Exploring the Electrochemical Detection Of 8-Oxoguanine Within Human Sperm

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A thesis submitted in fulfilment of the requirements of the Manchester Metropolitan University for the degree of Master of Science (by Research)

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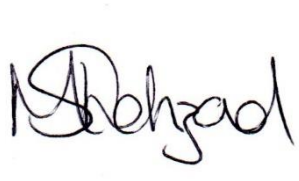
Declaration

I declare that the thesis submitted is my own work and I have maintained professional integrity during all aspects of my research degree and I have complied with the Institutional Code of Practice and Research Degree Regulations



Signed - Mahaah Shehzad

Date: 26th September 2017

Table of Contents

List of Figures and Tables

List of Abbreviations

µM	Micromolar
8-oxoGua	8-oxoGuanine
A	Adenine
AgCl	Silver Chloride
ART	Assisted reproductive technology
BER	Base excision repair
C	Cytosine
CASA	Computer Assisted Sperm Analysis
CO_2	Carbon Dioxide
ddH_2O	Double distilled water
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DPV	Differential Pulse Voltammetry
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
G	Guanine
H_2O_2	Hydrogen Peroxide
HPLC	High performance liquid chromatography
LMP	Low melting point
M	Molar
NMP	Normal melting point
OGG1	8-oxoguanine-DNA glycosylase
PBS	phosphate buffered saline
ROS	Reactive Oxygen Species
SDS	Sodium Dodecyl Sulfate
SOD	Superoxide Dismutase
SPE	Screen printed macroelectrodes
SPM	Sperm prep media

T	Thymine
TAC	Total antioxidant capacity
Tris-Cl	Tris Base and Hydrochloric Acid
UV	Ultraviolet
V	Voltage

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Abstract

The major causes of male factor infertility is poor sperm quality and DNA damage. Sperm DNA damage can impair sperm function, fertilization rates and is associated with increased miscarriage rates. Over the past decade, there has been increasing interest in the role of sperm nuclear DNA integrity in male factor infertility. Excessive production of reactive oxygen species (ROS) can cause irreversible DNA damage and form DNA lesions. The most common DNA lesion is 7,8- Dihyrdo-8-Oxoguanine (8-oxoGuanine), which derives from the oxidation of Guanine.

Measuring DNA damage in sperm is becoming an important procedure to assess sperm quality. Development of methods which quantify the level of 8-oxoGuanine (HPLC, LC-MS) and detect the level of DNA fragmentation (Comet) in a biological sample have become an area of interest. Recent technology (screen-printed electrodes- SPEs), has demonstrated a simple and cost-effective alternative of measuring 8-oxoGuanine.

This study aimed to investigate the use of SPEs, in the detection of 8-oxoGuanine in sperm DNA and seminal plasma, and to correlate degree of DNA fragmentation via the comet assay. These findings may enable future diagnostic techniques.

Sperm cells were separated from their seminal plasma, treated with a concentration gradient of H_2O_2. Sperm cells underwent DNA extraction and were stored for analysis via the comet assay. This study focused inducing oxidative damage, assessing DNA fragmentation in sperm cells and measuring the level of 8-oxoguanine in extracted sperm DNA and seminal plasma.

A significant difference was found in between the DNA % in tail and increasing concentrations of H_2O_2 in the comet assay. There was no detection of 8-oxoguanine in H_2O_2 treated sperm DNA, however 8-oxoguanine was detectable in seminal plasma when using the SPEs. Further work using the SPEs to detect 8-oxoguanine in a biological matrix is required.

1 Introduction

Sperm cells are formed from spermatogonial germ cells within the testis during the process of spermatogenesis. During this process, sperm histones are replaced with protamines, which bind more tightly to DNA, compacting the chromatin, thus improving DNA resistance to denaturation. Sperm nuclear DNA integrity is important for both sperm function and early embryogenesis. Sperm DNA damage can cause embryo defects and increase the rate of miscarriages. Three of the main causes of Sperm DNA damage are reactive oxygen species (ROS), abnormal chromatin packing and apoptosis (programmed cell death) (Gunes et al., 2015) (Robinson et al., 2012).

1.1 The Sperm Cell

The sperm contains three main parts; the head, the midpiece and the tail. It has the ability to deliver the haploid paternal genome to the oocyte during fertilisation. The head of the sperm contains the tightly compacted haploid DNA along with the acrosome which contains hydrolytic enzymes such as acrosin that digest the zona pellucida enabling sperm-oocyte fusion. The midpiece contains mitochondria for the production of ATP and energy for motility. The tail executes the movement propelling the sperm through the female reproductive tract towards the oocyte prior to fertilisation (Alberts et al., 2002; Coward and Wells, 2013)

1.1.1 Spermatogenesis

Sperm cells are produced in the testis during spermatogenesis, which begins at puberty. Spermatogenesis is a complex cellular process which produces the male haploid germ cells from diploid spermatogonial stem cells, it contains 3 phases: spermatogonial (mitosis), spermatocyte (meiosis) and spermatid phases (spermiogenesis). The entire process takes a minimum of 70 days to complete. Starting with spermatogonia in the testis, these cells undergo repeated mitotic divisions into primary spermatocytes, followed by meiosis I to develop into secondary spermatocytes and divide once again via meiosis II to produce round haploid spermatids. The next stage is spermiogenesis where the round haploid

spermatids transform into flagellated, highly condensed spermatozoa. Spermatozoa require further differention and maturation to enable motility (Coward and Wells, 2013).

1.1.2 Sperm function

The journey sperm cells take to fertilise an oocyte depends on a series of biochemical and physiological changes; capacitation and hyperactivation. Capacitation encompasses a set of structural and metabolic changes that sperm undergoes within the female tract, a prerequisite for the acrosome reaction. The efflux of cholesterol causes an influx of Ca^{2+} ions and carbonate which in turn elevates the level of cAMP, adenylyl cyclase, protein kinase. Tyrosine kinases and proteins migrate to the head of sperm where they are phosphorylated. The capacitated sperm demonstrates hyperactive, vigorous motility that can penetrate through the cumulous cells, zona pellucida and undergo the acrosome reaction and bind to the oocyte to complete fertilisation. (Yoshida et al., 2008; Coward and Wells, 2013)

1.2 Male infertility and ROS

Male factor infertility accounts for between 40 to 50% of infertile couples seeking ART in the UK. The major causes of male factor infertility are poor sperm quality and DNA damage (Kumar and Singh, 2015). Many abnormalities with semen parameters (such as decreased motility, morphology and count) can be attributed to conditions such as varicocele, environmental and lifestyle exposures, obesity or diabetes (Walczak–Jedrzejowska et al., 2013; Schulte et al., 2010). Over the past decade, there has been increasing interest in the role of sperm nuclear DNA integrity, in male factor infertility (Schulte et al., 2010). Sperm DNA damage can impair sperm function and fertilization rates, in addition link with increased miscarriage rates, (Robinson et al., 2012).

ROS has important roles in normal sperm function, including both capacitation and the acrosome reaction (Agarwal et al., 2014). However, the presence of excessive ROS production can cause irreversible DNA damage in sperm (Schulte

et al., 2010). Prolonged exposure to high levels of ROS can result in decreased sperm viability, motility and defects within the mid-piece (Pasqualotto et al., 2000). Formation of DNA lesions can lead to genomic instability within sperm and an increase in DNA fragmentation levels (Santiso et al., 2010). Sperm are particularly vulnerable, as their cell membrane comprises of large amounts of lipids (unsaturated fatty acids) which can be easily oxidized via the process of lipid peroxidation (Walczak–Jedrzejowska et al., 2013).

There are several mechanisms whereby ROS can cause infertility, firstly ROS damages the sperm membrane through lipid peroxidation, reducing both sperm motility and sperm-oocyte binding (Figure 1.2). Additionally, a loss of ATP (adenosine triphosphate) from the lipid peroxidation causes damage to axons and deteriorates sperm viability (Tremellen, 2008; Agarwal et al., 2014).

1.3 Reactive Oxygen Species

Oxidative stress is the most common cause of DNA damage, it is mediated by ROS. These are a group of highly reactive molecules, that are by-products of normal oxygen metabolism, both of which are readily produced in the body (via redox reactions) (Tremellen, 2008). The species include Oxygen ions (O_2), free radicals i.e. superoxide (O), hydroxyl (-OH) radicals and hydrogen peroxide (H_2O_2) each of which can have potentially severe effects on an individual's health. (Morrell, 2008). During the production of energy, oxygen is reduced and free radicals form (Figure 1.1), these molecules contain one or more unpaired electron. Free radicals search for any chemical reactions, to dismiss their unpaired electron, this reaction results in the oxidation of amino acids, carbohydrates and within chromosomal and mitochondrial DNA. (Tremellen, 2008).

Presence of both ROS and antioxidants form an innate mechanism which provides a natural defence against oxidative stress. Excess production of either component will disrupt this balance, resulting in the deterioration of sperm viability

and motility. Occasionally, the ROS-Antioxidant imbalance can be amended by making lifestyle changes i.e. quitting smoking (Agarwal et al., 2014)

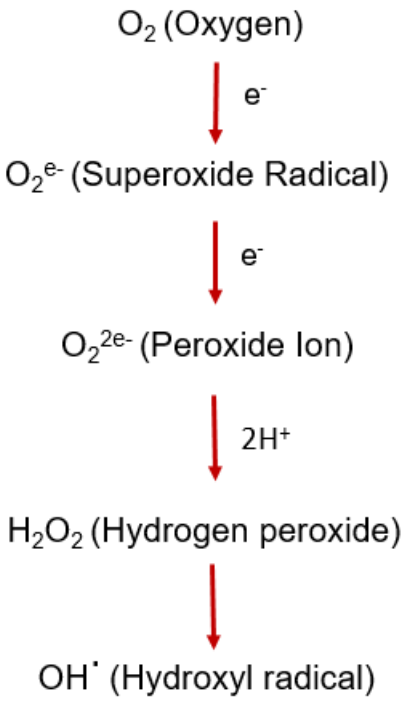

Figure 1.1: *Generation of Reactive Oxygen Species during energy transfer - adapted from (Das et al., 2014).*

1.3.1 ROS and Semen Antioxidants

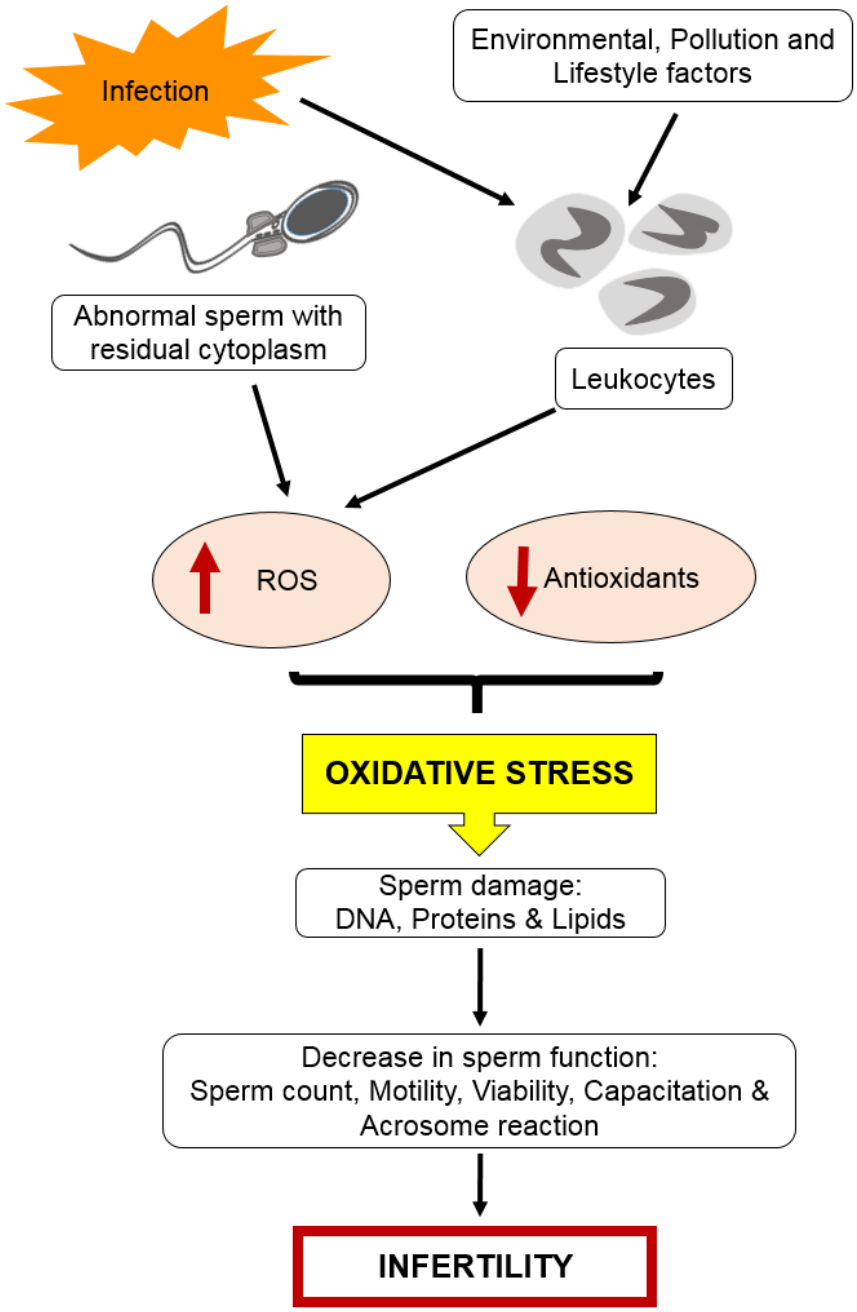

Figure 1.2 : *Effect of Reactive Oxygen Species on male fertility (adapted from Walczak–Jedrzejowska et al., 2013)*

The main constituents of semen include sperm cells and seminal plasma. Semen can also contain other cells – round cells (spermatocytes) and leukocytes. The number of leukocytes can fluctuate depending on environment, sexual abstinence and the presence of varicocele. Leukocytes, in particularly neutrophils can produce elevated levels of ROS when under specific conditions, they play a crucial role in the body's defence against any foreign infections

(Walczak–Jedrzejowska et al., 2013). The seminal plasma is full of antioxidant properties, capable of scavenging ROS and preventing sperm cells from severe damage (Pasqualotto et al., 2000). The main antioxidant system combines enzymatic and non-enzymatic factors to provide defence against ROS. Superoxide dismutase (SOD), catalases, glutathione peroxidase and ascorbic acid are the main antioxidants, which in combination represents the total antioxidant capacity (TAC). The TAC can provide a direct indication of oxidative stress. Research has found a positive correlation between semen parameters and TAC in abnormal samples, further research has found that defective sperm function caused by oxidative stress is recognized as a marker for male infertility as indicated by Pahune et al., 2013.

1.3.2 The role of DNA damage in fertility

The current method to identify male infertility is through a standard semen analysis, analysing the volume, concentration, motility and morphology. However, according to (Schulte et al., 2010) around 15% of patients who were diagnosed with male infertility had a normal semen analysis, therefore additional testing into the integrity of sperm DNA would be a better identifier of male factor infertility. Increased levels of DNA damage are often seen in the sperm of infertile men, this can have a negative effect on fertilisation rates and embryo development. In the past, there have been numerous studies that have researched into effect of sperm DNA damage on fertility rates, for example a meta-analysis was conducted and found a significant increase in miscarriages in patients with high DNA damage when compared to those with lower levels of DNA damage (Coughlan et al., 2015).

1.4 Formation of DNA adducts

The oxidation of DNA from the ROS produces DNA lesions, those of which can be directly associated with mutations and genetic damage (Brett, 2000). Failure to repair this damage could play a significant role in pro-mutagenic events, neurodegenerative diseases, age related diseases and more importantly disrupt gene expression during embryo development (Singh et al., 2011; Smith et al., 2013). Furthermore, as ROS is a crucial part of a mechanism whereby neutrophils

destroy pathogens, this could provide evidence, which links seminal leukocytes to increased levels of oxidative stress, resulting in male factor infertility. (Tremellen, 2008).

DNA continually undergoes damage when excess ROS is being produced; oxidative species can react with individual cell components and form DNA lesions, which can contribute to additional genomic damage.

Presence of these lesions can lead to base modifications, double and single strand breaks and activation of oncogenes (Soultanakis et al., 2000; Janssen et al., 2001). There are approximately 20 major DNA adducts which have been characterised from oxidative stress, each of which has its own repair pathway (Helbock et al., 1998).

As guanine has the lowest redox potential, it becomes more susceptible to oxidation out of the four bases. The most common DNA lesion that results from oxidative stress is 7,8-Dihyrdo-8-Oxoguanine it is commonly referred to as 8-oxoGuanine (8-oxoGua), this compound stems from the oxidation of guanine, whereby an oxygen atom attaches itself to guanine in the C8 position, as depicted in Figure 1.3.

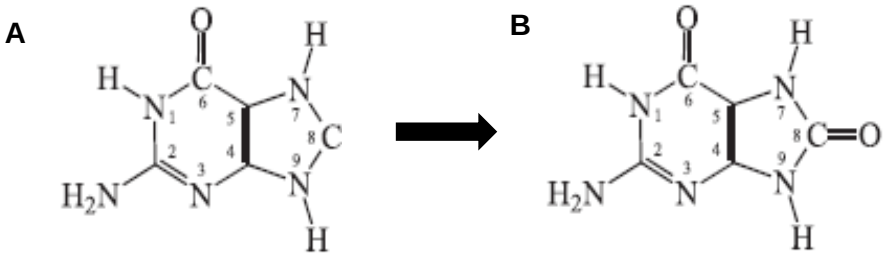

Figure 1.3: *The transformation of (A) Guanine to (B) 8-Oxoguanine (Brett, 2000)*

Oxidation to DNA by the activity of ROS can result in DNA injury. This could occur due to a spontaneous transversion of G → T. Additionally a G:C to T:A mutation could potentially initiate the failure of normal cellular function and result in disease (Brett, 2000). When 8-oxoGua is present within DNA, it can be considered a biomarker for both oxidative stress and DNA damage, numerous techniques have been developed to generate an innovative method for its detection (Persinger et al., 2001)

As each adduct has its own base excision repair (BER) pathway, the DNA lesion must be rapidly identified and its associated damaged base must be excised from the surrounding DNA (Singh et al., 2011). Pro-longed exposure to 8-oxoGua *in vivo* can lead to genomic instability and the fragmentation of DNA. 8-oxoguanine-DNA glycosylase (OGG1) is a repair enzyme that is responsible for the removal of 8-oxoGua from DNA, it works by using lyase activity to cleave the chain at specific points, removing the compound completely. The excision site is then processed by BER proteins, restoring the G:C base pairings. (OGG1 8-oxoguanine DNA glycosylase [Homo sapiens (human)] - Gene - NCBI, 2017) (Janssen et al., 2001).

8-oxoGua is not the only adduct that is derived from the reaction of Guanine and ROS. Both 6-diamino-4-hydroxy-5-formamidopyrimidine (Fapyguanine) and 7,8-dihydro-8-oxo-2'-deoxyguanosine (oxo^{8}dG) have been identified as products of oxidative DNA damage (Shigenaga et al., 1994).

1.5 Common methods of measuring levels of 8-oxoGuanine and DNA damage

Measuring the levels of 8-oxoGua and DNA damage in sperm is becoming an important procedure to assess sperm quality. There have been numerous developments over the past decade, to find methods, which quantify the level of 8-oxoGuanine and detect the level of DNA fragmentation within a biological sample. The overall aim of this method was to generate a technique to measure highly sensitive levels of oxidative DNA damage and 8-oxoGua within specific cells or fluids (Shigenaga et al., 1994).

High Performance Liquid Chromatography (HPLC)

High Performance Liquid Chromatography has been one of the more favourable methods to measure levels of 8-oxoGua; it requires highly skilled personnel to conduct. According to Herbert *et al*.,1996 using HPLC for the detection of 8-oxoGua has proven to show sufficient analysis of oxidised DNA (Herbert et al., 1996). HPLC can be coupled with different methods of detection i.e. UV, electrochemical, mass spectrometry and fluorescent, to deliver rapid, sensitive

and selective quantification of a compound. Analysis of 8-oxoGua within biological samples requires additional preparation i.e. DNA isolation, monoclonal antibody-based immunoaffinity purification, moreover, both have been shown to offer an innovative approach in assessing oxidative damage *in vivo* (Shigenaga et al., 1994).

Limitations of these methods

There are limitations for this method of detecting 8-oxoG, which include example isolating DNA from its biological compound could stimulate the formation of additional DNA lesions. This could present a problem if increased levels of 8-oxoGua are detected because of the method used for DNA isolation rather than from the sample itself. Moreover, the techniques mentioned fail to reveal the precise location and distribution of oxidative DNA damage within cells. The use of advanced immunocytochemistry and modified antibodies could provide insight into the exact positioning of these DNA lesions (Soultanakis et al., 2000).

Comet Assay

The Comet assay is a single cell gel electrophoresis assay which can measure both single and double stranded DNA damage. It begins with sperm cells being layered on pre-coated agarose glass slides, lysed with detergents to form nucleoids of DNA, linked to the nuclear matrix and then run through the gel via an electrophoresis stage. This assay revolves around visualizing the migration of DNA strands within a single cell. The negatively charged damaged DNA would migrate towards the anode, allowing the formation of a comet (Figure 1.4), whereas undamaged DNA would have minimal fragmentation and remain intact (Olive and Banath, 2006).

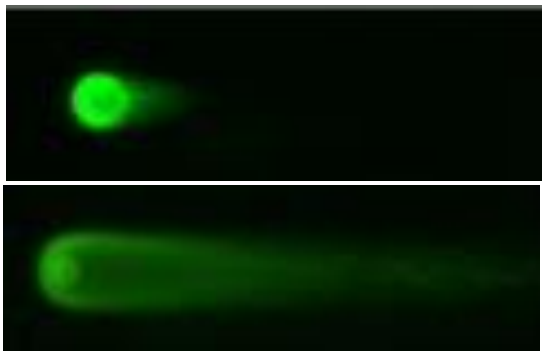

Figure 1.4: *Results from Comet assay - from undamaged DNA (top) and damaged DNA (bottom) (Gyori et al., 2014)*

Once cells are electrophoresed they are fluorescently stained, which under a fluorescent microscope can be picked up and analysed. The degree of DNA damage is determined by the length of the tail i.e. DNA fragmentation trailing behind.

OpenComet is a software used as a plugin on ImageJ, the user can select which images to input in the system, and further discard any background noise which arises moreover, it generates an Excel sheet containing data from 16 parameters (as described in Table 1.1) for each individual comet cell.

When analysing the comet parameters, (Gyori et al., 2014) suggested that DNA% in Tail, Tail Moment and Olive Moment, are the three most important parameters which give the best representation of overall DNA damage. The comet assay is a relatively simple method which is suitable for routine analysis within the clinical setting, however, from start to finish the assay is extremely time consuming and comprises of some overnight steps (Morris et al., 2002).

Table 1.1: *Summary of comet parameters computed by Open Comet. Three measures are given to assess DNA damage (Tail DNA%, tail moment and Olive moment) along with other useful shape and intensity measurements Modified version from (Gyori et al., 2014).*

Parameter	Description
Comet area	Number of pixels in the comet
Comet length	Length of the comet region in pixels
Comet DNA content	Sum of pixel intensities inside the comet
Comet average intensity	Comet DNA content divided by comet size
Head area	Number of pixels inside the head
Head diameter	Length of the head in pixels
Head DNA content	Sum of pixel intensities inside the head
Head average intensity	Head DNA content divided by head size
Head DNA%	Head DNA content as a percentage of comet DNA content
Tail area	Number of pixels in the tail
Tail length	Length of the tail in pixels
Tail DNA content	Sum of pixel intensities inside the tail
Tail average	Tail DNA content divided by tail size
Tail DNA%	Tail DNA content as a percentage of comet DNA content
Tail moment	Tail length times Tail DNA%
Olive moment	Product of tail DNA% and the distance between the intensity-weighted centroids of head and tail

1.6 Methods for 8-oxoGuanine detection

One method of increasing application for 8-oxoGua detection is the use of electrochemistry. Glassy carbon electrodes have been used in the past to determine levels of components within biological fluids. The glassy carbon strips were used alongside a counter (platinum wire) and reference (saturated calomel) electrode in a standard three-electrode configuration. All analyses were conducted using electrochemical software (Brett, 2000; Rebelo et al., 2004) .

Recently, the use of Screen printed macroelectrodes (SPEs) to detect 8-oxoGua in biological samples has demonstrated its effectiveness at a concentration range of 0.1 – 12µM (Bernalte et al., 2017). This provides a simple and cost effective method of measuring levels of 8-oxoGua in relation to DNA oxidation. Screen printed macroelectrodes (SPEs) are batch printed and composed of a flexible polyester base, coated with a carbon-graphite ink, a silver/ silver chloride (AgCl) paste (reference electrode) and a dielectric paste to cover connections. SPEs contain a reference electrode, working electrode and a counter electrode (as depicted in Figure1.5), each of which plays an important role in the quantification of 8-oxoGua.

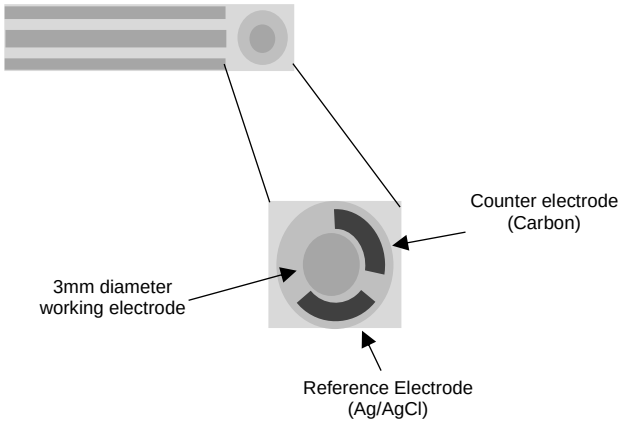

***Figure 1.5**: Screen printed electrode (SPE)*

Samples of extracted DNA within a biological fluid are pipetted onto a working electrode of an SPE. These are connected to a power pack and computer, the power pack can pass an electrical current down the connections and towards the working electrode, whereby the computer, which uses Palmsens – PSTrace software, conducts electrochemical measurements using differential pulse

voltammetry or cyclic voltammetry. In turn creating a graph which can display the level of 8-oxoGua within a sample, respective of the amount of H_2O_2 added.

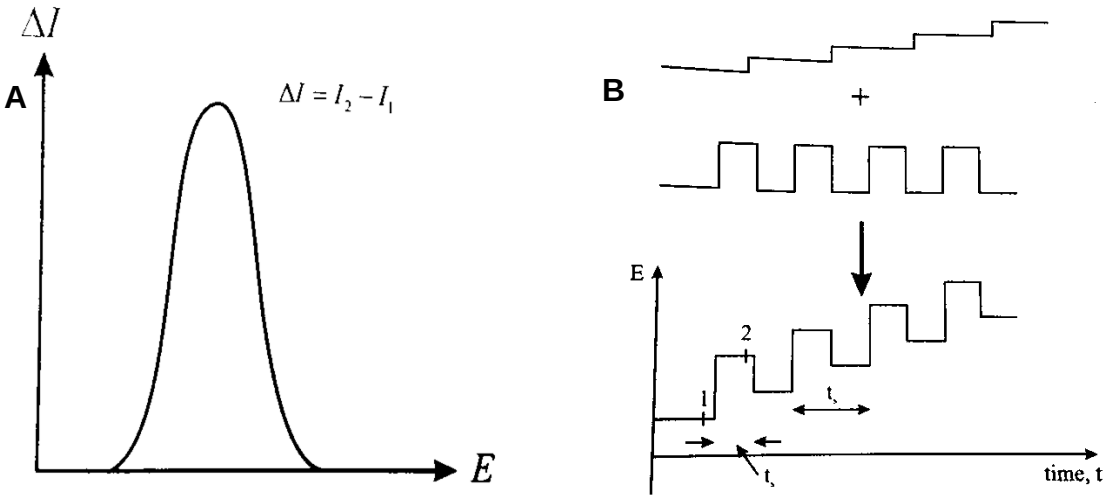

Figure 1.6 : *Voltammetric diagram from a DPV scan (A) and Waveform of pulses overlaid on a staircase (B) (Compton and Banks, 2010).*

Differential pulse voltammetry (DPV) is a method which is able to detect very low limits i.e. 10^{-7}M (Compton and Banks, 2010). The use of DPV allows the potential waveform found during the experiment to be superimposed on a staircase (Figure 1.6).

When using this type of voltammetry, the current before the end of each pulse and the current before the pulse is applied, are measured. The difference between these two values are plotted against a staircase potential – forming a peak shaped graph as depicted in Figr.

On the other hand cyclic voltammetry is slightly different, gathering large amounts of qualitative data about the electrochemical reactions, it is often the initial experiment to be performed, as it evaluates the effect of the suspension media on the redox processes by scanning linearly the potential of a stationary working electrode. (Wang, 2006).

The application of screen-printed electrodes to detect DNA adducts such as 8-oxoGua, has the potential to deliver a rapid and cost-effective analytical method which can be used in clinical practice and diagnosis (Bernalte et al., 2017).

1.7 Aims & objectives

Aims:

- The overall aim of the present study is to investigate the use of SPEs in the detection of 8-oxoGua from sperm DNA samples and seminal plasma, and to correlate degree of DNA fragmentation to levels of 8-oxoGua.

Objectives:

- Human sperm acquired from healthy volunteers will undergo treatment with H_2O_2.
- DNA damage from treated sperm will be assessed via comet assay.
- Extraction of DNA from H_2O_2 treated sperm cells using phenol-chloroform.
- Seminal plasma and extracted DNA will be electrochemically analysed using screen-printed macroelectrodes.
- Any association between levels of 8-oxoGua will be evaluated to determine future work.

2 Methods

2.1 Materials

Materials from each method were obtained as described.

Reagents	**Supplier**	**Catalogue Number**
Agarose, Low Gelling Temperature	Sigma	A9414
8-hydroxyguanine (8-oxoguanine)	Enzo Life Sciences	ALX-480-091-M010
Guanine		
Certified Molecular Biology Agarose	Bio-Rad	1613101
Chloroform	Sigma	C7559
Dimethyl Sulfoxide	Sigma	D8418
Dithiothreitol (DTT)	Sigma	D9779
Dulbecco's Modified Eagle's Medium (DMEM) High Glucose	Sigma	D1145
Ethanol	Sigma	51976
Ethylenediaminetetraacetic Acid (EDTA)	Sigma Aldrich	
Foetal Bovine Serum	Sigma	F0804
Hydrochloric Acid	Sigma	H1758
Hydrogen Peroxide (30%)	Thermo-Fisher Scientific	
Isolate II Genomic DNA Kit	Bioline	BIO-52065
Isopropanol	Sigma	I9516
L-Glutamine	Sigma	G5792
Penicillin-Streptomycin	Sigma	P4333
Phosphate buffered Saline	Oxoid	BR0014G
Proteinase K Solution	Qiagen	19133
Sodium Chloride		S3014

Sodium Dodecyl Sulfate (SDS)	Sigma	L3771
Sperm Freeze Media	Origio	10670010A
Sperm Preparation Media with Phenol Red	Origio	10705060A
SupraSperm® System	Origio	10922060A
Tris Base	Fisher Scientific	BP152-1
Triton™ X-100	Sigma	T8787
Trypan Blue	Thermo-Fisher Scientific	15250061
Trypsin	Sigma	T2600000
UltraPure™ Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)	Thermo-Fisher Scientific	15593031

Equipment
CASA counting chamber 20 microns – Cell Vision
Cell culture plates and T75 Flasks – Nunc by Fisher Scientific
Microlitre centrifuge - Hettich Laboratory
Cover slips 22mmx22mm
Eppendorf tubes – Starlabs
Fisherbrand™ Mini Vortex Mixer – Sigma Aldrich
Gilson Pipettes
Accublock™ Digital Dry Bath – Labnet International Inc.
Mr Frosty™ Freezing Container – Thermo Fisher Scientific
NUAIRE™ Autoflow Direct Heat CO_2 Incubator – Cell culture incubator
NUAIRE™ Biological Safety Cabinets – Class II safety cabinet
Olympus CX41 – Phase contrast microscope (CASA)
Pipette tips – Starlabs
Refrigerated 3-16PK centrifuge - Sigma
Sample collection pots – Sterilin UK
Scarstedt Falcon Tubes
SuperFrost Plus Slides – Thermo Scientific
Thermo scientific NanoDrop 2000 ONE Spectrophotometer

Thermo shaker
Zeiss Axio Imager Z1 – Fluorescent microscope

Software
AxioVision v4.8.2.0
Microsoft® Excel® 2013 version (15.0.4841.1000)
Sperminator® Pro Creative Diagnostics 1.0
Graphpad Prism v7.03
Open Comet v1.3.1
PalmSens PS Trace v4.7

The study was approved by the faculty ethics committee and full COSHH, Ethics, Research insurance and Risk Assessment forms are attached (Appendix A to Appendix D)

2.2 Semen procurement

Semen samples were produced from 10 consenting, normospermic donors (20-25 years) via masturbation, after 2-5 days of abstinence. Upon collection, samples stored at 37ºC for 30 minutes to allow for liquefaction to complete. All information and consent forms are attached (Appendix E)

2.2.1 Density gradient

To separate sperm cells from seminal plasma, a density gradient centrifugation (SupraSperm™, Origio) step was utilised. Here 1ml of neat semen was layered on top of layers of gradient media (80% and 55%) then centrifuged for 10 minutes at 500 x g (Figure 2.1).
The supernatant was removed and pellet was washed twice in Sperm Preparation Media (SPM) for 5 minutes at 300 x g and re-suspended in SPM.

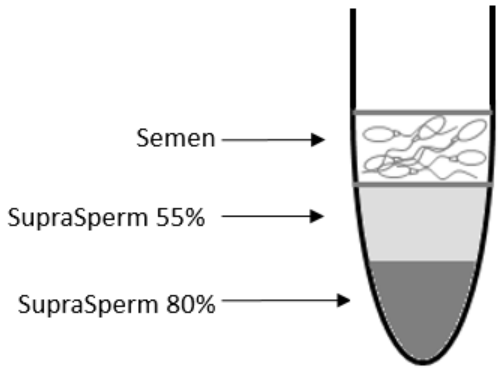

***Figure 2.1**: Density gradient diagram*

2.2.2 Computer assisted sperm analysis

To determine the semen concentration and motility and viability, 5µl of the cell suspension was pipetted into a 2-chamber 20 µm depth counting slides (Figure 2.2 B) and was analysed using the Computer Assisted Sperm Analyser (CASA – Sperminator, Procreative) (Figure 2.2 A).

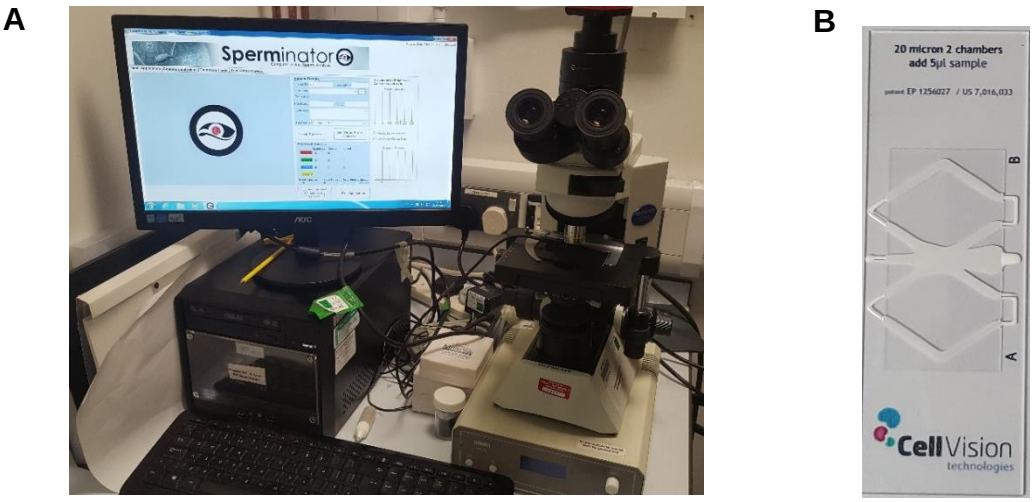

***Figure 2.2:** [A] Computer Assisted Sperm Analyser with Sperminator programme. [B] CASA slide.*

The CASA is comprised of a microscope with a heat controlled stage and a software, it can project a live image from diluted sperm/neat semen. The software used (Sperminator) can freeze a live image and asses the concentration, motility, and progressive velocity of sperm furthermore, allows the user to select/de-select other cells that are present. Sperm motility is measured using four categories – A: Progressive Motile (fast moving, forward direction) B: Slow Progressive

(slower moving, random direction) C: Non-progressive (twitching sperm), D: Immotile and the sperm concentration is calculated in million per millilitre (mill/ml).

2.3 Sperm Cells treatment with Hydrogen Peroxide (H_2O_2)

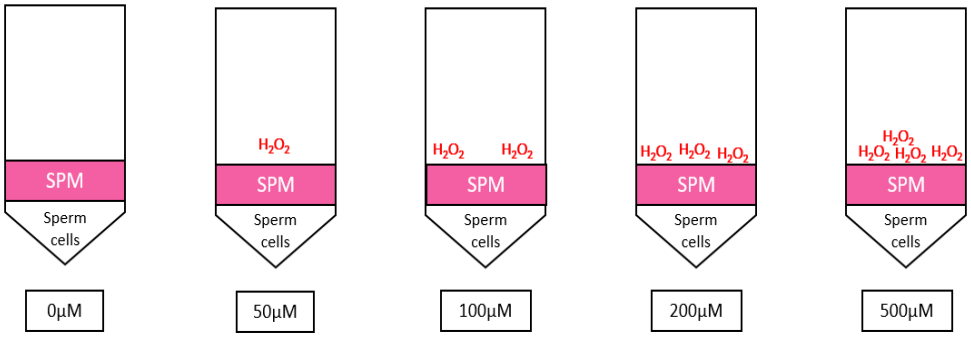

Figure 2.3: Treatment of sperm cells using a H_2O_2 concentration gradient. *SPM – Sperm Preparation Media.*

In regards to sperm cells, each tube contained an approximate concentration of 20 million cells ($20x10^6$/ml). Cells were treated with SPM and various concentrations of H_2O_2 from a stock solution of 10mM: 0µM, 50µM, 100µM, 200µM and 500µM – with 0µM being the non-treatment control (Figure 2.3). Cells were left to incubate for two hours in a 37°C - CO_2 incubator.

Following the incubation period, samples were centrifuged for 10 minutes at 12,000 x g. Supernatant and any remaining traces of H_2O_2 and media were removed, leaving a small white pellet.

2.4 Comet Assay

2.4.1 Optimization of conditions to detect DNA damage

To establish optimal conditions for H_2O_2 treatments and DNA damage detection using the comet assay (see Figure 2.7), several experimental conditions were set up. Incubation time, media type, number of cells and cryostorage of samples were all assessed to identify the optimal conditions required to maintain a high quality of Sperm DNA.

2.4.2 Optimization of storage

Cryostorage can affect sperm DNA integrity. Therefore, to establish which storage conditions were optimal to detect DNA damage using the comet assay, six experimental conditions were set up using different cryostorage conditions (Fraser et al., 2011).

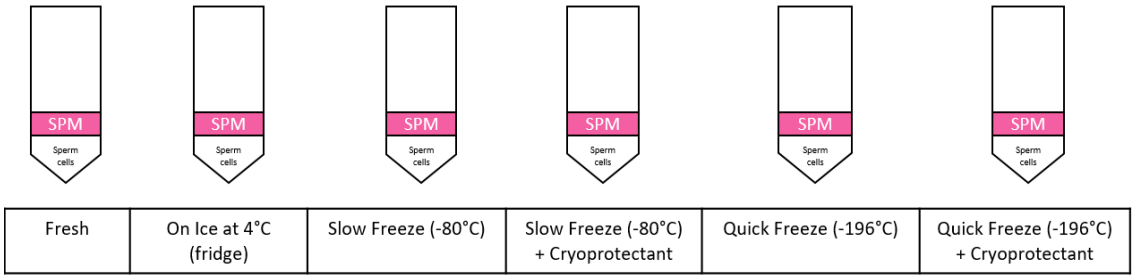

Figure 2.4: Samples were stored using different cyrostorage methods; *on Ice at 4ºC, slow samples were placed in an industrial -80ºC freezer and Quick samples were flash frozen in -196ºC liquid nitrogen. Cryoprotectant was added in two of the six conditions.*

Sperm cells were cryostored in the presence or absence of a cryoprotectant, and either underwent slow or fast freezing protocols (Figure 2.7). The degree of DNA damage was assessed using the comet assay in a fresh semen control and from samples after cryostorage (1-day post freezing).

2.4.3 Optimization of media and incubation time

The length of incubation and the type of media used may influence the stability of DNA and degree of fragmentation (Nabi et al., 2014). Therefore, to determine the best conditions, 18 experimental conditions were set up using various incubation times and different types of media. Sperm cells were treated with H_2O_2 in either SPM or PBS.

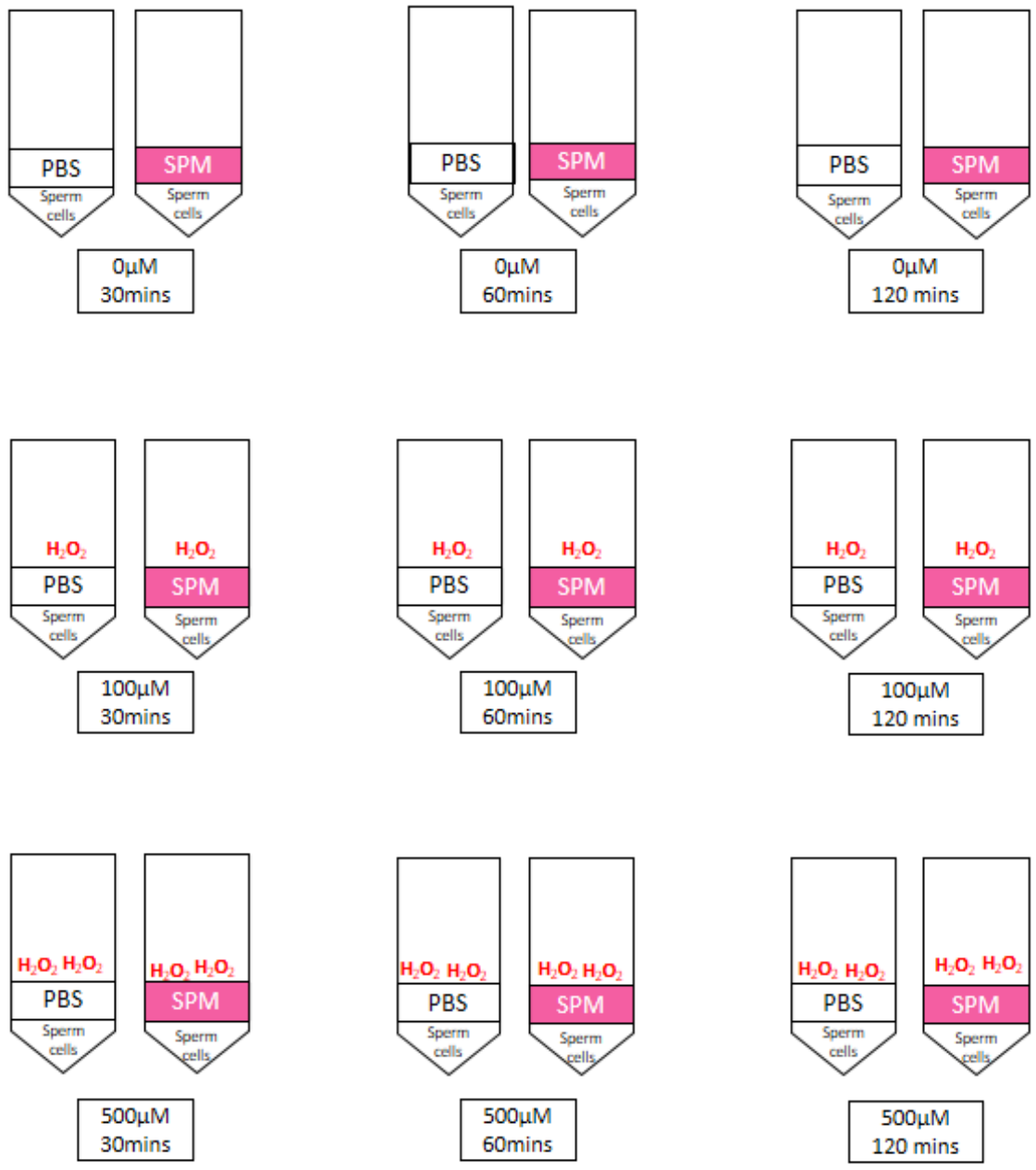

***Figure 2.5**: Eighteen experimental conditions using different types of media and length of incubation.*

Sperm cells (1million/ml) were added to the tubes and treated at different conditions. Sperm was treated with different concentrations of H_2O_2 (0µM, 100µM, 500µM) and incubation times (30 minutes to 120 mins). After treatment, samples were centrifuged to remove the media. The pellet of sperm cells were cryopreserved using freeze media (as per protocol – Origio). All samples were stored until used for Comet assay. (Figure 2.7).

2.4.4 Optimization of Cell number

To examine if the number of cells within the sample has a confounding effect, on the level of DNA damage detection, an optimisation step was performed. A gradient of cells with a concentration ranging from 1million/ml to 20 million/ml were prepared and treated with identical volumes of H_2O_2 - 500µM, prior to a 2-hour incubation period, as illustrated in Figure 2.6.

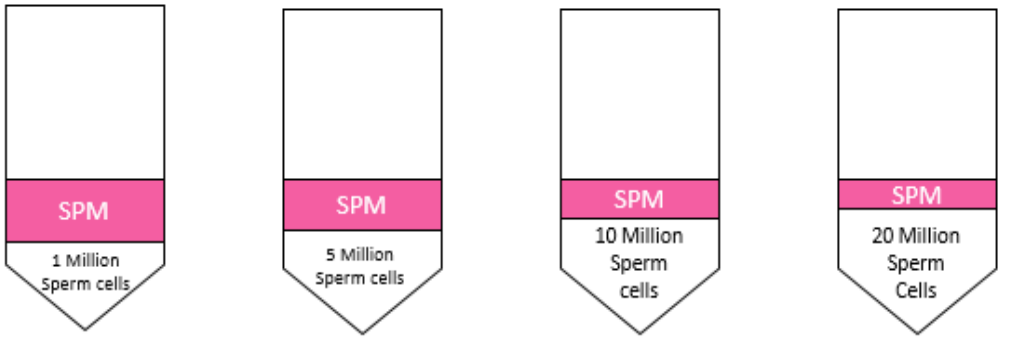

Figure 2.6: *Optimization of sperm cell number for Comet assay.*

2.4.5 Treatment with various concentrations of H_2O_2.

After optimisation, it was established that sperm cells, at a count of 20 million, treated in SPM for 2 hours, yielded the best conditions and were used as standards throughout the study.

For each of the three samples obtained, the neat semen was washed to separate cells from seminal plasma and concentration of sperm was calculated via CASA. The washed sperm was divided into five eppendorf tubes (20 million cells). Following on, each of the tubes were re-suspended in SPM, and treated with a concentration gradient of H_2O_2, which derived from a 10mM stock solution - as seen in Figure 2.4. Sample tubes remained in a 37^oC, CO_2 controlled incubator (Nuaire, UK) for 2 hours. After incubation, eppendorf tubes were centrifuged for 10 mins at 12 000 x g and supernatant was removed, cells were centrifuged once more to eliminate all traces of H_2O_2. Sperm pellets were re-suspended in equal volumes of SPM and freeze media. Samples were stored at -80^oC in preparation for the comet assay and electrochemical analysis.

2.5 Overview of Comet Assay

Comet assay used a standard method with slight adaptations. Each experiment was repeated in triplicate.

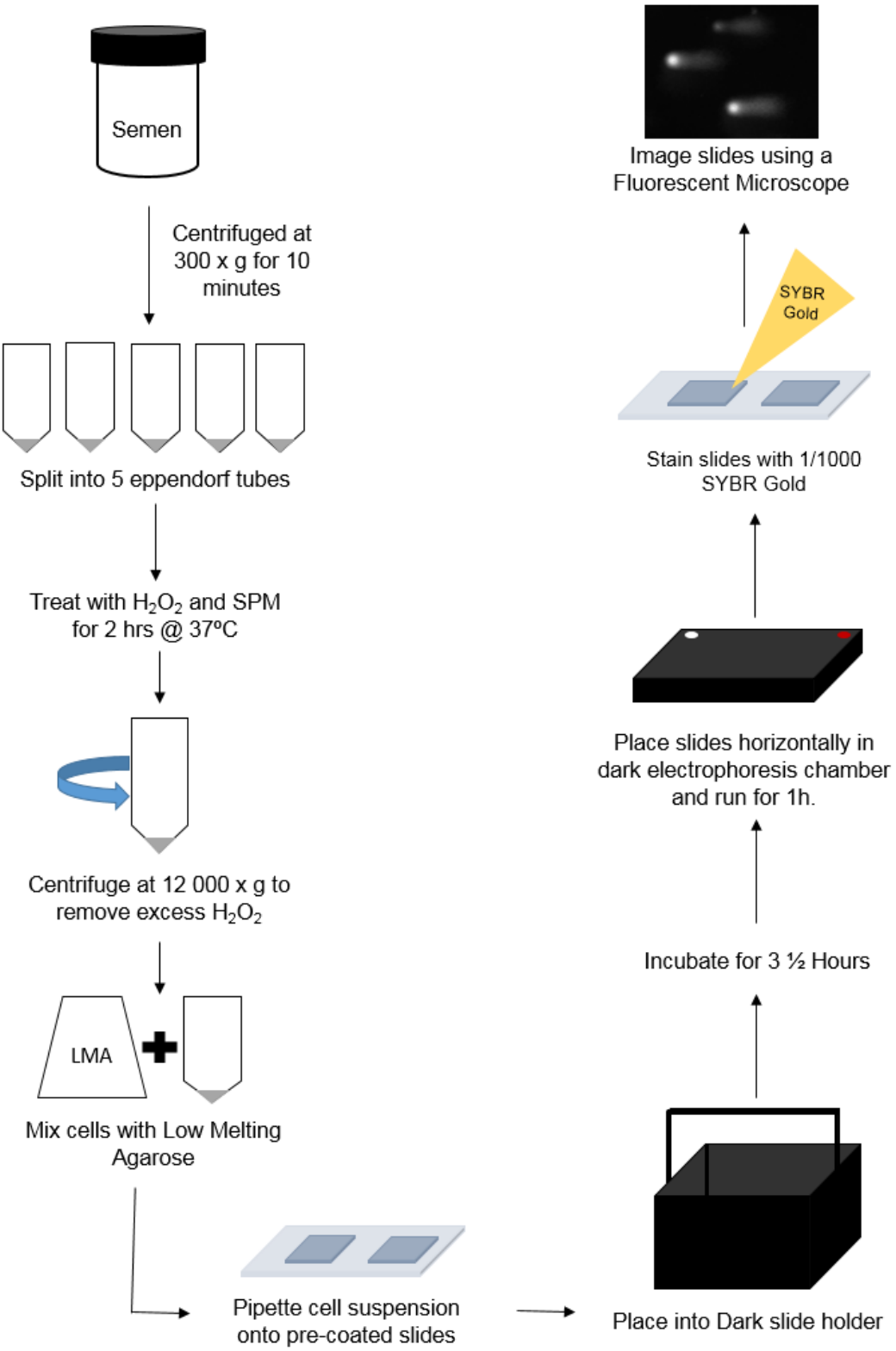

***Figure 2.7**: Overview of Comet Assay method*

Superfrost Plus Slides (Thermo Scientific) were pre-coated using 175µl of Normal Melting Point agarose (1%), which was covered with a coverslip (22mmx22mm) as shown in Figure 2.8. Slides were left to dry at room temperature for 30 minutes before removing the coverslips. Pre-coated slides were left overnight at room temperature protected from light.

Figure 2.8: *Microscope slides pre-coated with 1% Agarose.*

Lysis buffer, electrophoresis and neutralising buffers were prepared and stored at room temperature unless stated otherwise (Table 2.1)

Table 2.1: Comet buffer recipes

Lysis Buffer Preparation (1X), pH 10	
NaOH	8g
dH_2O	700ml
NaCl (2.5M)	146.1g
Disodium EDTA (100mM)	37.2g
Adjust to pH 10 and complete to 890ml with dH2O	
"Ready to Use" Lysis Buffer (1X)	
Lysis Buffer Preparation (1X), pH 10	178ml
Dimethyl Sulfoxide (DMSO)	10ml
Triton X-100	2ml
Store at 4°C	
Neutralisation Buffer (1X), pH 7.5	
Tris Base	60g
dH_2O	300ml
Adjust to pH 7.5 with HCl and complete to 500ml with dH_2O	
"Ready to Use" Neutralisation Buffer (1X)	
Neutralisation Buffer (1X), pH 7.5	80ml
dH_2O	120ml
Store at 4°C	
Electrophoresis Buffer (10X), pH 9	
Anhydrous Sodium Acetate	246g
Tris Base (10M)	121.14g
Adjust to pH 9 and Complete to 900ml with dH_2O	

2.5.1 Detection of H_2O_2 induced DNA damage using the Comet Assay

After optimal conditions were established and treated, the H_2O_2-induced sperm DNA damage was detected using the comet assay. Semen-free sperm were obtained via density gradient centrifugation and wash steps as described in section 2.2.1. Sperm were treated with H_2O_2 (see section 2.3) and stored until used for comet assay.

Frozen samples were thawed and centrifuged for 10 minutes at 12 000 x g to eradicate all possible traces of cryoprotectant, medium and H_2O_2. Cells were re-suspended in 500µl of PBS at a concentration of 1 x 10^6 cells.

Briefly the method was as follows. 30µl of the sperm cell suspension was mixed with 220µl of low melting point agarose (0.7%). Duplicate 75µl aliquots of this suspension was pipetted onto the pre-coated slides and covered with a coverslip. Once the gel had set, slides were immersed in lysis buffer (+ DMSO, 1% Triton X-100) at 4^{o}C for 1h in the dark. Once removed, slides were transferred to fresh lysis buffer with 10mM of DTT (Thermo Fisher, UK) for another hour at 4^{o}C. Dithiothreitol (DTT) is a reducing agent, which acts upon disulfide bonds within proteins. Afterwards the slides were transferred into fresh (room temperature) lysis buffer containing Proteinase K (Qiagen, UK) at 37^{o}C for 90 minutes. Slides were rinsed with dH_2O and placed horizontally in a dark electrophoresis tank, where they were covered with cold electrophoresis buffer, equilibrated for 20 minutes and electrophoresed for 1h at 13V, 120-125mA – during which the DNA fragments migrated towards the anode. Slides were washed in neutralising buffer, and left to dry overnight.

The following day, slides were rehydrated in dH_2O for 30 minutes and stained using 1/10000 SYBR Gold. After 45 minutes in completed darkness, slides were ready for imaging, using a fluorescent microscope at 20X magnification (Zeiss Axio Imager Z1).

2.6 Analysis of Comet Assay – Open comet

Images were analysed using the image-processing platform - ImageJ and Open Comet (plugin v.1.3.1). The images taken on the fluorescent microscope were inputted into Open Comet, this plugin rapidly generates the corresponding image with the head and tail of the comet highlighted. In addition, once all images are analysed, a spreadsheet containing the comet measurements (Table 1.1)

The software allows any abnormal comets or outliers, to be removed from the output spreadsheet. This offers a quick and efficient method to examine each comet, based on the intensity of the light emitted in relation to the amount of DNA present from the cell.

2.7 Detection of H_2O_2-induced 8-OxoGua formation

To examine if oxidative stress induced by H_2O_2 promotes 8-oxoGua formation on sperm DNA, sperm samples were treated with H_2O_2 as per section 2.3. After treatment, sperm DNA was extracted and used for analysis using SPEs.

2.7.1 DNA Extraction

DNA was extracted from sperm cells using the traditional phenol-chloroform method. In short, after cell preparation and treatment (section 2.2 -2.3), cells were re-suspended in 500µl cell lysis buffer (10% SDS, 0.5M EDTA, 1M Tris-Cl [pH8]) and Proteinase k solution [20mg/ml] (Qiagen, UK). Cells were left to incubate for 20 minutes in a thermo-shaker at 50^{o}C, 1000 rpm (Thermo Fisher Scientific, UK). The lysis buffer breaks down the cellular and nuclear membrane releasing its contents - including the DNA into the solution. The addition of the enzyme – proteinase K can digest contaminating proteins which are present in the sample.

Phenol:Chloroform:Isoamyl Alcohol [25:24:1] (Thermo Fisher Scientific, UK) was added and vortexed for 30 seconds . Cells were centrifuged for 15 minutes at 12,000 x g in a temperature controlled centrifuge (4^{o}C) (Sigma,UK)

The upper aqueous layer was transferred to a new 1.5ml Eppendorf and centrifuged twice, after each spin, supernatant was removed (500µl) and equal volumes of Chloroform and Cold Isopropanol were added consecutively. The final five-minute spin required the addition of 70% Ethanol, subsequently a white pellet became visible. Whilst keeping the pellet intact; the remaining ethanol was decanted. Samples were left to briefly air dry and genomic DNA was re-dissolved in TE buffer (Tris-Cl (1M), EDTA (0.5M) pH 8).

Measurements of double stranded DNA was quantified at 260/280nm using a Nanodrop One^{c} Spectrophotometer (Thermo Fisher Scientific Inc., USA). Samples were kept at -80^{o}C for further analysis. Results from the Phenol Chloroform DNA extraction are shown in Table.

Table 2.2: *Nanodrop Results showing the amount of DNA extracted from Sperm Cells including purity levels via phenol chloroform extraction method*

Sperm Sample (n = 3)	DNA (µg/100µl)	Standard Error	$A^{260/280}$
0µM	4.71	2.020531	1.79
50µM	4.87	1.527747	1.74
100µM	6.18	2.849821	1.61
200µM	6.21	1.893577	1.62
500µM	4.19	1.638132	1.69

2.8 Electrochemistry

Measurements of 8-oxoGua using SPEs were carried out in collaboration with Dr Elena Bernalte and Professor Craig E. Banks, Division of Chemistry and Environmental Science. The presence of 8-oxoGua on DNA from H_2O_2-treated sperm was analysed using screen-printed macroelectrodes (Figure 2.9)
Experiments were conducted using SPEs only (Figure 1.5). All electrochemical measurements were carried out using Palmsens (Palm Instruments BV, Netherlands) potentiostat controlled by software PSTrace 4.7. Electrochemical analysis was performed at room temperature as per (Bernalte et al, 2017)

Briefly, SPEs were electrochemically pre-conditioned by applying 10 scans in cyclic voltammetry between 0.0V and +1.0V at $50mVs^{-1}$ in PBS at pH 7.44. Pre-conditioning of SPEs removes residues that interfere with analysis. A standard of TE buffer was used, forming a baseline value, which treated samples can be compared to. Determination of parameters required constant manipulation to each variable, it was dependant on how the signal was influenced. Furthermore, optimisation was performed each time to obtain the maximum current and decide which parameters would be essential. Once these were identified, Differential Pulse Voltammetry (DPV) could be used. DPV is one of the most sensitive methodologies which can detect limits as low as 10^{-7}M (Compton Richard G, 2010; Bernalte et al., 2017).
DPV parameters were optimised meticulously, based on the oxidation of 8-oxoGua at the time of analysis. The final conditions used were nº of scans:10, step potential: 5mV, pulse amplitude: 90mV, pulse width:200ms and scan rate: $5mVs^{-1}$.

2.8.1 Sperm H_2O_2 treated samples

H_2O_2 treated DNA samples were thawed and vortexed briefly, 50µl of the solution was loaded onto the working electrode of an SPE, and the current was applied. Measurements of the current (µA) was taken before the end of each pulse and before the pulse was applied, the difference between these two values was then

calculated and plotted on a graph, against the potential (V) (Compton Richard G, 2010).

2.8.2 Electrochemistry using a biological sample (semen)

8-oxoGua can be detected in biological fluids such as urine and serum (Brett, 2000). In the present study, the levels of 8-oxoGua in seminal plasma after H_2O_2 treatment was investigated. A semen sample was obtained and aliquots of sperm-free seminal plasma was prepared by centrifuging the semen sample 300 x g for 10 minutes. A total count of 20 million sperm cells were added to each aliquot of seminal plasma and treated with H_2O_2 at 500 µM for 2 hours at 37°C (n = 3). The tubes were then centrifuged to remove the seminal plasma. Each sample was flash frozen in liquid nitrogen. The sperm pellet was re-suspended and stored (-80˚C) for Comet assay.

To detect levels of 8-oxoGua in the seminal plasma via SPEs, 50 µl of sample was loaded onto the working electrode, and underwent differential pulse voltammetry using conditions stated in section 2.8 . To control for viscosity and other factors that may interfere with detection (semen contains a complex matrix of proteins) a 1:10 dilution of the sample was prepared with ddH_2O analysis.

2.9 Statistical Analysis

All sets of data were statistically analysed used GraphPad Prism 7.03. Normality tests were conducted on all sets of data. Averages were calculated from the comet assay, using the following parameters: Percentage DNA in tail, Olive moment and Tail moment. Further calculations were required to find a significance between each optimization condition and experimental conditions. A one-way ANOVA or non-parametric equivalent (Kruskal-Wallis test) was used to analyse the relationship between DNA damage and levels of H_2O_2 treatment with a significance level of 0.05. Levels of 8-oxoguanine were also analysed using data which was generated from using electrochemical techniques.

3 Results

3.1 Comet Assay

Comet assay was used to investigate the level of DNA damage in sperm cells by assessing the amount of DNA fragmentation.

3.1.1 Optimisation of storage

Sperm samples were collected, treated with H_2O_2 and cryostored at -80°C before carrying out the comet assay. To ensure this cryostorage did not impact the integrity of the DNA (Fraser et al., 2011), optimisation experiments were performed to investigate the effect of cryostorage on sperm DNA fragmentation (Figure 3.1).

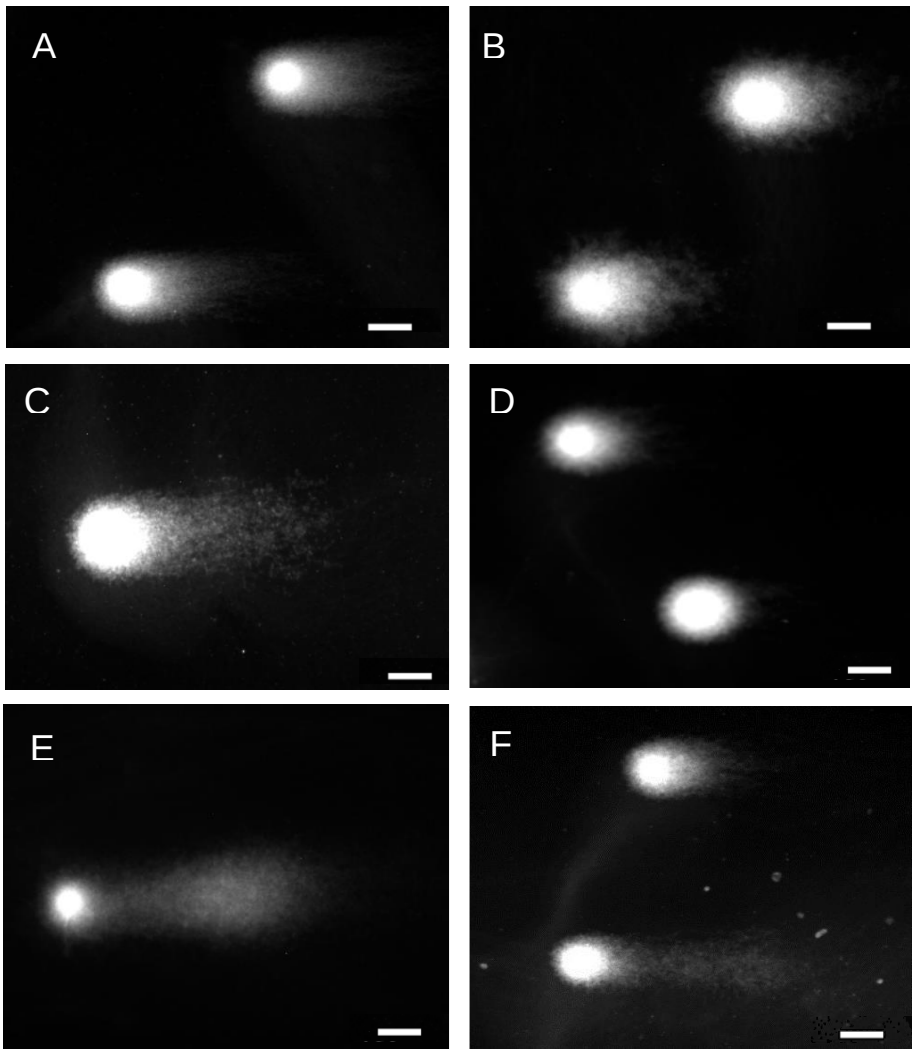

Figure 3.1: Images of Comets (A, B, C,D,E,F) in human sperm under different storage conditions. *[A] Fresh sperm (comet conducted on same day) [B] Sperm on Ice overnight at 4°C. [C] Sperm frozen at -80°C without cryoprotectant. [D] Sperm frozen at -80°C with cryoprotectant. [E] Sperm flash frozen at -196°C without cryoprotectant. [F] Sperm flash frozen at -196°C with cryoprotectant. Representative sample N= 4. Scale bar = 100µm*

Sperm cells displayed extended comet tails, indicating increased percentage of DNA damage. Using the neutral comet assay conditions, samples had a high percentage of DNA fragmentation throughout all storage conditions. From Figure 3.1. Tail lengths were significantly more extended in rapid (flash) frozen samples versus fresh samples (Figure 3.1 A, E, F). Furthermore, comet tails are shorter and more compacted in samples supplemented with cryoprotectant.

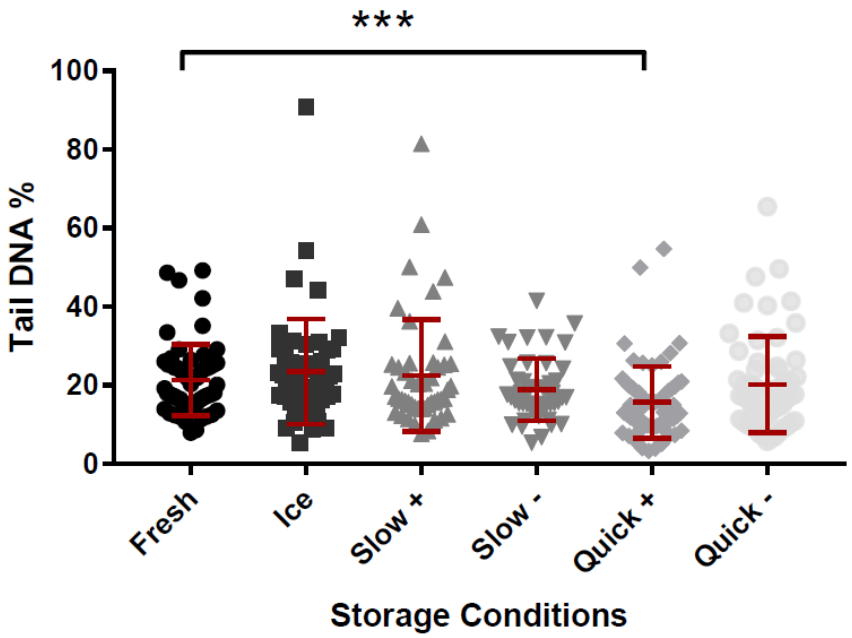

Figure 3.2: Storage condition optimisation. *Six different storage conditions were used, from a fresh sample to a quick freeze, with or without a cryoprotectant. N = 4, P value = 0.0003 (p < 0.05). * P < 0.05, ** P < 0.01, *** P < 0.001, **** p < 0.0001.*

A Kruskal Wallis test demonstrated a significant difference (p = 0.0003) between a fresh sample and a rapid (flash) frozen sample in liquid nitrogen with the addition of cryoprotectant (Quick +) i.e. Sperm freeze media - as depicted in Figure 3.2. Full values from Kruskal Wallis test can be seen in Appendix G.

3.1.2 Optimisation of Media and Incubation time

To investigate any confounding effects the media and incubation time may have on the stability and quality of nuclear sperm DNA, optimisation assays on both types of media and incubation times was carried out. In total 18 different conditions were performed. Figure 3.3 shows the comets derived from sperm treated in six of the 18 conditions. Refer to Appendix F for remaining experimental conditions.

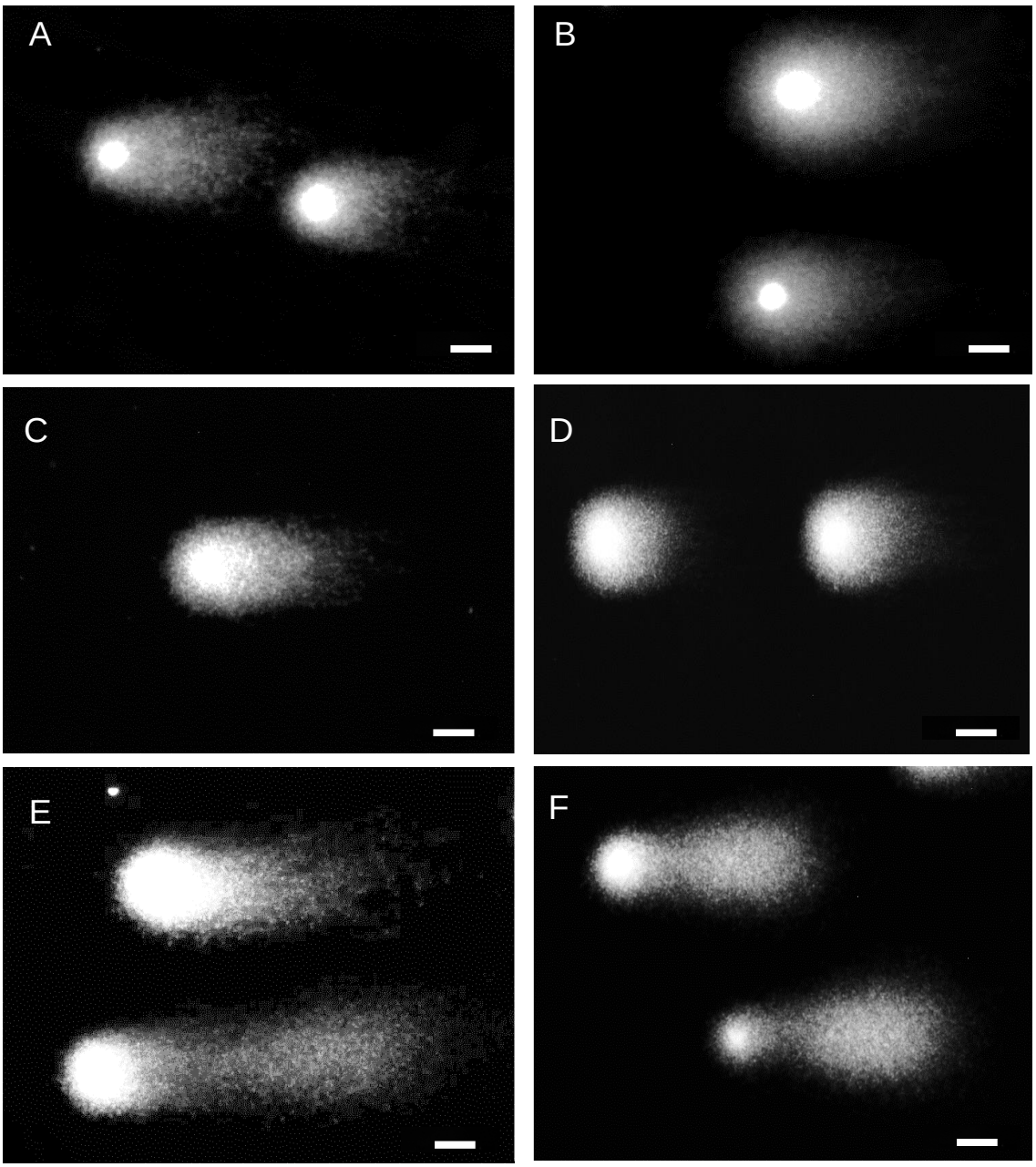

Figure 3.3: Neutral comet optimisation using different incubation times, types of media and H_2O_2 concentrations. *Slides were stained with SYBR Gold.* ***[A]*** *Human sperm treated in PBS at 0µM H_2O_2 for 30 minutes.* ***[B]*** *Sperm treated in SPM at 0µM H_2O_2 for 30 minutes.* ***[C]*** *Sperm treated in PBS at 100µM H_2O_2 for 60 minutes.* ***[D]*** *Sperm treated in SPM at 100µM H_2O_2 for 60 minutes.* ***[E]*** *Sperm treated in PBS at 500µM H_2O_2 for 120 minutes.* ***[F]*** *Sperm treated in SPM at 500µM H_2O_2 for 120 minutes. Scale bar = 100µm*

To investigate the different types of media and incubation time, samples were incubated in either sperm preparation media (SPM) or phosphate buffered solution (PBS), with either 0µM, 100µM or 500µM of H_2O_2. Furthermore, the duration of incubation ranged from 30 minutes to 120 minutes. Comet assay showed largely prolonged tails for the highest concentration (500µM of H_2O_2) and longest incubation time (120 minutes) (Figure 3.3 E, F). SPM was the better media to use as comets displayed a more intact, less granulated tail (Figure 3.3 B, D, F). Furthermore, from Figure 3.4 A, there is no effect from a 30-minute incubation period, as all significant differences noted are between 60 minute and 120-minute incubation periods.

All data sets required non-parametric analysis due to values lying outside of normal ranges. From using the Kruskal-Wallis statistical test, there were several significant differences between all conditions for each parameter. Table 3.1, 3.2 and 3.3 below, lists all significant p-values found, all of which correlate to Figure 3.4.

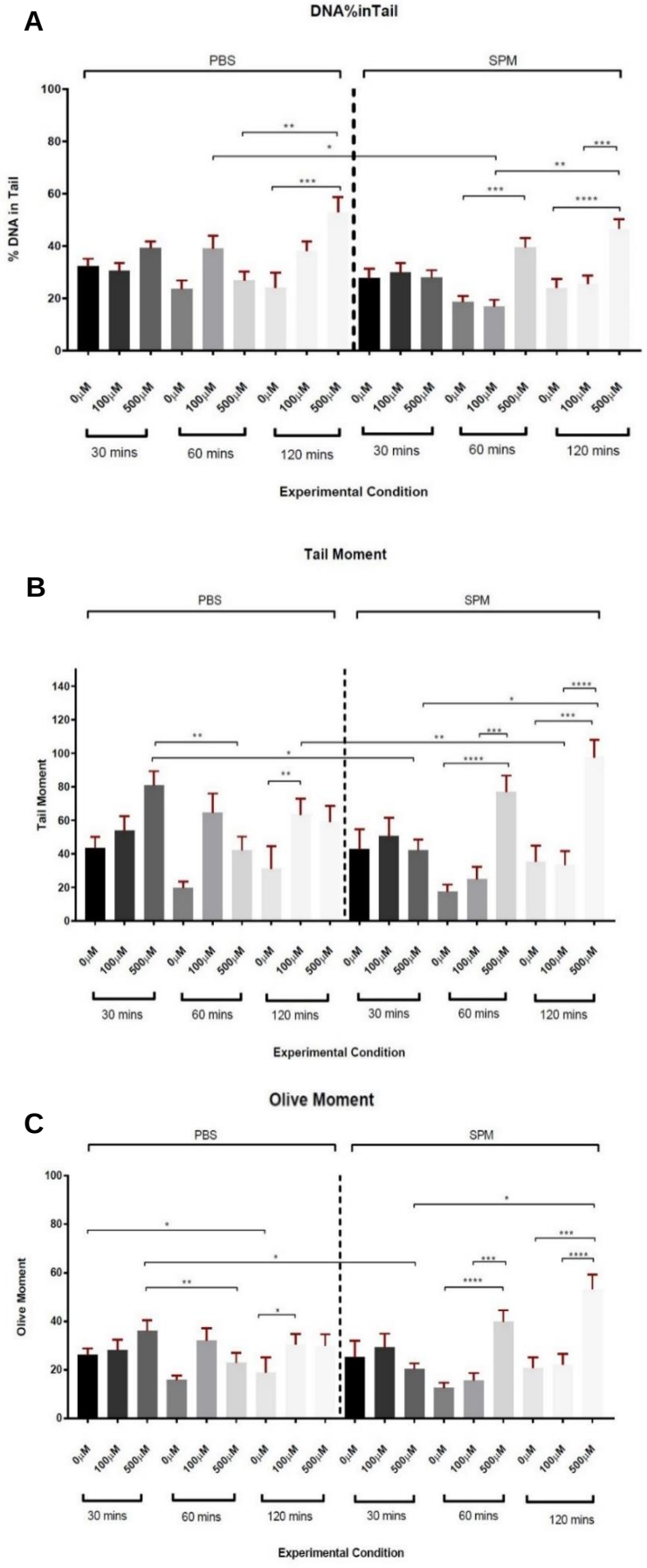

Figure 3.4: Optimisation of incubation period and type of media. *All results generated from each parameter: [A] DNA % in tail, [B] Tail moment and [C] Olive moment are plotted against each other. Significant difference observed p < 0.05. * P < 0.05, ** P < 0.01, ** P < 0.01, *** P < 0.001, **** p < 0.0001.*

***Table 3.1**: DNA % in tail p-values, for the optimisation of incubation time and type of media. (Figure 3.4 A)*

Dunn's multiple comparisons test	Mean Rank diff.	Significant?	Summary	Adjusted P Value
0.SPM.60 vs. 500.SPM.60	-206.3	Yes	***	0.0001
0.PBS.120 vs. 500.PBS.120	-275.3	Yes	***	0.0001
0.SPM.120 vs. 500.SPM.120	-212.2	Yes	***	0.0010
100.PBS.60 vs. 100.SPM.60	208.5	Yes	*	0.0206
100.SPM.60 vs. 500.SPM.60	-222.5	Yes	**	0.0011
100.SPM.120 vs. 500.SPM.120	-203.4	Yes	***	0.0003
500.PBS.60 vs. 500.PBS.120	-200.4	Yes	**	0.0038

***Table 3.2:** Tail moment p-values, for the optimisation of incubation time and type of media. (Figure 3.4, B)*

Dunn's multiple comparisons test	Mean Rank diff.	Significant?	Summary	Adjusted P Value
0.SPM.60 vs. 500.SPM.60	-245	Yes	****	<0.0001
0.PBS.120 vs. 100.PBS.120	-258	Yes	**	0.0040
0.SPM.120 vs. 500.SPM.120	-234	Yes	***	0.0001
100.SPM.60 vs. 500.SPM.60	-226	Yes	***	0.0008
100.PBS.120 vs. 100.SPM.120	214	Yes	**	0.0063
100.SPM.120 vs. 500.SPM.120	-264	Yes	****	<0.0001
500.PBS.30 vs. 500.SPM.30	163	Yes	*	0.0282
500.PBS.30 vs. 500.PBS.60	178	Yes	**	0.0089
500.SPM.30 vs. 500.SPM.120	-170	Yes	*	0.0195

***Table 3.3:** Olive moment p-values, for the optimisation of incubation time and type of media. (Figure3.4, C)*

Dunn's multiple comparisons test	Mean Rank diff.	Significant?	Summary	Adjusted P Value
0.PBS.30 vs. 0.PBS.120	212	Yes	*	0.0186
0.SPM.60 vs. 500.SPM.60	-261	Yes	****	<0.0001
0.PBS.120 vs. 100.PBS.120	-241	Yes	*	0.0138
0.SPM.120 vs. 500.SPM.120	-216	Yes	***	0.0007
100.SPM.60 vs. 500.SPM.60	-229	Yes	***	0.0006
100.SPM.120 vs. 500.SPM.120	-231	Yes	****	<0.0001
500.PBS.30 vs. 500.SPM.30	158	Yes	*	0.0434
500.PBS.30 vs. 500.PBS.60	179	Yes	**	0.0082
500.SPM.30 vs. 500.SPM.120	-171	Yes	*	0.0180

3.1.3 Optimisation of Cell number

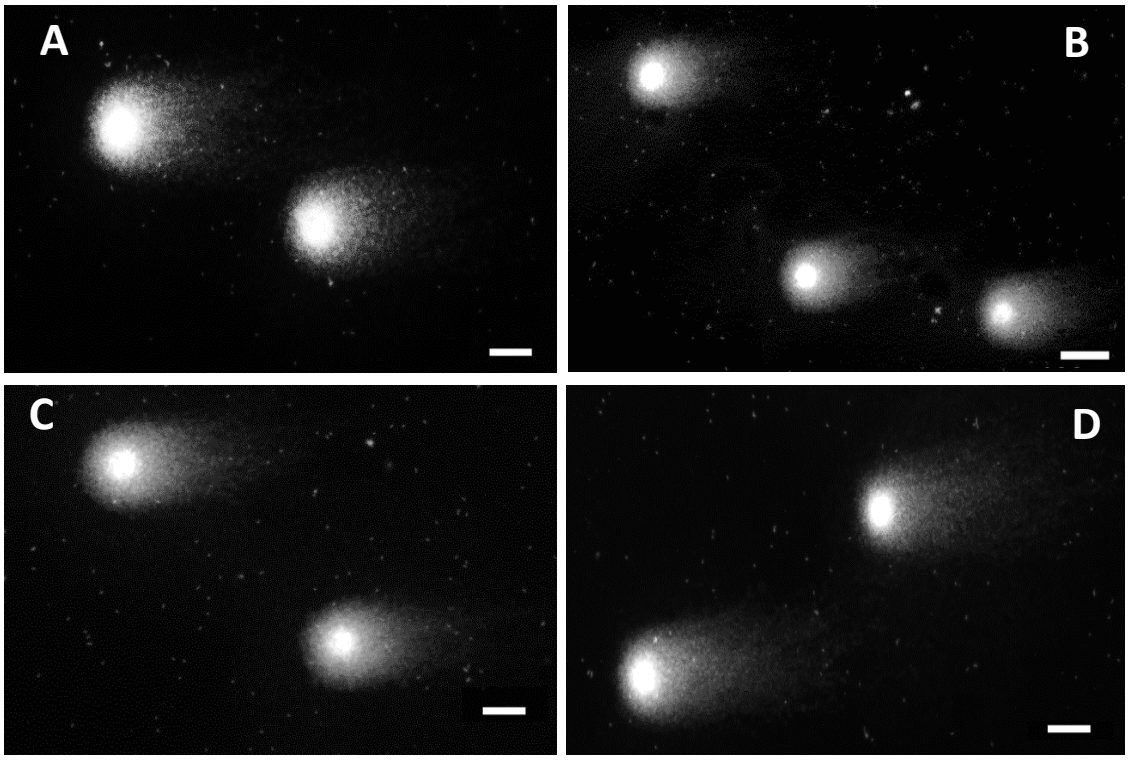

Figure 3.5: Neutral comet assay using different sperm cell numbers incubated for 2-hours with 500µM of H_2O_2. *[A] 1 million cells, [B] 5 million cells, [C]10million cells, [D] 20 million cells. Slides were stained with SYBR Gold. Scale bar = 100µm*

As the number of sperm cells treated may affect the outcome of the H_2O_2 treatments (Agarwal et al., 2014) a serial cell number (1, 5, 10, 20 million) underwent H_2O_2 treatment followed by processing for comet assay. The condition with the lowest concentration of cells (1million) were found to have the highest level of DNA damage, Figure 3.5 shows that treatment of sperm with H_2O_2 for those with a higher number of cells (20 million) had significantly lower levels of DNA damage when compared to the control.

Figure 3.6 shows the analysis of the cell number optimisation. A Mann-Whitney test was used as a non-parametric equivalent to a one-way ANOVA. The p-value was calculated using 1 million as the control. Difference between all the groups and 1 million is significant. P value = <0.0001 (1mill vs 5 mill), P = <0.0001 (1mill vs 10 mill) and P = 0.0429 (1mill vs 20mill). Full results from statistical tests can be seen in Appendix G.

The three parameters analysed (%DNA in Tail, Olive Moment, Tail Moment) are the most frequently used in literature (Gyori et al., 2014).

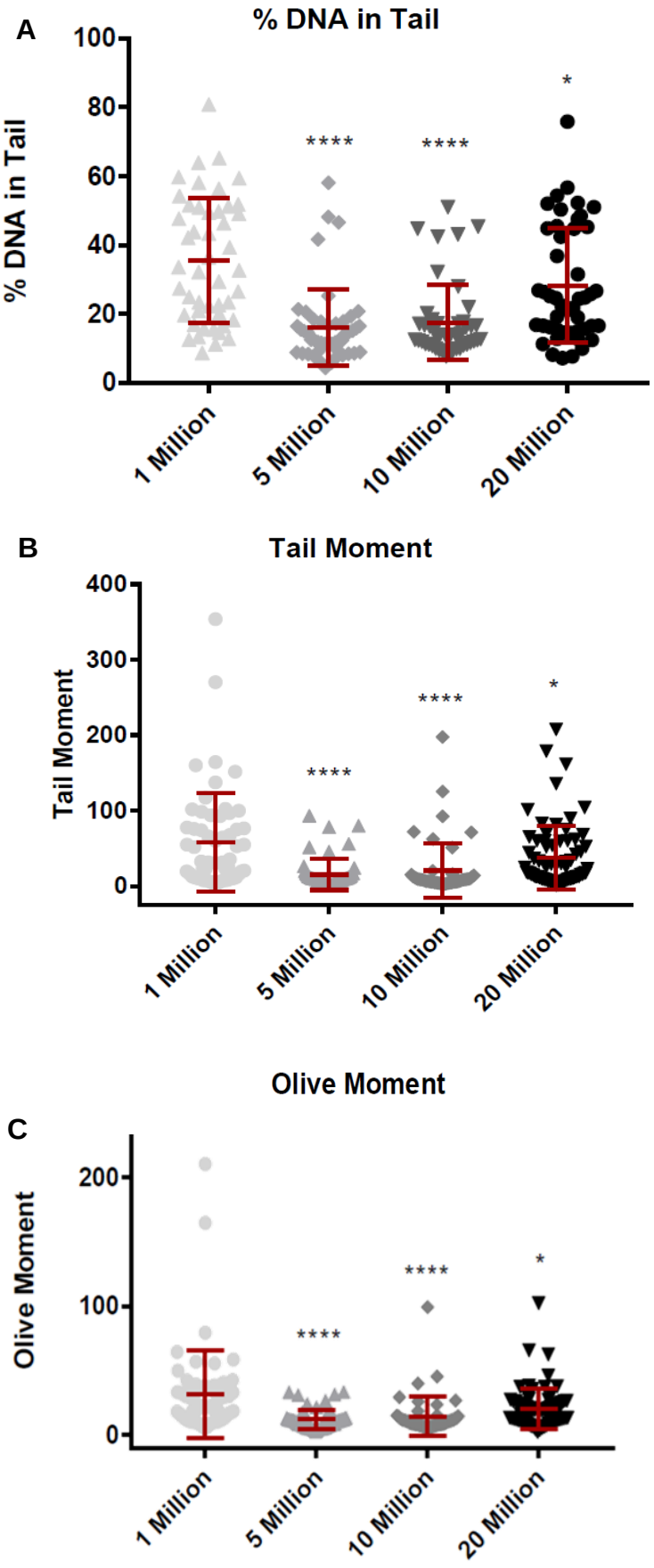

Figure 3.6: Statistical Analysis of Sperm Cell number using a non-parametric Mann-Whitney Test. *P-value (<0.05) was calculated using 1 million as the control. [A] % DNA within Comet Tail. [B] Tail moment = Tail length x % DNA in tail. [C] Olive moment: Tail DNA %*

3.1.4 Treatment of Sperm cells using H_2O_2

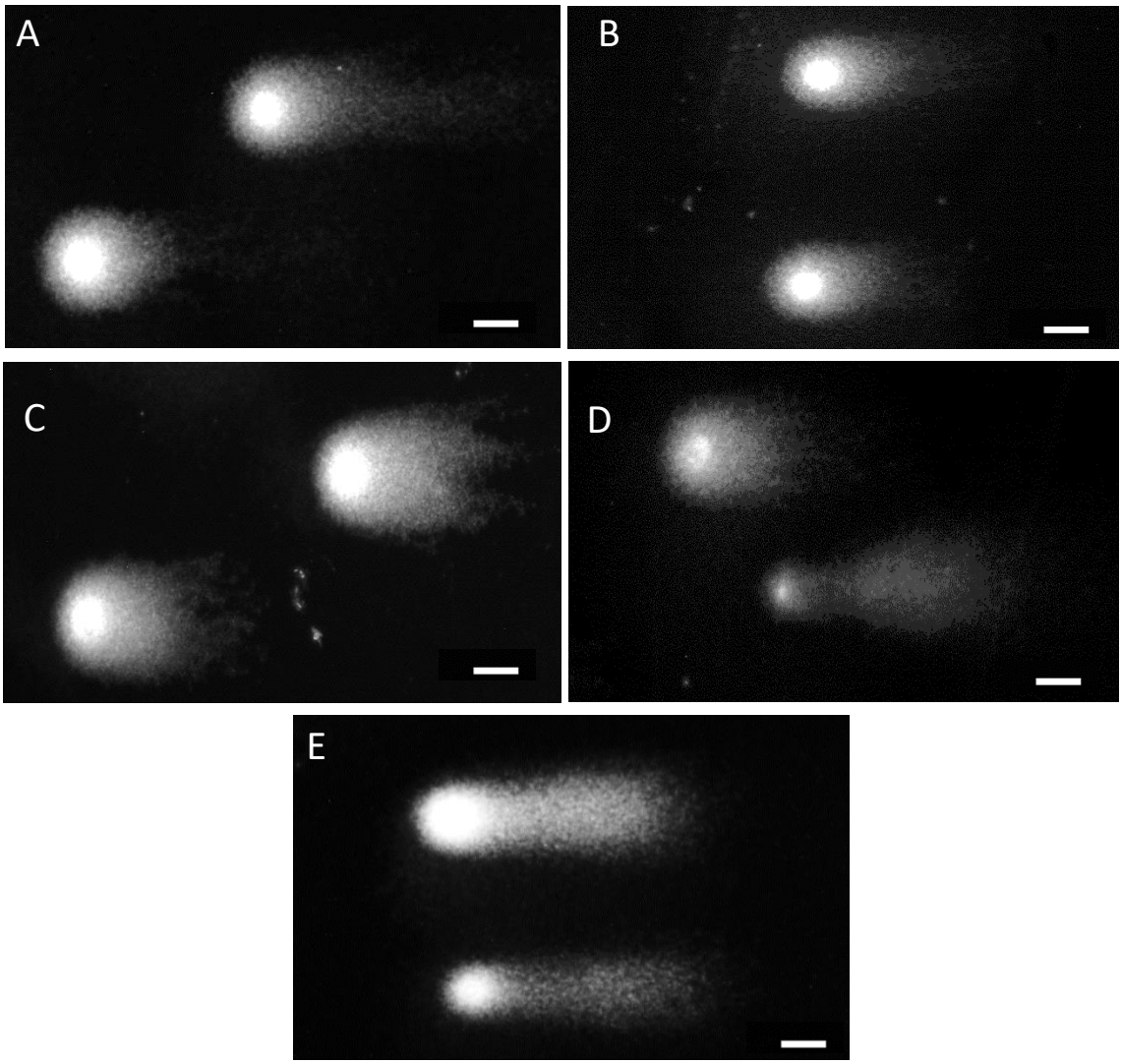

Figure 3.7: Comet images of sperm cells, treated in SPM for 2 hours then incubated in lysis buffer (1 hour) with DTT (1 hour) and Proteinase K (1.5 hours, Section 2.5.1). *Different H_2O_2 concentrations were used for each condition. [A] Cells treated with 0µM, [B] Cells treated with 50µM, [C] Cells treated with 100µM, [D] Cells treated with 200µM, [E] Cells treated with 500µM. Representative sample; n = 3. Scale bar = 100µm.*

Comet assay of human sperm (20 million cells per condition) showed an association between an increased level of DNA damage and the increase in H_2O_2 concentration (Figure 3.7, A-E). In Figure 3.7 A, the non-treatment control - minimal levels of DNA damage was observed in the comet tail. A dose response treatment of sperm was treated with H_2O_2 concentrations ranging from 50µM to 500µM (Figure 3.7, B to E). This was done to detect whether higher concentrations of H_2O_2 would have a direct correlation to the amount of DNA damage i.e. Percentage of DNA in tail; further imitating the increased level of ROS *in vivo*. In Figure 3.7, E there is a significantly large tail trailing behind the head of the comet; this specifies that highly concentrated levels of H_2O_2 can

successfully deteriorate DNA integrity by inducing DNA damage. Statistical analysis (Figure 3.8) showed a significant difference in percentage tail DNA between 0µM and 500µM H_2O_2 concentration. Indicating a positive correlation between the DNA % in tail and increasing H_2O_2 concentration. [Kruskal-Wallis; p value = 0.0001]. Full statistical results can be found in Appendix G

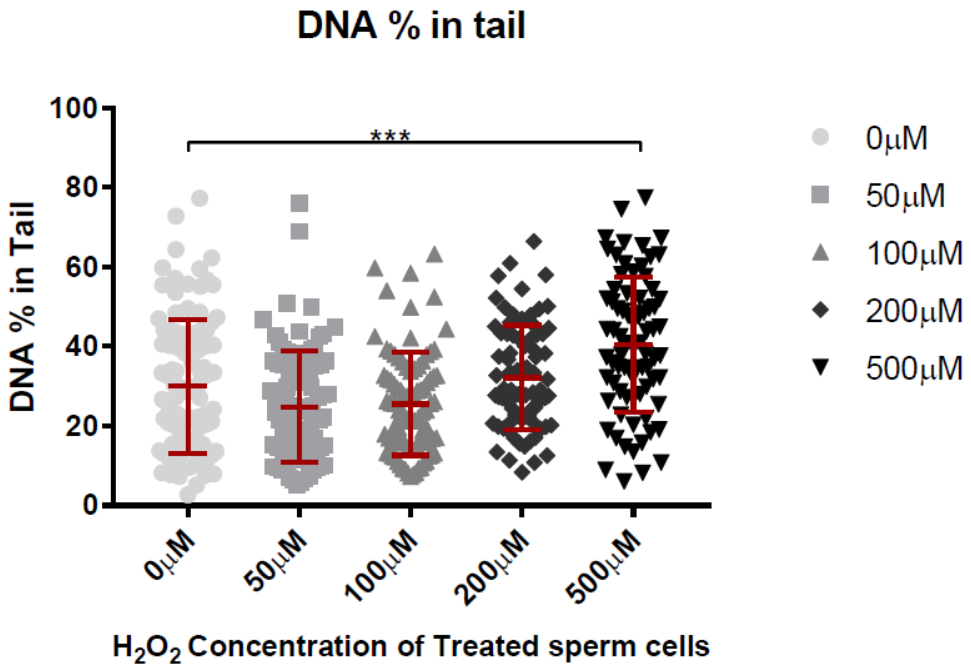

Figure 3.8: Statistical analysis of H_2O_2 treated sperm cells. *Treated for 120 minutes and prepared in Sperm Preparation Media (SPM) alongside a non-treatment control (0µM). A Kruskal-Wallis non-parametric test was utilised, finding a significant difference P < 0.05*

3.2 Electrochemistry

3.2.1 Electrochemical Analyses on H_2O_2 treated Sperm DNA

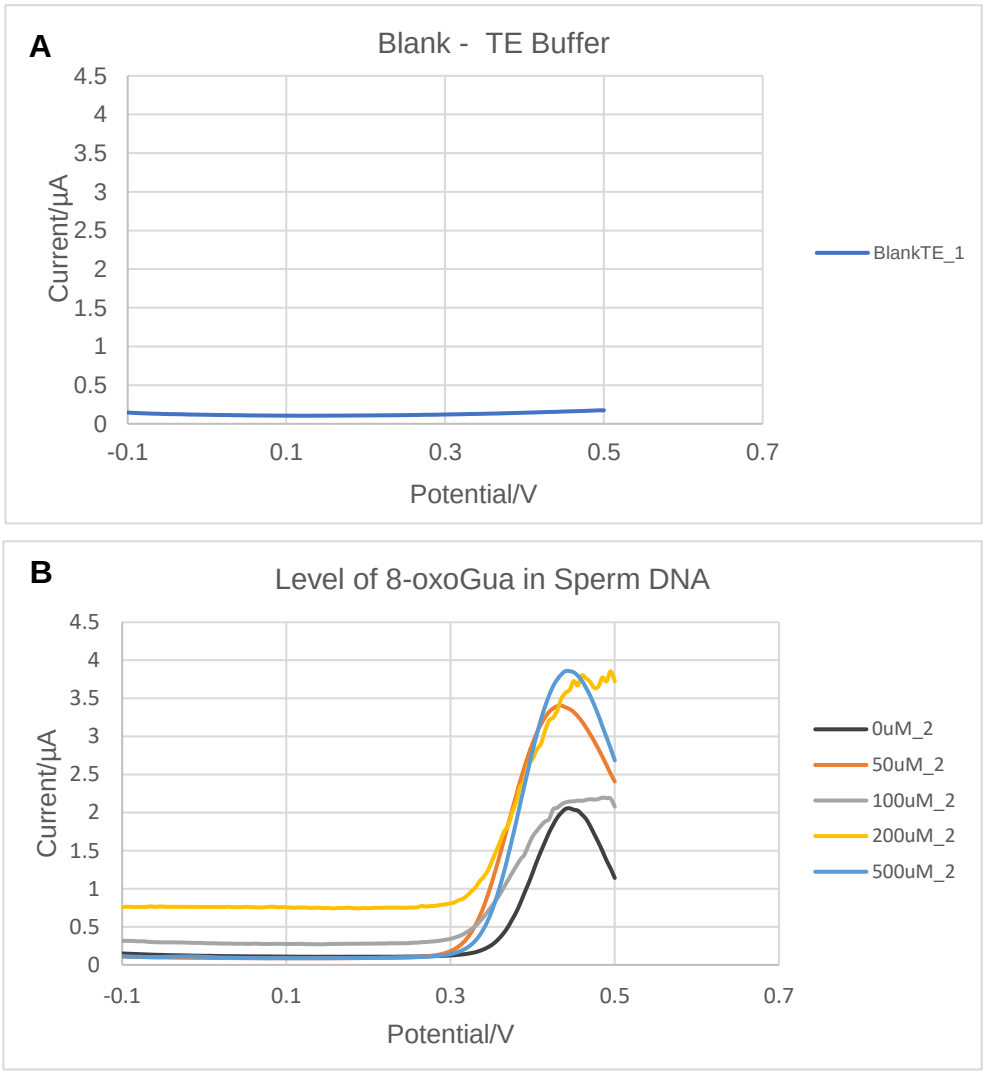

Figure 3.9: DPV measurements of extracted DNA using SPEs. *[A] TE buffer only [B] H2O2 treated extracted DNA from five different conditions*

SPEs were treated with TE buffer prior to experiment, the results (Figure 3.9 A). show that there was neither 8-oxoGua nor guanine in TE buffer. Figure 3.9-B shows the results of the electrochemical analysis from the five H_2O_2 treated samples. The SPEs were unable to detect 8-oxoGua within these biological samples, due to the absence of a peak at 0.1V. Furthermore, the electrodes could identify guanine within sperm DNA (large peak at 0.4V).

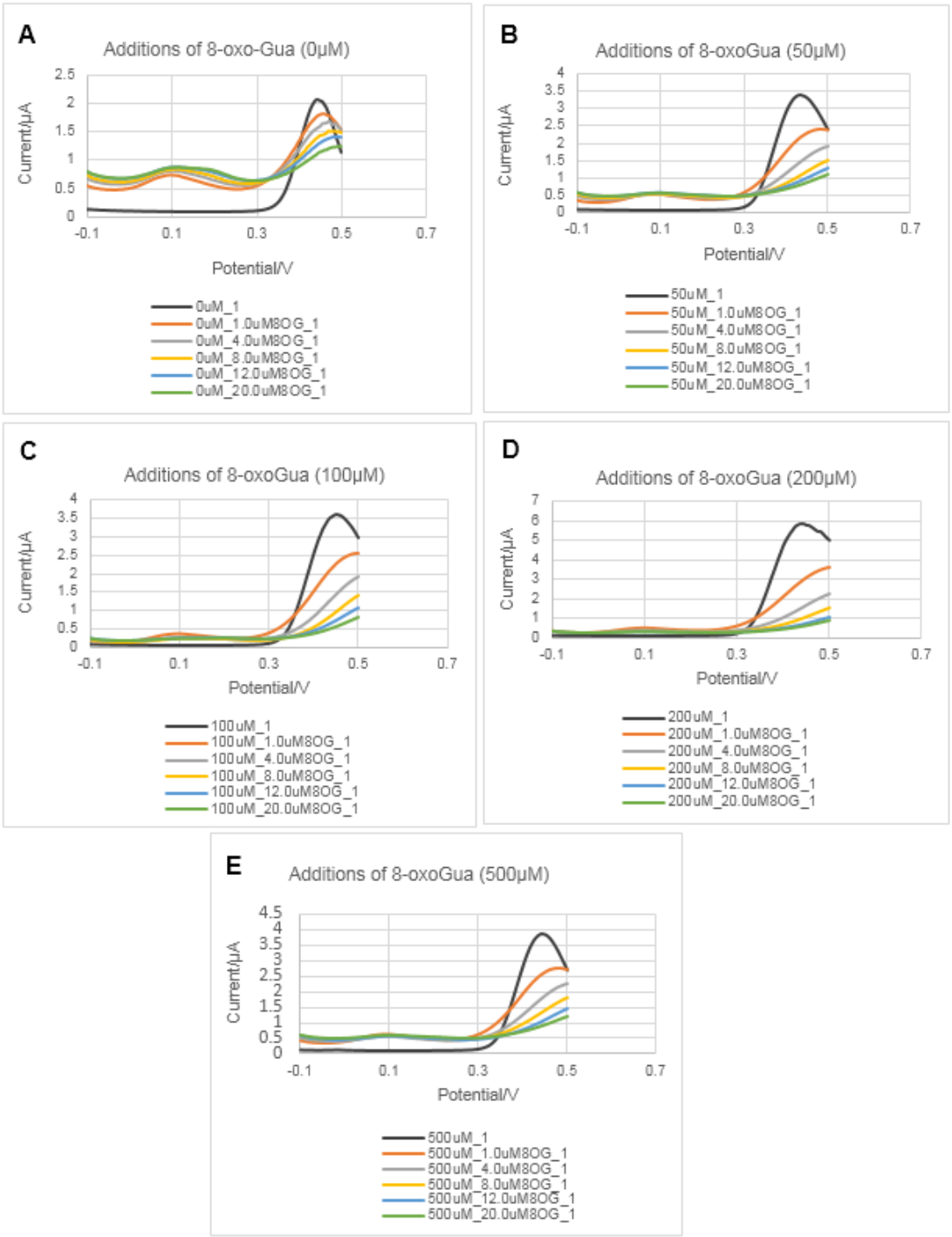

Figure 3.10: DPV electrochemical measurements from extracted sperm DNA, treated with a concentration gradient of H_2O_2 and spiked with various concentrations of pure 8-oxoGua (1 – 20 µM). *[A] No treatment control (0µM), [B] DNA treated with 50µM,* ***[C]*** *DNA treated with 100µM,* ***[D]*** *DNA treated with 200µM,* ***[E]*** *DNA treated with 500µM. Representative sample: N = 3.*

Samples were placed on the working electrode of an SPE and a current was passed through, the read outs generated from the PS Trace Palmsens software are shown in Figure 3.10, A-E. In all samples and conditions a larger peak seen at 0.4V approx. this indicates the presence of nucleobase Guanine in the sample (Bernalte et al., 2017). All H_2O_2 treated conditions (Figure 3.10), were unable to

detect 8-oxoGua in the extracted sperm DNA as indicated by the black line. Additionally, samples were spiked with known concentrations of pure 8-oxoGua and analysed using the SPE as shown by the coloured lines (Figure 3.10). With the addition of pure 8-oxoGua small peaks can be seen at 0.1V, which indicates the presence of 8-oxoGua.

3.3 Detection of 8-oxoGuanine with semen

3.3.1 H_2O_2 Treatment of Sperm cells within Semen

Semen containing $20x10^6$ sperm cells were either treated with H_2O_2 (500µM) or non-treated control. Using the neutral comet assay a visible difference between the two conditions (Figure 3.11).

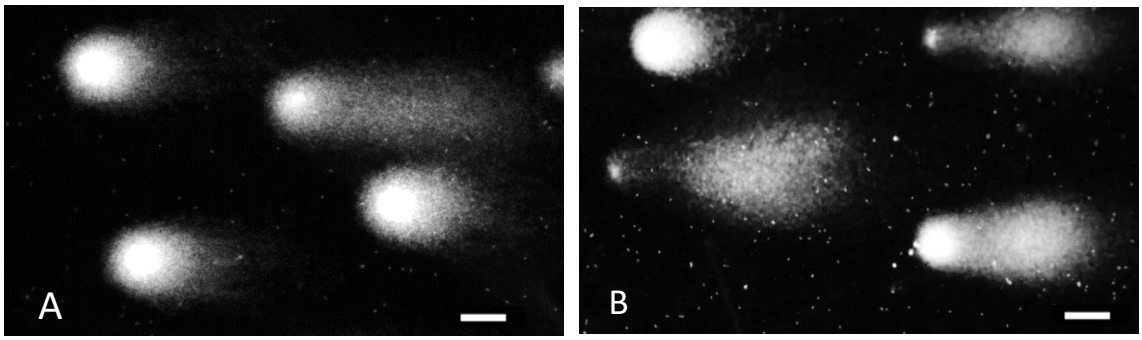

Figure 3.11: Comet assay from sperm cells incubated in their seminal plasma for 120 minutes*. [A] untreated sperm cells (control). [B] Human sperm cells treated with 500µM H2O2. Experimental condition repeated three times. Scale bar = 100µm*

Comet assay showed that sperm cells treated with 500 µM of H_2O_2 had elongated tails whereas in the control samples, DNA fragmentation was limited. The H_2O_2 exposure (500µM, n = 3, one semen sample) were compared to a non-treatment control to calculate the p-value (Kruskal-Wallis test). For each parameter, more than one significance difference is displayed (Figure 3.12)

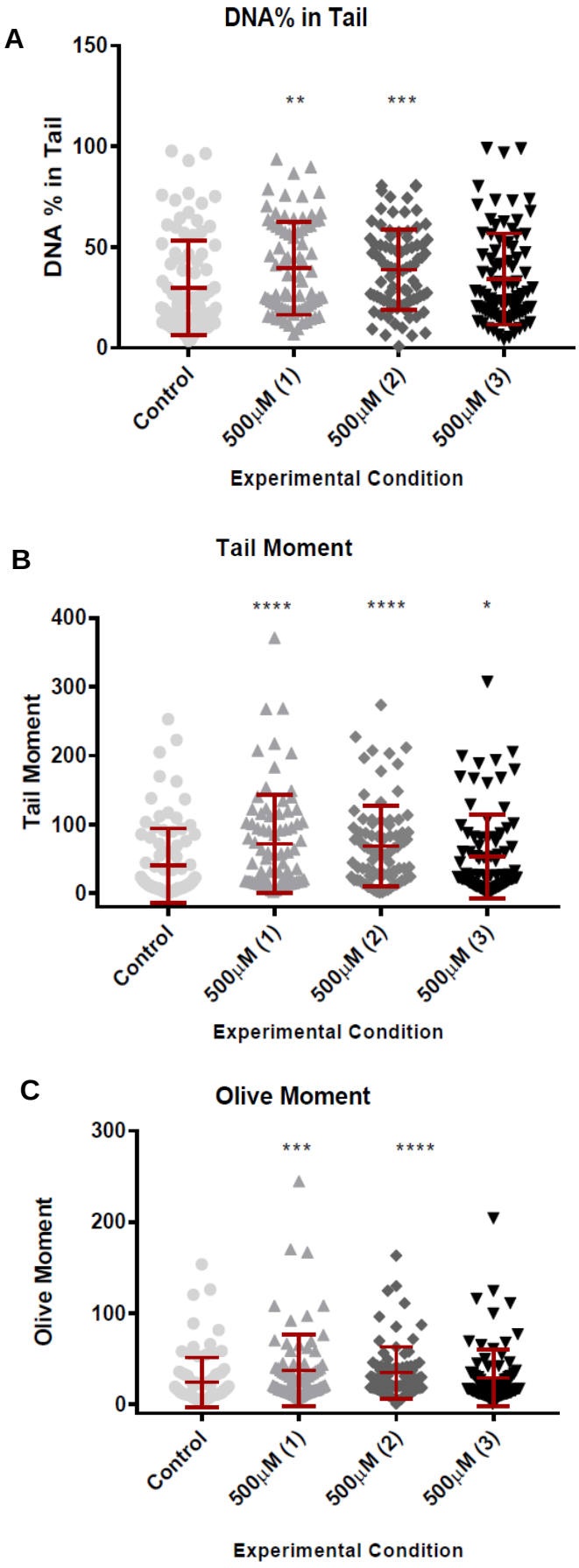

Figure 3.12: Analysis of comet assays using cells treated with H_2O_2 in semen (seminal plasma). *Kruskal-Wallis, P <0.05, N = 1, Exp. = 3 [A] % DNA in Tail – P value: 0.0015, 0.0004 [B] Tail moment – P value: <0.0001, <0.0001, 0.0333 [C] Olive moment – P value: 0.0003*

3.3.2 Electrochemical analysis of H_2O_2 treated cells in semen

Measurements of 8-oxoGua in sperm free seminal plasma, was carried out using the SPEs.

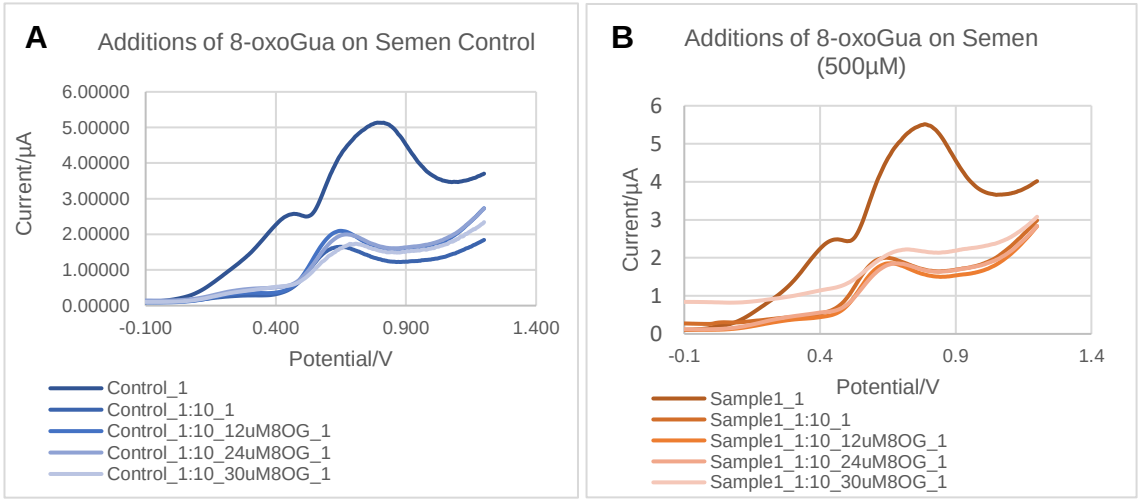

Figure 3.13: Electrochemical analysis of H_2O_2 treated and extracted DNA in semen. *N =1 Exp = 3. [A] No treatment control (0µM), [B] Treatment with 500µM H_2O_2*

Electrochemical analysis of seminal plasma, detected 8-oxoGua at 0.4V in undiluted samples and at 0.6V in diluted samples from using the SPEs. 8-oxoguanine was detected after spiking the diluted semen samples only (small peaks around 0.6V), this consolidated the detection of 8-oxoguanine in seminal plasma using the SPEs. The larger peaks in both Figure 3.13 A and B show the detection of Guanine other nucleotides which may be present in the semen sample. Seminal plasma required a 1:10 dilution,which resulted in the voltametric signal to shift to the right, denoting a decrease in pH. A recent study found that pH has a significant effect on DPV responses when detecting 8-oxoGua in PBS solution (Bernalte et al., 2017).

4 Discussion

The presence of high levels of reactive oxygen species (ROS) in relation to male infertility is well documented (Aitken et al., 2010). The involvement of oxidative sperm damage is due to the capacity of ROS to directly damage DNA and induce detrimental chemical and structural changes.

ROS-induced DNA damage can occur at all stages of spermatogenesis. Furthermore, increased sperm DNA damage is highly associated with, both decreased sperm function and fertilization rates (Robinson et al., 2012).

Exposure to ROS can generate various modified DNA bases. The most abundant adduct is 8-oxoGuanine (8-oxoGua) which plays a major role in mutagenesis and in carcinogenesis (Soultanakis et al., 2000; Singh et al., 2011)

The oxidation of guanine is enhanced in the presence of H_2O_2 (Kasai and Nishimura, 1984), therefore this study set out to detect levels of 8-oxoguanine in sperm DNA and seminal plasma after H_2O_2 exposure. The degree of DNA damage was assessed using the neutral comet assay, and the levels of 8-oxoGua was assessed using differential pulse voltammetry.

4.1 Oxidative DNA damage

4.1.1 Comet Assay

The comet assay is a common technique used to detect DNA fragmentation; it is a relatively simple and reliable method to measure double stranded DNA breaks in somatic and sperm cells (Lovell and Omori, 2008). The level of DNA fragmentation assessed by the comet assay is associated with lower fertilisation rates and increased miscarrige rates (Chi et al., 2011). The comet assay measures the degree of DNA fragmentation through an electrophoretic field – where smaller DNA fragments trail behind the cell's nuclear DNA. Comets with longer tails contain more dispersed DNA fragments i.e. higher levels of damage (Olive and Banath, 2006). Sperm DNA is compacted in the nucleus by its association with protamines, which make up a large percentage of the protein content of sperm (Gunes et al., 2015). To ensure they are removed and to

improve the sensitivity and reliability of the comet assay; DTT and Proteinase K are added to the lysis buffer prior incubation (Aoki et al., 2006). Proteinase K is more active at 37^{o}C whereas DTT breaks disulfide bonds between adjacent protamines at lower temperatures (4^{o}C).

The degree of DNA damage is determined by the pixel intensity within the comet tail, (Table 1.1). Studies have shown that the exact parameter to measure DNA damage is subjective; therefore, a combination of these parameters can help calculate this value. DNA percentage in the tail is preferred as values range from 0% to 100%, enabling comparisons between variables to be made. Tail moment and olive moment are common descriptors used alongside DNA percentage in tail to generate a true representation of DNA damage (Gyori et al., 2014; Lovell and Omori, 2008).

Following optimisation and dose-dependent treatment with H_2O_2, the degree of sperm DNA fragmentation was proportional to the concentration of H_2O_2 (Figure 3.7). The comet assay was utilised in this study to induce oxidative DNA damage and formation of 8-oxoGua (Mo et al., 1992).

The presence of H_2O_2 induces oxidative stress, which causes the formation of multiple DNA strand breaks. The increasing length of the comet tail is directly proportional to the number of DNA strand breaks. (Irvine et al., 2000)

4.1.2 Electrochemical analyses of 8-OxoGua in H_2O_2 treated sperm cells

Measuring 8-oxoGua in biological samples can be used as a biomarker for oxidative stress. 8-oxoGua can be detected using LC-MS and HPLC. This study attempted to use screen-printed electrodes to measure 8-oxoGua in sperm treated with H_2O_2. A recent study reported the use of SPEs to measure 8-oxoGua in the presence of other nucleobases (guanine and adenine) and in a solution of organic compounds. This study demonstrated a proof-of-concept process of detecting 8-oxoGua in background of other nucleobases and in a mixed organic solution (artificial semen) (Bernalte et al., 2017).

Previous methods such as glassy carbon strips have been used to detect levels of 8-oxoGua with a detection limit of 0.80µM (Brett et al., 2017; Brett, 2000) .

SPEs provide a simpler method to evaluate DNA damage by quantifying levels of 8-oxoGua as a biomarker of oxidative stress.

SPEs were employed to detect 8-oxoGua adducts in sperm cells treated with H_2O_2. Isolated DNA was placed on an SPE (as described in section 2.8.1). In the present study, 8-oxoGua was not detected in the extracted DNA. However, 8-oxoGua could be seen in the same DNA samples after being spiked with pure 8-oxoGua, to increase the overall concentration of 8-oxoGua within the DNA. This signifies that the SPEs were not sensitive enough to detect the low levels of 8-oxoGua within the H_2O_2. treated DNA. By adding pure 8-oxoGua to the pre-extracted DNA allowed a positive detection of 8-oxoGua at higher levels. This confirms higher detection limits and the functionality of the SPEs. (Figure 3.10).

The lack of 8-oxoGua detection from treated DNA at peak 0.1V (Figure 3.10) is likely due to low levels of 8-oxoGua in the samples. Furthermore, levels of 8-oxoGua can be presented as number of oxidised guanines per total number of guanines, which is about 1–5 per 10^6 in DNA (Collins et al., 2002). Therefore, the concentration of 8-oxoGua in cells represents the balance between formation and repair rates, which can fluctuate dramatically (Deng et al., 1997). As no levels of 8-oxoGua was detected in the DNA samples, SPE was used to analyse levels in seminal fluid.

The detection of 8-oxoGua in urine and seminal plasma has been carried out using HPLC-MS/MS (Lam et al., 2012). This study reported that free 8-oxoGua in seminal plasma was more detectable than from DNA adducts. However, HPLC-MS/MS requires expensive equipment and specialised training.

Studies using electrochemical detection of 8-oxoGua in biological fluids (urine and human plasma) have reported detection levels at below 100nM (Long et al., 1999). The simplicity and ease of using electrochemical analysis offers a straightforward and cost effective assay for measuring oxidative stress. In the current study, samples of semen containing $20x10^6$ were exposed to 500µM of H_2O_2, levels of 8-oxoGua was measured using SPE as per (Bernalte et al., 2017). To ensure oxidative damage of sperm DNA was present, comet assay was carried out on the sperm from this assay (Figure 3.11).

The maximum concentration of H_2O_2 to induce significant DNA damage was 500µM. This treatment exposure was carried out in semen. Sperm cells were

removed and processed for the comet assay. The levels of DNA fragmentation in H_2O_2 treated samples were more significant than untreated controls (Figure 3.11) – thus indicating the presence of oxidative damage. Determination of 8-oxoGua in seminal plasma was carried out as per (Bernalte et al., 2017).

SPE analysis of seminal plasma for 8-oxoGua generated a voltammetric signal, which corresponded to 8-oxoGua at 0.4V and 0.6V in undiluted and diluted samples respectively. This demonstrates that 8-oxoGua can be detected in seminal plasma – however, due to the limitation of samples tested (one semen sample) – these findings are inconclusive and more work is required.

Seminal plasma contains antioxidants, which protect the sperm from oxidative damage (Walczak–Jedrzejowska et al., 2013; Pahune et al., 2013). The lack of notable difference in 8-oxoGua in the control and treated conditions may be due to the antioxidant properties of semen.

4.2 Technical consideration and limitations

Development of a novel technique comes with challenges. The overall aim of this study was to develop a cost effective and simple assay to detect oxidative damage in sperm. The process was three-fold – (1) induce oxidative damage by exposure of sperm to H_2O_2 and measure DNA damage via comet, (2) detect 8-oxoGua in DNA undergone oxidative damage and (3) detect 8-oxoGua in seminal plasma.

4.2.1 DNA Extraction

Oxidative DNA damage may be induced during the DNA extraction processes (Helbock et al., 1998) therefore a number of extraction procedures were carried out. Phenol Chloroform was used to extract DNA from maximum of 20 million sperm cells. Phenol Chloroform, was the preferred method of extraction as this has been reported to reduce oxidative damage and in particular the induction of 8-oxoGua (Shigenaga et al., 1994) These findings are supported in another study where reduced 8-oxoGua levels were observed during phenol extraction of DNA in calf thymus (Helbock et al., 1998).

4.2.2 Comet assay optimisation

Four optimisation stages were required to achieve maximum specificity and sensitivity for the comet assay.

Two different media were used in this study, phosphate buffered saline (PBS) and sperm preparation medium (SPM). SPM is routinely used in IVF to culture sperm and contains antioxidants and vitamins that provide key nutrients for sperm survival (Henkel and Schill, 2003). High concentrations of bicarbonate assists the separation of motile sperm from immotile sperm, aiming to imitate conditions *in vivo* (Will et al., 2011).

Sperm cells were incubated with H_2O_2 for: 30 minutes, 60 minutes or 120 minutes, the treatment time with H_2O_2 and media was directly proportionate to the amount of DNA damage (Figure 3.3, 3.4). The number of cells within each experimental condition had a significant effect on the level of damage (Figure 3.5, 3.6).

Due to the experimental time scale, samples required freezing to carry out comet assay. Optimisation experiment investigated the level of DNA fragmentation from fresh or frozen sperm cells. From the two freezing methods used, a significant difference was found between fresh and rapid (flash) freezing. A slow freeze (-80^{o}C) method was utilised, which found no significant difference against fresh and rapid freezing (Figure 3.1, 3.2)

Optimal conditions for this investigation found treating 20 million sperm cells in SPM and H_2O_2 for 2 hours, and storing in a -80^{o}C freezer with cryoprotectant would be used throughout the study.

4.2.3 Electrochemical detection of 8-oxoGua

A lower detection limit for these SPEs was obtained at 0.33µM, this was significantly better than alternative electrode detection methods (i.e. graphite/ glassy carbon strips). However, even lower detection limits can be used in HPLC

(5nM) and this has enabled the detection of 8-oxoGua in several biological samples (Herbert et al., 1996; Lam et al., 2012).

Modification to SPEs using biochemical transducers, chemical mediators, or nanomaterials, could be implemented to improve the sensitivity and lower the detection limits of the SPEs (Bernalte et al., 2017).

Currently there is on-going studies looking into the clinical application of SPEs in a plethora of areas, in particular modifications to these SPEs which can improve the level of detection when using biological compounds within biological fluids(Couto et al., 2016)

Improvements to these electrodes may provide a successful clinical method of detection for hospital diagnosis, identifying potential risks of infertility within males.

5 Conclusion

The study focused on the detection of 8-oxoGuanine measurements within a biological fluid i.e. semen was investigated using electrochemistry. Sperm cells were exposed to a H_2O_2 treatments *in vitro* prior to analysis and did not detect 8-oxoGua when using the SPEs. Investigation into semen antioxidants found that 8-oxoGuanine could be induced from guanine by adding a potential (voltage) which is a novel finding. The comet assay was also explored and as expected an increase in H_2O_2 concentration was directly proportionate to the level of DNA damage.

Further work is required to develop a method which maintains a high yield and purity during the DNA extraction stage. Additionally, exploration into possible modifications of SPEs i.e. lowering the detection limits could assist the detection of 8-oxoguanine in cells, and help determine a potential correlation with DNA fragmentation levels. The results of this could create a modern day diagnostic technique linking sperm biology with electrochemical analyses however this remains to be discovered.

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Appendices

Appendix A

The MANCHESTER METROPOLITAN UNIVERSITY
Faculty of Science and Engineering
RISK ASSESSMENT COVER SHEET

FACULTY OF SCIENCE & ENGINEERING
SCHOOL: Healthcare Science
TITLE OF WORK: Using Electrochemical Quanitfication to measure levels of 8-Oxoguanine in Human sperm
LOCATION OF WORK (*LLn.nn*): 1st and 3rd Floor Molecular Biology Labs T3.01, a, b, c and d John Dalton Tower If off-site give contact details:
INTENDED ACTIVITIES (attach methods sheets (e.g. standard operating practices) and work schedules to this form): Conducting research to look levels of DNA damage (including 8-Oxoguanine) in Sperm DNA, for the completion of my MSc by research.
PERSONS AT RISK (including status [e.g. staff/student], for students please indicate course and level, for staff give contact email / phone number): • Undergraduate and Postgraduate Students • Academic, Technical and Research Staff • House services (i.e. cleaners) • Facilities and maintenance/service contractors • Participants • Any other users of the laboratory
HAZARDS (this should be a summary of the hazards anticipated – attach detailed assessments with appropriate risk control methods to this form): Biological: • Human Sperm • Handling of Hazard Group 1 & 2 biological substances • Culturing of mammalian cells Chemical: • Cryopreservation, of cells and other materials (-80°C Freezer or -196°C Liquid Nitrogen) • DNA extraction, creating cDNA and conducting PCR and Gel Electrophoresis • Analysis of samples using the FACS Verse machine (Flow cytometry) • Immunochemistry

- COMET Assay solutions, Total RNA purification kit components (B-mercaptoethanol; ethanol (70-100%), Human sperm DNA extraction kit, DNA methylation kit.

Physical/Mechanical/Electrical:

- Semen to be produced via masturbation
- Storing equipment above head height in laboratories,
- Centrifuges (Ultra/Mini/Bench top)
- Nano drop
- PCR and Gel Electrophoresis
- Use of Glassware and different temperatures (High/Low)
- Use of electrical equipment
- Correct handling and disposal of sharps

Are these hazards necessary in order to achieve the objectives of the activity? YES

Overall Hazard Rating: High

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION (appropriate COSHH data sheets / risk assessments must be attached to this form):

ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS.

NAME OF MATERIAL *Please provide also approximate quantity and concentration if applicable.*	HAZARD CLASS	HAZARD LABEL	Disposal *Hazardous materials must not be removed from laboratories. Disposal arrangements for the materials listed below in the location where the work is specified to be carried out are:*
Liquid Nitrogen	Warning		Leave small amounts of liquid nitrogen to evaporate in the fume hood. Never spill it on the floor and leave it to evaporate.
Lysis Solution	Warning		Can be poured down the sink
Column Preparation Solution	Warning		Can be poured down the sink
Proteinase K, from Tritirachium album	Danger		Dissolve or mix the material

			with a combustible solvent and burn in a chemical incinerator equipped with an afterburner and scrubber.
Buffer RLT (guanidinium thiocyanate)	Danger		Can be poured down the sink
Buffer RW1	Danger		Dispose of as hazardous waste in compliance with local and national regulations.
Ethanol (70-100%)	Danger/Flammable		To be diluted with lots of water and poured down the sink. Dispose of contaminated plastic ware in clinical waste bins. Dispose of remaining waste in organic waste disposal containers. Under fume hoods.
2-Mercaptoethanol	Danger		Do not pour down drain. This combustible material may be burned in a chemical incinerator equipped with an afterburner and scrubber
FACS Clean – contains bleach	Warning		This product is not considered a RCRA hazardous waste. Therefore flush used bleach solution down the sink with plenty of water.

Formaldehyde solution, 37% in water	Danger		Product This combustible material may be burned in a chemical incinerator equipped with an afterburner and scrubber. Offer surplus and non-recyclable solutions to a licensed disposal company. **It therefore needs to be collected for disposal.** Contaminated packaging Dispose of as unused product.
Human biological material	Warning	Biohazard 	- All contaminated materials, specimens and cultures must be decontaminated before disposal or cleaning for reuse -All biological waste should be incinerated
Paraformaldehyde.	Danger		Burn in a chemical incinerator equipped with an afterburner and scrubber but exert extra care in igniting, as this material is highly flammable. Offer surplus and non-recyclable solutions to a licensed disposal company.
Human Semen	Warning	Group 2 biological hazard 	-Contaminated consumables will be disposed of appropriately. E.g contaminated wastebins and contaminated sharps in sharp bins.

			-Benches and cabinets will be cleaned using either 70% IMS or 1% Virkon Samples will be stored at -80°C with participant ID number, and disposed of according to the HTA by incineration. -Lab coat and gloves will be worn at all times when handling samples and consumables.
Virkon (g)	Warning		Solid Virkon and any contaminated containers, including the original container will be disposed of as hazardous waste incinerated in clinical waste bags. Liquid virkon will be left overnight to decompose in a safe, designated area then disposed of down a designated sink with copious amounts of water.
RLRRL buffer	NA	NA	Dispose of kit components and contaminated plastic-ware in sealed containers in clinical waste bins.
B-mercaptoethanol	Danger		Dispose of contaminated plastic-ware in clinical waste bins. Do not pour waste down laboratory sink.
Dry Ice - Solid carbon dioxide pellets (dry ice) at a very low -78.5°C			Small quantities of dry ice can be left in the fume hood to sublimate. Any quantity can be returned to the dry ice bins
Sodium dodecyl sulphate (SDS) (µl)	Danger		Solid SDS (contained within the acrylamide gel) shall be placed in a biohazardous

			autoclavable bag and incinerated.
Triton X-100 (µl)	Warning		Dilute with plenty of water and pour down sink
Phenol-Chloroform	Danger		Dispose of contents/containers in accordance with local regulations. Contaminated packaging to be disposed of as unused product.
Hydrochloric Acid	Danger		If spilt; Soak up with inert absorbent material and dispose of as hazardous waste. Keep in suitable, closed containers for disposal. Do not pour down sink.
Sodium Hydroxide	Danger		Dissolve or mix material with a combustible solvent and burn in chemical incinerator. Do not pour down sink.
Potassium phosphate monobasic/dibasic	Warning		Dispose of contents/containers in accordance with local regulations. Do not pour down sink, or mix with household garbage.

RISK CONTROL METHODS (this should be a summary of the hazards anticipated – attach detailed assessments with appropriate risk control methods to this form):

Biological:

- Hazard Group 1&2 materials should be handled correctly, referring to Containment level 1&2 requirements.
- Gloves should be worn when handling biological samples and spillages should be cleaned with 1% Virkon/Trigene.
- Work space should be sterilised with Ethanol, before and after any work.

Chemical:

- Guidance should be followed as stated in appropriate COSHH forms.

Physical/Electrical/Mechanical:

- All electrical equipment should be PAT tested and use in accordance with SOPs
- Semen sample collection should follow steps to ensure patients safety during masturbation
- Centrifuges will be used in accordance with SOPs
- Sharps to be stored and disposed of in the correct manner
- Personal protective equipment (Gloves, goggles, lab coats) worn at all times in the laboratory

Hazard Rating with Control Methods: Medium

Will any specific **training** *be required?* Technical staff will provide laboratory training for general lab use, and research staff will be able to provide training for specific techniques.

Are there any specific **first aid** *issues?* No

PROCEDURE FOR EMERGENCY SHUT-DOWN (if applicable):

Switch off at mains and Unplug.

IF OFF-SITE INDICATE ANY OTHER ISSUES (e.g. associated with: individual's health and dietary requirements (obtain off-site health forms for all participating individuals and indicate where this information will be located); social activities, transportation, ID requirements; permissions for access and sampling).

N/A

	NAME	STAFF/STUDENT No.	DATE
Originator	Mahaah Shehzad	13116503	10/10/16
Supervisor (*XYZ*)	Michael Carroll	55039387	10/10/16
Technical Manager			
Divisional Safety Representative	Gethin Evans p.p. HoS	55061017	17/10/2016
DATE TO BE REVIEWED BY: Oct 2018			
SCHOOL REFERENCE NUMBER (XYZ/ddmmyy/LLn.nn): MS/17102016/GCMB1			

Please save the completed form as XYZ_ddmmyy_RA.doc and email to next signatory.

Appendix B

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY Science and Engineering	DIVISION Healthcare Science
TITLE OF ACTIVITY Immunocytochemistry	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable)**: Mahaah Shehzad **Student Course and Level (if applicable)**: MSc by Research **Supervisor/Staff Name and Status**: Dr Michael Carroll **Specific location (Room Number) where work is to be carried out**: John Dalton Tower	

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION	
HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
Paraformaldehyde	H228 Flammable solid. H302 + H332 Harmful if swallowed or if inhaled H315 Causes skin irritation. H317 May cause an allergic skin reaction. H318 Causes serious eye damage. H335 May cause respiratory irritation. H351 Suspected of causing cancer. P210 Keep away from heat/sparks/open flames/hot surfaces. - No smoking. P261 Avoid breathing dust. P280 Wear protective gloves/ eye protection/ face protection. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

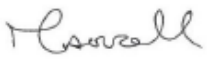
COSHH RISK ASSESSMENT SUMMARY

Good lab practices and working to appropriate SOPs to be implemented at all times while working in lab. Safety Spectacles, gloves, lab coat and other personal protective measures to be used whilst working. . Dispose of contaminated gloves after use in accordance with applicable good laboratory practices.

Paraformaldehyde will be kept well away from all sources of ignition.

DISPOSAL

Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are: Burn in a chemical incinerator equipped with an afterburner and scrubber but exert extra care in igniting as this material is highly flammable. Offer surplus and non-recyclable solutions to a licensed disposal company. Contaminated packaging should be disposed of as unused product.

DATE OF ASSESSMENT: 17/10/2016	**DATE TO BE REVIEWED BY:** Oct 2018
SIGNATURES OF ASSESSORS **Student:** Mahaah Shehzad  **Supervisor/Staff:** Michael Carroll 	**SIGNATURE ON BEHALF OF HEAD OF DIVISION** 
COSHH REFERENCE NUMBER: 48/1016/GHE	

NB: This COSHH Risk Assessment is valid only if the individual COSHH summary sheets are attached for the substances/materials listed, the signatures of the Assessors have been obtained, and the original COSHH Risk Assessment Form has been submitted to the H&S Coordinator. A COSHH Reference Number will be allocated and sent by e-mail to the Supervisor/Staff Assessor.

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY Science and Engineering	DIVISION Healthcare Science
TITLE OF ACTIVITY Handling human semen samples	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable)**: Mahaah Shehzad **Student Course and Level (if applicable)**: MSc by Research **Supervisor/Staff Name and Status:** Dr Michael Carroll **Specific location (Room Number) where work is to be carried out**: John Dalton Tower	

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION	
HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
Human Semen	**H315** – Causes skin irritation. **H317** – May cause an allergic skin reaction. **P201** – Obtain special instructions before use. **P272** – Contaminated work clothing should not be allowed out of the workplace. **P280** – Wear protective gloves/protective clothing/eye protection/face protection. **P281** – Use personal protective equipment as required. Hazard Group 2 Biological Material

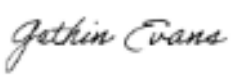
ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

COSHH RISK ASSESSMENT SUMMARY

-Contaminated consumables will be disposed of appropriately. E.g contaminated wastebins and contaminated sharps in sharp bins.
-Benches and cabinets will be cleaned using either 70% IMS or 1% Virkon
-Lab coat and gloves will be worn at all times when handling samples and consumables.
- Hazard Group 2 materials will be handled in accordance with Containment Level 2 requirements

DISPOSAL

Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are:

DATE OF ASSESSMENT: 17/10/2016	DATE TO BE REVIEWED BY: Oct 2018
SIGNATURES OF ASSESSORS **Student:** Mahaah Shehzad  **Supervisor/Staff:** Michael Carroll 	SIGNATURE ON BEHALF OF HEAD OF DIVISION 
COSHH REFERENCE NUMBER: 46/1016/GHE	

NB: This COSHH Risk Assessment is valid only if the individual COSHH summary sheets are attached for the substances/materials listed, the signatures of the Assessors have been obtained, and the original COSHH Risk Assessment Form has been submitted to the H&S Coordinator. A COSHH Reference Number will be allocated and sent by e-mail to the Supervisor/Staff Assessor.

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY **Science and Engineering**	**DIVISION** **Healthcare Science**
TITLE OF ACTIVITY Use of FACS machine	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable)**: Mahaah Shehzad **Student Course and Level (if applicable)**: MSc by Research **Supervisor/Staff Name and Status**: Dr Michael Carroll **Specific location (Room Number) where work is to be carried out**: John Dalton Tower	
HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION	
HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
FACS Clean - Warning	Hazard statements Causes skin irritation. Causes serious eye irritation. Precautionary statements Wear protective gloves/protective clothing/eye protection/face protection. Wash thoroughly after handling. IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Take off contaminated clothing and wash before reuse. If skin irritation occurs: Get medical advice/attention. If eye irritation persists: Get medical advice/attention. IF ON SKIN: Wash with plenty of soap and water.
Formaldehyde solution, 37% in water - Danger	Hazard statement(s) H301 + H311 + H331 Toxic if swallowed, in contact with skin or if inhaled H314 Causes severe skin burns and eye damage. H317 May cause an allergic skin reaction. H335 May cause respiratory irritation. H341 Suspected of causing genetic defects. H350 May cause cancer. H370 Causes damage to organs. Precautionary statement(s) P201 Obtain special instructions before use. P260 Do not breathe dust/ fume/ gas/ mist/ vapours/ spray. P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.

	P301 + P310 + P330 IF SWALLOWED: Immediately call a POISON CENTER or doctor/ physician. Rinse mouth. P303 + P361 + P353 IF ON SKIN (or hair): Take off immediately all contaminated clothing. Rinse skin with water/shower. P304 + P340 + P310 IF INHALED: Remove person to fresh air and keep comfortable for breathing. Immediately call a POISON CENTER or doctor/ physician. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P308 + P311 IF exposed or concerned: Call a POISON CENTER or doctor/ physician. P403 + P233 Store in a well-ventilated place. Keep container tightly closed.

ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

COSHH RISK ASSESSMENT SUMMARY

Safety spectacles, gloves, lab coat and other personal protective measures to be used whilst working in lab unless using a safety cabinet.
The laboratory spaces have access to suitable first aid equipment, eye washing and showers in case of accidents. All work shall be carried out cautiously and away from sources of ignition. Particular care ad detailed above and in the risk assessment must be taken with formaldehyde.

DISPOSAL

FACS Clean - This product is not considered a RCRA hazardous waste. Therefore flush used bleach solution down the sink with plenty of water.

Formaldehyde -
This combustible material may be burned in a chemical incinerator equipped with an afterburner and scrubber. It therefore needs to be collected for disposal.

Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are:

DATE OF ASSESSMENT: 17/10/2016	**DATE TO BE REVIEWED BY:** Oct 2018
SIGNATURES OF ASSESSORS **Student:** Mahaah Shehzad MShehzad	**SIGNATURE ON BEHALF OF HEAD OF DIVISION** Gethin Evans

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY Science and Engineering	DIVISION Healthcare Science
TITLE OF ACTIVITY DNA isolation and analysis. Including: DNA/RNA isolation, making cDNA, PCR and Gel Electrophoresis.	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable):** Mahaah Shehzad **Student Course and Level (if applicable):** MSc by Research **Supervisor/Staff Name and Status:** Dr Michael Carroll **Specific location (Room Number) where work is to be carried out:** John Dalton Tower	

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION	
HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
Lysis Solution	H315- Skin Irritation (Cat 2) H319- Eye Irritation (Cat 2) P280 Wear eye protection/ face protection. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P337 + P313 If eye irritation persists: Get medical advice/ attention.
Column Preparation Solution	H315- Skin Irritation (Cat 2) H319- Eye Irritation (Cat 2) P280 Wear eye protection/ face protection. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P337 + P313 If eye irritation persists: Get medical advice/ attention.
Proteinase K, from Tritirachium album	H315- Skin Irritation (Cat 2) H319- Eye Irritation (Cat 2) H334 May cause allergy or asthma symptoms or breathing difficulties if inhaled. H335 May cause respiratory irritation. P261 Avoid breathing dust. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P342 + P311 If experiencing respiratory symptoms: Call a POISON CENTER or doctor/ physician.

Buffer RLT (guanidinium thiocyanate)	H302 Harmful if swallowed. H314 Causes severe skin burns and eye damage. H412 Harmful to aquatic life with long lasting effects. P280 Wear protective gloves/ protective clothing/ eye protection/ face protection. P303 + P361 + P353 IF ON SKIN (or hair): Remove/ Take off immediately all contaminated clothing. Rinse skin with water/ shower. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P310 Immediately call a POISON CENTER or doctor/ physician. 2.
Buffer RW1	H226 Flammable liquid and vapour. H314 Causes severe skin burns and eye damage. P210 Keep away from heat/sparks/open flames/hot surfaces. - No smoking. P280 Wear protective gloves/ protective clothing/ eye protection/ face protection. P303 + P361 + P353 IF ON SKIN (or hair): Remove/ Take off immediately all contaminated clothing. Rinse skin with water/ shower. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P310 Immediately call a POISON CENTER or doctor/ physician.
Ethanol	H225 Highly flammable liquid and vapour. H319 Causes serious eye irritation. P210 Keep away from heat, hot surfaces, sparks, open flames and other Sigma-Aldrich - 24102 Page 2 of 16 ignition sources. No smoking. P280 Wear eye protection/ face protection. P305 + P351 + P338 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. P337 + P313 If eye irritation persists: Get medical advice/ attention. P403 + P235 Store in a well-ventilated place. Keep cool.
2-Mercaptoethanol	H301 + H331 Toxic if swallowed or if inhaled H310 Fatal in contact with skin. H315 Causes skin irritation. H317 May cause an allergic skin reaction. H318 Causes serious eye damage. H373 May cause damage to organs (Liver, Heart) through prolonged or repeated exposure if swallowed. H410 Very toxic to aquatic life with long lasting effects. P261 Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. P280 Wear protective gloves/ protective clothing/ eye protection/ face protection.

	P301 + P310 + P330 IF SWALLOWED: Immediately call a POISON CENTER or doctor/ physician. Rinse mouth. P302 + P352 + P310 IF ON SKIN: Wash with plenty of water. Immediately call a POISON CENTER or doctor/ physician. P305 + P351 + P338 + P310 IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing. Immediately call a POISON CENTER or doctor/ physician. P403 + P233 Store in a well-ventilated place. Keep container tightly closed.

ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

COSHH RISK ASSESSMENT SUMMARY

Good lab practices and working to appropriate SOPs to be implemented at all times while working in lab. Safety Spectacles, gloves, lab coat and other personal protective measures to be used whilst working in lab unless using a safety cabinet. Dispose of contaminated gloves after use in accordance with applicable good laboratory practices.

The laboratory spaces have access to suitable first aid equipment, eye washing and showers in case of accidents. All work shall be carried out cautiously and away from sources of ignition. Ethanol shall be kept away from heat, sparks, open frame, or hydrochloric acid.

The biggest risk associated with this activity is the use of 2-Mercaptoethanol, which is a category 3 risk. Protective gloves and clothing will be worn at all times during this activity.

If experiencing respiratory symptoms due to Proteinase K inhalation call a poison centre or physician.
If 2-Mercaptoethanol, RLT or RW1 buffers come into contact with skin or eyes, or are ingested call a poison centre or physician.

DISPOSAL

Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are:

Non-hazardous buffers will be poured into the sink. Gels, samples and hazardous substances will be placed into autoclave/ clinical waste bags for incineration.

DATE OF ASSESSMENT: 17/10/2016	**DATE TO BE REVIEWED BY:** Oct 2018
SIGNATURES OF ASSESSORS **Student:** Mahaah Shehzad	**SIGNATURE ON BEHALF OF HEAD OF DIVISION** Gethin Evans

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY Science and Engineering	DIVISION Healthcare Science
TITLE OF ACTIVITY Cryopreservation	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable)**: Mahaah Shehzad **Student Course and Level (if applicable)**: MSc by Research **Supervisor/Staff Name and Status**: Dr Michael Carroll **Specific location (Room Number) where work is to be carried out**: John Dalton Tower	

HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION

HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
Liquid Nitrogen	H281: Contains refrigerated gas; may cause cryogenic burns or injury P282: Wear cold insulating gloves/face shield/eye protection. P315: Get immediate medical advice/attention. P336: Thaw frosted parts with lukewarm water. Do no rub affected area. P403: Store in a well-ventilated place

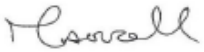
ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

COSHH RISK ASSESSMENT SUMMARY

Personal protective equipment will be worn at all times when handling liquid nitrogen as below:

Insulated gloves conforming to BS EN 511 (protective glove against cold) - gloves must be under the lab coat cuff and no skin should show. Eye protection should be to BS EN 166 standards and capable of protecting against cryogenic liquids. The visors must be clearly labelled as "suitable for use with cryogenic gases" Safety spectacles do not give adequate protection against splashes and are NOT suitable. Goggles protect only the eyes. A visor protects the eyes and the face. However it is still possible for liquid to splash up underneath the visor; the use of a visor with a chin guard should be considered if this is likely. Fully buttoned Howie neck lab coat
Jewellery – remove bracelets/rings etc as contact with metal will cause burns.

Liquid nitrogen dewars should be stored in appropriately ventilated rooms with oxygen level sensors.

DISPOSAL Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are: n/a	
DATE OF ASSESSMENT: 17/10/2016	**DATE TO BE REVIEWED BY:** Oct 2018
SIGNATURES OF ASSESSORS **Student:** Mahaah Shehzad  **Supervisor/Staff:** Michael Carroll 	**SIGNATURE ON BEHALF OF HEAD OF DIVISION** 
COSHH REFERENCE NUMBER: 44/1016/GHE	

NB: This COSHH Risk Assessment is valid only if the individual COSHH summary sheets are attached for the substances/materials listed, the signatures of the Assessors have been obtained, and the original COSHH Risk Assessment Form has been submitted to the H&S Coordinator. A COSHH Reference Number will be allocated and sent by e-mail to the Supervisor/Staff Assessor.

The MANCHESTER METROPOLITAN UNIVERSITY
COSHH RISK ASSESSMENT

FACULTY **Science and Engineering**	**DIVISION** **Healthcare Science**
TITLE OF ACTIVITY Cell Culture	
REASONS FOR ACTIVITY Research	
STATUS OF PERSONS UNDERTAKING ACTIVITY **Student Name (if applicable)**: Mahaah Shehzad **Student Course and Level (if applicable)**: MSc by Research **Supervisor/Staff Name and Status**: Dr Michael Carroll **Specific location (Room Number) where work is to be carried out**: John Dalton Tower	
HAZARDOUS SUBSTANCES/MATERIALS USED AND HAZARD CLASSIFICATION	
HAZARD NAME	HAZARD AND PRECAUTIONARY STATEMENTS
Trypan Blue	H351: Suspected of causing cancer P201: Obtain special instructions before use P202: Do not handle until all safety precautions have been read and understood P281: Use personal protective equipment as required P308 + P313: IF exposed or concerned get medical advice/attention
Virkon	H315: Causes skin irritation H318: Causes serious eye damage H401: Toxic to aquatic life H331: Toxic if inhaled P305 + P351 + P338: If in eyes rinse continuously with water for several minutes, Remove contact lenses if present and easy to do. Continue rinsing P281: Use personal protective equipment as required
Ethanol	H225: Highly flammable liquid and vapour H319: Causes serious eye irritation P210: Keep away from heat/sparks/open flames/hot surfaces – No smoking P280: Wear protective gloves/protective clothing/eye protection/face protection P305 + P351 + P338: If in eyes rinse continuously with water for several minutes, Remove contact lenses if present and easy to do. Continue rinsing P337 + P313: If eye irritation persists get medical advice/attention P403 + P235: Store in a well ventilated place, keep cool
Mitomycin C	H302: Harmful if swallowed H351: Suspected of causing cancer

	P280: Wear protective gloves/protective clothing/eye protection/face protection P301+P312+P330: If swallowed Call a poison centre or doctor/physician if you feel unwell rinse mouth

ALL CONTAINERS OF HAZARDOUS SUBSTANCES SHOULD BEAR THE CORRECT HAZARD WARNING LABELS

COSHH RISK ASSESSMENT SUMMARY

Good lab practices and working to appropriate SOPs to be implemented at all times while working in lab. Safety spectacles, gloves, lab coat and other personal protective measures to be used whilst working in lab unless using a safety cabinet. Dispose of contaminated gloves after use in accordance with applicable good laboratory practices.
Wash and dry hands. The laboratory spaces have access to suitable first aid equipment, eye washing and showers in case of accidents. All work shall be carried out cautiously and away from sources of ignition.
Ethanol shall be kept away from heat, sparks, open frame, or hydrochloric acid.
Virkon will only be handled in solid and liquid form whilst wearing a lab coat, gloves and eye protection.
Mitomycin will only be handled in solid and liquid form in a safety cabinet or tissue culture hood.

DISPOSAL

Hazardous materials must not be removed from the laboratories. Disposal arrangements in the specified location where work is to be carried out for the materials listed above are:

Liquid waste should aspirated in to a receptacle containing 1% Virkon for decontamination overnight. Decontaminated liquid waste can then rinsed down the drain with plenty of water. Plasticware should be decontaminated with 1% Virkon and disposed of in clinical waste bag. Solid Virkon waste, including the container, will be disposed of as hazardous waste i.e. placed in yellow clinical waste bags and incinerated.

DATE OF ASSESSMENT: 28.09.16	**DATE TO BE REVIEWED BY**: Oct 2018
SIGNATURES OF ASSESSORS **Student**: Mahaah Shehzad  **Supervisor/Staff**: Michael Carroll 	**SIGNATURE ON BEHALF OF HEAD OF DIVISION** 
COSHH REFERENCE NUMBER: 43/1016/GHE	

Appendix C

APPLICATION FOR ETHICAL APPROVAL

Introduction
All university activity must be reviewed for ethical approval. In particular, all undergraduate, postgraduate and staff research work, projects and taught programmes must obtain approval from their Faculty Academic Ethics committee (or delegated Departmental Ethics Committee).

APPLICATION PROCEDURE

The form should be completed legibly (preferably typed) and, so far as possible, in a way, which would enable a layperson to understand the aims and methods of the research. Every relevant section should be completed. Applicants should also include a copy of any proposed advert, information sheet, consent form and, if relevant, any questionnaire being used. The Principal Investigator should sign the application form. Supporting documents, together with one copy of the full protocol should be sent to the Administrator of the appropriate Faculty Academic Ethics Committee. **(Insert contact details)**

Your application will require external ethical approval by an NHS Research Ethics Committee if your research involves staff, patients or premises of the NHS (see guidance notes)

Work with children and vulnerable adults
You will be required to have a Criminal Disclosure, if your work involves children or vulnerable adults.

1. Details of Applicants	
1.1. Name of applicant (Principal Investigator): **Dr Michael Carroll**	
Telephone Number: ex 1231	
Email address: michael.carroll@mmu.ac.uk	
Status: Senior Lecturer in Reproductive Science	Postgraduate Student (Taught or Research) Staff
Department/School/Other Unit: School of Healthcare Science	
Programme of study (if applicable):	
Name of supervisor/Line manager: Prof. Craig Banks	

1.2. Co-Workers and their role in the project: (e.g. students, external collaborators, etc)

Muaamar Al-Khaiqani, visiting PhD student (MK)
(0161 247 1163; muaamar.al-khaiqani@postgrad.manchester.ac.uk)

Stéphane Berneau - PDRA (stephane.berneau@gmail.com)

Mahaah Shehzad - MRes Student (mahaahshehzad@gmail.com)

Dr Stéphane Berneau - PDRA, MMU (stephane.berneau@gmail.com)

Muaamar Al-Khaiqani – PhD student, University of Manchester.
(muaamar.alkhaiqani@postgrad.manchester.ac.uk)

2. Details of the Project

2.1. Title:

Detecting 8-Oxoguanine in Human Sperm using Electrochemical Analysis

2.2. Description of the Project: (please outline the background and the purpose of the research project, 250 words max)

Male factor infertility accounts for between 40 to 50% of infertile couples seeking ART in the UK. The major causes of male factor infertility are poor sperm quality and DNA damage
Sperm DNA damage can be caused by a number of factors including environment and lifestyle factors (e.g. exposure to chemicals, diet, obesity and smoking) and clinical conditions such as diabetes. Furthermore, errors in DNA packing and chromatin integrity can increase the risk of sperm DNA damage. Sperm DNA damage not only impair sperm function and fertilization, it has also been reported to increase miscarriage rates in patients with high sperm DNA damage compared with those with low DNA damage.
A major cause of sperm DNA damage is exposure to reactive oxygen species (ROS). Oxidation of sperm DNA can cause irreversible damage to sperm DNA. DNA oxidation generates numerous DNA lesions, the most common being 8-oxoguanine (8-oxoG), whereby a hydroxyl radical ($OH^{-)}$ binds to guanine. The presence of 8-oxoG have pro-mutagenic effects if not repaired and can lead to genomic instability and DNA fragmentation.
There are several assays employed to measure sperm DNA damage, such as COMET, Halo and TUNEL – However, these can be costly and time consuming. This project will investigate the use of electrochemistry to measure 8-oxoG in sperm.

1.1. Describe what type of study this is (e.g. qualitative or quantitative; also indicate how the data will be collected and analysed). Additional sheets may be attached.

We will undertake quantitative laboratory-based research to measure the sperm DNA damage. Semen samples will be provided from consented volunteers, who will produce the semen sample

from home and bring it to the laboratory. When necessary, volunteers may be required to produce a sample on site. This will be conducted in a designated room (phlebotomy room, first floor, IRM).

Sperm will be isolated from seminal plasma via density gradient (Suprasperm,Origio) and washed in Sperm Preparation Media (Origio).
Sperm DNA will be extracted using either an altered version of the phenol-chloroform method or another that utilises DTT and Beta-mercaptoethanol.
Electrochemistry will be used on the extracted DNA, via the screen printed electrodes (strips) to quantify and detect levels of 8-oxoguanine, formation of a standard curve in relation to the levels of 8-oxoguanine within a biological sample will be produced, and further compared this to a blank measurement.
Various concentrations of Hydrogen peroxide (H_2O_2) will be added to the sperm and left to incubate at fixed time points (2 hours, 6 hours, 8 hours and 24 hours), extraction of DNA will then follow and levels of 8-oxoG will be measured using the screen printed electrodes. Additionally comparisons will be made using results from both undamaged and damaged Sperm DNA.
Using the results found from the electrochemistry, DNA fragmentation from sperm will then be investigated using the COMET assay.

1.2. Are you going to use a questionnaire?
YES (Please attach a copy)
Attached (see appendix)

1.3. Start Date / Duration of project:
Nov 2016 – Nov 2017

1.4. Location of where the project and data collection will take place:
MMU

1.5. Nature/Source of funding
This study is funded by consumable fees supporting MS MRes

1.6. Are there any regulatory requirements?
NO

2. Details of Participants

2.1. How many?
10

2.2. Age: 18 - 30

2.3. Sex: Male

2.4. How will they be recruited? (Attach a copy of any proposed advertisement)
They will be recruited from amongst the University staff and student, using posters, announcement boards (Screens) and verbal announcements.

2.5. Status of participants: (e.g. students, public, colleagues, children, hospital patients, prisoners, including young offenders, participants with mental illness or learning difficulties.)
2.6.
General public, students and staff

2.7. Inclusion and exclusion from the project: (indicate the criteria to be applied).

Inclusion Criteria

- Age 18+
- Males
- Subjects with general good health

Exclusion Criteria

- Males who underwent vasectomies
- HIV + males
- Prepubescent males (including those under 18 years)

2.8. Payment to volunteers: (indicate any sums to be paid to volunteers).
No

2.9. Study information:
Have you provided a study information sheet for the participants?
YES (Please attach a copy)
See appendix

2.10. Consent:
(A written consent form for the study participants MUST be provided in all cases, unless the research is a questionnaire.)
Have you produced a written consent form for the participants to sign for your records?
YES (Please attach a copy)
See appendix

.

3. Risks and Hazards

3.1. Are there any risks to the researcher and/or participants?
(Give details of the procedures and processes to be undertaken, e.g., if the researcher is a lone-worker.)

Full risk assessments have been undertaken from both the protocols used at MMU and within the School of Healthcare Science. Each worker will be fully aware of risks associated.

Semen sample: There is a risk of infection when handling any human biological fluid and semen is no exception. Every precaution will be taken to minimise this risk with the use of sterile equipment and protective gloves.

3.2. State precautions to minimise the risks and possible adverse events:

Care will be taken when handling semen and spermatozoa. Through proper training, all techniques (molecular biology, cytology will be carried out to minimize any risk).
Laboratory coats, disposable gloves (and eye protection where Risk assessment of procedure indicates this as a control measure)

3.3. What discomfort (physical or psychological) danger or interference with normal activities might be suffered by the researcher and/or participant(s)? State precautions which will be taken to minimise them:

4. Ethical Issues

4.1. Please describe any ethical issues raised and how you intend to address these:
None

5. Safeguards/Procedural Compliance

5.1. Confidentiality:

5.1.1. Indicate what steps will be taken to safeguard the confidentiality of participant records. If the data is to be computerised, it will be necessary to ensure compliance with the requirements of the Data Protection Act 1998.

After consent, the participants are allocated a unique code. This code is used on the medical questionnaires. Both consent medical questionnaires are securely stored. All subsequent participant information will be referred to by a unique reference number. No identifying details will be on any additional paperwork.

5.1.2. If you are intending to make any kind of audio or visual recordings of the participants, please answer the following questions:

5.1.2.1. How long will the recordings be retained and how will they be stored?
None

5.1.2.2. How will they be destroyed at the end of the project?
None

5.1.2.3. What further use, if any, do you intend to make of the recordings?
None

5.2. The Human Tissue Act

The Human Tissue Act came into force in November 2004, and requires appropriate consent for, and regulates the removal, storage and use of all human tissue.

5.2.1. Does your project involve taking tissue samples, e.g., blood, urine, hair etc., from human subjects?
YES – human semen

5.2.2. Will this be discarded when the project is terminated?
YES
NO

If NO – Explain how the samples will be placed into a tissue bank under the Human Tissue Act regulations:

All semen and spermatozoa will be stored according to our standard practice that conforms to the Human Tissue Act. Samples will be labelled with the participant ID number and stored in the -80 freezer until analysis of nucleic acids, seminal contents and spermatozoa are required. A digital or written record of the sample location will be kept alongside copies of the signed, informed consent. Sample will be retained for a period of no more than 10 years.

For sperm usage and storage used solely for research, and not for licensed treatment (IVF), the HFEA Exemption Regulations (2009) are relevant. Effectively these regulations provide an exemption from the HFEA Act for certain defined activities and purposes. Research only storage and use of sperm is one of these, hence research donated sperm is effectively considered as non-licensable material under the HFE Act and can be stored and used on unlicensed premises.

5.3. Notification of Adverse Events (e.g., negative reaction, counsellor, etc):
(Indicate precautions taken to avoid adverse reactions.)

Please state the processes/procedures in place to respond to possible adverse reactions.

The procurement of semen carries very low adverse reactions. However, the procedures in place to deal with any adverse events have been explained where necessary above. We will also contact a University First Aid representative and emergency medical attention will be sought wherever necessary (e.g. in any instance where fainting may occur).
All adverse events will be reported to the Faculty Ethics Committee at the earliest opportunity.

In the case of clinical research, you will need to abide by specific guidance. This may include notification to GP and ethics committee. Please seek guidance for up to date advice, e.g., see the NRES website at http://www.nres.npsa.nhs.uk/

SIGNATURE OF PRINCIPAL INVESTIGATOR: M. Carroll	Date 15th Nov. 2016
SIGNATURE OF FACULTY'S HEAD OF ETHICS: Gethin Evans	Date: 19/12/2016

Appendix D

Research Insurance Checklist

Manchester Metropolitan University

ADMINISTRATIVE DETAILS

Lead Investigator Name (Title/Forename/Surname)	Dr M Carroll
Contact Email Address	Michael.carroll@mmu.ac.uk
Full Title of the Research	**Detecting 8-Oxoguanine in Human Sperm using Electrochemical Analysis**

SECTION 1 – TECHNIQUES, TESTING AND INTERVENTIONS

Does your research study involve:

✓ **Physically invasive techniques?**

This refers to any test in which the skin of the participant is broken or an implement is inserted into any opening of the human body (e.g. eyes, ears, nose, mouth, lungs, stomach, rectum, vagina and urethra) or involves the taking of body samples such as saliva, hair, urine, faeces, sputum, skin, nails, or taking biopsies of any form for any purpose, or any form of scanning such as DEXA scans, Ultrasound scans, MRI, fMRI, CT, or PET scanning.

☐ **Ingestion of food stuffs or drugs?**

This refers to the consumption of any substance which may impact on psychological or physical state. Substances may include but are not limited to food, beverages or drugs.

☐ **Physical testing?**

This refers to any test in which a participant must perform an action resulting in the use of any muscle of the body and/or involves the use of scanning procedures, eye-trackers, mounted body cameras, sensors or electrodes, or the taking of swabs from any cavity of the body, respiratory challenge testing or recording of peak flows, EEG, ECG, Exercise ECG, Treadmill work.

☐ **Psychological intervention?**

This refers to any test which purposely alters the mood of the participant or involves administering personality inventories, or any other form of psychological test.

OR

☐ **I confirm that my research does not fall into any of the above categories** ***(please go straight to Section 3)***

SECTION 2 – CLINICAL TRIALS INSURANCE

Please complete this section only if you ticked one of the boxes in Section 1.

Does your research study involve:

☐ **Pregnant persons as participants with procedures other than blood samples being taken from them?**

☐ **Children aged five or under with procedures other than blood samples being taken from them?**

☐ **Activities being undertaken by the lead investigator or any other member of the study team in a country outside of the UK?** ***If 'Yes', please refer to the 'Travel Insurance' guidance on Page 1 of this form.***

OR

✓ **I confirm that my research does not fall into any of the above categories**

SECTION 3 – OTHER HAZARDS

Does your research study involve:

☐ **Working with Hepatitis, Human T-Cell Lymphotropic Virus Type iii (HTLV iii), or Lymphadenopathy Associated Virus (LAV) or the mutants, derivatives or variations thereof or Acquired Immune Deficiency Syndrome (AIDS) or any syndrome or condition of a similar kind?**

☐ **Working with Transmissible Spongiform Encephalopathy (TSE), Creutzfeldt-Jakob Disease (CJD), variant Creutzfeldt-Jakob Disease (vCJD) or new variant Creutzfeldt-Jakob Disease (nvCJD)?**

☐ **Working in hazardous areas or high risk countries?** ***Please refer to the 'High Risk Countries' guidance on Page 1 of this form.***

☐ **Working with hazardous substances outside of a controlled environment?**

☐ **Working with persons with a history of violence, substance abuse or a criminal record?**

OR

✓ **I confirm that my research does not fall into any of the above categories**

Appendix E

Participant information sheet

Title of Study: *Investigating the effect of lifestyle and environment on human sperm.*

Study Background

Sperm are produced continuously in the testis through the process of spermatogenesis – the sperm cells are vulnerable to damage. This damage can result in faulty sperm and can cause infertility. One type pf damage is to the DNA found in the sperm head. Damaged DNA can result in increased miscarriages and is a major cause of male infertility. This study will develop a method to assess a specific type of DNA damage. This information will offer potential therapeutic options that may improve male infertility.

Who can take part?

Any male aged over 18 years old.

What is involved?

You will be required to provide a semen sample either at home or within a secure room at the school of healthcare science. You will produce this semen via masturbation. A full sample is required. You will be provided with a sterile container from which to deposit your specimen.
The semen sample will be assessed for parameters such as motility (how well the sperm are moving), morphology (the shape of the sperm cells) and concentration (the number of sperm per millilitre of seminal fluid). The sperm will then undergo a variety of biochemical and molecular biology tests. The samples will be stored at -80°C for further analysis.
Your sperm sample will NOT, at any time, be used for any assisted reproductive techniques and will ONLY be used for research or teaching purposes.

Please note that this is not a diagnostic test and you will not be informed of the quality of your semen/sperm.

Are there any risks in taking part in the study?

There may be a slight risk of fainting due to the physical activity of semen production. If you are providing the sample on site, the secured room can be locked from the inside. After an allotted time has elapsed, a study team member will knock on the door to ensure you are OK. If there is no answer, they will enter the room using a key to establish your status.

Participant informed consent. ID code .....

Name: .....

Date of Birth: ..... Age: .....

Project title:

Detecting 8-Oxoguanine in Human Sperm using Electrochemical Analysis

Principal Investigator:
Dr Michael Carroll

Ethics approval number:

I have read and understood the information sheet and all verbal explanation outlining the purpose of the study and the assessments involved.

Any questions I have about the study, or my participation in it, have been answered to my satisfaction. I understand that I do not have to take part and that I may decide to withdraw from the study at any point without having to give a reason. I understand that my sperm will not be used, at any time, for any form of assisted reproductive technique, such as fertilising human eggs or the creation of human embryos. I understand that my semen may be also used for teaching purposes. My concerns regarding this study have been answered and such further concerns as I have during the time of the study will be responded to. It has been made clear to me that, should I feel that my rights are being infringed or that my interests are otherwise being ignored, neglected or denied, I should inform the Chair of the Ethics Committee of the School of Healthcare Sciences, Manchester Metropolitan University, Oxford Road, Manchester, M1 5GD.

Signed _____ Date _____

I give my consent for semen collected from me during the course of this study to be retained in the study biobank at MMU for retrospective biochemical and molecular biology analysis.

Date

Signed _____ _____
Name (Print).....

Witnessed _____ Date _____

Semen Procurement Form.

Introduction

Please follow the instructions concerning the production of a semen sample.

As a fresh sample is essential for this test, it must be brought to the laboratory within one hour of being passed at home.

NOTE: USING A PUBLIC TOILET TO PASS YOUR SAMPLE IS AGAINST THE LAW.

Instructions for collecting the semen sample

You should abstain from intercourse or masturbation for three to four days prior to providing the specimen.

The sample must be obtained by masturbation (manual stimulation) and should be collected directly into the specimen container provided. A condom or artificial lubrication must not be used for semen collection, as it will kill the sperm.

The complete specimen is needed for this study, so if any is spilt you must tell us, as a repeat specimen may be required.

Label the specimen container with your full name, date of birth and the date and time the specimen was passed.

Delivery of your sample

Deliver the sample to the School of Healthcare Science within **one hour** of passing the sample.

Keep the sample at body temperature while being transported to the laboratory, for example by carrying it in an inside pocket. Excessive cold or heat can damage the sperm.

For further information contact:
Dr M. Carroll (michael.carroll@mmu.ac.uk)
Phone: 0161 247 1231

Specimen details

Please complete this form and bring it with you to the laboratory along with your sample and the request form.

Name:_____

Date of Birth: _____

Date of specimen Time passed:_____

Time specimen passed: _____

Abstinence (days): _____

I confirm that this semen specimen is mine and I consent to its use for the purpose of research and education.

Signed

_____

Medical Screening Questionnaire

It is important that the investigators are aware of any health conditions before participation in this research study. This information will be kept strictly confidential.

Please answer the following questions as accurately as possible.

Are you currently taking any prescribed medication? **YES/NO**

Are you currently attending your GP? **YES/NO**

Have you ever suffered from a cardiovascular problem? **YES/NO**
i.e. *high blood pressure, anaemia, heart attack etc*

Have you ever suffered from a neurological disorder? **YES/NO**
i.e. epilepsy, convulsions etc

Have you ever suffered from an endocrine disorder? **YES/NO** *i.e. diabetes etc*

Have you ever suffered from a chronic gastrointestinal disorder? **YES/NO**
i.e. Crohn's disease, irritable bowel syndrome etc

Have you ever suffered from a skin disorder? **YES/NO**
i.e. eczema etc

Do you suffer from any allergies? **YES/NO** *i.e. any medications, foods etc*

Have you had a vasectomy or any urological surrey? **YES/NO**
i.e. testicular surgery

Have you had Mumps? **YES/NO**

Have you had any testicular injuries / torsions? **YES/NO**

Do you knowingly have, or had a Sexually Transmitted Infection? **YES/NO**

If you have answered "yes" to any of these questions, please provide details below:

Study Procedures

The following is a brief description of the procedures and techniques that will be employed during this study.

Semen procurement:

Semen will be produced by masturbation in to sterile containers provided. On occasions where participants cannot provide a specimen from home they will be asked to produce a sample on site in a dedicated, secured room. The specimen container will be placed in the plastic bag with the completed 'semen procurement form'.

Semen analysis:

Semen analysis is carried out within 30 minutes of specimen production. Volume, pH and other physical characteristics may be noted. Sperm motility and concentration is measured and a sample of semen is smeared on to a glass slide for fixing and morphological analysis.

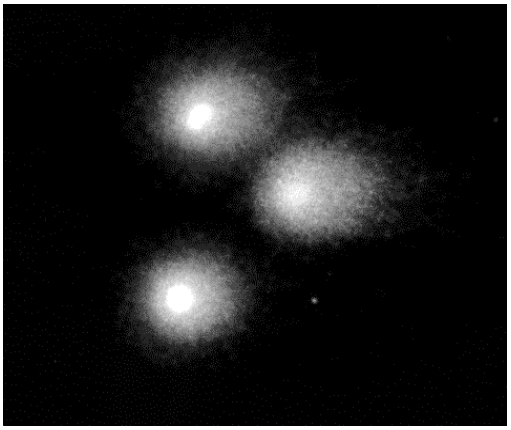
Molecular biology analysis:

DNA and RNA will be isolated from both fresh and frozen sperm cells using commercial kits (QIAGEN) and examined DNA integrity using a variety of techniques. The sperm DNA will be exposed to oxidative damage and this damage will be measures using electrochemistry.

Chromatin assays such as chromatin dispersion assay and comet assays will be conducted to investigate the integrity of the sperm nucleus after exposure to various compounds.

Appendix F

Comet Assay images – Optimisation of Incubation time and Media



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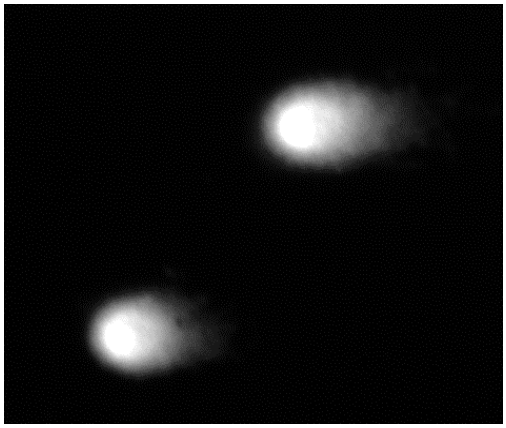

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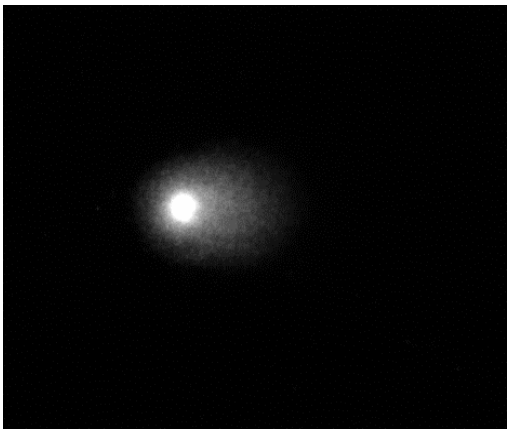

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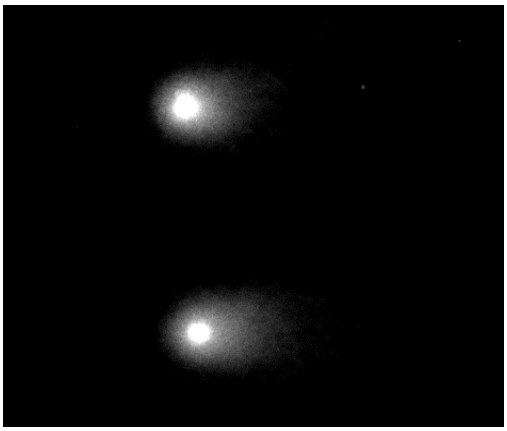

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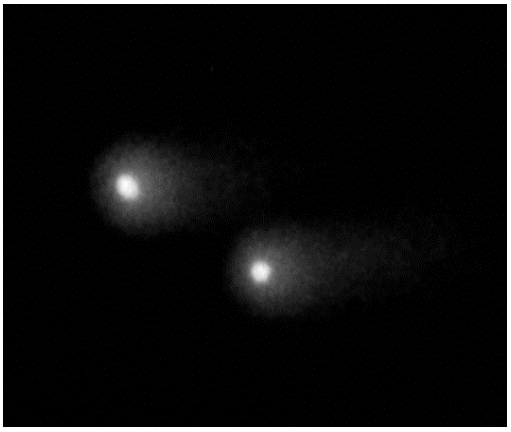

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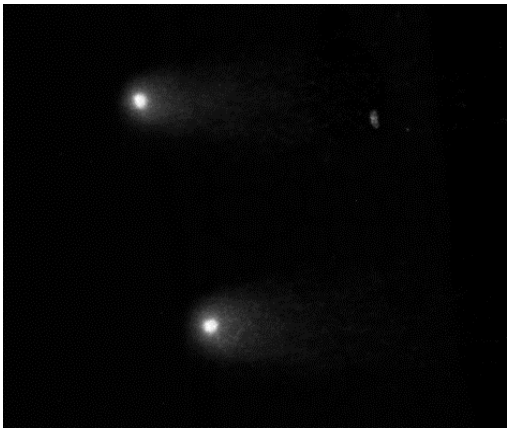

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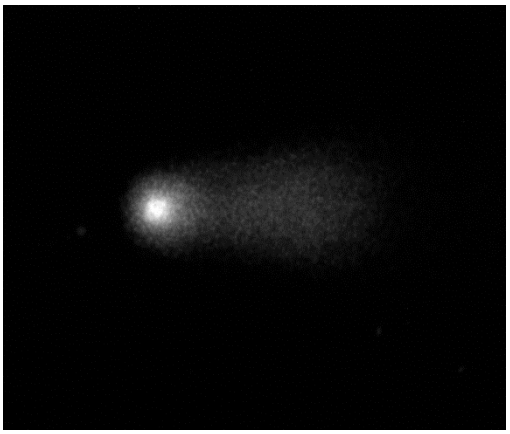
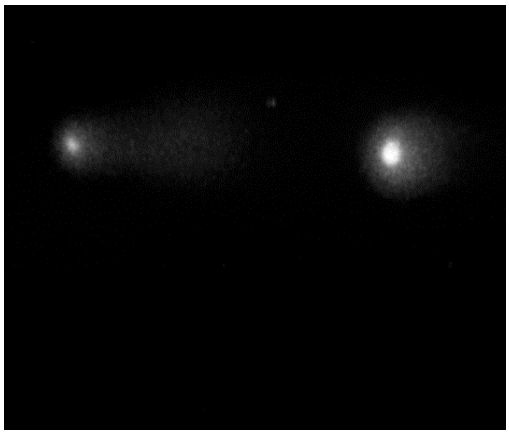
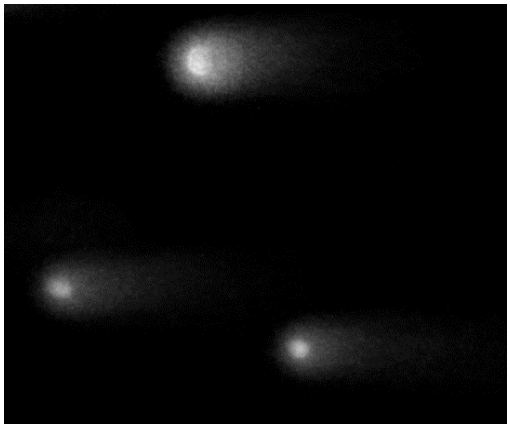


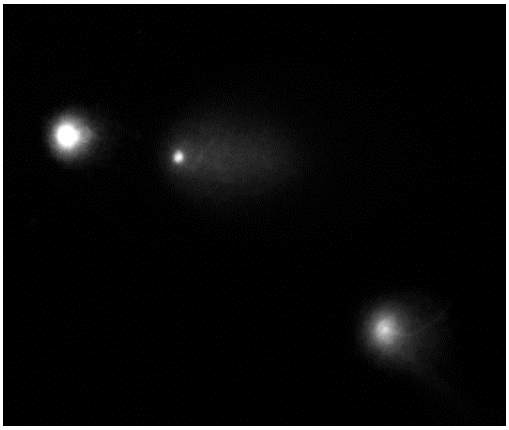
100µM, PBS, 120 minutes



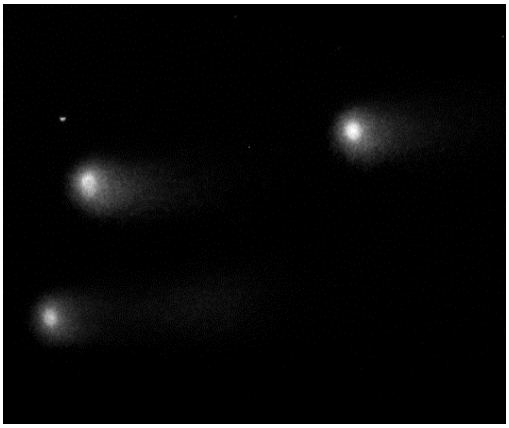
100µM, SPM, 120 minutes



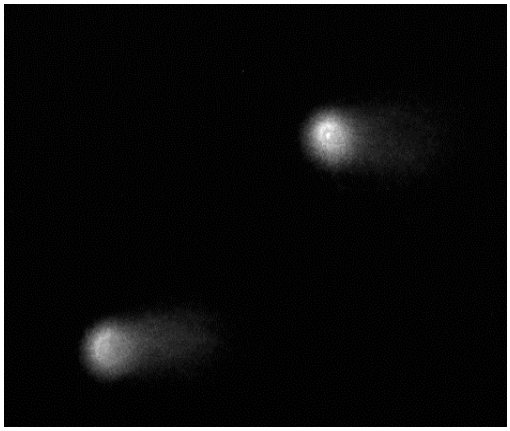
500µM, PBS,30 minutes



500µM, SPM, 30 minutes



500µM, PBS, 60 minutes



500µM, SPM, 60 minutes

Appendix G

Comet Assay Statistical results

Optimisation of Storage

Table 1: DNA % in Tail

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
Fresh vs. Ice	-12.85	No	ns	>0.9999	B	Ice
Fresh vs. Slow +	10.04	No	ns	>0.9999	C	Slow +
Fresh vs. Slow -	23.56	No	ns	>0.9999	D	Slow -
Fresh vs. Quick +	68.41	Yes	***	0.0003	E	Quick +
Fresh vs. Quick -	27.33	No	ns	0.6046	F	Quick -

Optimisation of Cell Number

Table 2: 1 mill vs 5mill – DNA % in tail

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	3003 , 1462
Mann-Whitney U	334
Difference between medians	
Median of column A	32.68, n=47
Median of column B	12.51, n=47
Difference: Actual	-20.17
Difference: Hodges-Lehmann	-17.06

Table 3: 1 mill vs 10 mill - DNA % in tail

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,C	2955 , 1510
Mann-Whitney U	382
Difference between medians	
Median of column A	32.68, n=47
Median of column C	13.95, n=47
Difference: Actual	-18.73
Difference: Hodges-Lehmann	-15.15

Table 4: 1mill vs 20 mill - DNA % in tail

Mann Whitney test	
P value	0.0429
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,D	2500 , 1965
Mann-Whitney U	837
Difference between medians	
Median of column A	32.68, n=47
Median of column D	24.49, n=47
Difference: Actual	-8.19
Difference: Hodges-Lehmann	-6.15

Table 5: 1 mill vs 5 mill - Tail Moment

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	4161 , 2055
Mann-Whitney U	624
Difference between medians	
Median of column A	32.12, n=58
Median of column B	9.66, n=53
Difference: Actual	-22.46
Difference: Hodges-Lehmann	-21.03

Table 6: 1 mill vs 10 mill - Tail Moment

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,C	3904 , 1768
Mann-Whitney U	591.5
Difference between medians	
Median of column A	32.12, n=58
Median of column C	8.035, n=48
Difference: Actual	-24.08
Difference: Hodges-Lehmann	-19.34

Table 7: 1 mill vs 20 mill - Tail Moment

Mann Whitney test	
P value	0.0533
Exact or approximate P value?	Exact
P value summary	ns
Significantly different (P < 0.05)?	No
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,D	4245 , 4401
Mann-Whitney U	1700
Difference between medians	
Median of column A	32.12, n=58
Median of column D	18.48, n=73
Difference: Actual	-13.64

Table 8: 1 mill vs 5 mill Olive Moment

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,B	4197 , 2020
Mann-Whitney U	588.5
Difference between medians	
Median of column A	23.2, n=58
Median of column B	11.08, n=53
Difference: Actual	-12.12
Difference: Hodges-Lehmann	-12.14

Table 9: 1 mill vs 10 mill - Olive Moment

Mann Whitney test	
P value	<0.0001
Exact or approximate P value?	Exact
P value summary	****
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,C	3897 , 1774
Mann-Whitney U	598
Difference between medians	
Median of column A	23.2, n=58
Median of column C	10.51, n=48
Difference: Actual	-12.69
Difference: Hodges-Lehmann	-11.25

Table 10: 1 mill vs 20 mill - Olive Moment

Mann Whitney test	
P value	0.0115
Exact or approximate P value?	Exact
P value summary	*
Significantly different (P < 0.05)?	Yes
One- or two-tailed P value?	Two-tailed
Sum of ranks in column A,D	4372 , 4275
Mann-Whitney U	1574
Difference between medians	
Median of column A	23.2, n=58
Median of column D	16.36, n=73
Difference: Actual	-6.835
Difference: Hodges-Lehmann	-5.805

H_2O_2 treated Sperm cells – Statistical Result

Table 11: DNA % in Tail

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
0µM vs. 50µM	41.2	No	ns	0.0922	B	50µM
0µM vs. 100µM	33.8	No	ns	0.2652	C	100µM
0µM vs. 200µM	-25.4	No	ns	0.7329	D	200µM
0µM vs. 500µM	-80.5	Yes	***	0.0001	E	500µM

Table 12: Tail Moment

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
0µM vs. 50µM	45.2	No	ns	0.0509	B	50µM
0µM vs. 100µM	30	No	ns	0.4133	C	100µM
0µM vs. 200µM	-23.4	No	ns	0.8870	D	200µM
0µM vs. 500µM	-85.9	Yes	****	<0.0001	E	500µM

Table 13: Olive Moment

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
0µM vs. 50µM	45.8	Yes	*	0.0461	B	50µM
0µM vs. 100µM	31.3	No	ns	0.3562	C	100µM
0µM vs. 200µM	-16.5	No	ns	>0.9999	D	200µM
0µM vs. 500µM	-75	Yes	***	0.0004	E	500µM

H_2O_2 treated Sperm cells – Descriptive Statistics (Mean)

Table 14: DNA % in Tail

Concentration	Mean
0µM	30
50µM	24.8
100µM	24.6
200µM	32.1
500µM	40.5

Table 15: Tail Moment

Concentration	Mean
0µM	50.1
50µM	35.4
100µM	36.8
200µM	52.4
500µM	84.1

Table 16: Olive Moment

Concentration	Mean
0µM	29.5
50µM	21.8
100µM	23.2
200µM	29
500µM	45.2

H_2O_2 treated sperm cells within seminal plasma – Statistical Result

Table 17: Seminal Plasma – DNA % in Tail

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
Control vs. 500µM (1)	-53.1	Yes	**	0.0015	B	500µM (1)
Control vs. 500µM (2)	-56.7	Yes	***	0.0004	C	500µM (2)
Control vs. 500µM (3)	-28.8	No	ns	0.1594	D	500µM (3)

Table 18: Seminal Plasma – Tail Moment

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
Control vs. 500µM (1)	-68.1	Yes	****	<0.0001	B	500µM (1)
Control vs. 500µM (2)	-73	Yes	****	<0.0001	C	500µM (2)
Control vs. 500µM (3)	-37.9	Yes	*	0.0333	D	500µM (3)

Table 19: Seminal Plasma – Olive Moment

Dunn's multiple comparisons test	Mean rank diff.	Significant?	Summary	Adjusted P Value	A-?	
Control vs. 500µM (1)	-60.1	Yes	***	0.0003	B	500µM (1)
Control vs. 500µM (2)	-70.8	Yes	****	<0.0001	C	500µM (2)
Control vs. 500µM (3)	-30.1	No	ns	0.1305	D	500µM (3)