DETERMINATION OF *IN VITRO* EFFECTS OF AQUEOUS EXTRACT OF *CAMELLIA SINENSIS* ON HUMAN SPERM FUNCTIONS

by

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[redacted]

DISSERTATION

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DECLARATION

I, Setumo Mmaphulane Abigail declare that the dissertation with the tittle ‘’**THE DETERMINATION OF THE EFFECTS OF THE AQUEOUS LEAF EXTRACT OF CAMELLIA SINENSIS ON HUMAN SPERM FUNCTION’’** is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references and that this work has not been submitted before for any other degree at any other institution.

Signed

Date 23/OCTOBER/2021

DEDICATION

Everything may be rushing on you, and everything may be too slow, just hold on and know you will reach your destiny eventually. This is dedicated to me and everyone who seems to be stagnant in life, no situation is permanent it shall be well.

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Thank you, God, for everything, I am grateful for the grace and love.

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ABSTRACT

Infertility, defined as the inability to conceive following one year of unprotected sexual intercourse, respectively affects 25% of couples globally. Oxidative stress (OS) has been greatly related to the idiopathic cause of infertility and *Camellia sinensis* contains antioxidants that may enhance reproductive functions. This study focussed on the effects of *Camellia sinensis* (green and black tea) on human sperm functions in both normal and abnormal samples. Semen samples (n= 59) collected from donors were liquefied, analysed, and classified as normal (n=40) and abnormal (n= 19) using the WHO criteria. Samples were washed and exposed to aqueous leaf extracts of green and black tea (0, 0.4, 4, 40, 405 µg/ml) for 1 hour. Human Tubular Fluid (HTF) served as the control. The respective sperm parameters were analysed (sperm motility, vitality, DNA fragmentation, mitochondrial membrane potential (MMP), capacitation and acrosome reaction (CTC) and reactive oxygen species (ROS). Green and black tea significantly increased vitality, and intact MMP, while it significantly reduced, CTC, and intracellular ROS as well as DNA fragmented spermatozoa in both normal and abnormal samples compared to the control ($p<0.05$). A significant increase in sperm CTC, ROS, with a decrease in sperm vitality, and intact MMP was observed in the abnormal compared to the normal samples ($p<0.05$). No significant change in motility was observed between normal and abnormal samples compared to their respective controls, in both green and black tea ($p>0.05$). *Camellia sinensis* improved human sperm function *in vitro* and may be attributed to its antioxidant activity.

KEY CONCEPTS

Antioxidants

Camellia sinensis (Green tea & Black tea)

Infertility

Oxidative stress

Spermatozoa

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RESEARCH OUTPUTS

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TERMINOLOGIES

Antioxidant refers to a substance that can trap free radicals before oxidative stress takes place (Canda, Oguntibeju & Marnewick, 2014). Antioxidants act as the first line of defence, scavenging or destroying the damaging effect caused by oxidative stress and reactive oxygen species (Walczak-Jedrzejowska, Wolski & Slowikowska-Hilczer, 2013).

Camellia sinensis is described as an evergreen species that looks like a shrub or small tree belonging to the *Camellia* genus of flowering plants and Theaceae family. It has popular names such as tea plant, tea shrub and tea tree (Kumar, 2018).

Infertility is when a couple fails to conceive following twelve months of regular sexual intercourse without the use of contraceptives or any traditional medication (Vander Borght & Wyns, 2018).

Reactive oxygen species (ROS) are known as highly reactive oxygen by-products with half-life times in the nano- to milli-second range (Wu, 2015).

Spermatozoa refers to a fully developed motile sex cell that can fertilise an egg cell naturally and that has a compact head and a flagellum for swimming (Malić Vončina, Golob, Ihan, Kopitar, Kolbezen & Zorn, 2016).

ABBREVIATIONS

ALH	-Average Lateral Head Displacement
ANOVA	-Analysis of Variation
ART	-Assisted Reproductive Technology
BCF	-Beat Across Frequency
BMREC	-Biomedical Research Ethics Committee
BSA	-Bovine Serum Albumin
CASA	-Computer Aided Semen Analysis
CSA	-Computed Sperm Analysis
Cov	-Coefficient of Variance
CTC	-Chloro-tetracycline
DABCO	-Diazabicyclo Octane
DHE	-Dihydroethidium
DNA	-Deoxyribonucleic Acid
Dutp	-Deoxynucleotidyl Transferase
E&A	-Eosin and Negrosin
Hcg	-Human-Chorionic Gonadotrophin
HCL	-Hydrochloric Acid
LIN	-Linearity
MMP	-Mitochondrial-Membrane Potential
OS	-Oxidative Stress
PR	-Progressive Motility
ROS	-Reactive Oxygen Species
SCA	-Sperm Class Analysis

SD	-Standard Deviation
SEM	-Standard Error of Mean
STR	-Straightness
TREC	-Turfloop Research Ethics Committee
TUNEL	-Transferase Mediated dUTP Biotin Nick End Labelling
USA	-United States of America
UWC	-University of the Western Cape
VAP	-Velocity Average Path
VCL	-Velocity Curvilinear
VSL	-Velocity Straight Line
WHO	-World Health Organisation

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CHAPTER 1: INTRODUCTION

1.1 Introduction

Infertility is when a couple is incapable of conceiving following regular, unprotected sexual intercourse without the use of contraceptives for one year and affects at least 50 - 80 million couples worldwide (Vander Borght, and Wyns., 2018). Infertility affects about 15% of couples globally, of which males are found to be solely responsible for 20-30% of the infertility cases and contribute to 50% of the overall cases (Agarwal, Mulgund, Hamada and Chyatte., 2015). In Africa, about 12-16% of couples are diagnosed with infertility, of which 20 - 40% is due to a male factor (Agarwal *et al*., 2015). Furthermore, the prevalence of sexual health problems among ageing male adults is rather high, and 20-30% of adult men are affected by at least one sexual dysfunction (Agarwal *et al*., 2015). Reproductive ability in males include the production of semen with normal spermatozoa in the acceptable number (quantity), together with the desire and ability to mate (Mohammadi, Nikzad, Taherian, Amini Mahabadi and Salehi., 2013).

Male infertility may arise due to a number of factors such as stroke, hypertension, blood vessel disorders, diabetes mellitus, oxidative stress in the testes, decreased testosterone production and secretion by the Leydig cells, genetic disorders, genital duct obstruction, varicocele, male impotence, decreased sperm production, decreased semen quality parameters, and erectile dysfunction (Chen, Shi, Huang, Li, Ma *et al*., 2019; Mohammadi, Nikzad, Taherian and Maabadi, 2013). Age related disorders also play a part in the causation of infertility (Vander, Borght and Wyns, 2018).

The generation of reactive oxygen species (ROS) has become a serious problem because of its harmful effects when its levels are high on sperm normal function and quality (Du Plessis, Agarwal, Halabi and Tvrda., 2015). Atoms or molecules that contain one or more unpaired electrons are called free radicals (Topal, Nar, Gocer, Kalin, Kocyigit *et al*., 2016). Oxidative stress has been known as the main cause of infertility due to excessive production of reactive oxygen species that eventually overpower the defence system, consequently damaging the spermatozoa (Beygi, Forouhari, Mahmoudi, Hayat and Nourimand., 2021). Oxidative stress plays an important part in infertility, as increased levels of ROS were detected in infertile patients with declined levels of seminal plasma and blood plasma antioxidants (Kang, Punjani, Lee, Li and Goldstein., 2021).The

idiopathic causes of male infertility can also be described as an early ageing of testis promoted by ischemia and oxidative stress (OS) in association with defective mitochondrial genome that controls oxidative phosphorylation (Ko, Sabanegh Jr and Agarwal., 2014).

Spermatozoa are some of the cells that require oxygen to survive, thus they live in aerobic conditions and are constantly exposed to oxidative stress (Bardaweel, Gul, Alzweiri, Ishaqat, ALSalamat *et al*., 2018). Oxygen is highly required for life support, and its metabolites such as ROS interfere with cell functioning and temper with the survival of the cells (Corcoran and Cotter, 2013). Besides the fluidity of the plasma membrane of the sperm that is attacked by OS, sperm deoxyribonucleic acid (DNA) integrity is also affected (Wright, Milne and Leeson, 2014). Therefore, there must be the disabling of ROS so that only a minimum amount required for normal cell function is kept. It is very important to have different types of antioxidants to keep control of ROS or oxidants to protect spermatozoa (Aitken, and Drevet., 2020).

There are several modern treatments such as assisted reproductive therapy (ART) that are used to address issues of male infertility, but these methods tend to be expensive and non-convenient to most of the masses (Bernhardt, 2000). Antioxidants play a critical role of scavenging free radicals. Studies show that lycopene, a lipid soluble carotenoid, may be important in maintaining fertility with its free-radical scavenging properties found in diet rich in tomatoe (Pakrashi, and Oehninger, 2014). It was suggested in a study by Raj, Selvakumar, Krishnamoorthy, Revathy, Elumalai *et al*., (2014) that lycopene may have a protective property against polychlorinated biphenyl induced epididymal toxicity. Studies conducted in broilers confirmed that the provision of antioxidants in diet serves as a simple method of administering antioxidant compounds and their activity to the bodies of individuals as a method of destroying free radicals, thereby managing infertility (Agarwal, Leisegang, Majzoub, Henkel, Finelli *et al*., 2021). Vitamin E is also another lipid soluble antioxidant which functions as a free radical chain reaction breaker. This is executed by the inhibition of polyunsaturated fatty acids peroxidation because in sperm, the levels of polyunsaturated fatty acids are notably high (Bolle, Evandri, and Saso., 2002). Hence, the application of these antioxidants could enhance fertility by increasing sperm motility and function (Azadi, Afzan, Karimi, Sinniah, and Kumara, 2015).

The antioxidant protection system of humans has been highly evolved to protect cells and organs against ROS (Aitken and Drevet., 2020). The evolution is made up of different

components, endogenous and exogenous, that function together to neutralize free radicals (Rhemrev, van Overveld, Haenen, Teerlink, Bast *et al*., 2000; Lazzarino, Listorti, Bilotta, Capozzolo, Amorini *et al*., 2019; O'Flaherty., 2014). Nutrient derived antioxidants like ascorbic acid (vitamin C), tocopherol and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds are some of the components that are included (Lazzarino, Listorti, Bilotta, Capozzolo, Amorini *et al*., 2019). Antioxidants dispose, scavenge, and suppress ROS formation (Bansal., 2015). There is an impairment of the seminal plasma non-enzymatic antioxidant capacity in infertile men than in fertile men, indicating there may be a relationship between decreased non-enzymatic antioxidant capacity and male infertility shown by some studies (Walczak-Jedrzejowska, Wolski and Slowikowska–Hilczer., 2013).

Camellia sinensis is known as a species of evergreen shrub whose leaves and leaf buds are used to produce tea and it is of the genus *Camellia* of flowering plants in the family Theaceae (Namita *et al.,* 2012). It has common names such as tea plant, tea shrub and tea tree (Namita *et al.,* 2012). *Camellia sinensis* is processed in different forms such as the green tea (unfermented), black tea (fermented and oxidized) and oolong (partially oxidized) as well as white tea (young leaves and minimally processed) (Perry., 2001; Dias., 2013). *Camellia sinensis* is rich in phytochemicals such as flavonoids, known as antioxidants (Azadi *et al*., 2015).

Camellia sinensis is traditionally used for urinary inconsistency, common cold, and suppression of anxiety as well as for sites of burns on the skin to avoid blisters formation (Akram, Hamid, Khalil, Ghaffar, Tayyaba, *et al*., 2014). Heart disease risk factors, micro vascular function, and skin tension may be improved by green tea with its several properties (Suzuki-Sugihara, Kishimoto, Saita, Taguchi, Kobayashi *et al*., 2016; Bogdanski, Suliburska, Szulinska, Stepien, Pupek-Musialik *et al*., 2012). Green tea is also a great anticancer agent and protects the liver as well (Yu, Deng, Lu, Liu, Li *et al*., 2014; Aboulwafa, Youssef, Gad, Altyar, Al-Azizi *et al*., 2019). Black tea is traditionally used as an aphrodisiac (Ratnasooriya and Fernando., 2008).

A higher percentage of sperm count, and motility was demonstrated in rats that consumed rooibos (*Aspalathus linearis)* and green tea (*Camellia sinensis)* as compared to the other groups (Awoniyi, Aboua, Marnewick, and Brooks, 2012). Also, an increased catalase activity was observed in rats that were supplemented with rooibos (a South African red

bush herbal infused tea originating from the family of legume) and green tea, as well as high levels of superoxide dismutase concentration and sperm glutathione (Morton, Julia F.,1983; Awoniyi, Aboua, Marnewick, and Brooks., 2012).The levels of ROS as well as lipid peroxidation showed a reduction tendency as compared to the control group when the rats were supplemented with rooibos and green tea (Awoniyi, Aboua, Marnewick, Du Plesis, and Brooks., 2011). Furthermore, rooibos and green tea extracts may increase antioxidants defence mechanisms and give protection against induced oxidative damage, improving sperm function and quality (Awoniyi, Aboua, Marnewick and Brooks., 2012).

In another study, the aqueous extracts of white tea and green tea were evaluated, and it was found that epigallocatechin-3-gallate was the most abundant catechin in white tea, as it was twice as much in the white tea extract (Dias, Alves, Tomás, Socorro, Silva and Oliveira., 2014). It was also found in another study about white tea antioxidant properties that antioxidant potential, lipid peroxidation, and viability of spermatozoa were positively affected the most with white tea extract supplementation (Dias, Alves, Tomás, Socorro, Silva and Oliveira., 2014). The media antioxidant potential had an increase mostly with white tea supplementation, which was in line with the decrease in lipid peroxidation as well as sperm antioxidant potential (Dias, Alves, Tomás, Socorro, Silva and Oliveira., 2014). The findings in this study indicate that white and green tea can restore the spermatozoal viability, positive effects on lipid peroxidation, as well as spermatozoa antioxidant potential with all of these more abundant in white tea than green tea (Dias, Alves, Tomás, Socorro, Silva and Oliveira, 2014).

Traditional practitioners in Sri Lanka recommend black tea for improvement of sexual function and for delaying ejaculation (Sharma, Joshi, Baldi, Khatri and Dube., 2013). *Camellia sinensis* was shown to improve rat sperm parameters (Das and Karmakar., 2015; Opuwari and Monsees., 2020a; 2020b). On the other hand, Opuwari and Monsees (2015) revealed that both green and black tea have anti-androgenic effects thereby reducing testosterone production in TM3 Leydig cells. Despite the extensive research studies done on animals, there is no evidence of research conducted on the effects of *Camellia sinensis* on human sperm functions.

1.2 Problem statement

With the increasing incidence of male infertility and the use of highly expensive methods of treatment such as assisted reproductive therapy as well as the associated side effects, there is a great need to provide alternative, cheap, and accessible treatment with little or no side effects. Medicinal plants naturally possess antioxidant properties that may be beneficial in improving sperm quality and fertility. *Camellia sinensis* with its antioxidant properties and other useful properties reduces oxidative stress and might thereby improve male sexual health problems. The plant is readily available and convenient to most of the population and with little or no side effects. There is no evidence of research conducted on the effects of *Camellia sinensis* on human sperm functions. Therefore, this study's aim is to determine the effects of *Camellia sinensis* on human sperm functions (motility, vitality, capacitation-and-acrosome reaction, mitochondrial membrane potential, reactive oxygen species and DNA fragmentation).

1.3 Purpose of the study

1.3.1 Aim

The study's aim was to determine the effects of *Camellia sinensis* (green and black tea) on male reproductive functions using human sperm.

1.3.2 Objectives

- To determine the effect of *Camellia sinensis* on sperm vitality, sperm motility and sperm mitochondrial membrane potential using eosin and negrosin stain, sperm class analysis (SCA) and DePsipher, respectively.
- To determine the effect of *Camellia sinensis on* oxidative stress in sperm by reactive oxygen species production and DNA fragmentation in sperm using dihydroethidium (DHE) stain and terminal deoxynucleotidyl transferase dUPT nick end labeling (TUNEL) assay, respectively.
- To determine the effect of *Camellia sinensis* on sperm acrosome reaction and capacitation using capacitation and acrosome reaction (CTC) assay.

- To compare the effects of *Camellia sinensis* in normal and abnormal human sperm.

1.3.3. Research question

What are the effects of *Camellia sinensis* on human sperm function *in vitro*?

CHAPTER 2: LITERATURE REVIEW

2.1 Infertility

A couple is said to be infertile when they are incapable of falling pregnant following one year of consistent unprotected sexual intercourse (Vander Borght, and Wyns., 2018; Fisher and Hammarberg., 2012; Gunes, Al-Sadaan and Agarwal., 2015). Another form of infertility is secondary infertility which is defined as the incapability of a couple to fall pregnant for the second time after they have successfully conceived for the first time. This secondary infertility shares many of the causes of primary infertility and has an incidence of about 70-80% of men (Vanlangenhove., 2018). At least one in six couples every year are diagnosed with infertility and accounts for 48.5 million couples per year globally (Agarwal, 2015; Tabong and Adongo., 2013). Sexual health problems are mostly common and highly prevalent among ageing male adults with statistics indicating that 20 – 30 % of male adults are faced with at least one sexual health disorder (Gunes, Al-Sadaan and Agarwal., 2015). The incidence of infertility is about 25% in couples who are trying to conceive, out of which 15% of them seek medical attention and 5% remain childless (Inhorn and Patrizio, 2015). Males contribute 50% to this childlessness (Agarwal *et al*., 2015).

There are environmental and lifestyle factors that contribute highly to male infertility, including cigarette smoking, alcohol intake and use of illicit drugs, obesity, psychological stress, advanced paternal age, dietary practices and coffee consumption (Durairajanayagam., 2018). Other factors including testicular heat stress, intense cycling training, lack of sleep and exposure to electromagnetic radiation from mobile phone use also play a role in male infertility (Durairajanayagam., 2018). The combination of environmental, lifestyle and dietary factors contribute wholly to poor sperm quality (Sharma, Biedenharn, Fedor and Agarwal., 2013). Other factors that reduce sperm quality are occupational exposure to various chemicals, heat, radiation and heavy metals (Sengupta., 2013). Furthermore, environmental oestrogen and pesticides have also been associated with spermatozoa damage (Sengupta., 2013). The male foetal and/or neonatal exposure to increased environmental oestrogen level is also considered to be associated with the decline in sperm number (Carré, Gatimel, Moreau Parinaud and Léandri., 2017). Cigarette smoking is known to be most common among men worldwide.

Smoke known as a somatic cell mutagen and carcinogen has a high chance of badly affecting the male reproductive health (Kovac, Khanna and Lipshultz., 2015). It is also determined that poor sperm function in sperm penetration assays is associated with cigarette smoking (Sharma, Harlev, Agarwal and Esteves., 2016).

Oxidative stress has been linked to the idiopathic causes of infertility due to the excessive production of ROS that eventually overpowers the antioxidant defence system, consequently damaging the spermatozoa (Agarwal, Aponte-Mellado, Premkumar, Shaman and Gupta., 2012). Idiopathic cause of male infertility can be described as an early ageing of testis promoted by ischemia in association with defective mitochondrial genome that controls oxidative phosphorylation (Ko, Sabanegh Jr and Agarwal., 2014). In industrialised communities, there has been an increasing decline in sperm count over the last few years (Carré, Gatimel, Moreau, Parinaud and Léandri., 2017). Age related disorders also play a role in the causation of infertility (Vander Borght and Wyns., 2018).

2.2 Causes of male infertility

2.2.1 Oxidative stress and reactive oxygen species

When oxidants are more than antioxidants, peroxidation products develop and results in OS (Birben, Sahiner, Sackesen, Erzurum and Kalayci., 2012). Oxidative stress has been associated with several diseases such as cancer, connective tissue disorders, ageing, infection, inflammation, HIV, as well as male infertility (Rahman, Hosen, Islam and Shekhar., 2012). There is normally a balance between ROS generation and scavenging in male reproductive tract. Consequently, a required amount for the regulation of normal sperm function is left in minimal amounts (Kratz and Piwowar., 2017). Those normal functions include sperm capacitation, acrosome reaction and sperm oocyte fusion (Kratz and Piwowar., 2017). When the production of ROS exceeds the minimal amounts required, the defence strategies of spermatozoa are tempered with and cannot perform their normal duties, resulting in OS (Costantini., 2014). This includes cellular components like lipids, proteins, nucleic acid, and sugars as they are potential OS targets (Costantini., 2014).

Oxygen is highly required for life support and its metabolites such as ROS interfere with cell functioning and tamper with the survival of the cell (Bardaweel, Gul, Alzweiri, Ishaqat, ALSalamat and Bashatwah., 2018). Spermatozoa is one of the cells that live in aerobic

conditions and is constantly exposed to oxygen paradox (Meyers., 2012). Therefore, there must be the disabling of ROS so that only a minimum amount required for normal cell function is kept. It is very important to have different types of antioxidants to keep control of ROS or oxidants in protection of spermatozoa (Ko, Sabanegh Jr and Agarwal., 2014). It is not only the fluidity of the plasma membrane of the sperm that is attacked by OS, the DNA integrity of the sperm nucleus is also affected (Aitken, Smith, Jobling, Baker and De Iuliis., 2014). The restricted distribution of cytoplasmic space as well as limited volume (which contains plenty of defensive enzymes) reduces significant antioxidant protection and result in oxidative attack (Wang and Hai., 2016). The susceptibility of sperm membrane lipids to OS is exaggerated by the significant amounts of polyunsaturated fatty acids, and it is further worsened due to the cells generating ROS actively to facilitate the increment of tyrosine phosphorylation associated with sperm capacitation (Aitken, Gibb, Baker, Drevet and Gharagozloo., 2016). It is under these conditions that, the sperm membrane lipids automatically turn an intrinsic apoptotic pathway characterised by mitochondrial ROS generation, loss of mitochondrial membrane potential, caspase activation, phosphatidylserine exposure and oxidative DNA damage (Aitken, Baker, and Nixon., 2015). Spermatozoa only possess the first enzyme in the base excision repair pathway, 8-oxoguanine DNA glycosylase in response to OS. The formation of a basic site which establishes the DNA backbone and generation of strand breaks is catalysed by this enzyme (Aitken, Gibb, Baker, Drevet and Gharagozloo., 2016).

The generation of ROS has become a major setback on sperm normal function and quality because of its harmful effects when its levels are high (Aitken, Smith, Jobling, Baker and De Iuliis., 2014). Recently, it has been reported that high levels of ROS are detected in semen samples of 25 to 40 percent of men who are diagnosed of infertility (Agarwal, Sharma, Sharma, Assidi, Abuzenadah, Alshahrani, Durairajanayagam and Sabanegh., 2014). Studies show that low ROS levels can improve the binding of human spermatozoa to zona pellucida, which is an effect reversed by glutathione reductase (Saraswat, Kharche and Jindal., 2014). In other studies, incubating spermatozoa with low levels of hydrogen peroxide was found to be able to stimulate sperm capacitation, hyper-activation, acrosome reaction, and oocyte fusion (MoraEsteves and Shin., 2013). Nitric oxide and superoxide anion have proven to be able to improve sperm capacitation and acrosome reaction. Although the mammalian spermatozoal physiology seems paradoxical, the low

levels are beneficial and high levels appear to be harmful to sperm cells (de Andrade, Arruda, Torres, Pieri, Leite, Celeghini, Oliveira, Gardés, Bussiere and Silva., 2018).

The prevention of ROS formation is the first line of defence against OS (Ighodaro and Akinloye., 2018). One of the examples is the binding of metal ions, iron, and copper ions specifically, that function to prevent ROS from starting a chain reaction (Tvrda, Peer, Sikka and Agarwal., 2015). Sperm lipid peroxidation (LPO) and DNA damage is mainly controlled by chelation of transition metals. Transition metals produce secondary and highly reactive oxidants when they become loosely packed, especially hydroxyl radical (Tvrda, Peer, Sikka and Agarwal., 2015). The main issue is for the chain reaction (a compound that carries an unpaired electron will react with another compound to form an unpaired electron) to be broken by the formation of non-radical end products because free radicals have the likelihood to move towards the chain reaction (Tvrda, Peer, Sikka and Agarwal., 2015). Vitamin E (α- tocopherol) is a chain-breaking antioxidant that inhibits LPO in membranes by scavenging peroxyl and alkoxyl radicals. External reducing agents such as ascorbate or thiols determine the ability of vitamin E to maintain a steady state rate of peroxyl radical reduction in the plasma membrane by recycling vitamin E (Franco, Chaveiro, Góis and da Silva., 2013). The damage caused by OS can be repaired in some instances but since spermatozoa do not have cytoplasmic enzyme systems required to carry out the repair process, they are unable to repair. This makes spermatozoa to be vulnerable to oxidative damage (Aitken, Smith, Jobling, Baker, and De Iuliis., 2014).

The few recognised causes of male infertility is the excessive generation of reactive oxygen species by abnormal spermatozoa and contaminating leukocytes (leukocytospermia) (Aitken and Baker., 2013). Other environmental factors include pesticides, exogenous oestrogens, and heavy metals, which negatively affect the formation of sperm since male sperm counts were declined (Sharma, Biedenharn, Fedor and Agarwal., 2013). With older people being the most affected, age may be considered as one additional factor that induce OS (Aitken, Smith, Jobling, Baker and De Iuliis., 2014).

2.2.2 Antioxidants and free radicals

Compounds that have oxidation inhibiting properties are known as antioxidants (Gulcin and Beydemir., 2013). The chemical reaction that yields free radicals that lead to chain reactions with cell damaging potential can be broken or terminated by antioxidants such as thiols and ascorbic acid (Agarwal, Virk, Ong, and Du Plessis., 2014). There are sources of different enzymatic and non-enzymatic antioxidants such as ascorbic acid that are contained in the seminal plasma, that serve as protection against oxidative stress in the spermatozoa (Song, Norkus and Lewis., 2006). Due to high density of the mitochondria with the possibility of leaking oxygen radicals in the cytoplasm, the scavenging oxygen ability of spermatozoa may be limited. Therefore, the capacity of the antioxidants must be present in the seminal fluid as well (Song, Norkus and Lewis.,2006). That is why, the protection against ROS and the prevention of other damages are of critical importance and they can be provided by both enzymatic and non-enzymatic antioxidants (Song, Norkus and Lewis., 2006).

There are overlapping antioxidants like glutathione and enzymes that are maintained to balance OS and are internally produced, or they use dietary antioxidants like vitamin C and vitamin E (Carocho and Ferreira., 2013). Antioxidants dispose, scavenge, and suppress ROS formation (Bansal., 2015). There is an impairment of the non-enzymatic antioxidant capacity of the seminal plasma in infertile, compared to fertile men, indicating a possible relationship between decreased non-enzymatic antioxidant capacity and male infertility (Walczak–Jedrzejowska, Wolski and Slowikowska–Hilczer., 2013).

The antioxidant protection system of humans has been highly evolved to protect the cells and organs against ROS (Henkel, Samanta, and Agarwal., 2018). The evolution is made up of different components, endogenous and exogenous, that function together to neutralise free radicals. Antioxidants taken in the form of food like ascorbic acid (vitamin C), tocopherol and tocotrienols (vitamin E), carotenoids, and other low molecular weight compounds are some of the components included (Henkel, Samanta, and Agarwal., 2018). The role of antioxidants and vitamins in infertility has been evaluated by several studies in association with male fertility. From these, a few have proven to be of benefit in antioxidant therapy in the treatment of male infertility (Yao and Mills., 2016). It was demonstrated by Yao and Mills. (2016) that vitamin C from diet has an effect that is beneficial to sperm DNA in males who smoke. vitamin C was tested in a placebo-controlled trial and demonstrated an improvement of sperm quality (Yao and Mills., 2016).

2.2.3 Oxidative Stress-Induced DNA damage

The characteristic tight packaging of the DNA and the antioxidant present in seminal plasma are the two factors that protect sperm DNA from oxidative insult (Bisht, Faiq, Tolahunase and Dada., 2017). The sperm was exposed to artificially produced ROS and resulted in a distinct rise in DNA damage with all bases affected, chromosomal rearrangements with base-free sites production, frameshifts, cross-links, and deletions (Simon, Emery and Carrell., 2017). The single and double strands DNA breaks with high frequencies are shown to be associated with OS (Agarwal, Virk, Ong and Du Plessis., 2014). Studies strongly suggest that high levels of ROS facilitate fragmentation of DNA, and this is frequently detected in spermatozoa of infertile men (Agarwal, Virk, Ong and Du Plessis., 2014).

The intactness of the sperm chromatin and the breakage of double and single strands at high frequencies has been shown to occur due to OS (Evenson, Djira, Kasperson, and Christianson., 2020). Furthermore, the sperm nuclear DNA damage has been shown to negatively affect natural fertility (Cho, Esteves and Agarwal., 2016). The sperm quality measure by its chromatin/DNA is an independent measure of sperm quality which gives better diagnostic and prognostic results than all other standard sperm parameters such as sperm concentration, motility, and morphology (Komiya, Kato, Kawauchi, Watanabe and Fuse., 2014).

2.2.4 Varicocele and DNA damage

It may be suggested that sperm dysfunction in varicocele is to some extent related to OS because in previous studies, it has been proven that ROS in sperm is higher in men with varicocele (Jensen, Østergren, Dupree, Ohl, Sønksen and Fode., 2017). Varicocele patients display a greater ROS generation and high levels of nitric oxide, which is related to the generation of ROS (Cho, Esteves and Agarwal., 2016). Damage to sperm DNA appears to be related to high levels of OS in semen in men with varicocele (Jensen, Østergren, Dupree, Ohl, Sønksen and Fode., 2017). About 15% of men in the overall population are with varicocele, with 19-41% of infertile men presenting with varicocele (Vanlangenhove., 2018). These statistics highly indicate that the decline in infertility among men could be caused by varicocele (Vanlangenhove., 2018). There are other experiences such as altered spermatogenesis, which may be related to several factors such as reflux of toxic metabolites from adrenal or renal origin, disturbed hormone status,

spermatic venous hypertension, testicular hypoxia secondary to stasis, and abnormal temperature regulation (Hamada, Esteves, and Agarwal., 2015).

Hamada, Esteves and Agarwal. (2015) demonstrated that there was a substantial reduction of DNA polymerase levels in extracts of testicular tissues from men who are infertile and having varicocele. It has been suggested that spermatogenesis of patients with varicocele may have deleterious effects from DNA polymerase activities reduction (Shiraishi, Matsuyama and Takihara., 2012). Seminal OS also play a role in sperm DNA damage since it was found that high levels of seminal OS and reduced total antioxidant capacity were detected in both men who are fertile and infertile, as well as men who are clinically diagnosed with varicocele (Wang, Zhang, Lin, Zhang and Zhang., 2012).

It is not clearly understood as to how varicocele affect and damage spermatogenesis and sperm quality (Cho, Esteves and Agarwal., 2016). In this regard, it can be said that OS is an important factor as increased levels of ROS were detected in patients who are infertile with varicocele as well as declined levels of seminal and blood plasma antioxidants (Cho, Esteves and Agarwal., 2016). There is a positive relationship between ROS levels and the degree of varicocele, and ROS levels may be expected to decline after varicocelectomy treatment (Cho, Esteves and Agarwal., 2016). Recent studies show a significant rise in sperm DNA fragmentation index as compared to healthy controls. Furthermore, it was noticed that there is a higher amount of OS among infertile patients with varicocele as compared to infertile patients with normal genital examination and controls (Choucair, Rachkidi, Raad, Saliba, Zeidan, Jounblat, Jaoude and Hazzouri., 2016). As it appears, there is a correlation between spermatozoal DNA damage and high semen OS levels in infertile patients with varicocele (Cho, Esteves and Agarwal., 2016). There is also an issue of apoptosis where a higher level is observed in spermatozoa of patients with varicocele than in healthy patients, which may result in significant DNA damage (Cho, Esteves and Agarwal., 2016).

2.3 Sperm structure and function

2.3.1 Male reproductive system

The male reproductive system (Figure 2.1) is made up of several sex organs that are involved in the human fertilisation process. These organs are situated on the outside of

the body and on the inside in the pelvic region (Sharma, Hanukoglu, and Hanukoglu., 2018). The main organs that take part in sexual intercourse with the aim of fertilising are the penis and the testicles (O'Grady., 2021). These two external genital organs work hand in hand in the production of semen and spermatozoa (O'Grady., 2021).

There are also internal organs in the male reproductive system which include the testis, epididymis, vas deferens, as well as accessory glands (O'Grady., 2021). The function of testis as an internal sex organ is to produce spermatozoa through mitotic division in the seminiferous tubules (Tabara, Shiraishi, Takii, Fujimoto, Nakai *et al*., 2021). It also functions in synthesising and secreting androgens that in turn regulate the functions of male reproduction (Darbandi, Darbandi, Agarwal, Sengupta, Durairajanayagam *et al*., 2018). The androgens are produced in the Leydig cells which are in the interstitium in between seminiferous tubules (Zirkin and Papadopoulos., 2018). When sperm is produced in the seminiferous tubules, it is then transported into the epididymis (Sharma and Hanukoglu., 2019). In that period of movement, the sperm matures and gets concentrated (Sharma and Hanukoglu., 2019). The main role of Vas deferens or sperm duct is to carry the mature spermatozoa from the epididymis to the ejaculatory duct (Comeau and Benhalima., 2018). The accessory glands of the male reproductive system (seminal vesicles, prostate gland and bulbourethral glands) provide fluid for lubricating the duct system and nourishment of sperm cells (Srivastava, Kumari, and Gond., 2020).

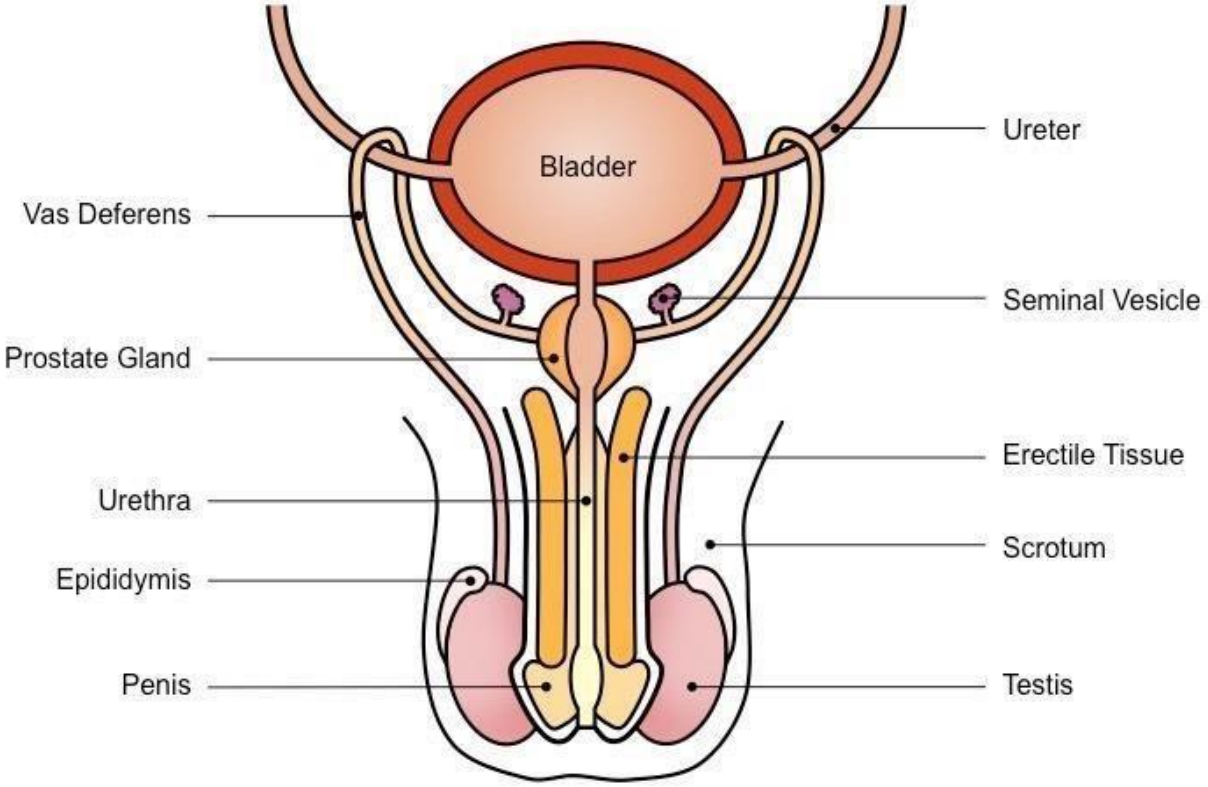


Figure 2.1: Male reproductive organs

https://www.w3spoint.com/wpcontent/uploads/2019/08/word-image-112.jpeg; accessed on 15 October 2021

2.3.2 Spermatogenesis and hormone regulation

Male sex cells or gametes are called spermatozoa and are produced in the testes (Justine., 2002). All sex cells (male and female) carry a total of twenty-three chromosomes each from meiosis (Justine., 2002). These cells are mainly involved in the reproduction process where there is interaction between male and female gametes (Richardson., 2013).

The process in which the haploid spermatozoa mature from germ cells in the testis is known as spermatogenesis, and it begins with stem cell division by mitosis to produce two types of cells (Jan, Hamer, Repping, de Rooij, van Pelt *et al*., 2012). The two cell types are A-dark and A-pale, which replenish the stem cells and type B, which differentiate into primary spermatocytes. This proceeds to a process called meiosis, where in meiosis I, the primary spermatocytes divide into secondary spermatocytes which further divide into two haploid spermatids through to meiosis II (Nishimura, and L'Hernault., 2017). The

result of these processes is the mature spermatozoa known as sperm cells (Nishimura, and L'Hernault., 2017; Figure 2.2).

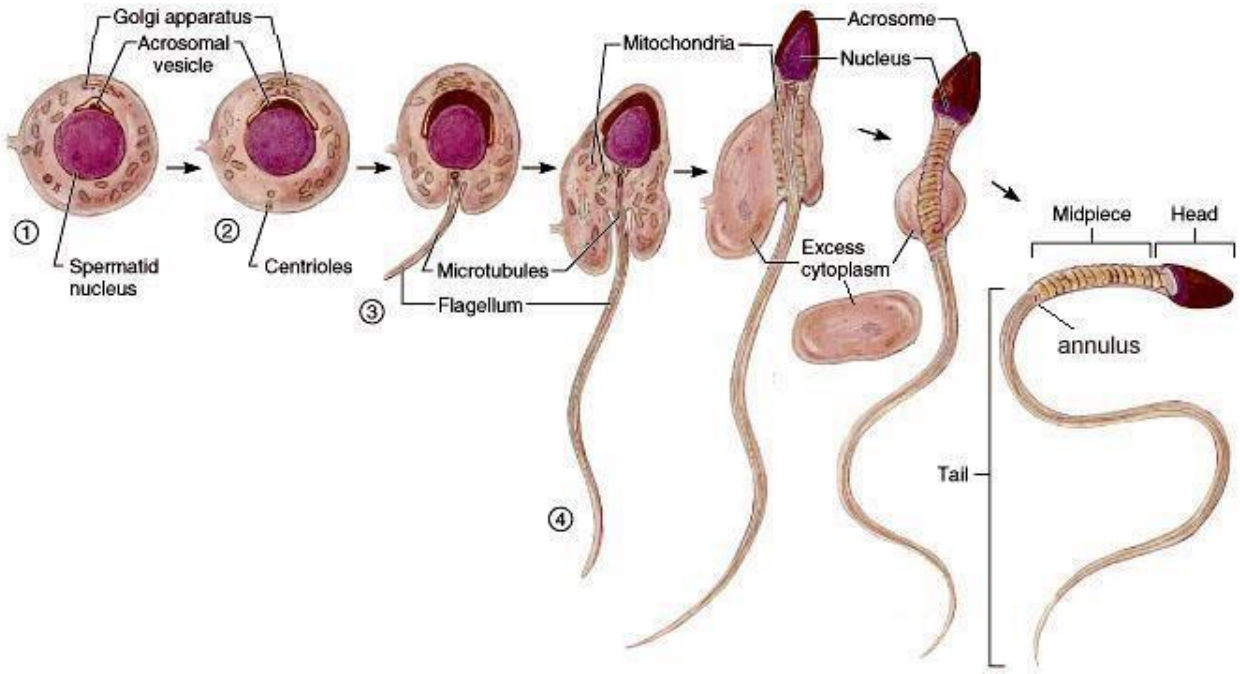


Figure 2.2: Spermatogenesis

https://image.slidesharecdn.com/spermatogenesis-171112112558/95/spermatogenesishttps://image.slidesharecdn.com/spermatogenesis171112112558/95/spermatogenesis-9-638.jpg?cb=15104861369-638.jpg?cb=1510486136 accessed on 15 October 2021

There are several hormones that are involved in the process of spermatogenesis which include testosterone, GnRH, and luteinising hormone (LH). The level of testosterone in blood is monitored by the hypothalamus which is a crucial role (Dohle, Arver, Bettocchi, Kliesch, Punab and Ronde., 2012). When testosterone levels are low, it is an indication that testicular activity is low. This stimulates the hypothalamus to release a releasing hormone called gonadotrophin releasing hormone (GnRH) through a negative feedback mechanism (Morelli, Sarchielli, Comeglio, Filippi, Vignozzi *et al*., 2014). This GnRH acts as a stimulus to produce follicle stimulating hormone (FSH) and luteinising hormone (LH) by flowing into the pituitary gland (Morelli *et al*., 2014; Figure 2.3). Thereafter, testosterone is produced in the testes by the Leydig cells through stimulation of LH from the anterior pituitary gland (Opuwari, Matshipi, Phaahla, Setumo, Moraswi *et al*., 2020; Figure 2.3).

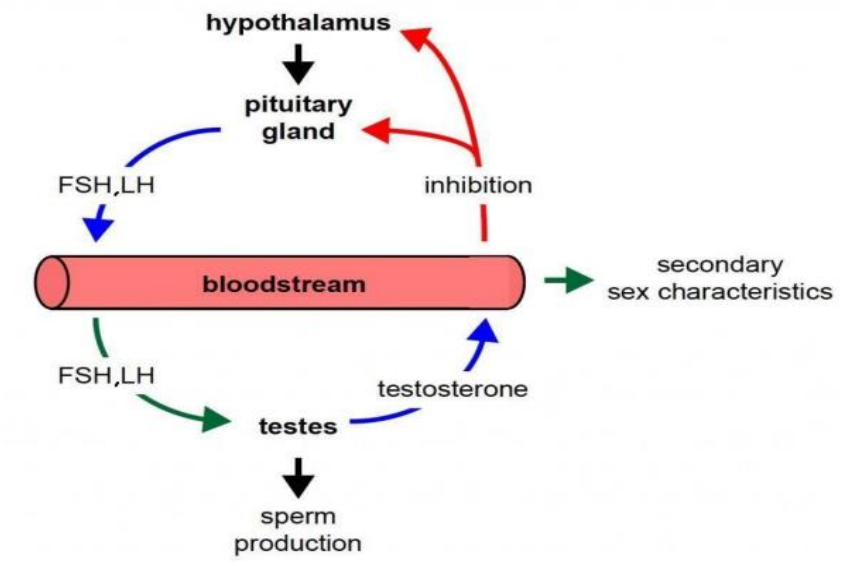


Figure 2.3: Hormone regulation of spermatogenesis

https://bio1220.biosci.gatech.edu/files/2018/10/male-reprouction-hormonal-regulationhttps://bio1220.biosci.gatech.edu/files/2018/10/male-reprouction-hormonalregulation-1024x815.png1024x815.png accessed on 15 October 2021

2.3.3 Impairment of sperm motility and vitality

Sperm motility may be described as the proper movement of sperm cells through the reproductive tract of a female to be able to reach the egg for fertilisation (Suarez., 2016). Reduced sperm motility and increased formation of ROS have been associated with decreased axonemal protein phosphorylation and sperm immobilisation, which are both related with reduced membrane fluidity necessary for fusion of oocyte (Walczak–Jedrzejowska, Wolski and Slowikowska–Hilczer., 2013).

It is hypothesised that the movement of the sperm tail takes place when the microtubules that are powered by adenosine triphosphate slide past one another, moving by means of their dynein arms (Lin and Nicastro., 2018). These dynein arms are the actual ATP molecules (Lin and Nicastro., 2018). This technique is used to give an explanation to the motility of the 9+2 sperm tails, cilia and flagella. It is of clinical usefulness to determine several glycosidases in semen and to assess them (Li, Sun, Ni, Shi, Wang, Isa, Ge, Jiang, Fan, Ma and Yang., 2020). There is a relationship between alpha-glycosiderase and

motility, which is that the abnormal function of the epididymis is directly proportional to the decreased production of alpha glycosiderase (Li, *et al*., 2020).

2.3.4 Capacitation and acrosome reaction

Capacitation is the required step to render spermatozoa competent for fertilisation; and it is the second last step in mammalian spermatozoa maturation (Gervasi, and Visconti., 2016). This process takes place after ejaculation *in vivo* by entering the female reproductive tract (Gervasi and Visconti., 2016). The physical appearance of the spermatozoa before and after capacitation is normal and mature, as only physiological changes occur during this process (Puga Molina, Luque, Balestrini, Marín-Briggiler, Romarowski and Buffone., 2018). The secretion of sterol-binding albumin, lipoproteins, proteolytic enzymes, and glycosidic enzymes like heparin is aided by the uterus in the steps of capacitation (Sajeevadathan., 2018).

The acrosome cap and acrosome make the acrosome region, which is a structure that is cap-like, that covers the frontal half of the sperm head and is formed during the biogenesis of the acrosome accompanied by spermatid differentiation during spermiogenesis (Wang, *et al*., 2014). The Golgi complex, for which the acrosome is a product of, has several contents, including acrosin enzyme found in the acrosomal matrix, and there are polysaccharides like mannose, hexamine, and galactose present in the acrosome as well (Wang, *et al*., 2014). The space between the nuclear membrane and plasma membrane is solely occupied by the acrosome. The acrosome consists of two acrosomal membranes (an inner acrosomal membrane and an outer acrosomal membrane), of which the outer acrosomal membrane encircles the plasma membrane whereas the inner acrosomal membrane encircles the nuclear membrane (La Spina, Stival, Krapf, and Buffone., 2017).

For sperm to fertilise the female egg, it must first fuse with the plasma membrane of the female egg (Inoue, Hamada, Kamikubo, Hirata, Kataoka, *et al*., 2013). There are a few problems encountered with fusion and penetration of the egg cell by the sperm cell; therefore, the process of acrosome reaction helps in preparation of the sperm cell for fusion and penetration (Tilney., 2012). The zona pellucida of the egg is the one responsible for acrosome reaction initiation; and when it is approached by the sperm cell, the contents of the acrosome are exposed because of fusion between the plasma membrane and the outer acrosomal membrane surrounding the acrosome (Carroll., 2018). The enzyme is converted into acrosin which is an active form capable of acting on

the membrane. This procedure takes place when the glycoproteins of the ovum membrane encounter the acrosome (Tulsiani and Abou-Haila., 2012). In turn, the sperm cell can penetrate the egg for the process of fertilisation to take place (Tulsiani and Abou-Haila., 2012). These enzymes of the acrosome are known as lysosomal enzymes (Nelson., 2012).

The acrosome plays an important role during fertilisation such as recognition of the oocyte/egg (Liao, Chang, Liang, Chung, Wei *et al*., 2018). The sperm cell is stimulated by acrosome enzymes to move towards the egg by encountering diffusible molecules from the egg fluid. This process is called chemotaxis (Liao, *et al*., 2018). The navigation of sperm cell towards the egg is allowed by chemotaxis via chemical signals, as well as thermotaxis (Bahat, and Eisenbach., 2006). With this said, it can be deduced that chemotaxis is an important process that ensure that the process of fertilisation takes place within the correct species or same species where the target gamete is recognised by primary ligands (proteins) (Springate and Frasier., 2017). The acrosome reaction process is illustrated in the diagram below (Figure 2.4).

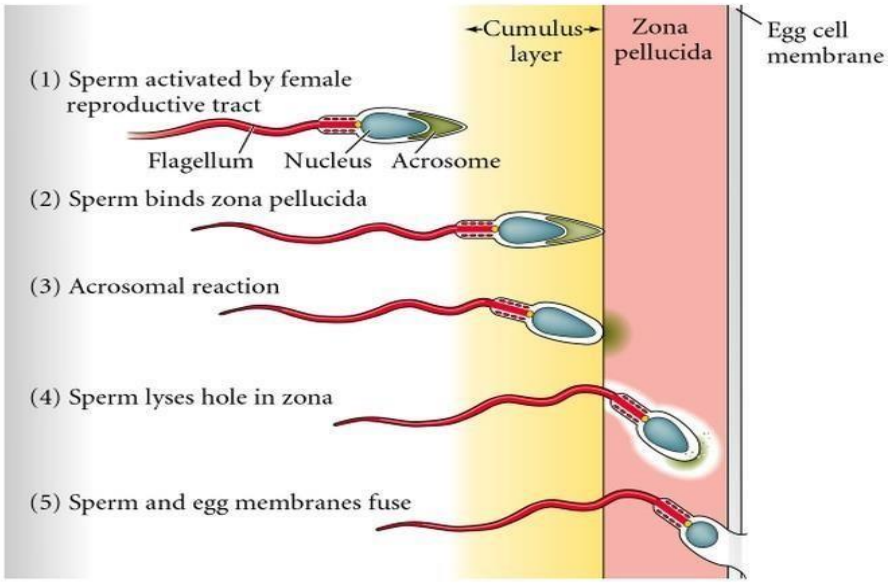


Figure 2.4: Acrosome reaction

http://courses.biology.utah.edu/bastiani/3230/DB%20Lecture/Lectures/a5fert.html accessed on 15 October 2021

2.4 *Camellia sinensis*

2.4.1 Description and definition

Camellia sinensis is described as an evergreen species that looks like a shrub or small tree belonging to the genus *Camellia* of flowering plants and family of Theaceae. Its common names include tea plant, tea shrub and tea tree (Kumar., 2018). The leaves of this plant are about 4-15 cm long and 2-5 cm wide with 7-8 petals and fresh leaves of the plant contain about 4% caffeine as well as related compounds such as theobromine (Ahmeda, Zangeneh and Zangeneh., 2020). Pressing the seeds of *Camellia sinensis* is used to make tea oil, a sweetish seasoning and cooking oil which is different from tea tree oil (oil that is used for medical and cosmetic reasons) (Ukwubile, Samagoro and Nuhu., 2018). *Camellia sinensis* is processed as black tea, white tea, green tea and oolong tea (Figure 2.4).

Figure 2.5: Different types of *Camellia sinensis*

https://i.pinimg.com/originals/4b/a4/d5/4ba4d5cbd68401723c4ce32933b77a82.jpg accessed on 15 October 2021

Green tea, black tea, and oolong as well as white tea are produced from *Camellia sinensis* based on the way they were processed (Jiang, Engelhardt, Thräne, Maiwald and Stark., 2015). Green tea is unfermented, while oolong is fermented and allowed to partially oxidise, while black tea is completely fermented and oxidised (Perry., 2001; Chilton, Burton and Reid., 2015). The young leaves of the tea and its buds covered with tiny, silvery hairs are used to prepare white tea (Rusak, Komes, Likić, Horžić and Kovač., 2008; Dias., 2013). Sunlight has an effect of increasing chlorophyll when in contact with the leaves. Therefore, the young leaves are kept away from contact with sunlight to maintain the white colour during growth (Rusak *et al*., 2008; Dias., 2013). The drying and steaming of white tea takes place all at once. Its compounds include alkaloids (caffeine and theobromine), polyphenols, amino acids, proteins, carbohydrates, volatile organic compounds, and trace elements (Çelik, 2006; Mahmood, Akhtar, and Khan., 2010; Namita *et al*., 2012). Green tea comprises of more phytochemicals compared to black and oolong tea (Lee, Lee, Chung, Kim, Kim *et al*., 2015).

Different tea qualities are based on how old the tree is or how long the tree has grown. The light green small leaves are originally harvested for tea and have some white hair underneath, whilst the older leaves are deep green in colour (Zhao, Li, Liu and Yang., 2014). These differences are the ones that determine the tea qualities since they have different chemical compositions (Zhao, Li, Liu, and Yang., 2014). It was found by Chinese scientists that *Camellia sinensis* contains about three billion base pairs after sequencing its genome. This was found to be more than most plants sequenced before (Wei, Yang, Wang, Zhao, Liu *et al*., 2018).

2.4.2 Cultivation

Camellia sinensis is largely and mainly cultivated in tropical and subtropical climates, in areas with at least 127 cm (50 inches) of rainfall a year; and they preferably rather grow in rich and moist areas with full to partial sunlight and can grow in hardiness zones 7-9 (Ahmed, Stepp, Orians, Griffin, Matyas *et al*., 2014). However, the clonal one is commercially cultivated from the equator to as far north as Cornwall and Scotland on the UK mainland (van Driem., 2019). Many high-quality teas are grown at high elevations, up

to 1,500 meters (4,900 feet), as the plants grow more slowly and acquire more flavour (Hames., 2014).

Tea plants have a potential of growing into a tree if left unobstructed, nonetheless, cultivated plants are pruned to waist height for ease of plucking (Wei, *et al*., 2018). There are two principal varieties that are used, these are the small leaved Chinese variety plant (*Camellia sinensis*) and the large-leaved Assamese plant (*Camellia sinensis* assamica) used primarily for black tea (Dias., 2013). Figure 2.6 shows different stages of tea making and young leaves of *Camellia sinensis* (Figure 2.6A), dried leaves and fermented leaves (Figure 2.6 C) as well as the ready to consume home tea preparation (Figure 2.6C).

Figure 2.6: Leaves of *Camellia sinensis*

(A) Dried *Camellia sinensis* leaves (B) Fermented *Camellia sinensis* leaves (C) In store *Camellia sinensis* tea

Available at:

https://noonline.2021cheapbest.com/content?c=tea%20plant&id=25 accessed on15 October 2021

2.4.3 Properties and health benefits of *Camellia sinensis*

Tea is the most common beverage after water and has been used as medicine for many years globally (Figueiroa, *et al*., 2009). Scientific studies are exploring and discovering what has long been known by Chinese people and other people throughout the world (Sharma, Joshi, Baldi, Khatri and Dube., 2013). A study proved that there was a significant decrease in total cholesterol and low-density lipoprotein after treatment with

150 mg of green tea daily for 3 months (Prasanth, Sivamaruthi, Chaiyasut and Tencomnao., 2019).

In Sri Lankan traditional native medicine, traditional doctors use black tea brew of *Camellia sinensis* for treatment of urinary inconsistency, common cold and to suppress anxiety (Srivastava and Pandey., 2015). It is also used for sites of burns in the skin to prevent blisters formation. Black tea is also used for delaying of ejaculation and to improve sexual function, as recommended by some traditional practitioners of Sri Lanka (Srivastava and Pandey., 2015). *Camellia sinensis* (black, white, green and oolong teas) has several health benefits, including anticancer, hepatoprotective activities, anti-inflammatory, analgesic, antipyretic, anti-allergic activities, asthma and allergy, antimicrobial and antiviral activity, cardiovascular activities anti-schistosomiasis as well as anti-parasitic activities (Adeneye., 2016).

2.4.4 Antioxidant properties of *Camellia sinensis*

White tea (WT) is shown to have a higher concentration of polyphenols in total, total catechins, caffeine, gallic acid, theobromine, epigallocatechin (EGC) and epigallocatechin-3-gallate (EGCG) compared to green tea, black tea, and oolong (Sanlier, Atik and Atik., 2018). These higher concentrations of the major constituents may somehow be related to higher antioxidant activities of WT (Dias, Carrageta, Alves, Oliveira and Silva., 2019). There are also reports on WT containing higher concentrations of catechins and lower theaflavins and thearubigins concentrations than black and green tea (Dias, Carrageta, Alves, Oliveira and Silva., 2019).

There is a major consumption of black tea in some Asian countries such as China and Japan, Western countries, and the Middle Eastern parts of Africa (Nataraj, Manivasagam, Thenmozhi, Essa, and Khan., 2016). Black tea is rich in antioxidants that can impair disease causing cells by free radicals' neutralisation (Naveed, BiBi, Kamboh, Suheryani, Kakar *et al*., 2018). There is clinical evidence by research that black tea has benefits on heart related and heart diseases, and counteract endothelial dysfunction (Spoorthi, Gautham, More, and Maiti., 2018). Reports show that black tea contains as much TF-3 and TF-4 rich in antioxidants just as green tea has (Bhattacharya, Chatterjee, Mandal, Mukhopadhyay, Basu *et al*., 2020). The compounds of these are so broad and extremely effective. Therefore, they are useful, low cost, no side effects and readily available to the mass communities (Shahidi and Ambigaipalan., 2015).

2.4.5 Reproductive health benefits of *Camellia sinensis*

These teas are also known for their reproductive health benefits in males as they improve some if not all sperm parameters. Green tea consumption has proven to rise sperm concentration and viability in male rats and has shown to be a safe option for reproductive, kidney and liver health (Opuwari and Monsees., 2020b). There was an observation of higher sperm count and motility for rats that were consuming rooibos and green tea (*Camellia sinensis*) as compared to the other groups (Awoniyi, Aboua, Marnewick, and Brooks., 2012). There was also an observation of a significantly higher catalase activity in rats that were supplemented with rooibos and green tea, as well as high levels of superoxide dismutase concentration and sperm glutathione (Awoniyi., 2010). The levels of ROS as well as lipid peroxidation showed a reduction tendency as compared to the control group where the rats were supplemented with rooibos and green tea (Awoniyi, Aboua, Marnewick, Du Plesis, and Brooks., 2011). This may suggest that rooibos and green tea extracts may increase antioxidants defence mechanisms and give protection against induced oxidative damage, improving sperm function and quality (Awoniyi, Aboua, Marnewick and Brooks., 2012).

In another study, the aqueous extracts of white tea and green tea were evaluated. It was found that Epigallocatechin-3-gallate (EGCG) was found to be the most abundant catechin, as it was twice as much in the white tea extract (Dias, Alves, Tomás, Socorro, Silva and Oliveira., 2014). There was also an evaluation for storage media of antioxidants where it was found that spermatozoa antioxidant potential, lipid peroxidation, and viability were positively affected the most with white tea extract supplementation (Dias *et al*., 2014). The media antioxidant potential had an increase mostly with white tea supplementation, which was in line with the decrease in lipid peroxidation as well as sperm antioxidant potential (Dias *et al*., 2014). The findings in this study indicate that both white tea and green tea can restore the spermatozoal viability, positive effects on lipid peroxidation as well as spermatozoa antioxidant potential with all of these more abundant in white tea than green tea (Opuwari, and Monsees., 2020 (a); Dias, *et al*., 2014).

Traditional practitioners in Sri Lanka recommend black tea for improvement of sexual function and for delaying ejaculation (Sharma, Joshi, Baldi, Khatri and Dube., 2013). *Camellia sinensis* was shown to improve rat sperm parameters (Das and Karmakar., 2015). Opuwari and Monsees (2015) revealed that both green and black tea had antiandrogenic effect by the reduced production of testosterone in TM3 Leydig cells.

Black tea is also customarily used as an aphrodisiac, which could be due to its ability to elevate testosterone level (JianFeng, PengYing, ChengWei, TaoTao, YunGui and KaoShan., 2012). In another study, the intake of black tea caused no significant change in the secretion of testosterone in male rats (Opuwari and Monsees., 2015). Furthermore, green tea extracts administered over 4 weeks changed rat testis histology and function with subsequent inhibition of spermatogenesis and steroidogenesis (Das and Karmakar., 2015). In contrast to these, Satoh, Sakamoto, Ogata, Nagai, and Mikuriya *et al*. (2002) reported a significant rise of plasma testosterone in rats treated for eight weeks with high doses of green tea extract.

Natural compounds have been used customarily to cure diseases. For instance, positive effects on reproductive results have been reported using products derived from tea (*Camellia sinensis*), (Martins, Alves, Bernardino, Dias, Silva *et al*., 2014). Tea is one of the natural compounds that have been used for treatment of diseases, including fertility. Human spermatozoa were studied using tea extract to see if there is any improvement on certain aspects of its quality such as capacitation (De Amicis, Santoro, Guido, Russo, and Aquila., 2012). This study showed improvement on phosphorylation and cholesterol efflux via the oestrogen receptor pathway (De Amicis *et al*.,2012). There was extreme oxidative stress that was induced on the mice and then later treated with tea extract to analyse the parameters such as sperm concentration, motility and MMP hyperthermia, which proved to be beneficial after twenty-eight days of stress induction (Abshenas, Babaei, Zarei, Allahbakhshi and Sharififar., 2011; Ding, Wang, Wu, Zhao, Zhang, *et al*., 2015). A combination of two different types of teas, green and black tea seemed to improve ART at room temperature after incubation *in vitro* (Dias, *et al*. (2014). With that, it is deduced that as much as both teas show improvement, there is evidence that white tea has better antioxidant with ferric reducing power as compared to green tea with similar proportions of concentrations (Dias *et al*., 2014). The oral intake of white tea showed improvement on protein oxidation, lipid peroxidation, morphology, as well as sperm motility. The oral consumption of white tea showed improvement in testicular antioxidant power, reduced lipid peroxidation, protein oxidation, sperm motility and sperm morphology (Oliveira, Tomas, Dias, Martins, Rato, Alves *et al*., 2015). Therefore, this study's aims is to determine the *in vitro* effects of aqueous extract of *Camellia sinensis* on human sperm function.

CHAPTER 3: RESEARCH METHODOLOGY

3.1 Introduction

This chapter's purpose is to give a description of the methodology that was used in the study. The chapter includes details of the research methods, research design, sampling, inclusion criteria, study site, data collection, ethical considerations, and data analysis.

3.2 Research method

This is an experimental study with the use of quantitative methods.

3.2.1 Experimental study

An experimental study is a prospective study where an intervention is allocated to different groups which are followed-up over time to identify those who develop the outcome under consideration (Omair., 2014). This study is an experimental study since spermatozoa were incubated in different concentrations of *Camellia sinensis* (green and black tea).

3.2.2 Quantitative study

Quantitative method is a type of research that involves identifying variables (Creswell and Clark., 2017). The information acquired must be measurable as a quantity and expressed numerically. This study was quantitative because at the end, the results obtained were expressed as quantities. There were measurements of sperm viability, mitochondrial membrane potential, sperm motility, capacitation and acrosome reaction, reactive oxygen species and DNA fragmentation following incubation with different concentrations of *Camellia sinensis* (green and black tea).

3.3 Research experimental design

3.3.1 Research setting

The *in vitro* experimental procedure as well as the extraction of the plant was carried out at the University of the Western Cape, Department of Medical Biosciences, Andrology Research Laboratory (Figure 3.1).

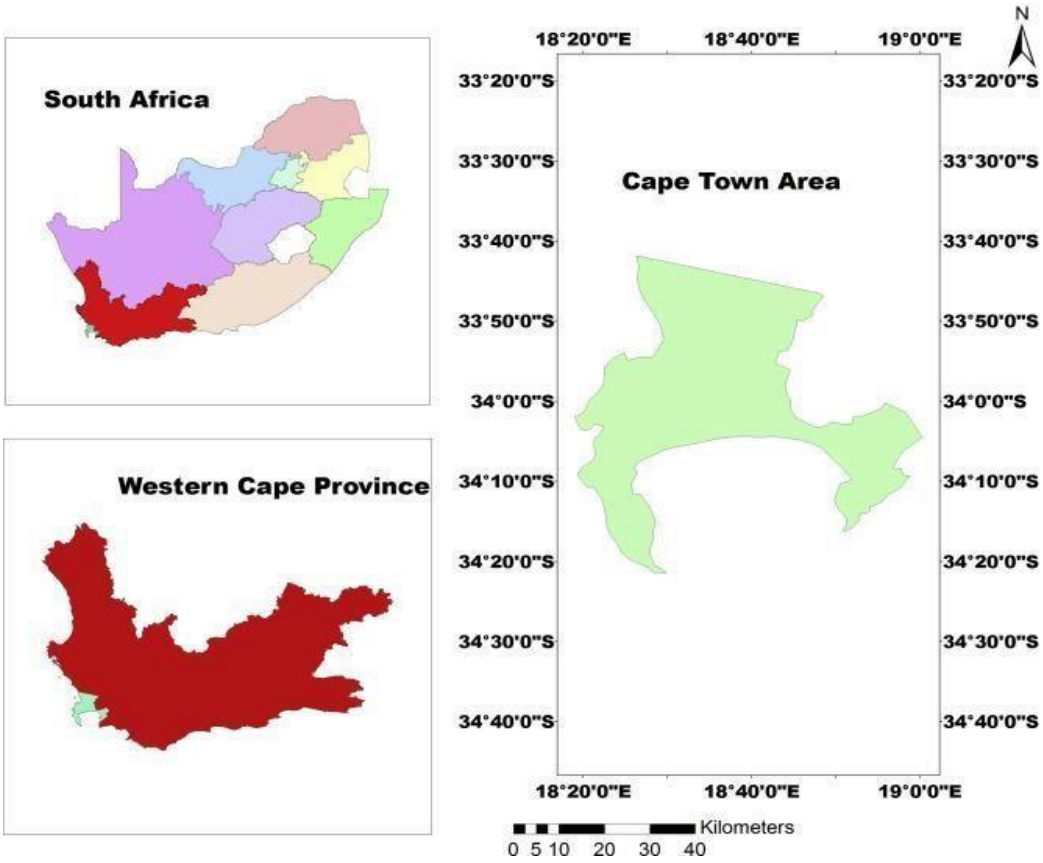


Figure 3.1: South African map showing Western Cape Province as the study area.

https://ars.els-cdn.com/content/image/1-s2.0-S2405844019358086-gr1.jpg accessed 15 October 2021

3.3.2 Sampling

3.3.2.1 Population

Population sampling refers to the method of selecting a subset of subjects that are representative of a whole population (Emanuel, Kapur, and Do., 2017). The population in this study consisted of 59 (total) voluntary donor semen samples. From the samples, WHO criteria were used to determine normal and abnormal samples. All samples with sperm concentration <15 million/ml, progressive motility <32%, and total motility <40% were regarded as abnormal; and all samples with above the stated criteria were regarded as normal (WHO., 2010). Out of the 59 semen samples, 19 were found to be abnormal and 40 were found to be normal. The donors were partaking in a semen donor programme at the University of the Western Cape , Department of Medical Biosciences, Andrology Research Laboratory. Upon signing informed consent forms, human semen was obtained

by convenient sampling which is a non-probability sampling that includes samples being drawn from that part of the population that is close to hand.

3.3.2.2 Sampling method

Participant selection was done using convenient selection. The size of participants was determined using Cochran's formula (Singh and Masuku., 2013):

$$n_0 = \frac{Z^2 pq}{e^2}$$

Where:

- $\mathbf{n_0}$ = sample size
- **Z** = Z value for the anticipated level of confidence
- **p** = approximate proportion of the population that has the attribute in question
- **q** = 1-p
- **e** = anticipated level of precision (margin of error)

$$no = \frac{(1.65)^2 (0.25) (1-0.25)}{(0.1)^2}$$

no= 51.046…

no= 51 participants

3.3.2.3 Source and plant preparation

Freshly boiled (100 ℃) distilled water was added to green or black tea (Five Roses™, Cape Town, South Africa) (2g/100ml) for 5 minutes (Opuwari and Monsees., 2015). The aqueous extract was then filtered using a cheese cloth and Whatman's filter paper (no. 4 and no. 1, respectively; Whatman, Madestone, England) using a vacuum system, and was allowed to cool to room temperature. The filtrate was first frozen at −20℃ then freeze dried using the freeze-drying system and stored in a cool dry place until ready to use.

The average yield of the extract was calculated from three preparations (green tea: 3.6016g/l; black tea: 3.6020g/l). Six (6) cups (150 ml each) is the recommended intake of tea per day (Saito, Inoue, Sawada, Shimazu, Yamaji *et al*., 2015) and to determine the concentrations (*x*) of black tea and green tea to be used for this study, the following were considered for an average 80 kg man:

- *Camellia sinensis* recommended dose = 6 cups/day
- 1 cup =150 ml
- Average human weight =80 kg (where 1kg=1ml)
- Average extract yield of green tea = 3.6016g/l)
- Average extract yield of black tea= 3.6020g/l

Green tea

$\frac{x}{1\ cup} = \frac{average\ yield\ of\ extract}{1000\ ml}$=

$$x = \frac{150 * 3.6016}{1000}$$

$$x = 0.54024\ \text{g/ml}$$

$$Recommended\ daily\ intake = 5.4024\ x\ 6 = 3.24144g$$

$$Recommended\ concentration\ based\ on\ an\ 80kg\ man = \frac{3.24144\ g}{80000\ ml}$$

Where 1kg = 1000 ml

$$Recommended\ concentration\ based\ on\ an\ 80\ kg\ man = 0.000040518\ \text{g/ml}$$

40.518 µg/ml

Black tea

$\frac{x}{1\ cup} = \frac{average\ yield\ of\ extract}{1000\ ml}$=

$$x = \frac{150 * 3.6020}{1000}$$

$$x = 0.5403 \text{ g/ml}$$

$$Recommended\ daily\ intake = 5.403\ x\ 6 = 3.2418g$$

$$Recommended\ concentration\ based\ on\ an\ 80kg\ man = \frac{3.2418g}{80000\ ml}$$

Where 1kg = 1000 ml

$$Recommended\ concentration\ based\ on\ an\ 80\ kg\ man = 0.0000405225 \text{ g/ml}$$

Thus, the concentrations of green tea and black tea used for this study were 0.4, 4.0, 40, and 405 µg/ml.

The freeze-dried extract was then reconstituted in human tubular fluid (HTF) to the final concentrations determined (0 µg/ml, 0.4 µg/ml, 4.0 µg/ml, 40 µg/ml and 405 µg/ml) for both green and black tea plant extracts, where the HTF media served as the control.

3.4 Data collection

3.4.1 Source and preparation of human semen sample

Semen samples were collected from healthy volunteers participating in the semen donor programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape.

Upon the signing of informed consent forms, human semen was attained by convenient sampling from 59 men (18-45 years). The semen was collected by masturbation into sterile vials following 3-5 days of sexual abstinence. Semen samples were incubated at 37^{o}C for 10 to 20 minutes to enable liquefaction to take place within the first one hour of attainment. Afterwards, 2µl of liquefied semen was transferred in a Leja slide (Microptic, Bacerlona, Spain) in a pre-warmed incubator at 37^{o}C to determine baseline sperm concentration and motility using Sperm Class Analyzer (SCA Evolution SL, Microptic, Bacerlona, Spain). From this semen analysis, the sample was classified as normal (sperm concentration >15 million/ml, progressive motility >32%, and total motility >40%); and where the sample did not meet any of the criteria set, it was classified as abnormal sample (sperm concentration <15 million/ml, progressive motility <32%, and total motility <40%) following the World Health Organization criteria (WHO., 2010).

3.4.2 Preparation of human tubular fluid medium

The HTF medium is described as the synthetic medium essential for preparation and spermatozoa washing due to its ionic composition that resembles the composition of natural HTF, which delays cell death and allows sperm parameters to be carried out at optimal time (Quinn *et al*., 1985). Therefore, in this study the HTF medium was the one chosen and preferred. Some of the substances that compose the HTF medium mimic the compounds found in the fallopian tube. The substances are: 101.60 mM NaCl, 4.69 mM KCl, 2.04 mM $CaCl_2.2H_2O$, 0.02 mM $MgSO_4.7H_2O$, 0.37 mM KH_2PO_4, phenol red (dye indicator), 25 mM $NaHCO_3$, 2.78 mM glucose (anhydrous), 0.33 mM sodium pyruvate, 21.40 mM sodium lactate (60% syrup), penicillin, streptomycin, 20 mM HEPES (Merck Millipore, Tullagreen, Carrigtwohill, Ireland). These substances were dissolved in distilled water and once completely dissolved, osmolarity was regulated to 280 mOsmol/kg. Lastly, 10 mg/ml bovine serum albumin (BSA) was added prior to making use of the medium (HTFBSA) (Sigma Aldrich, St Louis, MO, USA). The medium was sterilised by filtration through a 0.22 µm filter (Takalani, Adefolaju, Henkel, and Opuwari., 2021).

3.4.3 Experimental procedure for semen analysis

Calculations were done to ensure that a uniform concentration (15 million/ml) of spermatozoa is maintained throughout the study, with reference to the concentration of the spermatozoa of the sample. Subsequently, the semen was diluted 1:5 with human tubular fluid- bovine serum albumin (HTF-BSA) (Moichela, Adefolaju, Henkel and Opuwari, 2020) and centrifuged for 10 minutes at 300 xg (Labortechnik, Wehingen, Germany). The supernatant was discarded, and the pellet re-suspended in HTF-BSA. Suspensions of spermatozoa in HTF-BSA were next incubated with different concentrations of aqueous extract of *Camellia sinensis (*green and black tea) for 1 hour at 37ºC. Afterwards, suspensions were analysed for various semen parameters as indicated in Figure 3.2:

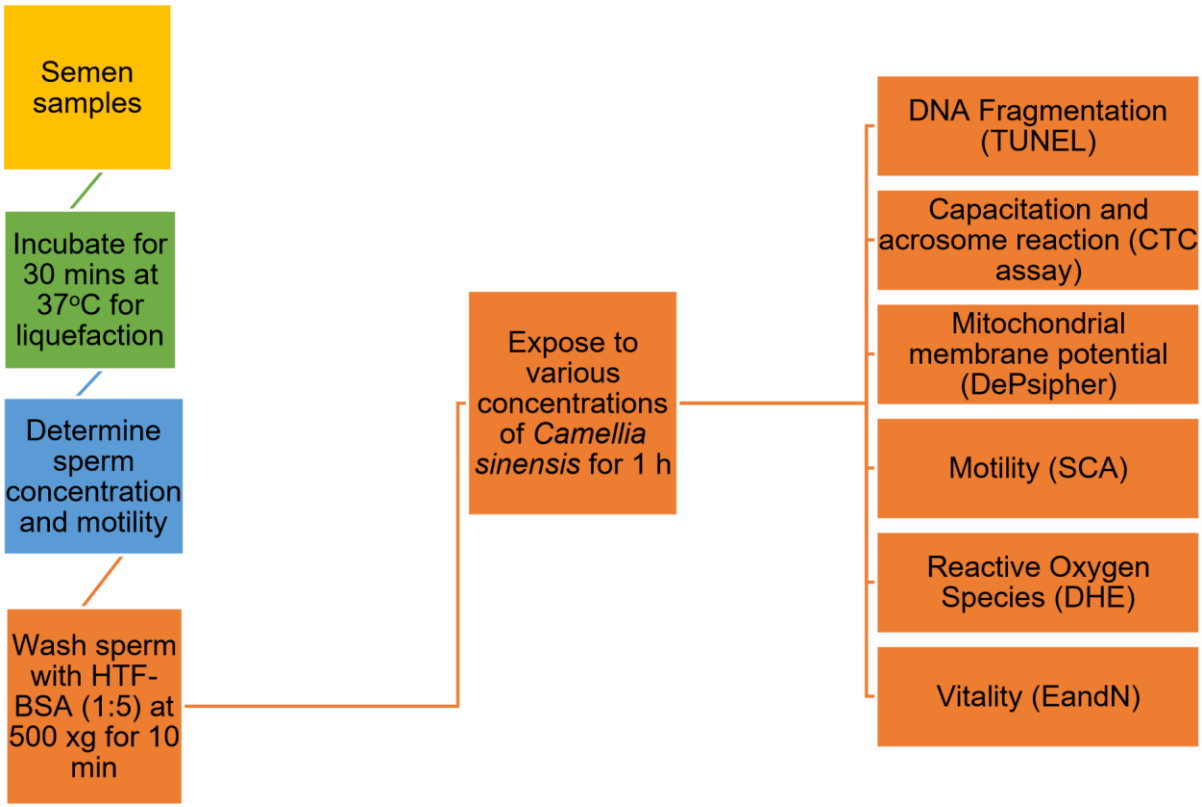


Figure 3.2: Experimental procedure: TUNEL (transferase-mediated dUTP-biotin nick end labelling), CTC (capacitation and acrosome reaction), SCA (sperm class analysis), DHE (dihydroethidium), E&N (eosin and negrosin), and HTF-BSA (human tubular fluid – bovine serum albumin).

3.4.3.1 Sperm motility

Sperm cell motility was determined using the Sperm Class Analyzer 5.0 (SCA Evolution, Microptic S.L Barcelona, Spain) (Figure 3.3). After 1- hour exposure with the various concentrations of *Camellia sinensis* at 37°C, 2µl of each sperm suspension was placed on Leja slides. Motility of at least 360 spermatozoa was analysed at 37°C according to the criteria established by the WHO (WHO, 2010) with a Nikon Microscope (Nikon Instruments Inc., America) at 100X Phase Contrast 1. The kinematic parameters that were investigated are: total motility, progressive motility, velocity curve line (VCL), velocity straight line (VSL), velocity average path (VAP), linearity (LIN), straightness (STR), beat cross frequency (BCF), amplitude of lateral head displacement (ALH) (Figure 3.4 and Table 3.1).

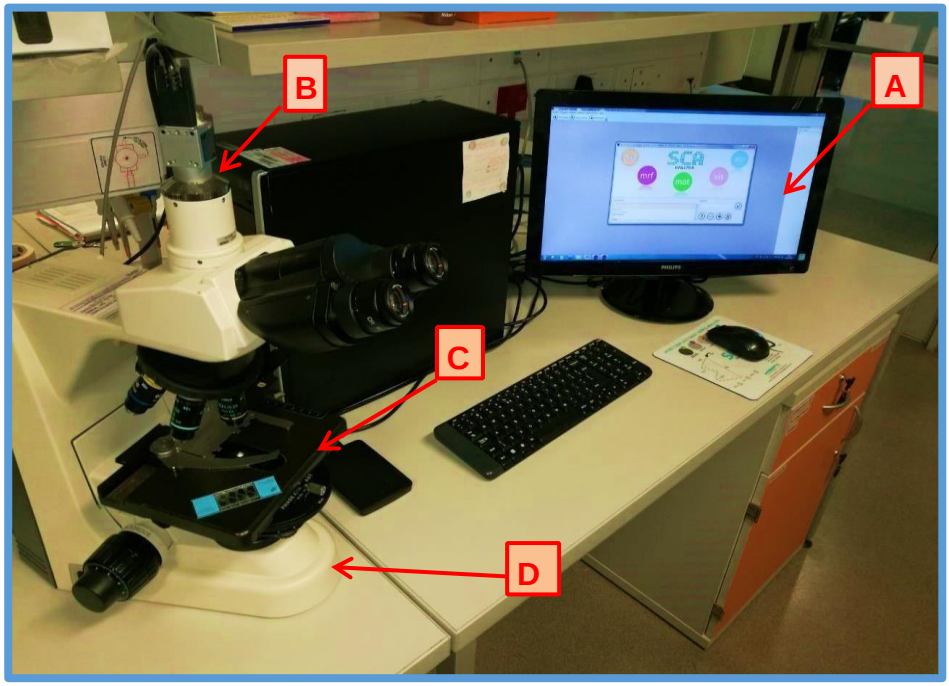


Figure 3.3: Equipment used for assessment of semen parameters as well as computer aided semen analysis (CASA) analysis of sperm motility. A. Computer with SCA® software for CASA analysis, B. Basler A312fc digital camera, C. Heated stage, D. Nikon Eclipse 50i microscope.

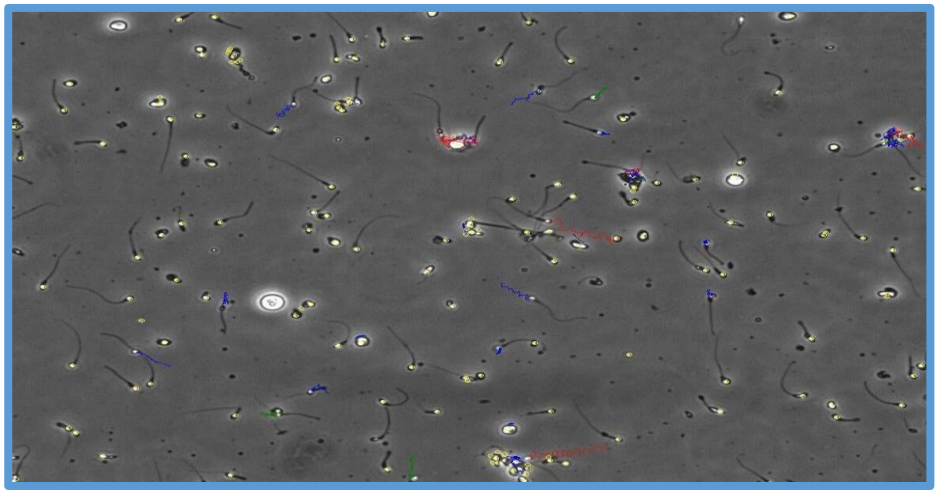


Figure 3.4: Determination of sperm concentration and motility

Table 3.1: Sperm kinematic parameters

SPERM PARAMETER	MEASUREMENT OF MOVEMENT
Total motility (%)	Spermatozoa showing movement; (types a+b+c)
Progressive motility (%)	Spermatozoa that move either linearly or in a circle regardless of speed; ≥ 5 µm/s but < 25 µm/s (type a+b)
Rapid progressive motility (%)	Spermatozoa that move actively, either linearly or in a large circle with a speed of > 80 µm/s
Medium progressive motility (%)	Spermatozoa that move actively, either linearly or in a large circle with a speed between > 50 µm/s and < 80 µm/s
Non-progressive motility (%)	Spermatozoa tail movement seen but no net space gain (movement < 5 µm/s)
Velocity curve line (VCL) (µm)	Time-averaged velocity of a sperm head along its actual curvilinear path
Velocity straight line (VSL) (µm)	Time-average velocity of a sperm head along the straight line between its first detected position and its last
Velocity average path (VAP) (µm)	Time-average velocity of a sperm head along its average path
Linearity (LIN) (%)	Linearity of the curvilinear path = VSL/VCL
Straightness (STR) (%)	Linearity of the average path = VSL/VAP
Beat across frequency (BCF) (Hz)	Magnitude of lateral displacement of a sperm head about its average path
Amplitude of lateral displacement (ALH) (µm)	Magnitude of lateral displacement of a sperm head about its average path

Type a = rapid progressive motility (WHO, 1990)

Type b = slow or sluggish progressive motility (WHO, 1990)

Type c = non-progressive motility (WHO, 1990)

3.4.3.2 Sperm vitality

One-step eosin-negrosin (E& N) staining technique was utilised to investigate the effect of *Camellia sinensis* on sperm vitality according to WHO (2010). The staining solution was then made by dissolving 0.67 g of eosin Y and 0.9 g of NaCl in 100 ml of distilled water with moderate heating,10g of nigrosin was then added, and the solution was brought to the boil. The solution was then filtered through filter paper to remove coarse and gelatinous precipitates. Then the filtered solution was stored in a dark glass bottle at room temperature until use. After 1-hour incubation with various concentrations of *Camellia sinensis* at 37^{o}C, the sperm suspension was mixed with E & N stain (1:1) in an Eppendorf

vial. A smear was then made on a glass slide and left to air dry. Afterwards, the slides were viewed with a 100X oil immersion objective in the bright field using a light microscope. A total of 200 spermatozoa were counted and the percentage of live sperm calculated. Dead sperm appeared red or purple and live sperm appeared white (Figure 3.5).

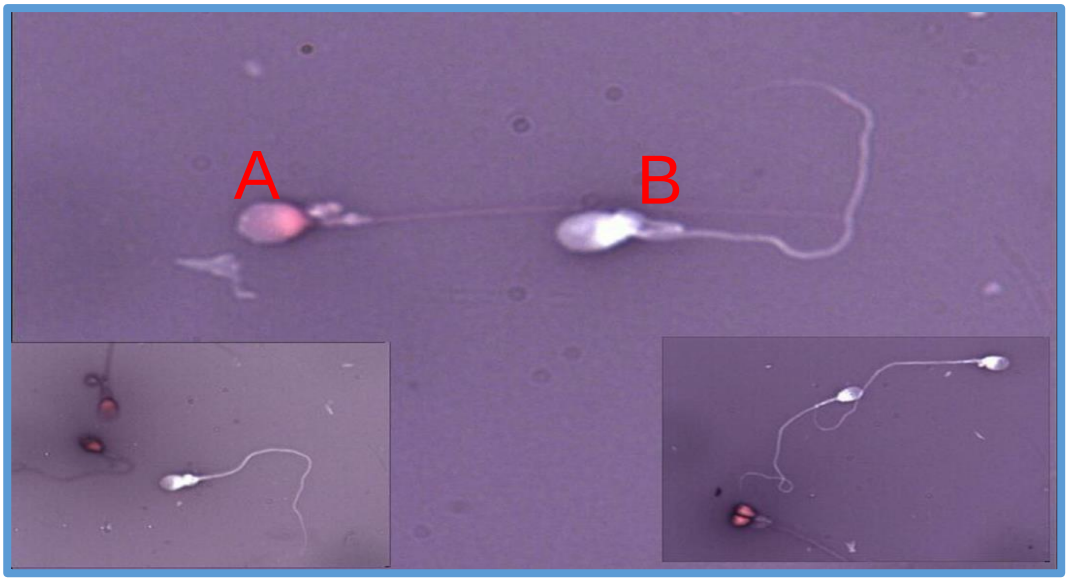


Figure 3.5: Eosin-nigrosin vitality stain of sperm. A= dead spermatozoon and B= live spermatozoa.

3.4.3.3 Sperm capacitation and acrosome reaction

The chlortetracycline (CTC) fluorescence assay procedure (Green, Cockle, Watson, Fraser,1996) was used to assess the form of capacitation and acrosome reaction. After 1-hour incubation of aqueous extracts of *Camellia sinensis,* sperm suspension was treated in Hoechst 33258 (Sigma). A stock staining solution of Hoechst 33258 (100 mg/mL) was prepared in distilled water. This solution may be stored at 4ºC for one month. The stock solution was diluted 1:1000 in HTF and then with sperm suspensions in HTF-BSA (1:100) before use. Thereafter, the samples were incubated at room temperature for two minutes before being washed by centrifugation with 4 ml of 2% polyvinylpyrrolidone (PVP40) in HTF at 900 xg for five minutes. The CTC solution (pH 7.8) was prepared on the day of use and contained 750 µM CTC in a buffer of 130 mM NaCl, 5 mM cysteine in 20 mM Tris-HCl. The bottle containing the solution was kept wrapped in foil at 4oC until use.

Hoechst-treated spermatozoa were mixed with CTC solution and 12.5% w/v paraformaldehyde in 0.5 M Tris-HCl (pH 7.4; 1:1:0.2), respectively. Subsequently, 10 µl

of the suspension was placed on a slide and one drop of 0.22 M 1.4 diazabicyclo (2.2.2) octane (DABCO) dissolved in glycerol: PBS (9:1) was mixed in carefully to delay the fading of the fluorescence. Slides were then viewed with a 100x oil immersion objective using a fluorescent microscope (Zeiss, Oberkochen, Germany). Then 200 live cells (Hoechst-negative) were evaluated for CTC staining patterns as follows: even fluorescence over the entire head (characteristic of non-capacitated; acrosome-intact cells), fluorescence-free band in the post-acrosomal region (characteristic of capacitated; acrosome-intact cells) and cloudy or absent fluorescence over the sperm head (characteristic of capacitated; acrosome-reacted cells) (Figures 3.6 and 3.7).

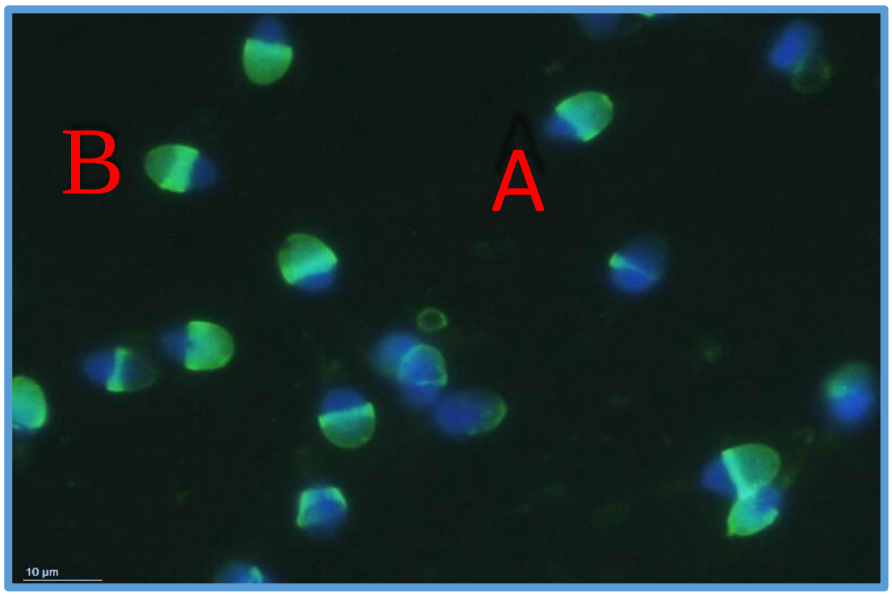


Figure 3.6: Human spermatozoa acrosome reaction detection. A. Acrosome reacted spermatozoon B. Acrosome intact spermatozoon.

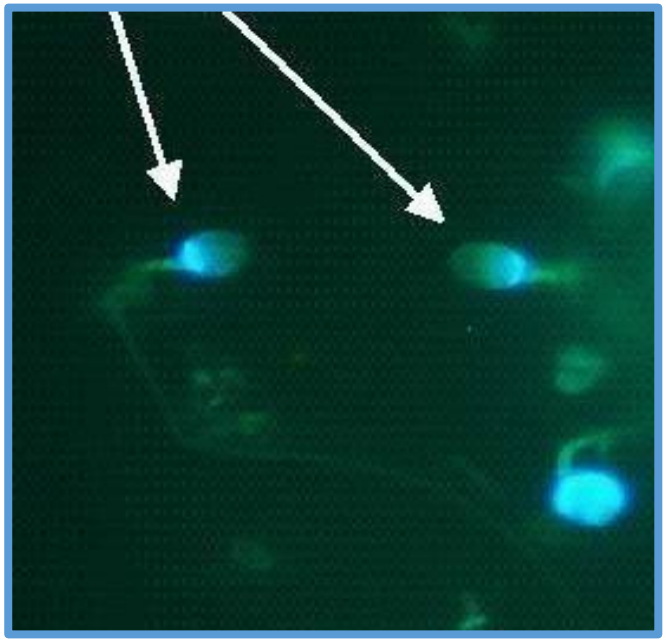


Figure 3.7: Capacitated acrosome reacted spermatozoa, the arrows are pointing to capacitated acrosome-reacted spermatozoa.

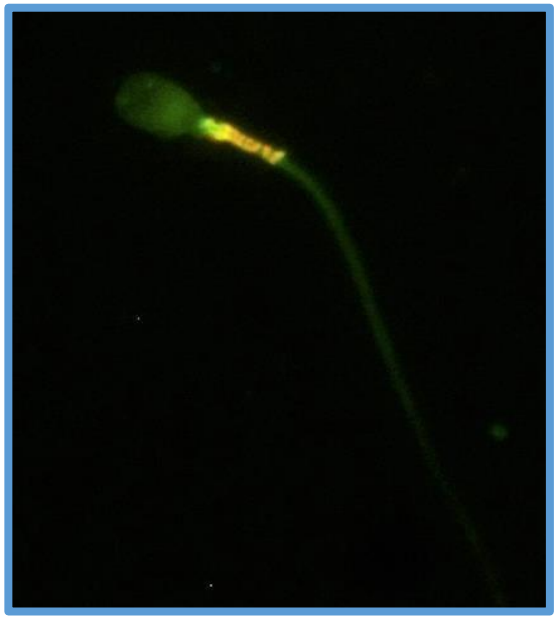
3.4.3.4 Sperm mitochondrial membrane potential

Sperm intact MMP was determined by means of a lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA). The following amendment was implemented from the protocol provided by the manufacturer. The reaction buffer was diluted with pre-warmed distilled water (1:10) at 30^{o}C, and 20 µl of stabiliser was added per millilitre of buffer with 1 µl of DePsipher dye. Thereafter, the solution was added to 500 µl of prepared reaction buffer, mixed thoroughly and centrifuged for one minute at 300 xg (Labortechnik, B A 55 Wehingen, Germany). The supernatant was transferred into a test tube and immediately used.

After 1-hour incubation of the semen with different concentrations of the aqueous extract of *Camellia sinensis*, intact mitochondrial membrane potential (MMP) in sperm was determined with the use a lipophilic cationic dye (DePsipher™, Trevigen, Minneapolis, USA), following the instructions provided by the manufacturer. Spermatozoa were observed using a fluorescence microscope with a 488 nm excitation filter (Zeiss) at ×400 magnification. Sperm showing intense red/orange fluorescence was considered healthy with intact MMP, while those with disrupted MMP fluoresced green were considered damaged. A total number of 200 spermatozoa were counted and the number with intact

MMP recorded as a percentage of the total number of sperm counted (Figure 3.8 A and B).

A.



B.

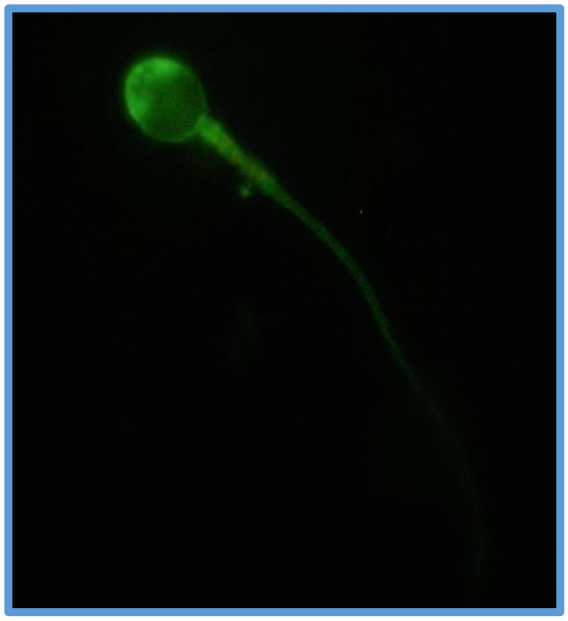


Figure 3.8: Spermatozoa Mitochondrial membrane potential (MMP), A. Mitochondrial membrane intact spermatozoon B. Mitochondrial membrane disrupted spermatozoon.

3.4.3.5 Sperm reactive oxygen species

Reactive oxygen species production was determined by use of dihydroethidium (DHE; Molecular Probes, Eugene, OR,

USA) as fluorescing probe according to Mupfiga, Fisher, Kruger, and Henkel (2013). A stock solution was prepared using 20 µM DHE in PBS, with pH adjusted to 7.4.

Following incubation of sperm samples with the different concentrations of aqueous extract of *Camellia sinensis* for 1hr at 37°C, an aliquot of 100 µl of spermatozoa was centrifuged for 10 min at 500xg (Labortechnik, Wehingen, Germany). Afterwards, the supernatant was discarded, and the pellet resuspended in 100 µl PBS and 20 µl DHE stock and then incubated for 15 min at 37°C. Thereafter, 10 µl of each sample was placed on a slide and viewed under oil immersion using an epifluorescence microscope with 488 nm excitation and 590 emission filters (Zeiss, Oberkochen, Germany). Bright orange fluorescing sperm are indicative of extreme ROS production. The percentage of ROS-positive sperm was calculated from no less than 200 spermatozoa (Figure 3.9). A total of 200 spermatozoa were analysed and the result represented as the percentage of ROS-positive spermatozoa.

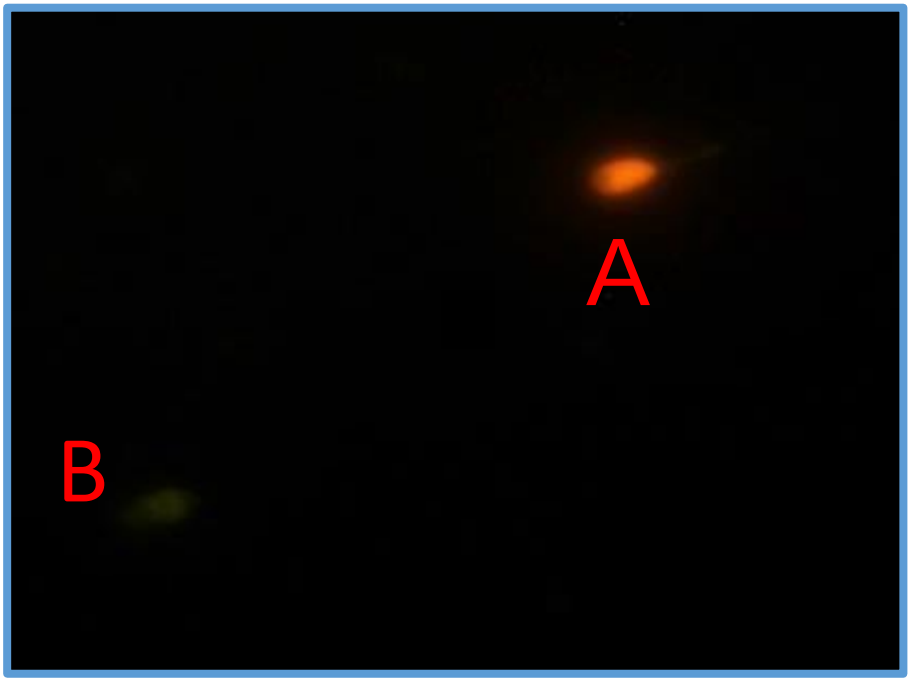


Figure 3.9: Dihydroethidium (DHE) stained spermatozoa A. ROS positive spermatozoa B. ROS negative spermatozoa.

3.4.3.6 Sperm DNA fragmentation

DNA fragmentation was measured by using the terminal deoxynucleotidyl transferasemediated dUTP-biotin nick end labelling (TUNEL) (Dead EndTM; Fluorometric, Promega, Madison, USA) assay (Henkel, 2005). The TUNEL assay measures DNA damage directly by detection of single and double DNA strand breaks (Sharma, Masaki and Agarwal, 2013). The DNA strand breaks are noticed in an enzymatic reaction through labelling the free 3'-OH and the altered nucleotides with terminal deoxynucleotidyl transferase.

After incubation with the different concentrations of aqueous extracts of *Camellia sinensis* at 37°C, 100 µl sperm suspension was added to 100 µl phosphate buffered saline (PBS) (Oxoid Ltd., Hampshire, England) and subjected to centrifugation at 300 xg (Labortechnik, Wehingen, Germany) for 10 minutes. The resultant pellets were resuspended, and wet smears were made on a StarFrost™ slide (Knittel Gläser, Braunschweig, Germany) and left to air dry at RT. The slides were then fixed in 4% formaldehyde in PBS (pH 7.4) for 25 minutes at 4°C. After fixing slides, they were then washed in PBS for five minutes at RT and permeabilised in 0.2% Triton™ X-100 (SigmaAldrich, St. Louis) in PBS for five minutes at RT. Thereafter, the slides were rinsed two times in PBS for five minutes each time at RT, and 100 µl of equilibration buffer was added to each slide and allowed to equilibrate for 10 minutes. Thereafter, 20 µl of TUNEL reagent (DeadEnd™, Promega, Madison, WI, USA) was added to each slide and cooled with a plastic cover slip (Promega). After incubation for 60 minutes at 37°C in a humidified chamber protected from light, the reaction was halted by immersion in 2× SSC (Promega) for 15 minutes. Slides were then washed in PBS three times, and more than 200 randomly selected sperm were immediately analysed with the use of a fluorescence microscope (Zeiss, Oberkochen, Germany) with a 488 nm excitation filter and a 510–530 nm emission filter at 400x magnification with an oil immersion objective. Sperm with normal DNA showed only light background staining (TUNEL-negative), while sperm with fragmented DNA exhibited a bright green fluorescence (TUNEL-positive). At least 200 spermatozoa were counted, and TUNEL-positive sperm were recorded as a percentage of the total number of sperm per field (Figure 3.10).

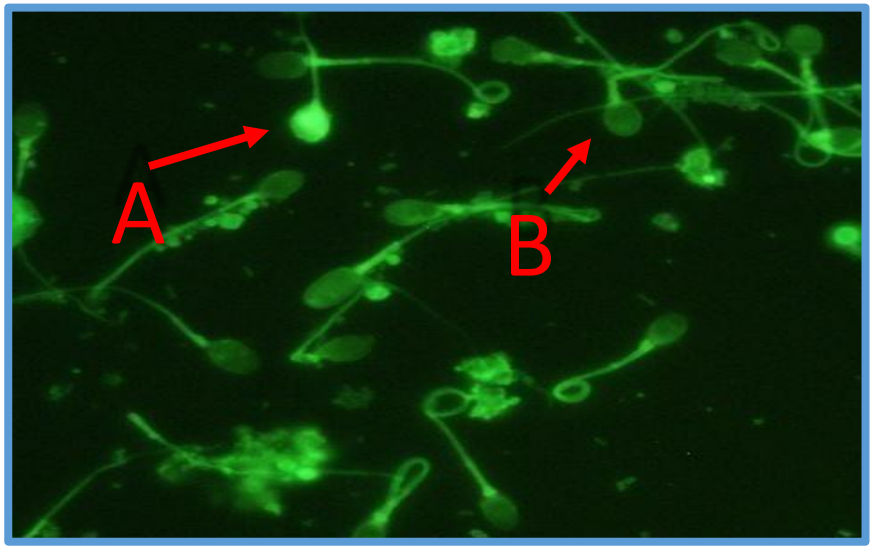


Figure 3.10: Determination of DNA fragmentation using TUNEL assay, A. Sperm with fragmented DNA exhibited a bright colour (TUNEL-positive); B. Sperm with normal DNA showed only a slight background staining (TUNEL-negative)

3.5 Ethical considerations

3.5.1 Ethical clearance and approval

Ethics clearance certificate was obtained from Turfloop Research Ethics Committee (TREC/393/2.19:PG), University of Limpopo and the Biomedical Research Ethics Committee (BMREC BM18/3/17), University of the Western Cape.

3.5.2 Protection from physical harm

The risk associated with this study was minimal as the subject donated semen by masturbation after three to five days of sexual abstinence. The obtained semen was then used for the exposure to aqueous extract of *Camellia sinensis,* and all semen analysis was then be performed.

3.5.3 Waste disposal

When the experiments were done, remaining semen was disposed of as bio-hazardous waste into red bins and stored in biohazard boxes. The boxes were then collected by the Cape Waste Management Company (Wasteman) and thereafter destroyed by incineration. The waste generated during plant preparation at the University of the Western Cape was also discarded safely and collected by the waste company wasteman.

3.5.4 Anonymity and confidentiality

There were no patient or donor names that were disclosed in the data recording process or to anyone in the distribution of the results. Donor codes were given to the respective donors as a way of protecting their identity in the semen donation process. Consent forms were presented to the subjects in three different languages, English, Afrikaans and Xhosa (Appendix C, D &E) to avoid language barriers.

3.4.5. Informed consent

Informed consent forms that were available in English, Afrikaans and Xhosa (appendix C-E) were given to participants to sign following an explanation of the aim, and objective of research as well as the procedure and risk associated with it.

3.6 Data analyses

3.6.1 Reliability, validity, and bias

3.6.1.1 Reliability

Reliability is the degree to which experiments, tests or measuring procedures produce similar results on repeated trials (Payne and Lundberg., 2014). Reliability of the results obtained was determined in duplicates to obtain repeatable results.

3.6.1.2 Validity

Validity is defined by the degree to which any measuring instrument measures what it is supposed to measure (Heale and Twycross., 2015). Validity of the tests was determined using specific and sensitive machinery, as well as following the manufacturer's instructions for the kits used.

3.6.1.3 Quality assurance

To avoid any form of contamination during this study, a sterile environment was maintained by the donors as well as the personnel involved in the experiments. All the equipment was handled according to the sterility protocol as well correctly according to how it is supposed to be used (Bal-Price and Coecke., 2011). Laboratory rules were always followed to ensure safety and sterility.

3.6.1.4 Bias

Bias is a form of systematic error that can affect scientific studies and lead to a wrong measure of association (Saposnik, Redelmeier, Ruff and Tobler., 2016). Convenient sampling was used, and bias cannot be avoided, but it was minimised by making use of extra participants in case some do not show up and quit in the process.

- Methodological bias was avoided by conducting the experiments in duplicates whilst obtaining similar results. To avoid analytical variation, standard deviation (SD), mean and the coefficient of variance was calculated.
- Statistical bias was avoided using appropriate statistical tests.

CHAPTER 4: RESULTS

4.1 Determination of the *in vitro* effects of the aqueous leaf extract of *Camellia sinensis* on human sperm function

An informed consent was attained from all the donors involved in the study. Human semen samples were obtained from volunteering donors (n=59) participating in the semen donation programme at the Andrology Research Laboratory in the Department of Medical Biosciences, University of the Western Cape. Samples were classified as normal and abnormal and incubated with various concentrations of the aqueous leaf extract of *Camellia sinensis* (green and black tea) for 1-hour. Different sperm parameters were analysed, and data obtained were analysed using GraphPad Prism (version 5, Graphed Software Inc; California). The results were presented descriptively by demonstrating the aqueous leaf extract of *Camellia sinensis' **effects*** on human sperm function for normal and abnormal samples.

4.2 Summary statistics of baseline parameters of semen samples

Following the liquefaction of semen samples, baseline sperm concentration and motility was obtained using CASA. Samples were classified as normal or abnormal using the WHO (2010) criteria. Cut off values for normal samples was sperm concentration >15 million/ml, progressive motility >32%, and total motility >40%) and abnormal as sperm concentration <15 million/ml, progressive motility <32%, and/or total motility <40%) (WHO, 2010). Table 4.1 reveals the baseline parameters for the samples used in this study. Baseline analysis for normal and abnormal samples are respectively demonstrated as semen volume (Mean: 3.1 ± 0.2; Min: 1.5; Max: 5.5 ml) and (Mean: 2.4 ± 0.1; Min: 1.6 ; Max: 3.4 ml), sperm concentration (Mean: 59.5 ± 1.6; Min: 40; Max: 80 million/ml) and (Mean: 13.2 ± 2.4; Min: 0.4; Max: 40 million/ml), total motility (Mean: 64.7 ± 2.8; Min: 35.9; Max: 98.1 %) and (Mean: 53.8 ± 5.4; Min: 2.5; Max: 91%) and progressive motility (Mean: 47.9 ± 2.8; Min: 29.9; Max: 95.5 %) and (Mean: 35.9 ± 5.1; Min: 0.8; Max: 71.7%).

Table 4.1: Baseline parameters of semen samples

Baseline parameter	Group of subjects per baseline parameter	Concentration $(x10^6/ml)$	Total motility (%)	Progressive motility (%)	Volume (ml)
Minimum	Normal	40	35.9	29.9	1.5
	Abnormal	0.4	2.5	0.8	1.6
Median	Normal	59.5	62.6	40.5	3.0
	Abnormal	11.5	54.7	37.5	2.5
Maximum	Normal	80	98.1	95.4	5.5
	Abnormal	40	91.0	71.7	3.4
Mean	Normal	59.5	64.7	47.9	3.1
	Abnormal	13.2	53.8	35.9	2.4
SD	Normal	9.9	18.0	17.9	1.0
	Abnormal	10.2	23.5	22.2	0.5
SEM	Normal	1.6	2.8	2.8	0.2
	Abnormal	2.4	5.4	5.1	0.1

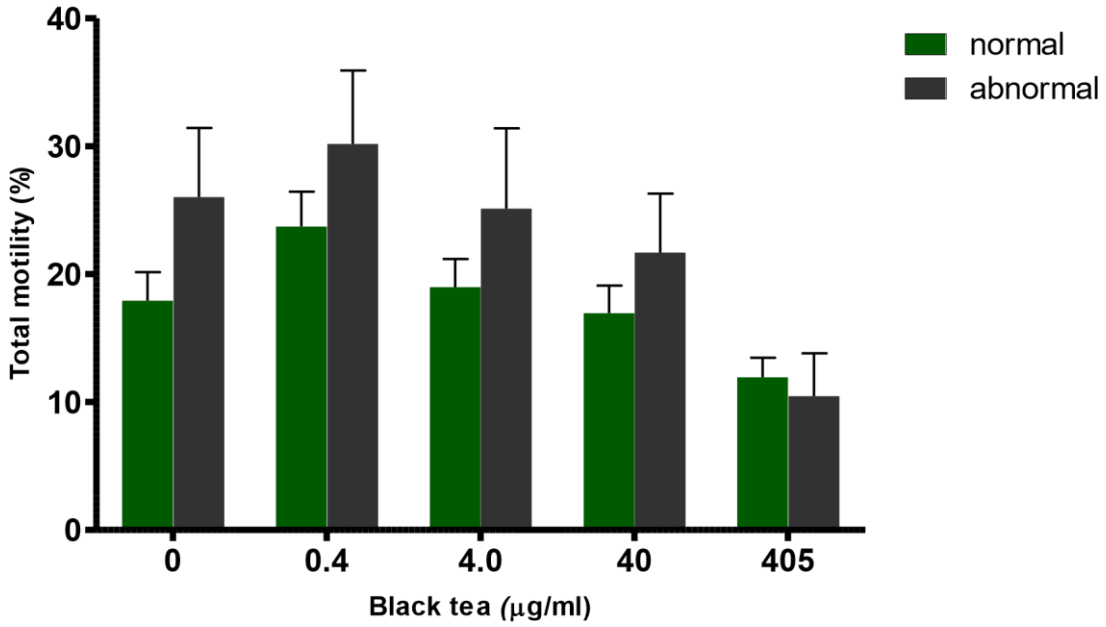
SD = standard deviation; SEM= standard error of mean no. of samples: normal=40 abnormal=19

4.3 Effect of the aqueous leaf extract of *Camellia sinensis* on human sperm motility parameters

Semen samples from a total of 59 donors were collected, washed, classified as normal (n=40) and abnormal (n=19), and treated at various concentrations of aqueous extracts of black and green tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hour. Figures 4.1- 4.4, Tables 4.2 and 4.3 show the results on the effects of black and green tea on the various sperm motility and kinematic parameters analysed.

4.3.1 Effect of the aqueous leaf extract of black and green tea on human sperm motility

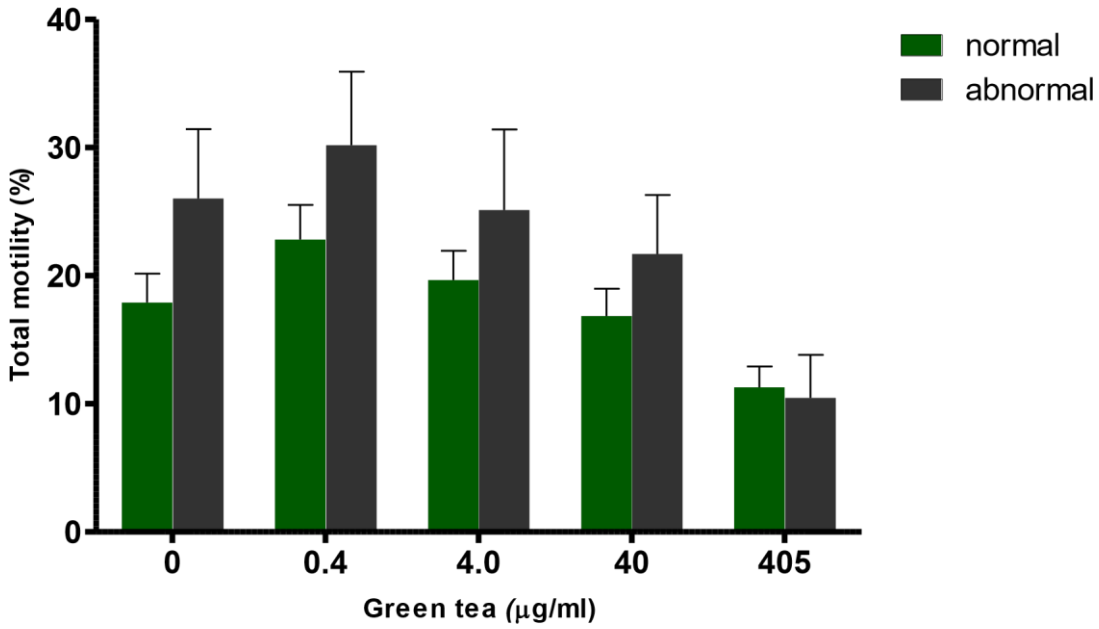
Figure 4.1 demonstrates the effects of black tea on total motility following exposure to different concentrations of black tea (0, 0.4, 4.0, 40, 405 µg/ml) for 1 hour. There was a non-significant decrease in sperm motility in both normal and abnormal samples ($p>0.05$). Also, there was no significant difference between normal and abnormal samples after incubation with black tea ($p>0.05$). Repeated measure ANOVA test showed no linear trend in the normal samples as well as the abnormal samples ($p>0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.1: Effects of black tea on human sperm total motility.

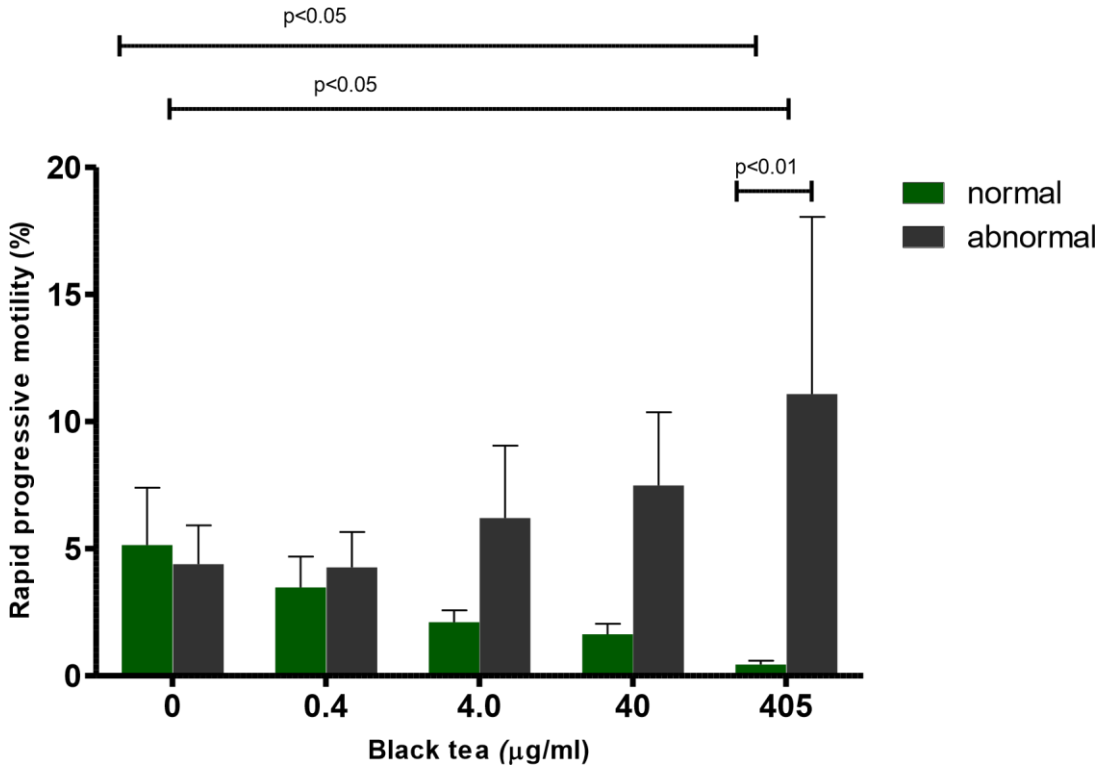
Figure 4.2 demonstrates the effects of green tea on total motility following exposure to different concentrations of green tea (0, 0.4, 4.0, 40, 405 $\mu g/ml$) for 1 hour. There was no significant difference between the normal and abnormal samples after incubation with green tea ($p>0.05$). There was no significant difference between total motility of both normal and abnormal samples as compared to their respective controls. Repeated measure ANOVA test showed no linear trend in the normal samples as well as the abnormal samples ($p>0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.2: Effects of green tea on human sperm total motility.

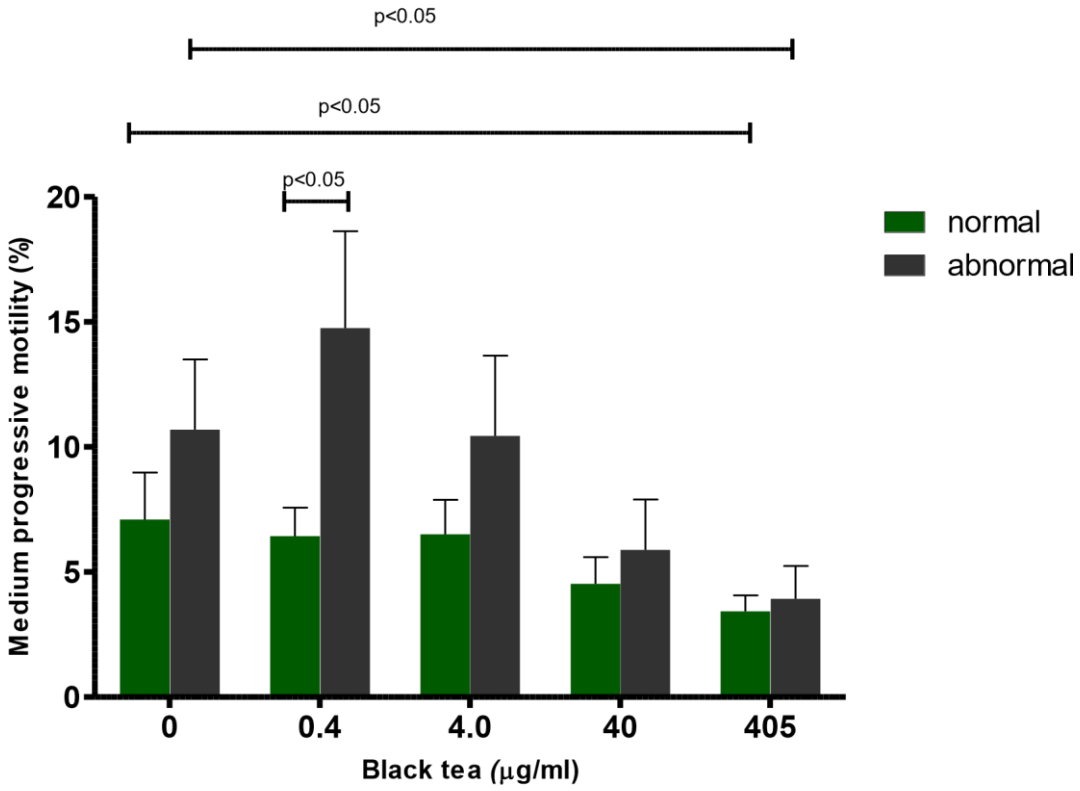
Figure 4.3a showed that the percentage of spermatozoa with rapid progressive motility (type A) was significantly higher in the abnormal group compared to normal group at the highest concentration ($p < 0.01$, Two- way ANOVA analysis). Also, the treatment of human sperm with aqueous extract of black tea showed a concentration–dependent increase in the percentage of rapid progressive sperm (type A) in the abnormal samples, while a dose-dependent decrease was detected in the normal sperm samples ($p<0.05$). At concentration 405 $\mu g/ml$, rapid progressive motility in abnormal samples was significantly higher than in the normal samples ($p<0.01$). Repeated measures ANOVA also showed a trend with increasing concentrations in the normal samples as well as the abnormal samples for type A.



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.3 (a): Effects of black tea on human sperm rapid progressive motility.

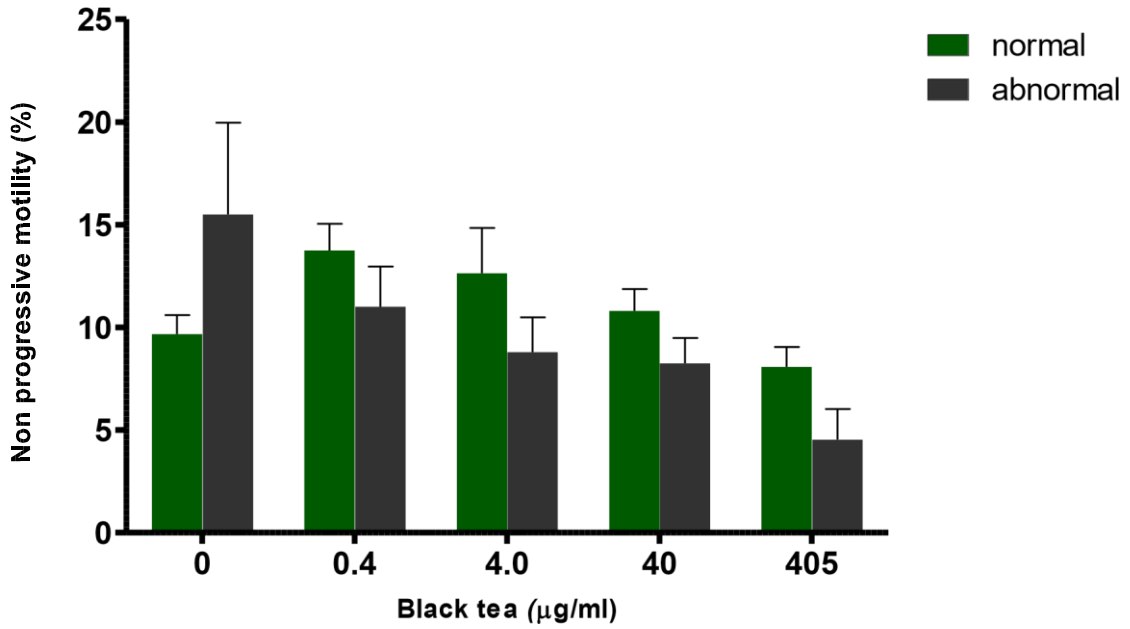
A dose-dependent decrease in the percentage of medium progressive motility (type B) was observed in normal sperm samples treated with black tea ($p<0.05$), while a dose-dependent decrease in abnormal samples treated with black tea ($p<0.05$) was observed. A trend was also observed with repeated measure ANOVA for type B normal samples ($p<0.05$) where the dose-dependent decrease was observed. There is a significant difference between normal and abnormal samples at concentration 0.4 $\mu g/ml$ where the abnormal is higher than the normal ($p<0.05$) (Figure 4.3b).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.3 (b): Effects of black tea on human sperm medium progressive motility.

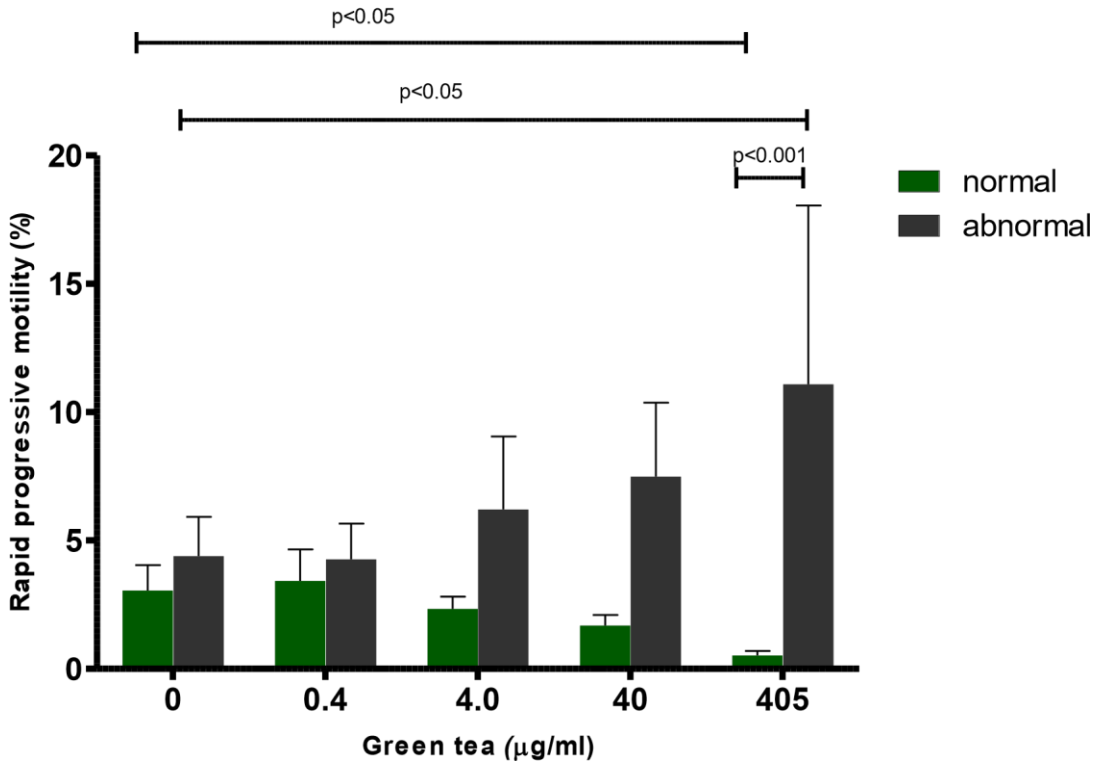
Figure 4.3c showed that after exposure to black tea for 1 hour, there was no significant difference between normal and abnormal sperm in non-progressive sperm observed (type C) ($p>0.05$; Two-way ANOVA). The percentage of non-progressive spermatozoa remained unchanged for both normal and abnormal sperm samples as compared to the control ($p>0.05$; Figure 4.3c). There was no trend observed for type C ($p>0.05$) with the repeated measure ANOVA trend analysis.



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.3 (c): Effects of black tea on human sperm non-progressive motility.

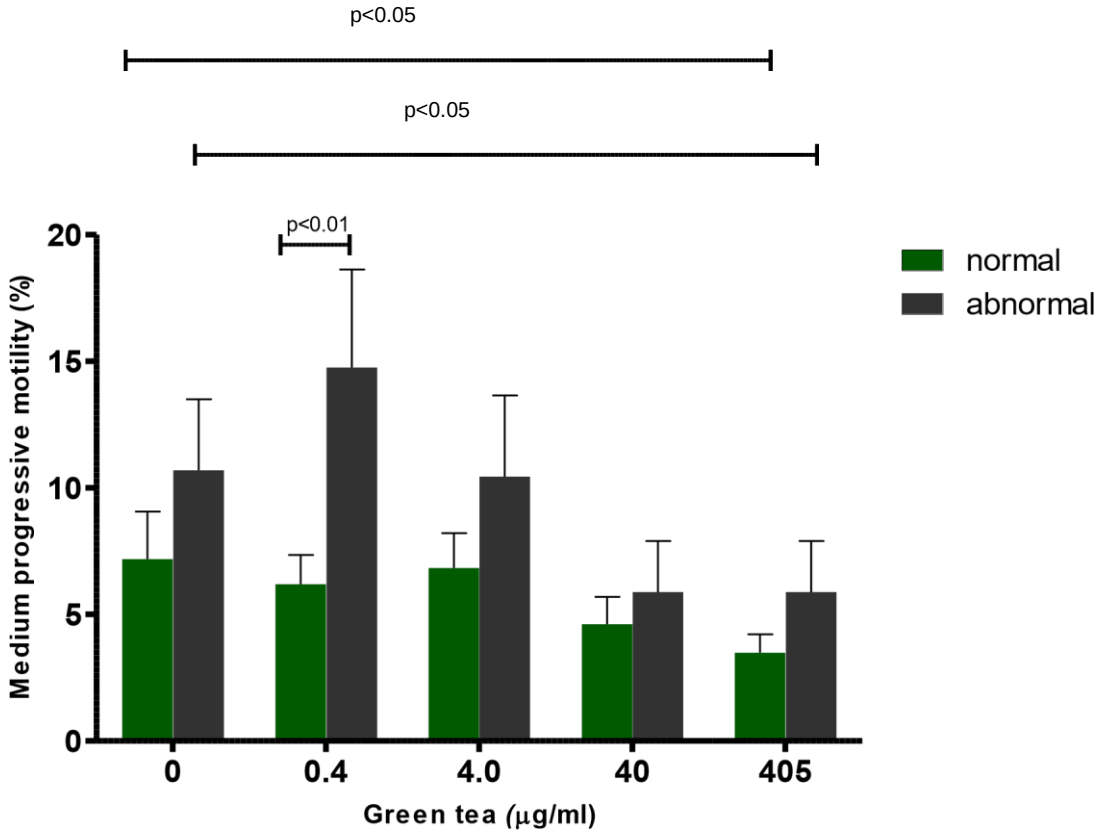
Treatment of human spermatozoa with aqueous extract of green tea showed a concentration–dependent increase in the percentage of rapid progressive sperm (type A) in the abnormal samples, while a dose-dependent decrease was detected in the normal sperm samples ($p<0.05$; Figure 4.4a). At concentration 405 $\mu g/ml$, rapid progressive motility in the abnormal sample was significantly higher than in the normal samples ($p<0.001$; Figure 4.4a). Repeated measures ANOVA also showed a trend with increasing concentrations in the normal samples as well as the abnormal samples for type A ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.4(a): Effects of green tea on human sperm rapid progressive motility.

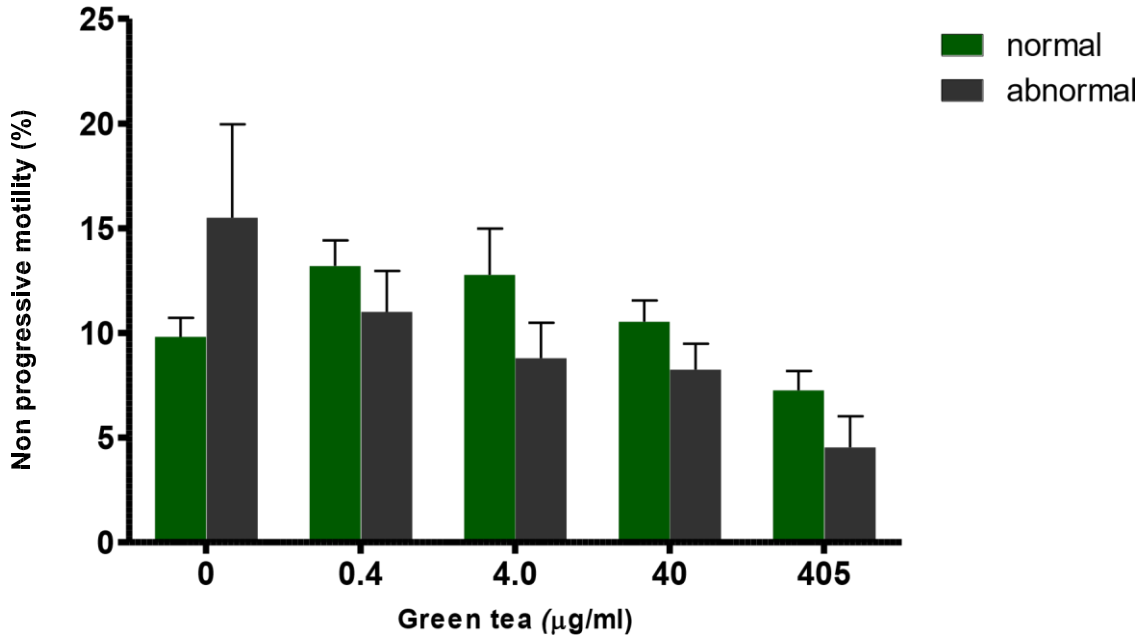
Figure 4.4(b) below shows a dose-dependent decrease in the percentage of medium progressive motility (type B) in normal and abnormal sperm samples treated with green tea ($p<0.05$). There is a significant difference between normal and abnormal at concentration $0.4\mu g/ml$, a significantly higher percentage of type B spermatozoa was detected in the abnormal compared to the normal samples ($p<0.01$). A negative trend was also observed with repeated measure ANOVA for type B normal samples ($p<0.05$) with increasing concentration.



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.4 (b): Effects of green tea on human sperm medium progressive motility.

After exposure of human spermatozoa to the various concentration of aqueous green tea extract for 1 hour, no significant change in the percentage of non-progressive spermatozoa was observed compared to the controls for both normal and abnormal groups ($p>0.05$). Also, there was no significant difference in the percentage of nonprogressive (type C) sperm observed between normal and abnormal spermatozoa (Figure 4.4c; $p>0.05$; Two-way ANOVA). There was no trend observed for type C spermatozoa ($p>0.05$) with the repeated measure ANOVA trend analysis.



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.4 (c): Effects of green tea on human sperm non-progressive motility.

Table 4.2 shows the results of aqueous extract of black tea. No significant changes were observed in most sperm kinematic parameters (VCL, VSL, VAP, LIN, STR and BCF) in both normal and abnormal samples compared to their respective controls ($p>0.05$). Also, for most sperm kinematic parameters, there was no considerable difference between the normal and abnormal samples at the respective concentrations ($p>0.05$) except for ALH, in which a significant decrease in the percentage of ALH was observed in the normal and abnormal groups compared to the control ($p<0.05$). In addition, the abnormal group had a significantly higher percentage of ALH compared to the normal group ($p<0.05$).

Table 4.2: Sperm kinematic motility parameters following incubation with increasing concentration of aqueous leaf extract of black tea for 1 hour.

Kinematics	Groups	0 µg/ml	0.4 µg/ml	4.0 µg/ml	40 µg/ml	405 µg/ml	p value
VCL ($\mu m\ s^{-1}$)	Normal	74.3±2.2[a]	72.4±2.3[a]	70.4±3.0[a]	72.7±2.4[a]	55.0±4.6[a]	>0.05
	Abnormal	73.1±4.5[a]	85.4±7.4[a]	85.8±4.8[b]	81.6±3.2[a]	55.4±8.8[a]	>0.05
VSL ($\mu m\ s^{-1}$)	Normal	27.7±1.7[a]	26.7±1.6[a]	25.4±1.4[a]	28.1±1.2[a]	17.8±1.9[a]	>0.05
	Abnormal	30.7±2.9[a]	25.9±3.3[a]	31.4±3.5[a]	34.4±2.5[a]	26.2±6.9[a]	>0.05
VAP ($\mu m\ s^{-1}$)	Normal	37.5±1.5[a]	35.9±1.4[a]	34.7±1.5[a]	38.7±1.4[a]	27.1±2.3[a]	>0.05
	Abnormal	39.5±2.5[a]	38.3±2.4[a]	41.7±3.0[a]	43.5±2.4[a]	34.7±7.1[a]	>0.05
LIN (%)	Normal	37.3±1.5[a]	37.3±1.5[a]	34.1±1.4[a]	39.0±1.3[a]	26.1±2.6[a]	>0.05
	Abnormal	39.8±3.0[a]	32.4±3.5[a]	35.5±3.0[a]	41.3±2.1[a]	30.6±6.9[a]	>0.05
STR (%)	Normal	67.0±2.0[a]	65.9±2.0[a]	62.6±2.5[a]	67.9±1.9[a]	48.2±4.4[a]	>0.05
	Abnormal	70.3±3.7[a]	60.5±5.6[a]	64.5±4.1[a]	74.2±2.0[a]	46.4±8.2[a]	>0.05
BCF (HZ)	Normal	20.7±1.2[a]	19.7±0.7[a]	18.5±1.0[a]	19.0±0.6[a]	14.1±1.4[a]	>0.05
	Abnormal	25.4±2.7[a]	19.5±1.1[a]	19.5±22.7[a]	18.8±1.1[a]	12.3±2.1[a]	>0.05
ALH (μm)	Normal	20.7±1.2[a]	2.0±0.0[a]	2.2±0.1[a]	2.1±0.0[a]	1.6±0.1[a]	<0.001
	Abnormal	3.4±0.9[b]	2.3±2.7[b]	2.6±0.1[b]	2.3±0.0[b]	11.3±6.7[b]	<0.001

Data comprises of normal (n=40) and abnormal (n=19) of sperm samples which are expressed as mean±SEM.
Abbreviations: BCF-beat across frequency, LIN-linearity, STR-straightness, VAP-velocity average path; VCL-velocity curvilinear; VSL-velocity straight line; ALH-average lateral head displacement. The results follow the 1-hour incubation with the aqueous extract of black tea with increasing concentration (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The columns with similar alphabets indicate no significant difference, while those with different alphabets indicates a significant effect. No significant change for most sperm kinematic parameters was observed in the treatment groups compared to the control ($p>0.05$) except for ALH ($p<0.05$) in both groups. No significant difference between normal and abnormal samples was observed with VCL, VSL, VAP, LIN, STR, and BCF ($p>0.05$). A significant difference between normal and abnormal samples was observed at ALH ($p<0.05$) for all the concentrations as well as the control group. A significant difference was observed between abnormal and normal samples was observed with VCL at concentration 4.0 µg/ml**.** There is a significant difference between the values of samples treated with the plant extract and the control samples in all the stated parameters.

In Table 4.3, aqueous extract of green tea had no significant effect on most sperm kinematic parameters (VSL, VAP, LIN, STR and BCF) in both normal and abnormal samples ($p>0.05$). Also, there was no considerable difference between the normal and abnormal samples at the respective concentrations ($p>0.05$). However, a significant decrease was detected in the percentage of ALH in comparison to the control for both the normal and abnormal samples ($p<0.05$), as well as a significant decrease in VCL compared to the control for both normal and abnormal samples ($p<0.05$). It is also observed that the abnormal samples had a significantly higher ALH percentage except in the control, compared to the normal samples ($p<0.05$). With VCL, it is observed that the significant difference only appears in the highest concentration of 405 µg/ml and it is higher in the normal than in the abnormal samples.

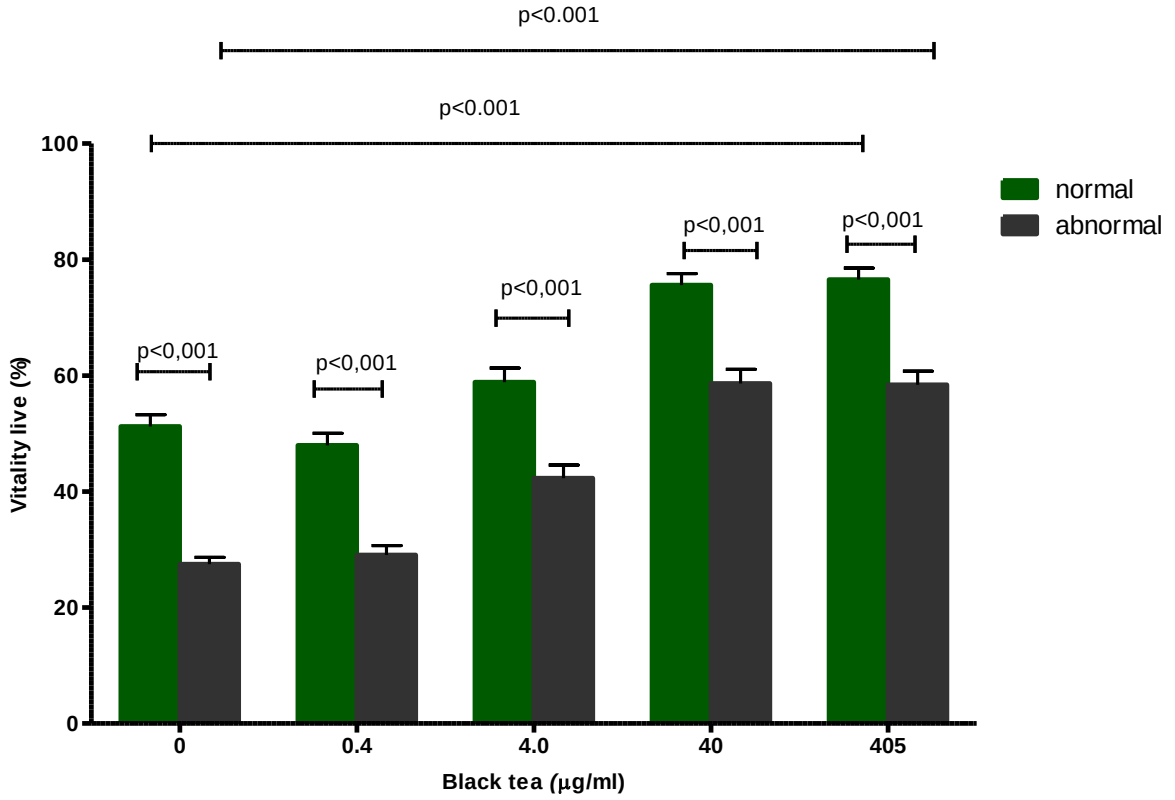
Table 4.3: Sperm kinematic motility parameters, following incubation with increasing concentration of leaf extract of green tea for 1 hour.

Kinematics	Groups	0 µg/ml	0.4 µg/ml	4.0 µg/ml	40 µg/ml	405 µg/ml	p value
VCL ($\mu m\ s^{-1}$)	Normal	74.4±2.2[a]	73.0±2.2[a]	70.0±2.9[a]	72.4±2.4[a]	57.9±4.2[a]	<0.05
	Abnormal	73.1±4.5[a]	85.4±7.4[a]	85.8±4.8[a]	81.6±3.2[a]	55.4±8.8[b]	<0.05
VSL ($\mu m\ s^{-1}$)	Normal	27.9±1.7[a]	26.7±1.5[a]	25.2±1.4[a]	26.9±1.1[a]	18.5±1.7[a]	>0.05
	Abnormal	30.7±2.9[a]	25.9±3.3[a]	31.4±3.5[a]	34.4±2.5[a]	26.2±6.9[a]	>0.05
VAP ($\mu m\ s^{-1}$)	Normal	38.1±1.5[a]	36.3±1.4[a]	34.5±1.4[a]	37.6±1.4[a]	28.7±2.1[a]	>0.05
	Abnormal	39.5±2.5[a]	38.3±2.4[a]	41.7±3.0[a]	43.5±2.4[a]	34.7±7.1[a]	>0.05
LIN (%)	Normal	38.2±1.7[a]	35.7±1.7[a]	34.2±1.4[a]	37.6±1.1[a]	27.5±2.3[a]	>0.05
	Abnormal	39.8±3.0[a]	32.4±3.5[a]	35.5±3.0[a]	41.3±2.1[a]	30.6±6.9[a]	>0.05
STR (%)	Normal	66.3±2.3[a]	65.7±2.0[a]	62.7±2.3[a]	66.9±1.8[a]	50.7±3.9[a]	>0.05
	Abnormal	70.3±3.7[a]	60.5±5.6[a]	64.5±4.1[a]	74.2±2.0[a]	46.4±8.2[a]	>0.05
BCF (HZ)	Normal	20.4±1.3[a]	19.7±0.7[a]	18.3±1.0[a]	18.7±0.7[a]	14.3±1.3[a]	>0.05
	Abnormal	25.4±2.7[a]	19.5±1.1[a]	19.5±1.5[a]	18.8±1.1[a]	12.3±2.1[a]	>0.05
ALH (μm)	Normal	3.6±1.2[a]	2.0±0.0[a]	2.2±0.1[a]	2.1±0.0[a]	1.7±0.1[a]	<0.001
	Abnormal	3.4±0.9[a]	2.3±0.1[b]	2.6±0.1[b]	2.3±0.0[b]	11.±6.7[b]	<0.001

Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. Abbreviations: BCF-beat across frequency; LIN-linearity; STR-straightness; VAP-velocity average path; VCL-velocity curvilinear; VSL-velocity straight line; ALH-average lateral head displacement. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The columns with similar alphabets indicate no significant difference, while those with different alphabets indicates a significant effect. No significant change for most kinematic parameters was observed in the treatment groups compared to the control ($p>0.05$) except for VCL and ALH ($p<0.05$) in both groups. No significant difference between normal and abnormal samples was observed with VSL, VAP, LIN, STR, and BCF ($p>0.05$). A significant difference between normal and abnormal samples was observed at VCL and ALH ($p<0.05$).

4.4 Effect of aqueous leaf extract of *Camellia sinensis* on human sperm vitality

Figure 4.5 below shows effects of aqueous extract of black tea on human sperm vitality following the exposure of human spermatozoa to the different concentrations of the extract (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr. The percentage of live sperm was significantly higher in the normal group compared to the abnormal group ($p < 0.001$; Two-way ANOVA analysis). Also, the treatment of human sperm with black tea resulted in a concentration–dependent increase in the percentage of live sperm for normal samples, and abnormal samples, compared to their respective controls ($p<0.001$). Repeated measures ANOVA also presented a linear trend with increasing concentrations in the normal as well as the abnormal samples ($p<0.05$).

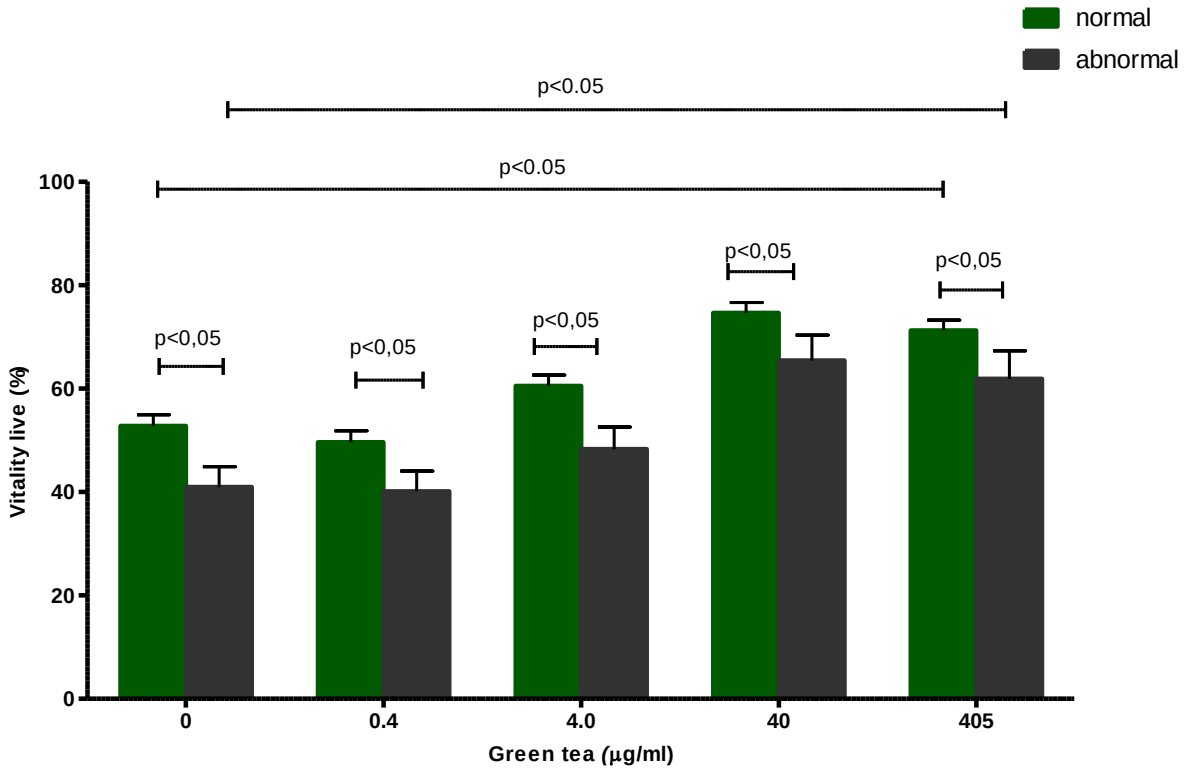


Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.5: Effect of aqueous leaf extract of black tea on human sperm vitality.

Figure 4.6 shows the effects of green tea on human sperm vitality following the exposure of human spermatozoa to the different concentrations of the aqueous green leaf extract (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr. The percentage of live sperm was significantly higher in the normal group compared to the abnormal group ($p<0.05$; Two- way ANOVA

analysis). Also, the treatment of human sperm with aqueous leaf extract showed a concentration–dependent increase in the percentage of live sperm for normal samples, and abnormal samples, compared to their respective controls ($p<0.05$). Repeated measures ANOVA also showed a linear trend with increasing concentrations in the normal as well as the abnormal samples ($p<0.05$).



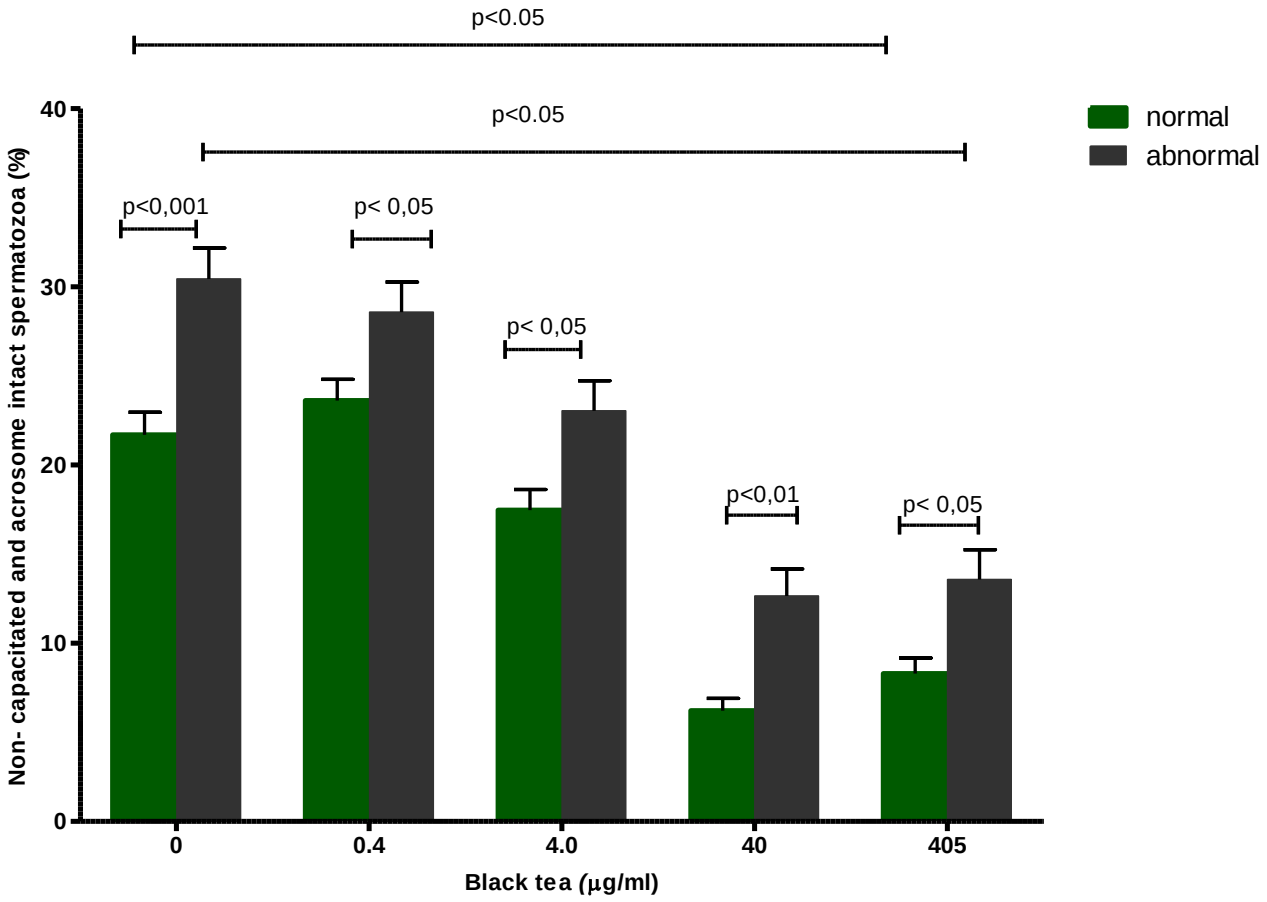
Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.6: Effect of aqueous leaf extract of green tea on human sperm vitality.

4.5 Effect of *Camellia sinensis* on sperm capacitation and acrosome reaction

Following the exposure of human spermatozoa to the different concentrations of black tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.7 below shows that the percentage of non-capacitated and acrosome intact sperm was significantly higher in the normal group compared to the abnormal group ($p<0.05$; Two- way ANOVA analysis). Also, the treatment of human sperm with aqueous extract of black tea resulted in a concentration–dependent decrease in the percentage of non-capacitated and intact sperm in both normal and abnormal sperm samples compared to their respective controls ($p<0.01$).

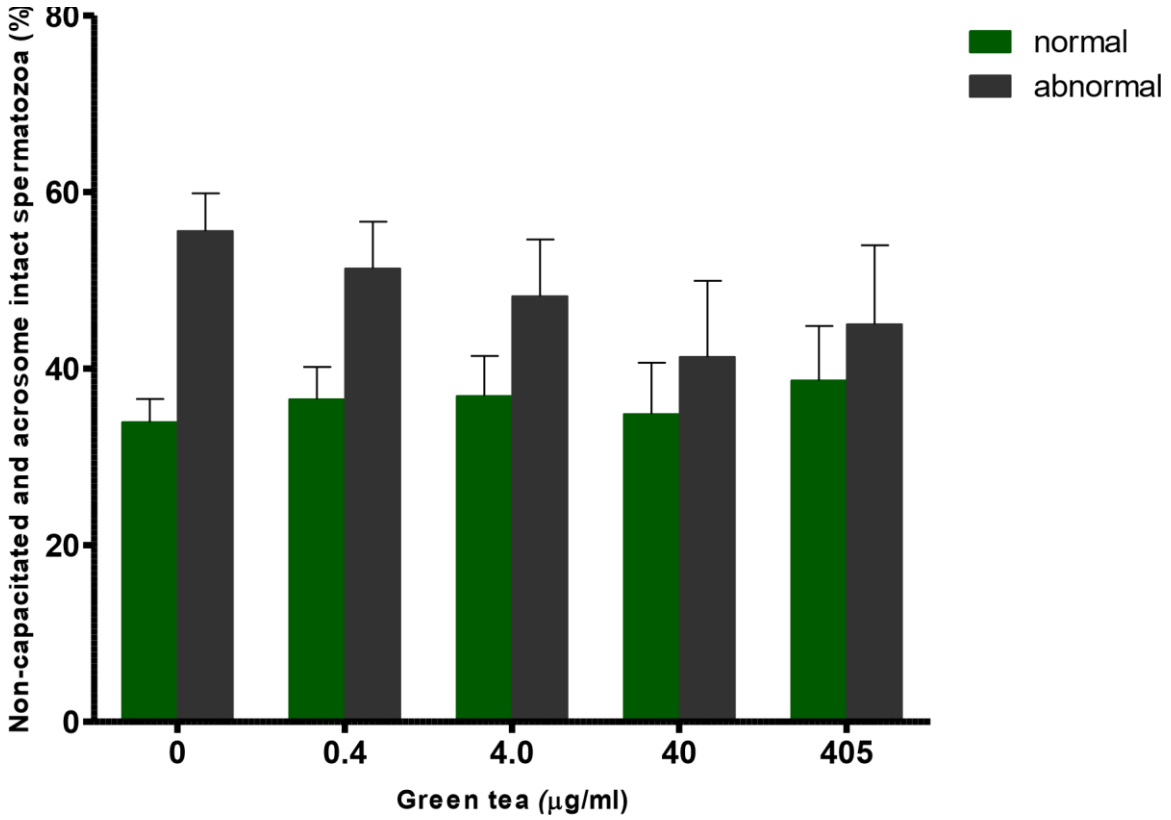
Repeated measures ANOVA also shows a decreasing trend with increasing concentrations in the normal as well as the abnormal samples (p<0.05).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.7: Effects of aqueous leaf extract of black tea on non-capacitated and acrosome intact spermatozoa.

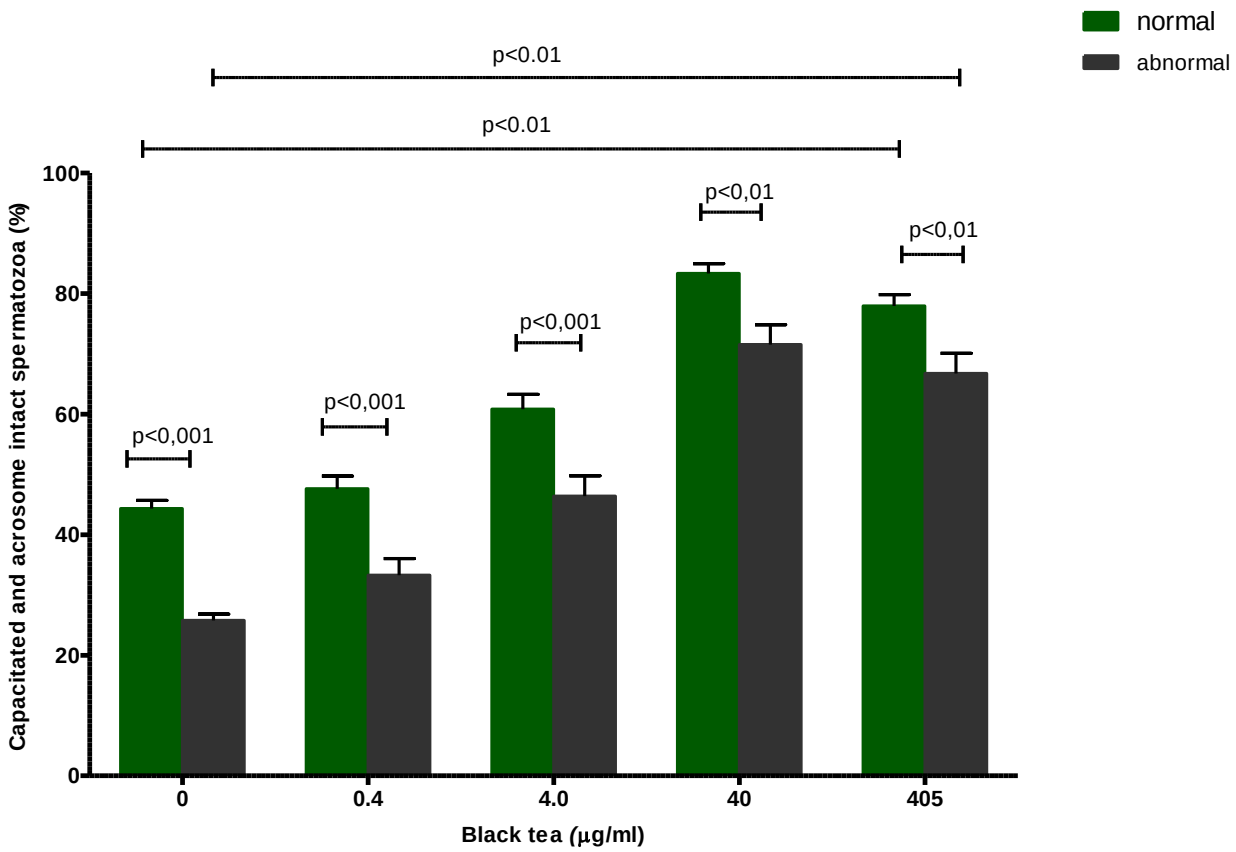
Figure 4.8 below shows that the treatment of human spermatozoa with different concentrations of aqueous leaf extract of green tea (0, 0.4, 4.0,40 and 405 µg/ml) for 1 hr resulted in no change in the percentage of non-capacitated and acrosome intact spermatozoa in the normal group compared to the abnormal groups (p>0.05). There was no significant change observed in the percentage of non-capacitated and intact sperm observed after the treatment with green tea leaf extract in the abnormal groups compared to its control (p>0.05). Repeated measures ANOVA showed no trend in both normal and abnormal samples (p>0.05).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.8: Effects of aqueous leaf extract of green tea on non-capacitated and acrosome intact human spermatozoa.

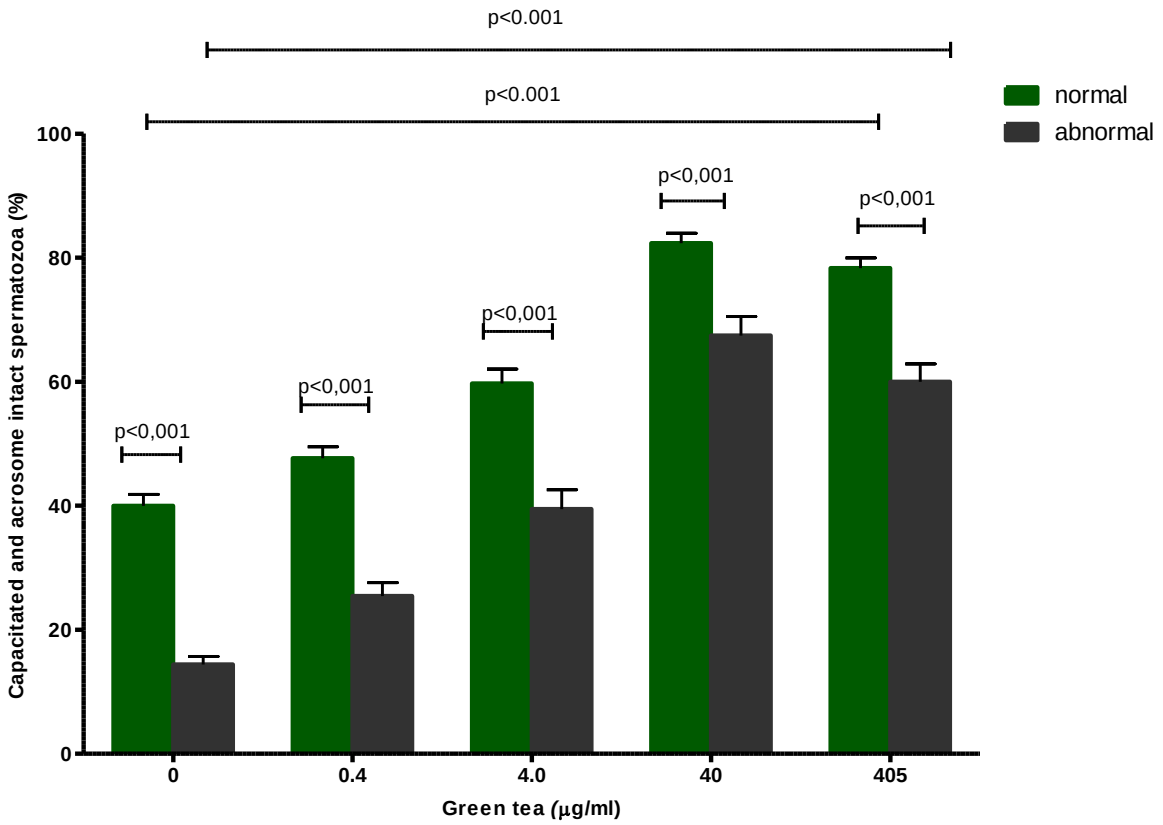
Following the exposure of human spermatozoa to the different concentrations of aqueous leaf extract of black tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.9 shows that the percentage of capacitated and acrosome intact spermatozoa was significantly higher in the normal group compared to the abnormal group ($p<0.01$; Two- way ANOVA analysis). Also, the treatment of human sperm with aqueous extract of black tea resulted in a concentration–dependent increase in the percentage of capacitated and intact sperm in both normal and abnormal sperm samples, compared to their respective controls ($p<0.01$). Repeated measures ANOVA also showed a significant positive trend with increasing concentrations of the extract in the normal as well as the abnormal samples ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.9: Effects of aqueous leaf extract of black tea on human sperm capacitation and acrosome intact spermatozoa.

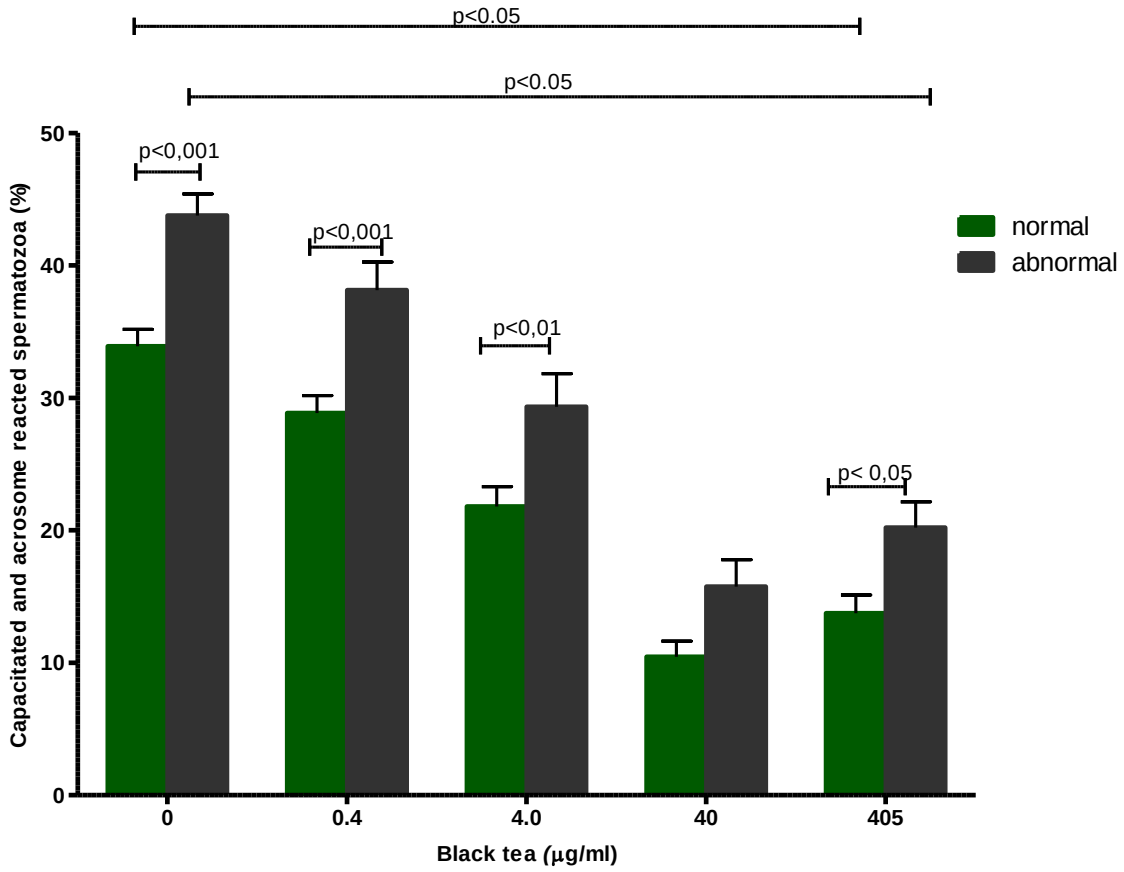
Following the exposure of human spermatozoa to the different concentrations of aqueous leaf extract of green tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.10 showed that the percentage of capacitated and acrosome intact spermatozoa was significantly higher in the normal group compared to the abnormal group ($p<0.001$; Two- way ANOVA analysis). Also, the treatment of human sperm with aqueous leaf extract of green tea resulted in a concentration –dependent increase in the percentage of capacitated and intact sperm in both normal and abnormal sperm samples compared to their respective controls ($p<0.001$). Repeated measures ANOVA also showed a positive significant trend with increasing concentrations of the extract in the normal as well as the abnormal samples ($p<0.001$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.10: Effects of aqueous leaf extract of green tea on human capacitated and acrosome intact sperm.

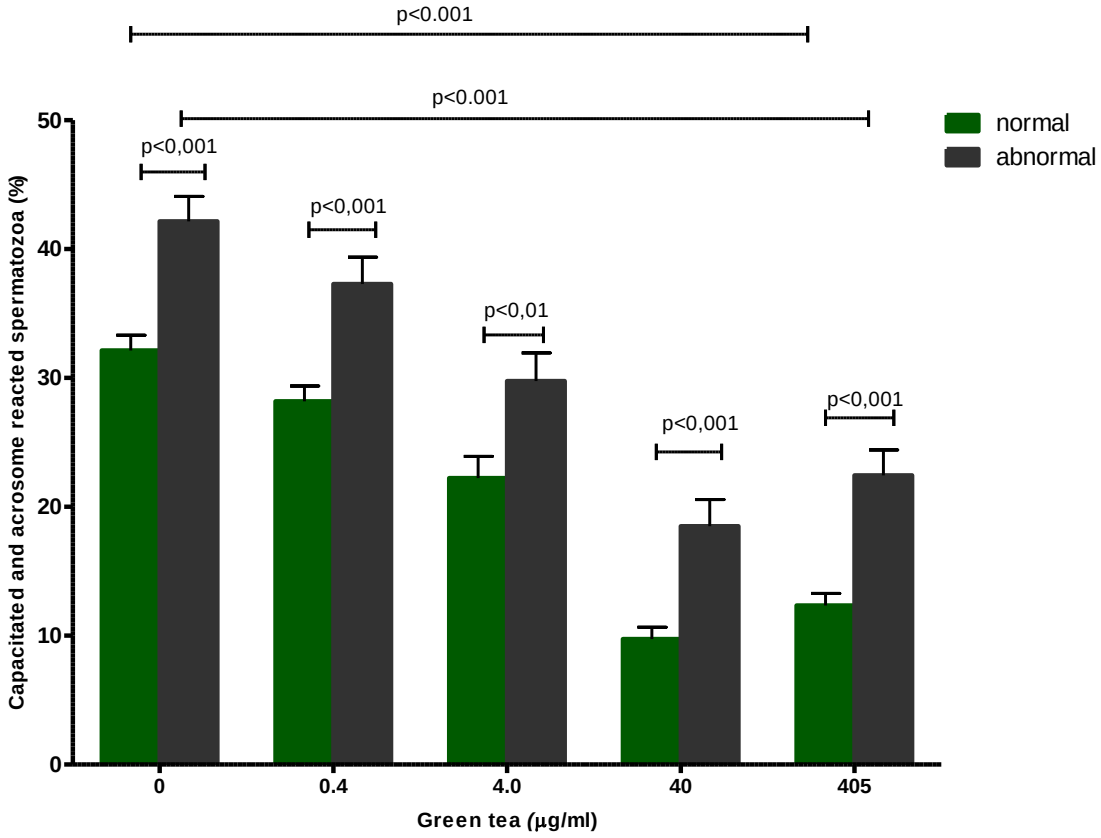
Following the exposure of human spermatozoa to the different concentrations of aqueous leaf extract of black tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.11 showed that the percentage of capacitated and acrosome reacted sperm was significantly higher in the normal group compared to abnormal group ($p < 0.05$; Two- way ANOVA analysis). Also, the treatment of human sperm with black tea resulted in a concentration –dependent decrease in the percentage of capacitated and reacted sperm in both normal and abnormal sperm samples compared to their respective controls ($p<0.05$). Repeated measures ANOVA also showed a negative trend with increasing concentrations in the normal as well as the abnormal samples ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.11: Effects of aqueous leaf extract of black tea on capacitated and acrosome reacted human sperm.

Figure 4.12 shows that the treatment of human spermatozoa with different concentrations of aqueous leaf extract of green tea (0 ,0.4, 4.0,40 and 405 µg/ml) for 1 hour resulted in a significant decrease in the percentage of capacitated and reacted spermatozoa in the normal samples compared to the abnormal samples ($p<0.001$). Also, there was a dose-dependent decrease in the percentage of capacitated and reacted sperm observed after treatment with green tea leaf extract in both normal and abnormal groups compared to their respective controls ($p< 0.001$). Repeated measures ANOVA showed a negative trend in the percentage of capacitated and reacted spermatozoa with increasing concentration in both normal and abnormal samples ($p<0.05$).

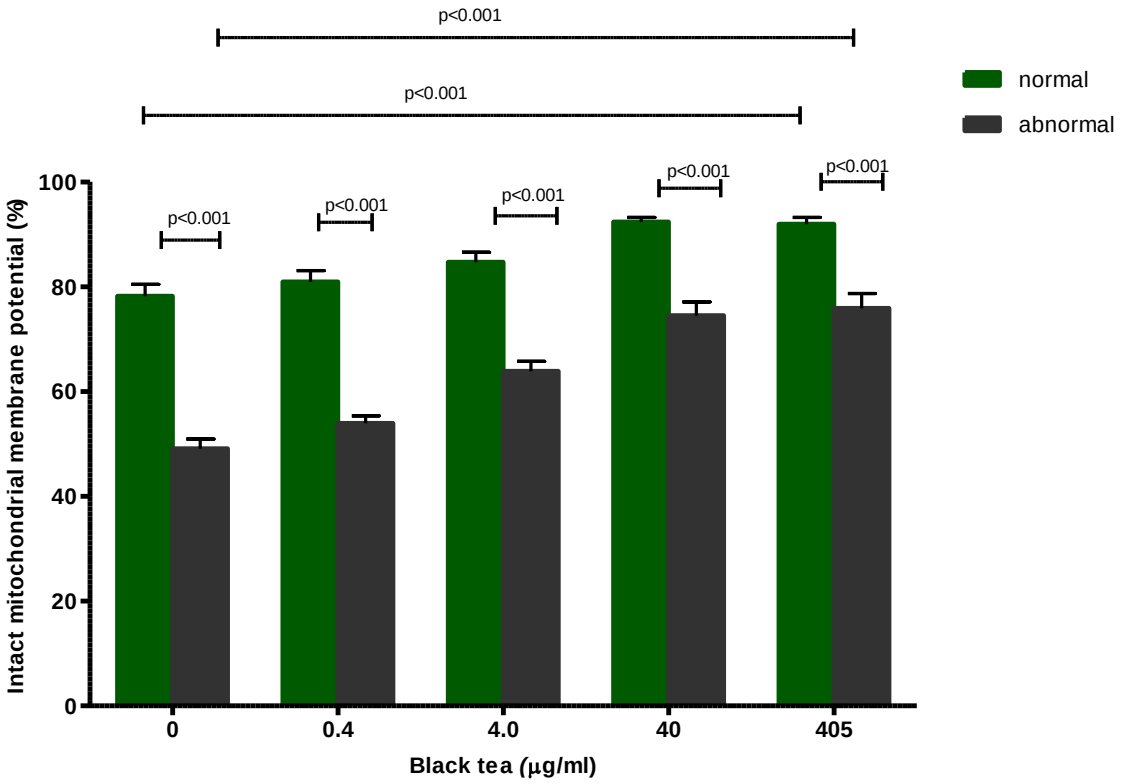


Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.12 Effects of aqueous leaf extract of green tea on capacitated and acrosome reacted human sperm.

4.6 Effect of *Camellia sinensis* on human sperm intact mitochondrial membrane potential

Following the exposure of human spermatozoa to the different concentrations of aqueous extract of black tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.13 below shows that the percentage of spermatozoa with intact mitochondrial membrane potential was significantly higher in the normal group compared to the abnormal group ($p< 0.001$; Two-way ANOVA analysis). Also, the treatment of human sperm with aqueous extract of black tea resulted in a concentration –dependent increase in the percentage of intact MMP spermatozoa in both normal and abnormal sperm samples compared to their respective controls ($p<0.001$). Repeated measures ANOVA also shows a trend with increasing concentrations in the normal as well as the abnormal samples ($p<0.05$).

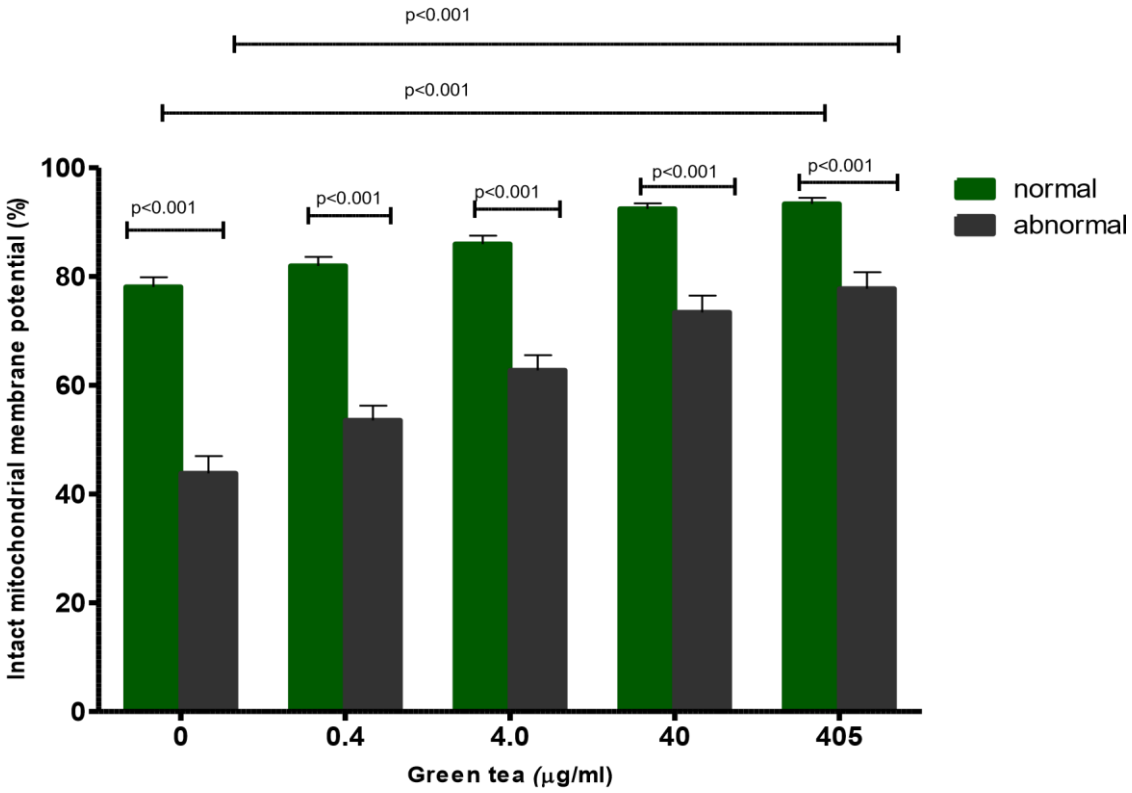


Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.13 Effects of aqueous leaf extract of black tea on human sperm intact mitochondrial membrane potential.

Figure 4.14 below shows the results of the treatment of human sperm with different concentrations of aqueous leaf extract of green tea (0, 0.4, 4.0,40 and 405 µg/ml) for 1hr. This showed a significantly higher percentage of intact mitochondrial membrane potential spermatozoa in the normal and abnormal groups compared to their respective controls ($p<0.001$). There was a dose-dependent increase in the percentage of intact mitochondrial membrane potential sperm observed after treatment with green tea leaf extract in both normal and abnormal groups compared to their respective controls ($p<0.001$). Furthermore, a significantly higher percentage of spermatozoa with intact MMP was observed in the normal groups compared to the abnormal groups ($p<0.001$).

Repeated measures ANOVA showed a trend of increasing intact sperm with increasing concentration ($p<0.05$) in both normal and abnormal samples.

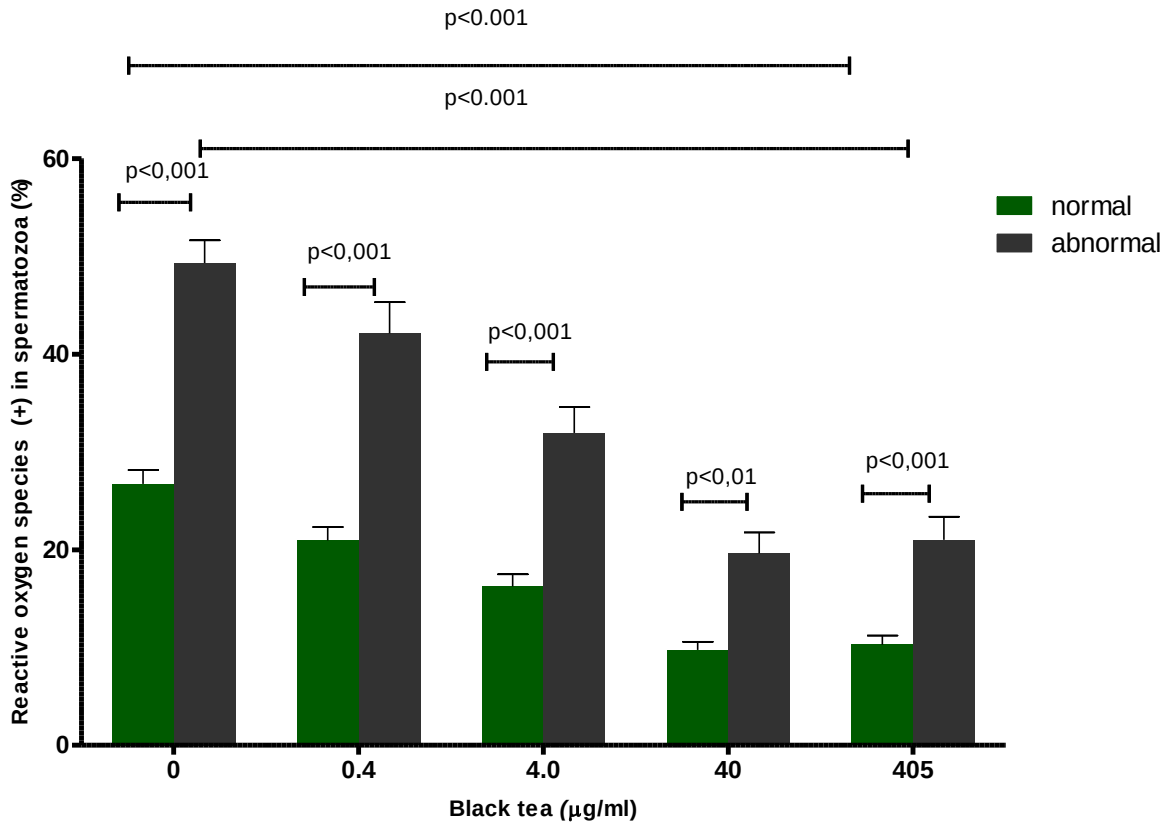


Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.14: Effects of aqueous leaf extract of green tea on human sperm intact mitochondrial membrane potential.

4.7 Effect of *Camellia sinensis* on intracellular reactive oxygen species in human spermatozoa

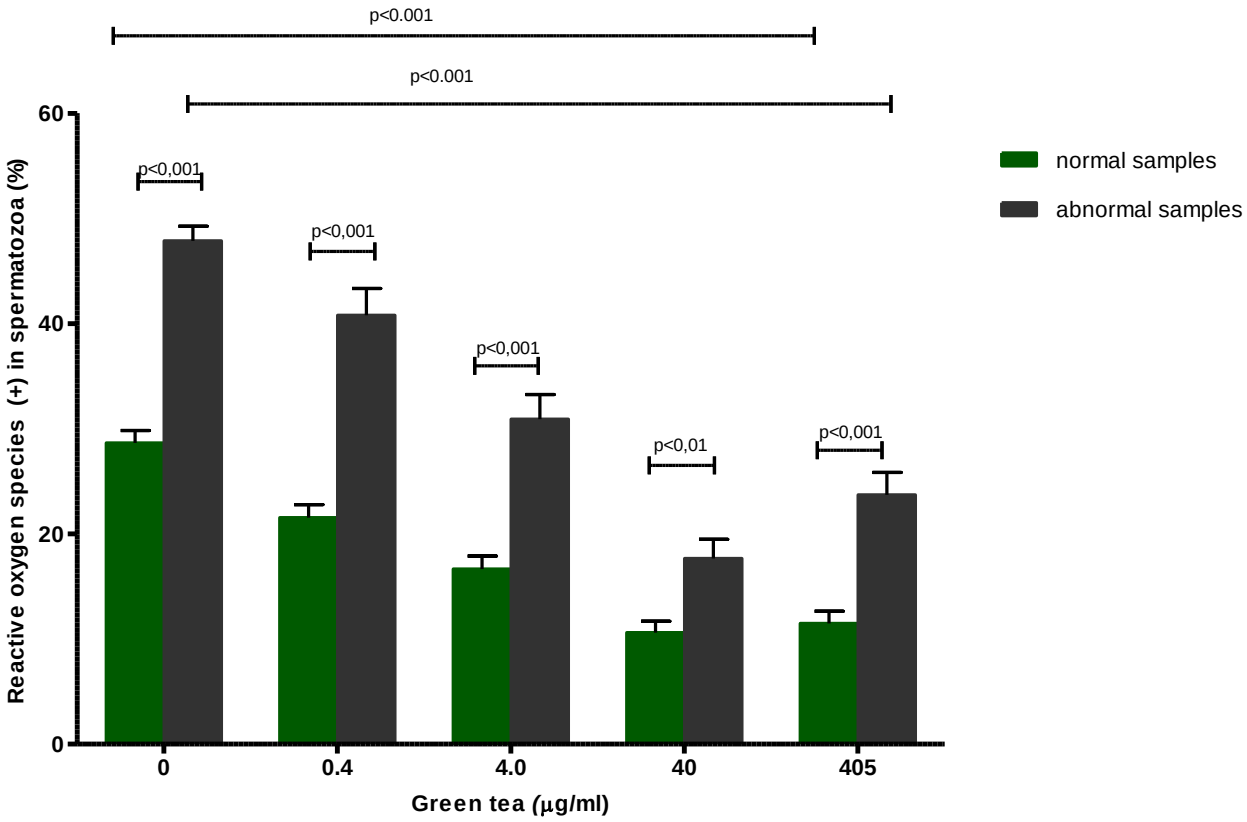
Following the exposure of human spermatozoa to the different concentrations of black tea (0, 0.4, 4.0, 40 and 405 µg/ml) for 1 hr, Figure 4.15 below shows that the percentage of positive reactive oxygen species spermatozoa was significantly higher in the abnormal group compared to normal group ($p<0.001$; Two- way ANOVA analysis). Also, the treatment of human sperm with aqueous extract of black tea resulted in a concentration –dependent decrease in the percentage of ROS+ spermatozoa in both normal and abnormal sperm samples compared to their respective controls ($p<0.001$). Repeated measures ANOVA also shows a negative trend with increasing concentrations in the normal as well as the abnormal samples ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.15: Effects of aqueous leaf extract of black tea on reactive oxygen species in human sperm.

Figure 4.16 demonstrates that the treatment of human sperm with different concentrations of aqueous leaf extract of green tea (0, 0.4, 4.0,40 and 405 µg/ml) for 1 hr resulted in a significantly lower percentage of positive reactive oxygen species spermatozoa in the normal and abnormal groups compared to their respective controls ($p<0.05$). A dose dependent decrease in the percentage of positive ROS spermatozoa was observed after treatment with green tea leaf extract in both the normal and abnormal groups compared to their respective controls ($p>0.001$). In addition, a significantly higher percentage of positive ROS was observed in the abnormal group compared to the normal group ($p<0.001$; Figure 4.14). Repeated measures ANOVA showed a negative trend with increasing concentration in both normal and abnormal samples ($p<0.05$).

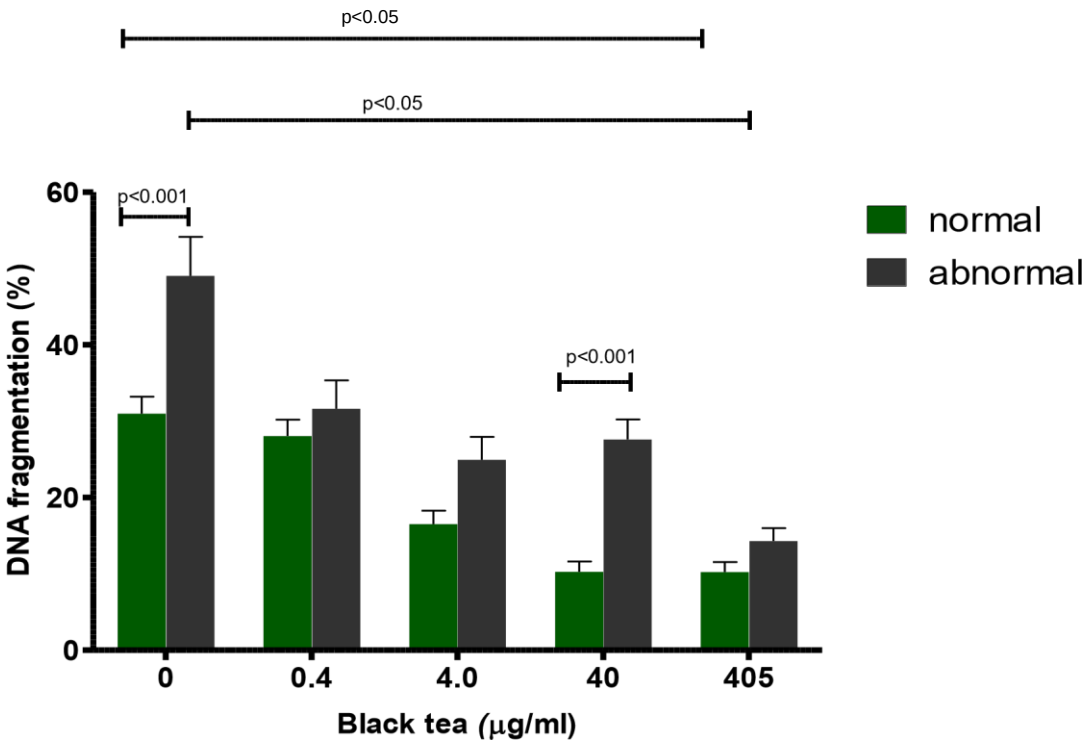


Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.16 Effects of aqueous leaf extract of green tea on reactive oxygen species in human sperm.

4.8 Effect of *Camellia sinensis* on DNA fragmentation in human spermatozoa

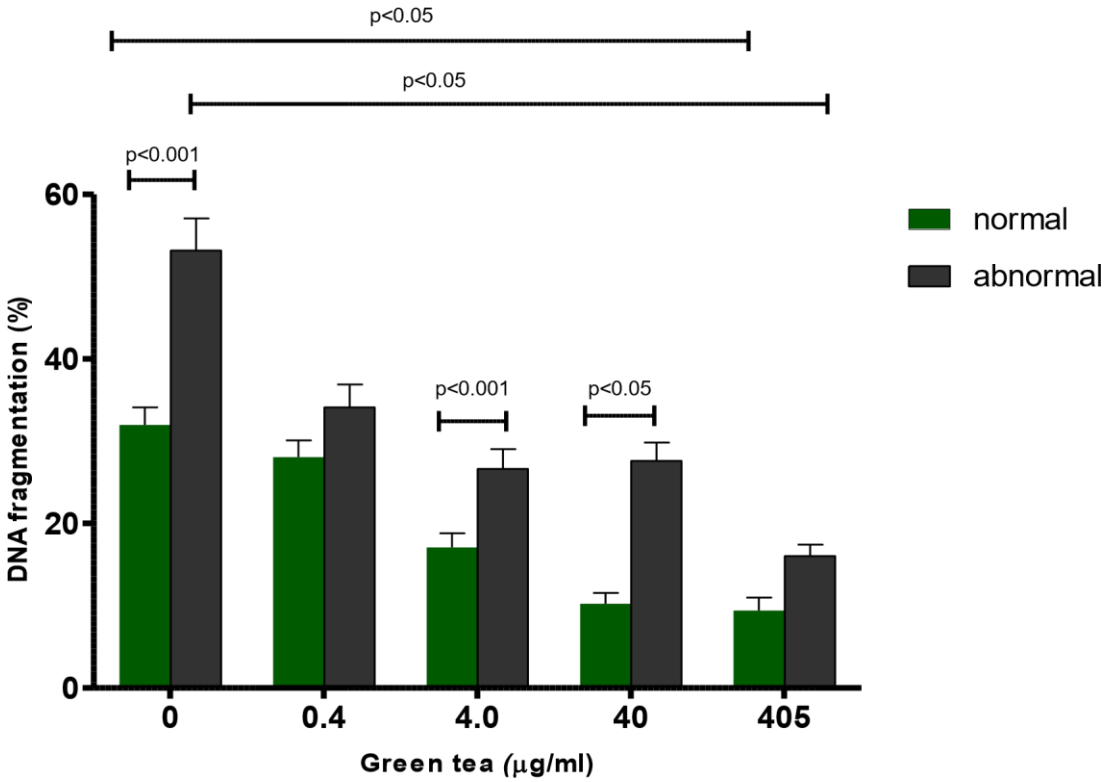
Figure 4.17 demonstrates that the treatment of human sperm with different concentrations of black tea (0 ,0.4, 4.0,40 and 405 µg/ml) for 1 hr resulted in a significantly decreased percentage of fragmented DNA spermatozoa in the normal and abnormal groups compared to their respective controls ($p<0.001$). Fragmented DNA spermatozoa percentage was significantly lower in the normal group at concentrations 0.4 µg/ml ($p<0.001$) and 40 µg/ml ($p<0.001$) than in the abnormal. A dose dependent decrease in the percentage of fragmented DNA spermatozoa was observed after treatment with black tea. Additionally, a significantly lower percentage of fragmented DNA spermatozoa was observed in the normal group as compared to the abnormal group at concentrations 0 µg/ml and 40 µg/ml ($p<0.001$). Repeated measures ANOVA showed a negative trend with increasing concentration in both normal and abnormal samples ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of black tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.17: Effects of black tea on human sperm DNA fragmentation.

Figure 4.18 demonstrates that the treatment of human sperm with different concentrations of green tea (0 ,0.4, 4.0,40 and 405 µg/ml) for 1 hr resulted in a significantly decreased percentage of fragmented DNA spermatozoa in the normal and abnormal groups compared to their respective controls ($p<0.001$). Fragmented DNA spermatozoa percentage was significantly lower in the normal group at concentrations 0 µg/ml ($p<0.001$); 4.0 µg/ml ($p<0.001$) and 40 µg/ml ($p<0.05$) than in the abnormal group. A dose dependent decrease in the percentage of fragmented DNA spermatozoa was observed after treatment with green tea. Additionally, a significantly lower percentage of fragmented DNA spermatozoa was observed in the normal group as compared to the abnormal group ($p<0.001$). Repeated measures ANOVA showed a trend of decreasing percentage of fragmented DNA with increasing concentration in both normal and abnormal samples ($p<0.05$).



Data comprises of normal (n=40) and abnormal (n=19) sperm samples which are expressed as mean±SEM. The results follow 1-hour incubation with the aqueous extract of green tea in different concentrations (0 µg/ml; 0.4 µg/ml; 4.0 µg/ml; 40 µg/ml and 405 µg/ml). The green bars represent normal samples, and the grey bars indicate abnormal samples.

Figure 4.18: Effects of green tea on human sperm DNA fragmentation.

CHAPTER 5: DISCUSSION

5.1 Discussion

Infertility is when a couple is incapable of falling pregnant following one year of unprotected sexual intercourse with the aim of falling pregnant (WHO., 2010; Fisher and Hammarberg, 2012; Gunes, Al-Sadaan and Agarwal., 2015). The prevalence of infertility indicates that one in six couples are affected every year (Brugo-Olmedo, Chillik and Kopelman., 2001). The incidence of infertility is about 25% in couples globally, out of which 15% of them seek medical attention and 5% remain childless after seeking medical treatment. Of this childlessness, the male factor contributes 50% (Agarwal *et al*., 2015).

There are several modern treatments such as assisted reproductive therapy that are used to address issues of male fertility, but these methods tend to be expensive and non-convenient to most of the masses (Sengupta, Agarwal, Pogrebetskaya, Roychoudhury, Durairajanayagam *et al*., 2018). From ancient times, it has been known that people used medicinal plants as their remedy for different illnesses. There are studies that narrate that in Southern Africa alone, the Khoisan people as well as the bushmen relied on plants as their food and medicine for every illness they came across (Herremans., 2004.). In the present day, there are rules and regulations that are used to govern the use of traditional medicine. The Traditional Health Practitioners Act, No. 22 of 2007 in South Africa is used to ensure safety and proper usage of traditional medicine (Louw and Duvenhage., 2016). The World Health Organization also had several studies on medicinal plants as the most affordable and readily available to all social classes in the world (WHO., 2004).

Currently, modern medication has taken over the health industry, making medicinal plants rare and not well-known (Lankatillake, Huynh, and Dias., 2019). However, in developing and some developed countries, the use of medicinal plants is rapidly returning as more people are going for cheaper medication (Sam., 2019). Medicinal plants are rapidly used in the production of the modern synthetic medication for healthcare services in the Western medication (Jamshidi-Kia, Lorigooini, and AminiKhoei., 2018). Some of these modern medications are derived from medicinal plants with modifications here and there with modern methods (Atanasov, Waltenberger, PferschyWenzig, Linder, Wawrosch *et al*., 2015). This is done because medicinal plants possess significant amounts of antioxidants and other useful chemical compounds that are useful in the precise

pharmacological concentrations manufacturing, and because many modern medicines were initially produced using medicinal plants (Prasad, Gupta, Kumar, Kumar, Wang *et al*., 2017). Several studies have proven that precisely these plants have good qualities (usually associated with their antioxidant properties) to improve and enhance the fertility parameters of males (Lampiao, Krom, and Plessis., 2008; Chikhoune, Stouvenel, IguerOuada, Hazzit, Schmitt *et al*., 2015; Mbemya, Vieira, Canafistula, Pessoa, and Rodrigues., 2017).

There are various antioxidants and enzymes that serve as the protective barrier for spermatozoa to prevent oxidative stress. Two well-known types are prevention antioxidants and scavenger antioxidants (Zhang, Duan, Song, Liu, Houn *et al*., 2021). Examples of prevention antioxidants include metal chelators and metal-binding proteins, which function to block new ROS formation. In contrast, scavenger antioxidants function to remove the already existing ROS (Zhang, Duan, Song, Liu, Houn *et al*., 2021). These mechanisms are naturally developed in the testes to protect them from damage of free radicals (Zhang, Duan, Song, Liu, Houn *et al*., 2021). There is a wide array of antioxidants categories that are developed in the Leydig cells that include enzymatic and nonenzymatic antioxidants (Nimse, and Pal., 2015). The examples of non-enzymatic antioxidants include glutathione, ascorbic acid, and tocopherol which function to break down and destroy free radicals (Nimse, and Pal., 2015). The enzymatic factors include resveratrol, melatonin, superoxide dismutase, glutathione peroxidase and catalase, which regulate testicular antioxidants inhibit oxidation (Nimse, and Pal., 2015). Some of the systems of these antioxidants can be found in plants such as *Camellia sinensis,* which may be used to lessen the effects of ROS on reproductive cells, thus, spermatozoa and improve fertility (Mahmoudi, Azizi, Abedini, Jahromi, Abidi *et al*., 2018).

Camellia sinensis (black, white, green and oolong teas) has several health benefits, including anticancer, hepatoprotective activities, anti-inflammatory, anti-schistosomiasis, anti-parasitic activities, and the body weight reduction effects (Aboulwafa, Youssef, Gad, Altyar, Al-Azizi *et al*., 2019. Adeneye., 2016; Izzreen, and Mohd Fadzelly., 2013). These teas are also known for their reproductive health benefits in males as they improve some, if not all, sperm parameters. Traditional practitioners in Sri Lanka recommend black tea for the improvement of sexual function and for delaying ejaculation (Sharma, Joshi, Baldi, Khatri and Dube, 2013). *Camellia sinensis* was shown to improve rat sperm parameters such as sperm motility, viability, and sperm concentration (Opuwari, and Monsees., 2013,

Das and Karmakar., 2015). Furthermore, black and green tea are known to elevate testosterone and are used traditionally as an aphrodisiac (JianFeng, PengYing, ChengWei, TaoTao, YunGui *et al*., 2012; Hamza, Al-Salmi, Laban, and El-Shenawy., 2020). Another study showed a significant change of testosterone secretion after treatment with black tea in male rats (Opuwari and Monsees, 2015). However, Opuwari and Monsees (2015) revealed that both green and black tea had anti-androgenic effect by the reduced production of testosterone in TM3 Leydig cells.

Given that motility is one of the main components of sperm quality, it was important that the extract be tested for its effects on sperm motility thereof. There are different factors that affect sperm motility such as DNA damage, ROS, lifestyle, as well as varicocele (Nowicka-Bauer, and Nixon., 2020). The study found that the aqueous extracts had no significant effects on total motility for both black and green in both normal and abnormal samples. Also, there was no significant difference between the normal and abnormal samples. In addition, both black and green tea extract significantly increased the percentage of rapid progressive motility at 405 μg/ml and medium progressive motility at 0.4 μg/ml in the abnormal samples. No further significant effects were observed in both normal and abnormal samples. It could be suggested that *Camellia sinensis* has some properties that improve sperm motility to some extent like in medium and rapid progressive motility, but in overall total motility, no improvement was observed. The increased rapid and medium progressive motility could indicate that the present speed of spermatozoa could be enhanced and would be able to have the required speed to fertilise. More research is needed to better understand the mechanism of action of the extract as well as its fertilising capabilities.

Sperm kinematic parameters are often a requirement for sperm movement measurement. Studies on sperm kinematics show that substances that enhance hyperactivated motility may improve kinematics such as velocity curvilinear (VCL), average lateral head displacement (ALH) as well as beat across frequency (BCF), but linear progression characters such as linearity (LIN), straightness (STR) as well as velocity straight line (VSL) may be reduced (Mortimer and Mortimer, 1990; Cancel, Lobdell, Mendola and Perreault., 2000; Adamkovicova, Toman, Martiniakova, Omelka, Babosova *et al*., 2016)., Following the treatment of human spermatozoa with the aqueous extract, there was no significant change in all the parameters except for ALH, which showed a significant increase in both the abnormal and normal samples for both green and black tea extracts

(Giaretta, Munerato, Yeste, Galeati and Spinaci., 2017). Studies show that kinematic parameters of sperm may have a relationship with fertilising capabilities of spermatozoa (Robayo, Montenegro, Valdes, and Cox., 2008). These kinematic parameters play an important role as markers in sperm function, with VCL and BCF as markers for sperm vitality, VSL, STR; and LIN as movement of sperm markers, and additionally, having STR and LIN as important markers for control of swimming arrays (Adamkovicova, Toman, Martiniakova, Omelka, Babosova *et al*., 2016). Kinematic parameters (VCL, VAP, ALH, and BCF) may be increased by any substance that enhances and increases motility hyperactivation (Moichela., 2021). It was observed in this study that the levels of VCL and ALH were significantly increased because of treatment with the plant extract *Camellia sinensis.* Other reasons for the improvement of these parameters may be the presence of antioxidant properties in the *Camellia sinensis* such as EGCG, calcium, and polyphenols (Roychoudhury, Agarwal, Virk, and Cho., 2017). This shows that *Camellia sinensis* may influence sperm motion, thereby improving motility and possibly, fertilising capacity (Collodel, Capitani, Baccetti, Pammolli, and Moretti., 2007; Boryshpolets, Kowalski, Dietrich, Dzyuba and Ciereszko., 2013). It is therefore necessary for more research to be done for further understanding.

Vitality is defined as the amount in percentage of sperm that is alive/living and healthy in a collected semen (Fu, Zhou, Liu, Li, Yao *et al*., 2014). This sperm must be able to move independently and live to its maximum duration. There is rarely a 100% live sperm present in a collected semen. This is due to the dead sperm taking long to reabsorb, making it present in the collected semen, thereby reducing the live sperm percentage (Fu *et al*., 2014). Other causes of low sperm vitality may be exposure to toxins, excessive ROS production and necrospermia (Agarwal, Sharma, Gupta, Boitrelle, Finelli *et al*., 2021). In this study, exposure of spermatozoa to different concentrations of *Camellia sinensis* (aqueous extract of black and green tea) resulted in a significant increase in the percentage of live spermatozoa in both normal and abnormal samples. A trend analysis also demonstrated a dose dependent increase in live sperm for both normal and abnormal samples. It could be suggested that *Camellia sinensis* could improve spermatozoal vitality, thereby possibly enhancing fertility.

When the amounts of reactive oxygen species are higher than the antioxidants, oxidative stress occurs (Aitken., 2017). Free radicals are normally produced as an indicator for the imbalance between ROS and antioxidants in the body (Aitken., 2017). The sperm cell

normally requires oxygen for metabolism, but the ROS as metabolites can be harmful to the cell (Dutta, Henkel, Sengupta, and Agarwal., 2020). When this happens, the natural antioxidants are the ones responsible for counteracting these deleterious effects (Dutta, Henkel, Sengupta, and Agarwal., 2020). Therefore, oxidative stress develops following the imbalance between the antioxidants and ROS generation (Dutta, Henkel, Sengupta, and Agarwal., 2020). There are several causes of this overproduction of ROS, including lifestyle choices, which may be associated with male infertility and its risk factors (Panner Selvam, Ambar, Agarwal, and Henkel., 2021). There is also a matter of vitamins A and E deficiency, which may also be associated with an increase in oxidative in the testes, which distracts the process of spermatogenesis and steroidogenesis (Akilah Amira, Kabel Ahmed, and Alharthi Huda., 2017). When these processes are interrupted, other biological molecules such as DNA, proteins and lipoproteins may be damaged (Ali, Martinez, and Parekh., 2021). It is therefore important that the excessive production of free radicals be avoided at all costs to maintain normal sperm cell functions (Ali, Martinez, and Parekh., 2021). In the current study, treatment with different concentrations of *Camellia sinensis* for an hour resulted in a significant decrease in the percentage of ROS in both normal and abnormal groups as compared to their respective controls. The abnormal samples showed to have a higher percentage of ROS than the normal samples in both green and black tea treated samples. A dose-dependent decrease in ROS production was also detected with the increasing concentration of the leaf extracts. It can be said that *Camellia sinensis* may have antioxidant properties that may improve the quality if spermatozoa by reducing the production if excessive ROS. When ROS production is reduced, oxidative stress may not occur, thus sperm normal functionality would be maintained (Nowicka-Bauer, and Nixon., 2020).

The mid piece of the spermatozoa consists of axosome and associated dense fibres, which are covered by the mitochondria responsible for ATP intracellular stores generation (Tremoen., 2018). Reactive oxygen species that are excessively produced may harm the mitochondrial membrane, thereby interfering with the normal functions, resulting in infertility (Darbandi, Darbandi, Agarwal, Sengupta, Durairajanayagam *et al*., 2018). The excess ROS produced also damages the ability of the sperm to go through capacitation, which is essential for fertility (De Luca, Colone, Gambioli, Stringaro, and Unfer., 2021). In this study, black and green tea treatment resulted in a significant increase in the percentage of intact membrane in both the normal and abnormal samples. In addition, a

dose-dependent increase was detected in both groups with increasing concentration of the plant extracts. The normal samples showed to have a higher percentage of intact MMP than the abnormal sample. This was observed in both green and black tea treated samples. This may suggest that *Camellia sinensis* may have a potential to improve mitochondrial membrane potential of human spermatozoa. Oxidative stress has detrimental effects on human normal sperm function, including MMP and DNA damage, resulting in sperm losing its potential to fertilise (Darr, Varner, Teague, Cortopassi, Datta *et al*., 2016). Studies show that ROS may be an indication of good mitochondrial function as a biomarker and may be pathologic when produced in excessive amounts (Darr, Varner, Teague, Cortopassi, Datta *et al*., 2016). As it is, ROS production enhances the hyperactivation of sperm mitochondria, and when in excess, it results in oxidative stress and loss of function (Darr, Varner, Teague, Cortopassi, Datta *et al*., 2016). As it is known through research, sperm motility is the primary parameter used to determine the quality of sperm. It is therefore important that sperm be supplied with enough ATP to maintain normal functioning of the human sperm (Agnihotri, Agrawal, Hakim, Vishwakarma, Narender *et al*, 2016). The energy supplied to the sperm is stored in the mitochondria of the sperm cell (Agnihotri, Agrawal, Hakim, Vishwakarma, Narender *et al*., 2016). This ATP is synthesised using proton concentration gradients and as well as electric potential gradients (Marchetti, Obert, Deffosez, Formstecher, Marchetti., 2002). Therefore, poor sperm motility may arise from the poor mitochondrial function and the inability to store enough ATP for effective sperm movement, thus affecting the fertilising potential of the sperm (Marchetti, Jouy, LeroyMartin, Defossez, Formstecher *et al*., 2004).

The ROS play a role of regulating the processes that take place during capacitation and this results in the success of spermatozoa fusion with the oocyte membrane (Baskaran, Finelli, Agarwal, and Henkel., 2021). The controlled levels of ROS assist in providing the fluidity of the membrane, which in turn maintains sperm acrosome reaction and enables the sperm to fertilise the oocyte (De Luca, Colone, Gambioli, Stringaro, and Unfer., 2021). When the levels of ROS are excessive, the normal capacitation process will be inhibited.

For spermatozoa to undergo acrosome reaction, it must first successfully undergo capacitation. Thus, spermatozoa that has undergone capacitation is liable to proceed to acrosome reaction (Baskaran, Finelli, Agarwal, and Henkel., 2021). Therefore, this emphasises that the balance between ROS production and its clearance is of high

importance as the effects of ROS on capacitation subsequently affects the acrosome reaction process (O'Flaherty., 2020). In this study, exposure of spermatozoa to different concentrations of *Camellia sinensis* (black tea extract) resulted in a significant decrease in the percentage of non-capacitated-and-acrosome intact spermatozoa in both the normal and abnormal groups in comparison to their respective controls. A dose-dependent decrease trend was detected with increasing concentration of the plant extract. For green tea, there was no significant change observed. On the other hand, both black and green tea leaf extracts significantly increased the percentage of capacitated and-acrosome intact spermatozoa in both normal and abnormal samples in comparison to their respective controls. The ANOVA trend test also indicated a dose dependent increase in the capacitated-and-acrosome intact spermatozoa for both normal and abnormal samples. For capacitated-and-acrosome reacted spermatozoa, green and black tea resulted in a significant decrease for both normal and abnormal groups compared to the respective control group. Abnormal samples showed to have a higher percentage on non-capacitated and acrosome intact spermatozoa than the normal samples in both black and green tea. For capacitated and acrosome intact spermatozoa, the normal samples were shown to have a higher percentage than the abnormal samples. In the capacitated and acrosome reacted category, the abnormal samples showed a higher percentage than the normal samples. This study resulted in both green and black tea decreasing acrosome reaction. This may suggest that black and green tea extracts may have properties that enhance fertility. However, there are some aspects of CTC that are still not improved as shown by the results of this study.

Sperm DNA is protected from oxidative stress by the tight packaging characteristic of the DNA and the availability of the antioxidant in the seminal plasma (Bisht, Faiq, Tolahunase and Dada., 2017). In one study, spermatozoa were exposed to artificial ROS. This resulted in the damage of DNA affecting all the bases, chromosomal rearrangements, frameshifts, and deletions as well as cross links (Simon, Emery and Carrell., 2017). There was breakage of the single and double strands of DNA with high frequencies which were shown to be strongly associated with oxidative stress (Agarwal, Virk, Ong and Du Plessis., 2014). There is a strong suggestion that the fragmentation of DNA is strongly associated with high levels of ROS (Agarwal, Virk, Ong and Du Plessis., 2014). This phenomenon is mainly observed in the spermatozoa of infertile men (Agarwal, Virk, Ong and Du Plessis., 2014). Excessive ROS production seems to alter the natural fertility by damaging nuclear

DNA (Agarwal and Said., 2003). Measuring the sperm quality using DNA as an independent measure seems to give better diagnostic and prognostic results than other parameters such as motility, sperm concentration, as well as morphology (Agarwal and Said, 2003). In this study, it was found that the *in vitro* treatment of human spermatozoa with the black and green tea extracts for an hour resulted in significantly decreased fragmented DNA in the normal and abnormal samples. Abnormal samples showed a higher percentage of fragmented DNA than the normal samples. The damaged spermatozoa DNA may be caused by DNA packing during the spermiogenesis process; and it may also be due to oxidative stress. Oxidative stress arises from the imbalance between ROS production and the antioxidant defence system (Zhaku, Agarwal, and Henkel., 2021). Studies show evidence of ROS being the reason for DNA single and double strand bonds breaking (Agarwal and Said., 2003). The bond breakage is usually observed in males who are diagnosed as infertile (Agarwal and Said., 2003). This may be an explanation to why in this study, the abnormal samples had a higher percentage of fragmented DNA as compared to the normal samples.

5.2 Conclusion and recommendations

In previous studies, it has been shown that *Camellia sinensis* is rich in antioxidants that play a significant role in correcting fertility (Aboulwafa, Youssef, Gad, Altyar, Al-Azizi *et al*., 2019). The antioxidants are the major phytochemicals found in this plant. They scavenge the free radicals which in turn prevent an increase in oxidative stress (Abd ElHack, Elnesr, Alagawany, Gado, Noreldin *et al*., 2020).

The study found that *Camellia sinensis* significantly reduced the levels of ROS in the samples treated with the aqueous plant extract for an hour. The incubation with the plant also significantly reduced DNA fragmentation and improved sperm mitochondrial integrity. This study further showed a significant improvement in sperm motility, vitality and capacitation and acrosome reaction. This may arise from the antioxidant properties of the plant that reduce oxidative stress, thereby maintaining a balance between the production of ROS and its removal. These discoveries may be an indication that *Camellia sinensis* has a potential of repairing the harmful effects of oxidative stress, thereby improving the capabilities of sperm to fertilise. *Camellia sinensis* is also readily available and affordable.

There are not enough studies that look at the effects of *Camellia sinensis* on human sperm function focusing on the parameters covered in this study. The mechanisms of action of this plant need to be researched further to get a better understanding. Therefore, further research needs to be conducted on different parts of the plant and its effects on human sperm function. This will give more insight on the capabilities of *Camellia sinensis* to improve sperm fertility.

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APPENDICES

APPENDIX A: TREC CERTIFICATE

TREC/393/2.19:PG

University of Limpopo
Department of Research Administration and Development
Private Bag X1106, Sovenga, 0727, South Africa
Tel: (015) 268 3935, Fax: (015) 268 2306, Email: anastasia.ngobe@ul.ac.za

TURFLOOP RESEARCH ETHICS COMMITTEE

ETHICS CLEARANCE CERTIFICATE

MEETING: 05 November 2019

PROJECT NUMBER: TREC/393/2019: PG

PROJECT:

Title:	Determination of in Vitro Effects of Aqueous Extract of Camellia Sinensis On Human Sperm Functions
Researcher:	MA Setumo
Supervisor:	Dr CS Opuwari
Co-Supervisor/s:	Prof R Henkel
	Mr SSR Choma
Degree:	Master of Science in Medical Science
School:	Health Care Sciences

PP.
PROF P MASOKO
CHAIRPERSON: TURFLOOP RESEARCH ETHICS COMMITTEE

The Turfloop Research Ethics Committee (TREC) is registered with the National Health Research Ethics Council, Registration Number: **REC-0310111-031**

Note:

i) **This Ethics Clearance Certificate will be valid for one (1) year, as from the abovementioned date. Application for annual renewal (or annual review) need to be received by TREC one month before lapse of this period.**

ii) **Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee, together with the Application for Amendment form.**

iii) **PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.**

APPENDIX B: BMRC CERTIFICATE

BMREC BM18/3/17

UNIVERSITY of the
WESTERN CAPE

Department of Institutional Advancement
University of the Western Cape
Robert Sobukwe Road
Bellville 7535
Republic of South Africa

27 August 2020

Dr CS Opuwari
Medical Biosciences
Faculty of Natural Sciences

Ethics Reference Number: BM18/3/17

Project Title: In vitro effects of Aspalathus linearis and Camellia sinesis on the human sperm functionality.

Approval Period: 24 July 2020 _ 24 July 2023

I hereby certify that the Biomedical Science Research Ethics Committee of the University of the Western Cape approved the scientific methodology and ethics ofthe above mentioned research project.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

Please remember to submit a progress report annually by 30 November for the duration of the project.

Permission to conduct the study must be submitted to BMREC for record-keeping.

The Committee must be informed of any serious adverse event and/or termination of the study.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

Director: Research Development
University of the Western Cape
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NHREC Registration Number BMREC-130416-050

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18 April 2019

Dr C Opuwari
Medical Biosciences
Faculty of Natural Sciences

Ethics Reference Number: BM18/3/17

Project Title: In vitro effects of *Aspalathus Linearis* and *Camellia sinesis* on the human sperm functionality.

Approval Period: 15 March 2019 – 15 March 2020

I hereby certify that the Biomedical Science Research Ethics Committee of the University of the Western Cape approved the scientific methodology and ethics of the above mentioned research project.

Any amendments, extension or other modifications to the protocol must be submitted to the Ethics Committee for approval.

Please remember to submit a progress report in good time for annual renewal.

The Committee must be informed of any serious adverse event and/or termination of the study.

Ms Patricia Josias
Research Ethics Committee Officer
University of the Western Cape

BMREC REGISTRATION NUMBER -130416-050

APPENDIX C: INFORMED CONSENT FORM (ENGLISH)

In vitro effects of Camellia sinensis on the Human sperm function

Information sheet and consent to participate in Research

To whom it may concern

You have been requested to consider participating in a scientific study on male sperm functions and medicinal plants. The aim of the study is to investigate the effects of *Camellia sinensis* on male reproductive functions using human sperm.

After collection of your semen samples masturbation, it will be exposed to various concentrations of the aqueous extract of *Camellia sinensis,* and the following objectives will be answered:

Can the aqueous leaf extract of *Camellia sinensis* or its components?

- Alter semen parameters such as sperm concentration, motility and vitality?
- Alter sperm fertilising capacity such as acrosome reaction, capacitation, and mitochondrial membrane potential?
- Have a protective function on sperm by reducing DNA fragmentation, and reactive oxygen species (ROS) production?
- Improve semen parameters in infertile men?

For those participating in the assisted reproductive treatment, after your semen has been processed for the treatment, you are scheduled for, a small portion of the semen sample (<100 µl) will be taken for these additional tests. This procedure will under no circumstances affect the assisted reproduction treatment or outcome as only the left-over samples shall be used. This will allow us to determine the possible effects of *Camellia sinensis* on human sperm, as to whether or not it can improve male fertility.

The current study will not involve any additional medical risk, discomfort or cost.

Participation in this scientific study is completely voluntary and participants are allowed to withdraw at any point in time until the data will be submitted for publication without any consequences. Withdrawal from the study should be done in a formally addressed email. In order to protect confidentiality of participants, a unique number will be assigned to each sample and will contain no personal information of the volunteers involved in the study. All data will be confidential and available to the scientific researchers involved as well as to participants if requested. The samples will be disposed of in the correct medical procedure by incineration once the data is captured.

The study has been ethically received by the UWC Biomedical Research Ethics Committee

(Approval number: _____)

In the event of any problems or concerns, additional information can be obtained directly from the doctor or scientific investigator through email:

Consent

I have been informed about the study titled "*In vitro* effects of *Camellia sinensis* on the Human sperm" by the scientific investigator.

I understand the purpose and procedure of the study.
I have been given an opportunity to ask questions about the study and have had answers to my satisfaction.

I declare that my participation in this study is entirely voluntary and that I may withdraw at any point in time until the data will be submitted for publication without any consequences and without affecting any treatment or care that I am entitled to.

If I have any further questions or query related to the study, I understand that I may contact the researchers.

If I have any questions or concerns about my rights as a study participant, or if I am concerned about any aspect of the study or researcher, then I may contact:

_____ _____

Signature of Participant Date

_____ _____

Signature of Investigator Date

APPENDIX D: INFORMED CONSENT FORM (AFRIKAANS)

DEELNAME INGELIGTE TOESTEMMING VORM

***In vitro* effekte van *Camellia sinensis* op die Menslike spermselle**

Inligtingsblad en toestemming om deel te neem aan Navorsing Aan

wie dit mag gaan,

U is versoek om te oorweeg om aan 'n wetenskaplike studie deel te neem oor manlike spermfunksies en medisinale plante. Die doel van hierdie studie is om die effekte van *Camellia sinensis* op manlike voortplantingsfunksies te ondersoek deur menslike sperma te gebruik.

Na die insameling van u semenmonstermasturbasie sal dit blootgestel word aan verskillende konsentrasies van die waterige uittreksel van *Camellia sinensis* en die volgende doelwitte sal ondersoek word:

- Om die effek van *Camellia sinensis* op spermmotiliteit te bepaal.
- Om die effek *Camellia sinensis* op sperm vitaliteit te bepaal.
- Om die effek van *Camellia sinensis* op kapasitasie en akrosome reaksie in spermatozoa te bepaal.
- Om die vermoë van *Camellia sinensis* te bepaal om spermselle teen DNAfragmentering te beskerm.
- Om die effek van *Camellia sinensis* op sperm mitochondriale membraanpotensiaal te bepaal.
- Om die effek van *Camellia sinensis* op die produksie van reaktiewe suurstofspesies in spermatozoa te bepaal.
- Om vas te stel of *Camellia sinensis* vrugbaarheid kan verbeter.

Vir diegene wat deelneem aan die bykomende voortplanting, nadat u sperma verwerk is vir die behandeling waarvoor u beplan word, sal 'n klein gedeelte van die semenmonster geneem word vir hierdie addisionele toetse. Hierdie prosedure sal onder geen omstandighede die geassisteerde voortplantingsbehandeling of uitslag beïnvloed nie aangesien slegs die oorblywende monsters gebruik sal word. Dit sal ons toelaat om die moontlike effekte van *Aspalathus linearis* op menslike sperma te bepaal, of dit manlike vrugbaarheid kan verbeter of nie.

Die huidige studie sal nie enige bykomende mediese risiko, ongemak of koste insluit nie. Deelname aan hierdie wetenskaplike studie is heeltemal vrywillig en deelnemers mag op enige tydstip terugtrek totdat die data sonder enige gevolge vir publikasie ingedien sal word. Onttrekking uit die studie moet gedoen word in 'n formele aangespreek e-pos. Ten einde die vertroulikheid van die deelnemers te beskerm, sal 'n unieke nommer aan elke monster toegeken word en sal geen persoonlike inligting van die vrywilligers wat by die studie betrokke is, bevat nie. Alle data sal vertroulik en beskikbaar wees vir die betrokke wetenskaplike navorsers sowel as die deelnemers indien dit aangevra word. Die monsters sal deur die verbranding in die korrekte mediese prosedure weggedoen word sodra die data gevang is.

Die studie is eties ontvang deur die UWC Biomediese Navorsingsetiekkomitee (Goedkeuringsnommer: _____)

In geval van enige probleme of probleme, kan addisionele inligting direk per e-pos by die dokter of wetenskaplike ondersoeker verkry word.

toestemming

Ek is op die hoogte van die studie getiteld "*In vitro* effekte van *Camellia sinensis* op die menslike sperm" deur die wetenskaplike ondersoeker.

Ek verstaan die doel en prosedure van die studie.

Ek het die geleentheid gekry om vrae oor die studie te vra en antwoorde tot my bevrediging gehad.

Ek verklaar dat my deelname aan hierdie studie heeltemal vrywillig is en dat ek op enige tydstip kan terugtrek totdat die data sonder enige gevolge vir publikasie voorgelê sal word sonder om enige behandeling of sorg waarvoor ek geregtig is, te beïnvloed.

As ek verdere vrae of navrae het wat verband hou met die studie, verstaan ek dat ek die navorsers kan kontak.

As ek enige vrae of kommer het oor my regte as studieleier, of as ek bekommerd is oor enige aspek van die studie of navorser, kan ek kontak:

_____ _____ Handtekening van

Deelnemer datum

_____ _____ Handtekening van

Ondersoeker datum

APPENDIX E: INFORMED CONSENT FORM (ISIXHOSA)

INKQUBO YOMSEBENZISWANO OQHUBILEYO

Imiphumo ye- *in vitro* ye- Camellia sinensis wiiselingi zesininzi zesintu

Iphepha leenkcukacha kunye nemvume yokuthatha inxaxheba kuPhando Kulowo ibhekisele kuye,

Uceliwe ukuba uthathe inxaxheba ekutheni uthathe inxaxheba kwisifundo sezenzululwazi kwimisebenzi yesilisa kunye nemithi yezityalo. Injongo yale sifundo kukuphanda iimpembelelo ze- *Camellia sinensis* kwimisebenzi yokuzala yoluntu isebenzisa isisu somntu.

Emva kokuqokelelwa kweesampula zakho zeesisampuli, ziza kubonakala kwiindawo ezahlukahlukeneyo ze- *Camellia sinensis* kunye neenjongo ezilandelayo ziya kuphandwa:

- Ukucacisa umphumo we- *Camellia sinensis* kwi-sperm motility.
- Ukuqaphela umphumo *Camellia sinensis. ulungelelaniso lobunzima* besilisa.
- Ukumisela umphumo we- *Camellia sinensis* kwi-capacitory kunye ne-acrosome reaction in spermatozoa.
- Ukuqaphela ikhono lika- *Camellia sinensis* ukukhusela i-cell spells malunga nokuhlukana kwe-DNA.
- Ukuqaphela umphumo we- *Camellia sinensis* kwi-membrane yesperm mitochondrial.
- Ukumisela umphumo we- *Camellia sinensis* kwimveliso yeentsholongwane ze-oksijeni esebenzayo kwi-spermatozoa. ✦ Ukuchonga ukuba i- *Camellia sinensis* inokuphucula ukuzala.

Kulabo bathatha inxaxheba ekuncedeni unyango lokuzala, emva kokuba umlenze wakho uphuculwa unyango oye ucwangciselwe kuyo, is sm isahlulo sonke sesampuli yesondlo siya kuthathwa ngenxa yezi mvavanyo ezongeziweyo. Le nkqubo ayiyi kuphazamiseka unyango okanye uphuhliso oluza kuncedisa nje kuphela ukuba kusetyenziswe iisampuli ezisele. Oku kuya kuvumela ukuba siqonde imiphumo ekhoyo ye- *Camellia sinensis* kwisperm yabantu, nokuba ingaba yenze ngcono ukuzala komntu okanye cha.

Uphononongo lwangoku aluyi kubandakanywa nayiphi na ingozi yonyango engaphezulu, ingakhathazeki okanye iindleko. Ukuthatha inxaxheba kulolu cwaningo lwezenzululwazi ngokuzithandela kwaye abathathi-nxaxheba bavunyelwe ukuhoxisa naliphi na ixesha ngexesha lokuba idatha ingeniswe ukupapashwa ngaphandle kwemiphumo. Ukurhoxiswa kweso sifundo kufuneka kwenziwe kwikhompyutha echongiweyo. Ukuze ukhusele imfihlo yabathathi-nxaxheba, inombolo ekhethekileyo iya kubelwa isampula nganye kwaye ayiyi kuqulethelwa ngolwazi oluqulethwe ngamavavolontiya abandakanyekayo kwisifundo. Yonke idatha iya kuba yimfihlo kwaye ifumaneke kubaphandi bezesayensi ababandakanyekayo kunye nabachaphazelekayo ukuba bayacelwa. Iisampuli ziya kulahlwa kwinkqubo efanelekileyo yezocwangco ngokutshatyalaliswa xa idatha ithathwa.

Uphononongo luye lwafunyanwa ngokomthetho yiKomidi yeeNkcazo zoBuchule beUWC (Inombolo yemvume: _____)

Xa kwenzeka nayiphi na ingxaki okanye iinkxalabo, ulwazi olongezelelweyo lunokufumaneka kwigqirha okanye uphando loosayensi ngokuchanekileyo nge-imeyile:

Mvume

Ndixelelwe ngophando oluthi "Iimpembelelo ze-*In vitro* ze- *Camellia sinensis* kunye kwiHuman sperm".

Ndiyayiqonda injongo nenkqubo yesifundo.

Ndinike ithuba lokuba ndibuze imibuzo malunga nokufunda kwaye ndifumene iimpendulo kwaneliseko lwam.

Ndiyaxela ukuba inxaxheba yam kulesi sifundo iphela ngokuzithandela kwaye ndiyakrhoxisa nanini na ixesha ngexesha le data lingafakwa ngencwadi ngaphandle kwayo nayiphi na imiphumo kwaye ngaphandle kokuchaphazela nayiphi na inyango okanye ukunakekelwa kwam.

Ukuba ndinemibuzo eminye okanye umbuzo ophathelene nesifundo, ndiyaqonda ukuba ndidibana nabaphandi.

Ukuba ngaba nayiphi na imibuzo okanye ukuxhalabisa ngamalungelo am nje njengomfundi othabatha inxaxheba, okanye ukuba ndixhalabele nayiphi na into yesifundo okanye umphandi, ngoko ndiyaqhagamshelana nayo: _____ _____ U tyikityo lwaBathathinxaxheba Umhla

_____ _____ Isayinwe oMphandi Umhla