THE EFFECT OF LIPID MODIFYING DRUGS ON MALE REPRODUCTIVE PARAMETERS

by

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Miss Bongekile T. Skosana

April 2019
Declaration

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Cebisa Bhadula

April 2019
Abstract
The risk of cardiovascular disease (CVD) is prevalent and on the increase globally. Lipid-lowering drugs, have been found to reduce CVD. They act by either reducing LDL or increasing HDL. Thus reducing the serum cholesterol which plays a pivotal role in male reproduction as it is a precursor for steroid hormone biosynthesis and forms an integral part of the sperm membrane. During spermatogenesis these hormones are necessary for normal sperm development and activation of genes in Sertoli cells, which promote differentiation of spermatogonia. The widespread prophylactic use of statins, especially by men of reproductive age, gives rise to concerns regarding the effect thereof on the male reproductive system.

Aim: To determine if lipid-modifying drugs (Simvastatin and Fenofibrate) have any effects on male reproductive parameters.

Methods: Male Wistar rats (n=60) were randomly divided into four groups and treated for 6 weeks as follows: Control, Simvastatin (0.5 mg/kg), Fenofibrate (100mg/kg) and Simvastatin + Fenofibrate (S+F). Sperm morphology was assessed using Computer-Aided Sperm Morphology Analysis (CASMA). The plasma concentration of Total Cholesterol and Triglycerides (TG) were analyzed by the veterinary section of PathCare, a private pathology company. Testosterone and Estradiol concentrations were measured by ELISA kits. Testicular and epididymal histomorphometrics were measured by staining the testis with H&E and quantified using Zeiss imaging software. Testicular oxidative status was assessed by measuring the activity of Catalase (CAT), Superoxide Dismutase (SOD) as well as lipid peroxidation using a microplate reader. Data was analyzed by GraphPad Prism® V5.00.
Results were expressed as Mean ± SEM using One-way ANOVA. p≤0.05 determine statistical significance.

**Results:** The body weight of animals was not significantly different between the groups (p=0.0753). However, the Simvastatin treated group presented with general increased body weight and significantly higher peritoneal fat compared to the Fenofibrate and S+F groups (p≤0.05). The Fenofibrate treated group had significantly higher fasted blood glucose levels compared to the Simvastatin group (p≤0.05). The total cholesterol and TG levels were generally reduced in treated animals compared to control animals. There were alterations observed in testosterone levels between the groups (p=0.0077). The S+F group receiving combination treatment had significantly lower testosterone levels compared to the Simvastatin (p<0.05) and Fenofibrate (p<0.05) groups, but did not differ from the control. There were no differences observed in sperm vitality when comparing the groups. The percentage of morphological normal spermatozoa was significantly lower in the Fenofibrate as well as S+F groups compared to the control group (p<0.05), while no differences were observed when comparing the Simvastatin group to the control group. When assessing testicular histomorphometricues, there were no significant differences found in seminiferous tubules’ area (p=0.0987), lumen diameter of the seminiferous tubules (p=0.914) and epithelial height (p=0.3401). When assessing the epididymal tubules’ parameters, luminal diameter did not show any significant differences (p=0.0620) and the mean heights of the epithelium also did not differ significantly (p=0.5101) between the treatment groups.
**Conclusion:** Short-term exposure to cholesterol-lowering drugs can alter male reproductive parameters, however, more studies using longer treatment regimens are needed. In the interim, it is advised that physicians treating men with infertility should take cognisance of this fact.
Opsomming

Die risiko vir kardiovaskulêre siekte (KVS) is algemeen en wêreldwyd aan die toeneem. Lipiedverlagende middels is voorheen bewys om die voorkoms van KVS te verminder. Hierdie middels tree op deur óf LDL te verminder of HDL te verhoog en gevolglik word serum cholesterol verlaag. Laasgenoemde speel ook ‘n sentrale rol in manlike reproduksie as ‘n voorloper vir steroïd hormoon biosintese en maak ‘n integrale deel uit van die sperm selmembraan. Gedurende spermatogenese word hierdie hormone ook benodig vir normale sperm ontwikkeling en aktivering van gene in Sertoli selle wat die differensiasie van spermatogonia bevorder. Die wydverspreide profilaktiese gebruik van statiene, veral deur mans in hul reproduktiewe ouderdom is kommerwekkend, veral oor die moontlike uitwerking daarvan op die manlike reproduktiewe stelsel.

Doelstelling: Om te bepaal of lipiedverlagende middels (Simvastatien and Fenofibraat) enige uitwerking op manlike reproduktiewe parameters het.

Mетодes: Manlike Wistar rotte (n = 60) is ewekansig verdeel in vier groepe en daarna vir ses weke behandeld soos volg: kontrole, Simvastatien (0.5 mg/kg), Fenofibraat (100mg/kg) en Simvastatien + Fenofibraat (S + F). Rekenaargesteunde spermmorfologie-analise (CASMA) is gebruik om sperm morfologie te evalueer. Die totale cholesterol en trigliseried (TG) konsentrasies van die rot plasma was ontleed deur die Veterinêre afdeling van PathCare, ’n privaat patologie maatskappy. Testosteroen en estradiol konsentrasies is met behulp van ELISA kits gemeet. Testikulêre en epididimale histomorfometrie is gemeet deur die testis met H&E te kleur. Daarna is dit gekwantifiseer met behulp van Zeiss beeldingsagteware. Testikulêre oksidatiewe status was geassesseer deur die ensiem aktiwiteit van
Katalase (CAT), Superoksied Dismutase (SOD) sowel as lipied peroksidasie te meet met behulp van 'n mikroplaat leser. Al die data was ontleed met behulp van GraphPad prisma® V5.00. Resultate is as gemiddelde ± SEM uitgedruk. Vir statistiese vergelykings is eenrigting ANOVA gebruik en 'n p-waarde <0.05 is gebruik om statistiese betekenisvolheid aan te dui.

Resultate: Daar was nie 'n beduidende verskil tussen die groepe diere se liggaamsgewigte nie (p = 0.0753), maar die simvastatien behandelde groep het 'n verhoogde algemene liggaamsgewig asook 'n aansienlik hoër totale peritoneale vet gehad in vergelyking met die Fenofibraat en S + F groepe (p<0.05). Die Fenofibraat behandelde groep het 'n hoër vastende bloedglukose vlak gehad in vergelyking met die simvastatien groep (p<0.05). Die totale cholesterol en TG vlakke was oor die algemeen verminder in die behandelde diere in vergelyking met kontrole diere. 'n Beduidende verskil is gevind in testosteroon vlakke tussen al die groepe (p=0.0077). Die S + F groep wat die kombinasie behandeling ontvang het, het 'n aansienlik laer testosteroon vlak gehad in vergelyking met die simvastatien (p<0.05) en Fenofibrate (p<0.05) groepe, maar het nie verskil van die kontrole groep nie. Geen verskille in sperm vitaliteite is tussen die groepe waargeneem nie. Die persentasie morfologies normale sperm selle was aansienlik hoër in die kontrole groep in vergelyking met die Fenofibraat en S + F behandelde groepe (p<0.05), maar nie in vergelyking met die Simvastatien groep nie. Met die evaluering van die testikulêre histomorfometrie is daar geen beduidende verskille gevind in die seminifereuse tubule area, luminale deursnit van die seminifereuse tubule en die epiteel hoogte nie. Daar was geen beduidende verskil tussen groepe met betrekking tot die luminale deursnee analise van epididimale tubule parameters nie. Met betrekking tot die gemiddelde lengtes
van die epiteel, was daar ook nie 'n beduidende verskil tussen die behandelde groepe nie.

Gevolgtrekking: Korttermyn blootstelling aan Cholesterol-verlagende middels kan manlike reproduktiewe parameters verander, maar studies met langer behandelingsregimens is nodig om hierdie stelling te ondersteun. In tussentyd word dit aanbeveel dat dokters wat onvrugbare mans behandeling van hierdie feit kennis neem.
Acknowledgement

I have taken great effort in the completion of this project. However, it would not have been possible without the kind support and help of many individuals. I would like to extend my sincere thanks to all of them.

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My sincere thanks and deepest appreciation goes to Almighty God through Jesus of Nazareth for his mercy, for giving me strength and faith to accomplish this project.

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### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>6-OHD</td>
<td>6-Hydroxydopamine</td>
</tr>
<tr>
<td>AEs</td>
<td>Adverse effects</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxytoluene</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum Albumin</td>
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<td>CASMA</td>
<td>Computer-Aided Sperm Morphology Analysis</td>
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<tr>
<td>CAT</td>
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<td>Cat.</td>
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<td>Coenzyme Q10</td>
</tr>
<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
</tr>
<tr>
<td>CD</td>
<td>Combined dyslipidemia</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>distilled water</td>
</tr>
<tr>
<td>deiH₂O</td>
<td>deionized water</td>
</tr>
<tr>
<td>DETAPAC</td>
<td>Diethylenetriaminepentaacetic acid</td>
</tr>
<tr>
<td>DHEA</td>
<td>Dehydroepiandrosterone</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acid</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>hematoxylin and eosin</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
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</tr>
<tr>
<td>HClO₄</td>
<td>Perchloric acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
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<td>HMG-CoA</td>
<td>Hydroxymethylglutaryl Coenzyme A</td>
</tr>
<tr>
<td>HMG-CoAr</td>
<td>Hydroxymethylglutaryl Coenzyme A Reductase</td>
</tr>
<tr>
<td>HSD</td>
<td>Hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate density lipoprotein</td>
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<td>KCl</td>
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<td>kPi</td>
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<td>LDL</td>
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<td>Malondialdehyde</td>
</tr>
<tr>
<td>MP</td>
<td>Mid-piece</td>
</tr>
<tr>
<td>PPARs</td>
<td>Proliferator-activated receptors</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S+F</td>
<td>Simvastatin and Fenofibrate</td>
</tr>
<tr>
<td>SCA</td>
<td>Sperm Class Analyser</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl Sulphate</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of mean</td>
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<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
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<tr>
<td>TBARS</td>
<td>Thiobarbituric acid reactive substances</td>
</tr>
<tr>
<td>tPA</td>
<td>Tissue Plasminogen Activator</td>
</tr>
<tr>
<td>TG</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TBA</td>
<td>Thiobarbituric Acid</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>VLDL</td>
<td>Very low density lipoprotein</td>
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**Units of Measurement**

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<tr>
<td>°C</td>
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<td>μg</td>
<td>Microgram</td>
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<tr>
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<td>L</td>
<td>Litre</td>
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<td>m²</td>
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<td>mmHg</td>
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<tr>
<td>pg</td>
<td>Picogram</td>
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**Symbols**

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<td>α</td>
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<tr>
<td>β</td>
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<td>[ ]</td>
<td>Concentration</td>
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Chapter 1: Introduction

1.1. Background

Infertility is the inability to conceive following at least a year of regular unprotected intercourse. The reasons are numerous and it may result from the inability of either or both partners to contribute to conception, or the inability of the female to carry a pregnancy to full term. Around 15% of the sexually active population, amounting to 48.5 million couples, are affected by infertility. Male infertility alludes to a male's inability to cause pregnancy in a fertile female. Males contribute between 40-60% to couple infertility cases and are observed to be solely responsible for 20-30% of these infertility cases. Approximately 7% of all men are affected by infertility and constitute 43% of the burden in Africa (Agarwal et al., 2015).

There are many causes for male infertility including semen abnormalities (Cooper et al., 2009), endocrine disorders (Islam & Trainer, 1998) as well as physical problems (Guo et al., 2017). Semen analysis is normally performed to provide an indication of a male’s fertility potential (diagnosis). However, this is merely a surrogate measure as true fertility potential can only be established once a successful pregnancy has been elicited.

Worldwide, the burden of chronic diseases such as cardiovascular diseases (CVDs), stroke, obesity as well as diabetes mellitus is increasing rapidly (WHO, 2002). Most deaths due to chronic diseases are attributable to coronary artery disease (CAD), which was responsible for 7.2 million deaths in 2003 (Bedi et al., 2006). CAD is the hardening of arteries due to atherosclerosis. Arterial stiffness is a known marker of the atherosclerotic burden (Yingchoncharoen et al., 2014). It is caused by damage to
the coronary arteries, allowing cholesterol to deposit in the tunica intima (the second layer of the blood vessel wall). This causes the recruitment of other fatty substances and the possible progression to the formation of a complex plaque, which subsequently can initiate an inflammatory response. The walls of coronary arteries harden due to plaque development, making arteries less compliant (i.e. more difficult for arteries to dilate and constrict in response to pressure changes) (Cecelja & Chowienczyk, 2012).

The damage to coronary arteries may be attributed to several factors. These include smoking, high blood pressure, dyslipidemia, diabetes or insulin resistance which may be exacerbated by an inactive way of life.

Cholesterol is an essential fatty substance, responsible for several functions including steroid hormone biosynthesis, vitamin D production as well as forming integral part of cell membranes. It is synthesized by all the cells, but the liver is the site of the highest rate of synthesis with increased mRNAs encoding multiple enzymes of cholesterol biosynthesis (Norton et al., 1998). Cholesterol is synthesized through the mevalonate pathway, a series of enzyme rated reactions. This pathway starts with the condensation of two Acetyl-Coenzyme A (CoA) molecules to form acetoacetyl-CoA, with subsequent condensation of Acetyl-CoA and acetoacetyl-CoA to form 3-hydroxy-5-methyl-glutarylcoenzyme A (HMG-CoA). 3-HMG-CoA is reduced to mevalonate by the enzyme HMG-CoA reductase, this is the rate limiting step of cholesterol biosynthesis. Mevalonate is further phosphorylated into activated isoprene that is polymerized to form squalene. Squalene is subsequently converted into cholesterol by cyclization and oxidation of methyl group (Mehta, 2013).
Lipoproteins are complex particles composed of multiple proteins which transport all hydrophobic lipids. They are composed of a central hydrophobic core of non-polar lipids, surrounded by a hydrophilic membrane. There are several classes of lipoproteins classified based on size, lipid composition, and apolipoproteins (Apo). These classes include chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL) as well as low density lipoproteins (LDL) (Feingold & Grunfeld, 2017).

Cholesterol is transported in the blood as low-density lipoproteins (LDL) and high-density lipoproteins (HDL). Total cholesterol (TC) is defined as the combination of HDL, LDL as well as triglycerides (TG) and in order for proper physiological functioning to occur, a specific level of TC is needed in the blood. However, should TC levels become excessive (reaching pathological levels) it may become harmful. In humans, a TC of less than 200 mg/dl is considered desirable. HDL, LDL and TG levels are considered optimal if the concentration in the blood is approximately 60 mg/dl, 100 mg/dl and 150 mg/dl respectively (Ma & Shieh, 2006). Triglycerides do not contain cholesterol, but are measured because it is the most common type of fat in the body and a high triglyceride level combined with high LDL or low HDL is linked with atherosclerosis (Welty, 2015).

CADs are on the increase and showing worrying trends. This is not only because a large portion of the population is affected, but also because CADs have started to appear earlier in life and can cause sudden death or disability without warning. CADs can be addressed and treated with lifestyle changes, drugs and certain medical procedures (Willett et al., 2002).
Lipid-modifying drugs are widely used to prevent the onset (primary) of CADs and also used as treatment after early diagnosis (secondary) of the disease. These drugs act by the reduction of the total amount of cholesterol in blood through decreasing the primary materials which deposit in the coronary arteries. A wide range of these medications are available and used solely or as a combination of 2 classes. Different classes of lipid-modifying drugs include niacin, statins, cholesterol absorption inhibitors (Ezetimibe), PCSK9 inhibitors, fibrates and bile acid sequestrants.

1.2. Problem statement

The risk of coronary artery disease is prevalent and still increasing globally. To mitigate against this, lipid-modifying drugs are routinely prescribed for disease prevention and have been the best selling prescription drugs globally. These drugs reduce the prevalence of CAD, have a favorable safety profile and are described as a proven lifesaving medication. However, like all other medications these drugs can also possess adverse effects (AEs). In light of the increasing prophylactic use of lipid-modifying drugs by men, especially those of reproductive age, it gives rise to concerns about the effect of these compounds on the male reproductive system (Elgendy et al., 2018). As far as the author is concerned, no study has ever been performed to evaluate the effect of Simvastatin and Fenofibrate and the combination thereof on male fertility parameters.

1.3. Aims

To ascertain whether two lipid-modifying drugs, statins and Fenofibrate, individually and in combination have any effects on reproduction parameters in male Wistar rats.
1.4. Specific objectives

1. To establish a male Wistar rat model in order to study the effect of lipid modifying drugs, that lower cholesterol levels, by feeding the animals jelly blocks containing one of the following treatments over a six week period: Simvastatin (0.5mg/kg), Fenofibrate (100mg/kg) or in combination.

2. To ascertain the effect of the drugs on male reproduction by:
   - Quantifying the level of lipids in the blood plasma.
   - Quantifying testosterone and estradiol levels.
   - Assessing sperm morphology.
   - Assessing sperm viability.
   - Assessing histological changes in the testis and epididymis.
   - Assessing testicular anti-oxidant status.

1.5. Significance of the study

This study will provide additional information about the possible effects of lipid lowering drugs on male reproductive parameters, assessing various reproductive parameters as well as trying to establish possible mechanisms of action.
Chapter 2: Literature review

2.1. Introduction

CAD is the leading cause of morbidity and mortality worldwide (Okrainec et al., 2004). It is characterised by atherosclerosis within the coronary arteries, due to deposition of cholesterol in the tunica intima. Men are being hospitalized for heart-related diseases at almost double the rate of women. Recent studies show a prevalence of 1.2% CAD cases in males of ≤45 years (Centers for Disease Control and Prevention, 2011 Medibank, 2015). Combined dyslipidaemia is characterised by increased LDL and TG, as well as reduced HDL concentrations in the blood. It is recognised as a prominent risk factor for atherosclerosis development in CAD (Yusuf et al., 2004). When TC levels become excessive (reaching pathological levels) it becomes harmful to the heart. In humans, a TC of less than 200 mg/dl is considered desirable, while HDL, LDL and TG levels are considered optimal if the concentration in the blood is around 60 mg/dl, 100 mg/dl and 150 mg/dl respectively (Ma & Shieh, 2006).

HDL is regarded as “good cholesterol” because of its composition as it has a great deal of protein and reduced proportion of cholesterol. It is also known to possess anti-inflammatory (Baker et al., 1999; Cockerill et al., 1995) antioxidant (Garner et al., 1998), anti-thrombotic (Bu et al., 2011) and anti-apoptotic (Nofer et al., 2001) effects that may reduce the risk of CAD in healthy humans. LDL contributes to fat build-up in arteries because it has a large amount of cholesterol and minimal proteins, therefore earning it the nickname “bad cholesterol” (Ravnskov, 2002).

Under normal physiological conditions, LDL is known to be a major transporter of cholesterol in the blood stream, while HDL clears the excess LDL in the blood stream.
through binding it after which it is subsequently broken down in the liver and excreted. These lipoproteins are important for normal functioning of the body, however elevated concentrations of LDL and low levels of HDL increase the risk factor of CAD.

2.2. Lipid modifying drugs

Lipid modifying drugs are groups of pharmaceuticals that are utilized for the treatment of elevated amounts of lipids, such as cholesterol, in the blood (hyperlipidaemia). There are a number of classes of hypolipidemic drugs. These drugs act by targeting different sites of lipid metabolism and differ in the effect which they have on cholesterol profiles as well as their AEs. Some classes of drugs may be more effective in lowering the LDL, while others may preferentially elevate HDL. Clinically, the decision on prescribing a specific drug depends on the patient's cholesterol profile, cardiovascular risk, as well as the liver and kidney functions, with a goal to balance the risks and benefits of the drugs. The most commonly prescribed class of lipid modifying drugs is statins (O'Keeffe et al., 2016), however, fibrates may be prescribed for patients who cannot manage their cholesterol through statins alone and is therefore sometimes used in combination with statins.

2.2.1. Statins

2.2.1.1. Introduction

Statins (3-hydroxy-5-methyl-glutaryl coenzyme A reductase inhibitors) are known to play a major role in inhibiting the accumulation of blood lipids, thereby preventing CAD (Liau, 2005). The 3-hydroxy-5-methyl-glutaryl coenzyme A reductase (HMG-CoAr) inhibitors are widely used for the primary and secondary prevention of atherosclerotic cardiovascular disease (Grundy, 2016), thus decreasing the prevalence of CAD. They are especially appropriate for lowering LDL, the cholesterol that is
associated with vascular diseases. Statins significantly delay the onset of atherosclerosis and reduce the risk of a serious vascular lesion, such as a heart attack or stroke (Liau, 2005). Statins also slow down the progression of disease, and therefore help to delay symptoms such as angina. They do not reverse the symptoms but can prevent them from aggravating (Lim, 2013). It has been noted that statins can lower LDL cholesterol (LDL-C) by 18-55% based on the type and dose of statins used (Laufs et al., 2016). A lower intensity statin is usually sufficient to reduce cholesterol levels adequately in most humans. If not a higher dosage thereof may be prescribed or shift to a higher intensity statin may be recommended (Raymond et al., 2015).

2.2.1.2. Application of statins

Statin therapy is frequently recommended for individuals who have familial hypercholesterolemia (high cholesterol levels as a result of a faulty inheritance) (Rodenburg et al., 2007), patients with pre-existing heart disease (Jackson et al., 2007), and those who are currently healthy, but are at high risk of developing heart disease in the future (Antonio et al., 2017).

Besides preventing elevated blood cholesterol, experimental and clinical data exist for other possible therapeutic uses for statins, including the treatment of immune and inflammatory disorders (Gilbert et al., 2017), and the use as anti-cancer drugs (Ciofu, 2012). Statins have also been explored as potential treatments for parasitic diseases such as trypanosomiasis, leishmaniosis, Chagas' disease and malaria (Parihar et al., 2016).
2.2.1.3. Benefits of using statins

Statins are beneficial to human health though their basic mechanism and pleiotropic effects. They improve blood flow by decreasing blood LDL concentration (Kapur & Musunuru, 2008; Parker et al., 2011), which subsequently removes fatty substances, such as cholesterol, from the bloodstream, thereby avoiding plaque formation. Statins are also reported to reduce the risk of narrowing arteries by keeping the smooth muscle lining of the arteries healthy and through reducing the fibrin (a protein involved in blood clot formation) deposit in the arteries (Haslinger et al., 2002; Mangat et al., 2007).

The atherosclerotic process is initiated when LDL accumulates in the intima, thereby activating plaque formation within the endothelium. This initiates an inflammatory response that promotes recruitment of monocytes and T-lymphocytes to the area of lipid accumulation. Previous studies have suggested that statins possess anti-inflammatory effects (Bu et al., 2011), owing to their ability to reduce the number of inflammatory cells within atherosclerotic plaques, thus reducing the chance of arterial damage. The mechanism is not yet clear, but it might involve the inhibition of monocytes which contribute to inflammatory cell recruitment (Niwa et al., 1996).

It is believed that statins assist by increasing the production of nitric oxide (NO) (Wolfrum et al., 2003), a molecule which stimulates vasodilation and subsequently improves blood flow. It is well known that increased blood flow in the pelvic area can lead to improved erections. It has thus been shown that statins could be a cheap and effective drug to treat erectile dysfunction (Cui et al., 2014).
Statins have been found to improve endothelial function in patients with acute coronary syndrome (Altun et al., 2014). Statins up-regulate the endothelial nitric oxide synthase (eNOs) and NAD(P)H oxidase activity (Wolfrum et al., 2003) through the inhibition of isoprenoids synthesis via inhibition of HMG-CoAr which is responsible for the conversion of HMG-CoA to mevalonate.

Independently of statin’s effect on the lipid profile, they are also shown to have anti-oxidant properties (Olsson et al., 2017). Statins have been reported to reduce Malondialdehyde (MDA) levels and increase superoxide dismutase (SOD) (Gong et al., 2012) as well as reducing the levels of reactive oxygen species (ROS) (Yoon et al., 2009).

2.2.1.4. Adverse effects

According to a statin survey, more than six in ten respondents (62%) discontinued their statin treatment due to AEs (Kapur & Musunuru, 2008). The best recognized and most commonly reported AEs of statins are muscle toxicity. One reason for this may be statins' interference with selenium-containing proteins. Selenoproteins, such as glutathione peroxidase, are crucial for preventing oxidative damage to muscle tissues (Di Stasi et al., 2010).

Statins also deplete the body of coenzyme Q10 (CoQ\textsubscript{10}), which accounts for many of their devastating results. CoQ\textsubscript{10} is used for energy production by every cell in the body, and is therefore vital for good health, high energy levels, longevity, and general quality of life (Saini, 2011). CoQ\textsubscript{10}'s is a critical component of cellular respiration and production of adenosine triphosphate (ATP). The presence of CoQ\textsubscript{10} can improve the
sperm kinetic features (Balercia et al., 2009). Reduced CoQ\textsubscript{10} may result in impaired sperm movement.

2.2.1.5. Statin’s mechanism of action

Statins act by competitively inhibiting HMG CoA Reductase, an enzyme responsible for the first committed step of the mevalonate pathway (\textit{Figure 2.1}), by blocking the conversion of HMG CoA to mevalonic acid. When administered statins are hydrolyzed to generate βδ-dihydroxy acid, an active metabolite structurally similar to HMG-CoA. After they are hydrolyzed, statins act by competing with HMG-CoA for HMG-CoAr. Forming a complex with catalytic portions of the enzyme by binding to the active site of HMG-CoAr and blocking access to the substrate from binding to the active site (Stancu & Sima, 2001).
Figure 2.1: Statins and the Mevalonate pathway. Statins act by inhibiting the conversion of HMG-CoA into mevalonic acid, thus reducing the production of isoprenoids, which results in low levels of cholesterol being produced. LDL=Low density lipoproteins

This pathway converts mevalonate into sterol isoprenoids, such as cholesterol which is an indispensable precursor of bile acids, lipoproteins, and steroid hormones, as well as a number of hydrophobic molecules and non-sterol isoprenoids. The intermediates play a pivotal role in physiological processes. By interrupting the synthesis of cholesterol, statins activate cell surface LDL receptors in the liver, leading to a foreseeable increased clearance of LDL from the bloodstream and a decrease in blood LDL levels (Rashid et al., 2005).
2.2.2. Fibrates

2.2.2.1. Introduction

Fibrates are a group of drugs derived from amphipathic (hydrophobic and hydrophilic) carboxylic acids that are mainly used to treat hypercholesterolemia. This group of drugs is composed of fibric acid subordinates, which lower the amount of TG in the blood by reducing the liver's production of VLDL and accelerating the removal of TG from the blood (Shipman et al., 2016). Additionally, fibrates have been shown to effectively increase HDL cholesterol levels by up to 15-25% (Moutzouri et al., 2010).

2.2.2.2. Applications of Fibrates

Fibrates are used to prevent pancreatitis by lowering blood TG in clinical trials (Shipman et al., 2016). They additionally have been utilized alone to avert heart attacks, particularly in patients with elevated blood TG and low HDL cholesterol levels.

2.2.2.3. Adverse effects

Fibrates are effective in lowering blood cholesterol and is said to be life-saving, but as with other drugs their pleiotropic effects, may negatively affect users. The administration of a fibrate is believed to induce myopathy (Ghosh et al., 2004), but the mechanism thereof is not clear. A previous study, also speculated that some cases may be due to metabolic changes, whereas others may be immune mediated (Le Quintrec & Le Quintrec, 1991). Fibrates administration has been reported to reduce the size of adipocyte (Jeong & Yoon, 2009) a critical regulator of systemic energy homeostasis. The reduction in the size of this tissue might negatively affect the energy metabolism in the whole body. This could be linked to the findings of Forcheron and colleagues who found that, Fenofibrate administration leads to a reduction in free fatty acid (FFA) levels, attributable to an increase in FFA clearance, predominantly via an increase in FFA oxidation in the muscle (Forcheron et al., 2002).
2.2.2.4. Mechanism of action

Fibrates act by activating peroxisome proliferator-activated receptors (PPARs), particularly PPARα. By activating PPARα, fibrates control the hereditary expression of various enzymes (McKeage et al., 2011). PPARα increases the levels of HDL by inducing the synthesis of ApoA-1 and A-2 (apoA-I and apoA-II). PPARα also induces lipoprotein lipolysis mediated by reduced hepatic apolipoprotein C3 (Apo C-III) production, resulting in reduced production of LDL particles. It also induces hepatic fatty acid (FA) uptake, thereof reducing the hepatic production of TG (Figure 2.2).

Figure 2.2: Fibrates mechanism of action. The mechanism through which Fibrates reduce cholesterol and triglycerides within the bloodstream by activation of PPARα. FFA=free fatty acids; LDL=Low density lipoprotein; HDL=High density lipoprotein; PPARα=proliferator-activated receptor alpha
2.2.3. Simvastatin and Fenofibrate

Simvastatin is a prodrug, which after administration is rapidly converted in the liver from inactive lactone to its acid form. It is an artificially derived fermented product of *Aspergillus terreus* (Subhan *et al.*, 2016). The drug was introduced for clinical use in 1989 and has been the most prescribed drug for primary prevention of CAD owing to their ability to reduce elevated lipid levels effectively with few side effects. In literature, Simvastatin has been shown to be highly effective in reducing LDL concentrations in dialysis patients (Masterson, 2002). This was further propagated by a clinical trial that found 16% LDL reduction in diabetic patients treated with 20–40 mg of Simvastatin with minimal tolerable AEs (Okeoghene & Alfred, 2013).

Fenofibrate is similarly a prodrug which is transformed into its active form, Fenofibric acid, in the liver. It has been widely used to treat patients with atherogenic dyslipidemia. It is a second line treatment used to reduce elevated levels of total cholesterol, LDL, TG and Apo B (McKeage *et al.*, 2011). It also increases HDL levels (Tsunoda *et al.*, 2016). The mechanism of action is known to be mediated through the binding of the fibric acid derivative to PPARα (Staels *et al.*, 1998), a transcriptional factor which plays key regulatory roles in fatty acid and cholesterol metabolism. As therapeutic effects, Fenofibrate and other PPARα agonists have been shown to cause a significant peroxisome proliferation, lipolysis, and increased synthesis of Apo AI and AII (Pawlak *et al.*, 2015). These drugs are usually recommended if statins do not achieve adequate cholesterol lowering and are to be used with a proper balanced diet or in combination with statins.

A previous study has shown that a combination therapy of Simvastatin and Fenofibrate might be more clinical beneficial in patients with combined dyslipidemia (Wang *et al.*, 2015).
2003). This was substantiated by studies which found that co-administration of Fenofibrate and Simvastatin has been shown to exert improvement in both HDL and LDL, as well as TG levels. Combination treatment furthermore minimized AEs on patients with hyperlipidemia and elevated TGs (Ellen & McPherson, 1998; Grundy et al., 2005). The recommended dosage to improve the overall lipid profile is 20 mg of Simvastatin with 160 mg of Fenofibrate daily (Grundy et al., 2005; Muhlestein et al., 2006).

2.3. Cholesterol and Male Reproductive Parameters

2.3.1. Introduction
Cholesterol is a key molecule with an important role in various physiological processes. It is of particular importance to the reproductive system as it is the common precursor for steroid hormone synthesis (Whitfield et al., 2015). It is also a fundamental constituent of the sperm plasma membrane lipid bilayer. Cholesterol is furthermore necessary for sperm to be able to undergo capacitation following ejaculation into the female tract before fertilizing the oocyte (Ickowicz et al., 2012).

2.3.2. Cholesterol Role in Sex Hormone Biosynthesis
Sex hormones are the group of hormones which are cholesterol derivatives which in males are primarily synthesized and secreted by the testes (Stephen et al., 2008). Sex hormone synthesis is controlled by the release of gonadotropin-releasing hormone (GnRH) from the hypothalamus (Harrison et al., 2004) which stimulates GnRH receptors in the pituitary gland to release the luteinizing hormone (LH) as well as follicle stimulating hormone (FSH) (Marques et al., 2018). LH then binds to Leydig cells, which stimulates the expression of steroidogenic acute regulatory protein (StAR). StAR promotes the uptake of cholesterol, mainly as LDL, into the inner mitochondria

16
and initiates steroidogenesis (Miller & Bose, 2011). Cholesterol is then converted to pregnenolone by the action of P450 side chain cleavage enzyme (P450scc) and subsequently converted to dehydroepiandrosterone (DHEA) in a two-step process mediated by 17,20-lyase (17α-hydroxylase). Because Leydig cells express high levels of 3-beta-Hydroxysteroid dehydrogenase (3β-HSD) and 17-beta-Hydroxysteroid dehydrogenase (17β-HSD), DHEA is rapidly converted to testosterone via the intermediates androstenediol and androstenedione, see Figure 2.3. Reduced levels of LDL achieved by lipid lowering drugs may lead to AEs because there will be reduced free cholesterol delivered across the cell membranes. Very low levels of LDL may impair the production of steroid hormones that are necessary for sexual and reproductive function in males (Olsson et al., 2017).
Figure 2.3: Sex hormone biosynthesis pathway in the male gonads.
Cholesterol taken up by StAR into the inner mitochondria is converted into
pregneolone which is subsequently converted into progestogens to produce steroid
hormones.
P450scc=P450 side chain cleavage; StAR=Steroidogenic acute regulatory protein;
DHEA=dehydroepiandrosterone; 3β-HSD= 3-beta-Hydroxysteroid dehydrogenase;
17β-HSD= 17-beta-Hydroxysteroid dehydrogenase

2.3.3. Cholesterol and Membranes
Cholesterol is a lipid which can be found within the cell membrane. It is synthesized
through a complex series of enzymatic steps in the endoplasmic reticulum and is
eventually transported through the Golgi apparatus to the plasma membrane (Fagone
& Jackowski, 2009). The role of cholesterol is to help provide the cell membrane with
extra support owing to its higher rigidity compared to the phospholipids and glycolipids
in the membrane. This is structural change occurs through immobilizing some of the
lipid molecules around them, which makes the cell membrane stronger and harder for
small molecules to pass through the membrane (Corvera et al., 1992). The presence
of cholesterol allows the cell membrane to be strong enough to contain the cell and
serve as an effective barrier to ions. Despite the fact that cholesterol is more rigid than some of its neighbouring lipids (phospholipids and glycolipids), which keeps the cell membrane fluid. By generating some extra spaces between the lipids, cholesterol prevents lipids from gelling together into their crystalline state. This allows lipids to move freely through the membrane as needed (Tabas, 2002).

Lipid rafts are made up of high amounts of cholesterol and sphingolipids. These rafts allow some sections of the membrane to be distinct from other areas (Simons & Sampaio, 2011). Lipid rafts are important for many cellular actions such as exporting proteins out of the cell as well as anchoring specific proteins in the membrane and keep protein clusters together.

2.3.4. Sperm Cell Membrane
It is known that cholesterol enhances lipid bilayer of the cells. Cholesterol has been found to have stabilizing effect on the plasma membrane by imposing conformational order on lipids (Leahy & Gadella, 2015). Cholesterol’s ability to control the lipid bilayer results in the control of mechanical membrane stiffness without compromising fluidity, thickness and permeability to water (Müller et al., 2008). Due to the stabilizing properties of cholesterol, it has been linked to capacitation and the ability to survive cryopreservation (Davis, 1980) This was further propagated by a theory stating that the presence of cholesterol in the sperm membrane helps a cell to tolerate adverse conditions (Mandal et al., 2014). It is often suggested that loss of cholesterol directly affects the sperm plasma membrane lipid bilayer and make it fusogenic (permeable to foreign molecules).
Conclusion

Most of the morbidity and mortality cases are due to CADs, characterized by arteriosclerosis. People use lipid modifying drugs for primary and secondary treatment of CADs. There are several classes of lipid modifying drugs, statins and fibrates are the mostly prescribed classes to be used solely or in combination. These drugs act by either elevating HDL or lowering LDL and TG levels in the blood, thereby resulting in low levels of blood TC. Cholesterol plays a pivotal role in male reproduction as it is a precursor for steroid hormone biosynthesis. During spermatogenesis these hormones are necessary for normal sperm development and activation of genes in Sertoli cells, which promote differentiation of spermatogonia. Cholesterol is also of importance in forming an integral part of the sperm membrane. Therefore, the use of these drugs might have a potential to alter male reproduction.

It is the purpose of this dissertation to assess the interaction between these two entities and analyse whether these drugs will be of benefit or detriment to male reproductive potential.
Chapter 3: Study design and Methodology

3.1 Design

Ethical clearance for this study was obtained from the Stellenbosch University Animal Ethics Committee (Ethical Number: SU-ACUD16-00111). The study was conducted according to “The Revised South African National Standard for the animal care and use for Scientific Purposes” (South African Bureau of Standards, SANS 10386, 2008). A total number of 60 male Wistar rats, weighing between 150 and 220g were used for this study. They were randomly assigned to 4 groups; control, Simvastatin, Fenofibrate, and Fenofibrate & Simvastatin (Figure 3.1), which were given jelly blocks with or without the addition of 0.5 mg/kg Simvastatin, 100mg/kg Fenofibrate or a combination of the two for 6 weeks respectively.

Figure 3.1: Study design. A total number of sixty male Wistar rats were randomly assigned into four treatment groups and treated for 6 weeks with lipid-modifying drugs. After 6 weeks the rats were sacrificed, body and testicular weights were recorded, spermatozoa was used for morphology & viability analysis while testis was used for histology and oxidative status and the plasma collected was used for lipid profiles and hormone analysis.
3.2. Sample collection

After 6 weeks of treatment, animals were sacrificed by euthanasia (intraperitoneal injection of 160mg/kg pentobarbital) and exsanguination. Body mass was also recorded at this point in time. Blood was collected from the thoracic cavity using EDTA blood tubes before centrifuging it at 1000xg for 10 minutes at 4°C within 30 minutes of collection. The plasma was removed and stored in liquid nitrogen for subsequent hormone analysis. The testis and epididymides were carefully removed, rinsed, weighed, and appropriately stored or prepared for further analysis.

3.3. Sperm retrieval

The epididymides harvested from each rat were placed in a petri dish containing 5ml solution of Hams F-10 nutrient medium (Sigma Chemicals, St Louis, MO, USA) supplemented with 3% Bovine serum Albumin (BSA) (Rosche Diagnostics GmbH Mannheim, Germany) at 37°C. The caudal portion of each epididymis was isolated by using a fine pointed dissection scissor and placed in 2ml of 3% HAMS-BSA solution. It was subsequently cut into radial sections and left in the medium for 5 minutes, with occasional agitation to facilitate the release of spermatozoa into the media (Figure 3.2).
Figure 3.2: Rat sperm collection from the caudal region of epididymis. Left epididymis was placed into a 5ml HAMS-BSA solution (B) and the rat sperm was retrieved by cutting the caudal portion of the epididymis into 2ml of HAMS-BSA solution (A).

3.4. Sperm analysis

Sperm quality of all groups was assessed by means of sperm viability and morphology analysis.

3.4.1. Sperm viability

Sperm viability (percentage of live vs. dead cells) was analyzed by a dye-exclusion technique using Eosin-Nigrosin stain (Sigma-Aldrich, St Louis, MO, USA). The sperm solution was mixed with Eosin and Nigrosin in a 1:2:3 (A 10μl of sperm solution was added and mixed with 20μl Eosin and 30μl Nigrosin) ratio. Smears were made by placing 10μl of the mixture on the end of double frosted ends microscope slide (25.4X76.2mm, 1.0mm-1.2mm thick). Using a plain slide, the mixture was spread across the frosted slide and allowed to dry at room temperature for a minimum of 24 hours. After 24 hours the slides dried and cover slips (0.13-0.17mm thick) were fixed.
with DPX mounting medium (Dako CA, USA). Two viability slides were prepared for each rat.

Eosin penetrates the membranes of dead cells, staining them purple and the live cells remain white, while Nigrosin provides the background counterstaining. Live and dead cells were visualized by light microscopy (Nikon Eclipse E200, Tokyo, Japan) with a 40x objective at 400x magnification, after which 200 spermatozoa were counted manually using a laboratory counter. The number of viable (live) spermatozoa was expressed as a percentage of the total number of spermatozoa counted.

3.4.2. Sperm morphology

To assess sperm morphology, 10μL of sperm solution was extracted from prepared sperm suspensions and smeared onto double frosted ends microscope slides (25.4X76.2mm, 1.0mm-1.2mm thick). The slides were allowed to air dry at room temperature for 24 hours before being stained with SpermBlue® dye (Microptic, Barcelona, Spain) according to the manufacturer’s guidelines (Vander Horst and Maree, 2009). The slides were immersed into a Coplin jar with SpermBlue® fixative (Microptic, Barcelona, Spain) for 10 minutes. The slides were then removed from the jar and excess fixative was allowed to drain. After excess fixative was drained the slides were dipped into a Coplin jar with SpermBlue® dye for 15 minutes after which it was dipped into distilled water for 5 seconds to remove excess stain. The slides were then left to dry at room temperature overnight. The stained dry slides were mounted with a cover slip using DPX mounting medium (Dako CA, USA).

Sperm morphology was analyzed by means of Computer-Aided Sperm Morphology Analysis (CASMA) using the Sperm Class Analyser V5.0 (SCA) (Microptic, Barcelona, Spain), software for visualization and quantification as described by Van der Horst et
al. (van der Horst et al., 2018). Bright field optics employing a 60x objective, i.e., 600x magnification, and blue filter on a Nikon E200 microscope (IMP, Cape Town, South Africa). Software settings were as follows: contrast and brightness were optimized for complete thresholding of the sperm head and mid-piece (MP). A minimum of 50 randomly selected sperm per rat from various systematically obtained microscopic fields were analyzed. Sperm images were captured digitally using a Basler 312fc firewire camera (Microptic, S.L., Barcelona, Spain) and analyzed automatically using the SCA system’s Rat morphology module.

The SCA software automatically analyzed the head and MP morphometrics. To determine if the head was normal, head length (ARC), width, perimeter, surface area and roughness were measured. Measurements of the MP included width, area and angle of insertion of the flagellum to the head. The distance from the anterior tip of acrosome to the posterior part of head (chord length), was measured and the linearity was calculated (Figure 3.3). The software automatically used the above mentioned morphometric dimensions to detect weather the sperm is morphological normal or abnormal (Figure 3.4).
Figure 3.3: Morphometric parameters of rat sperm as measured by CASMA. Illustration of rat sperm head (Blue) and midpiece (green) morphometries accurately measured by CASMA to detect if the sperm is morphological normal or abnormal. (van der Horst et al., 2018).

LIN=Linearity; CASMA=Computer-Aided Sperm Morphology Analysis.

Figure 3.4: Morphological normal and abnormal sperm as measured by CASMA. An illustration of how CASMA show a normal or abnormal spermatozoa.

CASMA=Computer-Aided Sperm Morphology Analysis.

3.5. Lipid profiling

The plasma concentrations of Total Cholesterol and Triglycerides were analyzed by the veterinary section of PathCare, a private pathology company.
3.6. Hormone analysis (Testosterone and Estradiol Levels)

The serum samples collected and stored as described in under section 3.2 were used for testosterone and estradiol hormone assays using an enzyme linked immunosorbent (ELISA) kit for either Testosterone (T; Elabscience, Cat. E-EL-0072) or Estradiol (E; Elabscience, Cat. E-EL-0065).

Samples were allowed to thaw at room temperature for ±2 hours. All the reagents were brought to room temperature 60 minutes before use. After they were completely defrosted, samples were mixed thoroughly using a vortex. The standards from the ELISA kit were centrifuged at 100xg for 1 minute and mixed thoroughly with a pipette. The samples and standards were added into the wells, 50µl each. Immediately thereafter 50µl of HPR-labeled Testosterone/Estradiol was added to each well and incubated for 1 hour at 37°C. The solution in the plate wells was aspirated and 350µl of wash buffer was added to each well and soaked for 60 second using ImmunoWash™ microplate washer (BIO-RAD). This wash step was repeated 3 times, thereafter microplate was pat dried with a paper towel. Subsequently, 50µl of substrate A and substrate B was added to each well and the plate was incubated at 37°C for 15 minutes in a Thermoshaker (AOSHENG) with shading light. Thereafter, 50µl of stop solution was added and the optical density (OD) was immediately determined at 450nm using the iMark™ microplate reader (BIO-RAD).
Figure 3. 5: Testosterone standard curve

Figure 3. 6: Estradiol standard curve
3.7. Anti-Oxidant status analysis

3.7.1. Lysate preparation
Approximately 50mg of testicular tissue, stored in liquid nitrogen, were procured and used for lysate preparation. The tissue was placed into a microcentrifuge tube containing an equal amount (50mg) of 0.5mm zirconium oxide beads (Biocom Biotech) for homogenization and 100µl of ice cold lysis buffer (50mM Sodium Phosphate, 0.5% (w/v) Triton X-100, pH 7.5) was added. The samples were homogenized with a Bullet blender® 24 (Next Advance, Inc. New York) at speed 9 for 3x1 minute periods with 1 minute rest intervals in-between. The volume of the lysis buffer was immediately topped up to 500µl by adding 400µl of lysis buffer. The samples were then allowed to incubate on ice for 30 minutes and were centrifuged at 15000 rpm for 20 min at 4°C. Supernatants were transferred into clean cryotubes and stored at -80°C until further analysis within one month.

3.7.2. Protein quantity determination
The protein quantity of the testicular tissue homogenate was determined with the Bicinchoninic acid (BCA) protein assay kit. BSA (1mg/ml) was used to prepare the standard curve as shown in Table 3.1. The samples were diluted with deionized water (deiH₂O) to ensure that the protein concentration was within the linear range of the standard protein concentration (200-1000 μg/ml). A BCA working reagent was prepared from a combination of reagent A (BCA solution, SIGMA Cat. B9643-1L-KC) and reagent B (copper (II) sulfate pentahydrate 4 % solution, SIGMA Cat. C2284-25mL-KC) prepared in a 50:1 ratio. The diluted standard (25µl) as well as samples (25µl) were pipetted in triplicate into a 96-well flat bottom Greiner clear plate. A 200
μl volume of the BCA working reagent was added to each well. The plate was shaken for 10 seconds on a vortex plate (Labnet International, Inc) to allow proper mixing. Thereafter, the plate was incubated on an ACCUBLOCK™ digital dry bath (Labnet International, Inc) at 37°C for 30 minutes. The absorbance was subsequently read at 562nm in a FLUOstar® Omega Microplate Reader. All calculations for the different antioxidant enzyme assays, catalase (CAT) and SOD, were normalized and standardized according to the BCA protein concentration.

**Table 3.1: BSA standards preparation**

<table>
<thead>
<tr>
<th>[BSA] (mg/ml)</th>
<th>Volume of deiH₂O (μl)</th>
<th>Volume of BSA (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>0.2</td>
<td>80</td>
<td>20</td>
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<tr>
<td>0.4</td>
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<td>0.6</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>0.8</td>
<td>20</td>
<td>80</td>
</tr>
</tbody>
</table>

BSA = Bovine Serum Albumin; deiH₂O = deionized water
3.7.3. Catalase activity

CAT assay buffer (50mM Potassium phosphate; pH 7.0) was prepared by adding 21.1ml of 1M Monopotassium phosphate (monobasic) and 28.9ml of 1M dipotassium phosphate (dibasic) into 1L of deionized water, and the PH was adjusted to 7.0 and stored at -4°C. Hydrogen peroxide (H$_2$O$_2$) stock solution was prepared immediately before assaying by adding 34µl of H$_2$O$_2$ in 10ml of CAT assay buffer and covered in foil to prevent oxidation by light.

The testis homogenates were diluted to 0.1µg/µl protein in CAT buffer using BCA values calculated as mentioned in 3.7.2. The CAT buffer was used as a blank, 5µl from the diluted tissue lysates was added in triplicate into the 96 well ultraviolet (UV) plate, followed by 170µl of assay buffer. Immediately before reading the plate the reaction was initiated by adding 50µl of H$_2$O$_2$ stock solution to all the wells and the absorbance was measured over a 5min period in order to determine the linear decrease over time, at 240nm in a FLUOstar® Omega Microplate Reader. The molar extinction coefficient (43.6 M$^{-1}$cm$^{-1}$) adjusted for the well pathlength was used to determine CAT activity (H$_2$O$_2$ consumed in µmole /min/µg protein).

3.7.4. Superoxide dismutase activity

Diethylenetriaminepentaacetic acid (DETAPAC; SIGMA Cat. D6518-5G) stock was prepared by adding 4mg in 10ml of SOD buffer (50mM Na-Pi buffer, pH7.4) and stored at -20°C. Tissue lysates were diluted with deionized water to 0.1µg/µl. 6-Hydroxydopamine (6-OHD, Sigma Cat. 162957-1G) was freshly prepared by adding 50µl of 70% Perchloric acid (HClO$_4$, Sigma Cat. 77230-10mL) into 10ml of double-distilled water (ddH$_2$O). 6-OHD (0.4mg) was added to the solution, whereafter it was wrapped in foil to prevent light oxidation and used as soon as possible. The diluted tissue lysates (5µl) were
aliquoted into a clear 96-well F-bottom microplate. To this 10µl of the SOD buffer was added and 170µl of DETAPAC stock solution. Immediately before plate reading, 15µl of 6-OHD solution was added and the auto-oxidation was recorded at 490nm for 4 minutes in 1 minute intervals using the FLUOstar® Omega microplate reader.

3.7.5. Lipid Peroxidation
Oxidizing agents can alter lipid structure, thereby creating lipid peroxides that result in the formation of MDA, which can be measured as Thiobarbituric Acid Reactive Substances (TBARS). In the presence of heat and acid, MDA reacts with Thiobarbituric Acid (TBA) to produce a coloured end-product that absorbs light at 530-540 nm. The intensity of the colour at 532nm corresponds to the level of lipid peroxidation in the sample. Unknown samples are compared to the standard curve.

3.7.5.1. Lysate preparation
Potassium phosphatase (kPi) buffer (50mM pH 7.5) was diluted to 0.1M. The working buffer was prepared by adding 1.15% Kcl to 0.1M kPi buffer (230mg Kcl in 20ml kPi buffer). Thin sections (±50mg) of testis tissue were cut and transferred into microcentrifuge tubes with an equal amount of 0.5mm zirconium oxide beads and 100µl of KclkPi buffer was added. The samples were homogenized for 3 minutes with a bullet blender as described previously in 3.7.1. The volume of KclkPi buffer was topped up to 500µl by adding 400µl of KclkPi buffer and supernatants were immediately transferred into clean cryotubes and stored at -80°C for further TBARS analysis.
3.7.5.2. Protein quantity determination

Sodium dodecyl sulphate (SDS) of 2% was prepared by adding 2g of SDS to 100ml ddH$_2$O. The standards were prepared by diluting BSA with 2% SDS as shown in the previous Table 3.1. Samples were diluted to a concentration of 0.1μg/μl in SDS. The diluted samples (25μl) and standards (25μl) were pipetted into a 96-well plate. A BCA working reagent was prepared from a combination of reagent A (BCA solution, SIGMA Cat. B9643-1L-KC) and reagent B (copper (II) sulfate pentahydrate 4 % solution, SIGMA Cat. C2284-25mL-KC) prepared in a 50:1 ratio. The diluted standard (25μl) as well as samples (25μl) were pipetted in triplicate into a 96-well flat bottom Greiner clear plate. A 200μl volume of the BCA working reagent was added to each well. The plate was shaken for 10 seconds on a vortex plate (Labnet International, Inc) to allow proper mixing. Thereafter, the plate was incubated on a heating block at 37°C for 30 minutes. The absorbance was subsequently read at 562 nm in a FLUOstar® Omega Microplate Reader.

3.7.5.3. Thiobarbituric Acid Reactive Substances

On the day of assay, the previously stored tissue lysates were allowed to thaw over ice for approximately 1 hour. Trichloroacetic acid (TCA, SIGMA Cat. T6399-500G) stock solution (10%) was prepared by adding 5g TCA in 50ml deiH$_2$O and stored at -4°C. TBA stock was prepared by adding 335mg of TBA (TBA, SIGMA Cat. T5500-100G) into 50ml deiH$_2$O, dissolved by heat at ~45°C for ±15minutes and cooled at room temperature. To prepare Butylated hydroxytoluene (BHT) 80mg of BHT (8%BHT, SIGMA Cat. W218405-1KG-K) was added into 1ml ethanol. The working solution was made by adding 5ml of 10% TCA, 62.5μl BHT solution and 44.94ml of ddH$_2$O and stored on ice. MDA stock was prepared by adding 1.23μl of commercial MDA to 10ml
ddH₂O. The stock solution (500µl) was further diluted to 0.25µg/µl with 1.5ml ddH₂O. MDA standards were prepared according to Table 3.2. Tissue lysates and standards (100µl each) were added in glass tubes followed by 1ml of 2% SDS solution and mixed with a vortex. TCA-BHT (2ml) working solution was subsequently added into the tubes, mixed with vortexing and incubated for 10 minutes. Thereafter, 2ml of TBA was added before covering with marbles and incubated in a water bath at 95°C for 60 minutes. The tubes were then removed and cooled on ice for 15 minutes before centrifuging at 3000rmp for 15minutes at 4°C. The supernatants were removed into clean microcentrifuge tubes. The supernatants (250µl) were pipetted into wells of a F-bottom clear microplate and the output was immediately measured at 532nm using a FLUOstar® Omega microplate reader.
Table 3.2: MDA standards preparation

<table>
<thead>
<tr>
<th>Tube</th>
<th>MDA (µl)</th>
<th>DeiH₂O (µl)</th>
<th>[MDA] (µM/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td>0</td>
<td>1000</td>
<td>0</td>
</tr>
<tr>
<td>b)</td>
<td>2.5</td>
<td>997.5</td>
<td>0.3125</td>
</tr>
<tr>
<td>c)</td>
<td>5</td>
<td>995</td>
<td>0.625</td>
</tr>
<tr>
<td>d)</td>
<td>10</td>
<td>990</td>
<td>1.25</td>
</tr>
<tr>
<td>e)</td>
<td>20</td>
<td>980</td>
<td>2.5</td>
</tr>
<tr>
<td>f)</td>
<td>40</td>
<td>960</td>
<td>5</td>
</tr>
<tr>
<td>g)</td>
<td>80</td>
<td>920</td>
<td>10</td>
</tr>
<tr>
<td>h)</td>
<td>200</td>
<td>800</td>
<td>25</td>
</tr>
<tr>
<td>i)</td>
<td>400</td>
<td>600</td>
<td>50</td>
</tr>
</tbody>
</table>

MDA = Malondialdehyde; DeiH₂O = deionized water
3.8. Histology

The testis and epididymis were fixed in 10% formalin solution (Kehahliet et al., 2016) for a minimum of 48 hours to allow complete fixation of the tissue (Figure 3.7). The tissue was cut into smaller pieces and placed in labelled embedding cassettes for tissue processing. This includes dehydration with a series of alcohols to ensure that water is removed, clearing with xylene and infiltration with paraffin wax using an automated processor (Duplex processor, Shandon Elliot) (Table 3.3). After processing the tissues, they were embedded in paraffin wax by placing the processed tissue piece in a metal embedding mould and filling the mould with wax at 60°C using a Leica EG1160 embedder. The wax was allowed to solidify on an iced surface and tissue blocks were obtained and kept at room temperatures until sectioning takes place. The blocks were cooled in a freezer ~2 hours prior to sectioning. Sections were cut (4μm thick) with a Leica RM 2125RT microtome. The sections were placed floating on warm water in a hot water bath (approximately 40°C) to allow stretching out. They were then attached to double frosted ends microscope slides (25.4X76.2mm, 1.0mm-1.2mm thick) and the slides were incubated in a warm oven to melt the wax off from tissue. These sections were stained with hematoxylin and eosin (H&E) and dehydrated with alcohol and xylene, as illustrated in Table 3.4, using a Leica Auto Stainer XL. The slides were covered with a cover slip (0.13-0.17mm thick) and fixed with DPX mounting medium (Dako CA, USA).

The testis and epididymis histomorphometric parameters were examined with the ZEISS imaging system Zen (Blue edition) V2.3Lite (Carl ZEISS microscopy, SA). The sections were viewed on a bright field Microscope Axio (Carl ZEISS Microscopy, SA) employing 10x objective (100x magnification). A total of 50 testis/epididymis tubule
images were analyzed. The images were captured using an Axiconcam 105 colour (Carl Zeiss Microscopy, SA), saved as CZI file and various parameters (*Figure 3.8*) were analyzed using Zen (Blue edition) V2.3Lite (Carl ZEISS Microscopy, SA).

*Figure 3.6: Testis fixed in formalin.* Testis tissues stored in formalin for histology analysis.
Table 3.3: Automated tissue processing procedure for histology purposes

<table>
<thead>
<tr>
<th>Step #</th>
<th>Solution</th>
<th>Incubation time (min)</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10% Formalin</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>2</td>
<td>70% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>3</td>
<td>96% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>4</td>
<td>96% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>5</td>
<td>99.9% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>6</td>
<td>99.9% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>7</td>
<td>99.9% Ethanol</td>
<td>30</td>
<td>Room temperature</td>
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<tr>
<td>8</td>
<td>Xylene</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>9</td>
<td>Xylene</td>
<td>30</td>
<td>Room temperature</td>
</tr>
<tr>
<td>10</td>
<td>Paraffin</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>11</td>
<td>Paraffin</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>Paraffin</td>
<td>60</td>
<td>60</td>
</tr>
</tbody>
</table>
**Table 3.4: Haematoxylin and eosin automated staining procedure**

<table>
<thead>
<tr>
<th>Step #</th>
<th>Solution</th>
<th>Time (min)</th>
<th>Repetitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60°C Oven</td>
<td>2</td>
<td>x1</td>
</tr>
<tr>
<td>2</td>
<td>Xylene</td>
<td>5</td>
<td>x2</td>
</tr>
<tr>
<td>3</td>
<td>99% Ethanol</td>
<td>2</td>
<td>x2</td>
</tr>
<tr>
<td>4</td>
<td>96% Ethanol</td>
<td>2</td>
<td>x1</td>
</tr>
<tr>
<td>5</td>
<td>70% Ethanol</td>
<td>2</td>
<td>x1</td>
</tr>
<tr>
<td>6</td>
<td>Tap water</td>
<td>2</td>
<td>x1</td>
</tr>
<tr>
<td>7</td>
<td>Haematoxylin</td>
<td>8</td>
<td>x1</td>
</tr>
<tr>
<td>8</td>
<td>Running water</td>
<td>5</td>
<td>x1</td>
</tr>
<tr>
<td>9</td>
<td>Eosin</td>
<td>4</td>
<td>x1</td>
</tr>
<tr>
<td>10</td>
<td>Running water</td>
<td>1</td>
<td>x1</td>
</tr>
<tr>
<td>11</td>
<td>70% Ethanol</td>
<td>0.5</td>
<td>x1</td>
</tr>
<tr>
<td>12</td>
<td>96% Ethanol</td>
<td>0.5</td>
<td>x2</td>
</tr>
<tr>
<td>13</td>
<td>99% Ethanol</td>
<td>0.5</td>
<td>x1</td>
</tr>
<tr>
<td>14</td>
<td>Xylene</td>
<td>1</td>
<td>x1</td>
</tr>
</tbody>
</table>
3.9. Data Analysis.

Statistical analysis was carried out on GraphPad Prism® v5.00 The results were expressed as mean ± standard error of mean (SEM). A Kolmogorov-Smirnov’s test was used to test normality and data distribution. Where data was normally distributed a One-way Analysis of Variance (ANOVA) was used to test significance followed by a Bonferroni’s multiple comparison test for comparison between groups. The Bonferoroni post hoc test was preferred to Dunnett’s test of multiple comparison, as the latter test only compare each of a number of treatments with a single control, while the Bonferroni test provided more information on difference between all treatment groups. Furthermore, no changes in attaining significance was observed when either of the two post hoc tests were applied. Where data was not normally distributed, Krukal-Wallis test was employed. P≤0.05 determined statistical significance.
Chapter 4: Results

Normal male Wistar rats were treated with lipid modifying drugs which act to reduce total serum cholesterol and triglycerides. The rats were divided into 4 groups: a control group, treated groups receiving 0.5mg/kg Simvastatin, 100mg/kg Fenofibrate or a combination of the two. Drug dosages were administered orally via jelly blocks, once daily, for 6 weeks. The effects of these three treatments on male reproductive parameters were assessed by intergroup comparison – a One Way ANOVA followed by Benfororoni test for multiple comparison. All the results and findings of this study were statistically analysed using GraphPad® Prism V5.00 and the data expressed as mean ± SEM will be presented in this chapter.
4.1. Biometrics

Biometric data of the animals in the four experimental groups are presented in Table 4.1. It is evident that total body weight as well as testicular weight did not differ between the treatment groups. Animals treated with Simvastatin had a higher peritoneal fat content compared to the Fenofibrate group. The fasted blood glucose levels of the Fenofibrate treated group was marginally higher than that of other groups and significantly higher than that of the Simvastatin treated group.

Table 4.1: Biometric measurements of different treatment groups (Mean ± SEM).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Fenofibrate</th>
<th>S+F</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>327.1±8.326</td>
<td>342.3±9.645</td>
<td>323.5±8.056</td>
<td>308.9±9.204</td>
<td>0.075</td>
</tr>
<tr>
<td>Testicular weight (g)</td>
<td>3.047±0.092</td>
<td>3.328±0.081</td>
<td>3.317±0.097</td>
<td>3.158±0.056</td>
<td>0.056</td>
</tr>
<tr>
<td>Peritoneal fat (g)</td>
<td>11.00±0.900</td>
<td>13.36±0.676ab</td>
<td>10.57±0.547a</td>
<td>9.64±0.634b</td>
<td>0.004</td>
</tr>
<tr>
<td>Glucose level (mmol/L)</td>
<td>8.075±0.401</td>
<td>7.183±0.201a</td>
<td>9.083±0.706a</td>
<td>7.567±0.218</td>
<td>0.020</td>
</tr>
</tbody>
</table>

N=14; S+F is combination of treatment with Simvastatin and Fenofibrate. Values on the same row that differ significantly are indicated by the same letter.
4.2. Lipid profiling

The lipid profiles of the animals are shown in Table 4.2. Unfortunately, due to lack of sample volume, the pathology laboratory was unable to perform the analysis in all animals and furthermore could not generate data for all parameters. Due to the limited data size, no statistical analysis could be performed, but there is a general reduction in cholesterol and TG that can be observed in all three active treatment groups compared to control.

Table 4.2: Concentration of lipids in four treatment groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Fenofibrate</th>
<th>S+F</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cholesterol] (mmol/L)</td>
<td>2.5</td>
<td>1.9</td>
<td>1.2</td>
<td>1.1</td>
</tr>
<tr>
<td>[Triglyceride] (mmol/L)</td>
<td>1.28</td>
<td>0.70</td>
<td>0.37</td>
<td>0.35</td>
</tr>
</tbody>
</table>

N=2; S+F is a combination treatment with Simvastatin and Fenofibrate.
4.3. Hormones

The different concentrations of testosterone (Figure 4.1) and estradiol (Figure 4.2) were measured in the blood plasma.

There were alterations observed in testosterone levels between the groups (p=0.0077). The S+F group receiving the combination treatment had significantly low testosterone levels compared to the Simvastatin (15.52±0.3550ng/ml vs. 17.15±0.3199ng/mL, p<0.05) and Fenofibrate (15.52±0.3550ng/ml vs. 16.24±1.217ng/mL, p<0.05) groups, but did not differ from the control.

![Figure 4.1: Plasma testosterone concentrations of different treatment groups (Mean ± SEM).](https://scholar.sun.ac.za)

N=14; *=p ≤ 0.05; S+F is a combination treatment with Simvastatin and Fenofibrate.
There was a significant difference when comparing the estradiol levels between the four treatment groups (p=0.0308). Estradiol levels (Figure 4.2) were also the lowest in the F+S group (550.2±38.96) and differed significantly from the Simvastatin group (685.2±40.09, p≤0.05).

![Figure 4.2: Plasma estradiol concentrations of the different treatment groups (Mean ± SEM).](image)

N=14; *=p ≤ 0.05; S+F is a combination treatment with Simvastatin and Fenofibrate.
4.4. Sperm parameters

4.4.1. Sperm Viability

As seen in Figure 4.3, no significant differences were observed in sperm viability as measured using the Eosin-Nigrosin dye exclusion staining technique, despite a lowering trend observed in the Simvastatin group.

![Bar chart showing sperm viability](chart.png)

**Figure 4.3: Percentage of viable spermatozoa (Mean ± SEM).**

N=14; *=p≤ 0.05; S+F is combination of treatment with Simvastatin and Fenofibrate.

4.4.2. Sperm morphology and morphometric measurements

The percentage of morphologically normal spermatozoa as well as various morphometric parameters were determined objectively by means of CASMA.

4.4.2.1. Sperm morphology

Significant differences (p=0.0011) were found in the percentage of morphologically normal spermatozoa as can be seen in Figure 4.4. Both Fenofibrate (p≤0.001) as well as S+F (P≤0.05) treatment significantly decreased the percentage of morphologically normal cells compared to control values. The simvastatin treated group also showed
a decrease in normal morphology. However, it did not differ significantly from the control values.

Figure 4.4: Percentage of morphologically normal spermatozoa (Mean ± SEM).

N=14; *=p≤ 0.05; S+F is combination of treatment with Simvastatin and Fenofibrate.

4.4.2.2. Normal shape

There were no significant differences (p=0.0621) found between the means of the percentage spermatozoa with normal shape as shown in Figure 4.5. However, Fenofibrate had significantly decreased morphologically normal shape spermatozoa compared to control animals (60.71±4,122% vs. 73.57±2,711%, p≤0.05). No differences were found when comparing Simvastatin and S+F groups to control.
4.4.2.3. Normal size

There were significant differences (p=0.0148) found between the percentage of spermatozoa with normal size between the groups, as illustrated in Figure 4.6. Fenofibrate had significantly lower percentages of normal sized spermatozoa compared to control (47.86±3.573% vs. 68.14±4.910%, p≤0.05) and also the S+F group (56.57±4.542% vs. 68.14±4.910%, p≤0.05). Furthermore, the Simvastatin group had a significantly higher percentage of normal size spermatozoa compared to the Fenofibrate group (62.29±4.515% vs. 47.86±3.573%, p≤0.001).
**Figure 4.6: Percentage of morphologically normal sized of spermatozoa (Mean ± SEM).**

N=14; *p≤ 0.05, ***p≤0.001; S+F is combination of treatment with Simvastatin and Fenofibrate.

### 4.4.2.4 Sperm morphometries

The morphometric parameters of the sperm head and MidPiece automatically measured by CASMA are presented in the Table 4.3. The head morphometry parameters measured included head length (ARC), width, perimeter, surface area, roughness, regularity and number of vacuoles. No significant differences for any of these parameters were found between the groups. Measurements of the MP, (width, angle of insertion, area) also showed no difference between the groups, except for MP area which showed a significant increase in the combination treatment (S+F) when compared to control (2,167±0,1272 μm² vs. 2,744±0,1390 μm², p=0.0314).
### Table 4.3: Sperm morphometric parameters (Mean ± SEM).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Fenofibrate</th>
<th>S+F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>H Length/ARC (μm)</td>
<td>21,64±0,20</td>
<td>21,33±0,13</td>
<td>21,31±0,12</td>
<td>21,48±0,18</td>
<td>0.4362</td>
</tr>
<tr>
<td>Head Width (μm)</td>
<td>1,48±0,027</td>
<td>1,48±0,04</td>
<td>1,54±0,04</td>
<td>1,54±1,54</td>
<td>0.3397</td>
</tr>
<tr>
<td>H Perimeter (μm)</td>
<td>46,16±0,39</td>
<td>45,64±0,29</td>
<td>45,90±0,25</td>
<td>46,15±0,31</td>
<td>0.6132</td>
</tr>
<tr>
<td>H Area (μm²)</td>
<td>18,67±0,3267</td>
<td>18,85±0,3495</td>
<td>19,43±0,3872</td>
<td>19,50±0,31</td>
<td>0.2360</td>
</tr>
<tr>
<td>Chord (μm)</td>
<td>11,53±0,15</td>
<td>11,39±0,15</td>
<td>11,61±0,14</td>
<td>11,28±0,14</td>
<td>0.4145</td>
</tr>
<tr>
<td>H Angle (degrees)</td>
<td>62,39±1,35</td>
<td>59,21±1,38</td>
<td>58,61±1,07</td>
<td>58,94±1,47</td>
<td>0.1595</td>
</tr>
<tr>
<td>H Linearity (%)</td>
<td>53,29±0,67</td>
<td>52,99±0,70</td>
<td>54,32±0,95</td>
<td>52,82±0,90</td>
<td>0.5411</td>
</tr>
<tr>
<td>H Roughness</td>
<td>0,11±0,00</td>
<td>0,1129±0,001</td>
<td>0,1171±0,00</td>
<td>0,18±0,00</td>
<td>0.1841</td>
</tr>
<tr>
<td>MP Width (μm)</td>
<td>0,68±0,02</td>
<td>0,71±0,01</td>
<td>0,71±0,01</td>
<td>0,72±0,00</td>
<td>0.1450</td>
</tr>
<tr>
<td>MP Angle (degrees)</td>
<td>13,52±0,61</td>
<td>13,35±0,72</td>
<td>13,27±0,62</td>
<td>12,95±0,70</td>
<td>0.9420</td>
</tr>
<tr>
<td>MP Area (μm²)</td>
<td>2,17±0,13</td>
<td>2,58±0,17</td>
<td>2,68±0,14</td>
<td>2,74±0,14*</td>
<td>0.0314</td>
</tr>
</tbody>
</table>

N=14; H=head; MP=mid-piece; S+F is a combination treatment with Simvastatin and Fenofibrate. *=p≤ 0.05 vs. control.
4.5. Histology

The testicular and epididymial quantitative histological parameters will be reported in this section.

4.5.1. Testicular histomorphometrics

Despite that all three groups treated with the lipid lowering drugs showed a relatively lower seminiferous tubule surface area compared to the controls, no significant differences (p=0.0987) were observed (Figure 4.7).

The luminal diameter of the seminiferous tubules in the testes also did not differ (p=0.914) between any of the treatment groups, (Figure 4.8).

The height of the seminiferous tubules epithelium did not show any significant differences (p=0.3401) between the treatment groups despite that the Simvastatin treated group displayed a much thicker epithelium in general (Figure 4.9).

![Figure 4.7: Seminiferous tubules' area (Mean ± SEM). N=2; S+F is a combination treatment with Simvastatin and Fenofibrate.](image-url)
4.5.2. Epididymis histomorphometric parameters

The luminal diameter of the epididymal tubules did not show any significant differences (p=0.0620) between any of the treatment groups (Figure 4.10).
The mean heights of the epididymal tubules’ epithelium also did not differ significantly \((p=0.5101)\) between the treatment groups \((\text{Figure 4.11})\).

**Figure 4.10**: Lumen diameter of the epididymal tubules \((\text{Mean ± SEM})\). \(N=2\); \(S+F\) is a combination treatment with Simvastatin and Fenofibrate.

**Figure 4.11**: Epithelial height of epididymal tubes \((\text{Mean ± SEM})\). \(N=2\); \(S+F\) is a combination treatment with Simvastatin and Fenofibrate.
4.6. Antioxidants status and lipid peroxidation

In order to acquire an idea of the antioxidant status of the testis in the control and treated animals, both CAT and SOD activities were measured. From Table 4.4, it is evident that only CAT activity (p=0.048) was significantly affected, but not SOD activity (p=0.9688). The combination group (S+F) had a much higher CAT activity than the Fenofibrate group (59.94±15.57µmole/min/µg vs. 20.77±5.809µmole/min/µg; p<0.05). There was no significant difference (p=0.3873) in MDA levels between the treatment groups.

Table 4.4: Testicular anti-oxidant activity and lipid peroxidation levels (Mean ± SEM)

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control</th>
<th>Simvastatin</th>
<th>Fenofibrate</th>
<th>S+F</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD Activity (units/mg protein)</td>
<td>25.88±5.164</td>
<td>28.74±4.643</td>
<td>27.69±4.275</td>
<td>26.16±4.458</td>
<td>0.9688</td>
</tr>
<tr>
<td>CAT Activity (µmole/min/µg)</td>
<td>40.89±6.986</td>
<td>31.08±7.001</td>
<td>20.77±5.809a</td>
<td>59.94±15.57a</td>
<td>0.0408</td>
</tr>
<tr>
<td>MDA (µM/mg)</td>
<td>9.70±1.392</td>
<td>13.22±1.296</td>
<td>15.51±4.158</td>
<td>12.58±1.143</td>
<td>0.3873</td>
</tr>
</tbody>
</table>

N=14; S+F is a combination treatment with Simvastatin and Fenofibrate. Values in the same row that differ significantly are indicated by the same letter.
Chapter 5: Discussion

Male infertility may be caused by several factors, which contribute to alterations of semen quality. Widely used lipid-modifying drugs such as Simvastatin and Fenofibrate are proven to be effective in lowering blood cholesterol. Cholesterol is a precursor of steroid hormone synthesis and forms an integral part of the sperm membrane (Sèdes et al., 2018). During spermatogenesis these hormones are necessary for normal sperm development as well as the activation of genes in Sertoli cells, which promote differentiation of spermatogonia. This novel study aimed to determine whether Simvastatin and Fenofibrate and the use of combination therapy have any effects on male reproductive parameters by treating sixty male Wistar rats with lipid-modifying drugs for six weeks.

5.1. Biometric Parameters

5.1.1. Body and Testicular Weight

The baseline biometric values of the four groups were similar in animals of the same age and weighing between 150-220g. After 6 weeks of treatment, there were no significant differences found in both body and testicular weight between the groups. However, there was an increasing trend in body weight of the Simvastatin treated group compared to the control group. These results were supported by a study of Agguirre and colleagues, which also found that statins increase body fat and weight as well as liver fat (Aguirre et al., 2013). This may be due to Simvastatin’s mechanism of action, which reduces the efficiency of HMG-CoAr, thereby causing liver cells to trap more LDL from the bloodstream. This was supported by a clinical trial studying genetics, which found that modifications occurring in the gene encoding for HMG-CoAr are associated with a higher body weight and marginally higher type 2 diabetes risk.
A previous study also showed that Fenofibrate is effective in weight loss of diabetic rats (Mancini et al., 2001). This can be explained by a study that observed reduced food intake in obese rats treated with Fenofibrate (Park et al., 2012). In this study Fenofibrate treated animals did not show any significant difference in body weight when compared to control. The disagreement between this study and literature may be due to the short-term treatment. The combination treatment of S+F did not have any effect on body weight compared to control. These results were similar to the outcome of a previous clinical trial (Koh et al., 2005) where no differences were found in the body weight of patients with combined dyslipidaemia when treated with Simvastatin and Fenofibrate.

5.1.2. Peritoneal Fat Weight
In this study there were no differences observed when comparing the peritoneal fat of the lipid-lowering treated rats to the control group. However, the Simvastatin treated group had generally higher peritoneal fat weights compared to the control, which was significantly higher when compared to the Fenofibrate and S+F treated groups. These findings were supported by a study of Aguirre et al., (2013) where several statins increased the accumulation of liver and body fat in Zucker rats, but the mechanism is not clear. However, it could be speculated that it might be through statins’ basic mechanism of clearing LDL from the bloodstream, thereby increasing the LDL receptors on the liver. Peritoneal fat is more unsafe in light of the fact that it secretes proteins that add to inflammation, atherosclerosis, dyslipidaemia, and hypertension. High fat mass has been found to be associated with prediabetes (Jung et al., 2016), which can possibly impair spermatogenesis.
5.1.3 Blood glucose
There were no significant differences observed on blood glucose when comparing the treated groups to the control groups. The Fenofibrate group showed a significant elevation in glucose levels compared to the Simvastatin group. Previous studies found that Fenofibrate increases insulin sensitivity (Yong et al., 1999) by reducing insulin clearance and insulin secretion (Ramakrishnan et al., 2016), however, the effect was not observed in this study. This study has shown that both Simvastatin and S+F do not affect fasting glucose levels. Meanwhile, a previous study found that over a period of 2 years, Simvastatin intake can increase glucose levels in diabetic as well as nondiabetic patients (Sukhija et al., 2009). This inconsistency may be due to the different treatment periods since the current study had only six weeks of treatment vs. the 2 years of treatment of the previously mentioned study.

5.2. Lipid Profile

5.2.1. Cholesterol
Simvastatin and Fenofibrate effectively reduce the risk of CVDs by lowering lipids and thereby reducing the cholesterol levels. In this study, reduced cholesterol levels were observed in the treated groups compared to the control group. However, the difference could not be statistically tested due to the small sample size. These results were substantiated by studies which found that lipid-lowering drugs are highly effective in reducing LDL levels in dialysis and diabetic patients (Masterson, 2002; Okeoghene & Alfred, 2013), TC and TG (McKeage et al., 2011) as well as increasing the HDL levels (Tsunoda et al., 2016). The results of this study provides evidence for the efficacy of short-term exposure to cholesterol-lowering drugs on prevention of CVDs.
5.2.2. Triglycerides
Although, the difference was not statistically tested due to small sample size as a result of small volume of the serum collected, the S+F group had reduced TG. These findings corresponds to the study of Zhao et al., (2016) which also found that using Fenofibrate as an additive to statins can reduce TG by 38.1%. These results are further supported by findings of another clinical trial, which responded that statins are effective in primary prevention of cardiovascular complications (Conly et al., 2011).

5.3. Hormone Concentration
5.3.1. Testosterone
Testosterone is the androgen in men chiefly responsible for sexual and reproductive functions. In several studies, testosterone has been shown to play a significant role in sperm production (O'Donnell et al., 2017; Sharpe et al., 1990). Testosterone is also said to act as a vasodilator in the penis to promote erection (Chamness et al., 1995).

In this study there were alterations observed in testosterone concentrations. However, from our results there were no significant differences found when comparing the control and treated groups’ testosterone levels. In a meta-analysis, statins were found to reduce testosterone levels in males (Schooling et al., 2013). The discrepancy between the results of the current study and those of early study regarding the concentrations of testosterone may be due to the negative feedback of steroidogenesis. When cholesterol produces small quantities of testosterone, GnRH is stimulated to be released by the hypothalamus, which in turn stimulates the pituitary gland to release FSH and LH. LH and FSH then stimulate the testes to produce more
testosterone (Figure 5.1). However, in this study the S+F group had significantly decreased testosterone levels when compared to the Fenofibrate and Simvastatin treated groups. This may be attributed to the efficacy of the combination therapy to reduce cholesterol levels more than monotherapy (Tarantino et al., 2017). Very low cholesterol levels may down regulate the production of STAR (Sugawara & Fujimoto, 2004) resulting in reduced synthesis of steroid hormones. It is evident that the combination therapy negatively affects testosterone production and should be administered carefully, with regular assessment of testosterone levels, so that fertility is not affected.

![Figure 5.1: The hypothalamas-pituitary-gonadal testosterone feedback mechanism. Low testosterone levels stimulates hypothulumus to release GNRH to initiate the production of more testosterone.](image-url)
5.3.2. Estradiol

Estradiol is a sex hormone produced by Leydig cells in the testicular interstitium in males or aromatized from testosterone. It acts by regulating testosterone and it has been shown that estradiol can prevent sperm destruction (O'Donnell et al., 2001). Estradiol is involved in several male sexual functions including spermatogenesis (O'Donnell et al., 2001), libido (Ramasamy et al., 2014) and erectile function (Mancini et al., 2005). Despite estradiol’s involvement in spermatogenesis, supraphysiological levels can interfere at multiple levels (O'Donnell et al., 2001). It may act by increasing the synthesis of glycoproteins in Sertoli cells and Leydig cells and increases collagen synthesis and fat degeneration in the testicular connective tissue (Leavy et al., 2017).

In this study, there were alterations in estradiol concentrations when comparing the four groups treated with or without Simvastatin/Fenofibrate or the combination of the two. There is limited information about the effect of lipid modifying drugs on estradiol in the literature. Like all other steroids, in males, estradiol is produced from cholesterol carried by LDL, which is taken up by Leydig cells in the testes and used as a substrate for steroidogenesis. The lipid modifying drugs were expected to reduce the estradiol serum concentration since the lipids were reduced. However, the results from this study did not confirm the hypothesis. This can possibly be explained by the fact that testosterone, which was unaffected in this study, is converted to estradiol by aromatase enzyme. The group that received combination therapy had significantly lower estradiol concentrations compared to the Simvastatin treated group. This may be due to the fact that the combination therapy of Simvastatin and Fenofibrate lower lipids more than statins alone (Tarantino et al., 2017).
5.4. Sperm parameters

Drugs can affect male reproductive parameters through various mechanisms. They can directly or indirectly induce an impairment in spermatogenesis. The study by Pons-rejraji and co-workers found that using statins (Artovastatin) may adversely affect sperm parameters in healthy males by reducing the sperm number, vitality, total motility as well as increasing head, neck and MP deformities (Pons-rejraji et al., 2014). Contrarily a study of Purvis and colleagues found that simvastatin does not affect sperm quality in patients with heterozygous familial hypercholesterolaemia (Purvis et al., 1992). Sperm parameters are used as a measure of male fertility, thereof impaired sperm parameters may increase the risk of male infertility (WHO, 2010).

5.4.1. Sperm viability

Sperm viability is the measurement of live spermatozoa in the seminal fluid. The seminal plasma of a normal male has about 40 million spermatozoa per ml. However, not all spermatozoa in the seminal fluid are viable. For normal fertility about 58% or more of spermatozoa need to be viable (WHO, 2010).

In this study there were no differences found in sperm viability. There is controversy in the literature regarding the effect of lipid modifying drugs on sperm viability. A previous study showed that the use of Artovastatin by healthy men for five months may affect sperm viability (Pons-rejraji et al., 2014). The discrepancy might be due to the long-term treatment with a higher dosage of a high intensity drug compared to the current study. While another trial found that statins are not detrimental to semen parameters (Dobs et al., 2000). However, impaired lipid concentrations has been shown to negatively affect semen parameters (Schisterman et al., 2014). This was
further supported by the findings of Delashoub and co-workers who found that oral administration of different fibrates may reduce sperm viability in male Wistar rats (Delashoub et al., 2018). The differences were not observed in the current study, however, the fibrates used in the earlier study (Delashoub et al., 2018) were clofibrate and silafibrate. The higher daily dosage (20-40 mg/kg daily) could also be contributing to the discrepancy.

5.4.2. Sperm Morphology
Sperm morphology refers to the shape and size of spermatozoa. It is expressed as percentage of cells that appear normal in the semen. The percentage of morphologically normal spermatozoa has been found to be correlated with male fertility (Love, 2011). Spermatozoa of male Wistar rats is considered morphological normal when the average percentage of normal morphology is between 68% and 71% (van der Horst et al., 2018).

In this study there were no differences observed in head parameters between the groups. However, when comparing MP parameters, the MP area significantly increased in the S+F group compared to the control group. We further speculated that because MP contains sperm mitochondria the impaired MP may deplete mitochondrial DNA resulting in low ATP delivered to the flagellum for sperm to swim, and therefore could possibly reduce the percentage of total sperm motility. The percentage of morphological normal spermatozoa was significantly low in the Fenofibrate as well as S+F treated groups compared to the control group. No differences were observed when comparing the Simvastatin treated group with the control group. Use of statins has been proven to increase morphological abnormalities (Pons-rejraji et al., 2014). However, this was not observed in this study. The discrepancy regarding the effect of
statins on sperm morphology may be due to the fact that drugs used in the current study (Simvastatin) and the one used in the literature (Artovastatin) were different. The different statins have different pharmacokinetic properties (Chong et al., 2001) and belong to different intensity groups. High intensity statins (Artovastatin) are said to resemble more AEs compared to moderate intensity statins (Simvastatin) (Golomb & Evans, 2008).

5.5. Histomorphometry

5.5.1. Testicular Histomorphometry
In the present study, the histomorphometric evaluation of rat testis, showed that oral administration of lipid modifying drugs did not exert AEs on testis morphology. When evaluating seminiferous tubule area there was an increasing trend observed when comparing the treated groups to the control group. There were no differences observed in lumen diameter across the groups although the Simvastatin treated group showed an increased epithelial height, but the difference was not significant. A previous study found that statins induce various deleterious changes in the histological structure of the testes of adult male albino rat (Mostafa et al., 2015) where Artovastatin treated rats had significantly reduced epithelial height. The possible reason for the discrepancy may be due to the small sample size used in this study as well as the fact that the drug used in the earlier study belongs to a higher intensity group and the drug was administered orally via a gastric tube, while in the current study the rats were fed jelly blocks.

5.5.2. Epididymal Parameters
When spermatozoa leave the testis they are inactive and unable to fertilize ova. The spermatozoa need interaction with proteins in the epididymis for maturity, and to
acquire progressive motility as well as the ability to fertilize an oocyte (Cornwall, 2009). To ascertain if the epididymal structure was not altered by impaired cholesterol levels, epididymal parameters were measured. There were no differences observed in lumen diameter between the treated groups in this study. No significant differences were found in epididymal tubule epithelial height between the groups. However, the treated groups had generally thinner epithelium compared to the control group. From these results, it is evident that cholesterol-lowering drugs do not affect the structure of different of the epididymis.

5.6. Anti-Oxidant Status and Lipid Peroxidation

There were no differences in testicular SOD activity between the groups. When assessing CAT activity, a significant increase in the Fenofibrate treated group compared to the S+F was observed. Lipid peroxidation assessed by measuring the level of MDA did not have any difference between the groups. A previous study found that Fenofibrate enhances SOD and CAT activity and improves the abnormal TBARS levels in diabetic male Wistar rats (Eser et al., 2010). However, this was not found in the current study, controversy may be due to the fact that this study used healthy rats.
Chapter 6: Conclusion

Widely used lipid-modifying drugs are said to effectively reduce blood cholesterol which is essential for male reproductive function. In the present study, Simvastatin and Fenofibrate showed signs of the ability to lower cholesterol in a male Wistar rat model. This was observed in the reduction of total cholesterol and triglycerides in the treatment groups, but this cannot be statistically concluded based on the small number of samples that could be examined. The combination treatment tended to decrease cholesterol, and triglycerides better than the individual treatments, but it also drastically reduced normal sperm morphology and estradiol as well as increased sperm MP area. Therefore, whilst the administration of cholesterol-lowering drugs was generally positive for cardiovascular disease risk reduction, caution should be exercised when being prescribed to reproductive aged males. Reproductive hormones must be monitored during the intake of such drugs as cholesterol is necessary for their production. Lifestyle changes could also be an effective, and preferred, means of treatment as it is less likely to negatively affect hormone concentrations, and is a less detrimental method for the primary prevention of CVDs.

From these results it is evident that short-term exposure to Cholesterol-lowering drugs can alter male reproductive parameters, however more studies using longer treatment groups are needed. In the interim it is advised that physicians treating men with infertility should be cognizant of this fact.
Study Limitations

The study successfully reached the aims. However, there were parameters that could not be measured due to financial, time and sample size constraints. The parameters stated in future directions below could provide more conclusive evidence for the effect of lipid modifying drugs on male reproductive parameters.

Recommendations

- Increase sample size, for detailed and conclusive study a high study population size may be recommended.
- Measurement of lipid profile (LDL, HDL and VLDL) can provide more details about the mechanism of each drug.
- Measuring sperm motility can be more descriptive.
- Assess qualitative testicular and epididymal histology.
- Assess spermatogenesis stages to ascertain the effect of low cholesterol in sperm production.
- Apply treatment to a group of animals with increased cholesterol.
References


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