A mechanistic study on the mu-opioid receptor in human spermatozoa.

by Nadia van Niekerk

Thesis presented in fulfilment of the requirements for the degree Master of science in the faculty of Medicine and Health Sciences at Stellenbosch University

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April 2022

Declaration

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Abstract

Background: Infertility remains a complex and often unexplained phenomenon. The effect of opioids on male reproductive parameters is best understood by the suppression of the hypothalamic-pituitary-gonadal (HPG) axis. However, since the discovery of all three opioid receptor types on human spermatozoa, it has been hypothesized that the opioid system plays a regulatory role on sperm functional parameters. Literature regarding the possible mechanisms involved in these regulatory processes remain contradictory and scarce. Therefore, this study aimed to clarify the role of the mu-opioid receptor in human spermatozoa.

Methods: 30 semen samples were collected from healthy, human donors of reproductive age. Each sample was divided into 4 equal groups and treated accordingly. The study consisted of a control group, a codeine (opioid agonist) treated group, a naloxone (opioid antagonist) treated group, and a combination (codeine and naloxone) treated group. After a 3 hour incubation period, sperm functional parameters were assessed: Total motility, progressive motility and sperm kinematics were assessed using the Computer-Aided Sperm Analysis (CASA) system. Furthermore, viability, acrosome reaction and DNA fragmentation were investigated. By performing western blot analyses the total expression of A-kinase anchoring protein 3 (AKAP3) and protein kinase Cα (PKCα) were evaluated.

Results: No differences in sperm functional parameters were detected. It was observed that codeine influenced the acrosome reaction and DNA fragmentation negatively, and that naloxone seemed to alleviate these effects, but the results were not significant. Interestingly, the study found that naloxone significantly decreased the total protein expression of AKAP3 (1 ± 0.016 vs. 0.766 ± 0.025).

Conclusion: In order to clarify the role of opioid receptors on spermatozoa membranes, more research is necessary. The study results suggest that opioids may utilize sperm-specific signalling mechanisms downstream of the opioid receptors located on human spermatozoa. Understanding these mechanisms could prove to be invaluable in enhancing our understanding of infertility.

Opsomming

Agtergrond: Onvrugbaarheid bly 'n komplekse en dikwels onverklaarbare verskynsel. Tans word die effek van opioïede op manlike voortplantings parameters verklaar in terme van die onderdrukking van die hipotalamus-pituïtêre-gonadale (HPG)-as, maar sedert die ontdekking van al drie opioïedredeptor tipes op die menslike spermatozoa word daar gehipotiseer dat die opioïedstelsel moontlik ‘n direkte regulerende rol op spermfunksionele parameters speel. Literatuur rakende die moontlike meganismes betrokke by hierdie reguleringsprosesse bly teenstrydig en skaars. Daarom was die doel van hierdie studie om die rol van die mu-opioïedreseptor in menslike spermatosoa uit te klaar.

Metodes: Semenmonsters van 30 gesonde, menslike skenkers van reproduktiewe ouderdom was versamel. Elke monster was in 4 gelyke groepe verdeel en dienooreenkomstig behandel. Die studie het bestaan uit 'n kontrolegroep, 'n kodeïenbehandelde groep (opioeïd agonis), 'n naloksoonbehandelde groep (opioeïd antagonis) en 'n kombinasie (kodeïen en naloksoon) behandelde groep. Na 'n 3 uur inkubasieperiode is sperm funksionele parameters geassesseer: Totale beweeglikheid, progressiewe beweeglikheid en sperm kinematika was geassesseer deur gebruik te maak van die Rekenaargesteunde Sperm Analise (CASA) sisteem. Lewensvatbaarheid, akrosoomreaksie en DNA-fragmentasie was ondersoek. Die proteïen uitdrukking van AKAP3 en PKCα was geëvalueer met behulp van die Western klad tegniek.

Resultate: Daar was geen beduidende verskille tussen sperm funksionele parameters nie. Daar is waargeneem dat kodeïen die akrosoomreaksie en DNA-fragmentasie negatief beïnvloed het, en dat naloksoon blykbaar hierdie effekte verminder het, maar die resultate was nie betekenisvol nie. Interessant genoeg het die studie bevind dat naloksoon die totale uitdrukking van AKAP3 verminder het.

Gevolgtrekking: Om die rol van opioïedreseptore op spermatozoa membrane te verduidelik, is meer navorsing nodig. Die studieresultate dui daarop dat opioïede spermspesifieke seinmeganismes stroomaf van die opioïedreseptore, wat op menslike spermatosoa geleë is, kan gebruik. Om hierdie meganismes te verstaan, kan van onskatbare waarde wees om ons begrip van onvrugbaarheid te verbeter.

Acknowledgements

First and foremost, I honour my Lord and Saviour for bringing me thus far. All glory to God in the highest!

I would like to sincerely thank each of the following individuals for the part they have played in helping me to complete this degree:

Prof Stefan Du Plessis, thank you for your mentorship over the past few years. I will forever be grateful for the numerous opportunities I received to learn about the infertility field!

Dr Bongekile Skosana, for your continued patience and support ever since I joined the SURRG team.

Dr Erna Marais, for happily agreeing to be my co-supervisor in Prof Du Plessis' absence and for your willingness to help out wherever you can.

Prof Hans Strijdom, for your dedicated leadership of the Division of Medical Physiology and for your 'open-door' policy with all the students.

Lisa Marie Ferguson: We have truly walked side-by-side through the past few years, and I can honestly say I would never have gotten this far without you! Thank you for always cheering me on. We have some great memories together, and I will treasure them for years to come. If all I had to gain from completing this degree was your friendship – it would all be worth it!

Danelle Botha, for kindly helping me to understand and interpret my western blot results. She was a life-saver!

I would like to thank my parents, Herman and Eloise, for never wavering in their support of me. Thank you for believing in me and for always pointing me to the Lord. You have always done your best to give us kids the best possible opportunities and I will always be thankful for your labour of love.

And lastly (but certainly not in the least) thank you to Stephan, who has truly been my greatest supporter. Thank you for encouraging me in any way that you can – even if that means just bringing me chocolates! I appreciate you endlessly.

Dedication

I would like to dedicate this thesis to my parents:

Herman & Eloise van Niekerk

And to my late grandmother:

Pauline Anne van Niekerk

Mom and Dad, thank you for the opportunities you have given me. Your unwavering support and love have been the wind to my sails. I will forever be grateful to you for making this degree possible.

Ouma, whenever we spoke you would ask, without fail, about my studies. I wish I could phone you now and finally say “I did it, Oumie!”

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List of Abbreviations

5-HT	5-hydroxytryptamine
%	Percent
°C	Degrees celsius
α	Alpha
β	Beta
γ	Gamma
μl	Microlitre
μm	Micrometer
μm/s	Speed per second
μg	Microgram
AC	Adenylyl cyclase
AKAP3	A-kinase anchoring protein 3
ALH	Amplitude of the lateral displacement of the head
APN	Aminopeptidase N
APS	Ammonium persulfate
BCF	Beat-cross frequency
BSA	Bovine serum albumin
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CASA	Computer-aided sperm analysis
DAG	Diacylglycerol
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagles medium
DMSO	Dimethyl sulfoxide

DOR	Delta-opioid receptor / δ-opioid
DPDPE	d-Pen^2,d-Pen^5 enkephalin
ECL	Enhanced chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol tetra-acetic acid
FITC-PSA	Flourescein-conjugated pisum sativum agglutinin
FMHS	The Faculty of Medical and Health Sciences
FSH	Follicle stimulating hormone
Gα	G-protein, alpha subunit
Gβ	G-protein, beta subunit
Gγ	G-protein, gamma subunit
G$\beta\gamma$	G-protein, beta-gamma subunit
GABBA	Gamma amino butyric acid
GDP	Guanosine diphosphate
GIRK	G-protein coupled inwardly rectifying
GnRH	Gonadotropin-releasing hormone
GPCR's	G-protein coupled receptors
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate
HREC	Health Research Ethics Committee
HRP	Horseradish peroxidase
HPG	Hypothalamic-pituitary-gonadal
ICF	Informed consent form
IgG	Immunoglobulin G
IP_3	Inositol triphosphate

K^+	Potassium
kDa	Kilo dalton
KOR	Kappa-opioid receptor / κ-opioid
LIN	Linearity
LH	Luteinizing hormone
M	Molar
mA	Milliampere
ml	Milliliter
mM	Millimolar
mg/ml	Milligram per milliliter
MOR	Mu-opioid receptor / μ-opioid
n	Sample Size
Na^+	Sodium
NaCl	Sodium chloride
Na_3VO_4	Sodium orthovanadate
NEP	Endopeptidase neutral N
ng/ml	Nanogram per milliliter
OPIAD	Opioid-induced androgen deficiency
PAG	Periaqueductal grey
PBS	Phosphate-buffered saline
PDYN	Preprodynorphin
PENK	Preproenkephalin
pH	Potential of hydrogen
PI	Protease Inhibitor
PI3K	Phosphoinositide 3-kinases
PIP_2	Phosphatidylinositol 4,5-bisphosphate

$PIP_{3,4,5}$	Phosphatidylinositol-3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKCα	Protein kinase C alpha subunit
PLC	Phospholipase C
PLD	Phospholipase D
PMSF	Phenyl methyl sulfonyl fluoride
POMC	Proopiomelanocortin
PRB	Population reference bureau
Rpm	Revolutions per minute
RVM	Rostal ventromedial medulla
SCA	Sperm class analyzer
SD	Standard deviation
sDF	Sperm DNA fragmentation
SDS	Sodium dodecyl sulfate
SFK	SRC family tyrosine kinase
SS1	Stock solution 1
STR	Straightness
SURRG	Stellenbosch university reproductive research group
TB	Toluidine blue
TBS	Tris-buffered saline
TEMED	Tetrametylethylenediamine
V	Volt
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight-line velocity

WHO	World Health Organization
WOB	Wobble
WS1	Working Solution 1

CHAPTER 1

INTRODUCTION

Infertility is commonly defined as the inability to conceive after 12 or more months of regular, unprotected sexual intercourse. It is estimated that between 8-12% of all couples suffer from infertility (Ombelet et al., 2008). Additionally, it is well established that males are the sole contributors in 30-40% of all infertility cases (Cousineau and Domar, 2007). Despite the availability of significant scientific and medical interventions, approximately 30% of infertile couples are still diagnosed with unexplained or idiopathic infertility (Sadeghi, 2015). This alludes to a large gap in the existing knowledge with regards to infertility and its treatments and warrants more research.

Opioids are a well-known and widely-used class of analgesic medications (Norn, Kruse and Kruse, 2005). Cultivated from the opium poppy, modern opioid medications include the likes of morphine, codeine, fentanyl and oxycodone. Though powerful painkillers, these drugs are accompanied by various unwanted side effects. These include constipation, respiratory depression, nausea, sedation, physical dependence and tolerance (Benyamin *et al.*, 2008; Drobnis and Nangia, 2017). Furthermore, the intense feelings of euphoria associated with opioid usage has led to an ongoing and rapidly evolving, global abuse crisis (Volkow and Collins, 2017).

The discovery of high concentrations of endorphins and enkephalins in human semen and spermatozoa cells prompted the hypothesis that the opioid system plays a role in human reproduction (Foresta *et al.*, 1986; Kew, Muffly and Kilpatrick, 1990). Later, various groups described the presence of the mu, delta and kappa-opioid receptors on human spermatozoa for the first time (Agirregoitia *et al.*, 2006a; Albrizio *et al.*, 2006). One of the best understood effects of opioids on the reproductive system is the suppression of the hypothalamic–pituitary–gonadal (HPG) axis, when opioid receptor agonists bind to opioid receptors in the hypothalamus (Benyamin *et al.*, 2008). However, the effect of the opioid system on spermatozoa is poorly understood, and many contradictions exist in the current literature. The opioid system has been hypothesized to have a regulatory role on many of the functional sperm parameters, including motility, capacitation and acrosome reaction. Due to the gaps in the literature as well as the inconsistencies between studies, the mechanisms that are involved are still unclear. Therefore, this study aims to clarify the role of the mu-opioid receptor in human spermatozoa.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Globally, total fertility is decreasing at unprecedented rates. The World Population Data Sheet is released annually by the Population Reference Bureau (PRB), and tracks 24 population indicators for more than 200 countries and territories. According to the latest census done by the PRB, the total fertility rate (births per woman) has dropped from 3.2 in 1990, to 2.3 in 2021 (Population Reference Bureau, 2021). This is remarkable, as it is commonly accepted that a total fertility rate of 2.1 (births per woman) is required for a population to have a replacement level fertility (Vollset *et al.*, 2020). Furthermore, the PRB projects that by 2050, 39 countries will have fewer people than they do today, owing to a negative population growth. Though various factors influence global fertility rates, medical infertility contributes largely to these statistics and has been researched and reported on for many years (Whiteford and Gonzalez, 1995; Bongaarts, 2015; vander Borght and Wyns, 2018). Infertility is commonly defined as the inability to conceive after 12 or more months of regular, unprotected sexual intercourse (World Health Organization., 2010; Kumar and Singh, 2015). It is estimated that between 8-12% of all couples suffer from infertility (Ombelet *et al.*, 2008). Despite the availability of significant medical interventions, approximately 30% of infertile couples are still diagnosed with unexplained or idiopathic infertility (Sadeghi, 2015). This alludes to the growing need for additional research regarding infertility and its treatments. It is well established that males are the sole contributors in 30-40% of all infertility cases (Brugh and Lipshultz, 2004; Cousineau and Domar, 2007). Fertility parameters can be influenced by a variety of factors, for example; lifestyle, chronic diseases, hormonal imbalances and certain environmental factors. The use of various medications, including opioid medications, have been shown to affect reproductive parameters (Samplaski and Nangia, 2015; Machen and Sandlow, 2020).

Opioid medications have been used for thousands of years in treating acute and chronic pain, and have been documented in the use of surgical analgesia for several centuries (Norn, Kruse and Kruse, 2005). Cultivated from the opium poppy, modern opioid medications include the likes of morphine, codeine, fentanyl and oxycodone. Though powerful painkillers, these drugs are limited by the unwanted side effects that accompany long-term use. These include constipation, respiratory depression, nausea, sedation, physical dependence and tolerance (Benyamin *et al.*, 2008; Drobnis and Nangia, 2017). Furthermore, the intense feelings of euphoria associated with opioid usage has

led to an ongoing and rapidly evolving, global abuse crisis (Volkow and Collins, 2017). Opioids were hypothesized to have an effect on male reproductive parameters, after various groups noticed abnormal sperm quality in heroin addicts (Cicero *et al.*, 1975; Singer *et al.*, 1986; Ragni *et al.*, 1988). Since then, the long-term use of opioid analgesics have often been associated with hypogonadism, reduced sperm motility, low sperm count, poor morphology and increased DNA fragmentation (Safarinejad *et al.*, 2013; Böttcher *et al.*, 2017; Farag *et al.*, 2018). In order to understand the effect of these medications, it is necessary to give some background on the endogenous opioid system. This review will aim to elaborate on the effect of opioid analgesics on male reproductive parameters.

2.2 The endogenous opioid system:

The endogenous opioid system is known to play a major role in many aspects of normal physiology, neurobiology and even in some addictive disorders(Trigo *et al.*, 2010; Sobczak *et al.*, 2014). It consists of 3 families of receptors; the μ-opioid (mu-opioid receptor/MOR), δ-opioid (delta-opioid receptor/DOR), and κ-opioid (kappa-opioid receptor/KOR) receptors (Galligan and Akbarali, 2014). The system is completed by 3 families of endogenous opioid peptides, namely β-endorphins, enkephalins and dynorphins (Valentino and Volkow, 2018). These peptides are produced by proteolytic cleavage of large protein precursors known as proopiomelanocortin (POMC), preproenkephalin (PENK) and preprodynorphin (PDYN) (le Merrer *et al.*, 2009). All opioid peptides share a common amino (NH_2) terminal (Tyr-Gly-Gly-Phe signature sequence), by which they interact with opioid receptors. Opioid receptors and their peptides have a widespread distribution in the central and peripheral nervous systems and are particularly involved in pain modulation, reward, stress response and autonomic control circuits (Benarroch, 2012; Seeber *et al.*, 2019).

2.2.1 Opioid receptors

Opioid receptors belong to one of the largest, mammalian protein families in existence namely; the family of G-protein coupled receptors (GPCR's). Figure 2.1 depicts the structure of an opioid receptor. Structurally, opioid receptors consist of seven hydrophobic trans-membrane domains (TM1-7 on figure). There are three intracellular hydrophobic loops (i1-i3), three extracellular loops (e1-e3) and a glycosylated amino and a carboxyl terminus. The transmembrane domains TM5-7 have been shown to bind DOR ligands (Befort *et al.*, 1996). The transmembrane domain TM4 and the extracellular loop e2 bind ligands related to KOR, while the extracellular loop e1 has been shown to be a binding site for MOR (Surratt *et al.*, 1994). The intracellular loops (i1 and i3), the C-terminus

receptor fragment and the transmembrane domain TM5 all participate in various signal transduction pathways and participate in mediating opioid receptor G-protein interactions (Chan *et al.*, 2003).

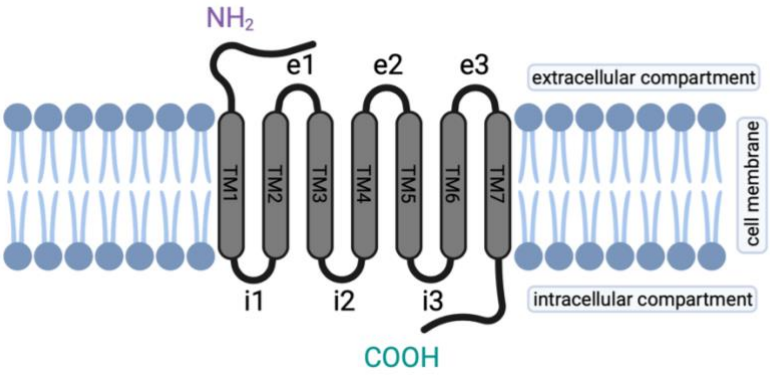

Figure 2.1: *The general structure of a G-protein coupled opioid receptor*
**Figure designed by author, using Biorender.*

When activated, opioid receptors follow a pattern that is characteristic for all GPCR's. Shortly, once a ligand binds to the opioid active site, the receptors' conformation changes, activating intracellular G-proteins. Each G-protein consists of three subunits: Alpha (Gα), Beta (Gβ), and Gamma (Gγ)(Standifer and Pasternak, 1997). Opioid receptors are coupled with Gαi (existing in 3 forms), Gαo (in A and B forms) and Gαz (Reisine *et al.*, 1996). The β and γ subunits form a heterodimer which is crucial for the function of Gα, as it enables proper conformation of Gα while ligand binding to the receptor (Law, Wong and Loh, 2003). G-protein activation involves dissociation of the Gα and G$\beta\gamma$ subunits, which is followed by the Gα subunit translocation and further interaction with Kir3 (G-protein-gated inwardly rectifying Potassium [K^+] channel)(Nagi and Pineyro, 2014). In addition, the release of Gα inhibits adenylyl cyclase activity, which causes a decrease in intracellular cyclic adenosine monophosphate (cAMP). The ultimate effect of releasing the G$\beta\gamma$ subunit is inhibition of voltage gated Calcium (Ca^{2+}) channels which in turn causes activation of Potassium (K^+) channels (Dupré *et al.*, 2009). The net effect is therefore a reduced intracellular cAMP, hyperpolarization of the cell and neurotransmitter release(Venkatakrishnan *et al.*, 2013). In some cases, opioid receptor activation has been found to result in elevation of intracellular Ca^{2+} concentrations and is believed to accomplish this by releasing Ca^{2+} from intracellular stores or enhancing calcium entry by a dihydropyridine-sensitive mechanism (Samways and Henderson, 2006). After the activation of the various intracellular effectors, guanosine triphosphate (GTP) is hydrolysed to guanosine diphosphate (GDP) and Gα loses its activity. It binds to the G$\beta\gamma$ subunit and the recovered complex is once again inactive.

2.2.2 Endogenous opioid peptides

Endogenous opioid peptides are naturally produced neurotransmitters that act as opioid receptor agonists (Frederickson and Geary, 1982). These peptides were first reported in the 1970's when they were discovered in the brain and cerebrospinal fluid (Hughes, 1975; Pasternak, Goodman and Snyder, 1975). These peptides are naturally produced in the central nervous system (CNS) and peripheral tissues such as the pituitary and adrenal glands (Snyder, Pasternak and Pert, 1975). As mentioned earlier, the three major endogenous opioid peptides (β-endorphin, enkephalins and dynorphins) result from the processing of three main precursors: POMC, PENK and PDYN. Each of these precursors has a unique anatomical distribution. The biosynthesis of POMC occurs mainly at the anterior and neuro-intermediate lobes of the pituitary gland. POMC is, among other things, responsible for the generation of β-endorphin (van Ree *et al.*, 2000). PENK is produced throughout the CNS, adrenal medulla and other peripheral tissues and is responsible for the synthesis of enkephalins (Kato, 2021), while PDYN was found to be produced in a widespread manner throughout the CNS and is responsible for the synthesis of dynorphins (Fang *et al.*, 2009). Interestingly, each of the endogenous opioids show a preference/increased affinity for a different receptor. β-endorphin for MOR, enkephalins for DOR and dynorphins for KOR (Gupta *et al.*, 2021).

One of the most-covered topics regarding endogenous opioid peptides is their effect on pain and analgesia. Opioid peptides have been found to mediate both ascending and descending pain pathways (Shenoy and Lui, 2021). An example of opioid-mediated pain suppression (descending pathway) can be seen in figure 2.2 and 2.3, which portrays an opioid agonist acting on a MOR in the periaqueductal grey (PAG) part of the midbrain. The PAG is a primary control centre for descending pain modulation and PAG neurons are under the influence of inhibitory neurotransmitter gamma-aminobutyric acid (GABA).

In the naïve state (figure 2.2), GABAergic interneurons located in the PAG fire continually thereby producing a steady release of GABA and inhibiting PAG output neurons. This disinhibits PAG projections to the rostal ventromedial medulla (RVM), meaning that the relay of sensory information between the brain and spinal cord is interrupted. Depending on the synaptic site (pre-/post-synaptic) of the receptor, opioids are able to activate different signalling cascades.

When a MOR agonist binds post-synaptically (figure 2.3), the dissociated Gα subunit activates G-protein coupled inwardly rectifying (GIRK) channels, which results in K^+ release and

hyperpolarization of the neuron. Additionally, adenylyl cyclase (AC) is inhibited which decreases intracellular cAMP production. Presynaptic binding (figure 2.3) of an opioid inhibits voltage dependent Ca^{2+} channels and activates voltage gated K^{+} channels. Overall, these two mechanisms succeed in blocking the release of GABA, thus activating the PAG.

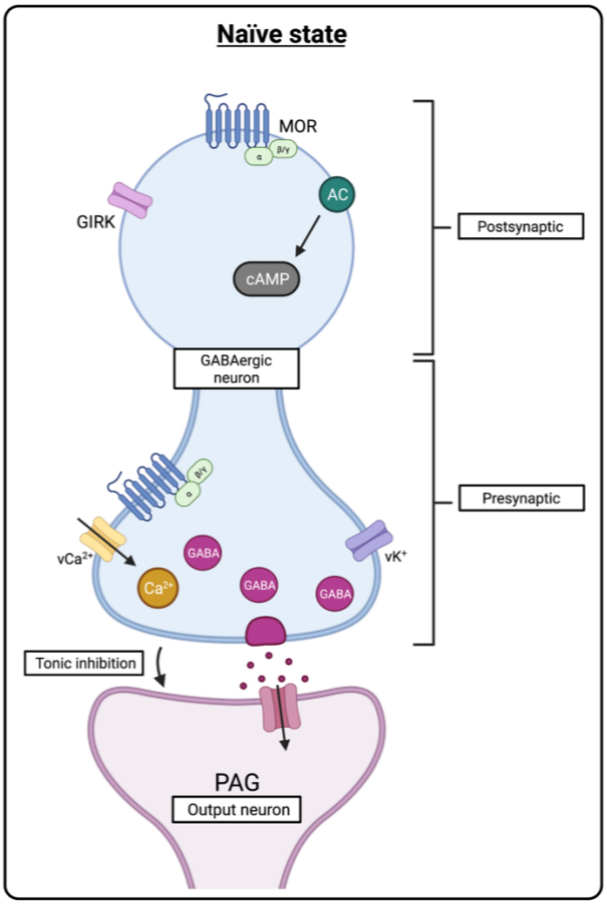

Figure 2.2: *The GABAergic neuron in its naïve state.*

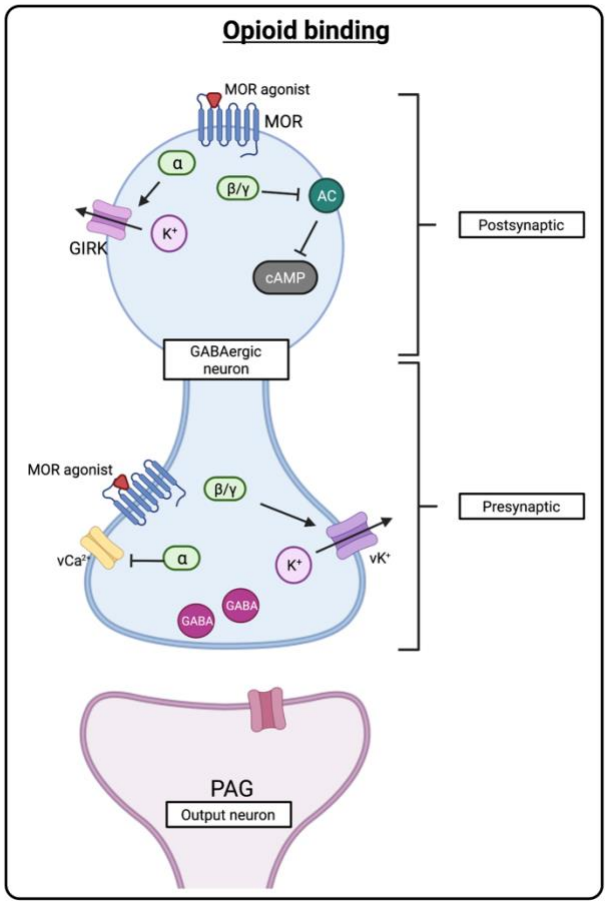

Figure 2.3: *The sequence of events in the GABAergic neuron when opioid binding takes place.*

Key: voltage gated potassium channels (vK^{+}), voltage gated calcium channels (vCa^{2+}), Adenylyl Cyclase (AC), G-protein coupled inwardly rectifying (GIRK) channel
**Figures adapted from* (Lueptow, Fakira and Bobeck, 2018). *Redesigned by author, using Biorender.*

This results in activation of serotonergic neurons in the greater nucleus raphe magnus and noradrenergic neurons in the (RVM) (Lueptow, Fakira and Bobeck, 2018). These neurotransmitters stimulate the 5-hydroxytryptamine (5-HT) and enkephalinergic interneurons in the spinal cord, which connects directly with the substantia gelatinosa of the dorsal horn. The result is a reduction of nociceptive transmission from the periphery to the thalamus (Williams, 2008a; Valentino and Volkow, 2018).

2.3 Exogenous opioids

Exogenous opioids, such as morphine, codeine and fentanyl, are substances that are introduced into the body and function as opioid receptor agonists (Dhaliwal and Gupta, 2021). Opioids have been used medicinally (as an analgesic) and recreationally (as a euphorigenic) throughout human history (Hamilton and Baskett, 2000; Presley and Lindsley, 2018). After the identification of the endogenous opioid system in the 1970's, the efficacy of opioids in treating illness was vastly improved. The advancements in modern medicinal chemistry and neuroscience were accompanied by the development of powerful new opioid drugs, such as heroin and morphine. Unfortunately, the beneficial effects exerted by these drugs, are eclipsed by some serious detrimental effects. Some of these unfavourable effects include analgesic tolerance, respiratory depression, nausea and vomiting, constipation, hyperalgesia and even transition to addiction (Inturrisi, 2002; Streicher and Bilsky, 2018). Presently, opioid medications remain an integral analgesic treatment for severe acute pain, perioperative pain and chronic pain (Rosenblum *et al.*, 2008). Accompanying the surge in chronic use of opioids, there has also been an increased occurrence of opioid associated endocrinopathy, which mostly manifests as an androgen deficiency (O'Rourke and Wosnitzer, 2016). This has been termed opioid-induced androgen deficiency (OPIAD) and leads to multiple central and peripheral effects, particularly on the sexual function of males. Of all the disadvantageous effects of chronic opioid use, the effects of opioid medications on the reproductive system is probably the least understood and investigated.

2.4 The endocrine effects of opioids

The regulatory effect of the opioid system on the hypothalamic-pituitary-gonadal (HPG) axis is well established (Dwyer and Quinton, 2019).

2.4.1 Hypogonadism

The HPG axis plays a crucial role in the development and maintenance of a healthy reproductive system (Fountas, van Uum and Karavitaki, 2020). Figure 2.4 illustrates the HPG-testosterone negative feedback loop that exists under homeostatic conditions. Under normal circumstance, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) which leads the pituitary gland to respond by releasing luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Elliott, Horton and Fibuch, 2011). LH and FSH are glycoproteins that are secreted by gonadotropic cells in the anterior pituitary (Bennett, 2013) and are responsible for the stimulation of Leydig and Sertoli cells. Leydig cells are largely responsible for the production of testosterone (Kenneth W.K., 2019).

Testosterone is a crucial, male hormone that is responsible for a variety of processes including regulating male sex characteristics, sex differentiation, spermatogenesis and fertility (Nassar and Leslie, 2021). A negative feedback loop is created, as testosterone directly affects the hypothalamus to decrease GnRH secretion. Hence, whenever serum testosterone levels exceed homeostatic levels, this automatic negative feedback loop is activated and GnRH secretion is decreased effectively pausing testosterone production.

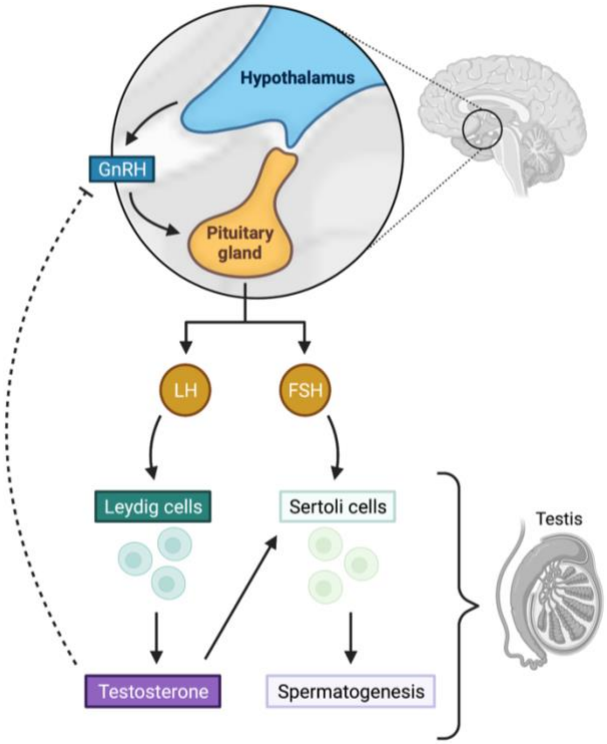

Figure 2.4: *Involvement of the HPG axis in Testosterone production by way of a negative feedback loop.*
**Figure designed by author, using Biorender.*

Opioids (endogenous and exogenous) act on the HPG axis by inhibiting the pulsatile release of GnRH. During chronic opioid use, the inhibition of GnRH is sustained for long periods of time. Inevitably, the decreased GnRH release causes the suppression of the HPG axis and lowered testosterone levels. This leads to various hypogonadal effects such as decreased libido, erectile dysfunction and loss of muscle mass. This effect has been confirmed in multiple studies (Rajagopal *et al.*, 2004; Burney and Garcia, 2012; Rubinstein, Carpenter and Minkoff, 2013), and is especially seen in instances where opioid analgesics are used to treat patients with chronic pain.

2.4.2 Effect of opioids on adrenal hormones:

The chronic use of exogenous opioids has been found to decrease adrenocorticotrophic hormone (ACTH) and cortisol levels (Antony, Alzaharani and El-Ghaiesh, 2020). Opioids have also been

shown to act on the hypothalamic-adrenal axis (HPA) by decreasing the production of dehydroepiandrosterone sulphate (DHEAS), an important precursor of testosterone, by the adrenals (Daniell, 2006; Aloisi *et al.*, 2011). Various studies have shown that the use of MOR agonists, such as morphine or methadone, are associated with an increase of prolactin by inhibiting dopamine release from the hypothalamus (Buss and Leppert, 2014; Merdin *et al.*, 2016). An excess of prolactin is associated with impaired gametogenesis and reduced production of GnRH, which may exacerbate the opioid-induced hypogonadism discussed previously.

2.5 The opioid system and male infertility

Spermatogenesis is a complex and highly coordinated process that is characterized by proliferation of spermatogonia as well as the meiosis and differentiation of developing spermatids into mature spermatozoa (Nishimura and L'Hernault, 2017). The presence of endogenous opioid peptides in the reproductive tracts of various species have been well-established (Shu-Dong *et al.*, 1982; Estomba *et al.*, 2016). Furthermore, the presence of these peptides has also been confirmed in human seminal plasma (Davidson *et al.*, 1989; El-Haggar *et al.*, 2006). The exact role that these peptides play within the reproductive system, remains unclear and necessitates further investigation.

2.5.1 Regulation of testicular function

Opioid precursors (POMC, PENK and PDYN) have been found to be expressed in the testes of rats and mice (Chen *et al.*, 1984; Wittert, Hope and Pyle, 1996). Since then, the presence of all three types of opioid receptors (MOR, KOR and DOR) have been identified in rat Sertoli cells (Fabbri *et al.*, 1985; Jenab and Morris, 2000). Literature indicates that opioids are involved in the local control of testicular function. Findings concur that chronic opioid exposure leads to testicular atrophy, altered testicular histomorphology, decreased serum testosterone levels and decreased spermatogenesis, among other things. Table 2.1 summarizes the effects of different opioids on testicular function, as reported by various studies. A study investigating the long-term effects of Tramadol (low affinity agonist of MOR) administration on epididymal sperm quality and testicular tissue in mice, found that Tramadol damaged the testicular germinal layer and led to the atrophy of seminiferous tubules (Azari *et al.*, 2014). The study also reported that spermatogenesis was impaired, due to the presence of large amounts of abnormal spermatozoa. These findings were corroborated by Nna et al., who reported reduced testicular antioxidant enzyme activity in addition to the aforementioned parameters (Nna *et al.*, 2017). An assessment of the sexual behaviour and fertility indices in male rabbits who were exposed to codeine in a chronic manner, showed that although

sexual libido was significantly increased, the copulatory efficiency, serum testosterone levels and fertility index were significantly lower in codeine-treated groups when compared to controls (A. F. Ajayi and Akhigbe, 2020). In a later study, the same group found that codeine caused testicular atrophy, altered testicular histomorphology and ultimately impaired spermatogenesis (Akhigbe and Ajayi, 2020). A case-control study performed on human patients measured the effect of opium-dependency on testicular volume and concluded that long-term opioid use leads to testicular atrophy (Cyrus *et al.*, 2012)

Table 2.1: *The effects of different opioids on testicular function as reported by various studies*

Opioid	Species	Study description	Findings	Reference
Tramadol	Mice (n=21)	In-Vivo: oral administration of different doses of Tramadol to mice. Sacrificed at 3, 6 and 12 weeks. Epididymal sperm quality and histopathological evaluations were done.	- Damage of testicular germinal layer - Damage to seminiferous tubules - Impaired spermatogenesis - The effects were dose-dependant	(Azari *et al.*, 2014)
Tramadol and other drugs	Rats (n=70)	In-Vivo: Five treatment groups (n=14) oral administration of drug for 8 weeks. 7 rats sacrificed after 8 weeks and remaining 8 left for another 8 weeks without treatment. Histopathological studies performed.	- Damage to seminiferous tubules - Leydig cell count reduced (implicates impaired spermatogenesis) - Reduced testicular antioxidant enzyme activities - Impaired spermatogenesis - Johnsen's mean testicular biopsy score (MJTBS) reduced	(Nna *et al.*, 2017)
Codeine	Rabbits (n=18)	In-Vivo: oral administration of different treatments administered to three treatment groups (n=6) for 6 weeks. They were paired with female rabbits and their sexual behaviours observed. They were sacrificed and various assays were performed.	- Sexual libido was increased - Copulatory efficiency decreased - Decreased serum testosterone levels (dose-dependent manner) - Fertility index decreased	(A. F. Ajayi and Akhigbe, 2020)
Codeine	Rabbits (n=21)	In-Vivo: Three treatment groups (n=7) were administered treatment orally for 6 weeks. Animals were sacrificed and various assays and histological procedures performed.	- Testicular atrophy - Alterations in testicular histomorphology - Decreased serum testosterone levels - Marked rise in oxidative markers - Elevated testicular enzymes - Testicular DNA fragmentation - Impaired spermatogenesis	(Akhigbe and Ajayi, 2020)
Various	Human (n=100)	Participants were opium dependents selected from an opioid dependency treatment clinic. The testes were measured by tensioning the scrotum skin, and using callipers to measure the dimensions of each testis.	- The testis volume in the opium dependent group was significantly lower (testicular atrophy) than that of the healthy controls.	(Cyrus *et al.*, 2012)

Morphine	Rats (n=48)	In-Vivo: Rats divided into 8 groups (n=6). Treated intraperitoneally for 14 days.	- Testicular atrophy - Decreased serum testosterone levels - Impaired spermatogenesis - Damage to seminiferous tubules - Damage of testicular germinal layer	(Eghoghosoa Akhigbe *et al.*, 2021)

As seen by the results of these studies, opioid analgesics reduce the structural and functional integrity of the primary sex organs. However, the mechanisms by which this is accomplished are poorly understood. One explanation of these detrimental effects may be the pronounced reduction of serum testosterone levels, by way of the HPG axis (discussed previously). Considering the presence of opioid receptors in animal testes, it may also point to opioid-induced alteration in metabolic processes that are associated with excessive tissue breakdown. Regardless, more research is crucial in order to obtain a better understanding of these mechanisms.

2.5.2 Semen

The discovery of endogenous opioid peptides in human semen first sparked an inquiry into the role of these peptides in reproduction. It was found that endogenous opioid peptides, such as enkephalins and endorphins were present in concentrations up to 12 times higher than detected in normal blood plasma (Sharp and Pekary, 1981; Fraioli *et al.*, 1984). Low seminal levels of the endogenous opioid metenkephalin has been associated with asthenozoospermic (reduced motility) patients (Fujisawa *et al.*, 1996). On the other hand, high concentrations of enkephalins and β-endorphins have also been found to reduce sperm motility (Rama Sastry *et al.*, 1982). This seems indicative of a closely-regulated, homeostatic system that affects sperm motility. While certain concentrations of opioid peptides are necessary to maintain sperm motility, very high concentrations of these peptides seem to be counter-productive.

Endogenous opioid peptide concentrations are controlled by enzymatic degradation. Some of these enzymes, such as aminopeptidase N (APN) and endopeptidase neutral N (NEP), were found to be present in both sperm and seminal fractions (Subirán *et al.*, 2008). The enzymatic activity of these peptidases was found to be especially high in semen when compared to other parts of the body (Fernández *et al.*, 2002). Interestingly, high levels of APN in human semen has been positively correlated with the percentage of dead/immotile sperm cells (Irazusta *et al.*, 2004), which seems to indicated that high APN activity may be related with necrozoospermia (dead sperm cells). Considering that spermatozoa are transcriptionally and translationally silent cells, this may suggest that sperm cells possess unique, sperm-specific signalling pathways. These enzymes may well act as endopeptidase activating or inactivating peptides or could even act as adhesion proteins, though

much more research is needed to confirm this. More recently, various groups have described the presence of the MOR, DOR and KOR on human spermatozoa for the first time (Agirregoitia *et al.*, 2006b; Albrizio *et al.*, 2006). This discovery strengthened the hypotheses that the opioid system plays a regulative role on spermatozoa fertilization potential. The in-vivo effect of opioids on the reproductive system is understood to work predominantly via the HPG axis, as discussed before. However, the exact mechanisms by which the opioid system affects spermatozoa are poorly understood. Furthermore, data on the in-vitro effect of various opioids on sperm functional parameters remain scarce and largely contradictory. Table 2.2 compares the outcomes of various in-vitro studies performed on the effect of opioids on spermatozoa.

Table 2.2: *A comparison of the in-vitro effects of various opioids on functional sperm parameters*

Opioid	Opioid receptor target	Species	Motility	Progressive motility	Capacitated cells	Acrosome reacted cells	Notes	Reference
DPDPE	DOR	Equine	↓	↓	↓	↑	Biphasic effect was seen	(Albrizio *et al.*, 2010)
Naltrindole (antagonist)			↓	↓	↓	↑		
Naloxone (antagonist)	MOR	Equine	No effect	↑ - low dose ↓ - high dose	↑	No effect	Biphasic and dose-dependent effect.	(Albrizio *et al.*, 2005)
Morphine	MOR	Human	↓	-	-	-	Pre-incubation with naloxone abolished negative effect of Morphine. Naltrindole effect: biphasic and insignificant after 3.5h.	(Agirregoitia *et al.*, 2006a)
Naloxone			No effect	-	-	-		
U50488H	KOR		No effect	-	-	-		
Nor-binaltrophimine			No effect	-	-	-		
DPDPE	DOR		No effect	-	-	-		
Naltrindole			↓	-	-	-		
Codeine	MOR	Human	↓	-	-	-	Effects were dose and time dependent. Also ↓ DNA and plasma membrane integrity	(Eghoghosoa Akhigbe *et al.*, 2021)
Remifentanil	MOR	Human	↓	-	-	-	-	(Xu *et al.*, 2011)
Naloxone			↑	-	-	-	Effect was seen after 35 minutes.	

U50488H	KOR	Human	-	↓	No effect	↓	-	(Urizar-Arenaza *et al.*, 2019)

There are some discrepancies between studies and few studies investigate the effects of opioids on parameters beyond motility. Albrizio and colleagues found that DOR agonist DPDPE decreases motility parameters, but Agirregoita and his team disputed this by reporting no effect on motility parameters. Interestingly, some studies found that opioids antagonists, such as naloxone and Naltrindole (DOR antagonist), affected sperm motility negatively in a dose-dependent way. One attempt to explain this is looking at previous studies that have reported seemingly contradictory effects of the endogenous opioid enkephalin, which has a high affinity for the DOR. High doses of enkephalin decreased sperm motility while lower dosages seemed necessary to maintain motility (Fraioli *et al.*, 1984; Fabbri *et al.*, 1989). This seems to indicate that the delta opioid receptor especially has a role to play in sperm motility. This could explain the dose-dependent effects of naloxone, due to the fact that it has more affinity for MOR than for DOR. Therefore, at low doses naloxone antagonizes mostly the MOR's but at higher doses is also able to displace DOR ligands and in doing so decreases sperm motility. More research is needed to elucidate these effects.

As seen in Table 2.2, studies suggest a biphasic effect of opioids. In equine spermatozoa, both a DOR agonist and antagonist immediately reduced total and progressive motility parameters. The biphasic effect showed up when these returned to non-significant levels after 180 minutes. The progressive motility of the sample incubated with DPDPE also stabilized, while the initial negative effect on total motility remained (Albrizio *et al.*, 2010). This could be indicative of the fact that opioid action on sperm is reversible.

2.6 The mechanisms of action

The signalling events that occur downstream of opioid receptors in spermatozoa remain uncharacterized. Very few studies have been done to elucidate these effects. One such study targeted the KOR and sought to establish the physiological effects as well as the signalling pathways downstream of the kappa opioid receptor. They observed that the KOR agonist, U50488H, provoked an increase in phosphorylated substrates of protein kinase C (PKC), and at the same time had an inhibitory effect on progressive motility and acrosome reaction (Urizar-Arenaza *et al.*, 2019). Upon this discovery, they further explored calcium-signalling pathways as both motility and acrosome reaction processes have been shown to be regulated by calcium (Kopf, 2002; E. R. S. Roldan and

Shi, 2007). They found the presence of both calcium dependent and calcium independent PKC, which coincides with other studies (Rotem *et al.*, 1992a; Jaiswal and Conti, 2003), and hypothesized that PKC might be involved in the regulation of a sperm-specific signalling cascade that is calcium independent. Interestingly, they also found a decrease in the phosphorylation levels of A-kinase anchoring protein 3 (AKAP3), a scaffold protein that is involved in the structure of the fibrous sheath and has been hypothesized to be involved in sperm motility regulation (Brown *et al.*, 2003).

As discussed in section 2.2.2, it is known that Opioid receptors are able to affect calcium channels in neuronal signalling, specifically by the inhibition of GABA release. However, it is necessary to investigate the sperm-specific cell-signalling mechanisms to understand calcium signalling in spermatozoa.

2.6.1 Sperm capacitation

Upon ejaculation, sperm undergo several biochemical changes, which are collectively known as capacitation. It involves a change in the sperm's motility pattern, termed hyperactivation, and the sperm's ability to undergo an acrosome reaction (Puga Molina *et al.*, 2018). Therefore, these changes prepare the sperm for successful binding and fertilization of an oocyte (Sutovsky and Manandhar, 2006). With enhanced motility and acrosome reaction (and therefore successful fertilization) dependent on this event, it is vital for male reproductive function. Capacitation, motility and acrosome reaction processes have been shown to be mediated by calcium pathways (Ickowicz, Finkelstein and Breitbart, 2012). Capacitation takes place after ejaculation and during the passage of sperm through the female reproductive tract. This process can be divided into two signalling events: fast and slow. The fast events ultimately result in the activation of the flagella, whereas the slow events result in changes to the pattern of sperm movement, known as hyperactivation (Visconti, 2009). The biochemical changes associated with sperm capacitation start with an efflux of cholesterol from the plasma membrane. This leads to hyperpolarization of the plasma membrane, an increase in the membrane fluidity and permeability to Ca^{2+} and HCO_3^- ions, changes in protein phosphorylation and protein kinase activity. In addition, an increase is seen in intracellular pH level, bicarbonate (HCO_3^-) concentrations, Ca^{2+} levels and cAMP levels (Arcelay *et al.*, 2004; Hernández-González *et al.*, 2006). The following schematic (figure 2.5) has been proposed to explain sperm capacitation. However, the mechanisms regarding these reactions are still poorly understood and more research is required.

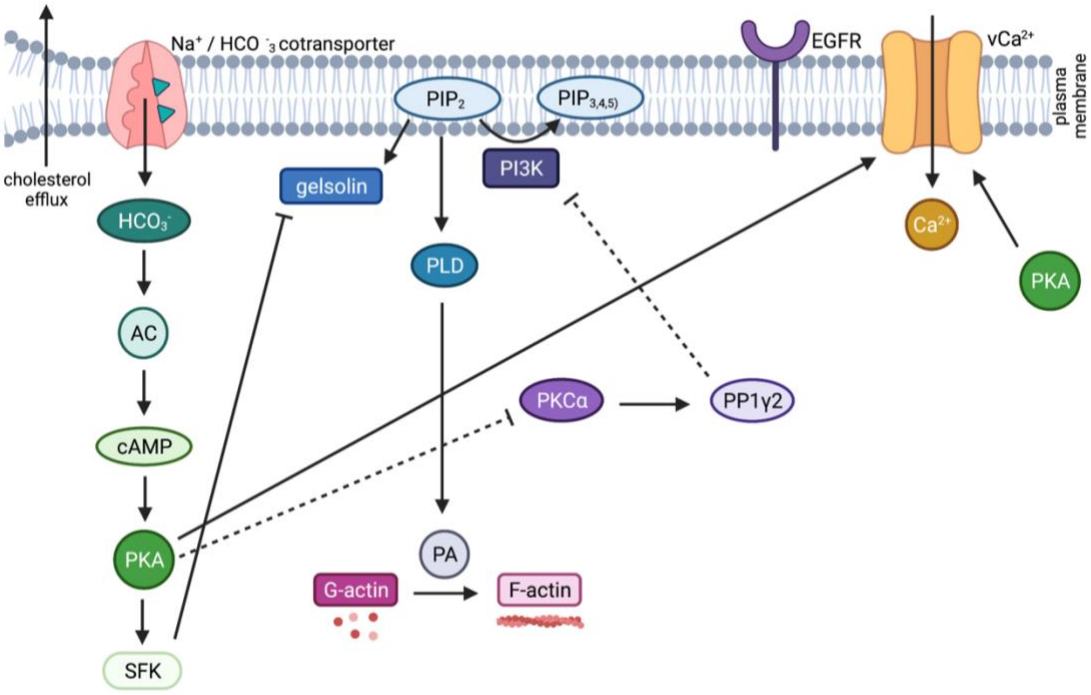

Figure 2.5: *A proposed mechanism of sperm capacitation*

**Figure adapted from* (Ickowicz, Finkelstein and Breitbart, 2012). *Redesigned by author, using Biorender*.

HCO_3^- enters the sperm by way a Na^+/HCO_3^- cotransporter (Demarco *et al.*, 2003). The rise in intracellular HCO_3^- activates enzymes that translocate membrane phospholipids, such as phosphatidylserine, and the result is a rapid collapse of the asymmetry of the sperm plasma membrane and a cholesterol efflux (Salicioni *et al.*, 2007; Visconti, 2009). The rise in HCO_3^- also activates soluble adenylyl cyclase (AC) which lead to cAMP and cAMP-dependent protein kinase A (PKA) activation. PKA activation modulates calcium channel response which produces membrane potential changes and ultimately increases intracellular Ca^{2+} concentrations (Wennemuth *et al.*, 2003). PKA is involved in the phosphorylation of various proteins and activates various protein kinases while inhibiting protein phosphatases – all this to finally increase the phosphorylation of Tyrosine residues (Bajpai and Doncel, 2003). Importantly, tyrosine phosphorylation is required for capacitation. In particular, PKA phosphorylates SRC family tyrosine kinases (SFKs), which phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP_2) bound gelsolin, thereby inactivating it (Jammey *et al.*, 1987). Gelsolin is an actin-severing protein, so its inhibition allows the formation of F-actin to continue. It has been reported that F-actin breakdown is necessary for the acrosome reaction to take place, which explains why gelsolin is later uninhibited (Gremm and Wegner, 2000).

PKC, which has been suggested to be involved in motility and acrosome reaction, is active at this stage of capacitation (Naor and Breitbart, 1997). PKCα phosphorylates protein phosphatase isoform

PP1γ2, which in turn causes phosphoinositide 3-kinases (PI3K) inhibition. However, as capacitation progresses the activation of PKA mediates the degradation of PKCα and PP1γ2 through a proteasome pathway, which relaxes the inhibition of PI3K. At the same time, $PIP_{2(4,5)}$ acts as a cofactor for Phospholipase D (PLD) activation, which stimulates actin polymerization. The regulation of cell motility has been shown to depend on PLD and actin polymerization (Cohen *et al.*, 2004; Itach *et al.*, 2012).

2.6.2 Acrosome reaction

During fertilization, there is interaction of the sperm and the egg cells. The spermatozoon is stimulated by the oocyte to undergo a process of regulated exocytosis, also called the acrosome reaction, which is characterized by the release of specific enzymes that are contained in the acrosomal granule. This process allows the spermatozoon to penetrate and (ultimately) fuse with the oocyte, thus completing fertilization (Florman, Jungnickel and Sutton, 2004; E. Roldan and Shi, 2007). The mechanism by which acrosome reaction occurs remains poorly understood. Figure 2.6 is an attempt on elucidating a possible mechanism for the acrosome reaction, based on literature. Please keep in mind that the events in figure 2.5 is a prerequisite for figure 2.6.

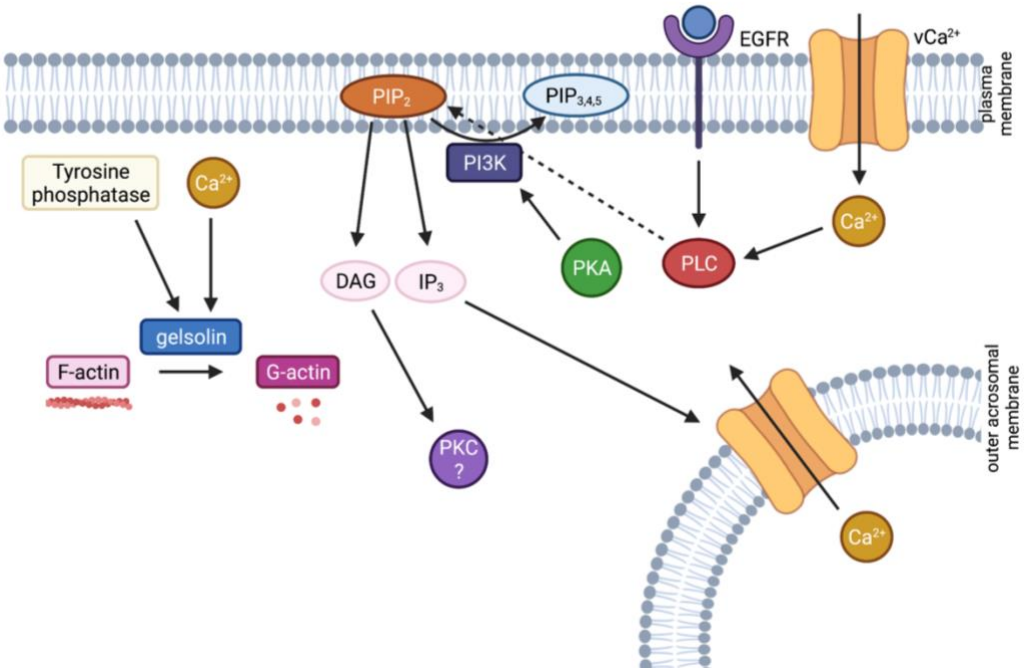

Figure 2.6: *A proposed mechanism for the acrosome reaction*

**Figure adapted from* (Ickowicz, Finkelstein and Breitbart, 2012). *Redesigned by author, using Biorender*.

When a spermatozoon comes into contact with the egg, it binds to the zona pellucida (ZP) and this leads to a variety of events. Among other things, there is an elevation in intracellular Ca^{2+} and an activation of the epidermal growth factor receptor (EGFR). EGFR is localized mostly on the acrosomal region of the spermatozoa, and has been shown to be involved in the acrosome reaction as well as sperm motility (Oliva-Hernández and Pérez-Gutiérrez, 2008; Breitbart and Etkovitz, 2011). This leads to the activation of phospholipase C (PLC) which causes multiple events. PLC hydrolyses PIP_2 and results in the release of gelsolin into the cytosol. In addition, the hydrolysis of PIP_2 result in inositol triphosphate (IP_3) formation, which mobilizes the acrosomal Ca^{2+} pool, and diacylglycerol (DAG) formation which activates PKC isoforms (O'Toole *et al.*, 1996; Spungin and Breitbart, 1996).

During this time, PKA successfully inhibits PKC, meaning that PI3K is now uninhibited. PI3K is primarily responsible for phosphatidylinositol-3,4,5-triphosphate (PIP_3) production, which then phosphorylates PIP_2 at the D-3 position of the inositol ring (Breitbart *et al.*, 2009). Phosphorylated PIP_2 releases gelsolin into the cytosol. Furthermore, PI3K's activity in the acrosome reaction has been suggested due to the presence of its catalytic and regulatory subunits in sperm (Fisher *et al.*, 1998; Etkovitz *et al.*, 2007).

Gelsolin is activated by the elevated levels of Ca^{2+} and undergoes tyrosine dephosphorylation by tyrosine phosphatases (Finkelstein, Etkovitz and Breitbart, 2010) The activated gelsolin is now able to facilitate the breakdown of F-actin, which ultimately results in the acrosome reaction.

2.7 Considering crosstalk between PKA and PKC

As described in the previous section both PKA and PKC have valuable roles to play in the proposed mechanisms for capacitation and acrosome reaction. PKA modulates calcium channel response, and is involved in the process to increase the phosphorylation of Tyrosine residues. PKC is involved in the acrosome reaction by inhibiting the effects of PI3K. PKA has an inhibitory role on the PKCα during these processes, undoing its inhibition of PI3K. Thus, the regulation of PI3K depends upon the crosstalk of PKA and PKC (Breitbart, Rubinstein and Etkovitz, 2006). A more in-depth understanding of these kinases is necessary to understand this crosstalk fully.

2.7.1 PKA

It is well known that PKA is involved in the regulation of sperm motility (Battistone *et al.*, 2013). Activation of the PKA catalytic subunit has been shown to increase flagellar beat frequency during capacitation (Carlson, Hille and Babcock, 2007). Specifically, PKA has been shown to mediate hyperactive motility, a process which occurs during capacitation (Shahar *et al.*, 2011). As discussed,

PKA is involved in various cascades and its phosphorylation of SFK's ultimately leads to actin polymerization – a process that has been deemed necessary for the development of hyperactive motility (Finkelstein *et al.*, 2013). Later, PKA indirectly stimulates the acrosome reaction by its inhibition of PKC. As recalled from the mechanisms explained before, PKC inhibits PI3K. Interestingly, it has been reported that the pharmacological inhibition of PI3K increases intracellular cAMP levels, thereby stimulating human sperm motility, and it leads to the tyrosine phosphorylation of A kinase anchoring protein 3 (AKAP3), which finally leads to the recruitment of PKA (Moss and Gerton, 2001; Brown *et al.*, 2003). This is significant, because it means that by PKC's inhibition of PI3K, cAMP levels rise (needed for PKA activation) and AKAP activation occurs - this ultimately leads to PKA activation. PKA eventually inhibits PKC, and the regulatory effect of PKC and PKA on each other is established.

2.7.2 PKA activation depends on AKAP3 phosphorylation

In general, the primary function of AKAPs is to bind the regulatory subunit of cAMP-dependent PKA and in doing so directs the kinase to discrete intracellular locations (Colledge and Scott, 1999). The AKAP family contains more than 50 structurally diverse members that have at least one PKA anchoring domain. In addition, AKAPs have unique localization signals and have the ability to form complexes with various signalling molecules (Rahamim Ben-Navi *et al.*, 2016). AKAP3 is a scaffold protein that is involved in the basic structure of the sperm fibrous sheath (Eddy, Toshimori and O'Brien, 2003). In addition to the PKA recruitment, it was demonstrated that AKAPs, specifically AKAP4 and AKAP3, are involved in regulating sperm motility (Miki *et al.*, 2002). Later it was shown that the influx of HCO_3^- seen during spermatozoa capacitation rapidly stimulates an increase in cAMP and tyrosine phosphorylation of AKAP3 (Luconi *et al.*, 2005). It has also been reported that diminished AKAP3 levels cause changes in the sperm proteome and mis-location of sperm proteins in mice (Xu *et al.*, 2020).

Urizar-Arenaza and colleagues found that AKAP3 phosphorylation was decreased after incubation with the KOR agonist (Urizar-Arenaza *et al.*, 2019). This is interesting because the study did not find any significant effects in the phosphorylated substrates of PKA. It is unclear how AKAP3 dephosphorylation has no effect on the phosphorylated substrates of PKA.

2.7.3 PKC

PKC exists in 11 isotypes, and these can be simultaneously present in a single cell. Three broad categories of PKC exist based on their requirements for activity and the structure of their regulatory

domains at the NH_2 terminus(Kalive *et al.*, 2010). Briefly, PKCα, βI, βII, and γ are dependent on DAG, phospholipids and calcium with their regulatory domains containing a C1 domain (binds DAG/PMA) as well as a C2 domain (binds anionic phospholipids in a calcium-dependent manner). Next the novel PKC's namely PKCδ, ε, μ, η and θ are DAG and phospholipid-dependent, but are calcium-independent. Lastly, the atypical PKC's are DAG and calcium-independent and include PKCζ and λ (Liu, Xie and Miki, 2006). The presence of most of the PKC isotopes in spermatozoa suggest multiple roles during gametogenesis and fertilization (Kalive *et al.*, 2010). PKCα has been shown to be involved in the inhibition of PI3K during capacitation. As discussed, the inhibition of PI3K ultimately causes gelsolin to remain bound to PIP_2, allowing actin polymerization and ultimately delaying the acrosome reaction. This is a direct result of crosstalk between PKA and PKC. The acrosome reaction is a calcium mediated reaction (Yanagimachi and Usui, 1974; Florman *et al.*, 1989). However, there have been reports that PKC may have the ability to induce the acrosome reaction in the absence of intracellular calcium (Rotem *et al.*, 1992b).

Recently, opioid receptor-mediated cardio-protection has become a subject of increasing interest. It has been shown that DOR activation is able to trigger intracellular survival signalling by stimulating PKC (Fryer *et al.*, 2001). This is significant, as it indicates that PKC can be influenced by opioid receptors and their agonists. When considering the findings by Urizar-Arenaza *et al.*, 2019 it is interesting that they reported an increase in the phosphorylated substrates of PKC, both calcium dependent (PKCα, PKCδ) and calcium independent (PKCι, PKCζ), and at the same time they noted inhibition of acrosome reaction. Since PKC is activated by opioid receptors elsewhere in the body, it might indicate that PKC could be activated in the same way in spermatozoa. Assuming that PKC will also inhibit PI3K in this instance, it could explain the inhibited acrosome reaction seen by Urizar-Arenaza and his colleagues. However, literature on the effects of opioids on spermatozoa functional parameters remain contradictory, with some studies reporting that opioids actually induce the acrosome reaction and so forth. In this instance it may be that high concentrations of PKC leads to the calcium-independent acrosome reaction that has been described by others (Bonaccorsi *et al.*, 1998). Nonetheless, more research is required in order to gain clarity on these mechanisms.

2.8 Study hypothesis, aims and objectives

Opioids are hypothesized to play a regulative role in the maintenance of sperm functional parameters, including that of motility, capacitation and acrosome reaction. It is anticipated that the opioid agonist will negatively affect sperm functional parameters, and that the antagonist will reverse these effects. Due to the significant gaps in the current literature as well as the inconsistencies

between studies, the exact effect of opioids on spermatozoa and the mechanisms by which they work are still unclear. Considering the global opioid abuse pandemic, it is crucial to gain an understanding of the effects of the corresponding system on reproduction. For this study, it was decided to focus primarily on the mu-opioid receptor.

The primary agonist for the MOR is morphine. However, morphine is a class A, schedule 2 drug, which makes it difficult to source. Codeine (also known as methylmorphine) occurs as a natural plant alkaloid in opium extracts and is commonly used to treat mild to moderate pain and cough symptoms. Codeine is converted to Morphine metabolites (morphine-3-glucuronide and morphine-6-glucuronide) inside the body, and since the primary site of action for Morphine is MOR (Williams, 2008b), this is also the case for codeine. Codeine is easier to obtain than morphine and thus, codeine was chosen as the MOR agonist for this study. Naloxone is an opioid receptor antagonist that is widely used to reverse opioid overdoses (Handal, Schauben and Salamone, 1983), and was therefore chosen as the opioid receptor antagonist for this study. The concentrations used (720ng/ml) were selected according to what the literature stipulates to be the plasma and seminal concentrations after codeine use (Findlay *et al.*, 1978; JC and WD, 1990; Zalata *et al.*, 1995). 720ng/ml is indicative of a a high pharmacological dose, compared to 360ng/ml which would be an average pharmacological dose. A previous study conducted in the SURRG laboratory compared different concentrations of codeine to a control. The concentrations used in this study representing chronic opioid use was based on the findings from an earlier study (unpublished data) conducted in the SURRG laboratories. The naloxone concentration was included in order to establish whether an opioid antagonist reversed/exacerbated any actions seen by the agonist. Therefore, the current study aimed to clarify the role of the mu-opioid receptor in human spermatozoa. The study objectives are as follows:

Objective 1:

To investigate the effect of the opioid receptor agonist (codeine) and antagonist (naloxone), both separately and combined, on sperm functional parameters. This will include total motility, progressive motility, sperm kinematics and sperm viability.

Objective 2:

To determine whether the opioid receptor agonist or antagonist had any effect on advanced sperm parameters; namely the rate of the acrosome reaction and levels of DNA fragmentation

Objective 3:

To determine overall expression of proteins of interest (AKAP3 and PKCα) by western blotting.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

The aim of this chapter is twofold: Firstly, to provide a detailed outline of the protocols employed in this study and, secondly, to give an overview of the materials used. Figure 3.1 briefly outlines the experimental procedures followed in this study. A total of 30 semen samples were collected (n=30). After undergoing a neat analysis, each sample was divided into 4 separate aliquots. Each aliquot was treated accordingly and incubated for a total of 180 minutes at 37°C. Sperm motility, kinematics, viability, acrosome reaction and DNA fragmentation were measured at 0 and 180 minutes respectively. Thereafter the remainder of these aliquots were frozen away in the -80°C for further analysis. The total protein expression for AKAP3 and PKC were measured by means of western blotting.

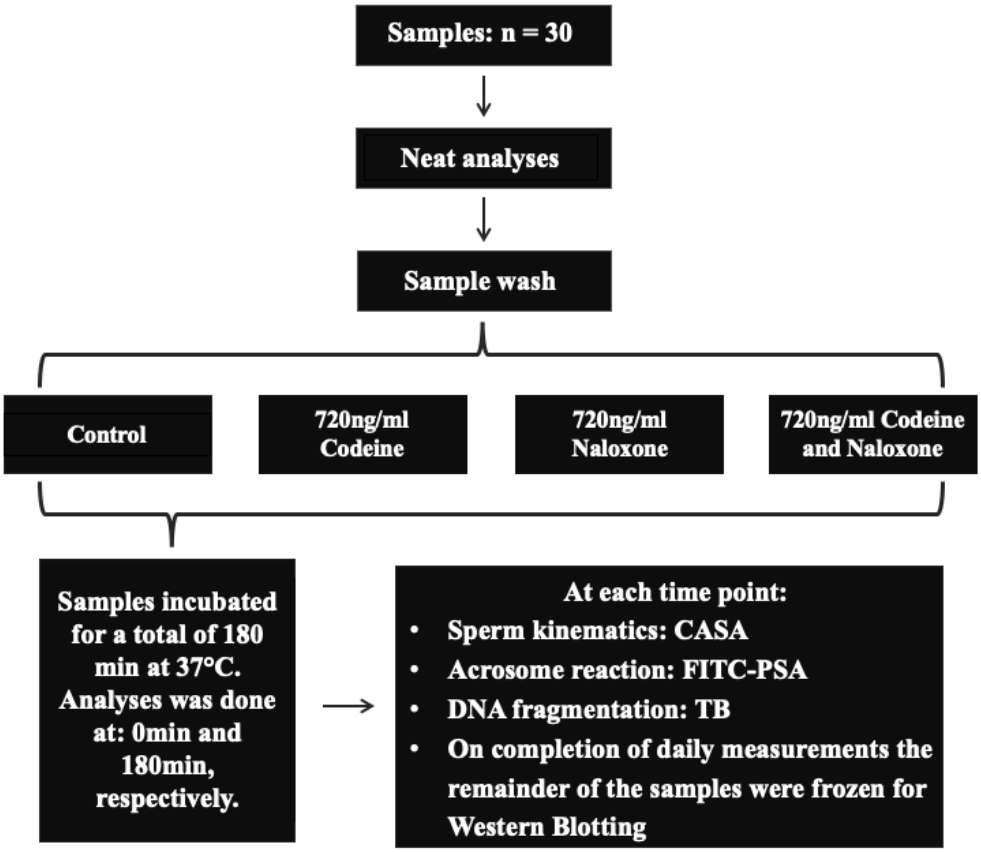

Figure 3.1: *Schematic of experimental procedure*
**Figure designed by author, using Microsoft PowerPoint.*

3.2 Ethical clearance

The study was granted institutional permission (service desk ID: IRPSD-1984) by the Institutional Review Board, while the Stellenbosch University Health Research Ethical Committee (HREC) granted ethical clearance (Ethical Reference number: S20/08/198) (see Appendix A). All study participants were informed that their participation was strictly voluntary, where after they were informed of the full research protocol and the details pertaining to the study. They were furthermore asked to sign an informed consent form (ICF) guaranteeing that donated samples would strictly be used for research in this specific study and will be disposed of accordingly following experimental use. Due to the sensitive nature of the research, participants were guaranteed anonymity and were assigned a unique donor code by which they were referred to throughout the study.

3.3 Participant recruitment

This study made use of volunteer donors of reproductive age (18-25), recruited from the student body of Stellenbosch University, Tygerberg Campus. Awareness about the study and the need for volunteer donors were created by distributing flyers at the Faculty of Medicine and Health Sciences (FMHS). As part of the recruitment process, volunteers were fully informed of the particulars of the study. Thereafter, participants were asked to complete a donor profile questionnaire, in order to establish the suitability of the volunteer. The following exclusion criteria were applied in order to eliminate confounding variables and the use of compromised samples:

- Individuals who smoke were excluded, as it was desired that the semen samples were not exposed to nicotine and/or cotinine prior to experiments.
- Individuals who practiced frequent and moderate to heavy alcohol consumption were excluded. For this specific study, participants were allowed to consume no more than 5 alcoholic beverages per week, and no more than 2 alcoholic beverages per day. Participants who consumed more than 5 alcoholic beverages per week and more than 2 alcoholic beverages per day were therefore excluded from the study. This was applied in order to preserve the integrity of the experiment.
- Individuals who were on chronic medication of any kind, were excluded.
- Individuals who participated in any recreational drug use were excluded.
- Chronic opioid users were excluded to ensure study integrity.

No socio-demographic and educational backgrounds were considered when accepting a participant. Volunteers were asked to provide informed consent and were assigned a unique donor code for anonymization purposes, which they were referred to throughout the study.

3.4 Data collection

In total, 30 semen samples were collected from volunteer donors between the ages of 18 and 25, at the FMHS. The minimum abstinence period was set at 24 hours, and the maximum abstinence period set to 7 days. Please note that this abstinence period implies abstaining from ejaculation; therefore, from sex, masturbation and any other activity that may cause ejaculation. Donations were scheduled via email and by making use of an app called Doodle. Donors were given access to a special donation room in close proximity to the Stellenbosch University Reproductive Research Group (SURRG) laboratory, which they could use for their own convenience. Semen samples are collected by means of masturbation into a sterile, wide-mouthed container – which is supplied by the SURRG lab. Participants were then required to place their samples into an incubator (37°C with 5% CO_2 and 95% humidity) located in the donation room. Prior to any analysis, samples were left in the incubator for a minimum of 30 minutes to allow liquefaction to occur.

3.4.1 Data collection in light of covid19:

In light of the Coronavirus pandemic, Stellenbosch University introduced its own online screening questionnaire that all students needed to fill in before being allowed on campus. This ensured that only 'low risk' individuals were allowed on campus. Furthermore, though the SURRG lab already had various protective measures in place - these measures were tightened to implement social distancing and to make the environment as safe as possible for both researcher and participant. The donation room was deep-cleaned, before and after a semen sample was donated. Special care was taken to sanitize all frequently used surfaces, such as the wash basin, door handles and incubator door, with extra care. Masks were deemed mandatory. Participants were strictly contacted via email and the Doodle app as mentioned before, so as to minimize face-to-face interaction. The donation room is equipped with a wash basin, hand soap as well as hand sanitizer and all participants were encouraged to practice good hygiene. When fetching the sample, the researcher (wearing personal protective equipment, such as a mask, a lab coat and gloves,) would clean the donation room, before removing the sample from the incubator and taking it to the SURRG lab. As the sample container was sealed, the researcher could also sanitize the outside of the container. In the unlikely event contact occurred between a researcher and a donor, they were asked to keep a minimal distance of 1.5 meters between them.

3.5 Standard semen analysis

After the liquefaction period, a standard semen analysis (described hereafter) was performed on each sample to assess semen quality and basic sperm parameters. This initial analysis, done on the unwashed sample, is also known as the neat analysis. In order to pass the neat analysis, the sample was required to adhere to the following criteria as set out by the WHO: A total volume of no less than 1.5 milliliters (ml), a total concentration more than or equal to 15×10^6 spermatozoa per ml, a total motility of 40% or higher and a progressive motility of 32% (WHO, 2010). The results of the neat analysis acted as a baseline of information about each sample. This analysis consists of both microscopic and macroscopic measurements. The macroscopic analyses primarily provide information on the ejaculate and include measuring the samples' volume, pH, viscosity, odor and color. In contrast, the microscopic analyses provide more information about the spermatozoa themselves. A combination of Phase-contrast microscopy and the Computer-Aided Sperm Analysis (CASA) program were used to determine the concentration, motility as well as various other kinematic parameters in the sample.

3.5.1 Volume

In a routine semen analysis, an accurate measurement of the sample volume is paramount to quantifying the total number of spermatozoa and non-sperm cells suspended in the ejaculate. The WHO assumes a lower reference limit of 1.5ml (5th centile, 95% confidence interval (CI) 1.4–1.7) for normal semen samples (WHO, 2010). Therefore, samples with a volume below this reference limit were excluded. Liquefied semen samples were transferred directly from the collection container to a pre-weighed, 15 ml falcon tube and thereafter weighed on the balance scale (Kern, Sigma-Aldrich, South Africa). The falcon tube volume was subtracted from the total volume in order to obtain the total semen volume. As per WHO guidelines, the volume was recorded in milliliters.

3.5.2 pH

The pH at ejaculation depends on the relative contribution of the alkaline seminal vesicular secretions and the acidic prostatic secretions. Therefore, measurement of the sample pH reflects the balance of the pH values between the different accessory glands. The WHO considers low pH values to be of clinical interest, as it could indicate a lack of alkaline seminal vesicular fluid. They have set a pH of 7.2 as the lower threshold value for normal semen samples (WHO, 2010). The pH was measured using pH test strips (pH indicator paper, Merck Millipore). Firstly, the sample container was gently swirled in order to make sure that the sample was well-mixed. A $2\mu l$ drop of semen was

spread evenly onto the pH strip. After approximately 30 seconds, the color was compared to the color wheel in order to obtain a pH reading. Samples with a pH below 7.2 were rejected.

3.5.3 Viscosity

A viscous ejaculate is different from an unliquified specimen, as its 'sticky' consistency does not change over time. A high sample viscosity can hinder the proper assessment of sperm motility and concentration. The sample viscosity was assessed by measuring the amount of time it took a $2\mu l$ drop of semen sample to fill up a 20-micron depth chamber slide (Leja 8 chamber slides, 2153 GN Nieuw Vennep, The Netherlands). The sample was considered too viscous if it took more than 10 seconds to fill up the chamber. To combat viscosity, samples were diluted with DMEM in a 1:1 ratio. A Pasteur pipette was used to gently mix the sample. If the sample remained too viscous for use, it was discarded.

3.5.4 Color

According to the WHO, a normal liquefied ejaculate has a "cream/grey-opalescent" color (WHO, 2010). It is important to note the color of the sample as it may indicate various abnormalities. For example, a less opaque color could indicate a very low sperm concentration or a sample may appear slightly yellow after long periods of abstinence. A red-brown appearance of ejaculate indicates the presence of red blood cells and is known as haemospermia. A deeper yellow ejaculate has also been associated with jaundice, drug use or taking certain vitamins (WHO, 2010). The semen color was determined visually by the researcher, and accordingly classified. Abnormally colored samples were not used in the study.

3.5.5 CASA-related sperm analyses

The concentration and motility of the samples were assessed by employment of the Computer-Aided-Sperm-Analysis (CASA) system, which consists of a computer, a microscope and a camera. The computer is equipped with an automated software system that tracks and analyses spermatozoa by taking high resolution images of them. This software is known as the Sperm Class Analyser (SCA) software system, version 6.3.0.59 (Microptic, S.L., Barcelona, Spain). The microscope used in the CASA-system is a Nikon Eclipse 50i Microscope (IMP, Cape Town, South Africa) and is equipped with a temperature-regulated stage and a Basler A312fc digital color camera (Microptic, S. L., Barcelona, Spain). The microscope was set to phase-contrast (Ph1), the 10x objective was used, the green filter was retained, the temperature-regulated stage was set to 37℃ and the brightness was

kept at 100 for all the CASA-related analyses. It is furthermore important to note that the CASA-system is able to measure concentration, motility and all the kinematics in conjunction with each other. It does this by capturing 50 images per second and generating all the data into a report. In order to obtain the most accurate results, a minimum of 5 randomly selected fields were captured per slide and at least 1000 spermatozoa were examined per slide.

3.5.5.1 Concentration

Sperm concentration is a routinely measured parameter, as it has proven essential in determining fertility potential. This measurement gives an indication of the number of spermatozoa per volume unit (mL) of the semen sample. The WHO has set a lower threshold value of 15 million spermatozoa cells per milliliter (M/ml) (WHO, 2010). Concentrations below 15M/ml are indicative of a condition known as Oligospermia (low sperm count). The CASA-system was used to analyze sample concentration. Following the liquefaction period, $2\mu l$ of each unwashed sample was pipetted into a specialized, $20\mu m$ Leja chamber slide. (These slides were pre-warmed to 37℃ on the heating stage in an attempt to minimize temperature fluctuations.) The SCA software setting was secured to "Human50," which optimizes specifically for human sperm. The sample was then observed under the microscope. At least 1000 spermatozoa were examined in each sample by capturing randomly selected fields. The concentration of spermatozoa was expressed as Million ($x10^6$) per ml.

Please note: As mentioned before, the CASA-system measures all the kinematics in conjunction using the same Leja chamber slide. Therefore, the above-mentioned method applies to all of the following kinematics:

3.5.5.2 Total motility

This measurement indicates the percentage of motile cells in a sperm sample, and is arguably among the most significant measurements. This stems from the fact that spermatozoa require motility to travel along the female reproductive tract so as to find and fertilize the oocyte (Donnelly *et al.*, 1998; Findeklee *et al.*, 2020). The WHO has stated that a total motility of 40% and above is to be accepted as normal (WHO, 2010). The motility of spermatozoa is expressed as a percentage.

3.5.5.3 Progressive motility

Spermatozoa are described to be progressively motile when they move actively in a straight line or in a large circle and covering a distance of at least $25\mu m$ in one second (WHO, 2010). The progressive motility is expressed as a percentage.

3.5.5.6 Curvilinear velocity (VCL)

This is a measurement of the time-averaged velocity and is done by considering the sperm head as it moves along the path traced out by the sperm. The VCL was expressed in micrometers per second (μm/s). The figure below randomly depicts a sperm trajectory, and seeks to explain how VCL and the kinematics in 3.5.5.7 through 3.5.5.14 are measured.

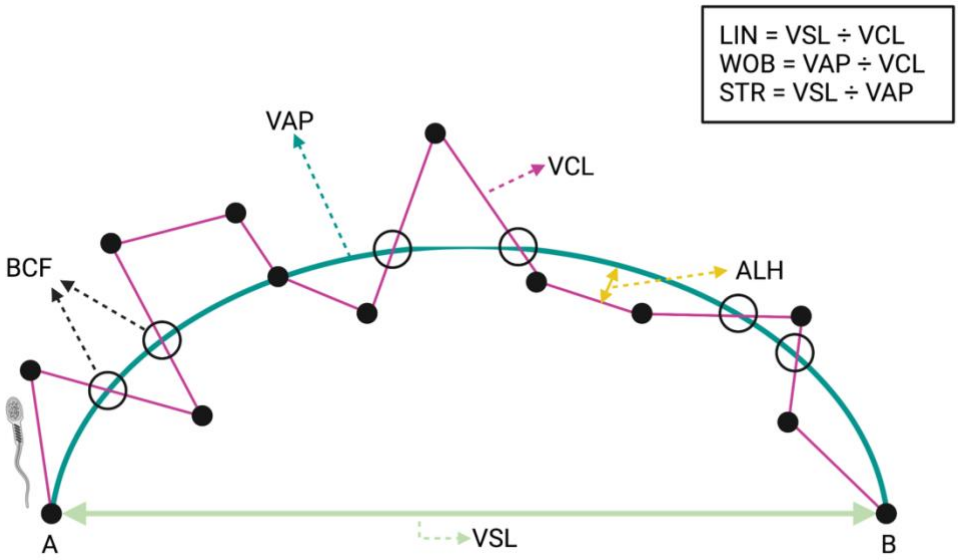

Figure 3.2: *A random sperm trajectory, indicating the various kinematic measurements.*
**Figure designed by author, using Biorender.*

3.5.5.7 Straight-line velocity (VSL)

This measurement calculates the velocity in a straight line between the first and the last points of the spermatozoa's path. The VSL was expressed as a micrometers per second (μm/s).

3.5.5.8 Average path velocity (VAP)

This measures the sperm head along its spatial average trajectory / is a calculation of the time-averaged velocity calculated along the average path of the spermatozoa. The VAP was expressed as micrometers per second (μm/s).

3.5.5.9 Amplitude of lateral head displacement (ALH)

Measures the magnitude of the lateral displacement of the sperm head about the average path. The ALH was expressed as distance covered over time (μm).

3.5.5.11 Linearity (LIN)

The CASA-system expresses the linearity of the curvilinear path by doing the following calculation: VSL ÷ VCL

3.5.5.12 Wobble (WOB)

By doing the following calculation, a measurement of oscillation of the curvilinear path about the average path is obtained: VAP ÷ VCL

3.5.5.13 Straightness (STR)

The linearity of the average path of spermatozoa is calculated by VSL ÷ VAP

3.5.5.14 Beat-cross frequency (BCF)

This can be explained as the average frequency at which the curvilinear path of a sperm cell crosses the average path.

3.6 Sample preparation

A sample was cleared for use once the neat analysis was completed and when it was affirmed that the sample adhered to the guidelines (described throughout 3.5) set out by the WHO. The sample then underwent preparation to get it ready for treatment.

Reagent: DMEM bought from Sigma-Aldrich.

Method: After passing the neat analysis, a single wash was conducted. The single wash is routinely done in order to remove seminal plasma and cellular debris. A volume of DMEM, in a 1:1 ratio to the semen sample, was added to the sample where after it was centrifuged at 1500 rpm for 15 minutes and at room temperature (22°C) in order to obtain a pellet. After discarding the supernatant with a Pasteur pipette, the pellet was resuspended and dissolved in 2-3 ml of DMEM. The concentration, motility and kinematics of the resuspended sample was measured once again as described throughout 3.5. At this point the sample was ready to be divided into 4 treatment groups. In order to keep track of treatment groups, the Eppendorf tubes were labelled as shown in figure 3.3. Each treatment group had a total volume of 1ml, which comprised of 20μl treatment and 980μl of

sample. In order to maintain as much consistency between samples as possible, all treatment groups were diluted to a concentration of 50M/ml spermatozoa. This was done by doing a calculation (shown below) for each individual sample, after obtaining the concentrations from the single wash.

$$C_1V_1 = C_2V_2$$

$$(Concentration\ after\ single\ wash)(V_1) = (50M/ml)(1ml)$$

(V_1) = the amount of washed sample needed to obtain a concentration of 50M/ml in a treatment tube with a volume of 1ml. The sample was diluted with the suitable amount of DMEM where necessary [1ml $-$ 20μl (treatment) - V_1= Amount of DMEM needed]. On the occasion that a semen sample with a neat concentration lower than 50M/ml was received, the sample was excluded from the study in order to maintain consistency. The sample was divided up between 4 Eppendorf tubes, each one annotating a different treatment group. The treatment was not yet added.

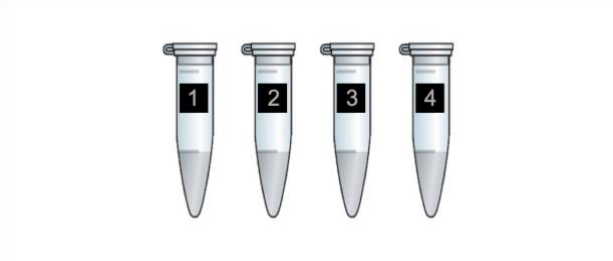

Figure 3.3: *Treatment groups. 1: Control; 2: Codeine 720ng/ml; 3: naloxone 720ng/ml; 4: Combination of codeine 720ng/ml and naloxone 720ng/ml.*
**Figure designed by author, using Biorender.*

3.7 Treatment preparation

The rational for the concentrations used in this study was briefly explained in section 2.8. As stated there, both existing literature and a previous study done in the SURRG lab were consulted before concluding on these concentrations. The in vitro effect of a high concentration of codeine (720ng/ml), an equally high concentration of naloxone (720ng/ml) and a combination of both these treatments (codeine 720ng/ml + naloxone 720ng/ml) were incubated and compared against a control group. The control group consisted of 980μl sample and 20μl phosphate buffered saline (PBS). PBS is a balanced, non-toxic salt solution often used for sperm washing in andrology laboratories (N.C. Martin *et al.*, 2006).

Reagents: Codeine-d_3 was acquired through the Cayman Chemical Company, USA. Naloxone hydrochloride dihydrate (hereafter referred to as naloxone) and PBS were purchased in powder form from Sigma-Aldrich. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich.

3.7.1 Codeine treatment preparation

A stock solution (SS1) was made by carefully mixing 100μl of the pure codeine-d_3 with 900μl of PBS in an Eppendorf tube. The concentration of the stock solution was worked out to be 100000ng/ml. From here a working solution (WS1) with a final volume of 1ml and a concentration of 36000ng/ml was made up as shown by the following calculation:

$$C_1V_1 = C_2V_2$$

$$(100000ng/ml)(V_1) = (36000ng/ml)(1ml)$$

$$V_1 = 360\mu l$$

$\therefore 360\mu l$ of SS1 was added to 640μl of PBS to obtain a working solution.

The following equation was done to determine how much of the working solution was needed to obtain a treatment concentration of 720ng/ml in 1ml of sample:

$$C_1V_1 = C_2V_2$$

$$(36000ng/ml)(V_1) = (720ng/ml)(1ml)$$

$$V_1 = 20\mu l$$

$\therefore 20\mu l$ of WS1 is needed for a final concentration of 720ng/ml in a 1ml solution.

In order to minimize free-thaw cycles, the working solution was divided up into 20 Eppendorf tubes containing 50μl of WS1. Each day one Eppendorf could be taken out, thawed and used for experimentation.

3.7.2 Naloxone treatment preparation

Naloxone (MW = 399.87 g/mol and m = 300mg) was bought in a powder form. In order to obtain a stock solution (SS1), 3mg naloxone was carefully weighed out and added to 1ml DMSO (concentration now 3mg/ml). A second stock solution (SS2) was created to get a lower concentration (1mg/ml):

$$C_1V_1 = C_2V_2$$

$$(3mg/ml)(V_1) = (1mg/ml)(1ml)$$

$$V_1 = 333\mu l$$

$\therefore 333\mu l$ of SS1 was added to $667\mu l$ PBS for a SS2 concentration of 1mg/ml in a 1ml solution.

In order to get a Working Solution concentration of 36000ng/ml the following calculation and dilution was done:

$$C_1V_1 = C_2V_2$$

$$(1000000ng/ml)(V_1) = (36000ng/ml)(1ml)$$

$$V_1 = 36\mu l$$

$\therefore 36\mu l$ of SS2 was added to $964\mu l$ PBS for a WS1 concentration of 36000ng/ml in a 1ml solution.

The following equation showed that $20\mu l$ of the naloxone WS1 would be needed for a final concentration of 720ng/ml in a total volume of 1ml:

$$C_1V_1 = C_2V_2$$

$$(36000ng/ml)(V_1) = (720ng/ml)(1ml)$$

$$V_1 = 20\mu l$$

The working solution was therefore divided into 20 Eppendorf tubes ($50\mu l$ in each) in order to minimize freeze-thaw cycles as much as possible. One Eppendorf was taken out, thawed and used for experimentation as needed.

3.8 Experimental procedure

Once the washed sample had been divided into the treatment groups, the treatment could be added. As described, $20\mu l$ of the appropriate treatment was added to the corresponding Eppendorf. The addition of the treatments were staggered in 5 minute increments, allowing the researcher to finish all the analyses of one treatment group before moving on to the next. Each treatment group was analyzed at 0 minutes and 180 minutes (the 0-minute measurement was taken to serve as an individual control value for each individual group). At each time point, sperm motility, concentration, kinematics, viability, acrosome reaction and DNA fragmentation were measured (refer back to figure 3.1). After the last analyses were done, each treatment group was centrifuged for 15 minutes at 1500rpm to obtain a pellet. The supernatant was discarded and the pellets were frozen away in the -80℃ freezer for biochemical analysis using western blotting (see section 3.12).

3.9 Assessment of cell viability

Assessing cell viability is worthwhile, as it is indicative of the number of live spermatozoa in a sample. By employing a dye-exclusion staining technique, the membrane integrity of the cells are checked. This method is based on the principle that when cells die, their plasma membrane loses their structural integrity, permitting the entry of membrane-impermeant stains (WHO, 2010). In contrast, viable cells remain unstained. This test can also serve as a check-up on the motility evaluation as there should be a correlation between the percentage of alive cells, and the motile cells (as measured by the CASA-system). The WHO has set a lower reference limit of 58% (5^{th} centile, 95% CI 55-63) for viable (membrane-intact) sperm. This study made use of an eosin-nigrosin staining technique. This technique uses nigrosine as a background stain to increase the contrast between the background and the sperm heads, making them easier to discern. In contrast, eosin penetrates the damaged membranes of the sperm cells, staining them pink. Viable cells are left unstained (white).

Reagents: Eosin (Sigma-Aldrich, St Louis, MO), Nigrosin (Sigma-Aldrich, St Louis, MO), DPX mounting medium (Sigma-Aldrich, Germany).

Method: A mixture was made by carefully pipetting $10\mu l$ eosin and $10\mu l$ nigrosin into an Eppendorf tube. At the appropriate time point, $10\mu l$ of the suitable sperm sample was added to the solution and mixed gently with the pipette tip. $10\mu l$ of the stained sample was then pipetted onto labelled, frosted slides and a smear was created (this was done in duplicate). The slides were then allowed to air-dry for a minimum of 8 hours. Once sufficiently dried, a drop of DPX mounting medium was placed onto the slide and a cover slip was mounted onto the slide. The slide was again left for a minimum of 8 hours before further analyses. Once the mounting medium had dried, the slide was ready to be viewed and analyzed on a Nikon Eclipse E200 light microscope. The microscope was set to 40x magnification with a blue filter, while using condenser setting A. Both viable (white) and non-viable (pink-stained) cells were counted on both of the slides (the control slide and the duplicate) in order to obtain an accurate average. This was done manually by using a desk counter and 100 cells were counted on each slide. The result was expressed as a percentage of viability.

3.10 Assessment of acrosome intactness

The acrosome reaction plays an essential role in the fertilization process by giving spermatozoa the ability of penetrating the zona pellucida in order to fuse with the oocyte (N. Crozet, 1994)It is therefore essential that the acrosome remains intact until it comes into contact with the proteins located on the zona pellucida.

Reagents: Fluorescein-conjugated Pisum sativum agglutinin (FITC-PSA), Phosphate-buffered saline (PBS) purchased from Abcam. Dako Fluorescence Mounting Medium purchased from Sigma-Aldrich.

Method: At the appropriate time point, 10μl of the suitable sample was pipetted onto a labelled, frosted slide and a smear was made. This was done in duplicate. Slides were left to air-dry for a minimum of 8 hours. Once sufficiently dried the slides were fixed in a 95% ethanol solution for 30 minutes, at 4℃. The slides were left to air-dry. The slides were taken into a dark room and the remainder of the experiment was carried out there, as FITC-PSA is light sensitive. After adding 450μl of PBS to 50μl of FITC-PSA, the solution was thoroughly vortexed to ensure adequate mixing. 10μl of this mixture was pipetted onto each slide and a smear was made. The slides were left in the dark room for 45 minutes, after which they were washed in distilled water in order to remove any excess staining. The slides were left to air dry. Hereafter, a drop of Dako Fluorescence Mounting Medium was positioned on the slide and a cover slip was mounted. Once the mounting medium had dried the slides could be viewed with a fluorescent microscope. The Nikon Eclipse 50i microscope, fitted with a green filter, and a 40x objective was set to the dark field (DF) with the FITC filter applied, in order to view the slides. The acrosomal region fluoresced bright green when the acrosome was intact. A dull acrosomal region was indicative of a reacted acrosome. A minimum of 100 cells were counted on each slide and spermatozoa were classified as either intact (bright-green) or reacted (dull). The results were expressed as a percentage.

3.11 Assessment of DNA fragmentation

DNA fragmentation in spermatozoa is classified as any chemical change that occurs in the normal structure of the sperm DNA (WHO, 2021). The toluidine blue staining technique determines the chromatin quality of spermatozoa by metachromatic staining of the sperm heads. This technique stains sperm heads with good chromatin quality light blue, while staining sperm heads with compromised chromatin integrity a deep violet color.

Reagents: 0,05% Toluidine Blue (TB) dissolved in 50% McIlvaine's citrate phosphate buffer, (pH 3.5, Merck) was purchased from Sigma-Aldrich. The acetone and ethanol for the 96% acetone-ethanol (ratio 1:1 and 4% dH_2O) solution was purchased separately from Merck and prepared in-house. 0,1 N HCl was prepared in-house and the components purchased from Merck. The DPX mounting medium and the immersion oil for microscopy (nd=1.515) was obtained from Merck.

Method: At each time point, 10μl of the appropriate sample was pipetted onto a labelled, frosted slide and a smear was made. The smear was allowed to dry for a minimum of 8 hours. Once the

smears were dry, the slides were fixed in freshly prepared 96% (1:1) acetone-ethanol, at 4°C for 60 minutes. The slides were once again allowed to air dry. Thereafter, slides were hydrolysed in 0,1 N HCl at 4°C for a total of 5 minutes. The slides were then rinsed in distilled water 3 times, for a duration of 1 minute each. The slides were stained at room temperature with a 0,05% TB solution for 4 minutes. The slides were then briefly rinsed in distilled water to remove the excess stain. The slides were allowed to dry overnight. The following day the slides were mounted using a drop of DPX mounting medium and a coverslip. The Leica CME light microscope was used to assess DNA fragmentation. Under the 100x objective and by using immersion oil, a total of 100 cells were counted in different areas of each slide. Fragmented cells were identified as deep violet/purple and unfragmented cells were identified as light blue. The values were recorded and expressed as percentages.

3.12 Western blotting

Western blotting is widely used in molecular biology as an immunodetection technique that distinguishes specific proteins in a sample of tissue homogenate. In short, this technique includes the separation of proteins according to size and utilizes a light emitting/non-radioactive method for the detection of immobilized specific antigens with antibodies conjugated with HorseRadish Peroxidase (HRP). Therefore, western blotting is able to provide information about the size of the protein as well as information of its expression.

3.12.1 Preparation of protein lysate

In order to measure the proteins of interest, the sperm cells had to be lysed so as to free the intracellular proteins. This was done with the help of a lysis buffer which contains chemicals that dismember the cell membranes. The buffer also contains protease and phosphatase inhibitors that serve to preserve cellular proteins from the degradation or dephosphorylation caused by these enzymes. In total, 30 human sperm samples were collected and treated as described in section 3.4 – 3.8. To ensure that a sufficient amount of protein could be obtained, samples were pooled into 3 groups. Therefore, a total of ten human sperm samples per treatment group were generated, so that there existed 3 new groups for each treatment. Table 3.1 illustrates this. Thus the biological repeats were n = 3 (includes pooled samples). The technical repeat for each protein was n=3, as we repeated each analysis 3 times on three separate blots.

Table 3.1: *Pooling of samples*

Control (C)	**Control 1** (sample C1-C10 pooled)	**Control 2** (sample C11-C20 pooled)	**Control 3** (sample C21-C30 pooled)
Codeine (Co)	**Codeine 1** (sample Co1-Co10 pooled)	**Codeine 2** (sample Co11-Co20 pooled)	**Codeine 3** (sample Co21-Co30 pooled)
Naloxone (N)	**Naloxone 1** (sample N1-N10 pooled)	**Naloxone 2** (sample N11-N20 pooled)	**Naloxone 3** (sample N21-N30 pooled)
Codeine + Naloxone (CN)	**Codeine + Naloxone 1** (sample CN1-CN10 pooled)	**Codeine + Naloxone 2** (sample CN11-CN20 pooled)	**Codeine + Naloxone 3** (sample CN21-CN30 pooled)

Reagents: Summarized in Table 3.2 are the reagents that were used to assemble the lysis buffer and the chemicals were obtained from Sigma-Aldrich or Merck. The bovine serum albumin (BSA) was purchased from Sigma-Aldrich. The Bradford solution was prepared in-house (0.12 mM Coomassie Brilliant Blue, 4.75% ethanol, 8.5% phosphoric acid) and the components purchased from Merck or Sigma-Aldrich.

Table 3.2: *Lysis buffer recipe*

Final concentration of Reagent	Stock concentration	Volume needed to make up 10 ml	Function
20 mM Tris-HCl (pH 7.5) **1 mM EGTA**	200mM 10mM	1 ml	Buffering salt Metalloprotease inhibitor
1 mM EDTA	100mM	100 μl	Metalloprotease inhibitor
150 mM NaCl	1M	1,5 ml	Buffering salt
1 $mM\ \beta - glycerophosphate$		0,002 g	Serine/Threonine phosphatase inhibitor
2,5 mM Tetrasodium-Pyrophosphate		0,01 g	Serine/Threonine phosphatase inhibitor
1mM Na_3VO_4	10mM	1 ml	Tyrosine phosphatase inhibitor
0.1% SDS	1%	1 ml	Detergent
50 μg/ml PMSF	100mM	30 μl	Serine/Cysteine protease inhibitor
0.5% Protease inhibitor (PI) cocktail		50 μl	Serine, Aminopeptidase and Cysteine protease inhibitors
1 % Triton X-100	10%	1 ml	Non-ionic membrane detergent

Top up to 10ml using dH_2O

*EGTA: ethylene glycol tetra-acetic acid; EDTA: ethylene diamine tetra-acetic acid; NaCl: Sodium Chloride; Na_3VO_4: Sodium Orthovanadate; SDS: Sodium Dodecyl Sulphate; PMSF: phenyl methyl sulfonyl fluoride.

Method: On the day that the lysates were prepared, sample pellets (see section 3.8) were taken out of the freezer and incubated at 37℃ for 30 minutes in order to thaw. While the samples were thawing, the Lysis buffer was prepared on ice, with the components indicated in Table 3.1. As described, the samples were pooled into three groups (hereafter referred to as samples) once fully thawed. Each sample was transferred to a labelled, 1.5ml Eppendorf tube containing 700μl of the ice-cold Lysis buffer as well as 7 stainless steel beads (1.6mm) (Next Advance, Inc, USA). Samples were homogenized by using a BulletBlender, set to level 8, at 4℃ in three consecutive cycles of 1 minute each and with a 5-minute rest period in between the homogenization steps. Samples were then centrifuged (BKC-TH16R high speed refrigerated centrifuge, Axiology labs, South Africa) for 20 minutes at 15000 rpm and at 4℃. The supernatant, containing the cytoplasmic proteins, of each sample was collected and transferred to new Eppendorf tubes. The supernatant will be referred to as stock samples henceforth.

The Bradford protein assay (Bradford, 1976) was used to determine the total concentration of protein for each lysate stock sample and was compared to a bovine serum albumin (BSA) standard concentration by using a spectrophotometer. In short, a 10x dilution was prepared for each stock sample using distilled water, the final volume being 100μl. ~~This was followed by performing a second 10x dilution of the first dilution to a final volume of 100μl.~~ Twice-filtered and diluted (5x with dH_2O) Bradford reagent (900μl) was added to duplicate BSA standards as well as the duplicate samples. By using a spectrophotometer (SPECTRONIC 20 GENESYS^{TM}) at a wavelength of 595nm, the absorption measures of the standards and the stock samples were taken. Since the spectrophotometer measures the absorption maximum of the protein-bound dye, the results are directly proportional to the amount of protein within each sample. After obtaining the averages of the duplicate values, BSA was used for the setup of the standard curve in order to calculate the unknown protein concentrations in the stock samples.

Finally, the protein lysates were assembled using the stock sample, 3 x Laemmli sample buffer (62.5mM Tris-HCl (pH 6.8), 4% SDS, 10% glycerol, 0.03% bromophenol blue, 5% β-mercaptoethanol)(Laemmli UK, 1970) and lysis buffer (used to top up to final volume). In pre-labelled Eppendorf tubes, the calculated volume of each stock sample was added to the calculated amount of Laemmli sample buffer and lysis buffer in a final volume of 150μl. In order to minimize

freeze-thaw cycles, and in order to get the most out of the lysates, the final lysate was divided into clearly labelled Eppendorf tubes with a final concentration of $5\mu g$ per $12\mu l$ each.

3.12.2 Protein separation and transfer

Reagents: Table 3.3 and 3.4 summarize the reagents used to prepare the hand-casted sodium dodecyl sulfate-polyacrylamid (SDS-PAGE) running gels. Components were purchased from Sigma-Aldrich or Merck. The running buffer (25mM Tris, 192mM glycine, 0.1% SDS) as well as the transfer buffer (25mM Tris, 192mM Glycine, 20% [v/v] Methanol) were made up in-house, and the chemicals purchased from Sigma-Aldrich or Merck.

Table 3.3: *Stain free 10% SDS-PAGE gel components*

Reagents	Stock	10%	Final concentration
dH_2O		4,85 ml	
2,2,2 Trichloroethanol (Stain Free)	99%	50 μl	0.5%
Tris-HCl (pH8.8)	1,5 M	2,5 ml	0.375 M
SDS	10%	100 μl	1%
Acrylamide/Bis-acrylamide	40%	2,5 ml	10%
APS	10%	50 μl	0.05%
TEMED	99%	20 μl	0.2%

*APS: Ammonium persulfate.

Table 3.4: *Stacking gel components*

Reagents	Stock	4%	Final concentration
dH_2O		3,05 ml	
Tris-HCl (pH 6.8)	0,5 M	1,25 ml	0.126 M
SDS	10%	50 μl	1%
Acrylamide/Bis-acrylamide	40%	500 μl	4%
APS	10%	25 μl	0.05%
TEMED	99%	10 μl	0.2%

Method: A 10% polyacrylamide (stain free), 15-well running gel was prepared as described in Table 3.3. After pouring the gel into the cast, a few drops of Butanol were added to the top in order to prevent oxidation of the gel and to allow it to set in a straight line. After a setting time of 30 minutes, the butanol was carefully washed off with distilled water. The 4% stacking gel (Table 3.4) was

poured into the cast, and 15-well combs were inserted to form the wells. After 15 minutes the combs were removed and the gels were deemed ready for use. In order to ensure proper unfolding of proteins, all protein lysates were boiled for 5 minutes prior to gel loading. The electrophoresis tank was assembled, and the tank filled with running buffer. Protein lysates ($5\mu g/12\mu l$) were loaded into the gel using a Hamilton pipette. The molecular weight marker (PageRuler^{TM} Prestained Protein Ladder: 10-180 kDa range) was loaded in lane 1. Figure 3.3 shows the layout of the gel.

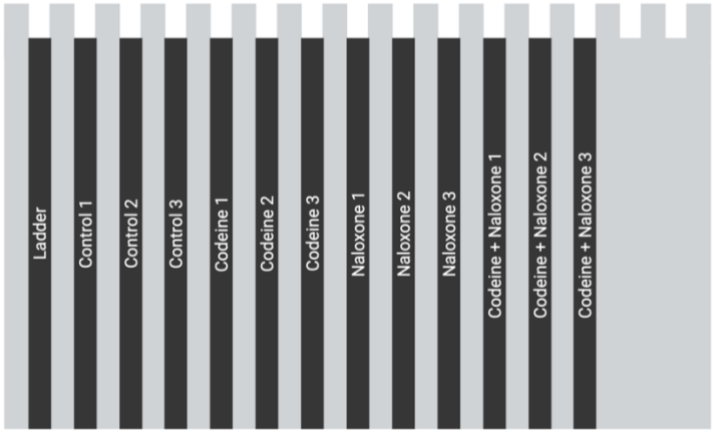

Figure 3.4: *Gel layout*
**Figure designed by author, using Biorender.*

The gels were subjected to an initial 100 V and 200 mA for 10 minutes. This was followed by a setting of 200 V and 200 mA for 50 minutes. During this process, proteins were separated according to molecular weight. After the running process, gels were activated and visualized using the ChemiDoc^{TM} MP Imaging System (Bio-Rad Laboratories, Inc.) to confirm that equal loading and separation of proteins took place. The next step involved transfer of the proteins to membranes (Millipore Immobilon-P Transfer Membrane, Merck) that had been briefly soaked in methanol and ice-cold transfer buffer. The membranes were sandwiched with the gels, and placed into a transfer tank along with ice-cold transfer buffer. The Trans-Blot Turbo^{TM} Transfer System (Bio-Rad) was set to 200V and 200mA for 60 minutes and was kept on ice throughout the transfer. Once completed, membranes were removed and visualized on the ChemiDoc system in order to make sure that all the proteins had transferred successfully. Images were saved to indicate total protein loading and were used for normalization. The membranes were then dipped in Methanol for 30 seconds in order to fix the proteins to the membrane.

3.12.3 Immunodetection of proteins

Reagents: The chemicals (1.37 M NaCl, 200mM Tris; pH7.6) used to make the 10x Tris-buffered saline (TBS) were purchased from Merck. Tween20 was purchased from Sigma-Aldrich. The

Clarity™ enhanced chemiluminescence (ECL) reagent was obtained from Bio-Rad Technologies. The Primary antibodies (Anti-AKAP3 antibody [ab170856]; Anti-PKC alpha antibody [ab32376]) as well as the secondary antibody (Goat Anti-Rabbit IgG H&L [HRP] ab97051) were purchased from Abcam.

Method: In order to prevent non-specific background binding, membranes were submerged in a blocking buffer consisting of 5% fat-free milk in 1x TBS-Tween (10% TBS and 1% Tween) and placed on a shaker for a duration of 2 hours at room temperature. Hereafter, membranes were washed three consecutive times with TBS-Tween for a duration of 5 minutes each. In order to minimize the amount of antibody needed, the membranes were narrowed (by cutting) above and below where the protein of interest would be located. Membrane pieces were then individually incubated with primary antibody overnight, on a shaker at 4℃. There were two proteins of interest, namely PKC alpha and AKAP3, and they were probed on different membranes. As seen in Table 3.5, both Anti-Akap3 and Anti-PKC were prepared in a 1:1000 dilution with 5% BSA-TBS-Tween. After primary incubation, the membranes were washed (three times for 5 minutes each) with TBS-Tween and thereafter incubated on the shaker with the secondary antibody at room temperature, for a duration of 1 hour. The secondary antibody was diluted with 5% BSA-TBS-Tween and made up in a 1:2000 ratio. Following secondary incubation, the membranes were washed in order to remove any unbound antibody. The membranes were then immersed into Clarity™ ECL reagent for 5 minutes before the ChemiDoc was used to expose the membrane.

Table 3.5: *Optimized dilutions for primary (rabbit) antibody*

Protein	Molecular weight (kilodalton)	Dilution	Incubation time	Supplier	Product code
AKAP3	95	1:1000 in BSA-TBS-Tween	18 hours	Abcam	ab170856
PKC*α*	75	1:1000 in BSA-TBS-Tween	18 hours	Abcam	ab32376

3.12.4 Protein normalization

Using Image Lab (version 6.0.1, Bio-Rad Technology Inc.), an automated total protein normalization was done, by using the stain-free membrane blot. This generated a normalized, raw pixel intensity per chemiluminescent protein band, which was then inserted into a Microsoft Excel spreadsheet. The

pixel intensities that corresponded to each treatment sample was calculated as a fold-change of the mean pixel intensity of the control samples.

3.13 Statistical analyses

For all the sperm functional parameters, GraphPad prism (version 9.2) was used to perform statistical analyses. A two-way analysis of variance (ANOVA) was done when two variables were compared to multiple groups, and was followed by Bonferroni's multiple-comparison test. A one-way ANOVA was done when a single variable was compared between multiple groups, and a Dunns' multiple comparison test was performed. The Shapiro-Wilk normality test was used to determine the normality of the data. Results were considered statistically significant if the probability value (p-value) was less than or equal to 0.05, and were expressed as the mean $\pm$ standard deviation (SD). Spearman's correlations were done on all sperm functional parameters, where the p-value was less than or equal to 0.05.

For western blot analysis, proteins were normalized using Image Lab (version 6.0.1, Bio-Rad Technology Inc.). Image Lab generates pixel densities automatically, and these were used to calculate a fold-change relative to the control conditions using Microsoft Excel. Hereafter, a one-way ANOVA with Shapiro-Wilk normality and Bonferroni post-hoc test were done in GraphPad prism.

CHAPTER 4

RESULTS

4.1 Introduction

The findings of this study will be presented in this chapter. The CASA-related analysis will be presented first, followed by Acrosome reaction, DNA fragmentation and western blotting results. The western blot results are represented as images, whereas the rest of the data will be represented graphically.

4.2 Treatment effects on motility and sperm kinematics

This section will describe how the functional sperm parameters were affected by the treatment of codeine, naloxone and the combination (codeine + naloxone) treatment, respectively. A total of 30 human semen samples were collected, treated and then analysed. Total motility, progressive motility, VCL, VAP, VSL, STR, LIN, WOB, ALH, and BCF were measured.

4.2.1 Total motility

A Two-way ANOVA was performed on the data, representing the effects of the different treatment groups on the total motility of spermatozoa and expressed as a percentage. As depicted in figure 4.1, no significant differences were detected was seen although a slight downward trend can be observed between time points.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significance was detected.

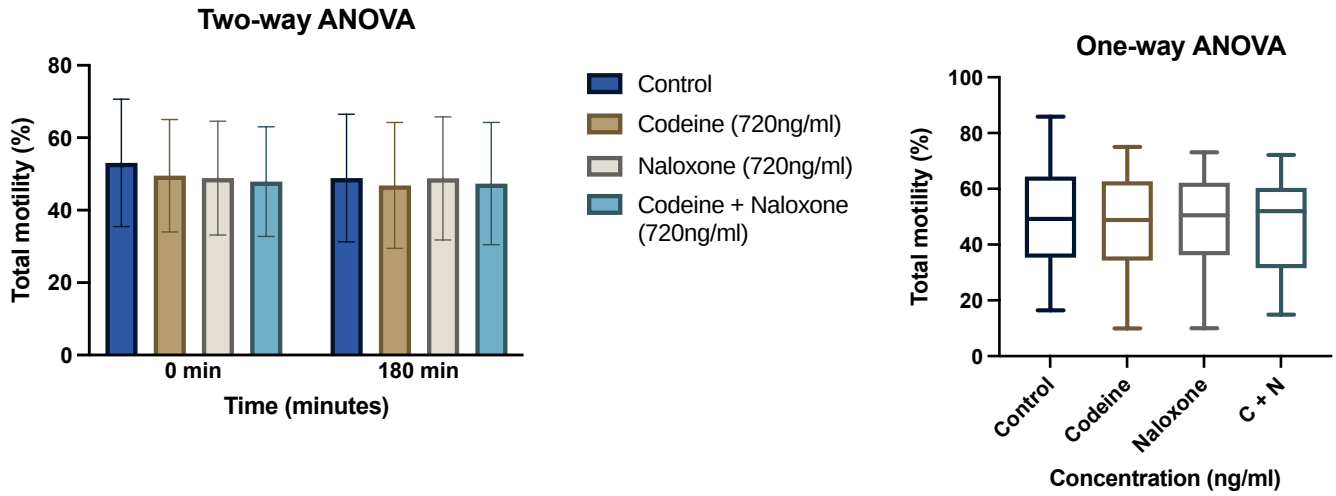

Figure 4.1: *The effect of codeine (opioid receptor agonist) and naloxone(opioid receptor antagonist), both separately and in combination, on total motility of spermatozoa*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.2 Progressive motility

Figure 4.2 shows a two-way ANOVA. It represents the effects of the respective treatments on the progressive motility of the sperm and is expressed as a percentage over time. No significant differences were seen, though a slight downward trend is observed.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

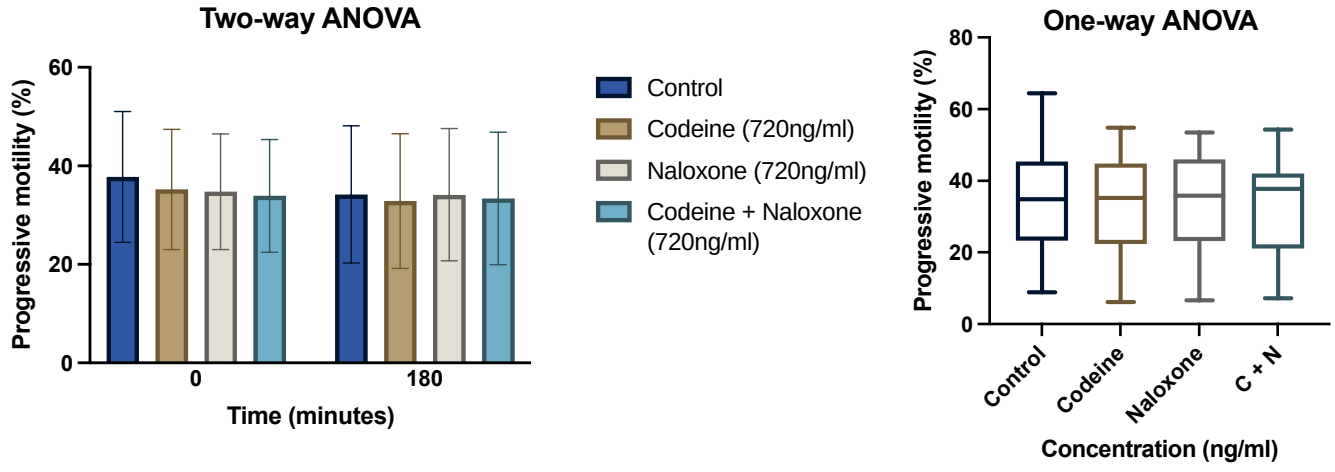

Figure 4.2: *The effect of codeine and naloxone, both separately and in combination, on progressive motility of spermatozoa*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.3 VCL

Figure 4.3 shows the result of a two-way ANOVA done on the effects of the treatments on the velocity along the curvilinear path (VCL) of spermatozoa, over time. The effect of each treatment on VCL is expressed as distance per time (μm/s). No significant differences were seen.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

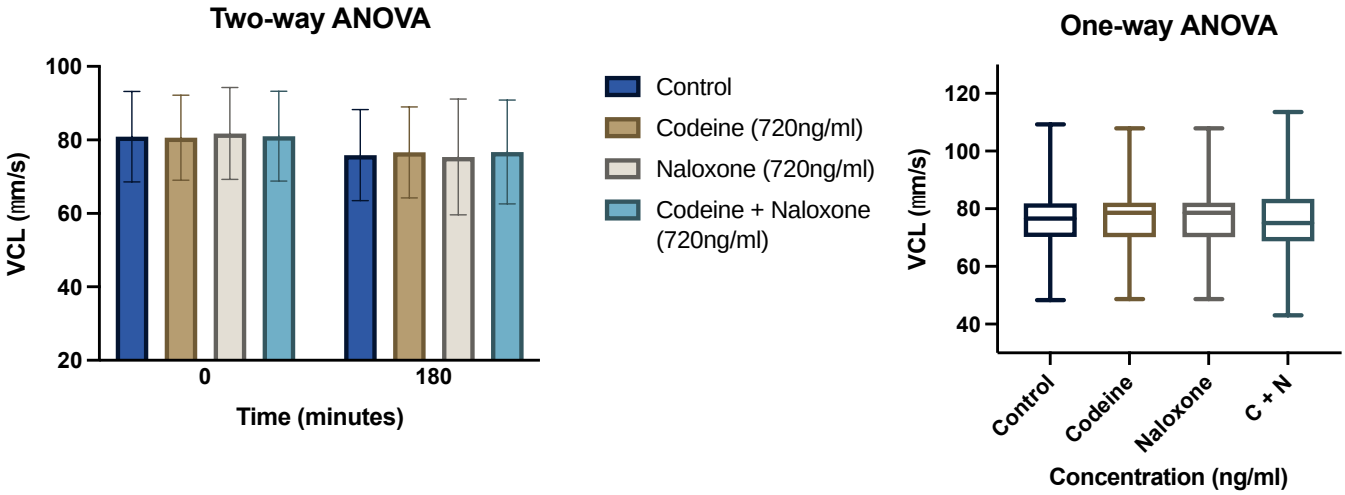

Figure 4.3: *The effect of codeine and naloxone, both separately and in combination, on VCL*
The two-way ANOVA is expressed as µm/s over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.4 VSL

Figure 4.4 shows a two-way ANOVA. It represents the effects of the respective treatments on the velocity along the straight-line path of the sperm over time, and is expressed as distance per time (μm/s). No significant differences were seen, though it is interesting to note the immediate dip caused by the codeine treatment group at 0 minutes.

The graph on the left in Figure 4.4 is a one-way ANOVA that depicts the effect of the treatment groups on motility after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

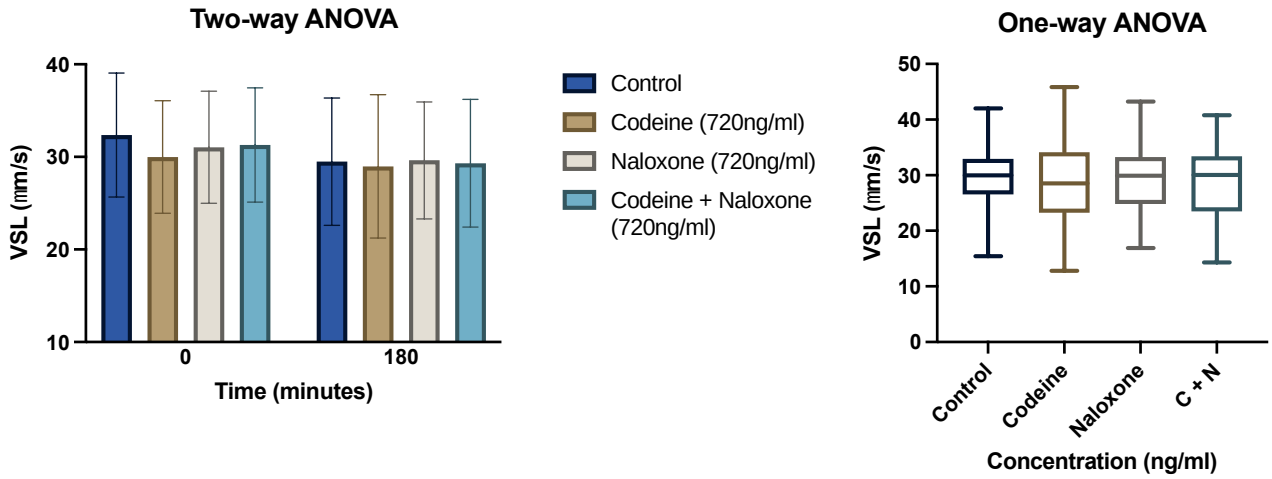

Figure 4.4: *The effect of codeine and naloxone, both separately and in combination, on VSL*
The two-way ANOVA is expressed as µm/s over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.5 VAP

A two-way ANOVA done on the effects of the treatments on the velocity along the average path of spermatozoa over time, is pictured in figure 4.5. The effects of the respective treatments on the VAP is expressed as speed per second (μm/s). No significant differences were seen.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

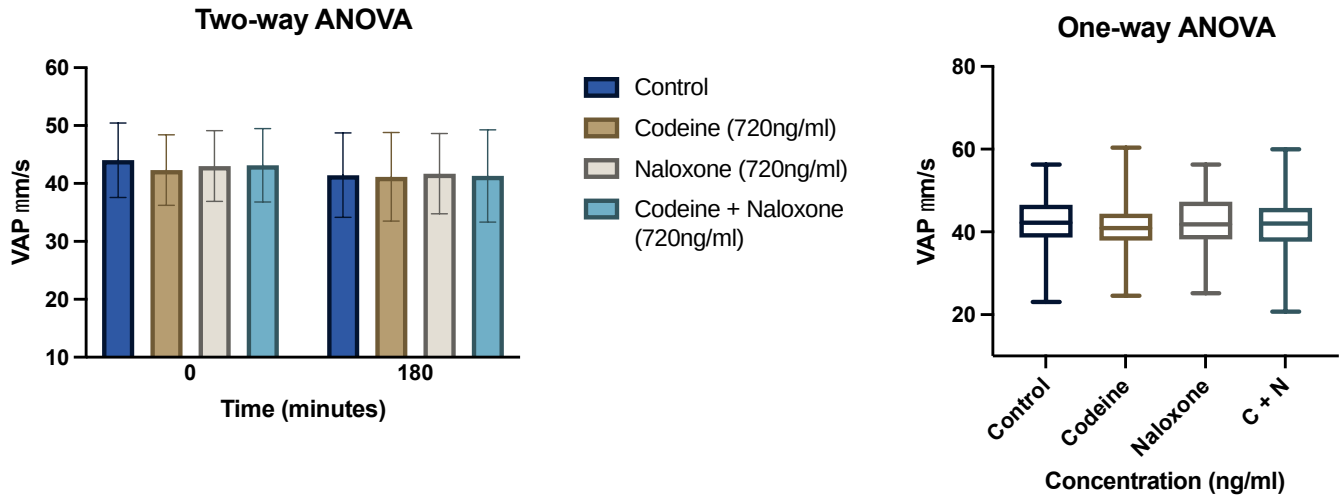

Figure 4.5: *The effect of codeine and naloxone, both separately and in combination, on VAP*
The two-way ANOVA is expressed as µm/s over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.6 STR

Figure 4.6 shows a two-way ANOVA. It represents the effects of the respective treatments on the straightness of the sperm over time, and is expressed as a percentage over time. No significant differences were seen.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

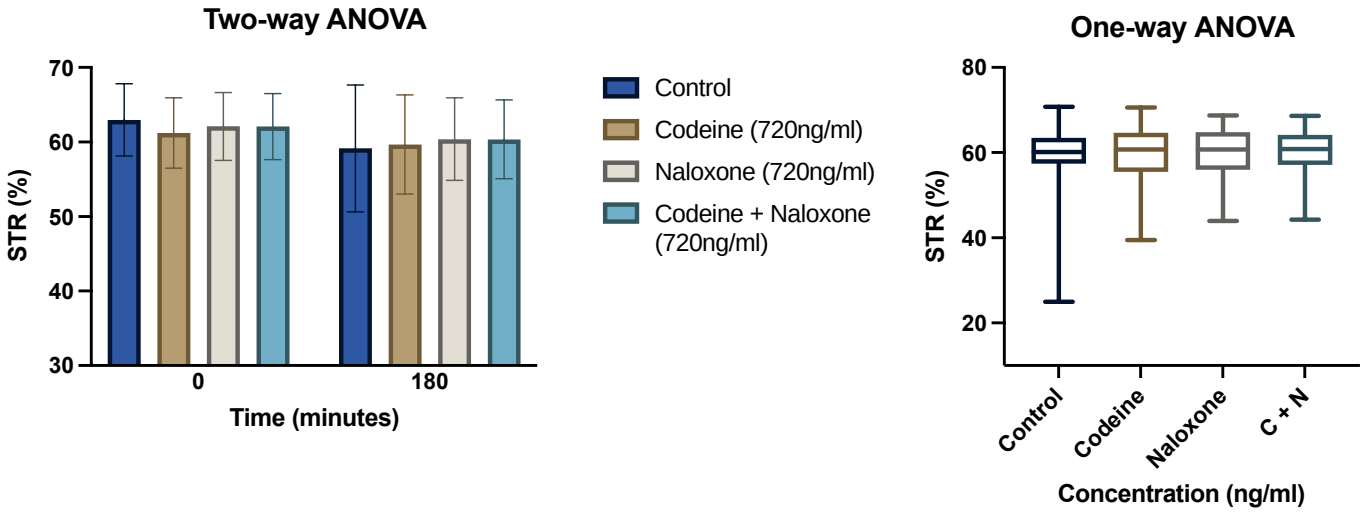

Figure 4.6: *The effect of codeine and naloxone, both separately and in combination, on STR*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.7 ALH

A two-way ANOVA performed on the effects of the treatments on the amplitude of the lateral displacement of the head of spermatozoa over time, is pictured in figure 4.7. The effects of the respective treatments on the ALH is expressed as distance over time (μm). No significant differences were observed.

The one-way ANOVA depicts the effect of the treatment groups on ALH, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

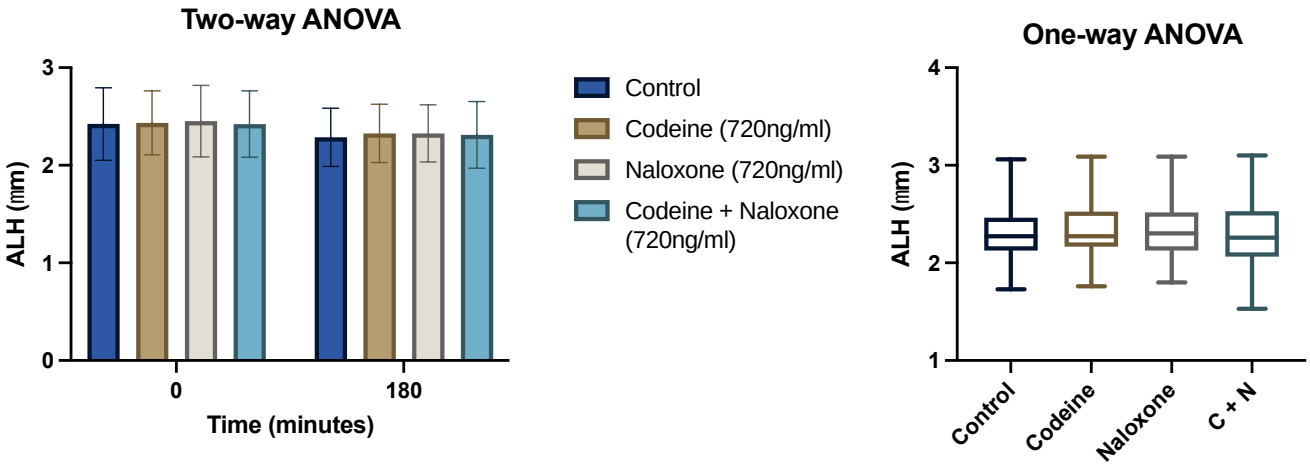

Figure 4.7: *The effect of codeine and naloxone, both separately and in combination, on ALH*
The two-way ANOVA is expressed as µm over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.8 LIN

A Two-way ANOVA was performed on the data; it represents the effects of the different treatment groups on the linearity of the spermatozoa and was expressed as a percentage over time. As depicted in Figure 4.8, no significant change was seen though it is interesting to note the immediate dip caused by the codeine treatment group at 0 minutes.

The one-way ANOVA depicts the effect of the treatment groups on LIN, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

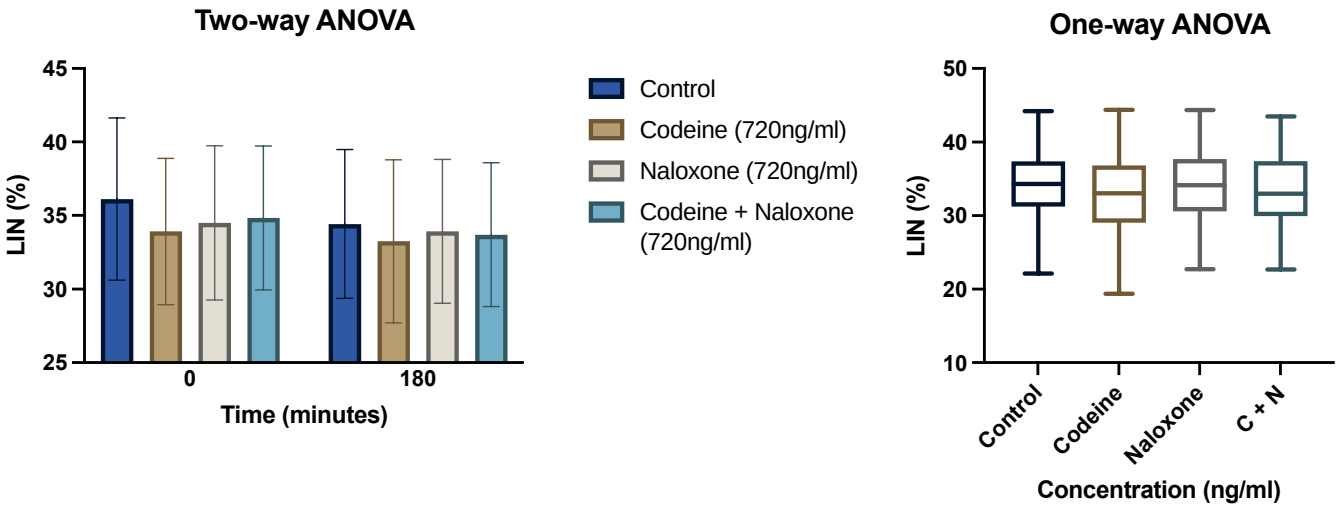

Figure 4.8: *The effect of codeine and naloxone, both separately and in combination, on LIN*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.9 WOB

Figure 4.9 depicts the result of a two-way ANOVA done on the effects of the different treatments on the wobble (oscillatory index) of spermatozoa, over time. The effect of each treatment on WOB is expressed as a percentage over time. No significant differences were seen.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

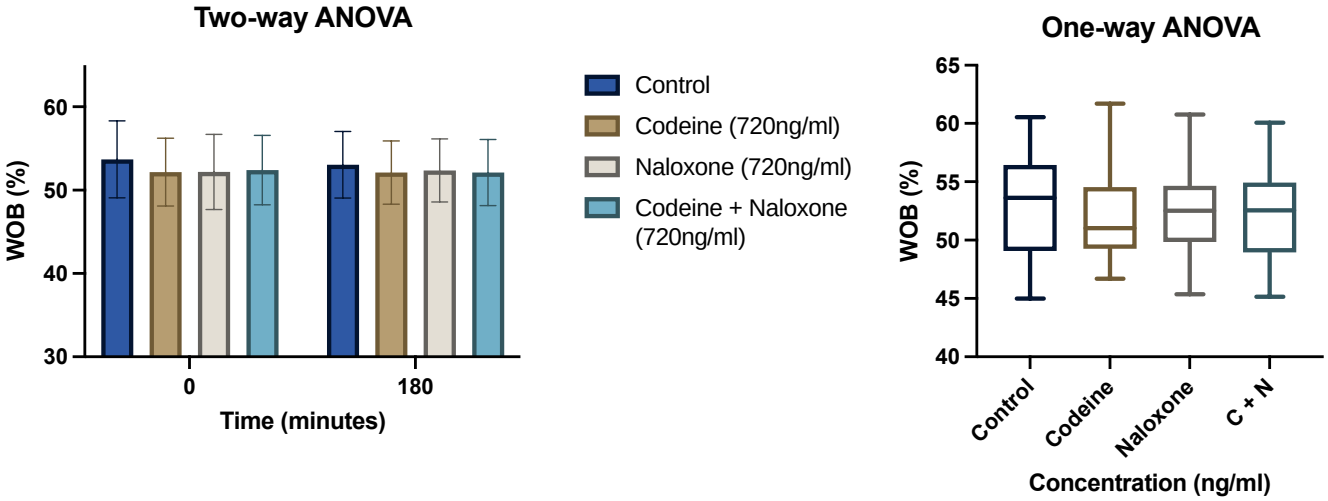

Figure 4.9: *The effect of codeine and naloxone, both separately and in combination, on WOB*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.2.10 BCF

A two-way ANOVA, done on the effects of the treatments on the beat cross frequency of the head of the spermatozoa over time, is pictured in figure 4.10. The effects of the respective treatments on the BCF is expressed as frequency over time (Hz). No significant differences were seen.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

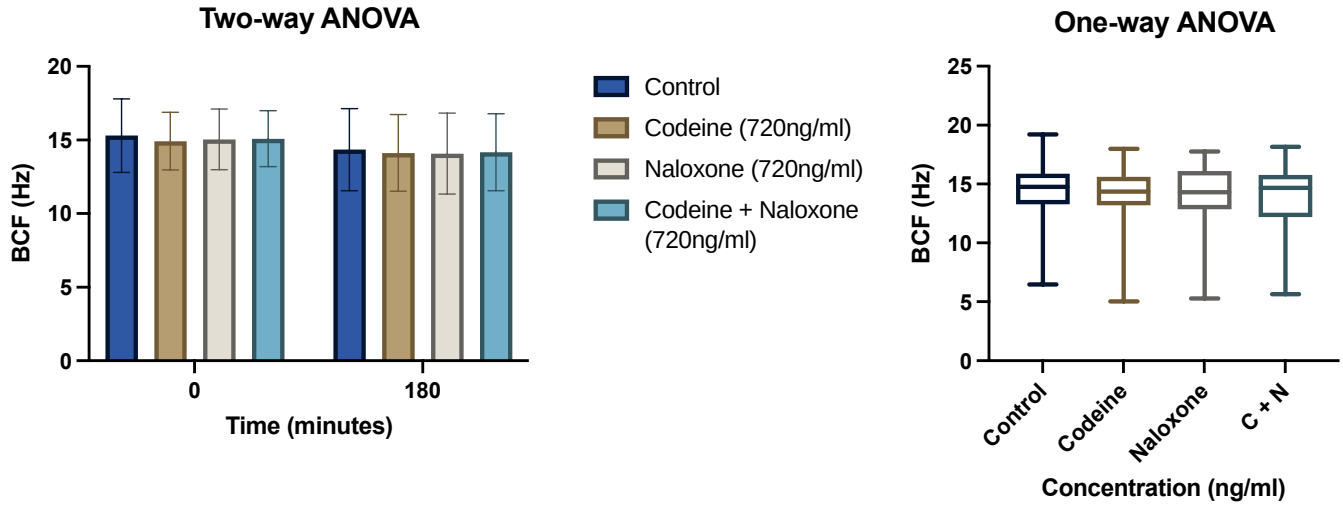

Figure 4.10: *The effect of codeine and naloxone, both separately and in combination, on BCF*
The two-way ANOVA is expressed as Hz over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 30; (C+N) is representative of the combination treatment (codeine + naloxone)

4.3 Treatment effects on cell viability

A two-way ANOVA was done on the effect of the different treatment groups on the viability of the spermatozoa, and was expressed as a percentage over time. The sample size for cell viability was 27, as the slides of the other 3 donors were damaged. As seen in figure 4.11 a slight downward trend can be observed, though these results were not significant.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

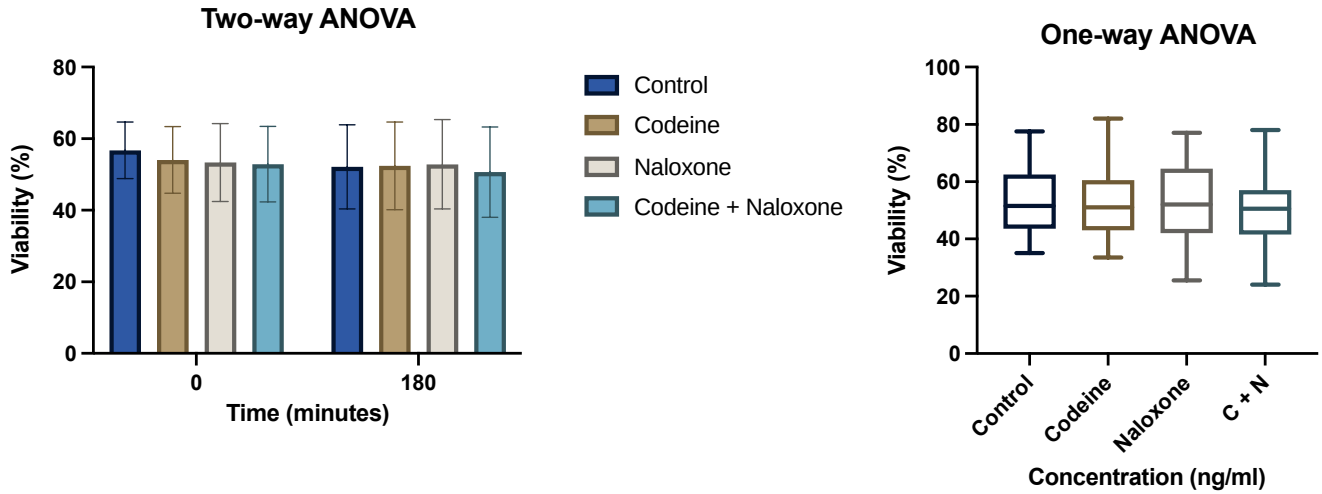

Figure 4.11: *The effect of codeine and naloxone, both separately and in combination, on cell viability*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 27; (C+N) is representative of the combination treatment (codeine + naloxone)

4.4 Treatment effects on the acrosome reaction

Figure 4.12 depicts a two-way ANOVA that was performed on the number of cells that experienced a reacted acrosome over time. This is expressed as a percentage over time. The sample size for this assessment is only 18, as unfortunately the slides of the other 12 donors were damaged. As seen in the figure below, the number of cells that displayed a reacted acrosome increased markedly from 0 to 180 minutes. From literature, it is known that the acrosome reaction starts to occur spontaneously after 180 minutes (Breitbart and Spungin, 1997), thus this increase is also seen in the control. Interestingly, the codeine treatment group showed the highest occurrence of reacted acrosomes whereas the naloxone group shows the lowest increase in reacted acrosomes. In the combined treatment group, it is interesting that the effect seems to be almost equal to that of the control – insinuating the possibility that the combination treatment had very little or no effect when compared to the control. These results were not significant.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

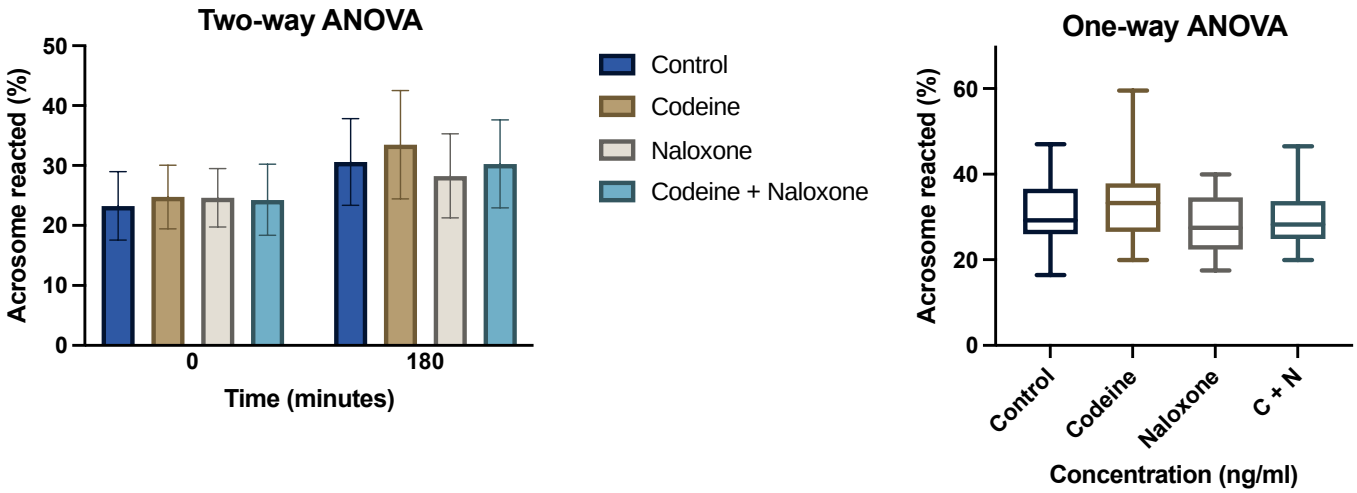

Figure 4.12: *The effect of codeine and naloxone, both separately and in combination, on the acrosome reaction*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 18; (C+N) is representative of the combination treatment (codeine + naloxone)

4.5 Treatment effects on DNA fragmentation

Depicted in figure 4.13 is a two-way ANOVA done on the effect of the different treatment groups on the DNA fragmentation. The results are expressed as a percentage over time. The sample size for DNA fragmentation was 27, as the slides of the other 3 donors were damaged. It is interesting to note that codeine seemed to have an immediate effect (at 0 minutes) on the DNA fragmentation, and that this initial effect did not seem to increase after 180 minutes. This effect seems to be mediated by naloxone, due to the combination treatment group not showing the same marked increase in DNA fragmentation. These effects were not significant.

The one-way ANOVA depicts the effect of the treatment groups on motility, after 180 minutes of incubation, and Bonferroni's multiple comparison test was performed. No significant differences were detected.

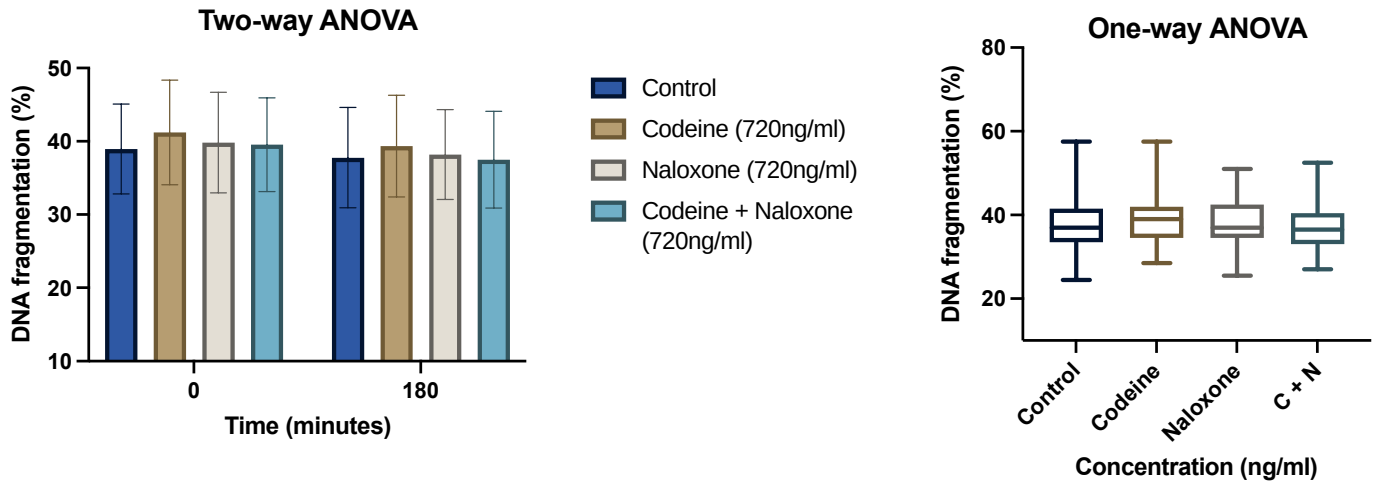

Figure 4.13: *The effect of codeine and naloxone, both separately and in combination, on DNA fragmentation*
The two-way ANOVA is expressed as a percentage over time
The one-way ANOVA, done at 180 minutes, shows the changes in percentage relative to treatment
n = 27; (C+N) is representative of the combination treatment (codeine + naloxone)

4.6 Correlations

Figures 4.14 to 4.18 shows a series of significant correlations that were performed, comparing various sperm functional parameters. The graphs display the r and p values of each individual correlation, and the parameters that are being compared can be read on the x and y axis. For example: Graph A (r = 0.9876; p = <0.0001) shows a significant, positive correlation between motility and progressive motility.

Motility correlated positively to all kinematic parameters, and the findings were significant. Interestingly, a significant, negative correlation was seen between motility and the acrosome reaction in graph K (r = -0.5898; p = <0.0001). A negative correlation was also observed between the acrosome reaction and progressive motility (graph V), VCL (graph AA), VAP (graph JJ), VSL (graph QQ), STR (graph VV), LIN (graph AAA), WOB (graph DDD), BCF (graph GGG) and viability (graph HHH). The correlation between DNA fragmentation and STR also showed a negative correlation as seen in graph WW. All other correlations were positive.

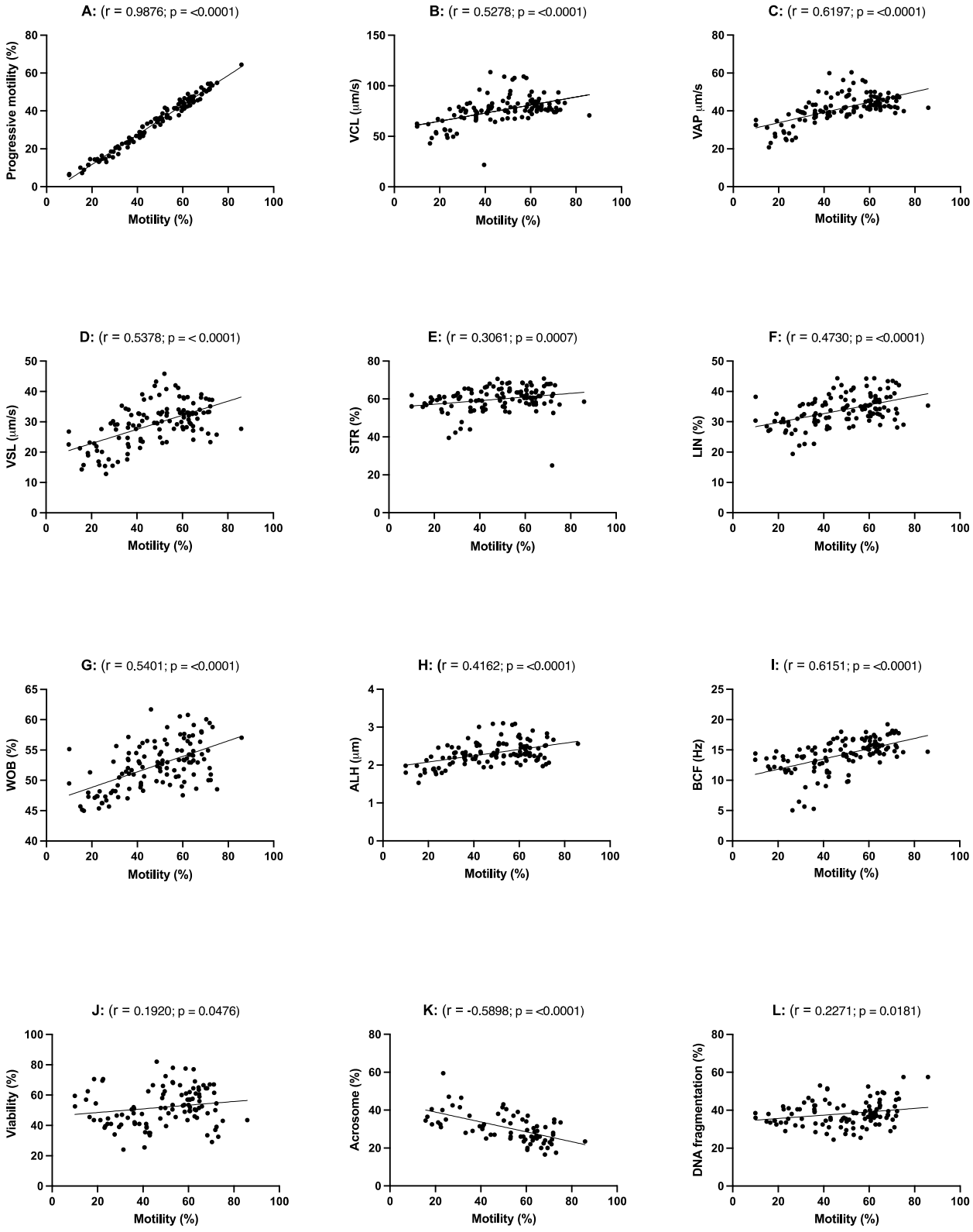

Figure 4.14: *Spearman's correlations of sperm functional parameters 1*

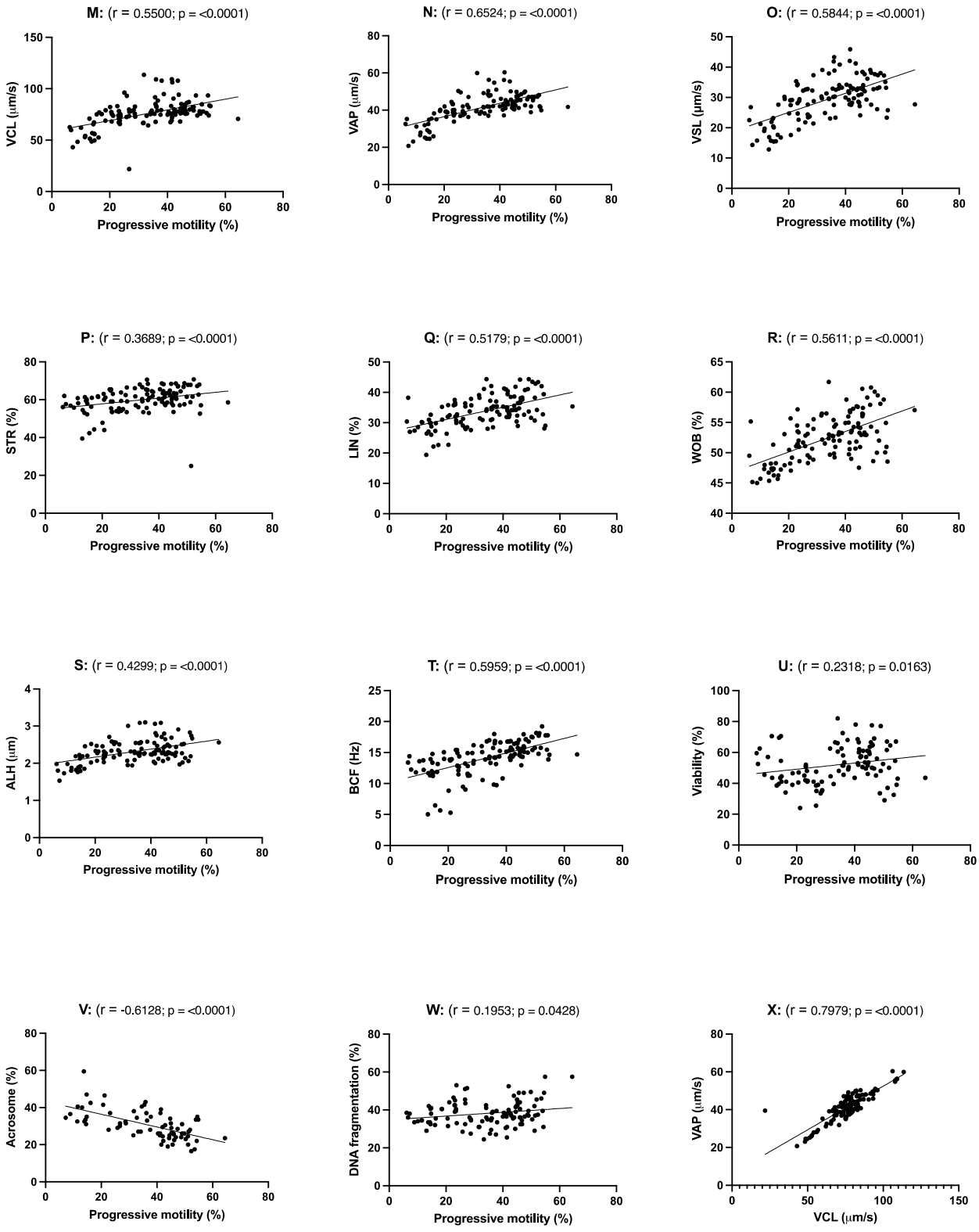

Figure 4.15: *Spearman's correlations of sperm functional parameters 2*

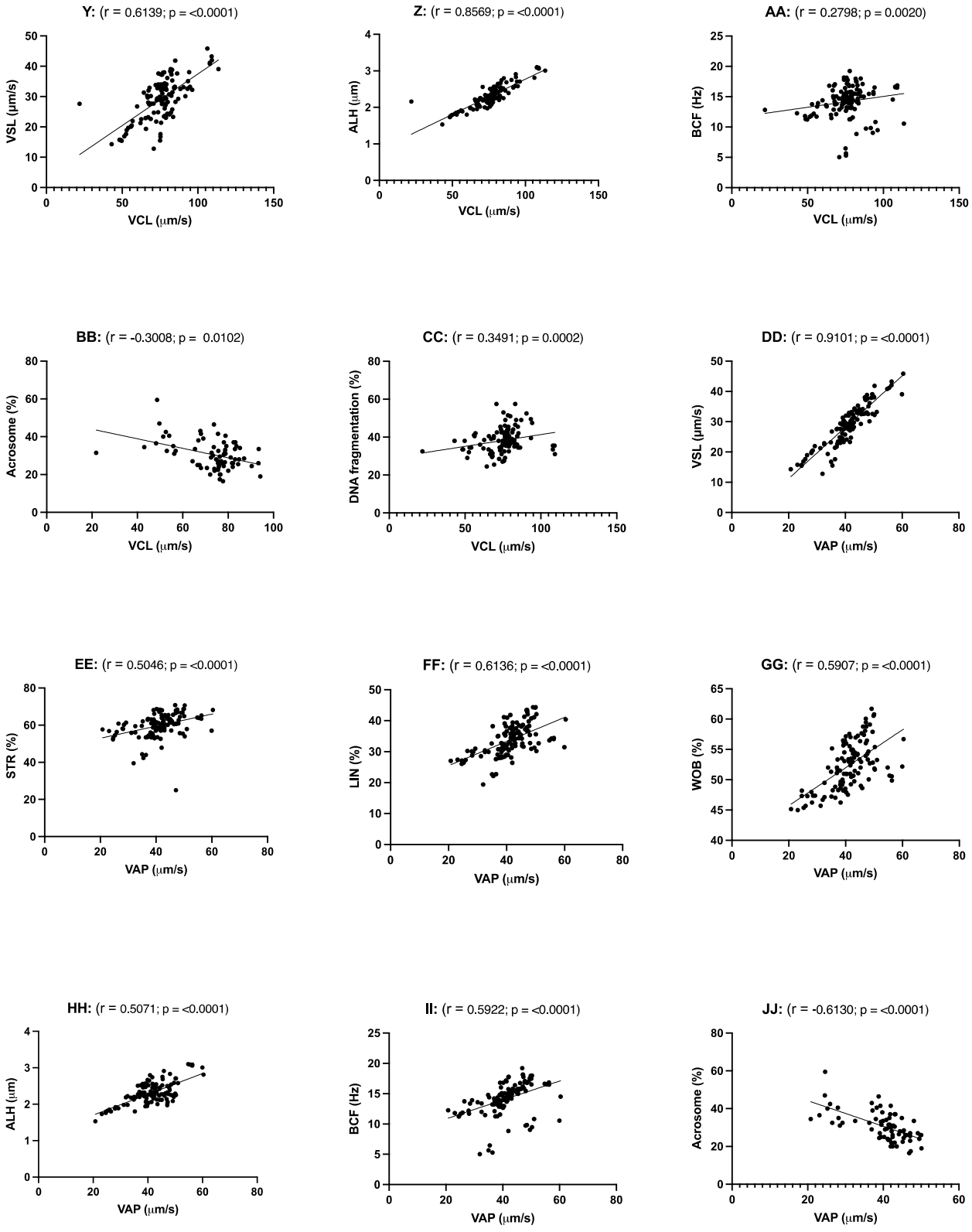

Figure 4.16: *Spearman's correlations of sperm functional parameters 3*

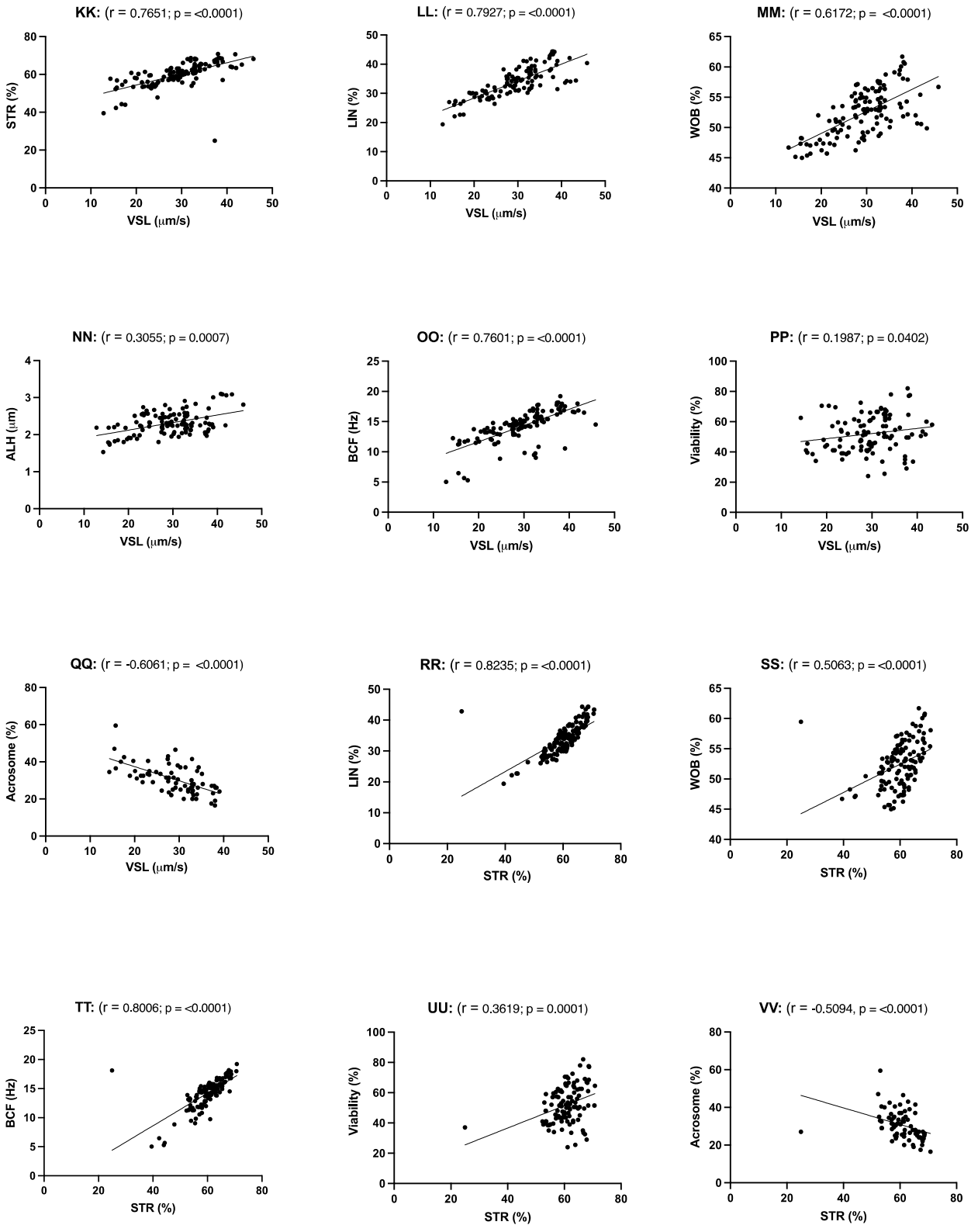

Figure 4.17: *Spearman's correlations of sperm functional parameters 4*

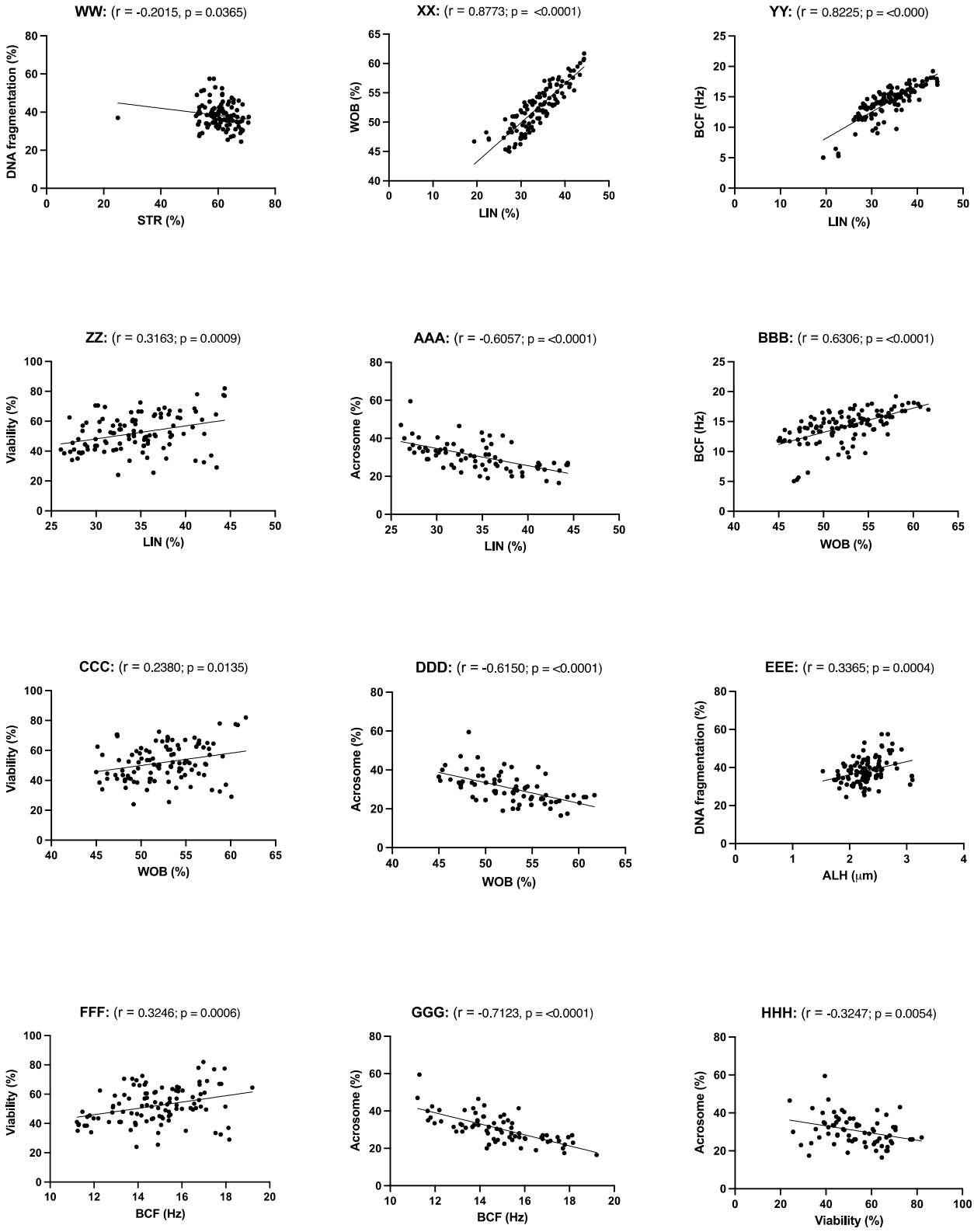

Figure 4.18: *Spearman's correlations of sperm functional parameters 5*

4.7 Western blot results

Presented in the following section are the western blotting results as observed when the total expression of AKAP3 (figures 4.19 – 4.21) and the total expression of PKCα (figure 4.22 – 4.24) were investigated.

4.7.1 AKAP3

Figure 4.19 is representative of the total protein of one of the membranes that were used to probe for the total AKAP3 expression. Figure 4.20 is representative of the final western blot, and AKAP3 can be seen at a molecular weight of 95kDa. The repeat membrane images can be found in Appendix B. Figure 4.21 depicts a one-way ANOVA, representing the total AKAP3 expression. There was a significant decrease ($p < 0.01$) in the total expression of AKAP3 in the group that was treated with naloxone compared to the control (0.766 ± 0.025 vs 1 ± 0.016, respectively).

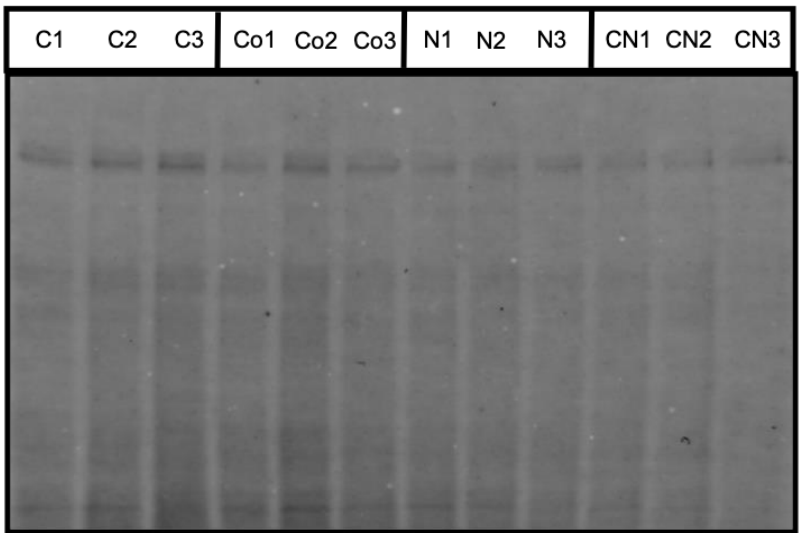

Figure 4.19: *A representative, total protein membrane for AKAP3*
Key: C: Control; Co: codeine; N: naloxone; CN: codeine + naloxone

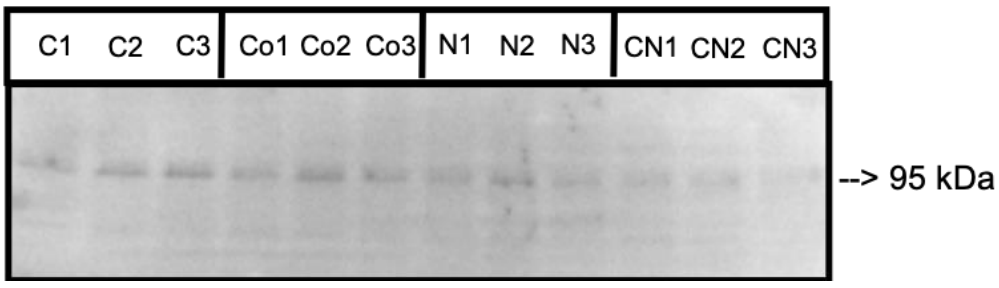

Figure 4.20: *A representative western blot of AKAP3 expression*
Key: C: Control; Co: codeine; N: naloxone; CN: codeine + naloxone

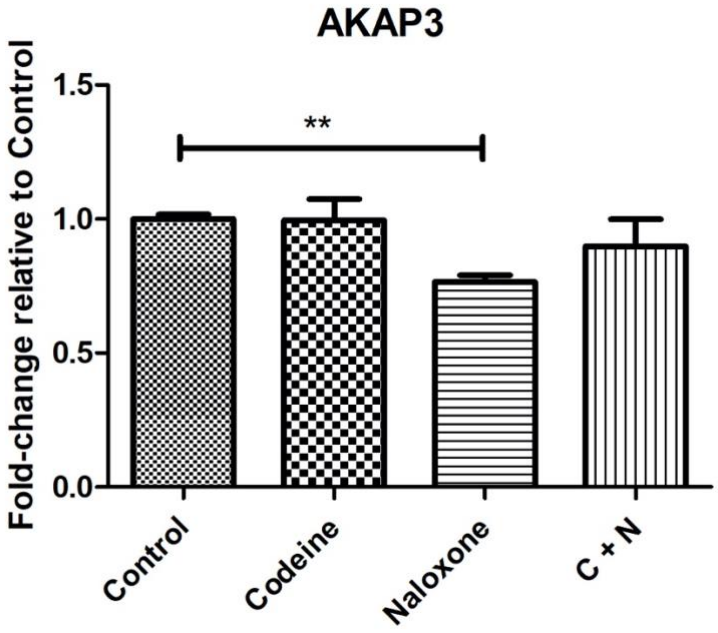

Figure 4.21: *The effect of codeine and naloxone, both separately and in combination, on the total expression of AKAP3*

Key: (C+N) is representative of the combination treatment (codeine + naloxone); all treatments 720ng/ml
*Each treatment group represents 3 biological repeats (n=3) on 3 different blots (3 technical repeats); normalized to total protein; one-way ANOVA with Shapiro-Wilk normality and Bonferroni post-hoc test; mean ± SD; **p <0.01*

4.7.2 PKCα

Figure 4.22 is representative of the total protein of one of the membranes that were used to probe for the total PKCα expression. Figure 4.23 is representative of the final western blot, and PKCα was detected at 75kDa. The repeat membrane images can be found in Appendix B. Figure 4.24 depicts the fold-change of total PKCα expression relative to controls. There were no significant changes (according to one-way ANOVA) in the total expression of PKCα in spermatozoa, after treatments. It is interesting to note that the total PKCα expression was highest in the naloxone-treated group, although not significant.

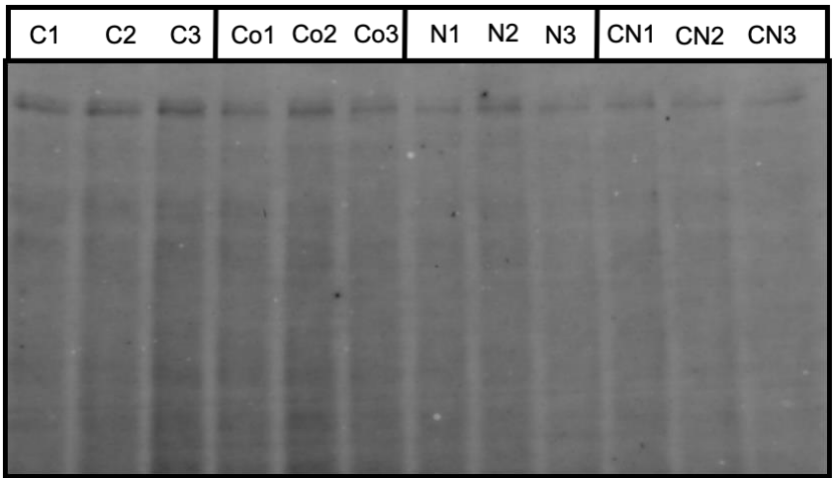

Figure 4.22: *A representative, total protein membrane for PKCα*
Key: C: Control; Co: codeine; N: naloxone; CN: codeine + naloxone

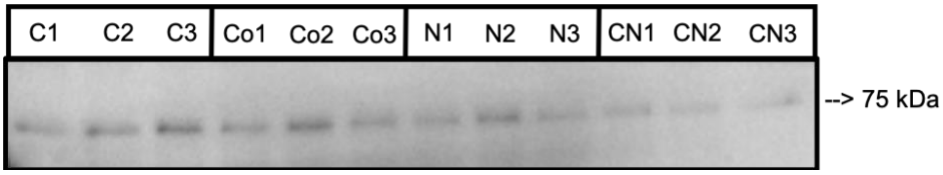

Figure 4.23: *A representative western blot of PKCα expression*
Key: C: Control; Co: codeine; N: naloxone; CN: codeine + naloxone

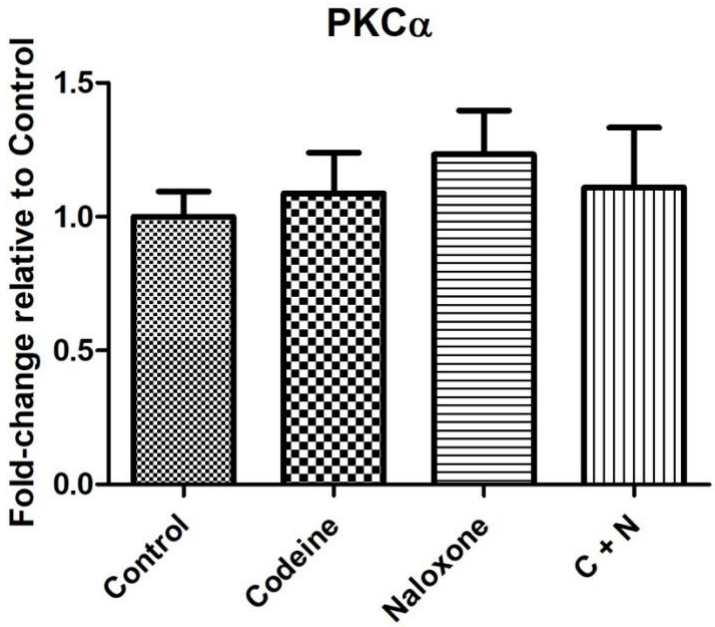

Figure 4.24: *The effect of codeine and naloxone, both separately and in combination, on the total expression of PKCα*
Key: (C+N) is representative of the combination treatment (codeine + naloxone)
Each treatment group represents 3 biological repeats (n=3) on 3 different blots (3 technical repeats); normalized to total protein; one-way ANOVA with Shapiro-Wilk normality and Bonferroni post-test; mean ± SD

CHAPTER 5

DISCUSSION

5.1 The effect of MOR modulation on sperm functional parameters

The first objective of the current study was to investigate the effect of the opioid receptor agonist and antagonist, both separately and combined, on sperm functional parameters. This included total motility, progressive motility, sperm kinematics and sperm viability. The results indicate that the respective treatment groups did not have a significant effect on these parameters, and are discussed below.

5.1.1 The effect of MOR modulation on total motility

Sperm motility is a prerequisite for normal sperm function and fertilization, since both the journey to reach the oocyte and penetration of the egg cell's extracellular matrix depends on sperm movement. A literature review revealed that very few studies have explored the effect of codeine, specifically, on human spermatozoa parameters. Recently, however, an *in-vivo* animal study investigated the chronic effects of codeine on rabbit sperm parameters and reported that sperm motility was significantly reduced (A. Ajayi and Akhigbe, 2020). This finding corresponds to the results of various *in-vivo* studies that used opioid receptor agonists other than codeine to demonstrate decreased sperm motility (Roshankhah, Gholami and Salahshoor, 2020). Furthermore, it is well-established that the sperm quality of patients or addicts of opioid medications is negatively altered (Ragni *et al.*, 1988; Safarinejad *et al.*, 2013). *In-vitro* studies on this topic are rare, but one such study found that MOR and DOR agonists decreased boar sperm motility at all concentrations, while a KOR agonist had no effect (Vicente-Carrillo, Álvarez-Rodríguez and Rodríguez-Martínez, 2016). This does not completely correspond to the findings that opioids increase equine sperm motility at low concentrations, but decreases it at high concentrations (Albrizio et al., 2010). Another study performed on human spermatozoa, found that different opioid analgesics had varying degrees of effects on sperm motility (Xu *et al.*, 2012). Another *in-vitro* study found that incubation of human spermatozoa with morphine only had a significant effect after 3 hours of incubation. Interestingly, this effect was eliminated by pre-incubation with naloxone (Agirregoitia *et al.*, 2006a). The same study found that incubation with the KOR and DOR agonists, respectively, resulted in an immediate reduction in overall motility. Considering this, the literature seems to indicate that the function of opioid receptors may differ between species. In the current study a decrease in total sperm motility was expected. However, no significant changes were seen in the total motility percentages. A slight

downward trend can be observed between the time points, but this effect is not significant. It is interesting to note that the treatment group incubated with naloxone in the current study seem to have the highest motility after 180 minutes, though this effect is not significant. The lack of significance could be attributed to the use of a 'weaker' MOR agonist. As seen in the study by Xu *et al.*, different types of opioids had different effects on sperm motility parameters. In addition, opioids have been said to differ in their structure and lipophilicity, signifying that their mode of action may differ (Mcmillan, 2018). Furthermore, it is known that Morphine (not codeine) is the principal MOR agonist (Contet, Kieffer and Befort, 2004). On the other hand, our findings disagree with that of Ajayi and Akhigbe (2020), who found codeine to negatively affect Rabbit sperm motility. It is furthermore possible that the incubation period limited the development of more significant results, as some studies saw significant effects after three hours. However, a previous study done in the SURRG lab indicated that codeine did not elicit significant effects after three hours, and thus informed the decision to only incubate for the specified time.

5.1.2 The effect of MOR modulation on progressive motility

Progressive motility refers to spermatozoa that are swimming in a straight line or in very large circles. The term generally denotes healthy functioning sperm, as they are able to swim 'progressively' towards their destination. Existing literature predominantly focuses on the effect of opioids on total motility. A study performed on equine spermatozoa reported an immediate decrease in progressive motility after the addition of the DOR agonist, DPDPE. This initial decrease in progressive motility was later restored, when progressive motility returned to near-control values at 180 minutes (Albrizio *et al.*, 2010). This indicates a biphasic effect and could mean that the initial decrease observed is temporary and is restored once the agonist has passed its half-life. Interestingly, some studies have also found that opioid antagonists affect progressive motility negatively (Refer back to Table 2.2). The results of the current study showed that the treatments had no significant effects on progressive motility, in contrast to some of the existing literature. As with the total motility results, a slight downward trend is seen but it is not significant. The reasons given for the lack of significance in total motility also apply here. In addition, it is possible that a biphasic effect occurred and it was not picked up as we only had two time points.

5.1.3 The effect of MOR modulation on sperm kinematics

Sperm kinematics may be identified as the "characteristics that describe sperm movement, as opposed to the proportion of motile cells" (Mortimer, van der Horst and Mortimer, 2015). These

measurements provide valuable insight into the way that the spermatozoa move. Literature on the effects of opioids on kinematic parameters are extremely rare. One such study found that Naltrindole, an opioid antagonist, affected VAP, VCL and VSL negatively and that these effects were dose-dependent (Albrizio *et al.*, 2010).

The current study found that neither the opioid agonist, nor the antagonist, had any significant effect on any of the kinematical sperm parameters. With all the parameters (VAP, VCL, VSL, STR, LIN, WOB, ALH, BCF) a slight downward trend is observed. In figure 4.4, which depicts the results of the treatments on the velocity along the straight-line path (VSL) it is interesting to note that the addition of codeine caused an immediate dip in this parameter at 0 minutes. The same effect was not seen as clearly in the combination treatment group, indicating that the addition of naloxone might have mediated this effect. But these results were not significant. When examining figure 4.8, which depicts the results of the treatments on the linearity (LIN), the same immediate dip is seen in the codeine-treated group. This effect is seemingly lessened in the combination treatment group. It is interesting to note these differences, but the effects were not significant.

5.1.4 The effect of MOR modulation on cell viability

The literature is clear that opioids of various kinds are able to influence spermatogenesis in mammals and humans (Sharp and Pekary, 1981; Esua, Uno and Ekaluo, 2018). Individuals who take opioids chronically, are almost always found to have decreased sperm cell integrity, and this effect has been mainly attributed to the action of opioids on the HPG axis. The effects of opioids on spermatozoa viability in an *in-vitro* setting is less clear. One study found viability to remain unaffected when incubated with an opioid agonist (Urizar-Arenaza et al., 2019). Considering the literature, therefore, it was anticipated that the treatment groups would not have a significant effect on sperm cell viability. The results of the current study correspond to this. Although a slight downward trend can be observed in figure 4.11, the effect was not significant. It is interesting to note that literature predicts that opioids decrease sperm motility, but not sperm viability when treated *in-vitro* (Olabarrieta *et al.*, 2020). This would make sense, as certain concentrations of opioids have also been said to have a temporal, immobilizing (biphasic) effect on spermatozoa in-vitro, not necessarily a necrotic effect (Xu *et al.*, 2012).

5.2 The effect of MOR modulation on advanced sperm parameters

The second objective of the study was to determine whether the opioid receptor agonist or antagonist had any effect on advanced sperm parameters; namely the rate of the acrosome reaction and levels of

DNA fragmentation. The results indicate that the respective treatment groups did not have a significant effect on these parameters, and are discussed below.

5.2.1 The effect of MOR modulation on acrosome reaction

The acrosome reaction is a crucial part of gamete interaction in all species, and has been referred to as a prerequisite event for a spermatozoon to pass through the zona pellucida and fuse with the oocyte (Patrat, Serres and Jouannet, 2000a). If a premature acrosome reaction occurs, it leaves the spermatozoon unable to penetrate the protective membranes of the oocyte and, in this way, fertilization is hindered. Literature in regards to the *in-vitro* effect of opioids on the sperm acrosome reaction, are largely contradictory. Referring back to Table 2.2, one study found that *in-vitro* incubation with the DOR agonist caused an increase in reacted acrosomes, while another study found that incubation with a KOR agonist decreased the number of acrosome reacted cells. Another study found that naloxone, an opioids receptor antagonist, also had a negative effect on the acrosome reaction. As seen in figure 4.12, the results showed that incubation with the respective treatments had no significant effect on the percentage of acrosome reactions seen in the treatment groups. Interestingly, when looking at the trends, the group treated with codeine showed the highest amount of reacted acrosomes at 0 minutes as well as after 180 minutes of incubation. At 180 minutes, the codeine treated group noticeably had the highest proportion of reacted acrosomes, whereas the naloxone treated group noticeably had the least amount. It is also interesting to note that the percentage of reacted acrosomes seen in the combination treatment group is in close comparison to that of the control. Though none of these effects were significant, it seems as though naloxone could have potential to challenge the effects of codeine. The lack of significance seen here may be, in part, due to the smaller sample size. As disclosed in section 4.4, there were a number of slides that were damaged and rendered un-interpretable.

5.2.2 The effect of MOR modulation on DNA fragmentation of spermatozoa

The WHO defines DNA fragmentation in spermatozoa as any chemical change that occurs in the normal structure of the sperm DNA (WHO, 2021). Sperm DNA fragmentation (sDF) can be triggered by many different processes including several pathological conditions, environmental conditions, oxidative stress and the defective packaging of DNA during spermatogenesis. The fertilization potential of a spermatozoon containing sDF is not necessarily impaired. However, research indicates that the presence of sDF may affect embryo development, implantation and successful pregnancies in both natural and assisted reproduction (Henkel *et al.*, 2003; Rex, Aagaard

and Fedder, 2017). Cases of infertility in normozoospermic men have also been related to sDF. According to the literature, DNA fragmentation is often observed in the sperm samples of men who use or abuse opioids chronically (Sansone *et al.*, 2018; Nazmara *et al.*, 2021). The *in-vitro* effects of opioids on the DNA fragmentation of spermatozoa are less clear. The current study did not show any significant effects of the respective treatments on the DNA fragmentation of the spermatozoa. However, a downward trend can be observed, with the codeine-treated group exhibiting the highest percentage of cells with fragmented DNA, as seen in figure 4.13. There is no noticeable difference between the time-points.

5.3 Correlation results

Correlations were performed to assess the statistical relationships between the functional spermatozoa parameters. As expected, the motility parameters correlated positively with all kinematic parameters. This means that changes in one of these parameters would result in a similar change in the other parameter. The positive correlation seen between cell viability and motility parameters makes sense, as it is known that sperm cells become immotile after necrosis (Lachaud *et al.*, 2004). Interestingly, the acrosome reaction parameters correlated negatively with all kinematic parameters. A negative correlation signifies that if one variable increases, the other will decrease and vice versa. Considering that spermatozoa have a decreased longevity after the acrosome reaction occurs, this makes sense (Patrat, Serres and Jouannet, 2000b). Literature affirms these results, as it has been shown that a negative correlation exists between motility parameters and the acrosome reaction (Parinaud et al., 1996). A negative correlation was also seen between DNA fragmentation and STR. Subsequently, it can be assumed that the level of DNA fragmentation directly influences the linearity of the average path of spermatozoa. This finding corresponds with literature, as increased DNA fragmentation has been associated with abnormal sperm movement (Oliveira *et al.*, 2010; Zribi *et al.*, 2011). It can be deduced that the relationships between the functional parameters in the current study were normal.

5.4 The effect of MOR modulation on proteins of interest

The third and final objective was to determine the overall expression of proteins of interest (AKAP3 and PKCα) by western blotting. The results indicate that the total expression of AKAP3 was significantly decreased by naloxone, while the overall expression of PKCα was not significantly affected. The results are discussed below.

The activity of opioid receptors in the pain modulation pathway is understood as explained in section 2.2.2. Among other things, the postsynaptic activation of the MOR leads to a decrease in adenylyl cyclase (AC) and eventually cAMP (figure 2.3). The MOR is also shown to inhibit voltage gated Ca^{2+} channels. Later (section 2.6.1 – 2.6.2) it is shown that the capacitation and acrosome reaction processes both depend on the activation of AC, which then activates cAMP and ultimately PKA. PKA activation leads to the activation of Ca^{2+} channels, and the Ca^{2+} influx ultimately results in capacitation and later the acrosome reaction (figure 2.5 and 2.6). Therefore, if the MOR functioned similarly in spermatozoa as it does in the brain, it could be hypothesized that a MOR agonist would inhibit sperm capacitation and acrosome reaction. Interestingly, as discussed in section 5.3, the treatment groups had no significant effects on the acrosome reaction. Considering this, AKAP3 was chosen as a protein of interest as the activation of PKA depends on AKAP3 phosphorylation (Rahamim Ben-Navi *et al.*, 2016). Due to the crosstalk between PKA and PKC (discussed in 2.7), as well as the involvement of PKCα, specifically, in sperm capacitation and acrosome reaction, PKCα was also a protein of interest.

5.4.1 AKAP3

In the current study a significant decrease in the total expression of AKAP3 in the group treated with the MOR antagonist, naloxone (figure 4.21), was observed.

As discussed in the literature review, AKAP3 is a scaffold protein that has been shown to be involved in the structure of the sperm fibrous sheath (Brown *et al.*, 2003). Furthermore, it is involved in sperm fertility by binding cAMP-dependent PKA. Therefore, a decreased total expression of AKAP3 could indicate a decrease in the sequestering of PKA. These observations strengthen the argument that sperm-specific signalling mechanisms involving AKAP3 exist down-stream of the opioid receptors. Referring back to figure 2.4, PKA has an inhibiting effect on PKC, which ultimately leads to the acrosome reaction. However, if PKA expression is decreased, PKC inhibition is alleviated which would ultimately inhibit the acrosome reaction. When analysing the effects of naloxone on the acrosome reaction (figure 4.12) the percentage of cells that underwent an acrosome reaction is lower than that of the control – though these effects were not significant. Taking this into consideration, the possibility arises that AKAP3 expression plays a regulatory role in the acrosome reaction. Urizar-Arenaza and colleagues found that AKAP3 showed decreased levels of phosphorylation after treatment with the KOR agonist (Urizar-Arenaza et al., 2019). Considering their findings, it is interesting that the current study found that the total expression of AKAP3 was significantly affected by naloxone, the opioid receptor antagonist, and not by the agonist. Further

studies will be necessary to investigate the AKAP3 phosphorylation in addition to the total expression. It is interesting to note that the decrease in total AKAP3 expression was reversed, in the combination treatment group (figure 4.21).

5.4.2 PKCα

In the current study the MOR modulators had no significant effect on the total expression of PKCα in spermatozoa (figure 4.24). Interestingly, all treatment groups showed (non-significant) increases in the total expression of PKCα, when compared to the control - with naloxone showing the highest increase. Considering that a significant decrease in the AKAP3 expression was seen in the same group (naloxone-treated), this observation is interesting. As discussed in section 5.6.1, the decreased AKAP3 expression could lead to decreased sequestering of PKA. Ultimately the result could be a decreased inhibition of PKCα (Itach *et al.*, 2012). Therefore, this increase in the total expression of PKCα could be expected. However, these effects were not significant.

Urizar-Arenaza and colleagues found that the KOR agonist, U50488H, increased phosphorylated substrates of PKC (Urizar-Arenaza et al., 2019). The current study showed that codeine caused a slight, insignificant increase in the total expression of PKCα, but interestingly the greatest increase in expression was seen in the naloxone-treated group. It is unclear why the MOR antagonist, as opposed to the agonist, resulted in these effects. More research is required to fully understand the effects of the opioid system on the signalling system in human spermatozoa.

CHAPTER 6

CONCLUSION

Considering the contradictory effects shown in the literature, it is clear that there is not a good understanding of the opioid system's role in human spermatozoa. Therefore, it is maintained that further research is necessary to understand the effects that opioids have on spermatozoa. Elucidating these effects and understanding the mechanism behind them could provide invaluable insight into an area of unexplained infertility cases. It may also lead to new therapeutic procedures, especially in how opioids are dispensed in reproductive-aged individuals. In addition, reversal of the negative reproductive effects that have been seen in those who use opioids chronically (for example cancer patients) who may still wish to have a chance to reproduce, can be explored.

As observed in our results, the error bars are consistently high throughout the study – indicating high individual variability. It suggests a much larger sample size is required. Additionally, some individuals may be more prone to the adverse effects of opioids than others. Furthermore, though the investigations of the sperm functional parameters were not significant, some results did show noticeable and interesting effects. The acrosome reaction and DNA fragmentation proved especially interesting. Furthermore, the western blotting results affirm the hypothesis that sperm-specific signalling mechanisms exist downstream of the mu opioid receptors located on human spermatozoa. Understanding these mechanisms could prove to be invaluable in enhancing our understanding of infertility.

Future studies

It is possible that the lack of effects were due to the use of a "weaker" opioid receptor agonist, namely codeine. We suggest either revising the concentrations that were used in our study, or using a different opioid receptor agonist. Furthermore, future studies could focus on a different opioid receptor (KOR or DOR). It is possible that these two receptors, rather than MOR, may be responsible for the effects of opioids on spermatozoa that has been seen in literature. Utilization of larger sample size, in order to combat the high variability between donors more effectively, is suggested. It would be interesting to investigate the total and phosphorylated levels of PKA, in addition to the proteins investigated in this study. This would aid in elucidating the underlying intracellular events.

Considering the large standard deviation of the current study's results, it is recommended that future studies take care to define the sperm population selected for study. This would be advantageous, as it

has been suggested that various sperm fractions do not respond in the same manner to treatments. Additional sperm functions may also be assessed, such as sperm hyperactivation.

Study limitations

As a result of the Covid-19 pandemic and the lockdown measurements that were taken, this study suffered from severe time-constraints. This greatly impacted the number of donors (n=30) that we were able to recruit and as a result, the *in-vitro* experiments were underpowered. Furthermore, some of the slides for viability, the acrosome reaction and the DNA fragmentation results were affected by the extended inaccessibility to them and were rendered un-assessable. The results of these measurements are therefore underpowered. Although the concentrations used were obtained from literature, it would be advisable to re-assess these concentrations upon further study.

Sperm subpopulations such as rapid progressive, medium progressive and non-progressive were not assessed, and could have provided valuable insight into the effects of the treatment on spermatozoa.

The low n value also had an impact in the western blotting analyses. In order to obtain sufficient protein for performing the western blots, sperm samples had to be pooled. Even though 10 samples were pooled together, the bands on the western blots remained faint. In order to generate more definitive results, more samples would need to be pooled together to obtain a better protein concentration. Another limitation regarding the western blot analysis was that we were unable to blot the phosphorylated AKAP3 and PKCα which could have clarified the mechanisms. In addition, during the move to the new Biomedical Sciences building many antibodies were damaged as a result of thawing. This impacted the amount of proteins investigated in the study.

CHAPTER 7

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Appendix A

Approval Notice

New Application

25/01/2021

Project ID :11642

HREC Reference No: S20/08/198

Project Title: A mechanistic study on the mu-opioid receptor in human spermatozoa

Dear Ms Nadia Van Niekerk

The Response received on 08/01/2021 was reviewed and approved by members of Health Research Ethics Committee via expedited review procedures on 25/01/2021.

Please note the following information about your approved research protocol:

Protocol Approval Date: 25 January 2021

Protocol Expiry Date: 24 January 2022

Please remember to use your Project ID 11642 and Ethics Reference Number S20/08/198 on any documents or correspondence with the HREC concerning your research protocol.

Please note that the HREC has the prerogative and authority to ask further questions, seek additional information, require further modifications, or monitor the conduct of your research and the consent process.

After Ethical Review

Translation of the informed consent document(s) to the language(s) applicable to your study participants should now be submitted to the HREC.

Please note you can submit your progress report through the online ethics application process, available at: Links Application Form Direct Link and the application should be submitted to the HREC before the year has expired. Please see *Forms and Instructions* on our HREC website (www.sun.ac.za/healthresearchethics) for guidance on how to submit a progress report.

The HREC will then consider the continuation of the project for a further year (if necessary). Annually a number of projects may be selected randomly for an external audit.

Please note that for studies involving the use of questionnaires, the final copy should be uploaded on Infonetica.

Provincial and City of Cape Town Approval

Please note that for research at a primary or secondary healthcare facility, permission must still be obtained from the relevant authorities (Western Cape Department of Health and/or City Health) to conduct the research as stated in the protocol. Please consult the Western Cape Government website for access to the online Health Research Approval Process, see: https://www.westerncape.gov.za/general-publication/health-research-approval-process. Research that will be conducted at any tertiary academic institution requires approval from the relevant hospital manager. Ethics approval is required BEFORE approval can be obtained from these health authorities.

We wish you the best as you conduct your research.

For standard HREC forms and instructions, please visit: Forms and Instructions on our HREC website https://applyethics.sun.ac.za/ProjectView/Index/11642

If you have any questions or need further assistance, please contact the HREC office at 021 938 9677.

Yours sincerely,

Mrs. Melody Shana

Coordinator

HREC1

National Health Research Ethics Council (NHREC) Registration Number:

Appendix B

Additional information: Western blot Analysis

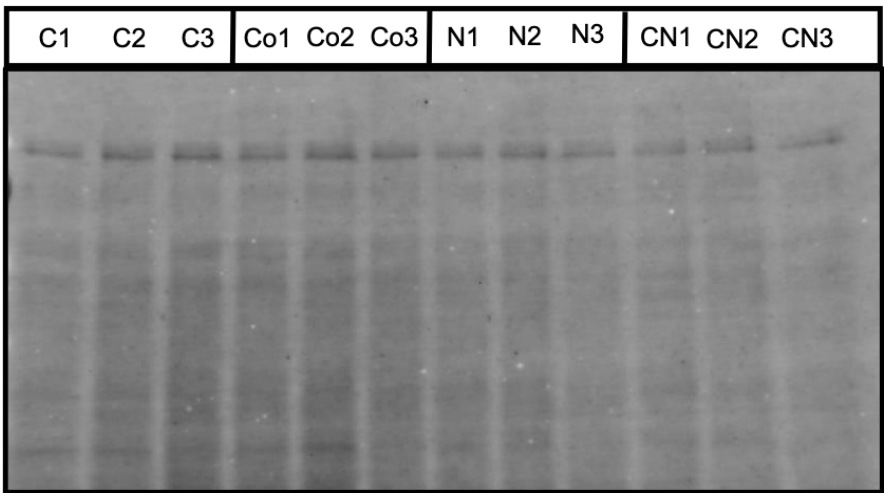

Figure B1: *Membrane used for AKAP3 normalization (2nd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

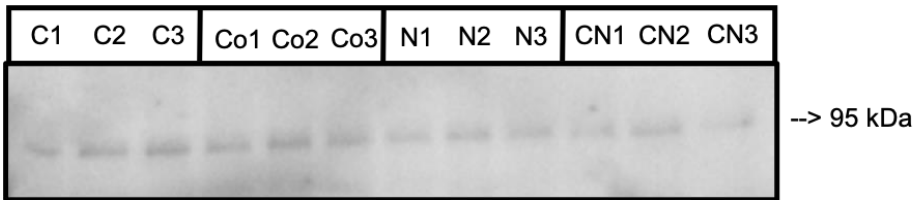

Figure B2: *Western blot membrane for AKAP3 (2nd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

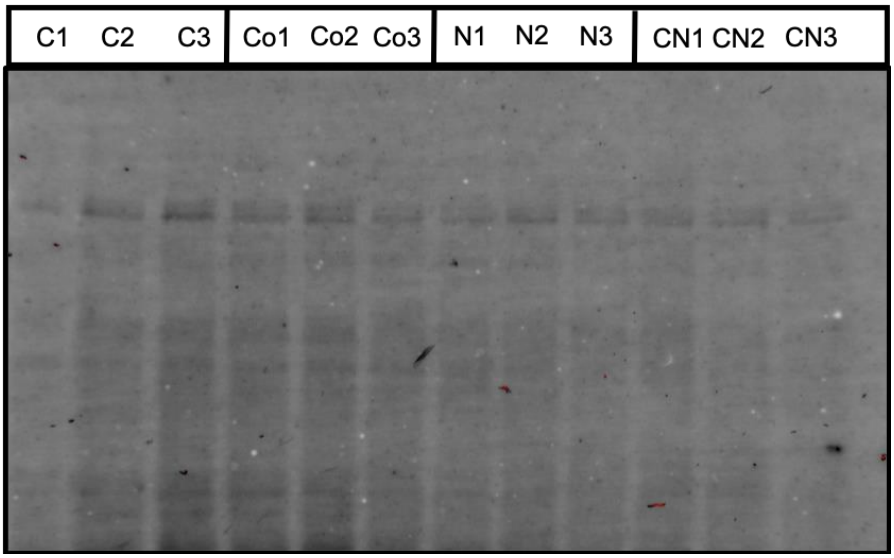

Figure B3: *Membrane used for AKAP3 normalization (3rd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

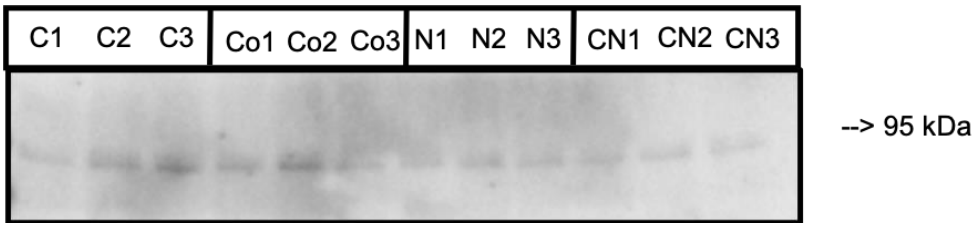

Figure B4: *Western blot membrane for AKAP3 (3rd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

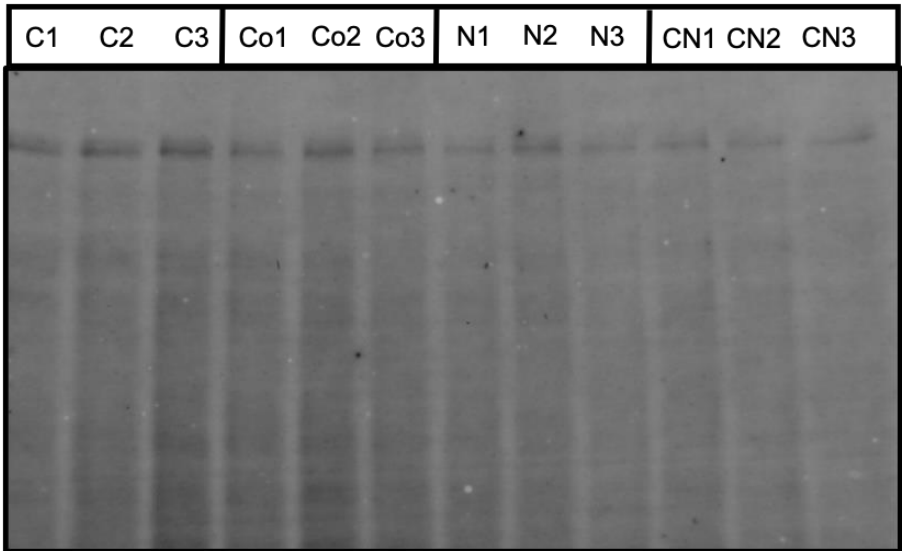

Figure B5: *Membrane used for PKCα normalization (2nd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

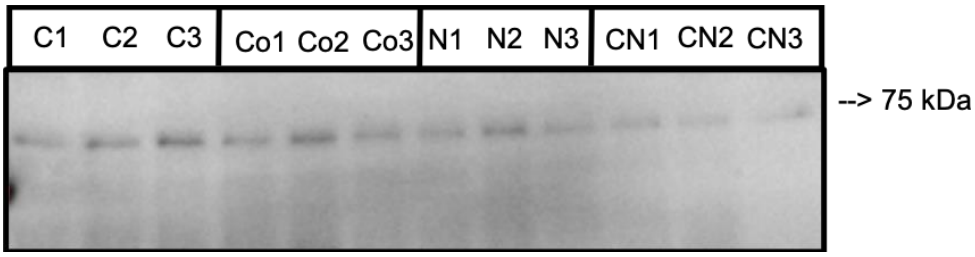

Figure B6: *Western blot membrane for PKCα normalization (2nd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

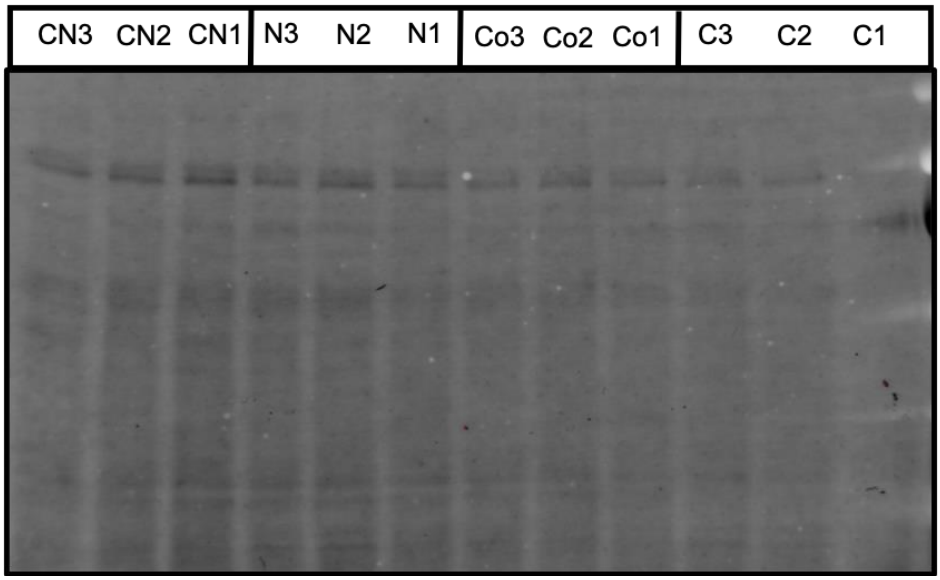

Figure B7: *Membrane used for PKCα normalization (3rd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone

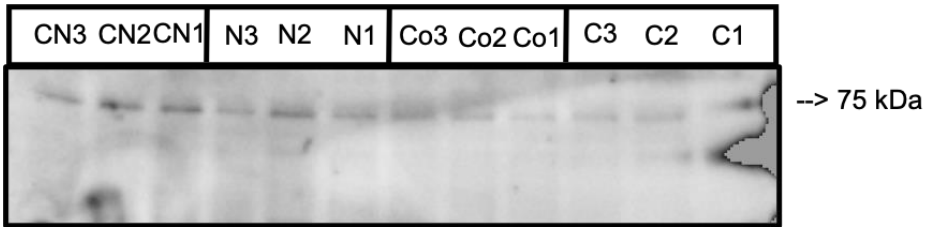

Figure B8: *Western blot membrane for PKCα (3rd repeat)*

Key: C: Control; Co: Codeine; N: Naloxone; CN: Codeine + Naloxone