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The effects of oxidative stress and age on human spermatozoa

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THE EFFECTS OF OXIDATIVE STRESS AND AGE ON HUMAN SPERMATOZOA

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ABSTRACT

Male infertility is now recognised as a significant factor in couples having difficulty conceiving. The impact of maternal age has long been known as a limiting factor, however recent research indicates that advancing paternal age can also negatively impact on a couple's chances of conception. One of the major contributing causes of male infertility has now been linked to spermatozoa exposure to reactive oxygen species (ROS). Such exposure induces oxidative stress when coupled with reduced total antioxidant capacity (TAC). Measures of both ROS and TAC are used as tests of oxidative stress status (OSS) which are used together to give further insight into male fertility status. Current research has revealed that the level of DNA fragmentation increases with age and, due to their composition, the sperm plasma membrane, DNA double helix and single stranded DNA are highly susceptible to ROS attack. ROS cause high levels of lipid peroxidation (LPO) that ultimately damages the plasma membrane and interferes with its vital functions. ROS also readily attack the purine and pyrimidine bases of DNA resulting in DNA damage. It is hypothesized that advancing paternal age will impact negatively of levels of ROS, TAC and DNA fragmentation in ageing men. The aim of this study was to determine the relationship between ROS, TAC and DNA damage on spermatozoon viability in relation to age.

Ejaculated semen samples from 54 men undergoing infertility assessment were collected and divided into men aged ≥40 years (n=16) and men aged ≤39 years (n=38). Samples were examined for their level of ROS and TAC as a possible indication of oxidative stress status. Samples were also assessed for DNA fragmentation and damage using TUNEL.

Statistical analysis consisted of independent t-tests, chi-square tests and multivariate logistic regression. Analysis found no significant associations between ROS, TAC, TUNEL and age. Significant differences were observed between abnormal sperm motility and age ($p<0.05$; t-test and

regression analysis) and between abnormal sperm concentration and TUNEL ($p<0.05$; chi-square test and regression analysis).

The results obtained in this present study did not support the hypothesis that there is an association between age, ROS, TAC and TUNEL. Research has identified a negative correlation between the effects of ROS and reduced TAC and advancing age on male fertility. ROS, either alone or in combination with reduced TAC, has also been directly associated with an increase in DNA damage. An increase in the incidence of certain childhood cancers and disease has been linked to spermatozoon DNA damage. Future research should be directed toward exploring the influences of these factors in relation to advancing paternal age given the potential benefits extend beyond the couple and to any future offspring.

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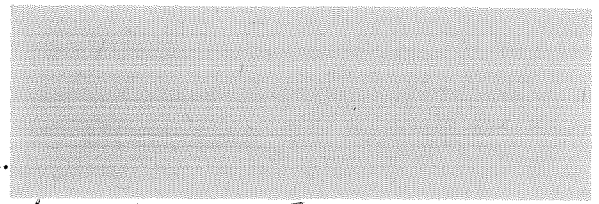
Signed. 

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ABBREVIATIONS

ADP	Adenosine diphosphate
ART	Artificial reproductive technology
ATP	Adenosine triphosphate
DHA	Docosahexaenoic acid
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
H_2O_2	Hydrogen peroxide
ICSI	Intracytoplasmic sperm injection
IVF	In Vitro fertilization
LPO	Lipid peroxidation
mL	Millilitre
mM	Millimole
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
$O_2^{\bullet-}$	Superoxide anion
OAT	Oligoasthenoteratozoospermic
$OH^{\bullet}$	Hydroxyl radicals
OS	Oxidative stress
OSS	Oxidative stress status
PBS	Phosphate buffered saline
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
$ROO^{\bullet}$	Peroxyl radicals
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TdT	Terminal deoxynucleotidyl transferase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
WHO	World Health Organization
ZP3	Zona pellucida 3 glycoprotein

CHAPTER 1:

LITERATURE REVIEW

1.1 INTRODUCTION

One in seven couples has difficulty conceiving during their reproductive lives with male factor infertility implicated in between 20-80% of these cases (Agarwal et al., 2004; Pirrello et al., 2005; Tremellen, 2008). It has been suggested that male subfertility is increasing (Male Infertility, 2005). Environmental, physiological and genetic factors have all been associated with reduced sperm function and unexplained male factor infertility (Sikka, 2001). The accurate identification of possible factors leading to infertility requires vast knowledge of the conditions deemed favourable for normal sperm development and function (Sikka, 2001). At present it is more common for couples to delay parenthood well into their thirties, without considering that their fertility status may be reduced to the point where assistance is required (Lampic et al., 2006; Sloter, Schmid, Marchetti et al., 2006; Tough et al., 2006). While the impact of age on female fertility has long been realized (Schmid, Eskanazi et al., 2007) only now are we starting to investigate male fertility. Advanced paternal age at the time of conception has now been associated with a number of diseases such as schizophrenia, multiple endocrine neoplasia and achondroplasia as well as with decreased semen parameters, chromosomal abnormalities and reduced fertility (Baker & Aitken, 2005; Lambert, Masson & Fisch, 2006; Plastira, Msaouel, Angelopoulou et al., 2007; Schmid et al., 2007).

Aetiologies implicated in male infertility include gene mutations, aneuploidy, infectious diseases, ejaculatory duct occlusion, varicocoele, radiation,

chemotherapy and erectile dysfunction (Ollero et al., 2001; Smith et al., 2006). Whilst many of these conditions are treatable, there remain cases where no immediate identification of the fertility problem can be made. It is well documented that older men take longer to produce pregnancy with an increasing proportion undergoing treatment (Ng et al., 2004). Recent research appears to focus primarily on the effects of reactive oxygen species (ROS), antioxidants, DNA damage and age on the fertilizing capabilities of human spermatozoa (Ebisch et al., 2007; Plastira et al., 2007; Tremellen, 2005).

1.1.1 Spermatozoon Anatomy

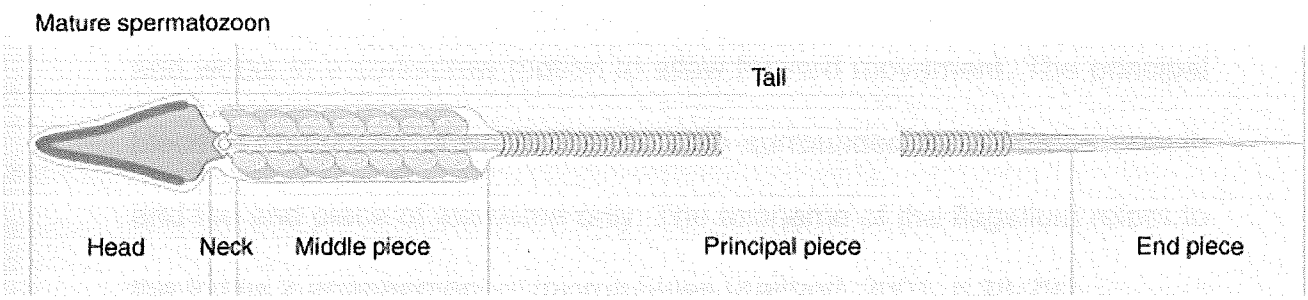
1.1.1.1 The Head

The anatomy of human sperm is presented in Figure 1. The head of the spermatozoon is composed of a tightly condensed and compact nucleus, containing DNA, and an acrosomal cap. The spermatozoon nucleus undergoes considerable reorganisation during the latter stages of spermatogenesis where approximately 85% of nuclear histones are replaced with nuclear protamines (Kalthoff, 2001, p.58; Boe-Hansen et al., 2006). Protamines are proteins containing a high content of positively charged amino acids (Oliva, 2006) and provide multiple functions. These include the generation of a more condensed paternal genome resulting in a more compact and hydrodynamic head, protection of the genome by making it largely inaccessible to nucleases or mutagens present in the surrounding environment and removal of epigenetic information from the paternal genome allowing for reprogramming in the oocyte. Also in some

unknown manner protamines participate in imprinting of the genome during spermatogenesis (Oliva, 2006).

Another stage in spermatogenesis involves the reduction or 'pinching off' of cytoplasm. This results in a mature spermatozoon with a characteristic shape containing little cytoplasm. The cytoplasm is a major reservoir of cellular defense mechanisms and in the immature spermatozoa aids in normal cell maintenance and function. As the majority of the cytoplasm is removed mature spermatozoa are left with few cytoplasmic defense mechanisms (Ebisch et al., 2007).

Figure 1 Spermatozoon Anatomy



(Ref: Young & Heath, 2002)

1.1.1.2 Plasma Membrane

The plasma membrane encases the head and contains a specific combination of phospholipids, cholesterol and proteins to ensure its optimal functioning (Kalthoff, 2001, p58). In normal intact functional plasma membranes the negatively charged membrane phospholipid phosphatidylserine (PS) is located on the inner side of the plasma membrane. At the onset of membrane disturbance PS is translocated to the

outer surface of the plasma membrane (Glander & Schaller, 2000) and is considered a marker of reduced sperm function.

1.1.1.3 The Midpiece

The midpiece of spermatozoa is rich in mitochondria and strategically placed at the base of the tail which utilizes the majority of the ATP generated (Kalthoff, 2001, p.58). Mitochondrial activity in the midpiece provides the ATP required to power the tail (Martini, 2001, p.1025). As a result of this localization the midpiece becomes more fragile and is the major target site for damage by reactive oxygen species (ROS; see section 2).

1.1.1.4 The Tail

The tail (also known as the flagellum) consists of a principal and end piece and works in a corkscrew motion to allow forward movement. The principal piece is composed of an axonemal core surrounded by a fibrous sheath and the end piece of axoneme only. The axoneme of the flagellum refers to the 9 plus 2 arrangement of microtubules (Kalthoff, 2001, p.28-29).

1.1.2 Capacitation

Capacitation refers to a series of structural and functional changes occurring in spermatozoa that begins after the removal of stabilizing factors, one of which is cholesterol, from seminal plasma and ends when spermatozoa are capable of responding to zona pellucida ligands on the oocyte by undergoing the acrosome reaction (De Jonge, 2005). Cholesterol within seminal plasma appears to impose a regulatory function on

capacitation and be a major seminal plasma inhibitor. Investigations have revealed that, on transit through the cervical mucous, the sperm plasma membrane undergoes further remodeling and loses both cholesterol and glycerol-phospholipids (De Jonge, 2005). Hyperactivation seems to occur immediately prior to the acrosome reaction and describes an apparent change in characteristic sperm motility. There is clear evidence that several critical components need to be present for initiation and optimization of capacitation (De Jonge, 2005).

1.1.3 Acrosome Reaction

Spermatozoa undergo the acrosome reaction in response to the zona pellucida, the oocytes extracellular matrix (Herrero, Lamirande & Gagnon, 2003). Capacitated sperm bind, at their head and equatorial segments (Kalthoff, 2001, p.84), by way of a sperm membrane binding site to the ZP3 glycoprotein receptor on the zona pellucida. This association triggers an influx of calcium which amongst other functions causes the completion of meiosis II in the oocyte and activates enzymes to accelerate protein synthesis (Martini, 2001, p.1067). Once bound, acrosomal vesicles are exocytosed and digest portions of the cumulus, corona radiata and zona pellucida. At this point reorganization of the plasma membrane allows the fusion of the sperm and oocyte plasma membranes and aids in the preclusion of polyspermy (Kalthoff, 2001, p. 85-93).

1.2 REACTIVE OXYGEN SPECIES (ROS)

Reactive oxygen species or oxygen free radicals are highly reactive compounds that contain one or more unpaired electrons. Such compounds include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), peroxyl radicals ($ROO^{\bullet}$) and hydroxyl radicals ($OH^{\bullet}$) (Sikka, 2001; Tremellen, 2008; Sanocka & Kurpisz, 2004). Tremellen (2008) points out that leukocytes and sperm are the two primary sources of free radicals within semen.

Male germ cells at various stages produce ROS and their production is essential at specifically controlled concentrations for normal sperm function (Sikka, 2001). Studies have found that there is a negative association between ROS concentration and morphology, motility, seminal plasma and ejaculate volume, sperm vitality, membrane integrity, DNA fragmentation, total antioxidant concentration (TAC) and fertilization rate (Ebisch et al., Gil-Guzman et al., 2001; 2007; Hammadeh et al., 2006; Lopes et al., 1998).

Immature spermatozoa show excessive ROS production, which is directly associated with DNA damage in mature spermatozoa (Hammadeh et al., 2006; Moustafa et al., 2004). Both superoxide and the hydroxyl radical ($OH^{\bullet}$), being the most toxic ROS (Sikka, 2001), are mutagenic. ROS production is highest in immature spermatocytes and lowest in mature spermatozoa and immature germ cells (Gil-Guzman et al., 2001). Leukocyte production of ROS in seminal plasma is also of importance as they have been reported to produce anywhere from 100- to 1000- fold

higher amounts than that of spermatozoa even at capacitation (Moustafa et al., 2004; Tremellen, 2008)

It is becoming increasingly apparent that spermatozoa ROS production is significant in the aetiology of infertility. Both ROS produced by leukocytes and spermatozoa impose a negative effect on sperm DNA integrity however, it is the ROS produced by spermatozoa that is the most significant (Tremellen, 2008). This is believed to be a result of the close proximity between the ROS production and sperm DNA. Free radicals attack the purine and pyrimidine bases and deoxyribose backbone of the DNA double helix (Tremellen, 2008). The primary mode of action of ROS is believed to be a rapid loss in intracellular ATP resulting in axonemal damage and reduced sperm motility. Hydrogen peroxide (H_2O_2) is the primary reactive oxygen species (ROS) to interact with mitochondrial ATP generation (Sikka, 2001). The inhibition of sperm mitochondrial function in semen samples from infertile patients containing high superoxide levels has been observed (Sikka, 2001). Interference with plasma membrane function via peroxidation of unsaturated fatty acids is believed to be another mode of action. Due to the pivotal role of the plasma membrane this interference renders the sperm dysfunctional (Donnelly et al., 1999; Ebisch et al., 2007; Sikka, 2001; Tavilani et al., 2008).

ROS are involved in the processes of sperm capacitation, hyperactivation, acrosome reaction (Lopes et al., 1998) and fertilization (Agarwal et al., 2004; Gil-Guzman et al., 2001). Hydrogen peroxide stimulates the

acrosome reaction and hyperactivation of sperm and is also important in low levels for sperm-oocyte fusion (Tremellen, 2008). Further to this, abnormally high ROS production interferes with sperm-zona pellucida binding, sperm-oocyte fusion (Appasamy et al., 2007; Tremellen, 2008) and the capacity to regulate intracellular calcium levels (Barroso et al., 2000).

1.2.1 Lipid Peroxidation

The plasma membrane is rich in polyunsaturated fatty acids (PUFA) and therefore susceptible to ROS attack resulting in lipid peroxidation (LPO) (Ebisch et al., 2007; Sanocka & Kurpisz, 2004; Sikka, 2001; Tremellen, 2008). Docosahexaenoic acid (DHA) is a major PUFA in spermatozoa and accounts for as much as 30% of phospholipid bound fatty acid and 73% of PUFA (Ollero et al., 2001; Sanocha & Kurpisz, 2004). DHA aids in maintaining membrane fluidity and is the main substrate for LPO. The amounts of DHA and cholesterol are reduced during spermatogenesis which may be an important modification in reducing the susceptibility of mature spermatozoa to oxidative damage (Ollero et al., 2001). The significance of this appears to be that the optimal level of DHA is retained so as to both optimize membrane fluidity and protect against oxidative damage (Ollero et al., 2001; Sanocka & Kurpisz, 2004).

The plasma membrane increases its permeability to electrolytes as a result of reduced membrane integrity from LPO. Essential reactions involved in LPO are catalysed by the presence of iron and copper ions. Membrane damage appears to be the most evident consequence of free radical

damage on sperm viability (Donnelly et al., 1999). The two most common pathways of LPO are non-enzymatic and enzymatic. Enzymatic LPO is both NADPH and ADP dependent (Sikka, 2001).

Lipid peroxidation of PUFA following exposure to oxidative stress (OS) results in a notable decline in sperm motility (Hammadeh et al., 2006). Vitamin E terminates lipid peroxidation chain reactions and vitamin C acts to also prevent oxidative damage to sperm DNA (Ford & Whittington, 1998).

1.3 ANTIOXIDANTS

The majority of vital antioxidant defense systems are present in sperm, however their effects are limited due to their minimal concentration. As sperm have been stripped of vigorous protective mechanisms against oxidative agents, spermatozoa are particularly vulnerable to peroxidative damage (Smith et al., 1996).

Antioxidant defense systems consist of both enzymatic and non-enzymatic components (Tremellen, 2008). Enzymatic antioxidants include superoxide dismutase (SOD), catalase, glutathione peroxidase, and reductase (Agarwal et al., 2004; Sikka, 2001; Tremellen, 2008). SOD and catalase both inactivate the superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2). Washed sperm appear to be more susceptible to toxic oxygen metabolites indicating a protective effect of seminal plasma on sperm integrity. Studies have revealed that semen samples from subfertile men exhibit lower antioxidant capacity and value than that of fertile men, confirming an

inverse relationship between antioxidant capacity and LPO exists (Smith et al., 1996).

There are many components of the non-enzymatic antioxidant defense systems. They include ascorbate (vitamin C), urate, α-tocopherol (vitamin E), pyruvate, albumins, β-carotene, ubiquinol, glutathione, amino acids taurine and hypotaurine, carnitine, carotenoids, flavenoids and prostasomes (Smith et al., 1996; Tremellen, 2008). These are all found in seminal plasma and act as scavengers of free radicals. Vitamin E is a major chain breaking antioxidant found in the sperm plasma membrane and suppresses LPO in a dose dependent manner. Albumin does not prevent LPO, only neutralizes lipid peroxides by being oxidized itself (Twigg et al., 1998; Tremellen, 2008; Aitken & Krausz, 2001). Prostasomes are extracellular organelles that are secreted by the prostate with one of their functions being fusion with leukocytes in the ejaculate to reduce their production of ROS (Tremellen, 2008). Observations also indicate that ROS production is dependent on NADPH. Not only does ROS generation by NADPH encourage LPO but also results in a marked increase in DNA damage. Albumin specifically reacts with peroxyl radicals and aids the slowing of peroxidative damage (Agarwal et al., 2004; Tremellen, 2008). At certain levels SOD enzymes hasten the removal of superoxide limiting their detrimental effects. Several studies have concluded that NADPH may serve as a substrate for SOD production in spermatozoa (Twigg et al., 1998).

Total antioxidant capacity (TAC) is a measure of oxidative stress status (OSS). As antioxidants react with free radicals to neutralize their effect, an excessive amount of ROS production diminishes antioxidant capacity resulting in oxidative stress (OS). Ford & Whittington (1998) note the TAC of seminal plasma is lower in subfertile men as opposed to fertile men. Aitken and Krausz (2001) further this by affirming an inverse correlation between fertility status and TAC exists. Treatment with antioxidant supplementation assists in cases where reduced TAC is the cause however antioxidant therapy appears to work within, at present, undefined limits (Tarin et al., 1998; Donnelly et al., 1999). At lower concentrations they protect against cellular damage and are even necessary for normal function, however at higher concentrations antioxidants add to cellular attack, dysfunction and pathological states (Sikka, 2001).

1.3.1 Oxidative Stress

Oxidative stress (OS) is a condition associated with the overproduction of ROS induced by oxygen and oxygen derived oxidants (Sikka, 2001). It is increased by both a rise in ROS production and a reduction of cellular antioxidant capacity and is a key player in the aetiology of abnormal sperm function through mechanisms involving peroxidative damage to the plasma membrane (Twigg et al., 1998). OS is also known to affect the sperm genome by causing high frequencies of single and double DNA strand breaks (Hammadeh et al., 2006; Twigg et al., 1998).

Sperm are extremely susceptible to OS as a result of their capacity to yield ROS and the high content of polyunsaturated fatty acids in their plasma membrane (Donnelly et al., 1999; Hammadeh et al., 2006; Smith et al., 1996). H_2O_2 is a relatively stable ROS that readily crosses plasma and nuclear membranes. Peroxide derived ROS readily reacts with transition metals such as iron and copper that are abundant in normal biological processes. This results in altered signaling and tissue injury repair processes, consequently inducing cellular damage from the catalysis of the lipid peroxidation cascade (Aitken & Krausz, 2001). Nitric oxide (NO) has been shown to regulate several functions in the male reproductive tract and at differing concentrations has opposing effects on sperm motility. At lower concentrations it is proposed to play a role in sperm hyperactivation but at higher concentrations of NO can inhibit sperm motility (Sikka, 2001; Herrero, De Lamirande & Gagnon, 2003).

1.4 DNA DAMAGE

For the accurate transmission of genetic material it is essential that sperm DNA be of highest quality. In the last stages of spermatogenesis the nucleus is condensed and the distinctive shape of the sperm head is formed. The ability of mature sperm to undergo DNA repair is greatly diminished (Boe-Hansen et al., 2006; Donnelly et al., 1999) as is their ability to undergo programmed cell death. The long term effect of DNA damage is dependent not only on the extent and location of the damage but also on the ability of the oocyte or even early embryo to repair the damage (Boe-Hansen et al., 2006; Ollero et al., 2001; Plastira, 2007).

Immature spermatozoa exhibit increased DNA damage, alterations in protamination, chromatin packaging and excessive ROS production. Nuclear alterations observed in spermatozoa from infertile patients include abnormal chromatin structure, microdeletions, chromosomal rearrangements, aneuploidy and DNA strand breaks (Barroso et al., 2000; Moustafa et al., 2004). Aitken and Krausz (2001) remark that both mutagenic and promutagenic changes in the sperm genome have been extensively associated with advancing paternal age. Sperm function and infertility are closely linked to DNA damage. It is well established that infertile men exhibit increased chromosomal abnormalities (Black et al., 2000), however the source of such damage is the cause of much deliberation. A common supposition is that defective apoptosis may account for a remarkable percentage of spermatozoa with DNA damage. Both superoxide and $OH^{\cdot}$ are known to induce chromosome deletions, dicentrics and sister chromatid exchanges (Donnelly et al., 1999). DNA damage has been well linked to poor pregnancy outcome (Lambert, Masson & Fisch, 2006). Moustafa et al. (2004) and Aitken and Krausz (2001) present several hypotheses to explain the source of DNA damage in sperm. Firstly that it may be the result of improper packaging and ligation during spermiogenesis secondly, as a result of OS and finally it may be a side effect of apoptosis.

1.4.1 Apoptosis

Programmed cell death or apoptosis is essential for tissue remodeling. It is a normal process that occurs within somatic cells as well as amongst different sub-populations of spermatozoa where it plays a role in regulating sperm concentration, release and the elimination of defective spermatozoa (Ebisch et al., 2007; Ford, 2001). Abnormal apoptotic processes have been related to selected cases of infertility such as oligozoospermia and sperm dysfunction. Appasamy et al. (2007) note that OS is a mediator of apoptosis aiding in the removal of defective cells.

The presence of DNA nicks in spermatozoon could be a result of incomplete apoptosis. Expression of *Fas*, a cell surface protein, is higher in infertile men than men with normal semen parameters and believed to be associated with apoptosis (Moustafa et al., 2004). *Fas* expression on the plasma membrane is increased during the early stages of apoptosis. The inefficient removal of such marked cells in the male reproductive tract results in their presence in the ejaculate where they amplify ROS exposure to mature spermatozoa. In turn this damages both mitochondrial and nuclear membranes (Plastira et al., 2007; Schmid, Eskanazi et al., 2007) diminishing DNA protection. New diagnostic methods are now revealing that advancing paternal age is commonly associated with many autosomal dominant disorders such as achondroplasia, Aperts syndrome (Schmid, Eskanazi et al., 2007), Marfan syndrome and osteogenesis imperfecta (Lambert, Masson & Fisch, 2006).

1.5 MALE FERTILITY AND AGE

The functioning of all body systems declines with age and all are interconnected, a decline in one system will impact another. For example, degenerative changes such as a decrease in protein composition and reduced water content in the prostate affects the quality of semen produced (Lambert, Masson & Fisch, 2006). DNA damage is significantly higher in older men as opposed to younger men. The article Male Infertility (2005) reported that DNA damage was considerably higher in males aged over 45 years than all younger age groups, furthermore, it was doubled in over 45s when compared to men younger than 30 years of age.

There have been few studies addressing the fertility of older men while controlling for reduced female fertility. This means the results of such studies should be interpreted with caution as they may be influenced by lowered female fertility rather than be associated with male fertility and advancing age. Testosterone production is known to decline with age (Lambert, Masson & Fisch, 2006), usually in the sixth decade of life (Martini, 2001). This is both a variable and gradual process amongst individuals. While sperm production continues there is a reported decline in libido and sexual activity (Ng et al., 2004). Sloter et al. (2006) notes that, unlike women, there is no apparent age threshold in relation to sperm motility however there still appears to be a clear relationship between advancing paternal age and decreased sperm motility.

Results of the study by Ng et al. (2004) showed that the median semen volume and total sperm output reduced significantly in older non-infertile men (over the age of 50 years). The study also reported that the probability of obtaining normal values for ejaculate volume, sperm density and total sperm output was also significantly reduced in older men when compared with younger men. There was a significant increase in the frequency of abnormal sperm morphology and vitality especially involving sperm tail defects observed in this study, which may be a possible reflection of the complex cellular structural assembly process of the axoneme, resulting in increased opportunity for age-dependent induced damage (Ng et al., 2004).

Much research has been performed examining the role of antioxidants on reproduction, however little has been reported specifically on TAC in relation to age. When looking at ROS and OS a similar picture emerges where, too few studies have incorporated the added variable of age. Sloter et al. (2006) furthered this by stating that contributions from both the environment as well as the individuals age and diet are poorly understood. Clearly there is a need for further examination on such topics and is the basis for this study.

CHAPTER 2:

HYPOTHESIS and AIMS

2.1 HYPOTHESIS

Advancing age is related to increasing oxidative stress and reduced TAC, specifically past the age of 40 years.

2.2 AIMS OF THE STUDY

- The overall aim of the study is to ascertain the relationship between age and human spermatozoon viability.
- The specific aim of the study is to determine the relationship between the effects of ROS, antioxidants and DNA damage on human spermatozoon viability in relation to age.

CHAPTER 3:

MATERIALS and METHODS

3.1 Ethics Approval

Written approval for this study was granted by the King Edward Memorial Hospital Internal Ethics Committee (ECO5-43.1) and by the Edith Cowan University Faculty of Computing, Health and Science Ethics Sub-Committee for the Conduct of Human Research.

3.2 Semen Collection and Processing

Excess semen from samples collected for routine semen analyses or Assisted Reproductive Technology at Concept Fertility Centre, Subiaco, were utilized for this study. In total 54 semen samples were collected and used in the study. Samples were collected through masturbation into sterile specimen containers. Patient consent for the use of excess semen was obtained prior to use (refer to Appendix 1 for Consent Form).

A preliminary semen analysis was conducted by Concept staff in accordance with the World Health Organisation (WHO, 1999) guidelines. Parameters examined include whole semen volume, pH, consistency, appearance, concentration, motility and morphology.

Prior to commencement of TUNEL protocol, 5-20µl of neat sample was smeared on a silanized glass microscope slide (Superfrost Plus, Menzel-Glaser, Braunschweig), air dried and stored until analysis.

Sample processing was performed at the Concept Fertility Centre. Sample processing prior to commencement of the ROS assay involved centrifugation of liquefied semen samples at 300*g* for 10 minutes in 20ml Falcon tubes. Seminal plasma was aliquoted into a separate Falcon tube, later to be transferred to a 2ml microcentrifuge tube and frozen at -80C until later required. The pellet was resuspended in PBS, pH 7.4, at a concentration of $3x10^6$ sperm/ml. Tubes containing seminal plasma were transported on ice to Edith Cowan University, Joondalup. Tubes containing sperm suspension were transported in a paper bag and kept between 20-40°C.

Information was obtained using a semen analysis questionnaire (refer to Appendix 2) at the time of collection regarding cigarette smoking and antioxidant supplementation. Ten of the men reported smoking and 4 used antioxidant supplements. The distribution of these men between the age groups was equal and so they were included in the study.

3.3 ROS assay

The ROS assay was performed according to the procedure used by Smith et al (2006). Briefly, basal levels of ROS were will be measured using a chemiluminescence assay with luminol as the probe. 10µl of 5mM stock luminol in dimethylsulphoxide (DMSO) (refer to Appendix 3 for reagent preparation) was added to 500µl of prepared sperm suspension (refer to section 3.2). A negative control was prepared by the addition of 10µl of luminol to 500µl of PBS. Results were obtained from sample assessment

using a luminometer (FLUOstar OPTIMA) and expressed as counted photons per minute. Samples were processed in such a way as to maintain a similar time frame between centrifugation and luminescence measurement.

3.4 TAC assay

The Total Antioxidant Status Assay Kit, Cat. No. 615700 (Calbiochem) was utilized for performing the TAC assay. An Eppendorf BioPhotometer was set at 600nm absorbance and cuvettes (Eppendorf UVette) with a 1 cm path length used. The sample was thawed for 20 minutes at room temperature. Five minutes prior to use the diluted substrate and chromogen were equilibrated at 37°C. A Thermomixer Comfort was used to maintain temperature throughout the assay. A blank sample was prepared according to Table 1 found in manufacturers protocol (refer Appendix 3). After mixing, 600µl of sample was transferred to a cuvette and initial absorbance (A_0) read. The sample was transferred back into the microcentrifuge tube before 200µl of diluted substrate was added. It was mixed again, transferred to a cuvette and absorbance read at 3 minutes. A standard was prepared (as per Table 1 in the manufacturers protocol) and processed obtaining an initial measure and one at 3 minutes. Study samples were then processed in the same manner as for the blank and standard samples. Antioxidant concentration was determined following manufacturer's calculation of antioxidant status found in kit user protocol. The following equation was used to determine antioxidant levels in each of the samples.

$$\text{Antioxidant Concentration (mM)} = \frac{[\text{Standard}](\Delta A \text{ Blank} - \Delta A \text{ Sample})}{(\Delta A \text{ Blank} - \Delta A \text{ Standard})}$$

Where A = absorbance after 3 minutes
A_0 = initial absorbance
$\Delta A = A - A_0$
[Standard] = concentration of standard (mM)

Two standard and two blank samples were prepared for 15 study samples, the average was used in the calculation of the antioxidant concentration in relation to those 15 samples.

3.5 TUNEL assay

This assay was performed using the ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101 (Chemicon International, Australia). Slides were fixed with a 3:1 ratio of methanol/acetic acid solution. Slides were then washed in phosphate buffered saline (PBS) and permeabilised in PBS-0.1% Triton X-100 solution and washed in PBS. 75µl Equilibration buffer was applied for at least 10 seconds and excess removed before 55µl working strength terminal deoxynucleotidyl transferase (TdT) enzyme was applied, covered with a plastic coverslip and incubated at 37°C for one hour. Following this the slide was incubated in working strength stop/wash buffer and washed in 2 changes of PBS. Anti-digoxigenin peroxidase conjugate (65µl) was then applied and allowed to incubate at room temperature for 30 minutes. Slides were washed in PBS then 75µl peroxidase substrate applied and allowed to develop for 4 minutes. When sufficient colouring had occurred the slides were washed in 3 changes of dH_2O and air dried. Slides were then counterstained using 0.5% (w/v)

methyl green and washed in dH_20 and air dried before being viewed by brightfield microscopy (1000x magnification). Four hundred sperm were counted on each slide with DNA damaged sperm being expressed as a percentage. Sperm with DNA damage were stained brown and undamaged sperm were stained green (Figure 2). Negative controls were prepared following the above procedure but reaction buffer was used instead of the working strength TdT enzyme.

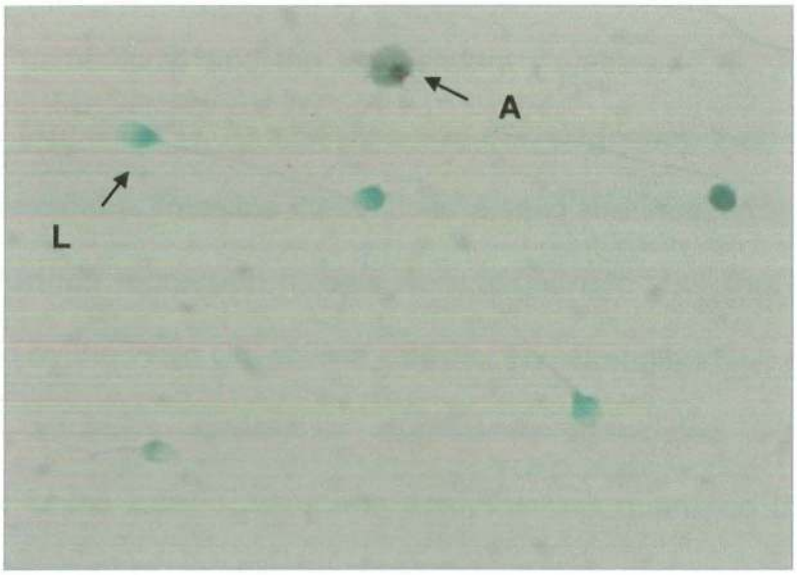


Figure 2: Pictomicrograph of the TUNEL assay Typical field of view for counting DNA damaged sperm. 1000x brightfield microscopy. A region of DNA fragmentation can be seen on the sperm labeled (A). The blue/green stained sperm (L) shows no signs of DNA damage. (Photograph courtesy of Concept Fertility Centre)

3.6 Statistical Analysis

Univariate analysis was used to assess differences between ROS, TAC, TUNEL and sperm parameters (motility, morphology, age and total sperm

concentration). Independent-group t-tests were performed in the comparison between categorical and continuous variables while the chi-square test for relatedness or independence was performed in the comparison of two categorical variables. Statistical analyses were performed using the student SPSS for Windows version 15.

Multivariate logistic regression was used for the examination of the relationships between outcome variables (total sperm concentration, motility, morphology) and the independent variables ROS, TAC, TUNEL and age (Appendix 7). An analyses was also performed between age and the study factors. Possible interactions among the independent variables included in the regression models were tested and statistical significance assessed by the Wald Chi-square statistic. The strength of the associations between variables statistically significantly associated with outcome variables in the logistic regression analyses was quantified by estimated odds ratios and 95% confidence intervals.

CHAPTER 4:

RESULTS

4.1 Semen Parameters

Semen parameters from samples used in this study are presented in Table1. Age was grouped into ≤39 yrs and ≥40 yrs, 16 of the 54 subjects were ≥40 yrs age and 38 were ≤39 yrs age. Morphological normality was considered to be met when ≥15% of the sample had normal morphology. The cut off for normal motility was ≥50%, greater than 50% of the sample was required to be motile. Total sperm concentration was normal if greater than 20 million per ml. These cut offs were based on standards according to the World Health Organisation (WHO, 1999).

Table 1. Semen Variables

	Age (Years)	*Ejaculate Volume (mL)*	*Total Sperm Concentration (million/mL)*	*Motility (% motile)*	*Morphology (% normal)*
≤ 39 yrs age	32.6 ± 0.6	3.6 ± 0.2	73.7 ± 6.1	62.9 ± 2.1	19.3 ± 1.6
Range	26 - 39	1.7 - 6.6	10 - 175	35.7 - 93.1	5 - 39
≥ 40 yrs age	45.7 ± 1.4	3.8 ± 0.37	79.7 ± 12.4	50.1 ± 4.7	20.4 ± 1.4
Range	40 - 59	1.6 - 7.1	18 - 214	16.7 - 78.6	14 - 26

Note: Values are the mean ± SEM and the range

Differences were observed between age groups in relation to mean total sperm concentration, ≥40 yrs exhibiting higher sperm counts (79.7±12.4) than ≤39 yrs (73.7±6.1), and motility, individuals ≤39 displaying higher motile counts (62.9±2.1) than ≥40 yrs (50.1±4.7). Conversely, older men had lower percentage of motility than the younger group.

Table 2. Study Factors

	Age (Years)	*ROS (counted photons per minute)*	*TAC ([AOX] mM)*	*TUNEL (% positive)*
≤ 39 yrs age	32.6 ± 0.6	494.7 ± 151.4	2.3 ± 0.14	5.9 ± 0.54
Range	26 - 39	4 - 5342	1.09 - 6	1.35 - 13.4
≥ 40 yrs age	45.7 ± 1.4	408.8 ± 137.2	2.1 ± 0.24	7.7 ± 0.86
Range	40 - 59	3.5 - 1825	0.21 - 5	3.7 - 14.2

Note: Values are the mean ± SEM and the range

4.2 Multivariate Logistic Regression Analyses

4.2.1 Age

Multivariate analyses between age, ROS, TAC and TUNEL presented no statistically significant associations.

Table 3. Results of Multivariate Logistic Regression Analysis Examining the Relationship between Study Factors and ≥40 yrs age

study factor	OR*	95% C.I.	Wald Chi-square	df	p-value
ROS	1	0.99 - 1.001	0.3	1	0.6
TAC	0.8	0.35 - 1.8	0.3	1	0.6
TUNEL 'normal' ref: 'abnormal'	2	0.4 - 9.1	0.8	1	0.4

*OR: Odds ratio

4.2.1.1 Motility

Statistical significance was observed between motility and age. Analysis predicts that the odds of displaying abnormal sperm motility are 5.4 times higher for men ≥40 years of age than for men ≤39 years. Statistical significance was also observed in univariate analysis (Appendix 7) between

sperm motility and age (p=0.014), men ≤39 years age displayed higher counts of motility than those ≥40 years age.

Table 4. Results of Multivariate Logistic Regression Analysis Examining the Relationship between Abnormal Sperm Motility and Study Factors

study factor	OR*	95% C.I.	Wald Chi-square	df	p-value
ROS	1	0.99 - 1.001	0.4	1	0.6
TAC	1.7	0.8 – 3.6	1.7	1	0.2
TUNEL 'normal' ref: 'abnormal'	3.8	0.7 - 21	2.4	1	0.1
Age '≤39' ref: '≥40'	5.4	1.2 – 23.5	5	1	0.03

*OR: Odds ratio

4.2.2 Sperm Concentration and Morphology

A statistically significant association was observed in the sperm concentration model with DNA damage as measured using TUNEL (presented in Table 5). Regression analyses predicted that for men with moderate levels (10–14.9%) for DNA fragmentation the odds of having low sperm concentration (count <20 mill/ml) is 38.7 times that than for men with insignificant levels (<10%) for DNA fragmentation. The 95% CI (2.1–727.2) is very wide and lacking precision which is most likely due to small sample size.

Table 5. Results of Multivariate Logistic Regression Analysis Examining the Relationship between Abnormal Sperm Concentration and Study Factors

study factor	OR*	95% C.I.	Wald Chi-square	df	p-value
ROS	1.001	0.9 - 1.002	1.9	1	0.2
TAC	1.9	0.5 - 7.6	0.9	1	0.3
TUNEL 'normal' ref: 'abnormal'	38.7	2.06 - 727.2	5.9	1	0.02
Age '≤39' ref: '≥40'	0.5	0.03 - 7.3	0.3	1	0.6

*OR: Odds ratio

Table 6. Results of Multivariate Logistic Regression Analysis Examining the Relationship between Abnormal Sperm Morphology and Study Factors

study factor	OR*	95% C.I.	Wald Chi-square	df	p-value
ROS	1	0.99 - 1.001	0.001	1	0.97
TAC	1.1	0.5 - 2.8	0.09	1	0.8
TUNEL 'normal' ref: 'abnormal'	1.8	0.25 - 12.6	0.3	1	0.6
Age '≤39' ref: '≥40'	0.4	0.04 - 4.1	0.6	1	0.4

*OR: Odds ratio

Statistically significant mean difference across groups was observed between total sperm concentration and DNA fragmentation, $p<0.001$ (refer to Appendix 7), with men in the ≥40 year age group having higher levels of DNA fragmentation, 7.7±0.86 as opposed to 5.9±0.54. Analyses conducted using multivariate logistic regression for sperm morphology did not reveal any significant associations (Table 6).

4.3 Limitations and Bias

Given that associations between ROS, TAC, DNA fragmentation and age were not observed in this study, it may have been because the statistical power was limited due to a small sample size of 54. Green (2005) suggests sample size for regression analyses requires a case:predictor ratio of 10:1 which is also recommended by Peduzzi et al (1996). In wanting to include 4 predictors and assess interaction terms at the very least 4 x 10 cases would have been required (Peduzzi et al, 1996), and if following recommendations by Green (1991) then a minimum sample size of 108 would have been required to assess associations with 4 predictors. For testing the overall model a minimum sample size of at least 84 would have been required. This was not possible in the time available for sample collection.

The study samples may have been biased as a result of the method of collection. All samples were excess samples from routine sperm analysis or ART. Samples are more likely to exhibit abnormal qualities than samples that had been collected randomly from the general population and so could be considered non-representative. The study group ≥40 years age was under-represented.

CHAPTER 5:

DISCUSSION

5.1 Discussion

Male factor infertility is now recognized as a significant factor for couples having difficulty conceiving (Agarwal et al., 2004; Pirrelo et al., 2005; Tremellen, 2008), particularly with current trends showing couples are delaying parenthood well into their thirties (Collins & Crosignani, 2005; Ng et al., 2004). Female fertility has long been established as declining markedly with age, particularly by the fourth decade of life (Plastira et al., 2007; Schmid et al., 2007) and it has now become apparent that male fertility declines with advancing age, albeit not as dramatically as in women (Sloter et al., 2006). There is a growing body of evidence that reactive oxygen species, total antioxidant capacity and DNA fragmentation play a very significant role in the aetiology of male infertility. Even though current research is focusing on the effects of advancing paternal age, few associations have been definitively established (Nanassy & Carrell, 2008).

Older men are expected to display higher levels of DNA fragmentation (Aitken & Krausz, 2001) as a result not only of replicative errors but also from the added effects of oxidative damage over time. If antioxidant defense mechanisms are lacking there is greater exposure of DNA to ROS and a greater possibility of ROS induced DNA damage. The study by Barroso et al. (2000) demonstrated a positive correlation between the generation of ROS and DNA fragmentation. In the present study men ≥40 years exhibited higher levels of DNA fragmentation consistent with previous reports. Recently a study by Angelopoulou, Plastira and Msaouel (2007) studied the ageing effects on DNA fragmentation. They observed a

significant correlation between age and TUNEL positive levels. They compared 61 oligoasthenoteratozoospermic (OAT) patients, divided into 20–34 year olds and 35–50 year olds, to 49 healthy fertile controls with the same age group divisions. TUNEL positive results were on average higher for both patient groups as opposed to the controls with the average of the 35-50 year patient group being significantly higher. Plastira et al (2007) observed similar findings in their investigation of OAT patients, older patients with OAT displaying higher levels of DNA fragmentation as assessed by TUNEL assay.

In the present study the mean value of TAC was lower in the ≥40 year age group compared with the younger group and unexpectedly the mean value of ROS was higher for the ≤39 year age group as opposed to the ≥40 year age group. As Tremellen (2008) notes male factor infertility, regardless of age, has been linked with oxidative stress by several studies. It is a combination of both an increase in ROS production and a reduction in TAC. Moustafa et al. (2004) conducted a study examining the relationship between ROS and TAC in a group of patients that were randomly selected from attending a male infertility clinic and a group of controls that were donors. Moustafa et al. (2004) observed that patients had a significantly higher ROS level than donors, however they observed no significant difference of TAC between groups. Another study by Gil-Guzman et al. (2001) compared ROS and TAC levels in a group of male patients undergoing infertility screening and a group of normozoospermic donors. Observations found a significantly lower TAC in patients and a lower mean

average of ROS levels in donors. Unfortunately these studies did not examine the relationship of TAC and ROS with paternal age. The above studies by Moustafa et al. (2004) and Gil-Guzman et al. (2001) involved only 50 and 48 samples respectively.

Reductions in ejaculate volume, sperm motility and morphology have been noted with advancing age (Nanassy & Carrell, 2008). Data from the present study showed an overall reduction in sperm motility in ≥40 age group although no relationship with ejaculate volume, it being or sperm morphology was observed.

Data collected on the samples from smokers showed comparable readings of ROS and TUNEL across both groups when compared to those who were non-smokers. It should be noted however that the TAC values obtained were not a contributing factor of reduced fertility. Values fell either in the expected range of 1.3-1.77mM (refer Appendix 4) or higher, indicative of high seminal plasma antioxidant concentration. The aim of this present study was to investigate the relationship between age and its influences on ROS, TAC and DNA fragmentation. The results obtained did not support the hypothesis that an interaction exists. However associations at a non-significant level were observed between age and TAC with statistically significant findings between motility and age and between total sperm concentration and DNA fragmentation were found.

5.2 Future Investigations

Present research is uncovering areas of significant concern with regard to male infertility. Links between DNA fragmentation and advancing age resulting in sub fertility have been well documented (Aitken & Krausz, 2001; Barroso et al., 2000; Lambert, Masson & Fisch, 2006; Moustafa et al., 2004). Reactive oxygen species, particularly when associated with oxidative stress, have been shown to increase the level of DNA damage found in spermatozoa (Barroso et al., 2000; Donnelly et al., 1999; Lambert, Masson & Fisch, 2006) and when using standard analyses is not necessarily readily identifiable. Furthermore, total antioxidant capacity is associated with both ROS and DNA damage (Baker & Aitken, 2005; Sanocka & Kurpisz, 2004; Tremellen, 2008) where it only exacerbates the problem. Conclusive evidence needs to be established in linking these factors to reduced male fertility particularly concerning advancing age with the potential range of benefits falling anywhere between higher success rates with ART procedures of IVF and ICSI to a reduction in the incidence of childhood disorders resulting from germ line aberrations.

5.3 Conclusion

Susceptibility of spermatozoa to oxidative stress is intensified by the capacity for them to produce ROS themselves. Oxidative stress, resulting from excess reactive oxygen species generation and reduced total antioxidant capacity, not only appears to disrupt the fertilizing capacity of the sperm but also to attack both nuclear and mitochondrial DNA. The

ability of an embryo to both repair this damage and survive to term appears to be negatively associated with the level of DNA fragmentation in the germ line of spermatozoa. Natural protective mechanisms exist to prevent spermatozoa with damaged genomes from participating in the fertilization process however with the advent of ICSI these barriers can be bypassed resulting in a higher incidence of poor pregnancy outcomes and even childhood disease. This highlights the importance of further identifying significant interactions between age and other factors such as ROS, TAC and DNA damage implicated in the aetiology of male factor infertility. Benefits of this approach would enable a more individual diagnosis and treatment and have the potential to reduce the transmission of childhood diseases at the genomic level.

CHAPTER 6:

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CHAPTER 7:

APPENDICES

7.1 Appendix 1

Consent Form

BACKGROUND

Concept Fertility Centre would like to use some of your sperm for a research project. Normally, only a portion of your sperm is used for a semen analysis or ART preparation and the remainder is discarded. We would like to use this surplus sperm to study the effects of age on sperm quality. The causes of male infertility include genetic problems, chromosome abnormalities, infectious diseases, blockages, radiation, chemotherapy and erectile dysfunction. It is well documented that older men take longer to produce pregnancy with an increasing proportion undergoing assisted reproduction. Recent research has focused on the effects of oxidative stress on human sperm quality. Normal sperm production requires a balance between reactive oxygen species and antioxidants. Imbalances can cause oxidative stress to occur which can have a significant effect on normal human sperm function. This research will provide us with valuable information that will increase our knowledge of the factors that effect sperm function and may therefore benefit couples with male factor infertility. Your sample will not be identifiable in this research.

I.....have
(Given Names) (Surname)

- read the information above explaining the study entitled "The effects of age on human sperm oxidative stress"
- I have read and understood the information given to me. Any questions I have asked have been answered to my satisfaction. I agree to allow my sperm to be used for this research.
- I agree that research data gathered from the results of this study may be published, provided that names are not used.
- I am aware that I may withdraw this consent at any time, in writing to the Scientific Director.

If you have any complaints regarding the conduct of this study please refer them to Dr Geoff Masters, Director of Clinical Services, who can be contacted on 9340 8222.

Dated .....day of .....20.....

Signature.....

I, ... have explained the above to the signatory who has
(Investigators full name)

stated that he understood the same. Signature.....

7.2 Appendix 2

Semen Analysis Questionnaire

Please take a few minutes to complete these questions. Your answers will help us interpret your semen analysis results.

1. Is the sample jar labeled with **YOUR** name and date of birth?
   Please circle the correct answer Yes No

2. What time did ejaculation occur? .....

3. Did you collect the whole ejaculate?
   Please circle the correct answer Yes No

   If no – what portion was missed first middle end

4. How many days has it been since your last ejaculation? .....

5. Have you suffered from any illness in the last 3 months that raised your body temperature?
   Please circle the correct answer Yes No
   Comments:.....

   .....

6. Have you taken any medications during the last 3 months?
   Please circle the correct answer Yes No
   Comments:.....

   .....

7. Have you taken any antioxidant dietary supplementation during the last 3 months?
   Please circle the correct answer Yes No

   If yes please indicate the type and dosage .....

8. Have you smoked cigarettes during the last 3 months?
   Please circle the correct answer Yes No

   If yes please indicate the number per day .....

Name: .....Signature:.....

*** Please place the completed questionnaire in the paper bag with your sample jar and take to the collection cupboard.***

7.3 Appendix 3

ROS Assay Reagent Preparation

Dimethylsulphoxide (DMSO) F_W : 78.13

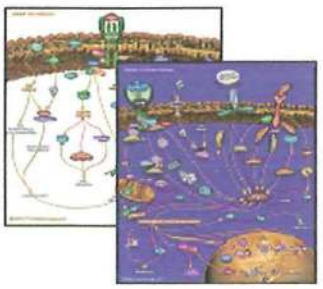
Luminol F_W : 177.16

1. Weigh out 0.468g of Luminol.
2. Add 1.3ml of DMSO to produce a 2M stock Luminol in DMSO solution.
3. Aliquot 25µl of stock luminol in DMSO into a sterile 2ml microcentrifuge.
4. Add 975µl of DMSO to produce a 5mM working strength Luminol in DMSO solution.
5. DMSO was stored wrapped in aluminium foil due to its light sensitivity.
6. Stock and working strength solution was made freshly daily.

7.4 Appendix 4

TAC Kit User Protocol (Calbiochem)

Visit our Interactive Pathways™ at
www.calbiochem.com/pathways



Calbiochem®

User Protocol 615700 Rev. 20-July-06 JSW

Total Antioxidant Status Assay Kit
Cat. No. 615700

Note that this user protocol is not lot-specific and is representative of the current specifications for this product. Please consult the vial label and the certificate of analysis for information on specific lots. Also note that shipping conditions may differ from storage conditions. Full details are available at www.calbiochem.com.

Size
1 kit

Form
50 Tests.

Format
Cuvette

Detection Method
Colorimetric

Storage
Upon arrival store the entire kit contents at 4°C. **Note: The concentration of the standard is lot-specific. Please refer to the vial label for lot-specific concentration.**

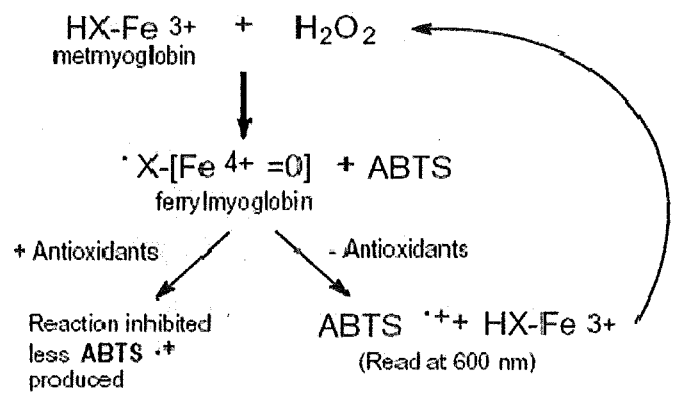
Intended Use
The Calbiochem® Total Antioxidant Status Assay Kit is designed to assay antioxidant levels in serum or plasma samples. Additionally, the assay may be used to measure the antioxidant potential of (suitably solubilized) food and drug samples.

Background
Free radicals are highly reactive molecules generated by the biochemical redox reactions that occur as part of normal cell metabolism, and by exposure to environmental factors such as UV light, cigarette smoke, environmental pollutants and gamma radiation. Once formed, free radicals attack cell structures in the body. As a result, free radicals have been implicated in numerous diseases, including cancer, atherosclerosis, rheumatoid arthritis, diabetes, liver damage, and central nervous system disorders.

Principle of the Assay

The Total Antioxidant Status Kit is dependent on antioxidants in the sample inhibiting the oxidation of ABTS™ (2,2'-Azino-di-[3-ethylbenz-thiazoline sulphonate]) substrate to ABTS™•+ product by metmyoglobin (a peroxidase). The amount of ABTS™•+ product can be monitored by reading the absorbance at 600 nm. Under the reaction conditions used, the antioxidants in the sample cause suppression of the absorbance at 600 nm to a degree that is proportional to their concentration.

Figure 1: Principle of the Assay



Materials Provided

- **Buffer:** 1 bottle, 100 ml, Phosphate Buffered Saline
- **Chromogen:** 5 vials, 10 ml each, Metmyoglobin and ABTS™
- **Substrate:** 2 vials, 5 ml each, stabilized H_2O_2
- **Standard:** 5 vials, 1 ml each, 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid*

***The concentration of the standard is lot specific. Please consult the vial label for the concentration.**

Materials Required but not Provided

- Spectrophotometer or automated analyzer capable of measuring absorbance at 600 nm. A temperature controlled cuvette holder is highly recommended.
- Cuvettes with 1-cm path length

Precautions and Recommendations

- All pipettes should be thoroughly cleaned before use. Water used in the assay must be as pure as possible, the minimum standard being double-deionized water.
- Ensure that all pipettes have been accurately calibrated and are dispensing the correct volume.
- Ensure that all glassware and disposables are free from contamination, especially from hypochlorite and detergents.
- Check the temperature of the equipment being used for incubation of the samples.
- Time the reaction carefully. The absorbance changes very rapidly, and any delay in taking the reading can lead to a substantial error.
- Ensure that there has been no loss of standard material upon opening the vial. The standard is supplied in lyophilized form, and the vials are, therefore, under vacuum. The stopper must be removed slowly to allow air to enter the vial gradually; otherwise there is a risk that material will be lost. In addition, before the stopper is fully removed, the bottle should be sharply tapped on the bench several times to dislodge any material adhering to the stopper. The stopper should then be removed and placed flat side down on the bench while the appropriate volume is dispensed into the vial for reconstitution. The stopper should then be replaced and the contents mixed, ensuring that the liquid comes into contact with the stopper, so that any material adhering to the stopper is dissolved.

Reagent Preparation

• Chromogen: Add 10 ml Buffer to each vial of Chromogen needed for the assay (10 tests per vial). After reconstitution, the Chromogen is stable for 2 days at 4°C or 8 h at room temperature.

• Substrate: Dilute each vial of Substrate needed for the assay by adding 7.5 ml Buffer. After dilution, the substrate is stable for 24 h at 4°C. NOTE: Some automated methods do not require substrate dilution.

• Standard: Add 1 ml distilled water to each vial of Standard needed for the assay. After reconstitution, the Standard is stable for 2 days at 4°C or 1 month at -20°C.

Detailed Protocol

1. Zero the spectrophotometer at 600 nm against air and set temperature to 37°C. Equilibrate the diluted Substrate and Chromogen at 37°C for 5 min just prior to use. NOTE: It is advisable to equilibrate only the amount of each reagent necessary for the assays to be performed. A temperature of 37°C must be maintained throughout the assay.
2. Add the following to a cuvette:

Table 1: Addition of Reagents

	Blank	STANDARD	SAMPLE
ddH_2O	20		
Standard		20	
Sample			20
Chromogen	1ml	1ml	1ml

3. Mix well and read the initial absorbance (A_o)
4. Add 200 µl diluted Substrate to each cuvette. Mix and start the timer simultaneously.
5. Read the absorbance after exactly 3 min (A)

Calculations

Calculation of Total Antioxidant Status

The antioxidant concentration of the Standard is used to calculate the antioxidant levels in the samples.

1. Determine the ΔA for the samples, standard, and blank. $\Delta A = A - A_o$
2. Calculate the concentration of antioxidants in the sample using the formula below:

Figure 2: Antioxidant Concentration Formula

$$\text{Antioxidant Concentration (mM)} = \frac{[\text{Standard}]\,(\Delta A\ \text{Blank} - \Delta A\ \text{Sample})}{(\Delta A\ \text{Blank} - \Delta A\ \text{Standard})}$$

NOTE: If the antioxidant concentration is greater than 2.5 mM dilute the sample with 0.9% NaCl and re-assay. Typical antioxidant levels in normal human plasma are 1.30-1.77 mM.

Sensitivity
Typically serum or plasma samples of 20 µl are sufficient when using the manual method. Samples of 5 µl are typically sufficient when using an automated analyzer. The assay is linear for antioxidant levels less than 2.5 mM.

Assay Range
0-25 nmol/10 µl plasma

Precision
Intra-assay coefficient of variation = 1.2% (mean = 1.37 mM; n = 20)
Inter-assay coefficient of variation = 2.4% (mean = 1.35 mM; n = 18).

Toxicity
MSDS available upon request.

References
Miller, N.J., et al. 1993. *Clin. Science* **84,** 407.

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7.5 Appendix 5

Protocol for TUNEL Assay using Apoptag kit and Grading System

DNA Fragmentation in Sperm using Apoptag kit

Making slides for DNA fragmentation staining

1. Etch a circle (approx 25mm diameter) on the back of 2 Superfrost Plus slides with a diamond pen. 1 of the slides will be used for staining and the 2nd a spare, unless used as a negative.
2. Write the patients identification number and date in ***pencil*** on the front of the slide.
3. Smear the appropriate volume of semen (see below) with the side of a pipette tip onto the front of the slide within the circle.

   For sperm counts > 40 mill/ml use 5µl

   For sperm counts 10-40 mill/ml use 10µl

   For sperm counts 5-10 mill/ml use 20µl

   For sperm counts < 5 mill/ml perform a spin direct on 1-2 ml of semen and use 20µl
4. Allow slides to dry.

Staining Slides

1. Remove 2 slides per patient from the slide box and place in a coplin jar containing 40 ml methanol/acetic acid (3:1 ratio) for 5 minutes at room temp.
2. Wash in PBS for 5 mins.
3. Permeabilise cells by incubating in PBS-0.1% Triton X-100 for 5 mins.
4. Wash in PBS 2 times for 5 mins. Remove equilibration buffer, Reaction buffer and Stop/Wash buffer concentrate from freezer. Remove TdT enzyme from freezer just prior to use.
5. Apply equilibration buffer to slides (75µl/5cm^2) and incubate at room temp for at least 10 seconds.

While this is incubating make up working strength TdT enzyme by adding 77µl Reaction buffer to 33µl TdT enzyme (enough for 2 slides).

6. Blot excess equilibration buffer off the slide and apply working strength TdT enzyme (55µl/slide) and cover with a plastic coverslip.
7. Place in 37°C incubator for 1 hour. During this incubation make up the Stop/Wash buffer by adding 1ml concentrate to 34ml of dH_2O in a coplin jar.
8. Remove coverslips carefully from slides and incubate in Stop/Wash buffer for 10 mins.
9. Wash slides in 2 changes of PBS for 5 minutes each in a coplin jar.
10. Blot off excess liquid and apply room temperature Anti-Digoxigenin Peroxidase conjugate (65µl/5cm^2). Incubate in a humidified chamber at room temp for 30 mins.
11. Wash slides in 2 changes of PBS for 5 mins each in a coplin jar. Make up Peroxidase substrate by adding 147µl DAB dilution buffer to 3µl DAB substrate for every 2 slides. **WARNING!** DAB is a carcinogen. Handle with gloves and dispose of all waste into yellow biohazard bags!!
12. Stain in substrate (75µl/slide) for 4 minutes at room temp, stopping reaction by washing in 3 changes of dH_2O at room temp. Airdry slides.
13. Counterstain slides in Methyl Green (0.5%) solution for 10 mins, followed by washing in 2 changes of dH_2O (3 quick dips each).
14. Airdry slides before viewing under microscope (100x oil immersion lens).
15. Count at least 400 sperm per slide and record results.

<u>Sperm DNA Fragmentation grading</u>

Less than 10% : Insignificant level

10 - 14.9% : Moderate level

15 – 19.9% : Significant level

More than 20% : Severe level

7.6 Appendix 6

TUNEL Assay Reagent Preparation

10X Phosphate Buffered Saline, pH 7.4 (PBS)

1. Dissolve 55g of Na_2HPO_4, 13.5g of NaH_2PO_4 and 117g of NaCl in 800ml of dH2O.
2. Adjust pH to 7.4 using NaOH or HCl.
3. Fill to a final volume of 1L using dH_2O.

PBS-0.1% Triton X-100

Mix 1ml of Triton X-100 concentrate to 99ml of PBS, pH 7.4. Store at 4°C for up to 1 month.

Working Strength TdT Enzyme

33µl of TdT enzyme was added to 77µl of Reaction buffer. Quantity sufficient for the treatment of two $5cm^2$ slides.
Both reagents are components of the Apoptag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101.

Working Strength Stop/Wash buffer

1ml of Stop/Wash concentrate was mixed with 34ml of dH_2O. Quantity sufficient to treat 5 slides in a coplin jar.
Stop/Wash buffer comes as a component of the Apoptag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101.

Peroxidase Substrate

Mix 3µl of 50X DAB substrate with 147µl of 1X DAB dilution buffer. Quantity sufficient to treat two $5cm^2$ slides.
Both reagents are components of the Apoptag® Plus Peroxidase *In Situ* Apoptosis Detection Kit, S7101.

0.5% Methyl Green Stain

1. Dissolve 1.36g of Sodium Acetate ($C_2H_3O_2Na \cdot 3H_2O$) in 80ml dH_2O. Adjust pH to 4 using acetic acid and make up to 100ml using dH_2O to produce a 0.1M Sodium acetate solution.
2. Dissolve 0.5g of Methyl green (crystal violet free, Sigma-Aldrich, M8884) in 100ml Sodium Acetate solution.
3. Filter through either a 0.45µm or 0.2µm filter.

7.7 Appendix 7

Results Univariate Models

Table 1. Univariate Analyses for Total Sperm Count

Study variables	Total Sperm Count Abnormal %	Total Sperm Count Normal %	Test for association p-value
ROS	7.4[a]	92.6[b]	0.119*
TAC	7.5[a]	92.5[c]	0.975*
Tunel : normal	2.3[d]	97.7[e]	0.001**
abnormal	33.3[f]	66.7[g]	
Age category: ≤ 39 years	7.9[f]	92.1[h]	0.833**
≥ 40 years	6.3[d]	93.8[i]	

n sample number of analysis [a]=4 [b]=50 [c]=49 [d]=1 [e]=43 [f]=3 [g]=6 [h]=35 [i]=15
* T test for mean difference across groups
** Chi-squared test for association

Table 2. Univariate Analyses for Sperm Morphology

Study variables	Sperm Morphology Abnormal % (n)	Sperm Morphology Normal % (n)	Test for association p-value
ROS	21.2[a]	78.8b	0.958*
TAC	21.2[a]	78.8b	0.995*
Tunel: normal	20[c]	80[e]	0.628**
abnormal	28.6[d]	71.4[c]	
Age category: ≤ 39 years	24[f]	76[g]	0.489**
≥ 40 years	12.5[h]	87.5[a]	

n sample number of analysis [a]=7 [b]=26 [c]=5 [d]=2 [e]=20 [f]=6 [g]=19 [h]=1
* T test for mean difference across groups
** Chi-squared test for association

Table 3. Univariate Analyses for Sperm Motility

Study variables	Sperm Motility Abnormal % (n)	Sperm Motility Normal % (n)	Test for association p-value
ROS	12.2[a]	77.8[b]	0.690*
TAC	22.6[a]	77.4[c]	0.610*
Tunel: normal	18.2[d]	81.8[e]	0.086**
abnormal	44.4[f]	55.6[g]	
Age category: ≤ 39 years	13.2[g]	86.8[h]	0.014**
≥ 40 years	43.8[i]	56.3[j]	

n sample number of analysis [a]=12 [b]=42 [c]=41 [d]=8 [e]=36 [f]=4 [g]=5 [h]=33 [i]=7 [j]=9
* T test for mean difference across groups
** Chi-squared test for association

Table 4. Univariate Analyses for Age

Study variables	≤ 39 years % (n)	≥ 40 years % (n)	Test for association p-value
ROS	70.4[a]	29.6[b]	0.733*
TAC	69.8[c]	30.2[b]	0.416*
Tunel: normal	72.7[d]	27.3[e]	0.307**
abnormal	55.6[f]	44.4[g]	

n sample number of analysis [a]=38 [b]=16 [c]=37 [d]=32 [e]=12 [f]=5 [g]=4
* T test for mean difference across groups
** Chi-squared test for association