Gould *et al*. 1978]. The success of this method has been reported for the Rhesus monkey [Wickings and Nieschlag 1980], Vervet monkey [Seier *et al*. 1989], Cynomologus monkeys [Yeung *et al*. 1989] and the Baboon [Cseh *et al*. 2000]. The electric current used in electro ejaculation does not fragment sperm DNA, but a problem is the poor motility of the cells [Rall 1993] due to the difference in the membrane properties compared to sperm collected by other means [Cseh *et al*. 2000].

Even though the technique of electro stimulation was satisfactory for this study, at times, some males produced low semen volumes and poor motility ejaculates. As some of the single males masturbate, it was difficult to ascertain their sexual abstinence at the time of sampling. Nonetheless, most of these samples were otherwise of good quality, with a high percentage of intact acrosomes, suggesting that their fertilization potential was not disrupted by the electro ejaculation process. Rectal probe electro ejaculation has been linked to retrograde ejaculations [Cseh *et al.* 2000], and this could explain the variation in ejaculates.

4.1 Hams F10 extender

There are many different culture media and treatments available for sperm recovery in clinical insemination programmes and for research purposes.

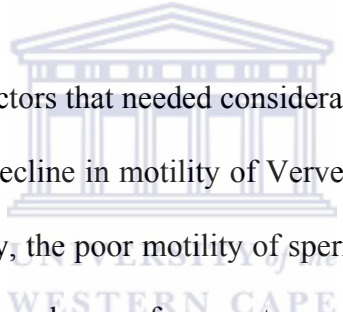
In many species, including the Vervet monkey, sperm motility declines rapidly when spermatozoa are maintained in semen [Seier *et al.* 1989]. It is preferable to use a suitable

medium that will extend the motility of the sperm and to imitate the environment of the female reproductive tract. Hams F10 extender medium is widely used to improve the motility of ejaculated spermatozoa and is developed specifically to support the clonal growth of a variety of cells with specific nutritional requirements (Sigma, Cape Town).

Therefore, Hams F10 was the culture medium used for the sperm swim-up technique in this study. The extender had to be freshly prepared for every experiment and pre-warmed at 37°C to prevent cold-shock to sperm. Hams F10 provided high-quality motile sperm throughout the experiment. This was consistent with other reports of motile sperm recovery for *in vitro* fertilization [Vijayakumar *et al*. 1987] and the best sperm responses for the same medium [Oliva *et al.* 1991]. One disadvantage of Hams F10 reported in the literature is that it may increase reactive oxygen species (ROS) [Gomez and Aitken 1996] and mammalian spermatozoa were, in fact, the first cell type in which this activity was described [Macleod 1943, Aitken and Fisher 1994]. Human spermatozoa are capable of generating O_2^- (super oxide) and H_2O_2 that is of fundamental biological importance in regulating the signal transduction pathways that control sperm capacitation [de Lamirande *et al.* 1997]. However, if the generation of these oxygen metabolites becomes elevated for any reason, this can result in oxidative stress that causes damage to the DNA and plasma membrane [Lopes *et al*. 1998, Baker and Aitken 2005]. Despite these concerns, it is unlikely that ROS generation via Hams F10 affected the results over one hour. The results of the control samples were consistent with a previous report of high percentage motility (>88%) and kinematic parameters, representing high quality sperm, when Hams F10 was used as culture medium [van der Horst *et al.* 1999].

4.2 Swim-up incubation

Under physiological conditions, ejaculated spermatozoa must be washed free from seminal plasma in order to undergo capacitation, which is necessary before they undergo the acrosome reaction [Wang *et al.* 1993]. Prolonged exposure to seminal plasma results in a marked decline in both motility and viability and spermatozoa are damaged by reactive oxygen species emanating from seminal leukocytes and damaged spermatozoa [Aitken and Clarkson 1988]. It is therefore important that spermatozoa must be separated from the seminal environment as soon as possible. The swim-up procedure accomplishes this separation, and enables the selection of populations of highly motile spermatozoa.



In this study, there were two factors that needed consideration concerning the sperm samples. The first factor was the rapid decline in motility of Vervet monkey sperm in seminal plasma [Seier *et al*. 1989] and secondly, the poor motility of sperm reported after electro ejaculation [Rall 1993]. Since the sperm membrane of spermatozoa can be damaged by centrifugation, cell debris and leukocytes, the direct swim-up method from semen was used [WHO 1999].

Most of the semen samples coagulated soon after ejaculation. The samples could not be left at room temperature to liquefy, as in the case of humans, because of the deterioration of semen quality after 30 minutes as reported by Seier *et al.* [1989]. No enzymatic liquefactions were used. Immediately after estimation of the motility and forward progression evaluations, Hams F10 was layered on top of the coagulum where motile spermatozoa then swam into the culture medium.

Both swim-up methods resulted in a highly motile sperm population that was required for the completion of each experiment. The success of the swim-up technique in this study showed agreement with previous findings that suggested this method offers the possibility of selecting spermatozoa with better motility, acrosomal integrity and the ability to undergo the acrosome reaction (AR) [Esteves *et al.* 2000].

4.3 Acrosomal integrity

Only spermatozoa with normal intact acrosomes undergo physiological acrosome reaction, which is essential for the spermatozoa to penetrate the zona pellucida and to fuse with the oolemma [Morales and Llanos 1996]. A normal sperm sample should contain a high proportion of spermatozoa with intact acrosomes [Tesarik 1989, Cummins *et al*. 1991, Henkel *et al*. 1993] and the data from this study demonstrated that fluorescence pattern I (intact acrosome) was the dominant characteristic for the control, (vehicle) propylene glycol 0.2% and GnRH treated groups (GnRH at 10^{-5}M, GnRH at 10^{-6}M and GnRH at 10^{-8}M). A striking feature of acrosome staining and scoring for the different fluorescent patterns is the large variability encountered from sample to sample leading to the large standard deviations. At times a trend seemed to appear in acrosome staining, but in this investigation the treated sperm samples was never statistically significantly different from the control or vehicle.

In Table 4.1, the data for acrosome loss in the controls of this study are compared to other reports for Vervet monkey sperm.

Table 4.1 Comparison of acrosome status reported for Vervet monkey sperm

Acrosome staining	Mean %	SD	n	Source
Intact acrosome	61.2	3.15	10	Conradie *et al*. 1994
	55.56	2.30	5	Seier *et al*. 2003
	97.11	4.80	6	De Villiers, 2006 (this thesis)
Equatorial staining	16.80	3.42	10	Conradie *et al*. 1994
	13.70	5.16	5	Seier *et al*. 2003
	1.31	3.37	6	De Villiers, 2006 (this thesis)
Absent acrosome	4.60	2.12	10	Conradie *et al*. 1994
	16.36	5.70	5	Seier *et al*. 2003
	1.58	3.22	6	De Villiers, 2006 (this thesis)

In this study, the percentage of intact acrosomes in the controls was much higher compared to previous reports for Vervet monkey sperm, while also demonstrating much lower figures for acrosome loss and absent acrosomes. The same trend was found in the GnRH treated groups. Two factors need to be considered when comparing these results. Firstly, a different staining method was used in this study and, secondly, the "patchy acrosome" variable was very difficult to distinguish from the bright pattern (intact acrosome). The uncertainty in distinguishing between intact acrosome and patchy acrosomes necessitated the investigator of this study to group patterns I and II together. This explains the discrepancy in the results as indicated in Table 4.1.

In conclusion, regardless of the difficulty in identifying the acrosome pattern II (patchy), the high numbers of intact acrosomes found in the treated groups suggest that the different concentrations of GnRH I & II had little or no effect on sperm plasma membrane composition and potentially on the functional changes in acrosome status. These results are

in agreement with Morales [1998] who indicated that GnRH had no effect on the acrosome reaction. The same author also reported that large concentrations of GnRH and Ca^{2+} antagonists showed different results regarding the acrosome reaction but progesterone treatment enhanced the acrosome effect. GnRH antagonists were not evaluated in this study.

4.4 Sperm motility

Sperm motility is a prerequisite for achieving fertilization. The male gametes must travel through cervical mucus and the female reproductive tract in order to penetrate the egg [Cooper 1986]. Thus, movement of sperm is a widely accepted parameter for assessment in male fertility.

4.4.1 Total percentage sperm motility

The percentage sperm motility in the controls of this study was comparable to previous reports for the Vervet monkey [Seier *et al*. 1989, Sankai *et al*. 1997 and van der Horst *et al*. 1999] and was not affected by high concentrations of GnRH I and II (10^{-5}M to 10^{-6}M). However, both GnRH I and II inhibited sperm motility (percentage) over an hour period at 10^{-8}M compared to the control values. It was particularly GnRH II, which depressed the percentage motile sperm from 90 ± 11.3 SD in the control to 49 ± 23.4 after one hour (10^{-8}M). This effect should, furthermore, be considered as biphasic in the context of a physiological response [Fourie 1998] given that Tripolin, a GnRH agonist, was shown to exert a double inhibitory-stimulatory action on cell growth when tested on two prostatic cell lines, depending on the dose and environmental conditions [Ravenna *et al.* 2000].

It is therefore evident that GnRH I and GnRH II *in vitro* inhibit the motility of Vervet monkey sperm at low/physiological levels (10^{-8}M). However, it has been previously reported that neither GnRH nor the antagonists at different concentrations affected the motility of human sperm *in vitro* [Morales *et al.* 1994]. Considering the fact that a functional type II GnRH receptor is present in primates but not in humans, the results of this study may accordingly reflect differences between species or provide the need for a new approach. The potential physiological significance of GnRH/sperm interaction and the percentage motility results will be discussed in conjunction with the CASMA results.

4.4.2 Computer Aided Sperm Motility Analysis (CASMA)

Only low concentrations of GnRH I and II (10^{-8}M) affected the kinematic parameters of sperm. These effects were evident from initial exposure to 60 minutes. There was a decline in most parameters (VCL, VSL, VAP, LIN, and ALH) but an increase in MAD over time after GnRH I and II exposure. The CASMA results therefore followed the same trend as the percentage motility results in this investigation.

A decrease in VCL, VSL and VAP reflects a decrease in the vigour of motility of a sperm population (Katz 1991, van der Horst 1995, van der Horst *et al.* 1999). Both GnRH I and II, particularly at low concentrations (10^{-8}M) decreased the vigour of sperm movement.

Alteration in LIN, ALH and MAD signifies that the pattern of sperm motion changed. A decrease in LIN and an increase in MAD show a decrease in sperm progression. In this investigation, LIN and ALH decreased but MAD increased after exposure to GnRH I and II

at low concentrations (10^{-8}M). When LIN decreases and MAD increases, sperm swim less progressively forward. When ALH decreases, there is a lower oscillation of the sperm head in relation to the VAP track.

It is evident from the above that sperm vigour, as well as forward sperm progression, was compromised when Vervet monkey sperm were exposed to low concentrations of GnRH I or II. The results of this investigation clearly show that most CASMA parameters are inhibited by GnRH I and II and these quantitative sperm motility parameters were significantly decreased. This will be discussed further under 4.5.4.

4.4.3 Sperm track analysis

The sperm track analysis represented the actual sperm swimming trajectories. Visual differences in representative sperm tracks cannot replace formal statistical models but are similar to ICONS and assist in visualizing differences in sperm swimming patterns and sperm morphology [e.g. Fourier blobs, Chernoff faces [Cleveland and McGill 1985], star symbol plot analysis [van der Horst *et al.* 1991, van der Horst 1995, van der Horst *et al.* 1999)]. ICONS are indispensable exploratory tools and once statistical differences have been established as in this investigation, greatly assist in visualizing/supporting differences in sperm tracks.

In this study, comparisons of sperm tracks representing different treatments supported the findings as verified statistically. In addition, "new patterns" of sperm motility emerged when sperm were exposed for one hour to GnRH I at 10^{-6}M as indicated in the Results

section (Fig.3.19B, p91). In this instance, the sperm motility pattern resembled the early stages of hyper activation. It may be possible that longer exposure of sperm to GnRH I at 10^{-6}M (>one hour) helps to trigger the hyper activated state. Unfortunately, sperm incubation in this study was restricted to one hour. It is thus hypothesized that GnRH I and II inhibit sperm motility at low concentrations (10^{-8}M) but may be stimulatory at higher concentrations after longer incubation. This hypothesis should be tested in future studies.

4.4.4 Hypothesis on GnRH I and II in relation to sperm motility development/inhibition and conclusion

Figure 4.1 (A to D) shows various GnRH receptor sites in the male and female reproductive systems. Virtually every compartment to which sperm and oocytes are exposed seems to be associated with some form of either GnRH and/or a GnRH receptor.

The importance of GnRH in spermatogenesis is well documented. A low affinity receptor for GnRH I, in some reproductive tissue, has been reported, as well as the presence of high-affinity specific binding sites and activity for GnRH II. One of these sites of action is the fertilizing sperm. A GnRH II receptor mRNA is expressed in the human sperm, although the functional protein is yet to be defined [van Biljon *et al.* 2002].

Investigators concluded that the composition of the extra cellular matrix of the female genital tract is the most favourable environment for sperm function/survival. They further stated that the sperm are able to respond to environmental cues within this milieu, and fertilization may not be limited to a random interaction between sperm and oocyte [Durkee *et al*. 1998]. Thus the female tract, and possibly the oocyte, "communicates" with the sperm by the elaboration

of the hypothesized factors. The GnRH found in the female genital tract could play a very important role in this unique mechanism. At some stage during transport, sperm might interact with GnRH at several possible sites along the male and female genital tract (see Figure 4.1). For example, GnRH produced by Sertoli cells could influence sperm during spermatogenesis, or during ejaculation the sperm cells may interact with GnRH present in seminal plasma.

During their transit throughout the oviduct, the sperm may cooperate with GnRH transferred by the products of ovulation. Reports indicate that fluids secreted by the upper female genital tract can have a role in synchronizing the acquisition of sperm fertilizing ability [Barratt and Cooke 1991] and that human follicular fluid (hFF) can result in a lower fertilizing ability by modulating sperm function [Munuce *et al*. 2004]. Based on these facts, and the presence of GnRH in hFF [Ying *et al*. 1981, Li *et al.* 1993], it is suggested that GnRH in the female reproductive tract could play a role in the selection phase of sperm prior to fertilization *in vivo*. The results of this study support this view, since they clearly demonstrated a sperm motility-inhibiting effect of a low-dose exposure of GnRH I & II on Vervet sperm *in vitro.*

It may furthermore be possible that GnRH I and II play a modulatory role in sperm motility during different phases of sperm maturation, as well as during their transit particularly in the female reproductive system. Before a sperm fertilizes the oocyte, it needs to undergo capacitation; otherwise the acrosome reaction cannot take place. One important landmark of capacitation is hyperactivation that is typically associated with a vast increase in VCL and ALH (200-300%) and a decline in LIN to less than 50% [Mortimer 1994]. It may be possible

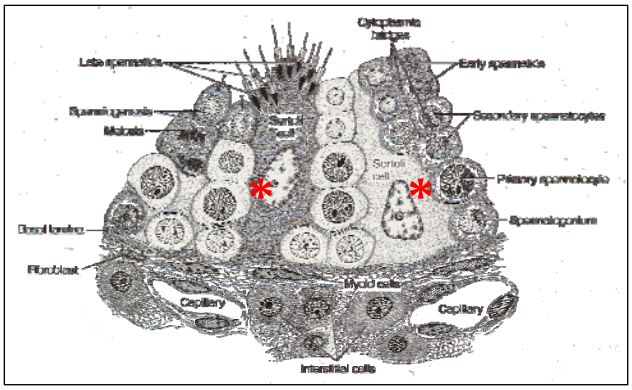
that higher concentrations of GnRH I and/or II may facilitate hyper activation over a longer exposure time than one hour.

However, the specific role of GnRH, and more importantly its mechanism of action within the sperm, remains to be clearly defined. There is currently no evidence for GnRH receptors on Vervet monkey sperm. Future studies should concentrate on the effect of a GnRH antagonist on sperm motility along with the potential presence of GnRH receptors on the sperm.

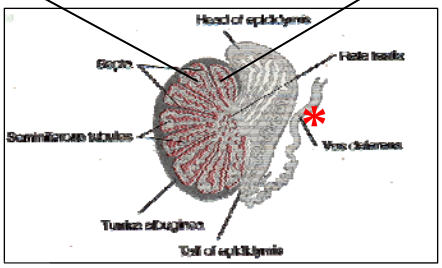
The Vervet monkey has proved to be a valuable model in reproductive physiology research. Should the presence of a GnRH receptor be confirmed in monkey sperm, this would provide an opportunity to investigate and address the many concerns regarding GnRH and sperm interaction.

* Testis, Sertoli cells, Spermatogenic cells and Leydig cells (rat & human) [Kadar *et al.* 1988, Bahk *et al.* 1995]
* Testicular germ cells (rat & mouse) [Bull *et al.* 2000]
* Prostate and seminal plasma (rat & human) [Azad *et al.* 1993, Sokol *et al.* 1985]
* Ejaculated sperm (human) [Morales 1998, van Biljon *et al*. 2002]

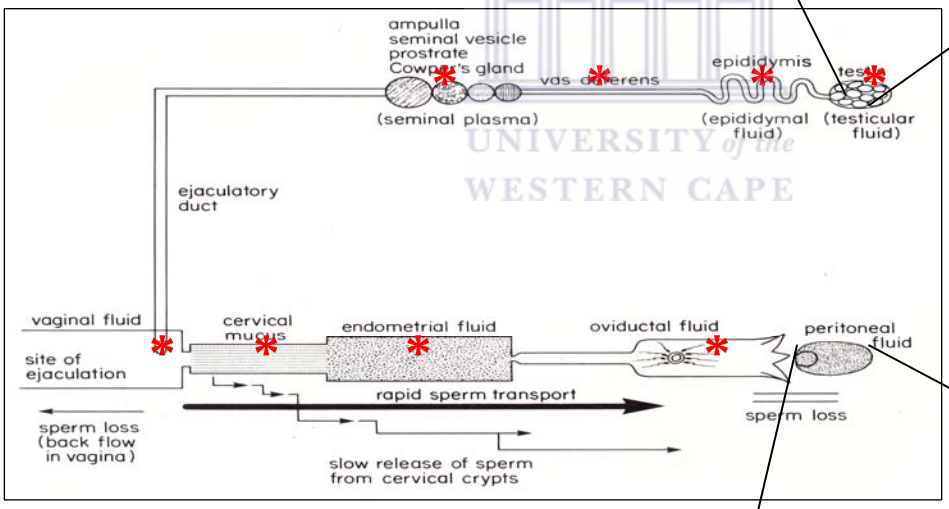
A



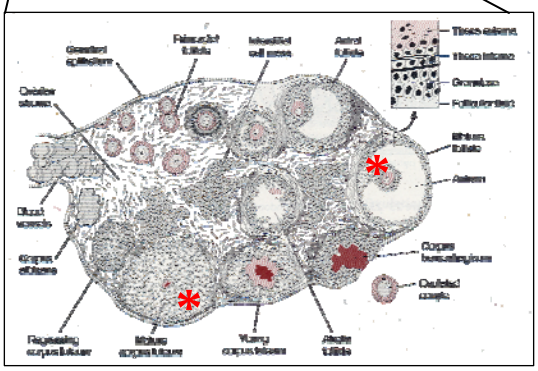
B



C



D



* Follicular fluid (human) [Ying *et al*. 1981]
* Ovary (baboon) and placenta (human) [Khodr and Siler-Khodr 1980, Aten *et al.* 1987, Siler-Khodr *et al.* 2003]
* Corpus luteum (human) [Bramley *et al.* 1985]
* Fallopian tube and endometrium (human) [Raga *et al.* 1998, Casan *et al.* 2000]
* Fertilization (human) [Morales 1998, Casan *et al.* 2000]

Figure 4.1 **GnRH sites (*) in the male and female reproductive systems. Sperm traveling through male and female genital ducts could be exposed to GnRH [Photomicrographs A, B & D: Ganong 1999, Diagram C: Hafez 1987]**

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