The effects of nanomaterials, in the presence and absence of serum proteins, on testicular cell metabolic processes and steroidogenesis

By

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Professor E.J. Pool

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Declaration

I, Ashley George Muller declare that the thesis entitled 'The effects of nanomaterials, in the presence and absence of serum proteins, on testicular cell metabolic processes and steroidogenesis' is my own work and has not been submitted for any degree or examination at any other university and that all sources of my information have been quoted as indicated in the text and/or list of reference.

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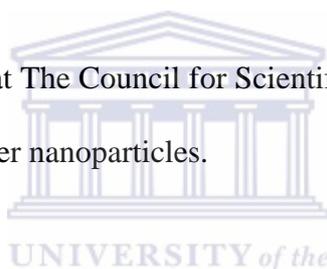
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List of abbreviations

°C	-	degrees Celsius
3β-HSD	-	3β-hydroxysteroid dehydrogenase-_Δ^4-_Δ^5 isomerase
17ß- HSD	-	17ß-hydroxysteroid dehydrogenase
µg/m^3	-	micrograms per cubic meter
µg	-	micrograms
µg/ml	-	micrograms per millilitre
µl	-	microliters
µl/well	-	microliters per well
µm	-	micrometers
ANOVA	-	analysis of variance
Ag	-	silver
Ag+	-	silver ions
Ag NPs	-	silver nanoparticles
ATP	-	adenosine triphosphate
Au NPs	-	gold nanoparticles
BBB	-	blood-brain barrier
BET	-	Brunauer, Emmet and Teller
BND	-	5-bromo-5-nitro-1, 3-dioxane
BSA	-	Bovine serum albumin
cAMP	-	cyclic adenosine 3',5'-cyclic monophosphate
cells/ml	-	cells per millilitre
cm^3	-	cubic centimeter
CO_2	-	carbon dioxide
DMF	-	dimethlyformamide
DNA	-	deoxyribonucleic acid
EDX	-	energy-dispersive X-ray
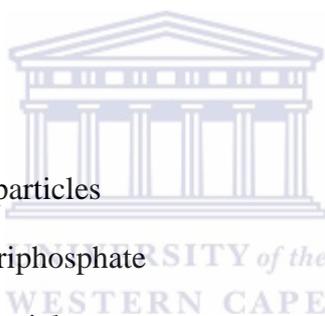
ELISA	-	enzyme linked immunosorbant assay
g/cm^3	-	grams per cubic centimeter
GSH	-	glutathione
h	-	hours
h/day	-	hours per day
H$_2$SO$_4$	-	sulphuric acid
hMSCs	-	human mesenchymal stem cells
IL-1	-	interleukin-1
IL-8	-	interleukin-8
kV	-	kilovolts
LH	-	luteinizing hormone
m^2/g	-	meters squared per gram
mg/kg	-	milligrams per kilogram
mg/ml	-	milligrams per millilitre
MIT	-	2-methyl-2H-isothiazol-3-one
ml	-	milliliter
MIP – 2	-	macrophage inhibitory protein
NaCl	-	sodium chloride
ng/g	-	nanograms per gram
ng/ml	-	nanograms per millilitre
nm	-	nanometers
NPs	-	nanoparticles
Ntera2	-	NT2, human testicular embryonic carcinoma cell line cells
OD	-	optical density
Ogg1−/− KO	-	8-oxoguanine DNA glycosylase knockout male mice
P450c17	-	cytochrome P450 17α- hydroxylase/C17-20 lyase
P450scc	-	cytochrome P450 side chain cleavage enzyme

particle/cm^3	-	particle per cubic centimeter
PVA	-	polyvinyl alcohol
PXRD	-	powder X-Ray diffraction
ROS	-	reactive oxygen species
SEM	-	scanning electron microscope
SOD	-	superoxide dismutase
StAR	-	Steroidogenic Acute Regulatory Protein
TEM	-	transmission electron microscopy
TiO2-NPs	-	titanium oxide nanoparticles
TMB	-	tetramethylbenzidine
TNF – α	-	tumor necrosis factor - alpha
UV-vis	-	ultraviolet- visible spectroscopy
WST	-	water soluble tetrazolium (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium)
XTT	-	2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5carboxanilide

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Abstract

Nanotechnology refers to the production and structural manipulation of materials between 10-100 nm ranges, known as nanomaterials, which display novel properties. Silver nanoparticles (Ag NPs) are said to be the most widely commercialised nanomaterial and it has found a wide range of applications in many different fields, including appliances and drug delivery systems. Due to their antimicrobial activity, believed to be attributed to the release of silver ions, they have been incorporated into many consumer products to serve as antibiotic agents. These applications lead to overexposure of organisms and the environment to silver which could eventually make its way into the human body via ingestion, inhalation and/or dermal contact and we don't fully comprehend the impact of Ag NPs inside the human body.

Silver nanoparticles were found to accumulate in any or all of the major organs and organ systems via circulatory and lymphatic system distribution after introduction to the human body via inhalation, ingestion, and/or injection. Various *in vitro* studies on different cell lines have concluded that silver nanoparticles induce oxidative stress, DNA damage, apoptosis, and/or necrosis. Diesel exhaust particles showed the ability to decrease sperm motility and gold nanoparticles showed a reduction in Leydig cell viability and steroid hormone production. The effect of Ag NP exposure on male fertility is however poorly understood and not enough data is available to fully assess risks posed by these particles on male reproduction. Therefore, it was the aim of this study to be the first to ascertain the effects of silver nanoparticles on testosterone production.

The Ag NPs used for this study have the following characteristics; purity ≥ 99.5%; 66.7 % of particles have a diameter between 20-40 nm in aqueous solution. Three month old male Balb/C mice were sacrificed and testicular cell cultures were prepared. The cells were subsequently treated with various concentrations of Ag NPs (with or without luteinizing hormone (LH)-treatment) and incubated for 4 hours. Testosterone secretion in the culture supernantant was then determined using a testosterone ELISA kit. Ag NPs (at 20 µg/ml) significantly ($p < 0.001$) decreased LH-stimulated testosterone production as compared to the control. This study showed that Ag NPs adversely affect testosterone synthesis *in vitro* and can therefore pose a risk for male reproduction.

Chapter 1: Literature Review

1.1. Nanotechnology: Overview

Nanotechnology refers to the production and structural manipulation of materials between 10-100 nm ranges, known as nanomaterials, which display novel properties (Braydich-Stolle et al., 2005). Nanomaterials can be grouped into five categories, namely carbon-based materials, polymers, metal-based materials, dendrimers, and composites and are found in different shapes, such as, rods, tubes, wires, different crystal forms, spheres or core-shell nanoparticles (NPs) (Lü et al., 2009; Kruszewski et al., 2011). The large surface-to-volume ratio in conjunction with other physiochemical properties, such as composition, surface chemistry and quantum confinement effects, make researchers believe that these nanomaterials may be harmful to the environment and biological organisms that live in it (Kruszewski et al., 2011). Despite this, many nanomaterials are continuously being manufactured for use in various industries.

1.2 Applications of silver nanoparticles

Silver nanoparticles (Ag NPs) are said to be the most widely commercialised nanomaterial and it has found a wide range of applications in many different fields (Braydich-Stolle et al., 2010). Xiu et al. (2012) states that the release of silver ions (Ag+) are exclusively responsible for the antimicrobial activity of Ag NPs. Due to their antimicrobial activity they have been incorporated into many consumer products to serve as antibiotic agents, including appliances such as washing machines and refrigerators, textiles, wound healing and medical products such as surgical instruments, contraceptive devices, bone prostheses as well as dental alloys (Stensberg et al., 2011; Tian et al., 2007; Kruszewski et al., 2011). Ag NPs

have also been used to treat diseases such as HIV subtype 1, where it was shown, *in vitro,* that Ag NPs, in the size range of 1-10 nm, bind to the HIV subtype 1 virus and thus prevent it from binding to the host cells (Elechiguerra et al., 2005). These applications, as drug delivery systems and treatments of diseases, require the need for a constant concentration of the drug (in these cases Ag NPs) in the blood for targeting of specific cells or organs (Moghimi et al., 2001; Panyam & Labhasetwar, 2003). This leads to overexposure of organisms and the environment to silver which could eventually make its way into the human body via ingestion, inhalation and/or dermal contact and we don't fully comprehend the impact of Ag NPs inside the human body (Kulthong et al., 2010). Benn & Westerhoff (2008) showed that Ag NPs that are incorporated into fabrics such as socks do leach out silver upon washing of the items. It was found that Ag NPs incorporated into antibacterial fabrics, such as wound dressings, are released upon interaction with artificial sweat and since Ag NPs are able to penetrate through the skin, it becomes paramount that the toxicological effects of Ag NPs be better understood (Kulthong et al., 2010; Larese et al., 2009). AshaRani et al. (2011) also discovered that Ag NPs causes haemolysis at concentrations of between 25-400 mg/ml, leading to further concerns with regards to wound dressings and other products that will come into contact with the skin.

1.3   Uptake of Ag NPs

Ag NP's cellular uptake is said to be fast and time-dependent. In as little as 2 hours almost all cells contain Ag NPs (Kruszewski et al., 2011). Gaiser et al. (2009) found that Ag NPs sized 35 nm were more readily found and taken up into the cytoplasm of C3A human hepatocytes or Caco-2 human intestinal epithelial cells than bulk silver particles (0.6-1.6 μm) after 2 hours and 24 hours of exposure. Lu et al. (2010) demonstrated, via transmission electron microscopy (TEM), that all forms of silver nanomaterials (colloidal and powder

spheres, colloidal and powder prisms) are assimilated in more or less the same manner. The uptake of both the 30 nm silver nanospheres and the 30 nm silver nanoprisms, into human HaCaT keratinocytes, accumulated in the nucleus in 7 hours. However, the surface charge of Ag NPs was shown to be a significant factor in its uptake into the cellular environment (Lesniak et al., 2005; Gregas et al., 2010). Previous studies found that positively or negatively charged Ag NPs (50 nm) were more readily taken up than their neutral counterparts (Lesniak et al., 2005; Gregas et al., 2010).

Studies have found that human mesenchymal stem cells internalised Ag NPs via macropinocytosis and clathrin-dependent endocytosis and this internalisation is influenced by different factors, including morphology and surface chemistry, as well as size and concentration (Greulich et al., 2010; Mailander & Landfester, 2009). Yen et al. (2009), however, found evidence that showed that coated and uncoated Ag NPs were taken up into J774 A1 macrophages via pinocytosis. Whichever mechanism is used for internalisation of Ag NPs, studies have shown that internalisation of these Ag NPs result in the production of reactive oxygen species (ROS), inflammation and ultimately cell death (Ahamed et al., 2008; Ahamed et al., 2010; AshaRani et al., 2009b). Bhakat et al. (2006) and Paz-Elizur et al. (2008) states that ROS has DNA damaging effects, such as causing a whole host of oxidised base lesions, both single-strand and double-strand breaks and abasic sites, which are ultimately mutagenic and/or cytotoxic. Both Hussain & Frazier (2003) and Li & Osborne (2008) stated that ROS synthesis in excessive amounts induce apoptosis in a number of cell culture models.

Asare et al. (2012) postulated that since Ag NPs are able to interact with nuclear material it can lead to DNA alterations, and if applied to the germline cells, it might affect spermatogenesis and fertility (AshaRani et al., 2009a; Kruszewski et al., 2011). This will

have devastating effects on the reproductive rate and population dynamics of the species affected.

VanWinkle et al. (2009) found that only smaller Ag NPs (< 20 nm) cross the cell membrane of rat type I-like alveolar epithelial cells and were uniformly distributed without agglomeration within the cytosol, mitochondria, and cell nucleus.

The toxicity of Ag NPs seems to be dictated by shape, size, surface chemistry and surface charge as these factors directly impacts on the extent, rate, location and/or timing of Ag+ release (Pal et al., 2007; Morones et al., 2005; Sotiriou & Pratsinis 2010; Ahamed et al., 2008; Lesniak et al., 2005; Gregas et al., 2010; Xiu et al., 2012).

1.4   Environmental impact of Ag NPs

Ag NP containing products leach the Ag NPs into the environment which may lead to bioaccumulation, food chain contamination and/or drinking water contamination (Karn et al., 2009; Gao et al., 2009). Kruszewski et al. (2011) states that the toxicological outcome of these Ag NPs lies in the nature of the NPs. The NPs will either end up in the soil if it is able to interact with natural materials or it can remain in the water where it can be exposed to various organisms. It thus becomes very relevant to understand the toxicity of Ag NPs on the organisms and the environment since they will most likely enter into the human food chain by some way or another. Several toxicological studies have been done on fish species.

AshaRani et al. (2008) exposed zebrafish (*Danio rerio*) embryos to either 5-20 nm BSA – or starch coated Ag NPs at a concentration of 5-100 µg/ml for 24, 48 & 72 hours post-fertilisation. The study revealed that the toxicity was dose-dependent and that it caused several embryonic malformations (AshaRani et al., 2008). Lee et al. (2007) also looked at

the Ag NP toxicity in Zebrafish embryos. They administered uncoated 5-46 nm Ag NPs at concentrations of 0.004-0.08 µg/ml for 24-120 hours and their results were similar to the results obtained by AshaRani et al. (2008).

Adult zebrafish assimilate Ag NPs through the gills which caused alterations in gene expression and increased the death rate (Griffitt et al., 2008; Griffitt et al., 2009). Wu et al. (2010) also discovered increased mortality in adult Japanese medaka (*Oryzias latipes*) exposed to 20-40 nm uncoated Ag NPs at concentrations between 0.5-8 µg/ml. Ag NPs are said to partially degrade *in vivo,* leading to release of Ag^+, but studies suggest that there are noticeable differences between the toxicity caused by Ag^+ as opposed to Ag NPs (Bar-Ilan et al., 2009; Chae et al., 2009; Gaiser et al., 2009). Kruszewski et al. (2011) proposes that this strengthens the argument that the release of Ag^+ is not the only factor in Ag NPs toxicity.
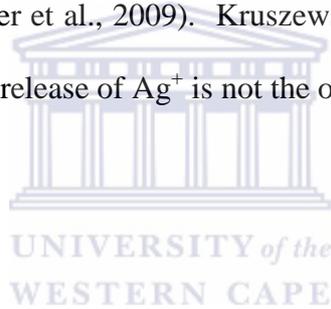
Table 1: A non-exhaustive summary of the environmental impact of Ag NPs

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP tested	Exposure duration	Outcome	References
Japanese medaka (*O. latipes*) at early-life stages	20-40	None	0.5-8 µg/ml fish 0.5-4 µg/ml eggs 0.1-1 µg/ml Embryos	48 h 168 h 60 days	Developmental retardation and morphological defects in embryonic larvae, toxicity in larval-juvenile medaka, histopathological changes in the larval eye	Wu et al., 2010
Zebrafish (*D. rerio*) Embryos	26, 44 and 216 in Medium	None	1 µg/ml	48 h	Toxicity, changes in gills morphology and gene expression	Griffitt et al., 2009; 2008
Zebrafish (*D. rerio*) Embryos	6-20	BSA and starch Coated	5-100 µg/ml	24, 48, 72 h postfertilization	Dose-dependent cytotoxicity, genotoxicity & metabolic arrest	AshaRani et al., 2008
Zebrafish (*D. rerio*) Embryos	5-46	None	0.004-0.08 µg/ml	24-120 h	Dose-dependent mortality and developmental abnormality in embryos	Lee et al., 2007

1.5   Studies on Ag NPs

1.5.1 *In vitro* studies using Ag NPs

The parameters which influence Ag NP toxicity include size, type, zeta potential, agglomeration and/or dispersion status as well as potential interaction with biomolecules (Asare et al., 2012). AshaRani et al. (2009b) used IMR-90 human lung fibroblast and U251 human glioblastoma cells and exposed them to 6-20 nm starch coated Ag NPs at concentrations of 25-400 µg/ml for 24, 48 and 72 hours. Their results indicated oxidative stress, DNA damage, apoptosis, necrosis and low ATP levels all suggesting metabolic arrest (AshaRani et al., 2009b). Schrand et al. (2008) exposed N2A murine neuroblastoma cells to 25 nm uncoated and polysaccharide coated Ag NPs at a concentration of 0.5-100 µg/ml for 24 hours. They found that this resulted in disruption of the actin cytoskeleton, oxidative stress degradation of mitochondrial integrity and decreased cell proliferation that continued even after the administration of nerve growth factor (Schrand et al., 2008). The administration of 3.08, 5.75 and 24.85 nm uncoated Ag NPs to J774 A1 macrophages at concentrations of 1-10 µg/ml for 24, 48 and 72 hours resulted in cytotoxicity only being seen in the cells exposed to the smaller Ag NPs (Yen et al., 2009) . This may suggest that only the smaller Ag NPs are able to enter the cells causing damage.

Greulich et al. (2009) administered 100 nm spherical Polyvinylpyrrolidone-coated Ag NPs to human mesenchymal stem cells (hMSCs) at a concentration of 0.05-50 µg/ml for 7 days. The results of which showed decreased cell proliferation and chemotaxis as well as increased release of IL-8. AshaRani et al. (2011) showed a concentration dependent toxic effect of both polyvinyl acetate- and starch-coated Ag NPs on human erythrocytes and haemagglutination resulting in swelling of cells, haemolysis and a loss in biconcavity.

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Reduced cell viability, DNA fragmentation and oxidative stress was evident when HT-1080 human fibrosarcoma cells derived from dermis, and A431 human skin carcinoma cells derived from the epidermis were treated with 7-20 nm uncoated Ag NPs at concentrations of 1.56-50 µg/ml for 24 hours (Arora et al., 2008). Kokura et al. (2010) observed no increase in cell death when they exposed human skin and HaCaT normal human keratinocytes to 730.5 nm cellulose gum coated Ag NPs at concentrations of 0.5-50 µg/ml (skin) and 0.002-0.02 µg/ml (keratinocytes) for 24 hours. It should be noted that the NPs used were quite large and at concentrations lower than that used in other studies. This again suggests that toxicity only occurs above a certain concentration and that since larger NPs are unable to enter the cell no toxicological effect is shown by them.

An interesting study by Kawata et al. (2009) found that 90 nm polyethylamine-stabilized Ag NPs were beneficial (increased cellular proliferation) to HepG2 human hepatocytes at concentrations under 1 µg/ml, but at concentrations greater than 1 µg/ml the effects became toxic. The opposite was discovered by Rosas-Hernández et al. (2009) who found that rat coronary endothelial cells exposed to low concentrations (1-10 µg/ml) of 45 nm uncoated Ag NPs showed inhibited proliferation, but at higher concentrations (50-100 µg/ml) the Ag NPs resulted in increased proliferation. Li et al. (2010) studied the effects of Ag NPs on embryonic development using mouse blastocysts as a model. Mouse blastocysts exposed to 13 nm uncoated Ag NPs at concentrations of 25 and 50 µM for 24 hours showed inhibition of cell proliferation and induction of apoptosis (Li et al., 2010). This may suggest that Ag NPs are detrimental to reproduction as it affects embryonic implantation and development.

The importance of surface chemistry on toxicity was shown when Ahamed et al. (2008) exposed mouse embryonic stem cells and mouse embryonic fibroblasts to 25 nm uncoated and polysaccharide (acacia gum)-coated Ag NPs and found that the coated Ag NPs exerted a greater effect on DNA damage and apoptosis than the uncoated Ag NPs. Murdock et al.

(2007) states that agglomeration tends to develop with the uncoated NPs while the coated NPs remain monodispersed. Ahamed et al. (2008) postulates that this could be the reason for the increased toxic effect of coated NPs, as the agglomerated uncoated NPs would most likely be too large to be internalised into the cells while the coated NPs are small enough to enter into and be distributed throughout the cell. Furthermore, Ahamed et al. (2008) found that there was an almost immediate increase in the expression of p53 protein upon exposure to the Ag NPs. Since p53 activation is closely linked to DNA damage, this then shows potential Ag NPs genotoxicity (Liu & Kulesz-Martin, 2001).

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Table 2: A non-exhaustive summary of in *vitro* studies using Ag NPs

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP Tested	Exposure duration	Outcome	References
Human erythrocytes	10.88 (PVA) 5.78 (starch)	PVA coated, starch coated	25-400 µg/ml	3h	haemagglutination resulting in swelling of cells, haemolysis and a loss in biconcavity	AshaRani et al., 2011
Human skin, HaCaT normal human keratinocytes	730.5	Cellulose gum-coated	0.5, 50 µg/ml (skin), 0.002-0.02 µg/ml (keratinocytes)	24h	No increase in cell death	Kokura et al., 2010
Mouse blastocysts	13	None	25 and 50 µM	24h	Inhibition of cell proliferation and induction of apoptosis	Li et al., 2010
IMR-90 human lung fibroblast, U251 human glioblastoma cells	6-20	Starch coated	0, 100, 200 and 400 µg/ml	2-3 hours	Chromosome instability and mitotic arrest in human cells. Upregulation of stress response genes. Calcium transients	AshaRani et al., 2009a
IMR-90 human lung fibroblast, U251 human glioblastoma cells	6-20	Starch Coated	25-400 µg/ml	24, 48, 72 h	Cytotoxicity, production of IL1, IL6, and TNF	AshaRani et al., 2009b
HepG2 human hepatoma cells	90	Polyethylenimine Stabilized	1-3 µg/ml	24h	Increased cell proliferation < 0.5 µg/ml cytotoxic > 1 µg/ml	Kawata et al., 2009

Table 2 continued

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP tested	Exposure duration	Outcome	References
Rat coronary endothelial cells	45	None	0.1-100 µg/ml	24 h	Inhibited proliferation (1–10 µg/ml), increased proliferation (50–100 µg/ml)	Rosas-Hernández et al., 2009
J774 A1 macrophages	3.08, 5.75, and 24.85	None	1-10 µg/ml	24, 48, 72 h	Cytotoxicity, production of IL1, IL6, and TNF	Yen et al., 2009
Mouse embryonic stem cells (mES), mouse embryonic fibroblasts (mEF)	25	Uncoated, polysaccharide coated	50 µg/ml	24, 48, 72 h	Coated NPs exerted greater DNA damage and apoptosis inducing effects than uncoated NPs	Ahamed at al., 2008
HT-1080 human fibrosarcoma cells derived from dermis, A431 human skin carcinoma cells derived from epidermis	7-20	None	1.56-50 µg/ml	24h	Reduced cell viability, DNA fragmentation and oxidative stress	Arora et al., 2008
N2A murine neuroblastoma cells	25	Uncoated, polysaccharide coated	0.5-100 µg/ml	24h	Oxidative stress, degradation of mitochondrial membrane integrity, disruption of actin Cytoskeleton	Schrand et al., 2008

Table 2 continued

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP tested	Exposure duration	Outcome	References
Human mesenchymal stem cells (hMSCs)	100	PVP coated	0.05-50 µg/ml	7 days	Cell proliferation and chemotaxis were decreased, IL8 release was increased	Wu et al., 2008

1.5.2 *In vivo* studies using Ag NPs

In vivo studies regarding Ag NPs are only limited to non-human organisms and limited in number (Kruszewski et al., 2011). McAuliffe et al. (2007) states that after the introduction of NPs into the human body via inhalation, ingestion, and/or injection, they can accumulate in any or all of the major organs and organ systems via circulatory and lymphatic system distribution (Panyala et al., 2008). Ag NPs cannot only traverse blood vessels, but they can also cross the biological barriers of the body, such as the blood-brain barrier and the blood-testes barrier (Lankveld et al., 2010; McAuliffe et al., 2007). Acute and chronic exposure to Ag NPs affects several physiological functions (Sung et al., 2011; Lankveld et al., 2010).

Lee et al. (2010) exposed C57BL/6 mice to Ag NPs and found that several genes linked with motor neuron disorders, neurodegenerative disease, and immune cell function in brain had been affected. Takenaka et al. (2001) compared the differences between 10nm and 100nm Ag NPs administered via inhalation and intratracheal exposure, resulting in the accumulation of Ag NPs in the blood and the lungs. Ji et al. (2007) showed a dose-related accumulation in lungs, brain, olfactory bulb and liver. Sprague-Dawley rats exposed via inhalation to Ag NPs exhibited no lesions discovered in the nasal cavity or lungs upon histopathological examination; however, there was a marked increase in growth of goblet cells, as well as an increase in neutral mucous production after the administration of high doses (Ji et al., 2007; Hyun et al., 2008). Sung et al. (2008) administered 18 nm Ag NPs 6 hours a day, 5 days per week for 90 days via inhalation to Sprague-Dawley rats and found that there was a decrease in the rats' tidal volume as well as an increase in bile duct hyperplasia and liver inflammation. Another study investigated the subchronic inhalation administration of 18-19 nm Ag NPs (6 hours per day, 5 days per week for 13 weeks) on Sprague-Dawley rats and found that the highest dose (3.0×10^6 particle/cm^3) resulted in macrophage accumulation in

the lungs, alveolar inflammation as well as increased bile duct hyperplasia (Sung et al., 2009).

The oral exposure of male and female Fischer F344 rats to 56 nm Ag NPs resulted in dose-dependent increases in cholesterol and alkaline phosphatase levels in blood, bile-duct hyperplasia, necrosis, fibrosis, and/or pigmentation changes in liver tissue (Kim et al., 2010). Histological changes in the intestinal mucosa as well as changes in the secretion of mucin was evident after the 28 day oral exposure (30, 300, 1000 mg/kg) of Sprague-Dawley rats to 60 nm Ag NPs (Jeong et al., 2010). Both Tang et al. (2008) and Tang et al. (2009) studied the difference between Ag NPs versus Ag microparticles in their ability to cross the blood-brain barrier and the ability to cross into the cardiovascular system after subcutaneous exposure, respectively. It was found that only the Ag NPs are able to cross the blood-brain barrier as well as cross over into the cardiovascular system upon subcutaneous administration, the latter ending with accumulation in various organs, including spleen, kidneys, brain, liver and lungs. Ag NPs showed a size-dependent accumulation pattern, where large particles (100 nm) were located in the spleen (~4000 ng/g organ), liver (~2000 ng/g organ), and lungs (~1000 ng/g organ) and smaller particles (20 nm) were found in the liver (~1000 ng/g organ), kidneys (~300 ng/g organ), and spleen (~200 ng/g organ) (Lankveld et al., 2010). Rahman et al. (2009) intraparentally exposed C57BL/6N mice to 29 nm Ag NPs (100, 500, and 1000 mg/kg) for 24 hours and this resulted in oxidative stress-related altered gene expression in the caudate, frontal cortex, and hippocampus regions of the brain.

Table 3: A non-exhaustive summary of in *vivo* studies using Ag NPs

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP Tested	Exposure duration	Outcome	References
Sprague-Dawley Rats	60	None	30, 300, 1000 mg/kg	Ingestion 28 days	Histological changes in the intestinal mucosa as well as changes in the secretion of mucin	Jeong et al., 2010
Fischer F344 rats	56	None	30, 125, and 500 mg/kg body weight	Ingestion 90 days	dose-dependent effects, such as an increase in cholesterol and alkaline phosphatase levels in blood, bile-duct hyperplasia, necrosis, fibrosis, and/or pigmentation changes in liver tissue	Kim et al., 2010
Rats	20, 80, 110	None	23.8, 26.4, 27.6 µg/ml	Intravenously	Size-dependent deposition in all major organs	Lankveld et al., 2010
C57BL/6 Mice	22	None	1.91×10^7 particles/cm3	Inhalation exposure: 6 h/day, 5 days/week, 14 days	affects several genes linked with motor neuron disorders, neurodegenerative disease, and immune cell function in brain	Lee et al., 2010
C57BL/6N Mice	29	None	100, 500, and 1000 mg/kg	Intraparentally 24 h	Oxidative stress-related altered gene expression in the caudate, frontal cortex, and hippocampus regions of the brain.	Rahman et al., 2009

Table 3 continued

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP Tested	Exposure duration	Outcome	References
Sprague-Dawley Rats	18-19	None	$0,6 \times 10^6$, 1.4×10^6, 3.0×10^6 particles/ cm^3	Inhalation exposure: 6 h/day, 5 days/ week, for 13 weeks	Macrophage accumulation in the lungs, alveolar inflammation as well as increased bile duct hyperplasia	Sung et al., 2009
Rats	50-100, 2-20 μm	None	62.8 mg/kg	Subcutaneously	AgNPs translocated to the blood circulation system and distributed throughout the main organs. Caused neuronal degeneration	Tang et al., 2009
Sprague-Dawley Rats	13-15	None	1.73×10^4, 1.27×10^5, 1.32×10^6 particles/ cm^3 μg/ml (61 μg /m^3)	Inhalation exposure: 6 h/day, 5 days/ week, for 28 days	Size and number of goblet cells containing neutral mucins increased in lungs	Hyun et al., 2008
Sprague-Dawley Rats	18	None	1.73×10^4, 1.27×10^5, 1.32×10^6 particles/ cm^3	Inhalation exposure: 6 h/day, 5 days/ week, for 90 days	Decrease in the rats' tidal volume chronic alveolar inflammation, including alveolaritis, granulomatous lesions, and alveolar wall thickening.	Sung et al., 2008
Rats	50-100, 2-20 μm	None	62.8 mg/kg	Subcutaneously	AgNPs traversed the blood–brain barrier (BBB) and move into the brain in the form of particles. Induction of neuronal degeneration and necrosis	Tang et al., 2008

Table 3 continued

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP Tested	Exposure duration	Outcome	References
Sprague-Dawley Rats	18	None	1.73×10^4 1.27×10^5 1.32×10^6 particles/ cm^3	Inhalation exposure: 6 h/day, 5 days/ week, for 28 days	Dose-related accumulation in lungs, brain, olfactory bulb and liver.	Ji et al., 2007
Fischer F344 rats	15	None	Inhalation 133 µg/m^3 $(3 \times 10^6 \text{ cm}^3)$ Intratracheal 50 µg AgNPs	24h	Dose dependent translocation to major organs	Takenaka et al., 2001

1.5.3 Studies of Ag NPs on male reproductive cells

Although a major cause for concern, there are not many studies done to assess the impact of silver nanoparticles on the male reproductive system. Asare et al. (2012) assessed the differences in cytotoxic and genotoxic effects of Ag NPs and titanium oxide nanoparticles (TiO2-NPs) on testicular cells by using 8-12 week old 8-oxoguanine DNA glycosylase knockout male mice (Ogg1−/− KO) and Ntera2 (NT2, human testicular embryonic carcinoma cell line) cells. Ag NPs (20 nm & 200 nm) and TiO2-NPs (21 nm) were fed to the Ogg1−/− KO mice together with their standard chow. The testicular cells were exposed to concentrations of 10, 50 and 100 µg/ml for 24, 48 and 72 hours. The Ogg1−/− KO genotype were used because they possess an impaired oxidative DNA damage repair system similar to human testicular cells and thereby they represent an appropriate model for human male reproductive toxicity assays. The study found that the Ag NPs had a more potent cytotoxic and cytostatic effect compared to the TiO2-NPs (Asare et al., 2012). The Ag NPs caused several effects in a time- and concentration dependent manner, including apoptosis, necrosis and as well as a decrease in proliferation. Asare et al. (2012) found a concentration dependent increase in DNA strand breaks with regards to the larger Ag NPs (200 nm). This DNA strand damaging effect may lead to alterations and mutations in the DNA, which could be passed onto offspring via genetic and epigenetic mechanisms, thus impacting on the future population of the affected species. The study also found that at the highest concentration (100 µg/ml) the metabolic activity of all three cell types were already reduced by an estimated 50% compared to the controls. In a study by Gromadzka-Ostrowska et al. (2012) they used 20 nm and 200 nm spherical Ag NPs on 96 adult 14 weeks old male Wistar rats (strain: Wistar Cmd: WI(WU)) that were divided into 4 groups who received either one single dose of 5 mg/kg or 10 mg/kg of 20 nm Ag NPs or 5 mg/kg of the 200 nm Ag NPs and a control group injected with 0.9% NaCl solution. They determined the acute effects of the

intravenous administration of Ag NPs on several parameters, including sperm count, abnormal spermatozoa frequency, germ cell DNA damage in sperm cells and testis morphometry. They found that the 20 nm Ag NPs caused germ cell DNA damage that peaked at 24 hours post injection, after which it decreased gradually for some time (7 and 28 days later). They postulated that the decreased effects were due to the removal of Ag NPs from the organism and DNA damage repair. They also noted that the highest DNA damage was found amongst the group that received the 20 nm Ag NPs treatments. It was postulated that the NP size effects could be due to the fact that smaller NPs are able to enter the cell more readily than the larger NPs. Liu et al. (2010) stated that the smaller NPs have a larger surface area, which is able to interact more with the environment and also increase the ion release. Although there were no differences in weight between the different subject groups, they found marked differences in the morphology of the testicles. These differences included seminiferous tubules with increased diameter, area and circumference in the animals exposed to 200 nm Ag NPs 28 days post injection. The evidence, they suggested, pointed to the theory that bioaccumulation of the 200 nm Ag NPs caused the seminiferous tubules to swell up. The authors argue that since Lankveld et al. (2010) stated that only 1% of the injected dose of Ag NPs accumulates in the testes no matter what the size, the amount of Ag NPs needed to bring about a negative effect on male fertility is very low. They also found that the treatment with Ag NPs resulted in decreased levels of testosterone as well as dihydrotestosterone measured at day 7 and day 28 post injection. In all the treated groups the number of abnormal spermatozoa (folded, amorphous spermatozoa, cells lacking or showing a small hook and cells with undulating or elongated heads) 28 days after injection, which is the time it takes for two cycles of germ cells to pass through the epididymis, was higher when compared with the number 24 hours post injection. This indicates a greater impact of Ag NPs on epididymis than the testes. They concluded that the toxicity of Ag NPs is more

apparent in the mature sperm cells in the epididymis than in the spermatozoa located in the seminiferous epithelium.

Braydich-Stolle et al. (2005) found that the effects described in other cell types were also true for mouse spermatogonia stem cells. They ascertained that Ag NPs exposure resulted in apopotosis, necrosis and mitochondrial dysfunction. Nanoparticles also showed the ability to decrease sperm motility and Gold NPs showed a reduction in Leydig cell viability and steroid hormone production (Wiwantitkit et al., 2009; Komatsu et al., 2008).

Takeda et al. (2009) showed that TiO2-NP's *in vivo* effects on spermatogenesis by decreasing epididymal sperm motility and inducing histopathological changes in mice testes. TiO2-NPs also caused a marked decrease in sperm density and motility as well as increased sperm abnormality and germ cell apoptosis (Guo et al., 2009). Ag NPs induced apoptosis in mouse embryos at the blastocyst stage, reduced implantation frequency as well as delayed post-implantation embryo development (Li et al., 2010). Ag NPs administration also decreases foetus viability (Philbrook et al., 2011). The effect of Ag NP exposure on male fertility is however poorly understood and not enough data is available to fully assess risks posed by these particles on male reproduction.

Table 4: A non-exhaustive summary of Studies of Ag NPs on male reproductive cells

Test species	Size (nm) of NP	Surface coating of NP	Concentration of NP Tested	Exposure duration	Outcome	References
8-12 week old male (Ogg1−/− KO) and Ntera2 cells	Ag NPs (20 nm & 200 nm) & TiO2-NPs (21 nm)	None	10, 50 and 100 µg/ml	Mice were fed the Ag NPs with their feed Cells exposed for 24, 48 and 72 hours	Ag NPs were more toxic than TiO2-NPs. Ag NPs caused several effects in a time- and concentration dependent manner, including apoptosis, necrosis and as well as a decrease in proliferation	Asare et al., 2012
96 adult 14 weeks old male Wistar rats (strain: Wistar Cmd: WI(WU))	20 and 200	None	One single dose of 5 mg/kg or 10 mg/kg of 20 nm Ag NPs or 5 mg/kg of the 200 nm Ag NPs and a control group injected with 0.9% NaCl solution	Intravenously	The 20 nm Ag NPs caused germ cell DNA damage that peaked at 24 hours post injection, after which it decreased gradually for some time (7 and 28 days later) Seminiferous tubules with increased diameter, area and circumference were evident in the animals exposed to 200 nm Ag NPs 28 days post injection.	Gromadzka-Ostrowska et al., 2012

Table 4 continued

Test species	Size (nm)	Surface coating	Concentration Tested	Exposure duration	Outcome	References
C18–4 cell line established from type A spermatogonia isolated from 6-day-old mouse testes	Silver (Ag-15 nm), molybdenum (MoO_3-30 nm), and aluminum (Al-30 nm) Cadmium oxide (positive control	None	5, 10, 25, 50, and 100 µg/ml 1, 2, 5, 10, and 25 µg/ml	48 hours	Ag NPs exposure resulted in apopotosis, necrosis and mitochondrial dysfunction	Braydich-Stolle et al., 2005
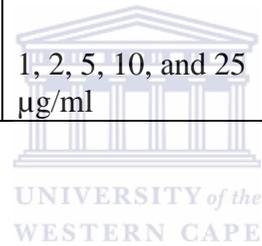
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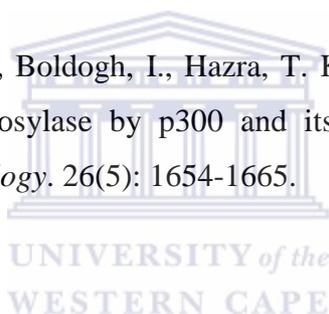
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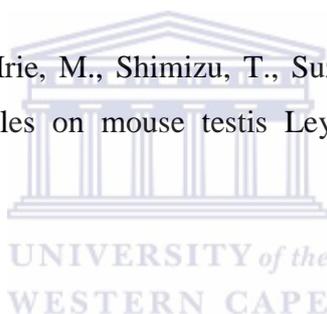
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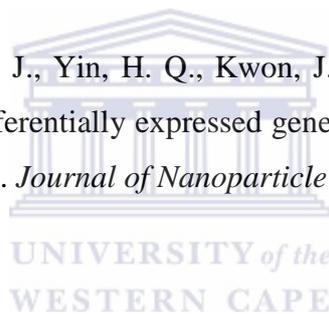
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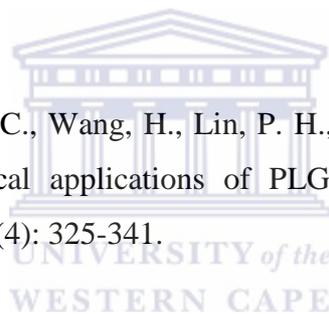
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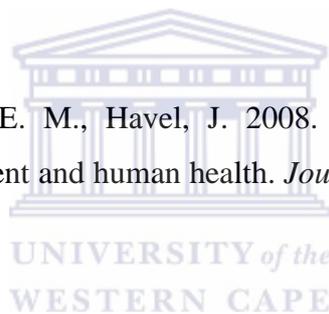
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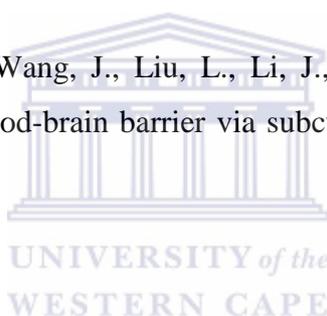
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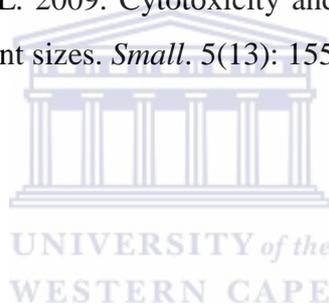
Chapter 2

2.1   Problem Statement

The toxicity studies done on Ag NPs have shown varying degrees of toxicological activity in different species, organs, as well as cell lines. Some studies suggest that Ag NPs possess a cytotoxic and pro-inflammatory effect resulting from the increased production of ROS, cytokine release and upregulation of heat shock proteins (Kruszewski et al., 2011; Daniel et al., 2010; Sung et al., 2009). Other studies point towards apoptosis and necrosis being an effect of Ag NP toxicity and that it also causes DNA damage and cell cycle progression disturbance (Asare et al., 2012; Lankoff et al., 2011; Wijnhoven et al., 2009). Bouwmeester et al. (2011), suggests that Ag NPs causes changes in the gene expression profile of cells, in particular the oxidative stress related genes. All the studies scrutinised point out that Ag NPs are toxic to the cells assessed in a time- and concentration dependent manner.

Consumer products, such as antibacterial socks and medical applications, such as potential anti-HIV agents, using Ag NPs have increased at an alarming rate, despite the lack of toxicological data on the effects of these Ag NPs on the human body. This is especially true concerning the effect of Ag NPs on the male reproductive system. Previous studies summarised in the literature review (Chapter 1) have pointed towards some degree of toxicity by Ag NPs on the male reproductive tract and suggest that Ag NPs could affect the offspring of the affected species and subsequent generations (Asare et al., 2012). Since we have already established the environmental impact of Ag NPs and that several species has been shown to internalise the Ag NPs directly from their environment, it can be postulated that not only can food supply dwindle, but humans can also get exposed to these Ag NPs by consuming the fauna that have internalised it (Griffitt et al., 2008; 2009).

A more pressing matter would be the effect these Ag NPs have on the quality of sperm and consequently, reproductive ability. Carlsen et al. (1992) states that semen quality has significantly decreased during a 50 year span, measured by a decrease in sperm count from 113x 10^6/ml in 1940 to 66x 10^6/ml in 1990 and a reduction in mean seminal volume from 3.40 ml to 2.75 ml in the same period among men with no history of infertility. Auger et al. (1995) found no decrease in seminal volume, but found a reduction in mean sperm concentration as well as a decrease in the percentages of normal and motile spermatozoa from 1973 to 1992. Both these articles show a remarkable decline in the quality of sperm even before the advent of nanoparticles in our everyday lives. It would be interesting to see whether Ag NPs will add on to the decline in sperm quality.

It is thus the aim of this study to determine whether Ag NPs affects spermatogenesis by assessing its influence on testosterone production.
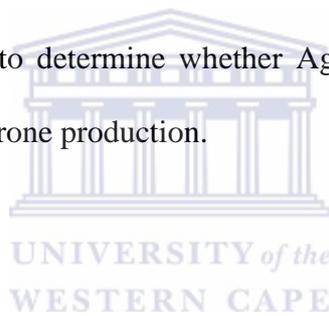
2.2 Hypothesis

H_0: Ag NPs has no effect on testosterone production by testicular cells.

H_1: Ag NPs affects testosterone production by testicular cells.

2.3 References

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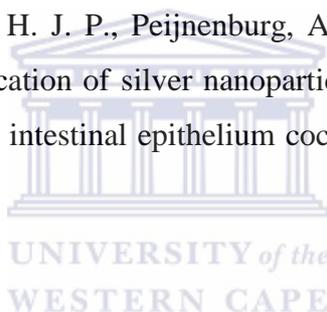
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Chapter 3: The effect of Silver Nanoparticles (Ag NPs) on testosterone production using primary testicular cell cultures (minced testes assay)

3.1 Abstract

This study aimed to ascertain the effects of Ag NPs on the male reproductive system *in vitro* by utilising mouse testicular cell cultures. Ag NPs are the most widely commercialised nanomaterial with a wide range of applications in many different fields. The Ag NPs used for this study have the following characteristics; purity ≥ 99.5%; 66.7 % of particles have a diameter between 20-40 nm in aqueous solution. Three month old male Balb/C mice were sacrificed and testicular cell cultures were prepared. Cells were subsequently treated with various concentrations of Ag NPs (with or without luteinizing hormone (LH)-treatment) and incubated for 4 hours. Testosterone secretion in the culture supernatant was then determined using a testosterone ELISA kit. Ag NPs (at 20 µg/ml) significantly ($p < 0.001$) decreased LH-stimulated testosterone production as compared to the control. Our study was not able to determine the mechanism of the anti-androgenic action. Additional studies are merited to ascertain the exact mechanisms involved.

3.2 Introduction

Ag NPs are said to be the most widely commercialised nanomaterial and it has found a wide range of applications in many different fields (Braydich-Stolle et al., 2010). Due to their antimicrobial activity they have been incorporated into many consumer products to serve as antibiotic agents, including appliances such as washing machines and refrigerators, textiles, wound healing and medical products such as surgical instruments, contraceptive devices, bone prostheses as well as dental alloys (Stensberg et al., 2011; Tian et al., 2007; Kruszewski

et al., 2011). The proliferation of applications of Ag NPs persists despite the lack of knowledge on the effects they have on the human body.

After exposure (oral, intravenous, dermal and inhalation) Ag NPs showed a size-independent distribution and accumulation in multiple organs, including spleen, liver, heart, brain, kidneys, lymph nodes, skin and testes (Kim et al., 2010; Lankveld et al., 2010; Sung et al., 2009). Toxicological studies based on other metal nanoparticles, such as gold nanoparticles (Au NPs) and titanium-oxide nanoparticles (TiO2-NP's) suggest that they decrease Leydig cell viability and steroid hormone production and that they decrease epididymal sperm motility and induce histopathological changes in mice testes, respectively (Komatsu et al., 2008; Takeda et al., 2009). The adverse effects of Ag NPs on the reproductive system are similar to those induced by other metal nanoparticles. Braydich-Stolle et al. (2010) showed that Ag NPs cause a decrease in the proliferation of spermatogonial stem cells. Gromadzka-Ostrowska et al. (2012) found that Ag NPs decreased the sperm count, increased abnormal spermatozoa and caused germ cell DNA damage in sperm cells and testis morphometry.

Testosterone is a major androgenic hormone that is responsible for, *inter alia*, the development of male reproductive structures, male secondary characteristics, as well as maintaining proper sperm cell production (Seeley et al., 2008; Martini et al., 2012). To our knowledge, there is no scientific data available on the effect of Ag NPs on testosterone production.

The US Environmental Protection Agency has recommended the sectioned or minced testes assay as the preferred method for screening the effect of substances on steroid hormone production and secretion (EPA, 2005). This assay has several advantages, including low cost, rapidity, simplicity, and the requirement of standard laboratory equipment and elementary laboratory training (EPA, 2005). Studies done by Ebrahim & Pool (2010)

showed that this assay can be used to monitor the effects of compounds on testosterone production.

The aim of this study is to utilise a minced testes assay to ascertain the effect of Ag NPs on steroidogenesis.

3.3   Materials and Methods

3.3.1 Reagents and chemicals

All chemicals, reagents, and solvents were purchased from Sigma (Germany), unless otherwise stated in the text. All reagents were of analytical grade.

3.3.2 Characterisation of silver nanoparticles (Ag NPs)

The Ag NPs in powder form (CAT no. 7440-22-4) was purchased from Sigma-Aldrich, South Africa. The manufacturer's specifications stated that the Ag NPs were <100 nm spherical nanoparticles with a specific surface area of 5.0 m^2/g, density of 10.49 g/cm^3 and a purity of 99.5 % based on trace metal analysis. Characterisation studies were undertaken to confirm the manufacturer's specifications.

3.3.2.1   Scanning Electron Microscopy (SEM) analysis of Ag NPs

SEM analysis was done using an EVO® MA15 Scanning Electron Microscope (SEM). A drop of Ag NPs suspension was pipetted onto the carbon surface of an SEM stub. Primary electron and/or secondary electron images were taken to identify the sample.

3.3.2.2 Energy-Dispersive X-ray (EDX) analysis of Ag NPs

The SEM had EDX spectrometry capabilities and this was used simultaneously during the SEM analysis in order to confirm the presence of Ag. The Ag NPs composition was quantified by EDX analysis using an Oxford Instruments® X-Max 20 mm^2 detector and Oxford INCA software (refer to Figure 3 under section 3.4.1.2).

3.3.2.3 Transmission Electron Microscopy (TEM) analysis of Ag NPs

Ag NPs' size and morphology were determined via TEM analysis using a JEOL 1200-EX II electron microscope at an accelerating voltage of 120 kV. A MegaView Camera employing Gatan Microscopic software with a resolution of 1376x1032, and 2 second exposure time was used to obtain images of the Ag NPs. A suspension of Ag NPs was dissolved in ethanol, and thereafter deposited onto copper grids and air-dried. Particle size distribution based on the TEM images was generated using ImageJ software.

3.3.2.4 Brunauer, Emmet and Teller (BET) analysis of Ag NPs

ASAP 2010 (Accelerated Surface Area and Porosimetry System; Micromeritics Instrument Corporation) was employed to determine the Ag NPs surface area. The Ag NP sample was degassed overnight at 100 °C before being analysed.

3.3.2.5 Powder X-Ray Diffraction (PXRD) analysis of Ag NPs

The crystalline nature of the Ag NPs was determined using PXRD (Panalytical X'pert Pro). The PXRD pattern was collected between angles of 2Θ from 3° to 90°.

3.3.2.6 Ultraviolet- visible (UV-vis) Spectroscopy of Ag NPs

A Helios Omega UV-VIS Spectrophotometer (Thermo Scientific) scanning from 400 to 1100 nm was used to measure light intensity as a function of wavelength. The Ag NP sample was suspended in dimethlyformamide (DMF) to prepare solutions for measurements.

3.3.3 Animals

Three month old, pathogen-free, male Balb/C mice were used for this study. Mice were purchased from the University of Cape Town Animal Unit (Cape Town, South Africa). The mice were housed in a well-ventilated animal house with a 12 hour light/dark cycle and fed standard mouse feed (Medical Research Council, Cape Town, South Africa) with free access to normal drinking water.

3.3.4 Cell culture

Mice were sacrificed by cervical dislocation. The testes were subsequently removed (aseptically), minced and then suspended in 10 ml serum-free medium which consisted of 1 % glutamax (Invitrogen), 1 % Penicillin/Streptomycin/Fungizone mix (Sigma), and 0.5 % Gentamicin (Sigma) in RPMI-1640 medium (Sigma). After allowing debris to settle, the supernatant containing cells were transferred to a new sterile tube. The cells were thereafter incubated at 37 ˚C with 5 % CO_2 for 1 hour. Following the incubation period, the cells were centrifuged at 40 000 x g for 10 minutes. The supernatant was then discarded and the cell pellet was resuspended in 10 ml serum-free medium and incubated at 37 ˚C with 5 % CO_2 for 30 minutes. The cells were centrifuged at 40 000 x g for 10 minutes and the supernatant obtained was again discarded. The cell pellet was then resuspended in 10 ml serum-free medium to an approximate concentration of 5 x 10^6 cells/ml for use in cell culture assays.

3.3.5 Preparation of Ag NPs

A 5 ml stock solution of 2mg/ml Ag NPs (Sigma) was prepared and then diluted with the serum-free medium described in the previous paragraph, to make up a 40 µg/ml sample to be used on the cell cultures. Due to the dilution factor associated with the protocol of the experiment, the final concentration of Ag NPs used was equal to 20 µg/ml.

3.3.6 Determination of the effect of Ag NPs on the production of testosterone

To determine the effect of the Ag NPs on the ability of the mouse testicular cells to produce testosterone, 100 µl of the serum-free medium was added to 6 columns of a 96 well plate (refer to Figure 1) in order to have replicates of 3 (3 columns LH minus & 3 columns LH plus). Stock Ag NP solution (100 µl at 40 µg/ml) was added to each well of the first row and then serial dilutions were performed to decrease the concentrations of Ag NPs in each subsequent row (refer to Figure 1). Prepared cell culture (50 µl per well) was then added to this mixture and the plate was incubated at 37 ˚C with 5 % CO_2 for 4 hours. An LH preparation, consisting of 0.04 % sheep pituitary LH (Sigma) in medium, consisting of 1 % glutamax (Invitrogen) and RPMI-1640 medium (Sigma), was prepared. This LH medium (50 µl per well) was added to the columns labelled LH Plus (columns 5-7). The medium (50 µl per well), consisting of 1 % glutamax (Invitrogen) and RPMI-1640 medium (Sigma) only, was added to the columns labelled LH Minus (columns 2-4). The plate was then allowed to incubate overnight at 37 ˚C with 5 % CO_2.

3.3.7 Testosterone production analysis using a testosterone ELISA

Following the overnight incubation, the plate was lightly shaken and the cells were allowed to settle. The top layer (20 µl) of each well was then transferred to a storage plate. A 4 x diluted wash buffer (180 µl) provided by the testosterone enzyme-linked immunosorbant (ELISA) kit (DRG Instruments, GmbH, Germany) was then added to these samples. These mixtures (25 µl per well) was then transferred to the ELISA plate and the rest of the assay was performed as per manufacturer's instructions (refer to Figure 2). All the required reagents were provided in the kit. The microtitre wells of the ELISA plate was coated with a monoclonal (mouse) antibody directed towards a unique antigenic site on the testosterone molecule. Standards or samples were added to the ELISA plate (25 µl/well). Subsequently, 200 µl/well of testosterone peroxidase conjugate was added for binding to the coated antibody. The contents in the wells were then incubated and mixed for 60 minutes. Afterwards, the wells were washed three times with 400 µl/well wash solution consisting of 5-bromo-5-nitro-1, 3-dioxane (BND) and 2-methyl-2H-isothiazol-3-one (MIT) and tapped dry. Then, 200 µl/well of the substrate solution, tetramethylbenzidine (TMB), was added. After mixing, the plate was allowed to incubate for 15 minutes at room temperature. The enzymatic reaction was stopped by adding 100 µl/well of the stop solution consisting of 0.5 M sulphuric acid (H_2SO_4). The optical density (OD) was read at 450 nm with a microtitre plate reader (Thermo Electron Corporation, Original Multiskan Ex). The 0 ng/ml standard results in maximum binding of the testosterone horseradish conjugate. All data was expressed as a percentage of 0 ng/ml standard. A standard curve was drawn using the results obtained for the testosterone standards. The testosterone concentrations of the samples tested were read using this curve.

Ag NP (µg/ml)	1	No LH added			LH added			8	9	10	11	12
		2	3	4	5	6	7					
A = 20												
B = 10												
C = 5												
D = 2.5												
E = 1.25												
F = 0.625												
G = 0												
H = Medium												
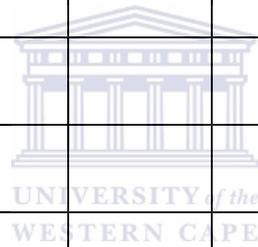
Figure 1: Plate layout for the determination of the effect of Ag NPs on testosterone production by testis cell cultures

	Standards of testosterone (ng/ml)	No LH added			LH added		
		2	3	4	5	6	7
A	16						
B	6						
C	2						
D	1						
E	0.5						
F	0.2						
G	0						
H							

Figure 2: Plate layout for testosterone ELISA

3.3.8 Statistical analysis

SigmaStat software (Systat Software Inc., USA) was used for statistical analysis. The experiments were performed nine (9) times in triplicate and data was statistically analysed via one-way ANOVA ($P < 0.001$) and regression analysis.

3.4   Results

3.4.1   Characterisation of silver nanoparticles (Ag NPs)

3.4.1.1   Scanning Electron Microscopy (SEM) analysis of Ag NPs

SEM analysis showed that the Ag NPs formed small, loosely packed aggregates of no more than 100 nm in size (refer to Figure 3).

3.4.1.2   Energy-Dispersive X-ray (EDX) analysis of Ag NPs

EDX analysis showed elemental Ag composition of Ag NPs (refer to Figure 3).
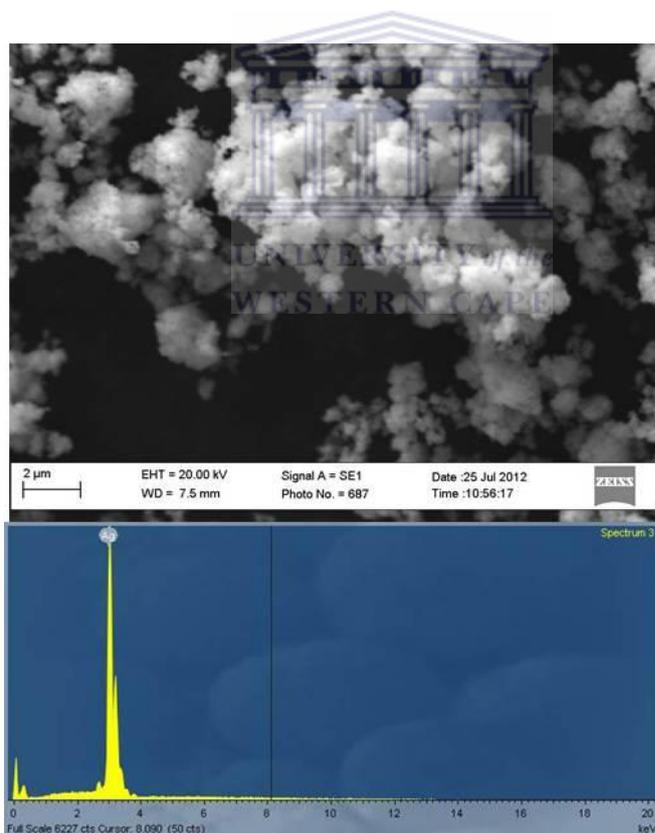
Figure 3: SEM image of Ag NPs showing spherical particles in the order of 100 nm (top) and its corresponding EDX spectrum showing elemental Ag composition (bottom)

3.4.1.3   Transmission Electron Microscopy (TEM) analysis of Ag NPs

3.4.1.3.1 TEM analysis of dry Ag NPs powder

Like SEM analysis, TEM analysis showed that the Ag NPs formed small, loosely packed aggregates of no more than 100 nm in size. Figure 4 can be consulted for the primary and aggregate size of Ag NPs in the dry state. The spherical nature of the Ag NPs was verified by TEM images. Particle size distribution revealed that the sample was mostly made up of smaller particles of the 10 nm range, with the second largest constituent of particles being located in the 20 nm range and then the rest of the sample being made up of larger particles in the 50-100 nm range.

3.4.1.3.2 TEM analysis of Ag NPs suspended in aqueous medium

Morphology and particle size distribution of Ag NPs were determined for Ag NP suspension under ambient conditions (average temperature = 14.6 °C). TEM analysis revealed the continuous aggregation of Ag NPs in suspension (refer to Figure 5). The particle size distribution also revealed that Ag NPs increased rapidly in size when suspended in an aqueous medium. Most of the Ag NPs were found in the 20-40 nm range in the suspended sample compared to the dry sample, which had most of the particles located in the 10-20 nm range (refer to Figure 5 & Figure 4, respectfully). The suspended samples also contained a smaller quantity of Ag NPs outside of the nm range (0,2-1 µm), effectively, no longer making them nanoparticles (refer to Figure 5).
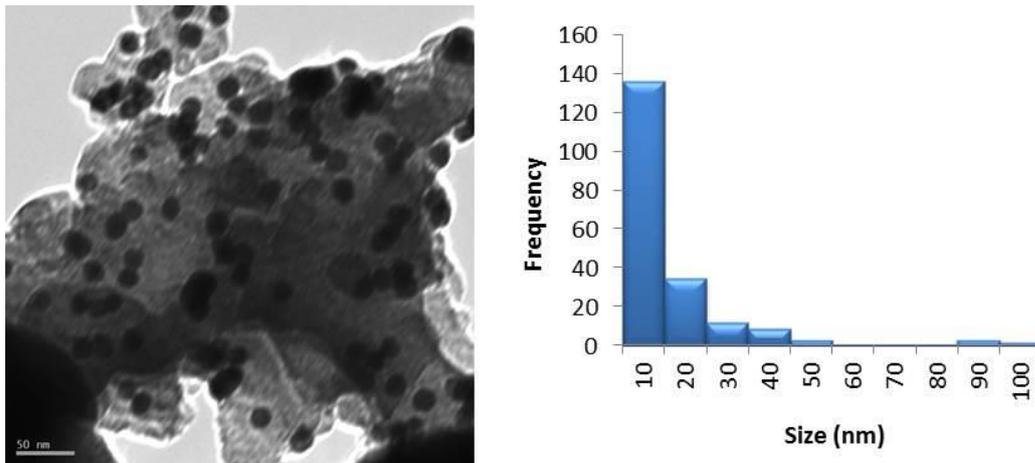
Figure 4: TEM image of Ag NPs taken at 30 000 magnitude (left). Particle size distribution of Ag NPs confirming that NPs were < 100 nm (right)
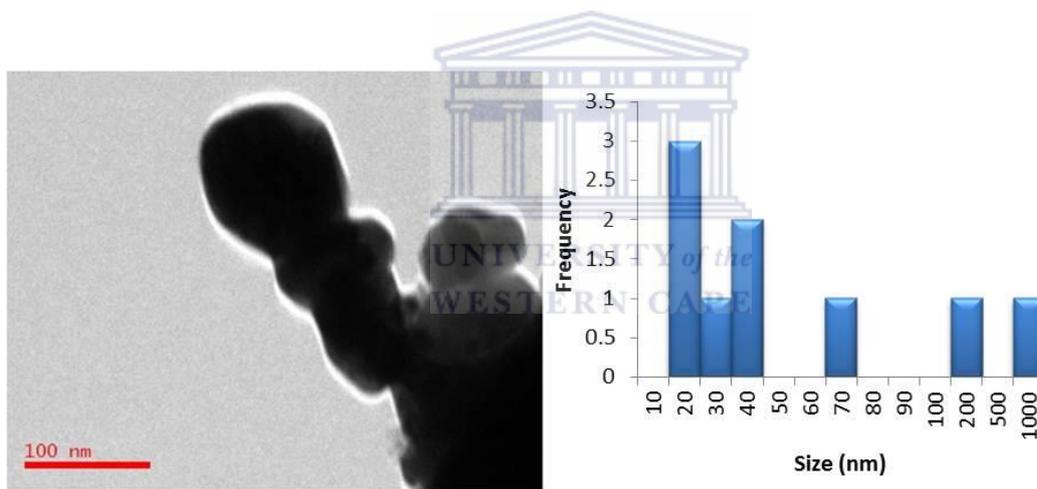

Figure 5: TEM image of Ag NPs in aqueous suspension taken at 20 000 magnification and associated histogram of particle distribution

3.4.1.4 Brunauer, Emmet and Teller (BET) analysis of Ag NPs

BET analysis revealed the surface area of the Ag NPs to be 7.5329 $m^2/g \pm 0.0028$ (refer to Figure 6).
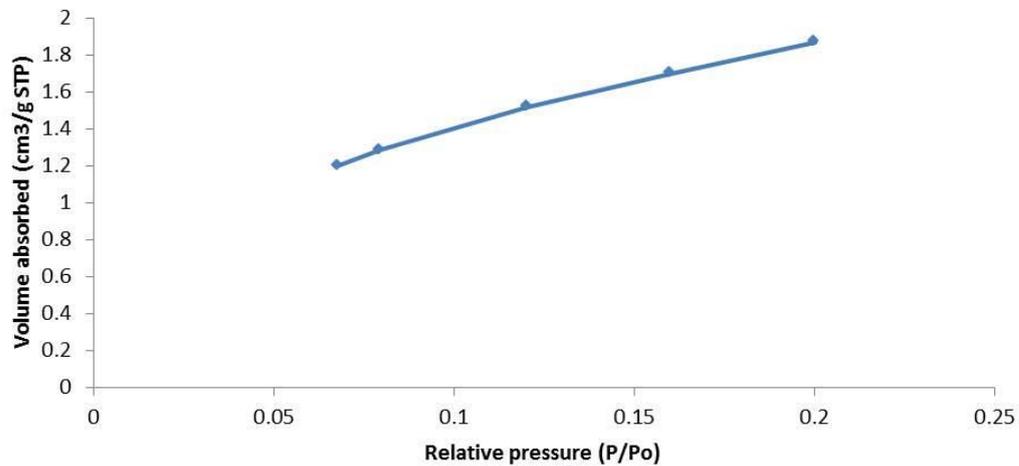

Figure 6: BET result

3.4.1.5 Powder X-Ray Diffraction (PXRD) analysis of Ag NPs

The PXRD pattern recorded for Ag NPs is shown in Figure 7, and demonstrated the crystalline nature of the Ag NPs.
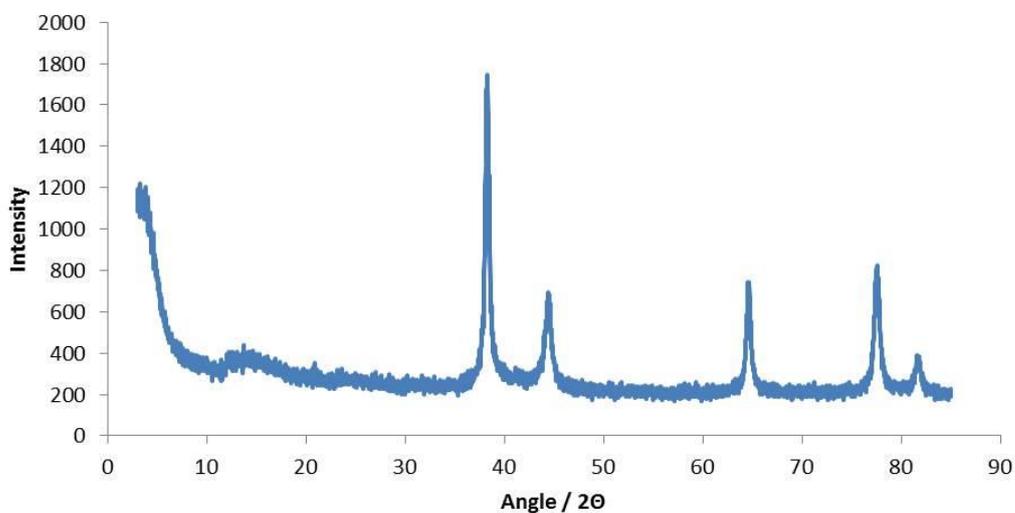
Figure 7: PXRD pattern of Ag NPs

3.4.1.6 Ultraviolet- visible (UV-vis) Spectroscopy of Ag NPs

The UV−vis absorption spectrum of the Ag NPs obtained is shown in Figure 8, with absorbance spectra at λ max = 422 nm.
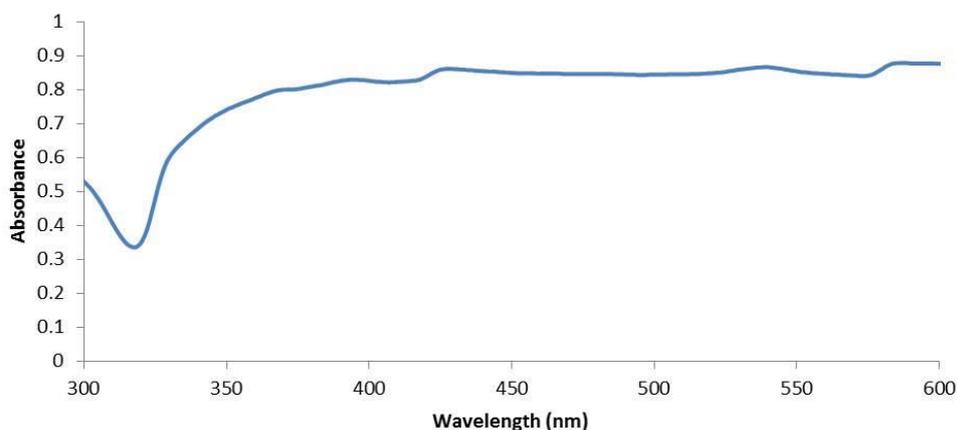
Figure 8: UV-vis spectrum of Ag NPs dissolved in deionized water

Test	Manufacturer's specifications	In-house characteristic
SEM	<100 nm	Loosely packed aggregates, <100 nm
EDX	99.5 % pure	99.5 % pure
TEM of dry powder	No data	Mainly < 20 nm (87.5 %), 30-50 nm (10.7 %), >50-100 nm (1.8 %)
TEM of aqueous med.	No data	Mainly 20 - 40 nm (66.7 %), >40-200 nm (11.1 %), >200-1000 nm (22.2 %)
BET	5 m^2/g	7.5329 m^2/g
PXRD	No data	Crystalline
UV-vis	No data	λ max = 422 nm.

Table 5: Summary of characteristics of Ag NPs used in this study

3.4.2 Effects of Ag NPs on testosterone production

Ag NPs significantly decreased ($P < 0.001$) LH-induced testosterone secretion at a concentration of 20 µg/ml as compared to the control (Figure 9). At 20 µg/ml, Ag NPs caused > 50% reduction in testosterone secretion in LH-stimulated cells as compared to the control. Cells incubated in the absence of LH secreted low levels of testosterone and showed no significant effect as compared to the control.
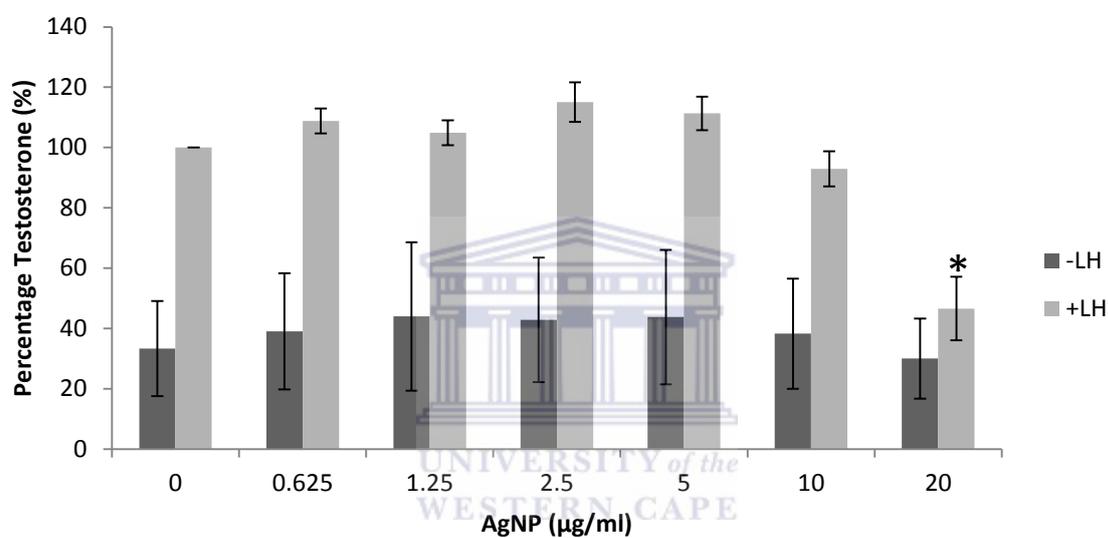

Figure 9: Effect of silver nanoparticles on testosterone secretion (with/without LH-treatment). Cells were treated for 4 hours and supernatants were screened thereafter for testosterone. (* indicates $P < 0.001$ relative to the rest of the nanoparticle-treated samples; error bars indicate Standard Error of the Mean n=9)

3.5   Discussion

Characterisation of the Ag NPs revealed that it indeed was spherical nanoparticles, which when in suspension is mostly found to be in the 20-40 nm range (refer to Figure 5). Analysis via EDX confirmed that the nanoparticles contained silver (refer to Figure 3).

Testosterone synthesis occurs under LH stimulation of Leydig cells during steroidogenesis. LH initiates this complex, multienzyme process when it binds to the membrane-bound LH receptors, stimulating the production of cAMP (Hales, 2002). Steroidogenic Acute Regulatory Protein (StAR) transfers cholesterol from the outer mitochondrial membrane into the inner mitochondrial membrane, a process which is cAMP-dependent (Stocco et al., 2000). This is the rate limiting step in steroidogenesis (Crivello & Jefcoate, 1980; Mori & Marsh, 1982; Privalle et al., 1983). Cholesterol is converted to pregnenolone via cytochrome P450 side chain cleavage enzyme (P450scc) (Stocco et al., 2000; Payne and Hales, 2004). After the dispersion of pregnenolone to the smooth endoplasmic reticulum, it is converted to progesterone by 3β-hydroxysteroid dehydrogenase-Δ^4-Δ^5 isomerase (3β-HSD) (Hales, 2002). Cytochrome P450 17α- hydroxylase/C17-20 lyase (P450c17) converts pregnenolone to 17α-hydroxypregnenolone and then to dehydroepiandrosterone (Chung et al., 1987). 17ß-hydroxysteroid dehydrogenase (17ß- HSD) then converts androstenedione to testosterone (Hales, 2002).
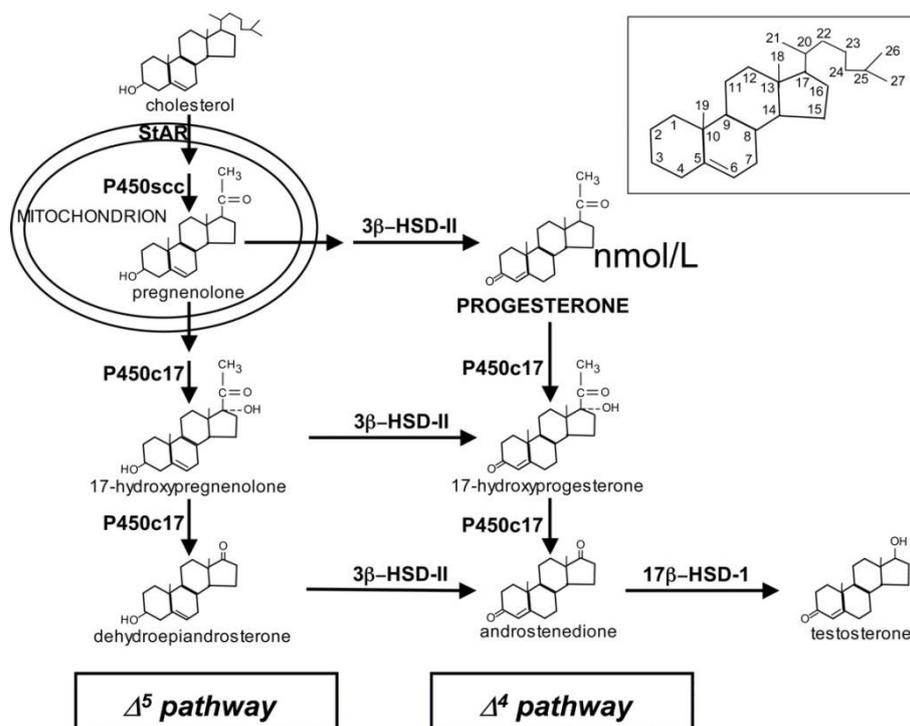
Figure 10: Summary of the steroidogenic pathway within the Leydig cells upon stimulus by LH (Bulun et al., 2005; Flück et al., 2003).

Our results indicated that the statistical significant (p<0.001) decrease in testosterone production only occurred at the highest concentration (20 µg/ml) of Ag NPs. The results obtained from the XTT and WST-1 assays were inconclusive (results not shown). The Ag NPs reacted with the XTT and WST-1 substrates and resulted in very high background readings. Our results do not suggest a mechanism of action, but there are several ways in which silver nanoparticles could be affecting the production of testosterone. Ag NP-induced decrease in testosterone production could be due to an inhibition of the steroidogenesis pathway, either by inhibiting StAR expression and/or action (cholesterol transport), cAMP, protein kinase A, or the activity of steroidogenic enzymes. Steroidogenesis is a complex, multienzyme process that can be disrupted at various points by various mechanisms. It only takes one of these points to be disrupted for testosterone production to be significantly decreased.

Steroidogenesis has been shown to be inhibited by inflammatory mediators, viz. cytokines and reactive oxygen species (ROS) (Hales, 2002) (refer to Figure 11). Hales et al. (1999) states that under normal physiological conditions cytokines and other inflammatory mediators play a positive role in the development and differentiation of Leydig cells. However, overexpression of pro-inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF–α), produced by activated macrophages has been shown to significantly inhibit Leydig cells and seem to act as transcriptional repressors of steroidogenic enzyme gene expression (Hong et al., 2004; Mauduit et al., 1998; Lin et al., 1991). ROS has even been associated with male infertility (Aitken & Krausz, 2001). ROS is continuously produced within the cell as a common by-product of metabolic processes, such as mitochondrial and microsomal electron transport reactions (Hales, 2002). Cells are usually protected from the harmful effects of these ROS by the cellular antioxidant systems, which includes, inter alia, superoxide dismutase (SOD), catalase and glutathione peroxidases (Finkel, 2003). Failure of the cellular oxidative systems to counteract the production of ROS, leads to an imbalance, known as oxidative stress (Finkel & Hollbrook, 2000). Oxidative stress is what has been cited to be detrimental to reproductive function (Ahotupa & Huhtaniemi, 1992).

Diemer et al. (2003) found that ROS causes inhibition of StAR protein expression and therefore prevented the transport of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane. As stated earlier this is the rate-limiting step in steroidogenesis (Crivello & Jefcoate, 1980; Mori & Marsh, 1982; Privalle et al., 1983). MA-10 tumor Leydig cells were subjected to varying concentrations of ROS (hydrogen peroxide & xanthine oxidase) (Diemer et al., 2003). It was found that ROS inhibited StAR protein expression and cAMP-mediated progesterone production in a dose-dependent manner (Diemer et al., 2003). ROS was found not to induce cell death and it had no effect on P450

side-chain cleavage enzyme protein levels (Diemer et al., 2003). The study found that ROS caused mitochondrial depolarisation and degeneracy of the mitochondrial membrane potential, which inhibited StAR protein and thus Steroidogenesis (Diemer et al., 2003). Murugesan et al. (2007) also found that ROS inhibited StAR protein expression.

AshaRani et al. (2009) assessed the toxicity of starch-coated Ag NPs using two human cell lines. Ag NPs induced mitochondrial damage, oxidative stress (radical oxygen species), and DNA damage in a dose dependent manner (AshaRani et al., 2009). The authors proposed the following mechanism of toxicity for Ag NPs: disruption of the mitochondrial respiratory chain, leading to production of radical oxygen species and interruption of ATP synthesis, which in turn cause DNA damage (AshaRani et al., 2009). Several other studies has also shown that Ag NPs induce reactive oxygen species (ROS) formation upon entry into the cell (Piao et al., 2011; Kim et al., 2009; Carlson et al., 2008).

Carlson et al. (2008) investigated the size-dependent cellular interactions of Ag NPs on alveolar macrophages. Ag NPs of 15 nm, 30 nm, and 55 nm were used to determine if ROS generation was size dependent. It was found that the 15 nm Ag NPs were the most toxic of the three Ag NPs tested (Carlson et al., 2008). Carlson et al. (2008) suggested that the 15 nm Ag NPs induced cytotoxicity of the cells via oxidative stress, as it caused a 10-fold increase in ROS levels in cells exposed to 50 µg/ml. The 15 nm Ag NPs also decreased cell viability, decreased mitochondrial membrane potential and completely depleted glutathione levels (GSH) (Carlson et al., 2008). It is interesting to note that the majority of the Ag NPs in our sample are located in the 10-20 nm range, which makes it possible that there could be a number of 15 nm sized Ag NPs within our sample. Carlson et al. (2008) also found that the Ag NPs activated the macrophages to release pro-inflammatory cytokines, such as tumor necrosis factor (TNF–α) and macrophage inhibitory protein (MIP–2).
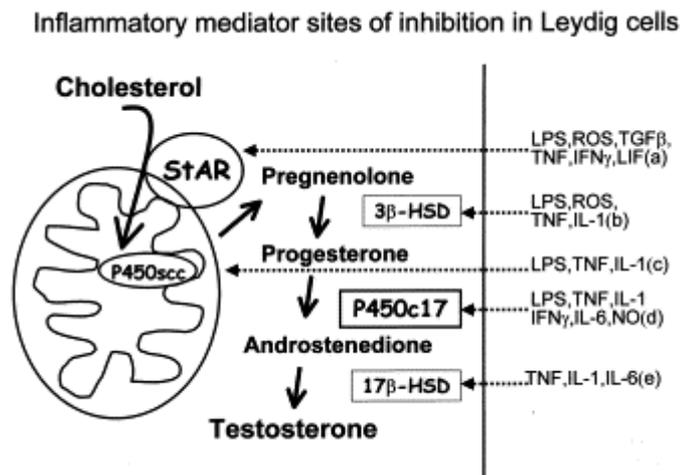
Figure 11: Inflammatory mediator sites of inhibition of Leydig cells (Hales, 2002). Refer to Hales (2002) for a detailed review on the inflammatory mediators that inhibit Leydig cell steroidogenesis.

Ag NPs in our study have been found to decrease testosterone. Low testosterone has not only been associated with negative effects on reproductive health, but has also been implemented in several diseases, such as Alzheimer's disease, anaemia, depressive illness, and type 2 diabetes (Ding et al., 2006; Ferrucci et al., 2006; Shores et al., 2004; Moffat et al., 2002). From the data obtained in this study, namely, inhibition of testosterone synthesis by Ag NPs, it can be concluded that Ag NPs may have several adverse effects on exposed males.

Apart from the detrimental effects on steroidogenesis, oxidative stress has also been linked to oxidative damage of spermatozoa, resulting in loss of sperm function (Aitken & Krausz, 2001). The high concentration of polyunsaturated fatty acids within the plasma membrane of human spermatozoa is what makes them so susceptible to oxidative stress (Jones et al., 1979). There is irrefutable evidence implicating oxidative stress with DNA damage in spermatozoa and male germ line, and this may be connected with pathology in the offspring (for a review see: Aitken & Krausz , 2001).

As with regards to the rest of the bodily functions and processes, steroidogenesis is also highly regulated and dependent on homeostasis being present. The right degree of expression of both cytokines and ROS seems to help regulate Leydig cell steroidogenesis. However, under pathological conditions resulting in the overexpression of both cytokines and ROS, there appears to be a disturbance of this homeostasis leading to an inhibition of steroidogenesis. Ag NPs have been shown to induce the overexpression of both cytokines (pro-inflammatory mediators) and ROS, leading to oxidative stress and as evidenced by this study, a decrease in Leydig cell testosterone production.

3.6   Conclusion

Male reproductive ability is reliant on the ability of the testes to manufacture sufficient levels of androgens (testosterone) and high volumes of viable sperm. The results from this study demonstrated that Ag NPs conspires to mitigate this capacity, as evidenced by the decrease in testosterone production at 20 µg/ml of Ag NPs. Our study was unable to impart a mechanism of action. However, literature has strongly suggested that oxidative stress, through ROS and cytokine overexpression, could be the reason for the negative effects on steroidogenesis. It would be interesting to see this hypothesis being tested in future studies.

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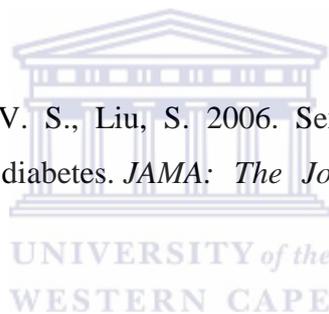
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Chapter 4: Further recommendations

This study is a necessary starting point for assessing the effects of Ag NPs on steroidogenesis. Therefore, it is only natural that further research questions would emerge from this study, if the results merit additional investigation. As the results showed clear evidence of Ag NPs interfering with steroidogenesis (decreasing testosterone synthesis), it is self-evident that further research is needed. There are two key issues that have to be investigated further, including the real mechanism by which Ag NPs inhibit testosterone production and the effect of serum proteins on this Ag NP-driven inhibition. However, for the successful investigation of these two issues, it seems critical that toxicological kits that are designed to be used in the investigation of nanoparticle toxicity needs to be developed.

4.1   The mechanism of action

It would be of great significance to determine the nature by which these Ag NPs exert their effects. Our study has deduced that the mechanism of Ag NPs toxicity could be driven by oxidative stress, causing ROS formation and cytokine expression. These products have been shown to affect several enzymes in the steroidogenesis pathway including StAR protein (Diemer et al., 2003). Since this is all speculation, empirical evidence still needs to be provided.

The US Environmental Protection Agency has recommended the sectioned or minced testes assay as the preferred method for screening the effect of substances on steroid hormone production and secretion (EPA, 2005). This assay has several advantages of which one is the ability to assess multiple endpoints. Multiple endpoints, such as testing progesterone, testosterone and estradiol could give better inside into the mechanism of action of Ag NPs

than just measuring testosterone alone. This might be easier to visualise with the aid of a diagram.
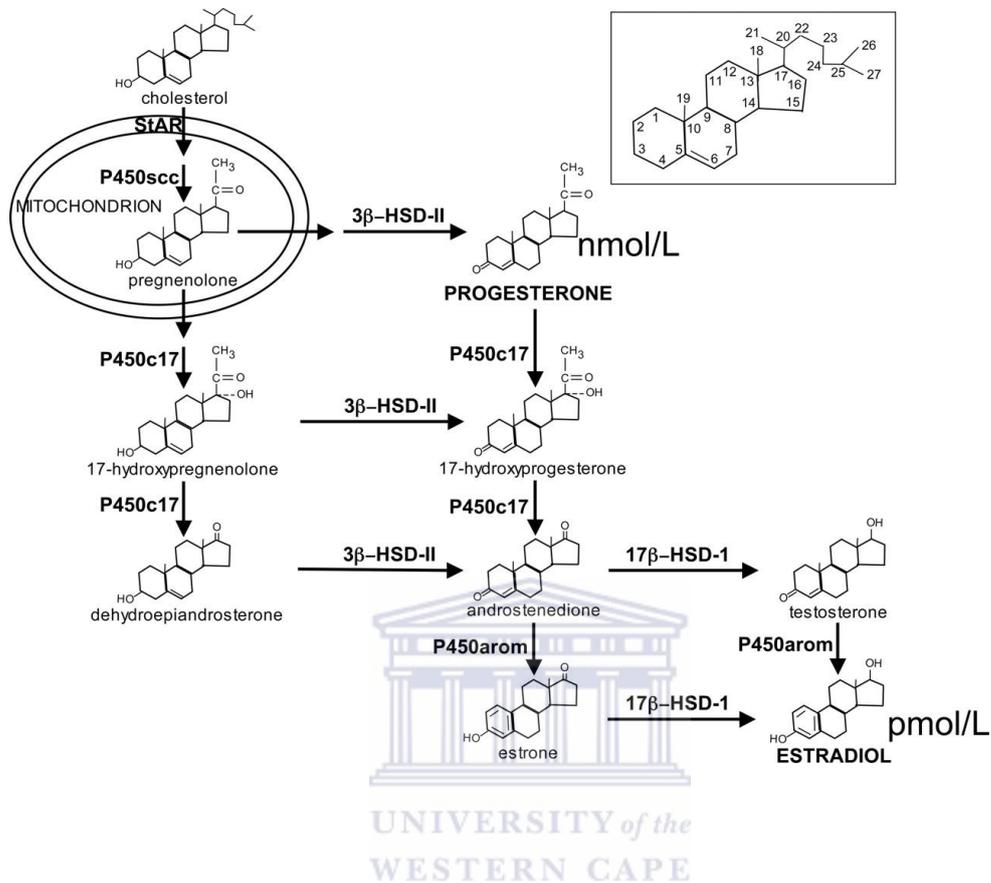

Figure 12: Steroidogenic pathway with multiple endpoints (Bulun et al., 2005)

Assessing the effects of Ag NPs on progesterone, testosterone, and estradiol would impart information on the particular step in the steroidogenesis pathway the Ag NPs act on. If progesterone levels are normal, but the testosterone levels are decreased, then perhaps the Ag NPs inhibit the effect of cytochrome P450 17α- hydroxylase/C17-20 lyase (P450c17) or 17ß-hydroxysteroid dehydrogenase (17ß- HSD). However, if it were detected that the estradiol levels are increased, then it would be assumed that the Ag NPs increases the activity of aromatase cytochrome P450 (P450arom). Therefore, the method of assessing multiple endpoints would give far greater insight into the mechanism of action or at least the sight of action, then just assessing one endpoint would.

4.2   Effect of serum proteins on nanomaterial toxicity

For the purposes of medical applications the nanomaterials are introduced into the host organism via parenteral administration. The body reacts against the NPs as foreign objects. There are many factors that dictate the kind of response the body mounts. The nanomaterials are immediately covered by proteins when they are introduced to a biological matrix, resulting in what is termed a protein "corona" (Cedervall et al., 2007; Lynch & Dawson, 2008). Aggarwal et al. (2009), states that protein coronas are complex and variable.

Opsonins, a component of the nanoparticle corona, are believed to increase the uptake of the coated nanoparticle by the cells of the reticulo-endothelial system (RES) (Patel, 1992; Chonn et al., 1992). This effect is due to the "molecular signature" created by the opsonins being present on the nanoparticle surface which plays a major role in recognition by the immune cells and internalisation route (Kiwada et al., 1987; Tyrrell et al., 1977). The route by which the nanoparticle is internalised has an effect on the rate of clearance of the nanoparticle and its contents from the bloodstream, its volume of distribution, organ deposition and rate and route of clearance from the body (Göppert & Müller, 2005a). For larger particles protein binding is already established as one of the most important factors influencing biodistribution (Dutta et al., 2007; Gessner et al., 2002; Göppert & Müller, 2005b)

Surface area seems to be one of the major determinants of the biological response to nanoparticles (Brown et al., 2001; Tran et al., 2000). As already mentioned before, nanoparticles have a larger surface-to-volume ratio than larger particles, which means more proteins will bind to the smaller nanoparticles (in relation to its mass) than to the larger particles (Aggarwal et al., 2009). Musteata et al. (2006) states that not only is protein binding of paramount importance to understanding the pharmacokinetics and pharmacodynamics of drugs in the body but it is also used to develop models to determine drug efficacy and

toxicity. As mentioned earlier particle properties such as shape, size, solubility, surface modifications (including targeting) and route of administration all influence the way nanoparticles are distributed throughout the body. Aggarwal et al. (2009) states that the missing link is in understanding the effect of these factors on the binding of serum proteins to the nanoparticles and how this in turn affects biodistribution, biocompatibility and therapeutic efficacy of nanoparticles. Although the mechanism of serum protein binding is not well known, it is known that the amount and type of proteins attached to the surface of the nanoparticle has an effect on the biodistribution of the nanoparticle (Aggarwal et al., 2009).

Nanoparticles are said to interact with approximately 50 proteins contained in the plasma of human blood (Aggarwal et al., 2009), as evidenced by numerous studies (Dobrovolskaia et al., 2009; Göppert & Müller, 2005c; Kim et al., 2007). Aggarwal et al. (2009) lists numerous nanoparticles and the types of serum proteins that have affinity to them (Table 1 in Aggarwal et al. 2009). Albumin, fibrinogen, IgG and IgM seem to be the most commonly found proteins on nanoparticles; appearing on the surfaces of several nanoparticles including liposomes, single- and double-walled carbon nanotubes, iron oxide nanoparticles, and Poly(D,l-lactic acid) nanoparticles, etc. Different opsonins have specific effects on the fate of the nanoparticle it is bound to. It is said that the binding of albumin to a nanoparticle increases its circulation time within the blood, while the binding of fibrinogen, IgG and complement are believed to promote phagocytosis and the subsequent elimination of the nanoparticles from the blood through the RES (Göppert & Müller, 2005c; Ogawara et al., 2004; Camner et al., 2002). Owens & Peppas (2006) and Roser et al. (1998) found that surface charge influences protein binding since neutral particles have a slower rate of opsonisation than their charged counterparts. Hydrophobic nanoparticles have a faster rate of opsonisation than hydrophilic nanoparticles (Owens & Peppas, 2006). Hydrophobicity is also said to impact on the degree of opsonisation as well as the type of proteins that bind to

the nanoparticles (Cedervall et al., 2007; Göppert & Müller, 2005a; Göppert & Müller, 2005b). Owens & Peppas (2006) found that several factors, including shape, size, morphology and surface curvature all influence the degree of opsonisation, but not the type of bound proteins.

It would be remarkable to see what effect the serum proteins would have on the seeming anti-androgenic effect of Ag NPs.

4.3   Development of cytotoxicity assays that are specific for nanoparticles

Kroll et al. (2009) made a review on some of the nanoparticles that interfere with different cytotoxic assays. Single-walled carbon nanotubes (SWCNTs) were found to form crystals with the MTT assay (Wörle-Knirsch et al., 2006). In another study, SWCNTs were found to increase WST-1 absorbance when the concentration of SWCNTs was increased (Casey et al., 2007). Our study encountered problems, when it was found that the WST-1 and XTT assays were not working when used with Ag NPs. Both of these assays require absorbance assessment at around 500 nm. Many nanoparticles absorb at this wavelength and could account for the problem we experienced (Kong et al., 2011).

To combat the possibility of interference by the nanoparticles, it is suggested that at least two or more independent cytotoxicity assays be performed (Kong et al., 2011; Wörle-Knirsch et al., 2006). It would also be advantageous, if cytotoxicity assays that are specific for nanoparticles could be developed.

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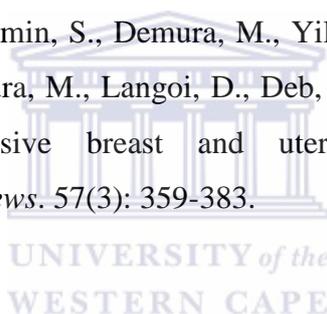
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Appendix 1:

TURNITIN REPORT

MSc Thesis submission *By Ashley Muller*

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- ID: 404544065
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