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# Semen Quality and Chemical Oxidative Stress; Quantification and Remediation

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SEMEN QUALITY AND CHEMICAL OXIDATIVE STRESS; QUANTIFICATION

AND REMEDIATION


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**DEDICATION**

I dedicate this thesis to the memory of my father Ali Saleem Bani-Hani; my great mother Fatima Al-kofahi; my wonderful wife Hala Abdallah for her support; my daughters Rowena and Leya; and to my brothers Najeeb, Rezig and Marzoog; my sisters Zuhor, Fadwa, Fadya, Wafa' and Noor for their believing in me.

# ACKNOWLEDGMENTS

SEMEN QUALITY AND CHEMICAL OXIDATIVE STRESS; QUANTIFICATION

AND REMEDIATION

SALEEM A. BANI-HANI

## ABSTRACT

The necessary multi-step process to prepare human semen for assisted reproduction (AR) such as *in vitro* fertilization (IVF) is known to induce oxidative stress in human spermatozoa and subsequent damage to the integrity of their cell components and, thus, to their function. Literature reports have viewed this as a potential link with the high frequency of birth defects among the IVF babies. In this work, we studied the effect of incubation and centrifugation on human sperm quality using established AR protocols. We found that *in vitro* incubation and centrifugation (20 min at 220$g$) of sperm generally degrade sperm quality in terms of motility and DNA oxidation. The total antioxidant capacity of the semen significantly decreased upon raising the centrifugation force from 220$g$ to 400$g$. We then studied the efficacy of supplements such as L-carnitine (LC) on remediating the oxidative stress. We found that supplementation with LC counteracts the overall damage of quality brought by the multi-step method of sperm processing. We determined the LC concentration range (0.1-1.0 mg/mL) with optimum oxidative stress remediation and which enhances qualities such as motility. Among the various oxidative stress species, we particularly focused on nitric oxide as a species with known dual functional and cytotoxic properties. We have demonstrated a method to monitor its dynamic concentrations in spermatozoa. The method enabled us to establish a link between motility and nitric oxide levels in spermatozoa. The method also allowed us to

establish that the LC-induced enhancement of human sperm motility is also accompanied with a simultaneous increase in NO production.

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# CHAPTER I

# INTRODCUTION AND OVERVIEW

## 1.1 Assisted reproductive technologies

Assisted reproductive technologies (ART) are the artificial techniques used to facilitate the pregnancy for couples who are trying to conceive without success. The most common ones are *In vitro* fertilization (IVF), Intracytoplasmic sperm injection (ICSI) and intrauterine insemination (IUI) [1; 2].

In IVF, the fertilization process, i.e. sperm and egg fusion, occurs outside the female's body in the Petri dish. The ultimate goal of this procedure is to increase the chance of fertilization that is not achieved through the normal intercourse. In IVF, the oocyte production is first induced using hormonal therapy. After ovulation, the eggs are retrieved from the female's ovary; in the meantime, sperm, fresh or cryopreserved, from

the male are prepared and added to the female's oocytes. The fertilized egg (gamete) is then incubated at 37ºC in the dark in highly sterilized conditions until the embryo is formed. After that, the embryo is transferred and placed in the center of female's uterus in order to achieve the pregnancy. In fact, several embryos are usually prepared and placed into the uterus so as to increase the success rate of the process.

The main primary steps of ovarian induction, oocyte retrieval technique, sperm preparations and embryo culture methods in IVF cycles are similar to those in ICSI cycles. Unlike IVF, in ICSI, the spermatozoa is selected based on certain criteria; spermatozoa of good motility and morphology is selected and injected artificially by the embryologist using a mico-needle inside the oocyte [3; 4] (figure 1-1). ICSI technique is mainly used to solve problems of severe male factor infertility as well as other problems that lead to failure of natural fertilization [3; 5].

In IUI, highly-motile washed sperm are injected into the female's uterus using a special plastic catheter [6]. The rationale of this method is to increase the number of highly-motile sperm at the site of fertilization, and hence increasing the probability of fertilization. IUI is used in cases where the sperm are unable, or barely able, to reach the oocyte in the female's body spontaneously. Such conditions are sexual dysfunction, asthenospermia (low sperm motility), and antagonistic cervical conditions such as increased cervical mucus thickness [6; 7; 8; 9].
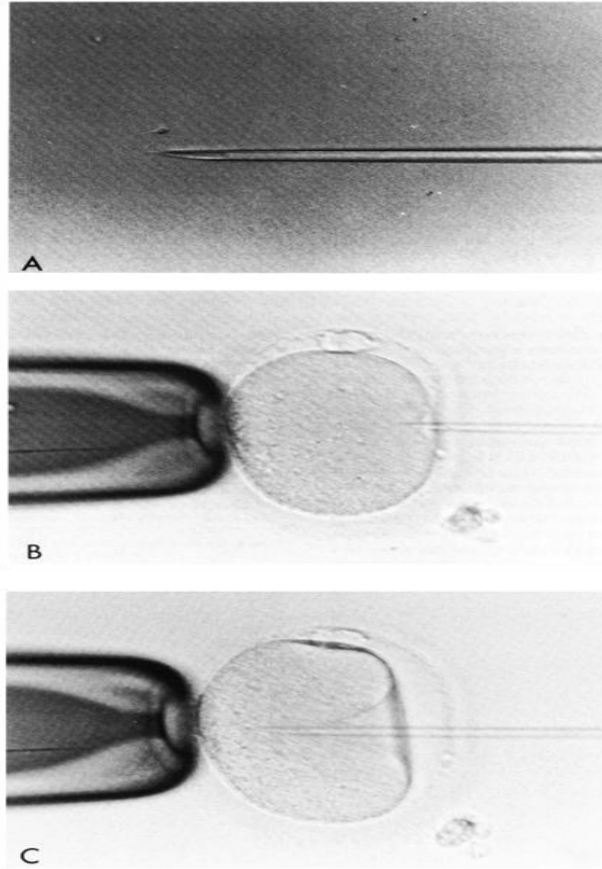
**Figure 1-1**: ICSI procedure. (A) the selected spermatozoa is immobilized and aspirated into a microinjection pipette;(B) the oocyte is secured using a holding pipette and a microinjection pipette; (C) the trapped spermatozoa is injected gently into the oocyte. (Adapted from M.C.Schiewe I, 1996).

## 1.2 Human semen processing for ART

Whether the sperm is fresh or cryopreserved, semen processing is routinely performed in all ART. Contemporary processing techniques of sperm preparation for ART require prolonged incubation as well as repeated centrifugation of human semen to separate sperm from the seminal plasma at speeds of 200-400g for 10 to 20 minutes. The processing of sperm should be gentle to allow the recovery of highly motile spermatozoa that are morphologically and functionally normal. Nevertheless, reports show that sperm centrifugation may generate low sperm quality, likely, by affecting the DNA integrity of sperm [10; 11]. New findings suggest that some birth defects occur more often among infants conceived with ART [12].

## 1.3 Reactive oxygen species and oxidative stress

Free radicals are highly reactive molecules or atoms that contain an unpaired electron. Among free radicals in biological systems, reactive oxygen species (ROS) are the most important due to their high reactivity in the body (figure 1-2). They are associated with the onset of a variety of diseases including cancer, arteriosclerosis, cardiovascular disease, infertility, and a host of inflammatory diseases [13]. The most important ROS in the human body are superoxide radical ($O_2^-\cdot$), hydroxyl radical ($\cdot OH$), Nitric oxide (NO), and the non-radical derivative hydrogen peroxide ($H_2O_2$) [14]. Endogenous entities such as mitochondria [15], peroxisomes, lipoxygenases, NADPH oxidase and cytochrome P450 as well as exogenous factors such as ultraviolet light, ionizing radiation, chemotherapeutics inflammatory cytokines, and environmental toxins are considered as to be among major factors that generate ROS in living cells [16].
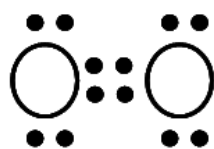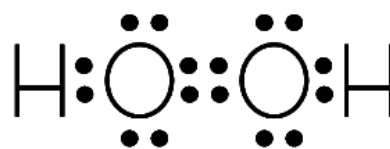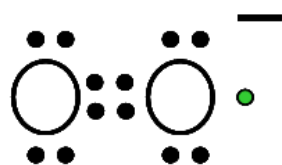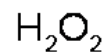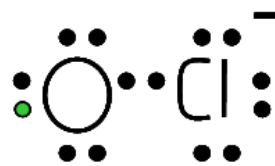
**Figure 1-2**: Lewis structure of dioxygen and some reactive oxygen species (ROS).

In live cells, the disturbance in the balance between oxidants and antioxidants in the favor of the former is termed as oxidative stress. Increased levels of free radicals such as $O_2^-\cdot$, NO and $\cdot$OH are capable of damaging DNA as well as the proteins and lipids, and ultimately leads to cell injury or cell death (Figure 1-3) [17; 18]. The mechanisms of the products formed by oxidative stress vary depending on the nature of free radical and its molecular target [18]. Some of oxidative products could be a rigid sign of oxidative stress and can be used as markers to assess the extent of the oxidative damage. For example, 8-oxo-guanine (8-oxodeoxyguanosine) serves as a sensitive marker to estimate the level of DNA damage caused by hydroxyl radicalS [19; 20].

## 1.4 Antioxidants role in human body

Antioxidants are substances capable of counteracting the free radical oxidative damage in body tissues [17]. They could be dietary, such as α-tocopherol (vitamin E) and ascorbic acid (vitamin C), or synthesized in the body like glutathione [21]. Antioxidants have attracted a great deal of attention for the defense they provide against oxidative stress [17]. Several studies showed that supplementation of dietary antioxidants such as vitamin E and β-carotene is associated with decreasing the risk of many diseases, such as heart diseases [22], cancer [23], Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis [24; 25; 26].

**1.5 Oxidative stress, antioxidants and human sperm quality in assisted reproduction**

Oxidative stress in human spermatozoa may be due to the lack of molecular defense mechanisms and increased sources of ROS [27]. Various studies have reported the role of oxidative stress in male infertility pathogenesis [28; 29]. Male infertility problems such as asthenozospermia and necrozoospermia may due to oxidative stress [27]. Similar to the effect of oxidative stress in other types of cell types, both sperm DNA and sperm membrane are affected negatively by oxidative stress (Figure 1-3), which consequently decreases the change of reproductive outcomes [27].

Sperm preparation for assisted reproductive technologies includes various routine steps such as incubation, cryopreservation and centrifugation. This induces oxidative stress and subsequently affects negatively the quality of sperm. The use of antioxidants to improve human sperm quality during processing for ART have been used extensively in the last 2 decades. Although there is a great evidence supporting the use of antioxidants to improve sperm quality in assisted reproduction [30; 31; 32; 33], optimal concentrations and the correct combination of the antioxidants should be cautiously standardized. In chapter III we will report on the efficacy of L-carnitine, a potent antioxidant, in improving sperm quality during centrifugation and prolonged incubation *in vitro* for use in ART protocols.
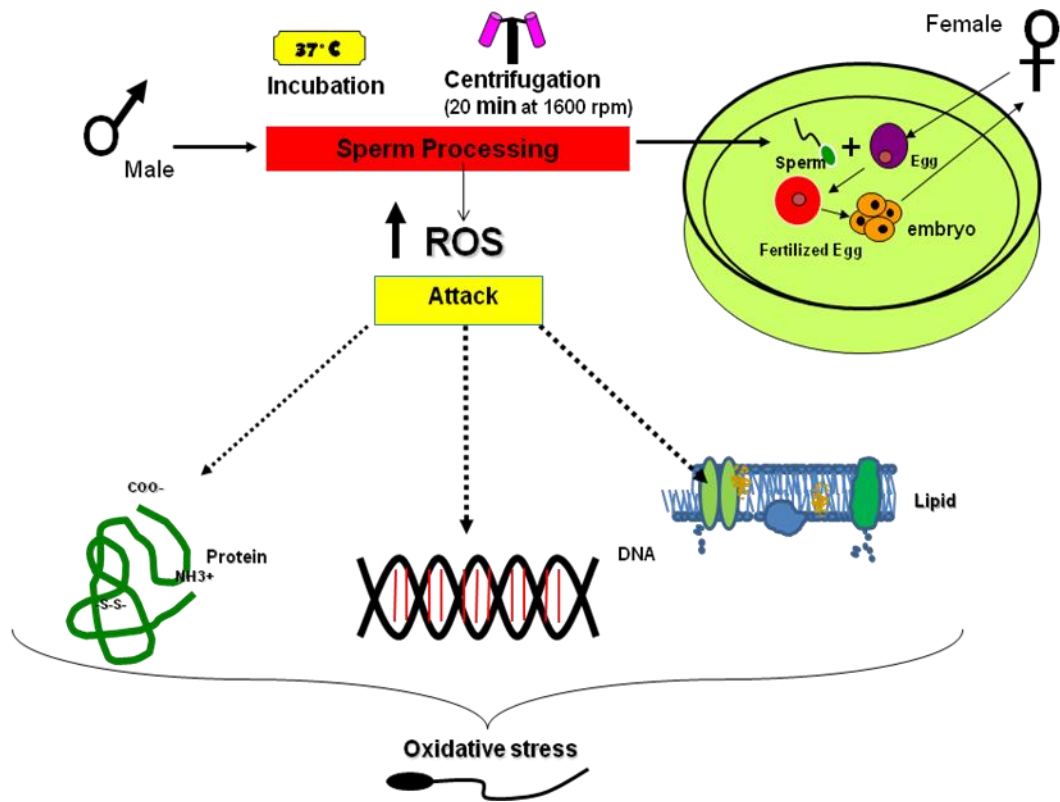
**Figure 1-3**: semen processing for ART such as IVF induces ROS against sperm.

## 1.6 Flow cytometry: an overview

In our experimental work, we intensively used flow cytometry to explore the effect of various factors affecting human spermatozoa in assisted reproduction. In addition, we standardized a method to detect the intracellular nitric oxide in human spermatozoa using flow cytometry. Therefore, there is a need to provide an overview of the main principles and fundamentals of this technique.

Flow cytometry is a technique for the analysis of various parameters of individual cells. It is used in a wide range of applications to measure different cell characteristics such as size, fluorescence, shape, viability, intracellular pH, intracellular redox state, and others. Besides, a variety of cell components can be determined using flow cytometry such as DNA, RNA, protein, Lipid, antigens, surface receptors, calcium and others. The flow cytometer achieves this analysis via passing a representative population of cells from the sample through a laser beam and capturing the emerged light from each individual cell as it passes through [34].

The main components of the flow cytometer are: (a) the fluidics (hydrodynamic focusing system). This system allows the chosen cell population to pass the laser beam one at a time (Figure 1-4). The intersection point between the laser beam and the cell is termed as interrogation point. (b) The laser, which represent the light source in the flow cytometer. (c) The optics, their main function is to gather the light from the cells after crossing the laser beam. (d) The detectors; they receive the accumulated light from the optics. (e) The computer system and other electronics, which convert the detector's signals into digital data and analyzes the data [34; 35; 36].

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At interrogation point, when the cell crosses the laser beam, two types of light scatter are formed: (a) forward scatter (FSC), which is the light, scattered in the forward direction, and (b) side scatter (SSC), which is the light scattered on the sides (Figure 1-4). In general, the forward scatter provides information about the size of the cell, whereas the side scatter provides information about characteristics within the cell such as granularity inside the cytoplasm, shape of nucleus, and membrane irregularity [37; 38; 39].
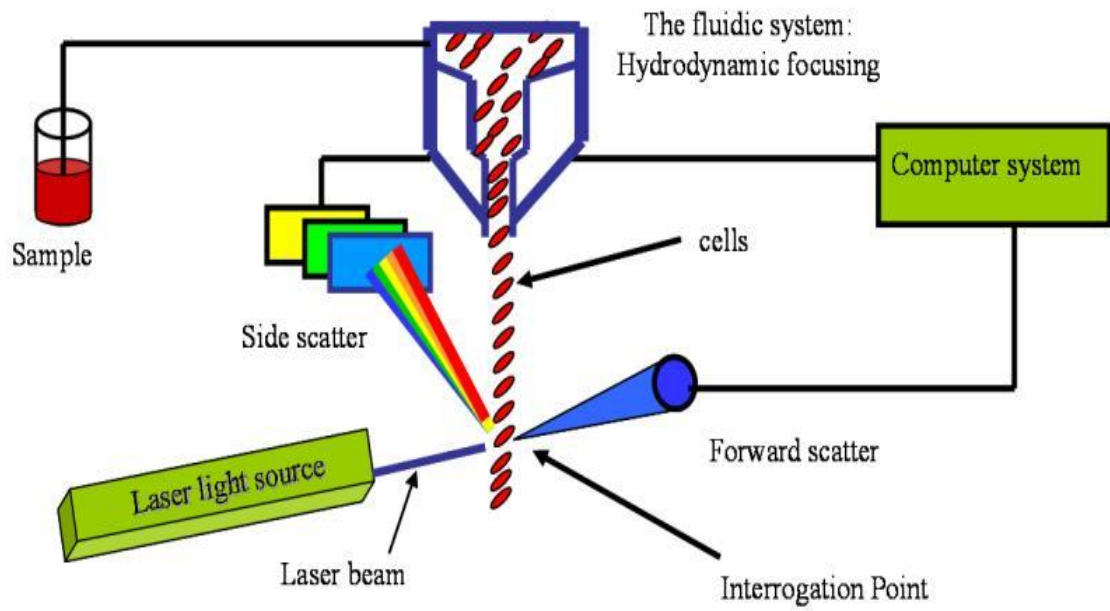
**Figure 1-4**: a representative scheme of flow cytometer system (see the text for details).

FSC is proportional to the size of cells that are passing the laser [38]. The bigger the cell size, the higher the FSC intensity. The detector converts the forward scattered light into a voltage pulse. Hence, the size of cell is finally proportional to the magnitude of the voltage pulse. The plot of cell count versus FSC is called forward scatter histogram (FSCH) (Figure 1-5). In this histogram, the larger cells appear on the right.

SSC is the light scattered at higher angles compared to the FSC. The main determinants of the angles of the scattered light are the structural complexity of the cell, as we have mentioned above. A separate detector in the flow cytometer, other than one used for FSC, is used to gather the side scattered light; it is usually placed 90° from the laser beam path. The plot of cell count versus SCC is also a one-dimensional histogram named side scatter histogram (SSCH) (Figure 1-6). It is similar to the FSCH, but it provides information about the cells characteristics under investigation.
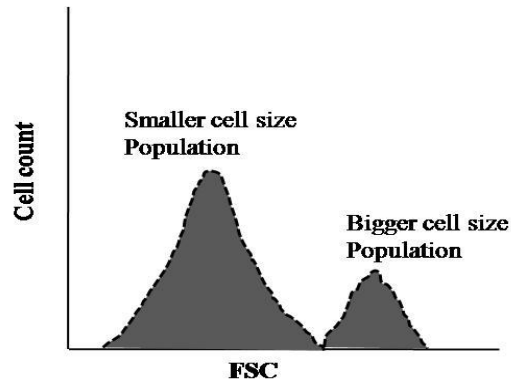
**Figure 1-5**: Flow cytometry forward scatter-histogram. Cells are sorted according to their sizes
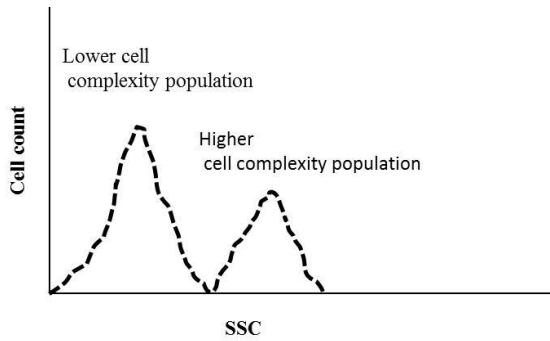


**Figure 1-6**: Flow cytometry side scatter-histogram. Cells are sorted according to their complexity fluorescence.
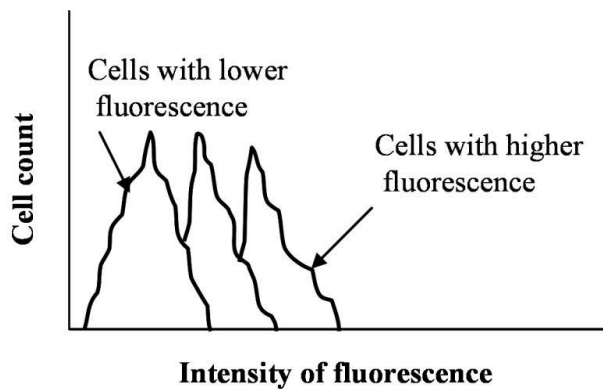


**Figure 1-7**: Flow cytometry fluorescence-histogram.

Fluorescence is another phenomenon used as handle to quantify a given property within cells. At the fundamental levels, it is a term used to describe light emission, mainly visible, from an excited fluorophore. The emission occurs when the fluorophore returns to its ground state from an excited state. Figure 1-8 is the Jablonski diagram (energy state diagram) of a fluorophore excitation and emission. The emitted light from a fluorophore occurs at a higher wavelength. For example, the fluorophore fluorescein isothiocyanate (FITC) that we used in this work to detect DNA oxidation can be excited with the blue light at 494 nm; while it emits the green light at 518 nm.

Fluorescence emitted from cells labeled with a fluorophore travels in the same path in the flow cytometer as the side scatter light. This path contains a number of light filters so as to deliver each range of wavelengths to the proper detector. Same as data collection in the cases of FSC and SSC, the light directed to the detector is translated into a voltage pulse. The more the light emitted from the cell, the higher the voltage pulse translated. The plot of intensity of fluorescence versus cell count is termed as flow cytometry fluorescence-histogram, which provides information about cell fluorescence that is increasing in the right direction (Figure 1-7).
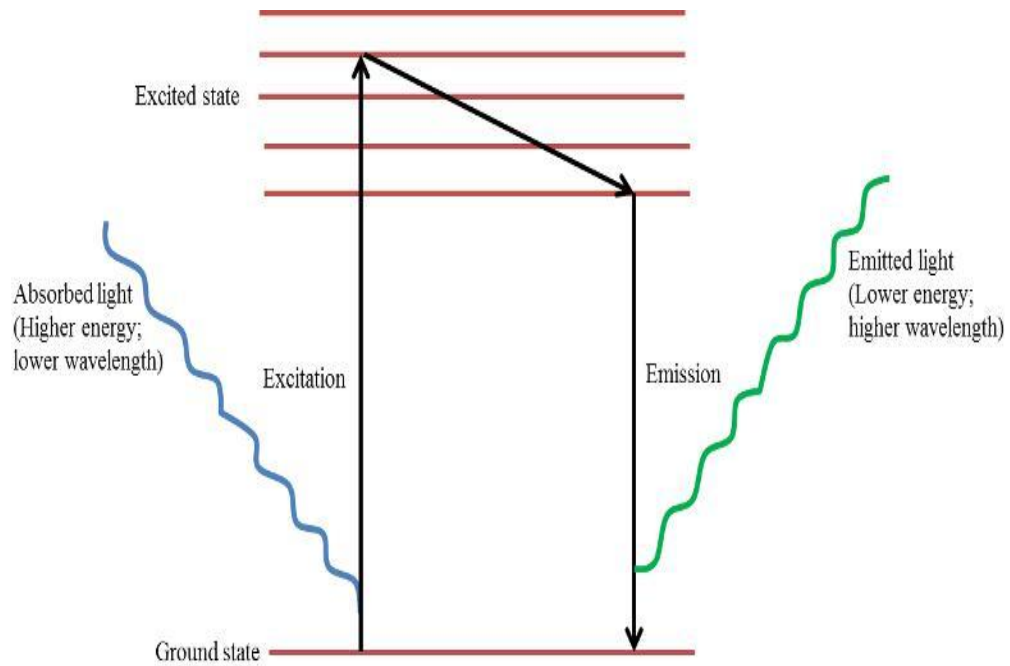
**Figure 1-8**: Jablonski diagram (Energy state diagram) - fluorophore excitation and emission. The fluorophore emission usually occurs at a lower wavelength; for example, the fluorophore Fluorescein isothiocyanate (FITC) can be excited at 494 nm, while it emits the light at 518 nm.

Forward scatter histogram is not necessarily showing the complexity of the cells. In other words, the peak population that appears in the FSCH could be a representative of multiple population. This problem can be resolved by looking at the data in the second dimension using a two-dimensional scatter plot or dot plot (Figure 1-9). In this plot (SSC versus FSC), we can look at both cellular parameters together: cell size and cell complexity.

Another important problem in flow cytometry is to analyze only the cells of interest. In many cases, cell fragments such as, small immature cells and debris, and sometimes bigger cells that are not of interest interfere with the measurement. This problem can be solved by establishing a threshold, or discriminator before start analyzing the cells. This will exclude interferences for compounds that are not of interest.
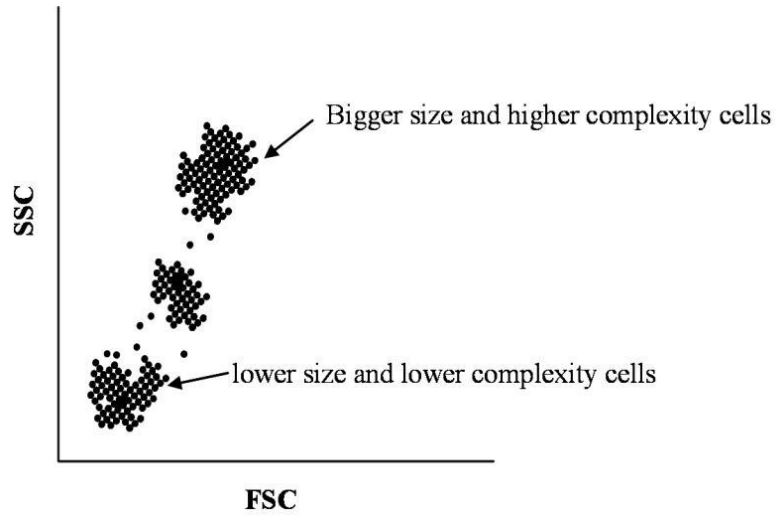
**Figure 1-9**: flow cytometry Scatter or dot plot. Cells are analyzed for both complexity and size.

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# CHAPTER II

# SEMEN QUALITY DURING PREPARATION FOR ASSITED REPRODUCTIVE TECHNOLIGIES

## 2.1 Introduction

Sperm centrifugation, required for sperm washing, is a routine technique during sperm processing for assisted reproduction technologies (ART). Reports from our laboratory showed a significant increase in reactive oxygen species (ROS) during sperm centrifugation. Studies have shown a decrease in fertilizing capacity of human spermatozoa, and an increase in the birth defects among the ART babies compared to the normal babies. Here, we examine the effect of centrifugation on total antioxidant capacity (TAC) of human semen on one hand, and on DNA oxidation, motility, and viability of sperm, on the other. We measured sperm motility, viability TAC and sperm DNA oxidative damage after centrifugation of semen samples for 20 min at $220g$. DNA oxidative damage in sperm was measured using both electrochemical and flow cytometry methods. Centrifugation significantly decreased sperm motility. The total antioxidant

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capacity of the semen significantly decreased upon raising the centrifugation force from 220$g$ to 400$g$. Concomitantly, the baseline of DNA oxidative damage in sperm increased after centrifugation. Together, sperm centrifugation during processing for ART increases oxidative stress in sperm, which negatively affects their quality. This could be one factor behind the negative reproductive outcomes.

## 2.2 Research methods and experimental design

In this chapter we aimed to examine the effect of centrifugation on sperm quality according to the assisted reproduction protocols. We used DNA oxidation, motility and viability of sperm, as well as total antioxidant capacity (TAC) of semen as measurement criteria to study this effect. The oxidative DNA damage in sperm was evaluated using both electrochemical and flow cytometry techniques. We also adapted an assay based on cupric reducing antioxidant capacity (CUPRAC) to measure the TAC in human semen [1].

### 2.2.1 Sample collection

The present study was approved by the institutional review board of Cleveland Clinic Hospital. Forty normospermic men were chosen and analyzed on the basis of normal semen analysis according to the World Health Organization (WHO, 1999) guidelines [2]. The effect of centrifugation on TAC of semen as well as DNA oxidation, motility and viability of sperm were examined. Samples were centrifuged at 1600 rpm (220$g$) for 20 minutes similar to sperm preparation protocols used in ART. Samples were collected by masturbation after a minimum of 48 to 72 hours of sexual abstinence. Following

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liquefaction at 37 ºC for 20 minutes, semen specimens were evaluated for volume, sperm concentration, total cell count, motility, and morphology.

## 2.2.2 Effect of centrifugation on sperm motility and viability

Two equal aliquots (5 X $10^6$ sperm) were prepared from each of the sixteen normospermic semen samples, and mixed with human tubal fluid (HTF; 1:1 vol. / vol.). The first aliquot was centrifuged at 1600 rpm at 25 ºC; while the second aliquot (control) was just incubated at the same temperature. Sperm motility and viability was measured after 20 min. A total amount of 5µL of the sample was used for evaluation of concentration and motility using a micro-cell slide chamber (Figure 2-1) (Conception Technologies, San Diego, CA).

Sperm viability was measured by the Eosin-Nigrosin staining method (Figure 2-2). To one drop of liquefied semen placed on a Boerner slide, two drops of 1% aqueous Eosin was added and mixed with a wooden stirrer for 15 seconds. Then, three drops of 10% aqueous Nigrosin were added and mixed well. Thin smear was made by pipetting 10µL onto a slide and allowed to air dry. Slides were mounted with a coverslip using Accu-mount media. Measurements were performed on two hundred sperm counted on each slide in duplicate sets using an X100 objective. Percentage of viable (unstained) sperm and non-viable (stained) were calculated.

**Motility:** (normal: >50%)

Motility (%) = Motile sperm X 100%
Total sperm

If we have one motile spermatozoa
In the first two rows, then the motility:
(1/7) (100%) = 14.3%

Disposable Hematocytometer

5 µl semen
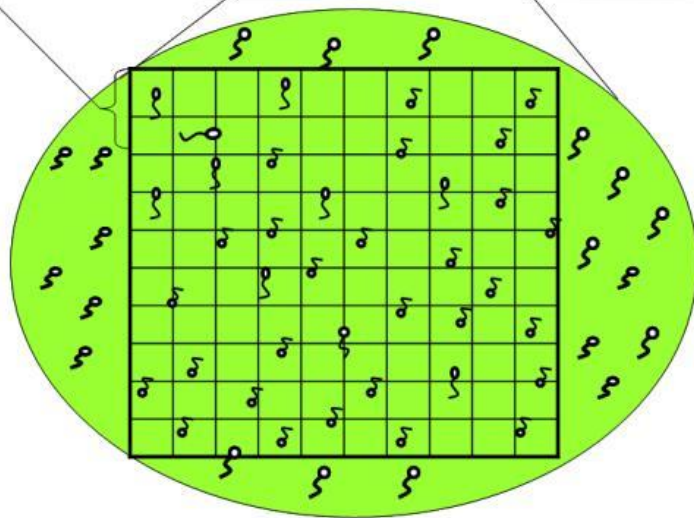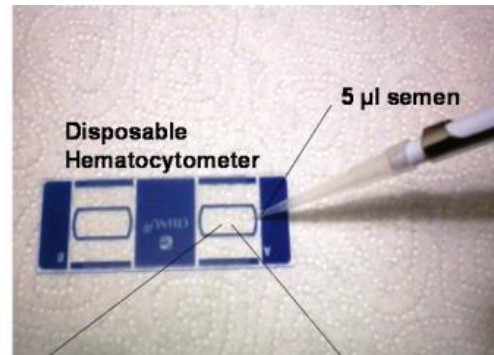
**Figure 2-1:** Sperm motility assessment. 5µL of semen sample is used to assess motility using a disposable micro-cell slide chamber (an example is shown in the figure).
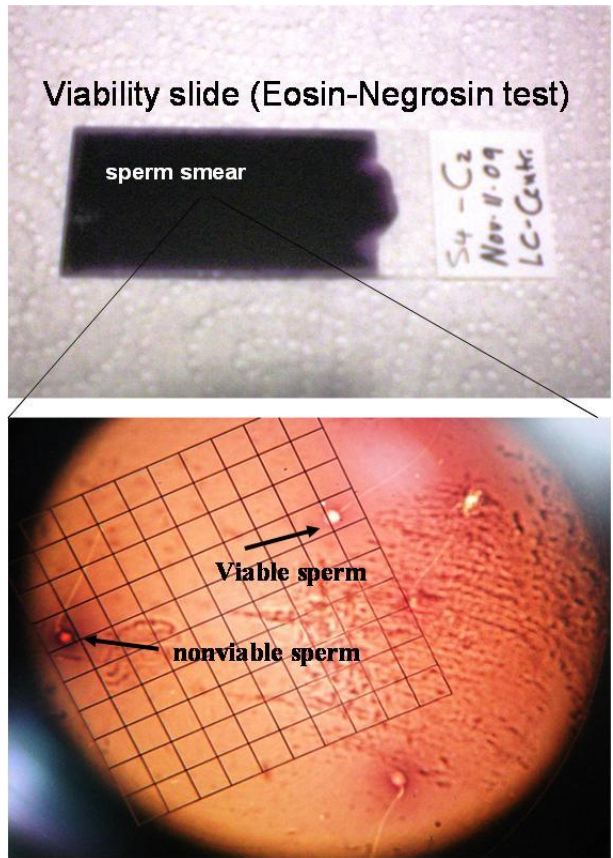
**Figure 2-2**: Sperm viability measurement using the Eosin-Nigrosin test. Stained sperm is considered viable, while unstained one (white) is considered non-viable.

## 2.2.3 Effect of centrifugation on total antioxidant capacity

Semen samples (n=10) from healthy donors, were divided into two aliquots; each contained 0.5 mL of semen. We centrifuged the first aliquot at 220$g$ for 20 min at 25 ℃, while the second aliquot (control) was incubated for 20 min at the same temperature. Semen samples (n=10), in another experimental set, were subjected to the same treatment as described above, but centrifuged at a higher centrifugal force (400$g$).

We adapted the cupric reducing antioxidant capacity (CUPRAC) method to measure the total antioxidant capacity in semen [1]. Briefly, 1 mL of working solution (CuCl$_2$ solution; 0.02 M), neocuproine alcoholic solution (0.0075 M) and 1-mL ammonium acetate (NH$_4$Ac) buffer solution (1:1:1 vol./vol./vol.) was mixed in a centrifugation tube with 12.5 µL of seminal plasma. All reaction tubes were centrifuged 3 min at 4000 rpm, and the entire supernatant was collected for use in absorbance measurements. The absorbance of the colored complex formed was measured against the reagent blank at 450 nm after 30 min incubation at room temperature (Figure 2-3) [3].
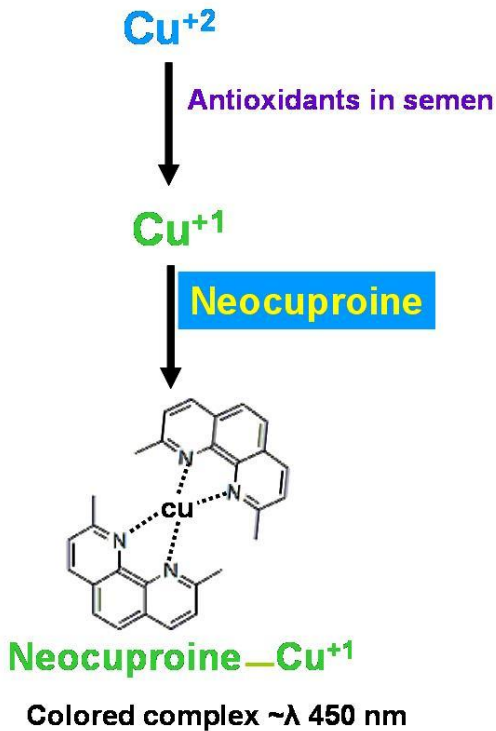
**Figure 2-3**: Cupric ion reducing antioxidant capacity (CUPRAC) principle. The colored complex (Neocuproine-Cu$^{+1}$) is formed after reducing cupric ion by semen antioxidants. The intensity of the color is proportional to the total antioxidant activity in semen.

## 2.2.4 Measurement of DNA oxidation by Flow cytometry

Semen samples (n=5) from healthy donors were divided into 2 aliquots (5 X $10^6$ sperm) and centrifuged at 1600 rpm for 20 min. at 25ºC. Controls were incubated for 20 min. at 25ºC. Oxidative damage to DNA was evaluated using the flow cytometric OxiDNA assay kit (Calbiochem, San Diego). The assay is based on fluorescent protein that binds to oxidized moieties on DNA (Figure 2-4). Briefly, both treated sperm samples and controls were washed in phosphate saline buffer, resuspended in 1% paraformaldehyde at a concentration of 2-4 x $10^6$ cell/ mL, and placed on ice for 15 to 30 minutes. Sperm were again washed and resuspended in 70% ice-cold ethanol by centrifugation at 300*g* for 5 minutes as per kit instructions. The ethanol supernatant was removed, and the sperm pellets were washed twice in the wash buffer and resuspended in 100 µL of the staining solution for 1 hour at room temperature in dark. The staining solution contained FITC-labeled protein conjugate in distilled water. All cells were further washed using rinse buffer, resuspended in 250 µL and incubated for 30 minutes in the dark on ice for flow cytometry measurements.

Data acquisition was performed within 30 min on a flow cytometer equipped with a 515-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). 10,000 cells were examined for each assay at a flow rate of 100 cells/ second. The FITC fluorescence was measured and data analysis was performed using FlowJo v4.4.4 software (Tree Star Inc., Ashland, OR).
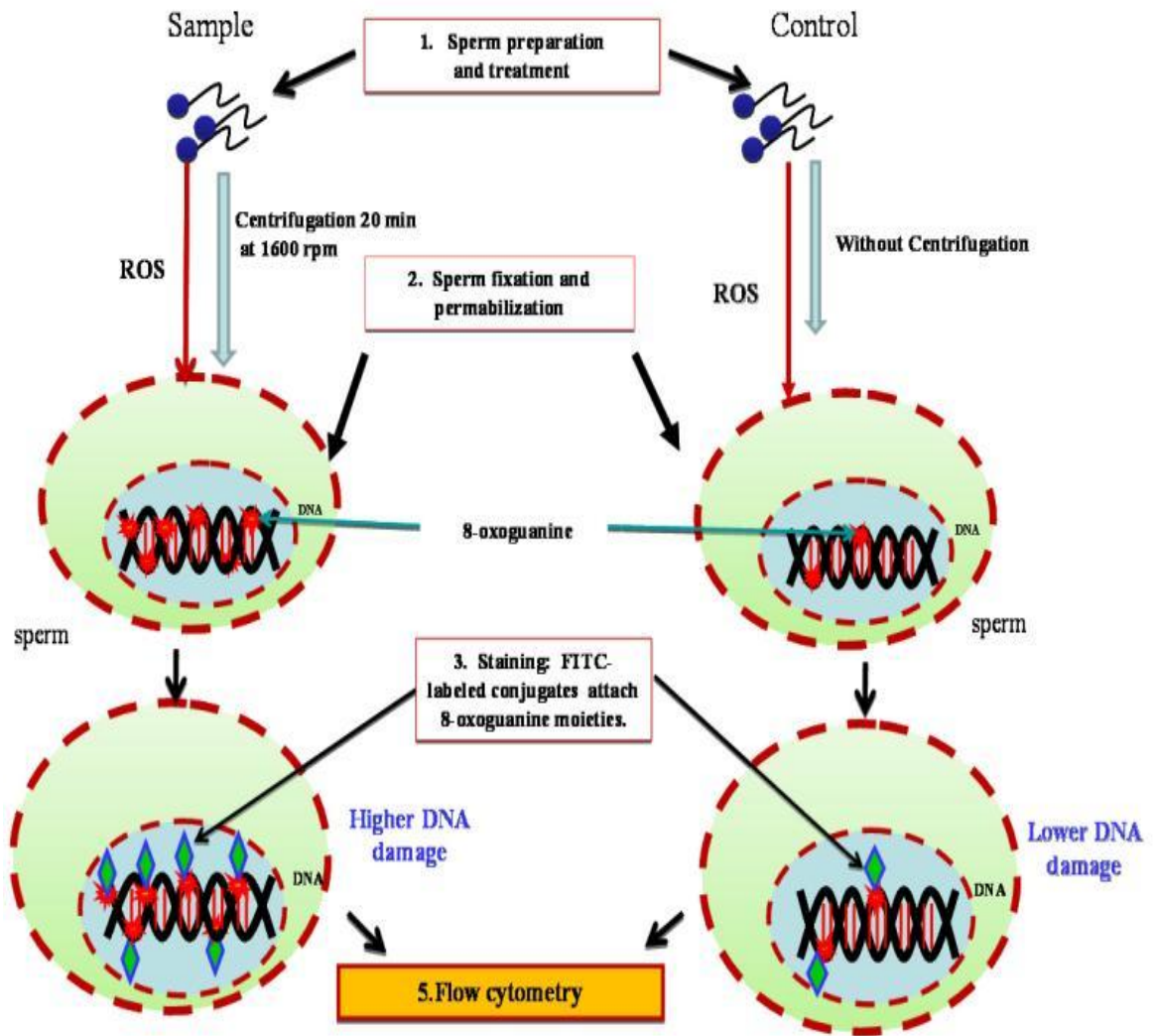
**Figure 2-4:** Experimental design of assessment of DNA oxidation using flow cytometry.

## 2.2.5 Measurement of DNA oxidation by Electrochemistry

Semen samples (n=5) from healthy donors were divided into 2 aliquots (sample and control). Each aliquot ($5 \times 10^6$ Sperm) was exposed to 20 min centrifugation at 1600 rpm under 25 ℃. Controls of the same sperm concentration were incubated for 20 min at 25℃ without centrifugation.

## 2.2.5.1 Sperm DNA Extraction

Cell lysis buffer (0.5 mL) and 60 µL of protinase K were added to both samples and controls and incubated overnight at 55 ℃. A 0.5 mL aliquot of chloroform: phenol mixture (1:1 vol./ vol.) was added and centrifuged for 5 min at 6000 rpm. The supernatant (aqueous layer) was collected, and 0.5 mL of chloroform was added, vortexed and centrifuged. The top layer was collected and mixed with 0.5 mL isopropyl alcohol to salt-out the white DNA pellet. The precipitated DNA was collected by centrifugation and the supernatant was gently decanted. Finally, the DNA was dissolved in 200 µL Tris-EDTA buffer and kept at 4℃ for future use.

## 2.2.5.2 DNA film preparation

DNA films were prepared by layer-by-layer electrostatic assembly method [4]. The method is simply based on adsorbing thin layers from two polyelectrolytes with opposite charge (in this case: poly (diallyldimethylammonium chloride) (PDDA) and the DNA sample under investigation). Disks of ordinary basal plane pyrolitic graphite (PG) were abraded with 400 grit silicon carbide (SiC) paper and sonicated in water for 1 min, then rinsed with water and dried. The graphitic surfaces of these electrodes are then

oxidized in 2.5% $K_2Cr_2O_7$/10% $HNO_3$ by scanning the potential once from 1.5V to 1.75V versus Ag/AgCl to form negative carboxylate groups on the surface [5]. Layers were formed by placing a drop of each adsorbate solution on the PG electrode for 15-20 min. The electrode was then rinsed with water to remove weakly adsorbed DNA and dried between adsorption steps. The times for steady-state adsorption, volume and concentration of each adsorbate solution were: (1) 15 min, 25 μL of 2 mg $mL^{-1}$ poly(diallyldimethylammonium chloride) (PDDA) in 50 mM NaCl; (2) 20 min, 25 μL of 0.1-0.2 mg $mL^{-1}$ DNA in 5 mM pH 7.1 Tris buffer and 0.50 M NaCl. Final DNA film architecture was denoted as the order of layer adsorption, (PDDA/DNA) $_2$. Alternate adsorption cycles were repeated until the desired number of layers was made. The number of layers of DNA/PDDA was optimized for the best time-sensitivity ratio. In this work films with 2 layers of DNA/PDDA ([DNA/PDDA]$_2$ films) were used (Figure 2-5).
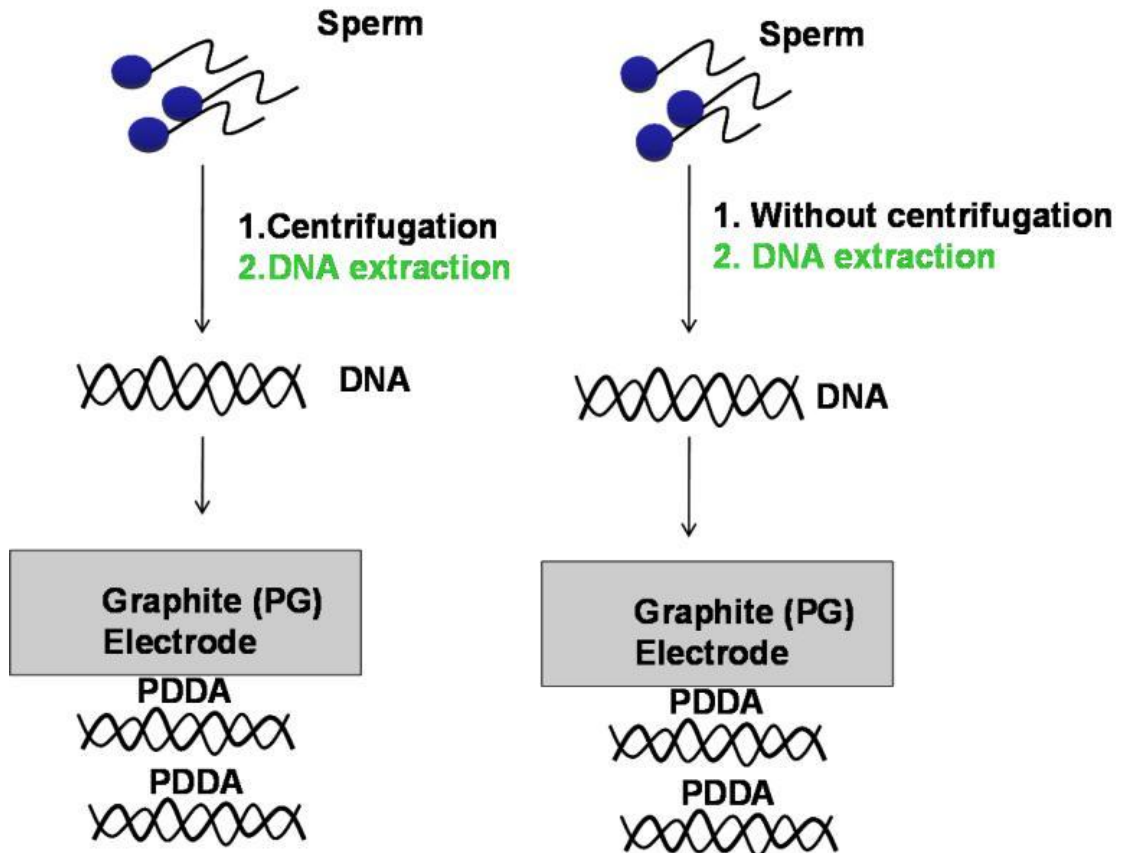
**Figure 2-5:** Electrochemical experiment design to explore the effect of sperm centrifugation on sperm DNA damage. Final DNA film architecture is denoted as the order of layer adsorption; (PDDA/DNA) $_2$.

### 2.2.5.3 Square Wave Voltametry

Square Wave Voltametry (SWV) was performed using CH electrochemical work station (CH Instruments 440A). The electrochemical cell employed an Ag/AgCl reference electrode, a platinum wire counter, and a film-coated PG disk as the working electrode. Unless otherwise noted, the SWV conditions were 4 mV step height, 25 mV pulse height, and 5 Hz. The electrolyte solution was 10 mM acetate buffer, pH 5.5, containing 50 mM NaCl and 50 µM Tris(2,2'-bipyridine)ruthenium(II), (Ru $(bpy)_3^{2+}$) as the reporting redox probe.

### 2.2.6 Statistical analysis

All values were reported as mean ± standard deviation. Difference was considered significant at $P < 0.05$ against the control group. Statistical analyses were operated using one-way analysis of variance (ANOVA) followed by an unpaired Student's $t$-test using the SPSS/PC computer software (SPSS 10.0.7, SPSS Inc.).

## 2.3 Results

### 2.3.1 Effect of sperm centrifugation on sperm motility and viability

Figure 2-6 shows the effect of semen centrifugation (at 220$g$, 20 min) on sperm motility and viability. Figure 1A shows the mean values ± standard deviations of sperm motility for both samples and controls (i.e. without centrifugation). Sperm motility was significantly different between centrifuged and uncentrifuged samples (24.0 ± 12.8 vs. 33.3 ± 14.2%) ($P < 0.05$; $t$-test). In contrast, no significant change was seen in sperm viability between centrifuged and uncentrifuged samples (34.2 ± 12.6 vs. 39.1 ± 9.7%, respectively; Fig.1B).
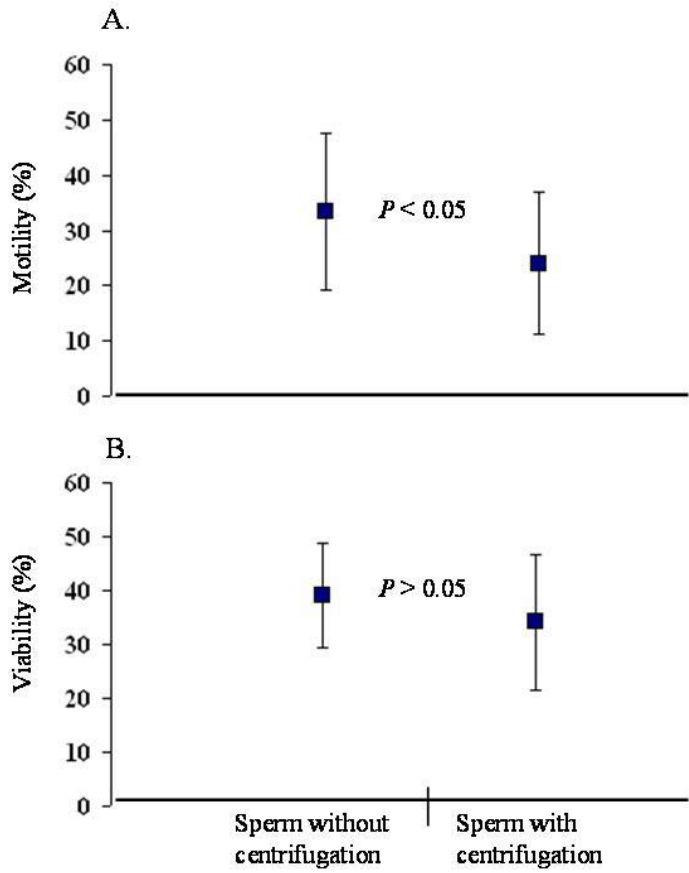
**Figure 2-6:** Effect of semen centrifugation on sperm motility (A) and viability (B). Sperm samples centrifuged 20 min at 1600 rpm; control assayed without centrifugation. Data are mean values ± standard deviations obtained from analysis of 16 semen samples.

**2.3.2 Effect of semen centrifugation on total antioxidant capacity of seminal plasma**

Figure 2-7 shows the effect of semen centrifugation on the total antioxidant activity (TAC) in seminal plasma as measured by the CUPRAC assay. The absorbance measured at 450 nm is directly proportional with the TAC of semen sample. We saw an insignificant difference ($P > 0.05$, $t$-test) in average absorbance ($0.67 \pm 0.08$ vs. $0.71 \pm 0.09$) at 450nm, and thus in total antioxidant capacity of seminal plasma in semen samples (exposed to 20 min centrifugation at $220g$) and the control, Figure 2A. On the other hand, we observed a significant deacrease in average absorbance ($0.78 \pm 0.1$ vs $0.62 \pm 0.03$) ($P < 0.05$; $t$-test) and thus in antioxidant activity in semen samples centrifuged at forces between $220g$ and $400g$ under the same conditions, Figure 2B.

**2.3.3 Effect of centrifugation on sperm DNA oxidation as evaluated by flow cytometry**

The effect of centrifugation on sperm DNA oxidation as evaluated by flow cytometry is shown in Figure 2-8. The flow cytometry analysis of FITC-labeled sperm showed that the intensity of the FITC fluorescence increased significantly ($P < 0.5$, $t$-test) upon centrifugation of sperm at $220g$ for 20 min. The intensity of fluorescence, represented as mean area of FITC-fluorescence $\pm$ standard deviation, for uncentrifuged and centrifuged sperm were $58.5 \pm 3.4$ vs $210.5 \pm 14.8$, respectively. This result shows that the onset of DNA oxidative stress is significant at forces as low as $220g$.
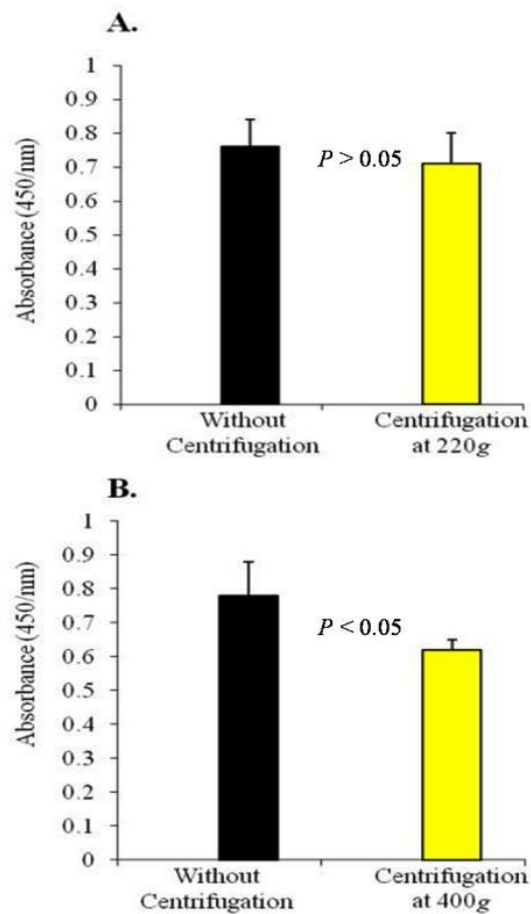
**Figure 2-7:** Effect of semen centrifugation on total antioxidant activity of seminal plasma as evaluated by Copper Reducing Antioxidant Capacity (CUPRAC). (A) Sperm centrifuged 20 min at 220$g$; (B) sperm centrifuged 20 min at 400$g$. Controls assayed without centrifugation. The absorbance at 450 nm is proportional to the total antioxidant activity in semen. $P$-value < 0.05 is considered statistically significant
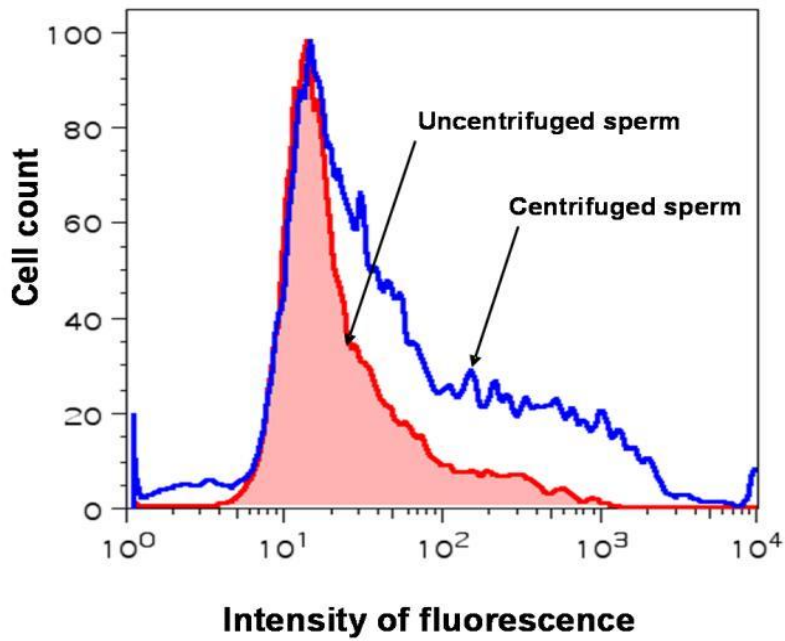
**Figure 2-8:** Effect of centrifugation on sperm DNA oxidation as evaluated by flow cytometry. Sperm centrifuged 20 minutes at 1600 rpm; control assays without centrifugation. The X-axis represents the intensity of FITC fluorescence. Data are representative of 4 independent experiments.

### 2.3.4 Effect of centrifugation on sperm DNA oxidation as evaluated by square wave voltammetry

DNA damage can also be detected through the -now standard- electrocatalytic oxidation of guanine bases by ruthenium tris-bipyridine as introduced by Thorp et al.[6; 7; 8]. Damaged DNA, with open double helices or broken strands, is expected to provide the reporter ruthenium redox probe higher access to guanine sites, and thus would generate oxidation currents commensurate with the extent of DNA damage. This criterion (i.e. large electrocatalytic currents equivalent to large DNA damage) is used here as such without explanation of the fundamental concepts, which can be found in numerous original papers using this method [6; 7; 8].  All DNA damage measurements are carried out in 10 mM acetate buffer, pH 5.5 solutions containing 50 mM NaCl and 50 $\mu$M Ru(bpy)$_3$$^{2+}$ as the reporter probe. All experiments are tripilcates and data points reported are means of 4 independent experiments.

Figure 2-9 shows the voltametric response of DNA modified electrode for both centrifuged and uncentrifuged sperm for the desired period of time. As seen in the figure, an increase in the oxidation current by 17.8% ($I_{centrifuged\ sperm}/I_{uncentrifuged\ sperm} = 1.178$), and thus in sperm DNA oxidative damage, was seen in sperm samples subjected to centrifugation (220$g$) compared with the controls (without centrifugation).

**Figure 2-9:** Square wave voltametry of (PDDA/DNA) $_2$ films on PG electrode in pH 5.5 buffer containing 50 μM Ru(bpy)$_3$ $^{2+}$. DNA samples were extracted form sperm exposed to 20 min centrifugation at 220$g$; control samples were DNA extracted form uncentrifuged sperm. Data are representative of 4 independent experiments.

## 2.4 Discussion

It has been demonstrated that centrifugation has an adverse effect on sperm recovery rates [9]. Centrifugation $< 330$ $g$ for about 15 min had insignificant effect on sperm motility. However a significant decrease in sperm motility was observed with centrifugation above $580g$ at the same length of time [10]. Our study shows that centrifugation forces as low as $220g$ for 20 min result in a significant decrease in sperm motility. We applied a longer centrifugation period (20 min) since this is an IVF protocol used in many andrology labs. A recent study reported by Lampiao *et al.* (2010) found that sperm centrifugation from 10 to 30 min at $400g$ also decreased sperm motility [11]. Our results are in agreement with these findings.

In an earlier study, we reported that centrifugation of motile sperm induces the generation of reactive oxygen species (ROS) [12]. In sperm washing procedures, depriving the sperm of seminal plasma –their antioxidant reservoir-, is a main contributing factor behind increased levels of ROS [13]. Induced ROS, mainly hydroxyl radicals, were shown to affect sperm function and motility [14]. The mechanism by which the elevated levels of ROS decreases sperm function and motility could be via increasing sperm's lipid peroxidation as well as direct oxidation of sperm DNA bases [14].

The effect of centrifugation on sperm viability was assessed using the one-step Eosin-Nigrosin test [15]. This test is principally based the membrane permeability for the supravital Eosin stain. Membrane injury allows the Eosin to enter inside the spermatozoa and stain it. The intensity of the color is proportional with the magnitude of the damage

[15]. The unstained spermatozoa (white-colored under the microscope) were considered viable, while the stained spermatozoa, regardless the intensity of the stain color, considered non-viable. Our results from this experiment show that viability is not affected by centrifugation (at least for a 20 min centrifugation period at 220$g$).

Namik *et al*. (2000), in their study measured sperm viability after cryopreservation-thawing cycles, using annexin V-Cy3 and eosin-Y staining. They noticed a significant variance between the percentages of sperm accepted as viable between both tests, in which, Eosin-Y staining test showed lower percent of live sperm compared to the annexin V-Cy3 test [16]. This variation is attributed to the different mechanisms through which these tests function. In the present study, we assessed the viability of sperm using the Eosin stain test that is based on the membrane permeability to Eosin. The mechanism by which this test categorizes the viable versus non-viable sperm could be a contributing factor behind the insignificant effect of centrifugation on sperm viability. Using other reliable viability tests may be needed for conclusive results on the effect of centrifugation on viability.

As a criterion of oxidative damage exposure, we have adapted the CUPRAC method, developed by Apak *et al*. (2008), to measure the total antioxidant capacity (TAC) in human semen. We used this assay because the reaction is carried out at nearly physiological conditions. In addition, the CUPRAC method is straightforward and reliable [1].

At 220$g$ centrifugation force, our results did not show a significant effect of centrifugation on TAC of semen samples compared to controls. However, we observed a significant decrease in semen TAC at 400$g$. It was reported that repeated sperm

centrifugation, the key step in sperm washing during processing for ART, leads to an excessive formation of ROS [12]. Along the same lines, a higher centrifugation force may induce more ROS formation [11]. Accordingly, the increased levels of ROS explain the decreased TAC that we observed in seminal plasma of sperm subjected to centrifugation at 400$g$.

Our flow cytometry experiment was designed to study the effect of centrifugation (20 min at 220$g$) on sperm DNA oxidation. Sperm samples were fixed, permabilized and the DNA oxidation was determined via specific detection of 8-oxoguanines, biomarkers of DNA oxidative damage, using FITC-labeled conjugate.

The results from this experiment showed an increase in the baseline of sperm DNA oxidation in the centrifuged sperm compared to the control sample.  It instructive to compare our findings to results obtained by other methods. In this regard, G.D. Smith *et al.* reported that  Sperm centrifuged during washing steps with HTF show an increase in the DNA fragmentation using sperm chromatin dispersion assay (SCD) [17]. Our findings are in line with these findings, and may indicate that the increase in DNA damage that we observe may be the result of increase in DNA breaks.

In live cells, including sperm, the presence of metal ions, such as ferrous and cupric ions, and hydrogen peroxide ($H_2O_2$) is known to produce hydroxyl radicals through what is termed as the Fenton reaction [18; 19]. Hydroxyl radicals are highly reactive oxygen species that induce lesions in DNA bases and deoxyribose sugars in double helix backbone, which leads to unwinding and DNA fragmentation and breaks [20].

In the electrochemical experiment, the $Ru(bpy)_3^{2+}$ redox probe reports on exposed guanine bases in DNA [21]. The $Ru(bpy)_3^{2+}$ is oxidized at the electrode surface to $Ru(bpy)_3^{3+}$. In the presence of exposed guanine bases, the oxidized ruthenium probe is reduced back to Ru (II) state (eq.1), which is again reoxidized at the electrode. This process produce a catalytic current proportional to the number of exposed guanine bases [22]. The double-stranded DNA shields guanine moieties from effective contact with $Ru(bpy)_3^{3+}$. Guanine becomes more available after chemical damage occurs in the DNA this provides more exposure, and thus, access to the resulting catalyst [23].

$$Ru(bpy)_3^{3+} + DNA(guanine) \longrightarrow Ru(bpy)_3^{2+} + DNA(guanine^+) \quad (Eq.1)$$
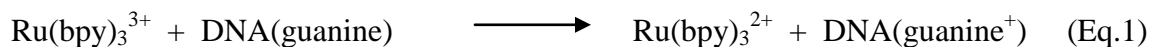
These results show an increase in the baseline of sperm DNA damage after centrifugation. This is confirms our results obtained with the flow cytometry method.

## 2.5 Conclusion

In summary, semen centrifugation during sperm preparation for ART may negatively affects sperm quality, via increasing baseline of sperm DNA oxidation and decreasing seminal TAC and sperm motility. This might be one factor behind the negative reproductive outcomes such as the increased birth defects among the IVF babies compared to the normal babies.

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# CHAPTER III


## L-CARNITINE AS PROTECTIVE MEDIA FOR SPERM IN ASSISTED REPRODUTIVE TECHNOLOGIES

**Summary**


In vitro incubation and centrifugation are known to decline human sperm quality. In our bodies, besides its antioxidant effects, L-carnitine facilitates the transport of the activated fatty acids from cytosol to the mitochondrial matrix and has antioxidant effects. Here, we investigated the effect of L-carnitine (LC) on human sperm motility, viability and DNA oxidation after incubation and centrifugation, following the sperm preparation protocols of assisted reproduction. Normospermic semen samples (n=55) were analyzed according to the World Health Organization (WHO) guidelines. LC concentrations that are not toxic to sperm using sperm motility and viability were standardized after 2- and 4-hour incubation at 37°C. Semen samples were also centrifuged for 20 min at 1600 rpm with the optimized LC concentration and analyzed for sperm motility, viability and DNA oxidation. Optimal improvement in sperm motility was seen at 0.5 mg/mL LC after

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incubation and centrifugation with 5 X $10^6$ sperm/mL. Higher concentration of LC (50 mg/mL) significantly decreased sperm motility and viability. LC did not alter the baseline of sperm DNA oxidation during both incubation and centrifugation. In conclusion, LC may enhance sperm motility following incubation and centrifugation, while it might not affect sperm viability and DNA oxidation.

## 3.1 Introduction

Infertility affects one in seven couples who are trying to conceive. Male factor infertility is involving in up to half of all infertile couples [1; 2]. Decreased semen quality, a measure of the ability of semen to achieve fertilization, is a primary cause of male infertility. Usually, poor semen quality is characterized by low sperm motility and viability. Assisted reproductive techniques (ART) such as intrauterine insemination (IUI), *in vitro* fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) are the most successful therapeutic means for male factor infertility.

Sperm preparation protocols used in ART involve sperm incubation and centrifugation. It was reported that sperm motility and viability decrease over time after ejaculation [3]. Further, a rapid decline in motility was noted after incubating periods lasting more than 4-hours at 37ºC [4]. A recent study has found that different centrifugation adversely affects sperm recovery [5]. Increased centrifugation speeds (> 500g) significantly increases the number of dead sperm [6]. Centrifugation has also been shown to increase the reactive oxygen species (ROS) formation in semen, which may affect sperm survival [7].

L-Carnitine (LC) is a naturally occurring molecule, derived from the amino acid lysine. In cellular systems, LC serves as a facilitator for the transport of activated fatty acids into the mitochondrial matrix, so that they can be broken down through β-oxidation to produce ATP [8]. A number of studies have examined the role of LC in reducing the cellular oxidative stress [9; 10; 11] (figure 3-1). Studies reported an association between lower levels of LC in semen and male infertility [12; 13]. Dietary supplementation with LC has been reported to improve sperm quality [14; 15]. Further, a positive correlation between free LC in human semen and sperm count and motility has been reported [16]. In addition, it was suggested that LC might be regarded as an index of androgenization [17]. *In vitro* as well as *in vivo* studies show that LC decreases the oxidative stress-induced DNA damage [18; 19; 20]. Furthermore, LC was reported to decrease actinomycin-D, hydrogen peroxide and TNF-alpha-induced DNA damage and improve the *in vitro* blastocyst development rate in mouse embryos [21].

The main goal of this study was first to standardize the LC doses that are not toxic to sperm, and examine LC effectiveness in improving sperm motility and viability, and decreasing sperm DNA oxidation induced by in vitro incubation and centrifugation.
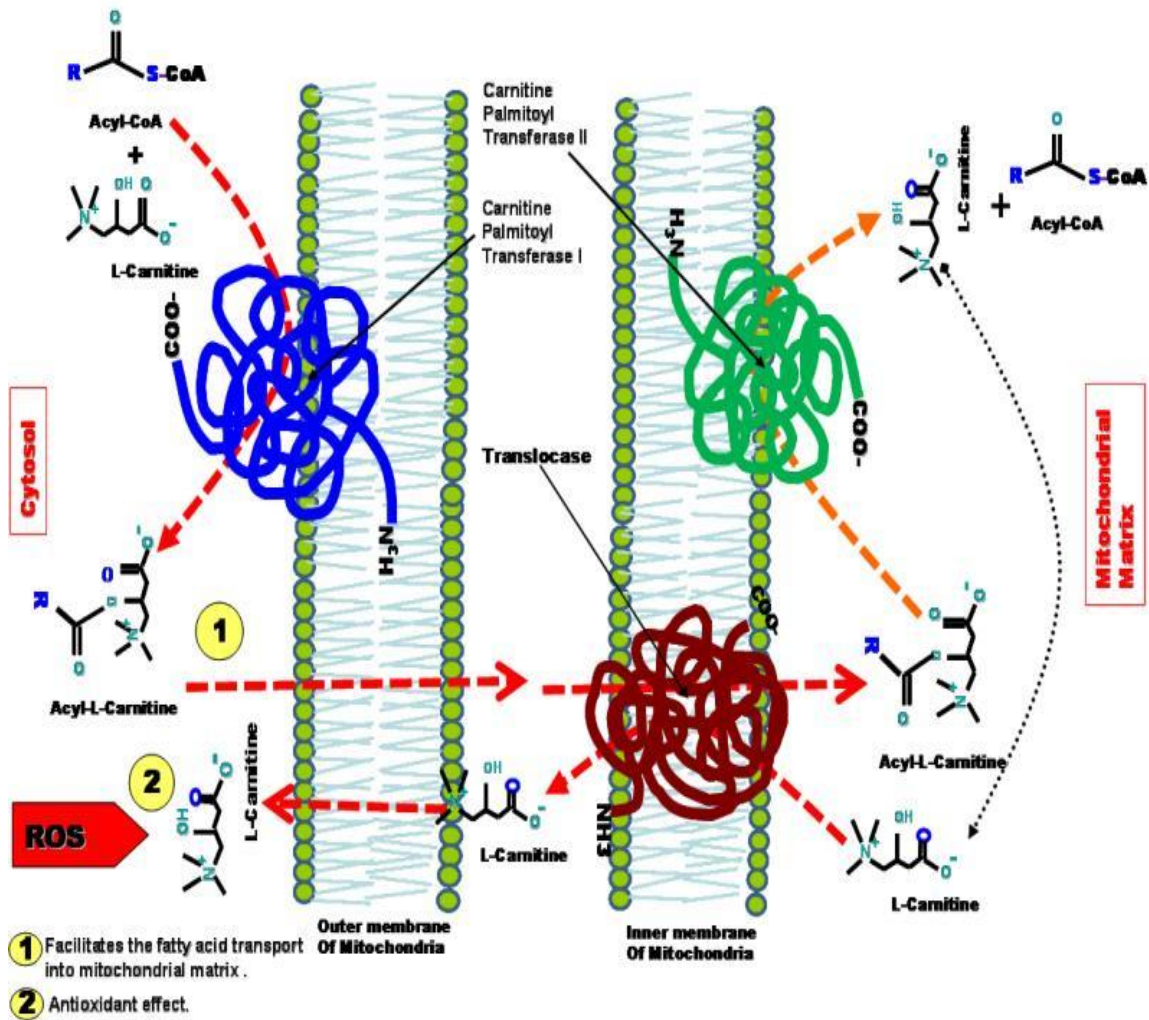
**Figure 3-1**: LC function. LC facilitates the fatty acid transport into mitochondrial matrix

and has an antioxidant effect.

## 3.2 Materials and methods

### 3.2.1 Sample collection

This study was approved by the institutional review board of Cleveland Clinic Hospital. Fifty five normozoospermic men were selected and analyzed on the basis of normal semen analysis according to the World Health Organization (WHO, 1999) guidelines (section 2.2.1), and used to study the effectiveness of LC on sperm motility, viability and sperm DNA oxidation during incubation and centrifugation. Samples were collected by masturbation following a minimum of 48 to 72 hours of sexual abstinence. Following liquefaction at 37 ℃ for 20 minutes, semen specimens were evaluated for volume, sperm concentration, total cell count, motility and morphology. A 5µL aliquot of the sample was used for evaluation of concentration and motility using a microcell slide chamber (Conception Technologies, San Diego, CA).

### 3.2.2 Effect of various LC concentrations on sperm motility and viability

Sixteen semen samples were examined. Each sample was divided into 6 equal aliquots; each contained $5 \times 10^6$ sperm/ mL. Samples were supplemented with various concentrations of LC dissolved in phosphate buffered saline, pH 7.4 (PBS). LC concentrations tested were 0.1, 0.5, 1.0, 10 and 50 mg/mL. The control contained PBS in place of LC. Sperm motility and viability were assessed after 2 and 4-hours incubation at 37ºC.

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### 3.2.3 Measurement of sperm viability

Sperm viability was measured by Eosin-Nigrosin staining (see page 25, chapter II)

### 3.2.4 Effect of LC on sperm motility and viability during centrifugation

Semen samples (n=19) were divided into two equal aliquots; each aliquot (5 x $10^6$ sperm) was supplemented with 0.5mg/mL of LC dissolved in human tubal fluid (HTF); control was supplemented with HTF in the place of LC. Sperm motility and viability were assessed after centrifugation at 1600 rpm for 20 min.

### 3.2.5 Effect of LC on sperm motility and viability for various sperm concentrations during sperm incubation

Semen samples from 12 donors were used to examine the optimal sperm concentration for studying the effects of LC. Sperm concentrations used were 5, 10, 20 and 40 X $10^6$/mL. Each aliquot was supplemented with 0.5 mg/mL LC dissolved in PBS. Control contained PBS in place of LC. Sperm motility and viability were measured immediately (time 0) and after 2h incubation at 37ºC.

### 3.2.6 Effect of LC on sperm DNA oxidation

Oxidative damage to DNA was evaluated using the flow cytometric OxiDNA assay kit (Calbiochem, San Diego). The assay is based on utilizing a direct fluorescent

protein binding method targeting oxidized sites in DNA. Briefly, sperm samples were treated with LC concentration at 0.5 and 50 mg/mL, incubated for 2h at 37ºC, washed twice in PBS, resuspended in 1% paraformaldehyde at a concentration of 2-4 X $10^6$ cell/mL, and placed on ice for 15 to 30 minutes. Sperm were again washed and resuspended in 70% ice-cold ethanol by centrifugation at X300$g$ for 5 minutes as per the kit instructions. The ethanol supernatant was removed, and the sperm pellets were washed twice in wash buffer and resuspended in 100 µL of the staining solution for 1 hour at room temperature in dark. The staining solution contained FITC-labeled protein conjugate and distilled water. All cells were further washed using rinse buffer, resuspended in 250 µL and incubated for 30 minutes in the dark on ice for flow cytometry measurements. Control samples without LC supplementation were subjected to the same assay. Negative controls were prepared without FITC staining, while the positive controls were prepared in the presence of 0.5 mM $H_2O_2$ and 0.25 mM $FeSO_4$. To study the effect of LC on sperm DNA oxidation during centrifugation, sperm samples (5x $10^6$ cell/mL) were treated with one volume HTF supplemented with LC concentration at 0.5 mg/mL, centrifuged for 20 min at 1600 rpm, then submitted to the same treatment as described above.

Data acquisition was performed within 30 min on a flow cytometer equipped with a 515-nm argon laser as a light source (FACScan; Becton Dickinson, San Jose, CA). 10,000 cells were examined for each assay at a flow rate of 100 cells/second. The FITC (log green fluorescence) was measured on FL1 channel. Data analysis was performed using FlowJo v4.4.4 software (Tree Star Inc., Ashland, OR).

### 3.2.7 Statistical analysis

All values were reported as mean ± S.D. Differences were considered significant at $P < 0.05$ against control group. Statistical analyses were operated using one-way analysis of variance (ANOVA) followed by an unpaired Student's $t$-test using the SPSS/PC computer software (SPSS 10.0.7, SPSS Inc.).

### 3.3 Results

### 3.3.1 Effect of LC on Sperm Motility and Viability on Incubation

The effect of various LC concentrations (0.1, 0.5, 1.0, 10, and 50 mg/mL) on sperm motility and viability was examined after 2 and 4-hour incubation periods at 37ºC (Figure 3-2). Sperm motility was significantly increased after LC supplementation at 0.1, 0.5 and 1.0 mg/mL after 2 h incubation (55.0% ± 16.9%, 58.4% ± 13.9% and 52.4% ± 19% respectively) compared with the control (39.4% ± 15%; $P < 0.05$) (Figure 3-2A). Semen samples supplemented with LC and evaluated after the 4-hours incubation period did not significantly increase in sperm motility compared with the control (Figure 3-2A). Semen supplementation with LC did not show a statistically significant increase in sperm viability at any concentration compared with the control at both incubation periods (Figure 3-2B). LC concentration of 50 mg/mL significantly reduced sperm motility and viability compared with the control at the two incubation periods ($P < 0.05$; Figure 3-2A-B).

### 3.3.2 Effect of LC on Sperm Motility and Viability during Sperm Centrifugation

Figure 3-3 shows the effect of LC on sperm motility and viability after sperm centrifugation for 20 min at 1600 rpm. Figure 3-3A shows the mean values ± standard deviations of sperm motility for semen samples centrifuged in presence of LC (0.5 mg/mL) and samples centrifuged in absence of LC (control). A significant difference ($P < 0.05$) in average sperm motility (37.5 ± 16.6 vs. 24.0 ± 12.8 %) was observed between sperm centrifuged with LC compared to the control. The viability was comparable

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between sperm samples treated with LC compared to those without LC treatment (38.0 ± 12.0 vs. 34.2 ± 12.6 %, respectively) (Figure 3-3B).
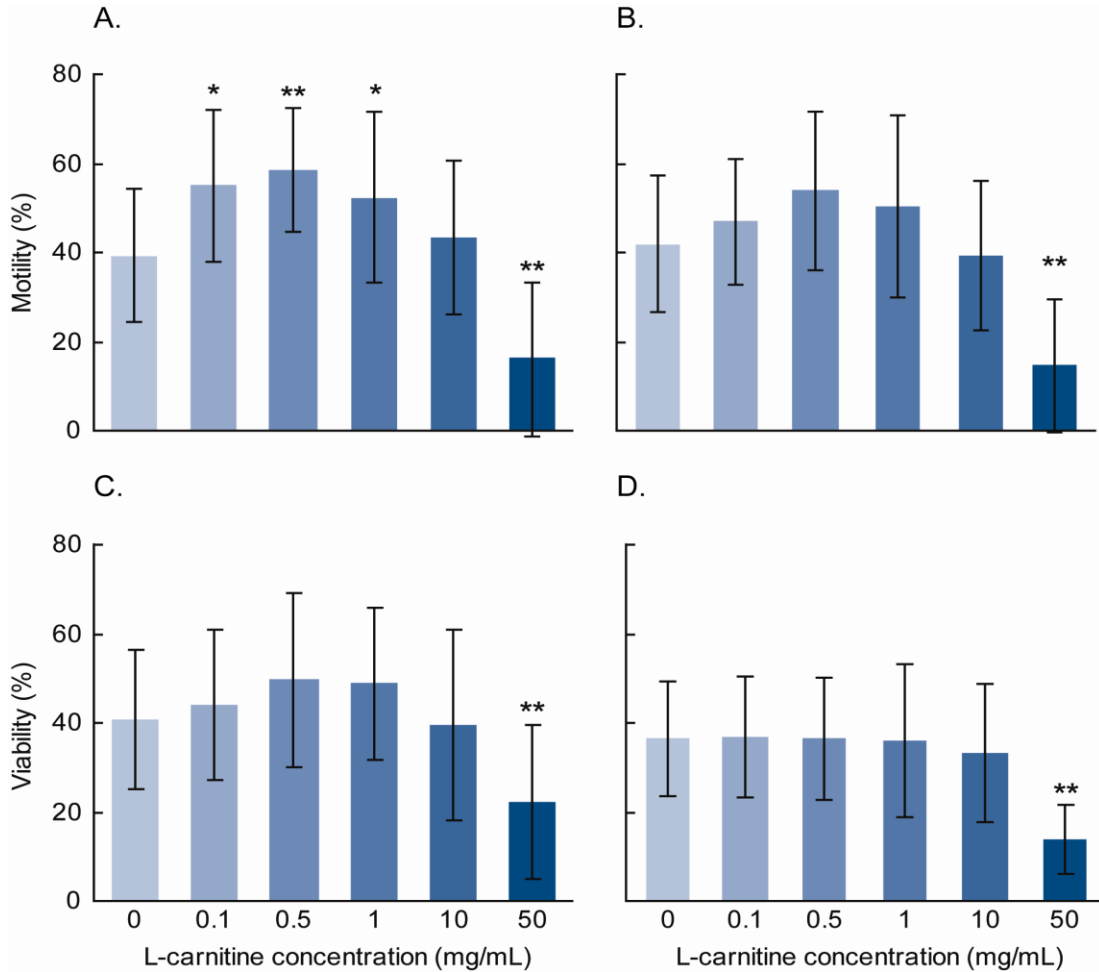


**Figure 3-2:** Effect of various *in vitro* incubation with L-carnitine concentrations at 37ºC on sperm motility for A: 2h and B: 4 h; viability C: 2 h and D: 4h. Data are mean values ± standard deviations of sperm motility and viability obtained from analysis of sixteen different semen samples. *P*-values for samples and their controls were measured for all LC concentrations (**P*-value < 0.05; ***P*-value <0.01); *P*<0.05 was considered statistically significant.
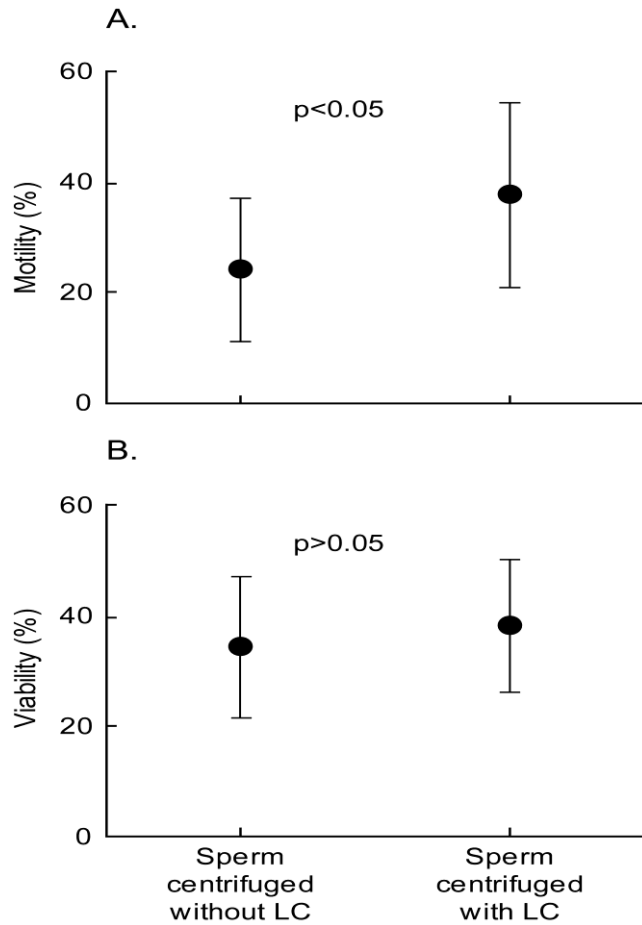
**Figure 3-3:** Effect of centrifugation on A: sperm motility with and without L-carnitine; B: sperm viability with and without L-carnitine. Sperm aliquots were centrifuged for 20 min at 1600 rpm. All data are mean values ± standard deviations. *P*<0.05 are considered statistically significant.

### 3.3.3 Effect of LC on Sperm Motility and Viability for various Sperm Concentrations

The effect of LC at 0.5 mg/mL concentration on sperm motility and viability was evaluated for various sperm concentrations (5, 10, 20, and 40 million/mL) (Figure 3-4). Control samples without LC supplementation were subjected to the same assay conditions. Motility and viability of sperm were assessed immediately after 2h incubation at 37°C. Statistically significant increase in sperm motility was observed in semen samples supplemented with LC compared with the control only for a sperm concentration of 5 x $10^6$/mL ($P < 0.05$; Figure 3-4A). No increase in sperm viability was seen after addition of LC at all sperm concentrations tested (Figure 3-4B).

### 3.3.4 Effect of LC on Sperm DNA Oxidation

The effect of LC on sperm DNA oxidation during incubation and centrifugation is shown in Figure 3-5. Flow cytometry analysis of FITC-labeled sperm showed that sperm treated with the LC at 0.5 and 50 mg/mL for 2h at 37 ºC did not increase the intensity of the FITC fluorescence, indicating an insignificant effect on the intrinsic baseline of DNA oxidation (Figure 3-5 A1-A2).

Figure 3-5B shows the effect of LC on the baseline of sperm DNA oxidation during centrifugation as evaluated by flow cytometry. Sperm samples centrifuged with LC at 0.5 mg/mL concentration did not show a significant difference in the intensity of FITC fluorescence, and thus in sperm DNA oxidation, compared to those without LC supplementation.
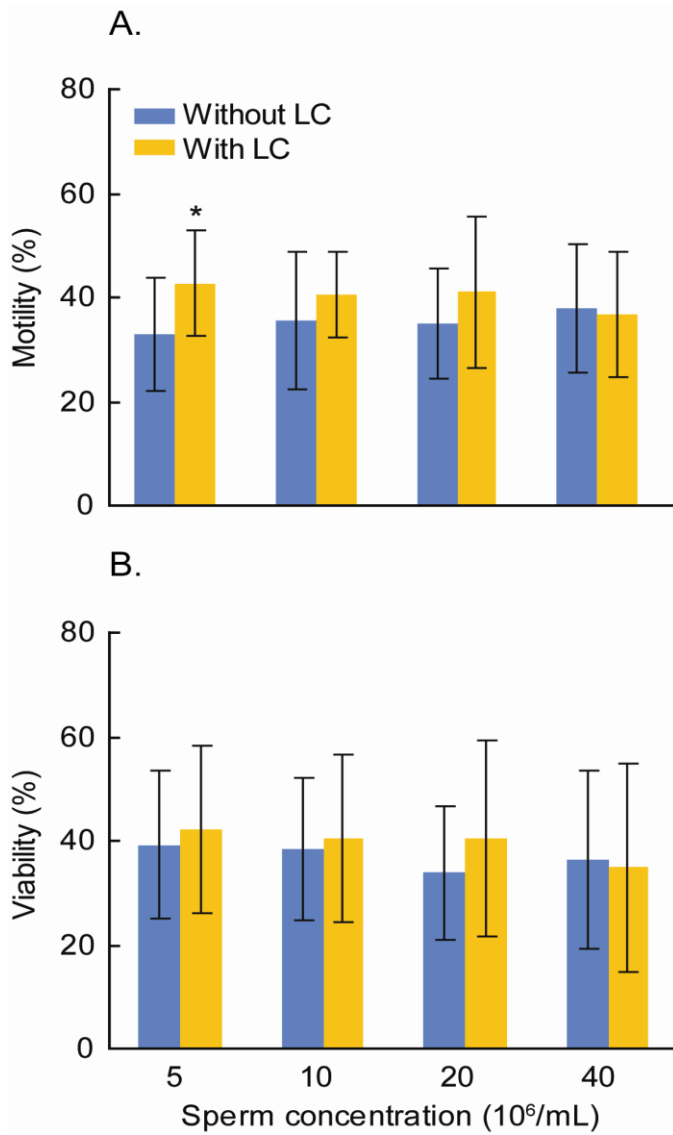
**Figure 3-4:** Effect of L-carnitine (0.5 mg/mL) on A: sperm motility and B: viability following incubation with various sperm concentration (5, 10, 20 and 40 X $10^6$/mL Motility and viability of sperm were assessed immediately after 2h *in vitro* incubation at 37°C. The histograms are mean values ± standard deviations obtained from analysis of twelve different semen samples (yellow). Controls (blue) were assayed without addition of L-carnitine. *P*-values for samples and their controls were measured for all sperm concentrations (* P-value < 0.05); *P*<0.05 was considered statistically significant.
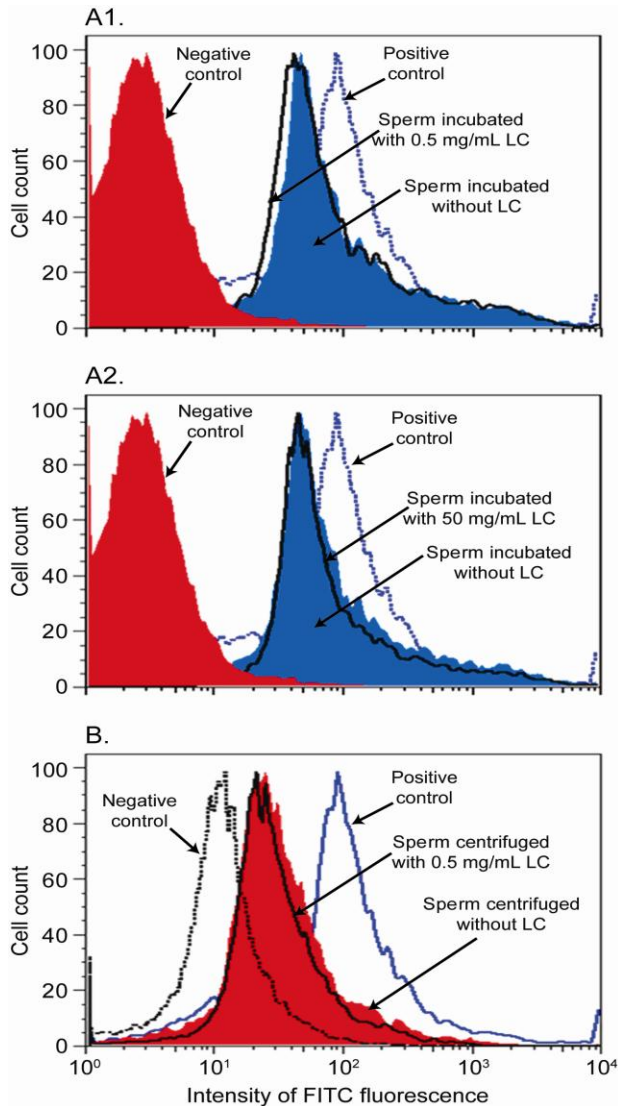
**Figure 3-5:** Effect of LC on sperm DNA oxidation during sperm incubation and centrifugation and evaluated by flow cytometry. A: Sperm incubated for 2h with 0.5 mg/mL (A1) and 50 mg/mL L-carnitine concentrations (A2) at 37ºC. B: Sperm centrifuged in the presence of 0.5 mg/mL LC for 20 min at 1600 rpm. Sample controls assayed in the absence of LC in both incubation and centrifugation. Negative controls were analyzed without FITC staining; positive controls contained $H_2O_2$-induced sperm DNA oxidative damage. The X-axis represents the intensity of sperm-labeled FITC fluorescence. Data are representative of 4 independent experiments.

## 3.4 Discussion

Studies examining effects of LC on sperm quality in general have focused on the effects of LC *in vivo*. Recent findings in this context show that administration of LC might antagonize the oxidative, as well as the pro-inflammatory, pathways that attenuate sperm motility [15; 23; 24]. In the present study, we have focused on the role of LC in improving sperm quality *in vitro*. In the first series of experiments, we standardized the LC concentrations, utilizing semen from normozoospermic donors and established that 0.1, 0.5 and 1.0 mg/mL concentrations were not toxic. Next, we examined sperm motility and viability after 2- and 4-hours of incubation.

To study the effect of LC on sperm motility and viability during sperm centrifugation, we used 0.5 mg/mL LC concentration. Sperm aliquots with and without LC (control) were centrifuged for 20 min at 1600 rpm. Sperm motility and viability were then assessed. Our results suggest that LC at the standardized concentrations was beneficial and improved sperm motility, in particular, after the 2h incubation period. A significant increase in sperm motility was observed in the centrifuged sperm supplemented with LC compared to the control. Similar enhancement in sperm motility *in vitro* has been reported after adding acetylcarnitine, an acetylated form of carnitine, to semen at 37ºC [25]. This positive effect of LC on sperm motility is due to its role in sperm metabolism as well as its antioxidant properties [26; 27]. LC facilitates the transport of long chain fatty acids across the inner membrane of mitochondria, so that they can be broken down through β-oxidation to produce ATP [8]; therefore, providing readily available energy for use by spermatozoa. Accumulation of the reactive oxygen species (ROS) in sperm leads to ATP depletion, lipid peroxidation and insufficient

axonemal phosphorylation [28]. The property of LC as a scavenger for ROS could thus be responsible for its positive effect on sperm motility.

The effect of LC on sperm viability was measured using the one-step Eosin-Nigrosin Test [29]. This test is primarily based on the structural integrity of the spermatozoa plasma membrane; damage in the membrane provides an access for Eosin to stain the entire spermatozoa or part of it based on the size of the damage [29]. A stained spermatozoa is considered non-viable, while the unstained one is counted as viable.

LC contributes in shielding sperm membranes against harmful ROS because of its antioxidant activity [27]. It also decreases the availability of lipids for peroxidation *via* easing the transport of fatty acids to the mitochondrial matrix for β-oxidation [30]. Neuman *et al.* (2002) reported that dietary supplementation with LC reduces sperm lipid peroxidation in roosters; this may preserve sperm membranes, thereby increasing their viability [31]. We therefore anticipated that semen supplementation with LC would improve sperm viability. Contrary to our expectation, we observed that semen supplementation with LC showed only a slight but statistically insignificant effect in improving sperm viability during incubation. Similar insignificant effects were reported earlier by Namik *et al.* (2000) who studied the influence of acetyl-L-carnitine on sperm membrane damage after cryopreservation-thawing cycles [32]. Similarly, using *in vivo* systems, it was found that dietary supplementation with LC did not improve the membrane integrity of pony stallion sperm [33]. A more recent study by Yeste *et al.* (2010) showed that the addition of LC to the diet of boar males had no effect on their sperm viability [34]. In contrast, De Rosa *et al.* (2005) noted that LC administration to infertile human males with poor sperm motility (<50%) was effective in improving

membrane integrity of the ejaculated sperm. In our *in vitro* study, we only added LC to semen samples with normal motility (>50%). A similar *in vitro* study, utilizing semen samples from infertile subjects is needed to assess LC effect on sperm viability during incubation and substantiate our findings.

Namik et al. (2000) used two assays eosin-Y staining and annexin V-Cy3 in their study to measure sperm viability after cryopreservation-thawing. These investigators observed significant discrepancy between the percentages of sperm marked as "live" between eosin-Y staining and annexin V-Cy3 assay. The eosin-Y staining showed lower percent of viable sperm compared to the annexin V-Cy3 assay [32]. These investigators attributed this discrepancy to the different mechanisms through which these assays operate. In our study, we measured sperm viability using the eosin-staining assay, which is based on the membrane permeability for the eosin stain. Therefore, the mechanism by which this assay recognizes live sperm could be a contributing factor in this insignificant effect of LC on sperm viability.

The influence of LC on sperm motility and viability of various sperm concentrations was also investigated (Figure 3). We supplemented different concentrations of sperm with LC at 0.5 mg/mL. Our results from this experiment suggest that LC was effective in increasing sperm motility only when utilizing sperm concentration of $5 \times 10^6$ cell/mL. At higher sperm concentrations, the decrease in the ratio of the sperm number against available LC molecules may explain the lack of effect in improving the sperm motility. On the other hand, sperm viability did not increase utilizing any of the sperm concentrations. These results are in agreement with our experiment above. This information may be exploited in exploring and designing

protocols that aim to improve sperm motility in patients with low sperm count and especially oligozoospermic patients.

LC has been shown to improve resistance to oxidative stress by decreasing DNA damage in *Ataxia telangiectasia* cells [35]. Moreover, Thangasamy *et al.* (2009) reported that LC significantly reduces DNA damage in lymphocytes of aged rats [18]. We also recently demonstrated the effectiveness of LC in reducing $H_2O_2$-induced DNA damage and improvement of *in vitro* blastocyst development rate in mouse embryos [21]. In the present study, we examined if LC had similar effects on human sperm DNA *in vitro*. We incubated the sperm samples with the standardized LC concentration 0.5 mg/mL, at 37ºC for 2h. We did not see the decrease in the extent of the sperm DNA oxidation (Figure 3-5-A1). Similarly, we did not see a significant effect of LC on the baseline of sperm DNA oxidation after centrifugation compared with control samples (i.e. centrifugation without LC) (Figure 3-5-B).

Studies *in vitro* show that semen supplementation with an antioxidant *does not* affect sperm DNA integrity. In fact, Donnelly et al. (1999) showed that neither addition of ascorbate nor addition of α-tocopherol to sperm preparation medium affected DNA integrity of sperm. However, both antioxidants provided protection against the $H_2O_2$-induced DNA damage in sperm [36]. Similar reports showed that addition of vitamin E to cryopreservation medium did not alter the post-thaw DNA fragmentation of sperm [37]. These results are in line with our observations, which don't show a significant effect of LC as an antioxidant LC in reducing DNA oxidative damage in sperm.

Very low concentrations of LC have been reported in azoospermic men compared with normospermic control [38]. Further, lower concentrations of LC were noted in oligoasthenozoospermic males, and LC supplementation was shown to improve semen quality in these people [39]. One explanation for the undetectable effect of LC on sperm DNA oxidation may be the fact that we used only semen samples from normozoospermic subjects in our study. Furthermore, contrary to majority of reported studies [21; 23; 35; 36], we measured the effect of LC on the baseline levels of oxidative DNA damage in sperm without any induction of oxidative damage as in other studies this might be another likely reason for the lack of significant effect of LC on sperm DNA oxidation. Another reason for lack of improvement in DNA oxidation may be the fact that mammalian sperm DNA is highly compacted (six-fold more highly condensed) compared to DNA in somatic cells [40]. Also, the majority of antioxidants in somatic cells are present within their cytoplasm, while in sperm, a significant amount of the antioxidants are present in seminal plasma outside the sperm because spermatozoa lose most of its cytoplasm during maturation [41]. Therefore, the differences in the DNA packaging and the distribution of antioxidants between spermatozoa and somatic cells might introduce variations in outcomes when measuring the effect of *in vitro* supplementation of antioxidants on DNA oxidation.

Given that LC is a substantial antioxidant [27; 42], the role of antioxidant therapy in improving male fertility could be a double-edged sword with unfavorable effects if the safety threshold dose is exceeded [42]. For example, extreme levels of the known antioxidant L-ascorbic acid are thought to be associated with a decline in fertility [43], even though it is the principal antioxidant in seminal plasma of fertile males [44]. Further

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research is needed before the role for antioxidants in the treatment of male infertility is established [45]. The *in vitro* studies on human sperm by Donnelly et al. (1999) showed that higher concentrations of L-ascorbic acid (> 20 µM) negatively affect sperm motility in both normozoospermic and asthenozoospermic samples. Our findings are in agreement with this concept, and we have shown that sperm quality *in vitro* is lower at high concentrations of LC.

It has been shown that cell treatment with high concentrations of antioxidants *in vitro* increases the levels of 8-oxoguanine moieties on DNA, a biomarker for DNA oxidative damage [46]. Increasing DNA oxidative damage may lead to apoptosis and cell death [47; 48]. We therefore examined if high LC concentration decreased *in vitro* sperm quality by inducing sperm DNA oxidation. We measured the 8-oxoguanine moieties in the sperm DNA after incubating the sperm with 50 mg/mL LC using flow cytometry. Our results suggest that this might not be the likely mechanism by which the high LC level affects negatively sperm quality *in vitro* (Figure 3-5-A2). Again, the fact that we used samples from normozoospermic subjects may contribute to the absence of significant effects in our work.

In conclusion, LC enhances sperm motility following *in vitro* incubation and centrifugation, while it might not significantly affect sperm viability and DNA oxidation baseline of sperm. Our results provide a solid ground to explore and design protocols to test potentially beneficial effect of LC supplementation for sperm prepared for ART.

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# CHAPTER IV


# NITRIC OXIDE IN HUMAN MALE REPRODUCTION

**Summary**

In this chapter, we provide a literature review of the role of nitric oxide (NO), one of the main reactive oxygen species, in human male reproduction. The goal of this review is to enrich our understanding of the molecular basis of the function of NO in human spermatozoa. In the beginning of this chapter, we discuss nitric oxide synthesis and mechanisms of action and function in cellular systems. Then, we provide a comprehensive literature review of the role of NO in human spermatozoa physiology and function, as well as its contribution as a marker in male infertility diagnosis. In addition, we provide an in-depth review of NO antagonistic effects on spermatozoa function and physiology. Moreover, we review the methods that are commonly used to measure NO in semen. The collective information from this literature survey points to the fact that some level of NO is vital for human sperm function and physiology and mainly for the motility

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of spermatozoa. However, increased levels of NO are considered detrimental to spermatozoa and contribute to male infertility.

## 4.1 Nitric oxide, function and synthesis

Nitric oxide (NO) is a diatomic free radical. It is similar to oxygen in its maximum solubility in water. NO is also a non-polar molecule and able to diffuse easily through cellular membranes [1]. One of the most distinctive chemical features of NO is that it is paramagnetic, does not have a tendency to make dimers at the standard conditions [2].

Currently, it is one of the extensively studied species in biomedicine because of its various functions in biological systems. It has been referred to as the endothelium-derived relaxing factor (EDRF) because it causes vasodilation via a cascade that leads to vascular smooth muscle relaxation [3; 4; 5]. In addition, nitric oxide prevents platelets aggregation [6], acts as a neurotransmitter [7; 8], and mediates macrophage bactericidal activity.

Reports have shown that NO has a major contribution in human reproduction. It is recognized as a key molecule in controlling hypothalamic-pituitary-gonadal axis [1]; it activates the release of luteinizing hormone-releasing hormone (LHRH), which triggers the pituitary gland to release the gonadotropins via activating nNOS in the pituitary [9]. In the gonad, it has a central role in inducing ovulation in human's female and in causing luteolysis [9]. NO has also a significant contribution in human's sexual behavior; in males, it induces the erection via generating cyclic guanosine monophosphate (cGMP) in

corpora cavernosa [10]. In females, it relaxes vaginal smooth muscle and induces vaginal secretions [9].
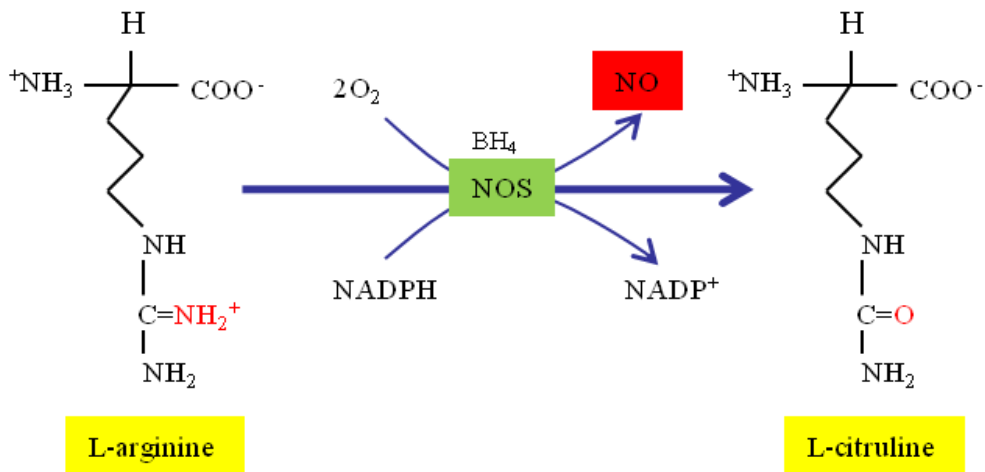


**Figure 4-1:** Synthesis of nitric oxide.

NO is synthesized by a family of enzymes called nitric oxide synthases (NOSs) from the oxidation of arginine. NO is released after a two-step catalytic cycle along with citrulline as a byproduct. $O_2$ and NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate, are required in this reaction (figure 4-1). Flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin, are also needed for the NOS reaction [11]. In tissues, NO has a very short half-life (3 to 10 seconds) because it reacts with oxygen ($O_2$) and and converts into nitrite and nitrate. It also reacts with superoxide ion ($O_2^{-\cdot}$) and thiol sites to yield peroxynitrite and nitrosothiols respectively.

## 4.2 NO synthases

Three isoenzymes of NO synthase have been identified. Endothelium NOS (eNOS), primarily present in endothelium, neuronal NOS (nNOS), primarily present in neural tissue, and inducible NOS (iNOS), can be expressed in various cells such as macrophages, monocytes, neutrophils and hepatocytes [11; 12].

The isoforms eNOS and nNOS are constitutive, synthesized usually at a steady rate in spite of physiologic need, and $Ca^{2+}$-calmodulin-dependent enzymes [11; 12] . iNOS is a $Ca^{2+}$-independent enzyme; it has specific inducers (e.g. inflammatory cytokines, tumor necrosis factor, bacterial endotoxins) which vary by cell type. These inducers increase iNOS synthesis, which can result in a large amount of NO being formed [13; 14; 15].

**4.3 NO and smooth muscle contraction:**

In endothelial cells, NO is synthesized by eNOS and diffuses into vascular smooth muscle (VSM), and activates the cytosolic –*soluble-* guanylate cyclase (sGC), which increases the production of cGMP, and consequently, protein kinase G (PKG). PKG then phosphorylates myosin light-chain kinase and leaves it inactive, thereby lowering smooth muscle contraction.

**4.4 Nitric oxide mechanisms of action mediated by peroxynitrite**

In biological systems, peroxynitrite, which results from the combination of NO with superoxide ion ($O_2$-**·**)**,** is a *double-edged sword* anion. It has both beneficial and detrimental actions. These actions, endogenous and exogenous, are mostly due to oxidation and nitration reactions with other biomolecules, mainly proteins, lipids and nucleic acids (Figure 4-2) [16]. The effects of peroxynitrite could be both direct and indirect. For example, peroxynitrite produces small concentrations of secondary NO when reacting with other biomolecules and promotes vasodilation, inhibition of platelet aggregation and hypotension [17].

*1. Role in bactericidal activity*

Normally, iNOS activity in macrophages is not high. In response to infection, by the effect of interferon-alpha and bacterial lipopolysaccharides (LPS), its synthesis increases resulting in higher NO production. NO combines with $O_2$-**·** and form peroxynitrite (ONOO$^-$), which, among other reaction pathways, breaks down in the presence of hydrogen ion into HO**·**, a highly bactericidal radical (scheme 1).
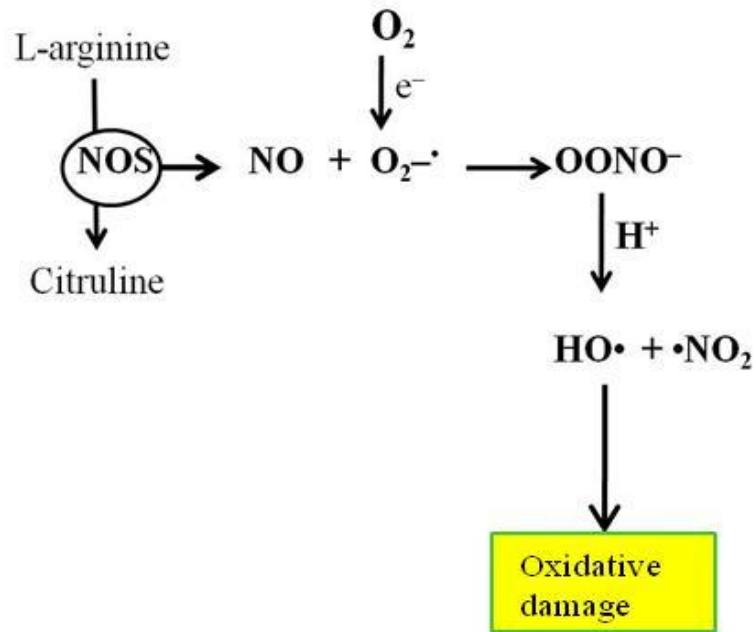
**Scheme 1**: Formation of hydroxyl radical from nitric oxide.

*2. Effects on proteins and enzymes*

Various proteins in humans and other biological systems are targets for peroxynitrite attacks. Oxidation and nitration of proteins may down-regulate the positive feedback cycles of injury and decreases the ability of the cell to fight against free radical damage. This will lead to antioxidant depletion, oxidative stress, and consequently, cell necrosis or apoptosis. Such cases are more likely when peroxynitrite attacks antioxidant enzymes such as the superoxide dismutase and glutathione reductase, as well as, other species of antioxidant defense mechanisms like glutathione and cysteine [16; 18-22]. Inhibition of cytosolic as well as membrane proteins and enzymes because of peroxynitrite attack leads to impairment of cell function, and subsequently disease onset (Table 4-1).

*3. Lipid oxidation and nitration by peroxynitrite*

The decomposition of peroxynitrite, in the presence of hydrogen ion, to form •OH and •$NO_2$ may initiate lipid peroxidation when it occurs in hydrophobic phases [16; 23]. The reaction results in Lipid peroxidation products such as malondialdehyde, hydroperoxide and conjugated diene [23], as well as in the nitro, nitrito-, nitrosoperoxo- and nitrated lipid oxidation products. Low-density lipoprotein (LDL) is also a target for peroxynitrite insult. LDL oxidation by peroxynitrite leads to the formation of $F_2$-isoprostanes [16; 24], a family of prostaglandin-like compounds formed via non-enzymatic lipid peroxidation.  Fatty acids nitration may secondarily inhibit protein function through thiol modifications [25]. Conversely, nitrated lipids can have anti-

inflammatory functions via *peroxisome proliferator-activated receptor gamma (*PPAR-γ)-dependent and independent pathways [16; 26].

*4. Effect on DNA*

DNA is also another target for peroxynitrite and its derived radicals. They can cause DNA base modification; for example, 8-oxoguanine and 8-nitroguanine are main products of purine oxidation [27]. Furthermore, it can activates $Ca^{2+}$-$Mg^{2+}$-endnonucleases [16]. In addition, peroxynitrite can oxidize deoxyribose sugar and lead to double and single strand breaks [16; 28]. Such DNA injury may lead to genotoxic damage, which may develop into pathological disorders. Peroxynitrite may also induce secondary DNA repair and activate the suicidal pathway including poly ADP-ribose polymerase activation (PARP-activation), and consequently cell necrosis (Figure 4-2) [29].

**Table 4-1**: Examples of cytosolic and membrane enzymes and proteins affected by peroxynitrite (adapted from Casaba Szabo *et al*., 2007).

| Enzyme or protein | Mechanism of action | Consequences and related health disorders | Ref. |
|---|---|---|---|
| Glyceraldehyde-3-phosphate dehydrogenase | Oxidation of cysteine residues | Impairment of cellular function | [30] |
| Tyrosine hydroxylase | Nitration of tyrosine residues | Impaired the production of dopamine in the dopaminergic neurons (e.g. in Parkinson disease) | [31] |
| Creatine kinase | Oxidation of cysteine residues | Impairment of myocytes function, which may leads to myocardial infarction or chronic heart failure. | [16; 32] |
| Calcium pumps: including sarco/edoplasmic reticulaum $Ca^{2+}$-ATPase (SERCA) | Tyrosine nitration and cysteine oxidation | Impairment of cellular calcium handling and cellular ionic balance, which is linked with heart failure. | [33; 34] |
| Epidermal growth factor receptor (EGF) | Dimerization, nitration and oxidation | Cellular signaling impairment including a possible pathway leading to insulin resistance | [16; 35] |
| Prostacyclin synthase | Oxidation and nitration | Prostaglandin formation inhibition. | [36] |
|  |  |  |  |
| Alpha-synuclein and microtubule related tau protein modification in CNS | Nitration and oxidation | Initiation pathogenesis of alpha-synuclein aggregates | [16; 37] |
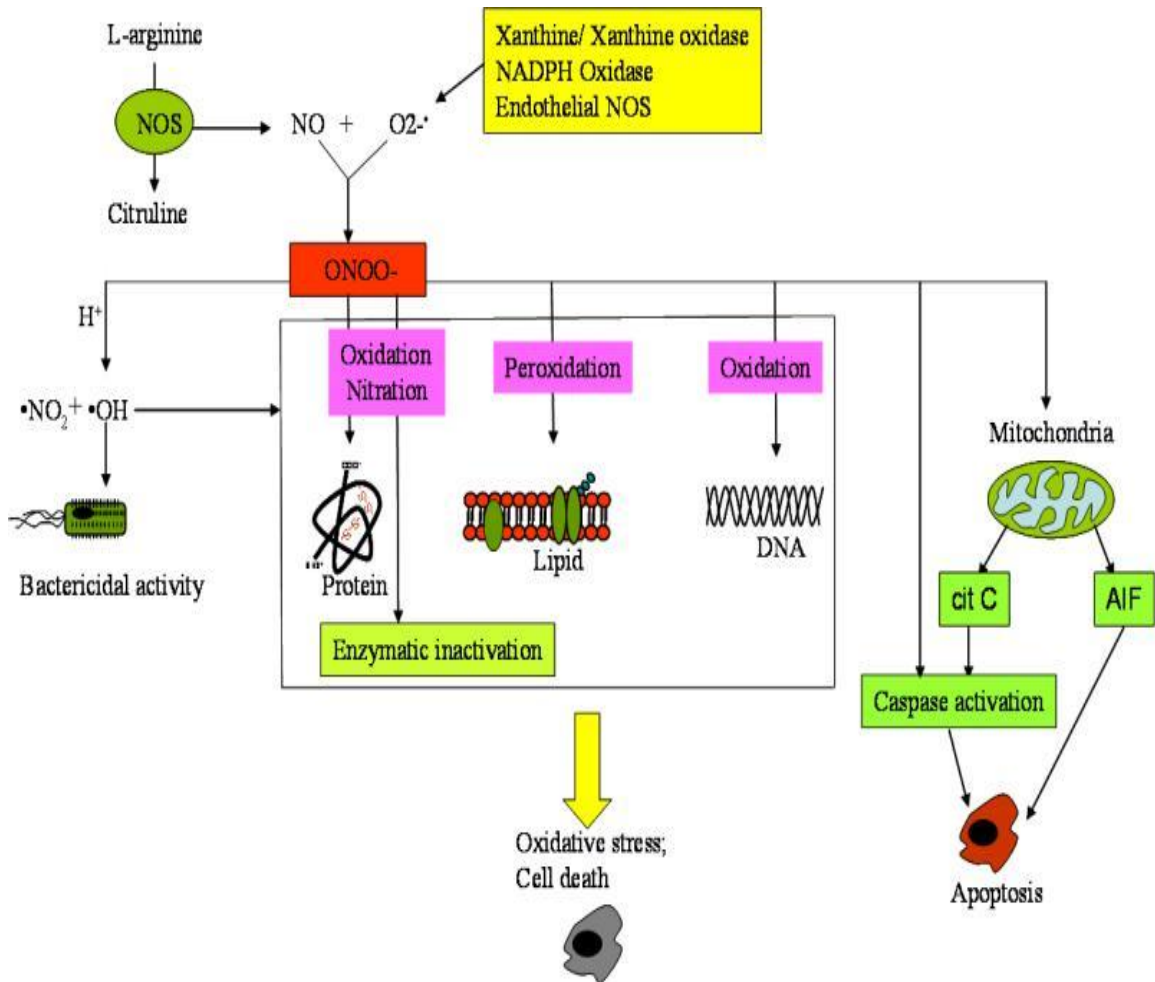
**Figure 4-2:** nitric oxide actions mediated by peroxynitrite**.**

5. *Effect on NOS cofactors*

Peroxynitrite can impair enzymatic cofactors directly or indirectly via free radical-dependent mechanisms. It can oxidizes tetrahydrobiopterin (BH4) (Figure 4-3), a cofactor of NOS enzyme that serves as an electron relay for timed redox reactions used in oxygen activation. The oxidation of BH4 leads to the inactive form quinonoid 5,6-dihydrobiopterin [38] which is subsequently converted to 7, 8-dihydrobiopterin. This oxidation affects negatively the function of NOS, and thus NO production [16]. Milstien and Katusic (1999) have suggested that abnormally low concentrations of BH4 may promote a cycle of its own destruction arbitrated by NOS-dependent formation of peroxynitrite; such mechanism might have a contribution in vascular endothelial dysfunction stimulated by oxidative stress [38].
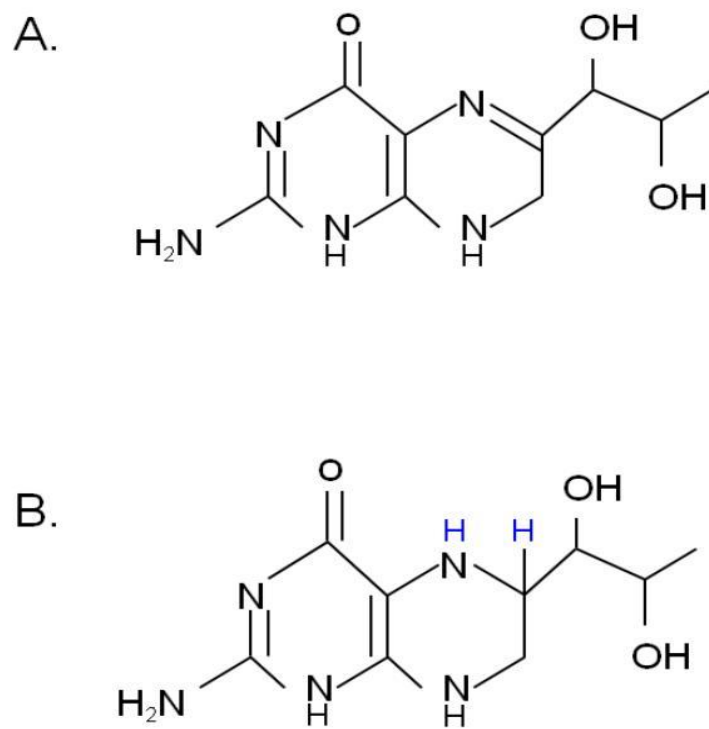
**Figure 4-3:** Chemical structure of tetrahydrobiopterin (A), and 7, 8-dihydrobiopterin (B).

## 4.5 Nitric oxide and male infertility

Studies have shown that NO has a major contribution in male reproduction [39-44]. Higher levels of nitric oxide were recognized in the semen of infertile men compared to fertile [42]. It was found that NO generation is very important in gamete interaction and fertilization route [45]. Xu *et al.* (2008) studied the relationship between nitric oxide synthase and nitric oxide with varicocele-based infertility; they found that NO mediates the decrease in semen quality induced by varicocele [46]. A recent study reported that the missense Glu298Asp polymorphism of eNOS gene is present at a higher frequency in infertile asthenozoospermic subjects [47].

Supplementation of asthenozoospermic infertile men ejaculates with Ginsenoside R (e) improves human's male fertility by increasing sperm motility [48]. The mechanism by which this occurs may involve NOS stimulation and enhanced NO synthesis [48]. Huang *et al.* (2006) studied the correlation between NO levels and sperm morphology; they found that low concentrations of NO are associated with defects in sperm morphology [49]. A very recent study showed that increased levels of NO is linked with a decrease in sperm function in asthenospermia, normospermia and leukocytospermia, whereas, lower levels of NO may contribute in the etiology of oligospermia [50].

## 4.6 Nitric oxide synthases in human spermatozoa

NOSs are ubiquitous, present in almost all tissues of the human body [51]. NOS activity has been verified in human testes, seminal vesicles, and prostates [52]. These findings imply a possible function of NO in spermatogenesis and the maturation of

sperm. It has been recognized that nitric oxide synthase (NOS) is present in human spermatozoa and NO production at endogenous levels is very important for adequate sperm function [48; 51; 53-55]. Lewis *et al.* (1996) reported that human spermatozoa express eNOS as well as nNOS [53]. At a later date (1998), Moria *et al.* confirmed that human spermatozoa express eNOS. The same group, using immunohistochemistry, detected the presence of eNOS in sperm and showed that the majority of sperm display postacrosomal and equatorial eNOS staining (head and midpiece). In 2000, Francavilla *et al.* concluded that constitutive NOS (eNOS and nNOS) plays a role in the capability of human sperm to fuse with oocyte in the *zona pellucida* (ZP), a glycoprotein membrane that surrounds the plasma membrane of an oocyte. They showed that inhibited constitutive NOS activity using $N^G$-nitro-l-arginine methyl ester (L-NAME) significantly decreased the oocyte fusion without affecting the ZP binding [56]. Newly published study showed that increased NOS activity is present in mature sperm compared to immature [57].

NOS activity was also studied in seminal plasma. Both calcium-dependent NOS activity as well as calcium-independent NOS activity were detected in human seminal plasma [58]. There was no association between NOS activity in seminal plasma and sperm motility and concentration [58]. Later, another study supported these findings by showing that seminal plasma nitrite does not correlate with sperm count and rapid-forward motility [59]. In addition, positive sperm culture did not increase the concentration of nitrite in seminal plasma [59].

On a related front, mRNA transcripts of eNOS and nNOS have been detected in semen samples, and they have a clinical significance in the diagnosis of fertilization [60].

For example, eNOS and nNOS transcripts were present in low motile sperm samples, while they are undetectable in the high motile ones [60].

## 4.7 Nitric oxide and sperm motility

Flagellum beating is very important to sperm motion; defects in sperm motility leads to infertility and ectopic pregnancy [61]. Several studies showed that NO production at endogenous levels is very important for adequate sperm motion [48; 51; 53-55]. Insulin, a central hormone in regulating carbohydrate metabolism in the human body, and leptin, an adipose-derived hormone, significantly increased *in vitro* motility and nitric oxide production in sperm [15] (Figure 4-4). Ankri *et al.* (2009) proposed that exposure of human spermatozoa to visible light may increase NO formation, which enhances the motility of sperm, and thus the rate of fertilization. According to the same group, this effect may be attributable to increase of light absorbance by the NOS coenzymes flavin and heme. Lately, an *in vitro* study observed that adding L-arginine to asthenospermic patient's semen samples increases NO production and sperm motility. This effect happens only in the presence of an active anion transport system [62].

It was also reported that sperm recovery is also altered upon using dissimilar methods to prepare sperm *in vitro*. Ding *et al.* (2002) used two different methods, Percoll and IxaPrep, to prepare human sperm *in vitro* and they found that the IxaPrep method provides a better recovery rate of sperm. They concluded that the better sperm quality by the IxaPrep method may be the result of decreased levels of NO generated, because increased levels of nitric oxide affects negatively the quality of the sperm [63].
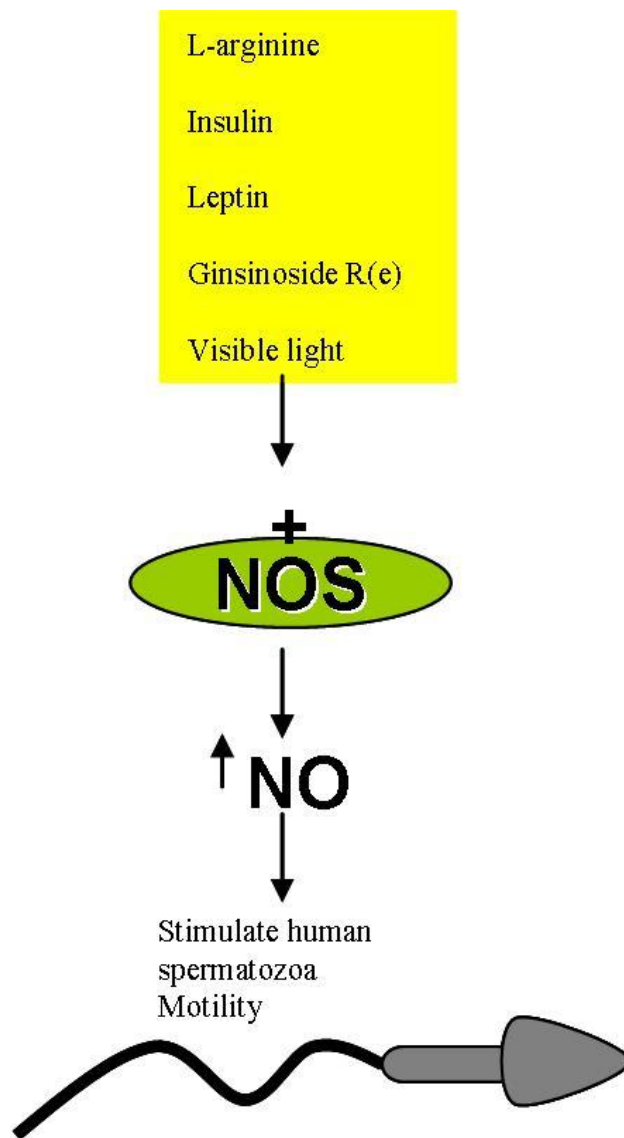
**Figure 4-4**: various compounds and factors increase sperm motility may be by increasing NO production.

## 4.8 Nitric oxide mechanisms of action in human sperm

Miraglia *et al.* (2010) showed that NO stimulates human spermatozoa motility by activation of soluble guanylate cyclase (sGC), subsequent production of cyclic guanosine monophosphate (cGMP) and activation of cGMP-dependent protein kinases (cGMP/protein kinase G signaling pathway) [64]. An earlier study suggested that NO induces acrosomal reaction in human spermatozoa via stimulating sGC, cGMP, and PKG activation, and extracellular $Ca^{2+}$ is required in this activation [65].

Alternatively, by a mechanism that does not require sGC activation; L. Lefievre *et al.* (2007) demonstrated that many proteins (signaling, regulatory, kinases, receptors, transport, structural and others) in human spermatozoa are possible targets for S-nitrosylation, a dynamic mechanism analogous to phosphorylation in which a nitrosyl group is added to a protein (25). In general, S-nitrosylation by nitric oxide is very important reaction in biological systems; it refers usually to the conversion of thiol groups, involving cysteine residues in proteins, to S-nitrosothiols, such examples are shown above (Table 4-1) (26, 27).

A subsequent study reported that NO has a key function in the gamete interaction and fertilization within human female's tract. It regulates the mobilization of stored $Ca^{2+}$ ions in human spermatozoa via protein S-nitrosylation, which ultimately lead to the regulation of flagellar beating. This action is synergistic with the action of progesterone (Figure 4-5) [66]. The pre-treatment of sperm with NO increased significantly the effect of progesterone on intracellular free calcium ions, possibly by binding on a membrane surface receptor, which activates $Ca^{2+}$ influx using a plasma membrane channels [45].

Increasing intracellular $Ca^{2+}$ induces mobilization of stored calcium via a $Ca^{2+}$-induced $Ca^{2+}$ release process (CICR).

On another front, reports have shown an association between apoptosis mechanisms in sperm and NO synthesis. For example, activation of apoptosis signaling in human spermatozoa is negatively correlated with NO production [57].

## 4.9 Role of nitric oxide in human sperm capacitation and acrosomal reaction

Sperm capacitation is an ultimate biochemical event that occurs typically in the female's reproductive tract after ejaculation. It is required for the spermatozoa to be competent for oocyte penetration. A number of studies reported that NO is important for human sperm capacitation [67; 68; 69]. It was indicated that NO is formed by capacitating human spermatozoa [70]. NO may act as a messenger via modulating the cAMP/protein tyrosine phosphorylation pathway involved in spermatozoa capacitation [70]. Herrero *et.al.* (2001) emphasized that capacitation is part of an oxidation process that involves NO. They demonstrated that the nitration of tyrosine in sperm proteins by NO takes place in capacitated human spermatozoa [71]. Later study found that NO regulates the phosphorylation of threonine-Glutamine-Tyrosine motif in human spermatozoa proteins of 80 and 105 kDa through capacitation [72]. Others have shown the effect of Ginsenoside Re, a bioactive component from ginseng, on sperm capacitation and acrosomal reaction occurs through NO-dependent cGMP/ protein kinase G signaling pathway [73]. De Lamirande and Lamothe (2009) showed a close interaction between NO and superoxide ion $(O_2\text{-}^{\bullet})$ during human sperm capacitation is needed for acquisition of fertility [69]. Inhibition of NO generation as well as other ROS by semenogelin, the

predominant protein in human semen, blocks sperm capicitation [74]. On the other hand, nitric oxide at low concentrations increased human sperm capacitation through enhancement of sperm-zona pillucida (ZP) binding capacity [75].
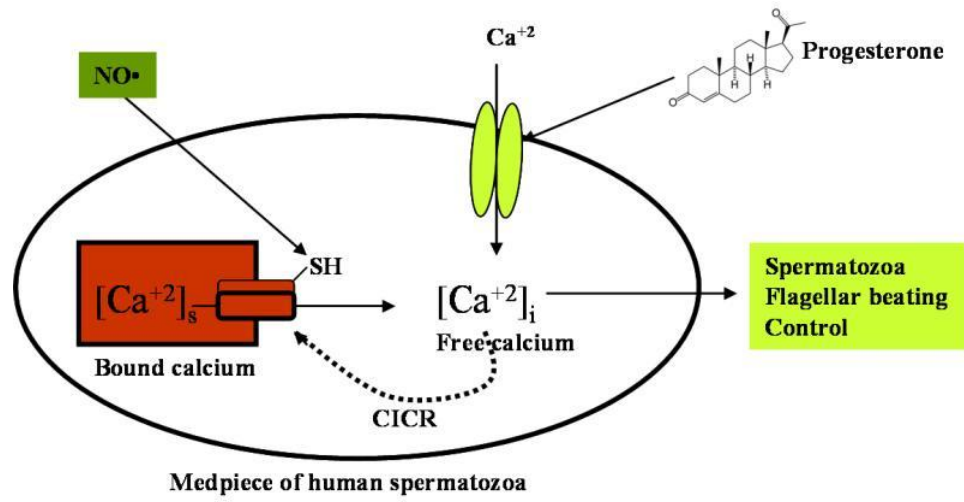
**Figure 4-5**: Synergistic effect of NO and progesterone (modified from Linda *et al.*, 2009).

**4.10 Adverse effects of NO on sperm physiology and function**

It has been shown that NO is vital for appropriate sperm function, while overproduction of this reactive free radical has also pathologic implications, particularly on the motility of sperm [55]. Induced oxidative stress to human sperm *in vitro* using high doses of $H_2O_2$ showed a significant increase in NO levels and a significant decrease in sperm motility [55; 76]. Rosselli *et al.* (1995) found that higher levels of nitric oxide decrease the quality of sperm *in vitro* [40]. Later study on varicocele suggested that nitric oxide synthase released within the dilated spermatic vein establishes a high oxidative stress state as a result of increased production of NO. Formation of peroxynitrite from the reaction of NO with superoxide ion also affects sperm function in varicocele patients [77]. On the other hand, no significant relationship was found between NO concentrations and the severity of athenozoospermia, oligoospermia, and abnormal sperm morphology in these people [78].

Treatment of purified spermatozoa with NO-doses from NO-donors such as S-nitroso-N-acetylpenicillamine and sodium nitroprusside decreased both sperm motility and viability [40; 42; 79]. The nitric oxide generator, SIN-1 (3-morpholinosydnonimine hydrochloride), was used as a spermicidal agent [80]. Adding the calcium ionophore A23187, an activator of both eNOS and nNOS, to human sperm medium increased significantly the production of NO [54] and, as a result, decreased sperm motility. Others showed that the overproduction of NO in the genital tract of infertile human subjects decreases the integrity of sperm DNA [81]. A later study demonstrated that TNF-alpha and IL-6, two cytokines involved in inflammation, decrease sperm motility possibly via inducing NO formation [82].

In contrast, melatonin (*N*-acetyl-5-methoxytryptamine), a hormone secreted by pineal gland and responsible for circadian rhythm, is able to scavenge directly and indirectly NO in human sperm. Accordingly, it was concluded that supplementation of sperm medium with melatonin might be able to counteract the adverse effects of NO on sperm [83].

## 4.11 Measurement of NO in semen

The most common method used to measure NO in human semen is *nitrite colorimetric assay*, also known as the Griess assay. In this assay, NO is assessed indirectly via measuring its breakdown product nitrite ($NO_2^-$), formed by spontaneous oxidation of NO [84]. The detection limit for this assay is 1 μM nitrite [84]. Sulfanilic acid first reacts with $NO_2^-$, in the semen sample to produce an intermediate diazonium, which then reacts with N-(1-naphthyl) ethylenediamine and form a stable azo dye, which absorbs light at 540nm [85]. To accurately measure all nitric oxide produced in the sample, some reports first convert all nitrate to nitrite using nitrate reductase enzyme, before using the Griess assay [85].

*Chemiluminescence* is another method commonly used to determine NO in semen. The detection is based on NO reaction with ozone ($O_3$), which leads to red light emission (Scheme 4-2). Photons detected from this reaction, using a photomultiplier tube are transformed to an electrical signal that is proportional to the amount of measured NO [63].

$$NO + O_3 \longrightarrow NO_2* + O_2$$

$$\downarrow$$

$$NO_2 + \boxed{\text{Light}}$$
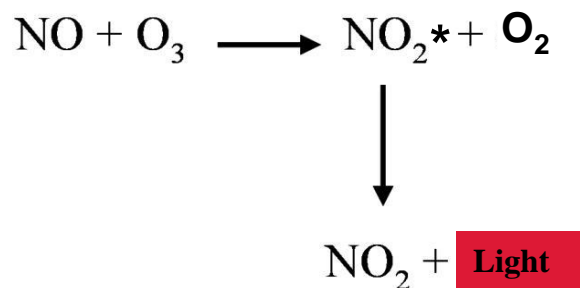
**Scheme 4-2:** reaction of NO with ozone.

Others have used other techniques to measure NO production at cellular level such as *electron paramagnetic resonance (EPR) spectroscopy and spin trapping* [70]. Such techniques are not commonly used in the andrology laboratories because they are more complicated and not readily available to common andrology laboratories [86; 87]. In the following chapter, we have standardized a direct method to measure nitric oxide in human spermatozoa using flow cytometry.

**4.12 Conclusion**

      Nitric oxide synthases, mainly the constitutive isoforms, are present in human spermatozoa. Physiologic levels of NO are vital for human sperm function and physiology, mainly for adequate flagellar beating and progressive motion. The effects of NO on human spermatozoa could be direct or indirect (mediated by peroxynitrite and/or its derivatives). Decreased levels of NO impair the function of spermatozoa. Likewise, excessive levels of NO contribute to oxidative stress and are detrimental to sperm quality. Although the evidence cited in this chapter shows that NO is very important for human sperm quality such as adequate sperm motility, many of its roles and mechanisms of action in sperm are yet to be fully unveiled.

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# CHAPTER V


# A NEW METHOD FOR MEASUREMENT OF NITRIC OXIDE PRODUCTION

# IN HUMAN SPERMATOZOA USING FLOW CYTOMETRY

**Summary**


Studies showed that nitric oxide (NO) has a major contribution in male reproduction. However, many of NO's mechanisms of action in sperm remain unclear; one important reason is that methods for direct NO measurement at the level single cells and/or collections of cells are not straightforward. Here we demonstrated a method to measure intracellular NO in human sperm by means of flow cytometry using NO-specific probe 4,5-diaminofluorescein-2-diacetate (DAF-2DA). Motile sperm are freshly prepared using a modified direct swim-up procedure. Both sperm and DAF-2DA concentrations are standardized. Sperm samples incubated with L-nitro-arginine methyl ester (L-NAME), a Nitric Oxide Synthase (NOS) inhibitor, showed lower fluorescence in a dose-dependent manner. Sperm samples supplemented with Diethylamine NONOate (DEA NONOate), a nitric oxide donor, showed higher fluorescence compared to the control. At last, we used this method to measure the effect of L-carnitine, a motility stimulant as we

have showed in the third chapter, on NO production in human spermatozoa. We found that LC may increases sperm motility via increasing the production of NO.

## 5.1 Introduction

Nitric oxide (NO) is one of the most studied molecules in biomedicine because of its various physiological functions. As we have reported in the previous chapter, it has been referred to as an endothelium-derived relaxing factor because it causes vasodilation via relaxing vascular smooth muscle [1; 2; 3]. In addition, it prevents platelets aggregation [4], acts as a neurotransmitter [5; 6], and mediates macrophage bactericidal activity [7].

Reports have shown that NO has a significant involvement in male reproduction [8; 9]. Various studies showed that endogenous NO is very important for sperm motion [10- 14]. NO generation was found to be crucial in gamete interaction and fertilization route [15]. A number of studies reported that NO is involved in human sperm capacitation [16; 17; 18]. In contrast, others have shown that higher levels of nitric oxide were observed in infertile men compared to control groups [19].

Although the evidence above points to the fact that NO is very important for human sperm physiology and function, its exact mechanisms of action in sperm are yet to be fully understood. The majority of measurement of NO in sperm relied on nitrite determinations using colorimetric assays. This may not relay an accurate picture on NO concentration and its fluctuations in spermatozoa. Methods that have the potential to follow NO concentration under varying conditions within cells are of particular importance to investigate the mechanisms of actions of NO in sperm function.

Analysis by flow cytometry has entered many andrology laboratories and has been used to evaluate sperm count and viability [20], to detect antisperm antibody [21; 22] and to study the chromatin structure of sperm [23]. The considerable advantage of flow cytometry is that millions of cells can be analyzed in seconds, providing a statistically more precise assessment of the level of analyte under consideration. Here, we demonstrated a method to detect the intracellular NO in human sperm using the NO-specific probe 4,5-diaminofluorescein-2-diacetate (DAF-2DA). The specific detection of NO and the sensitivity of fluorescence are coupled to the high throughput capability of flow cytometry; this provides a specific, sensitive, and fast method of monitor NO concentration in spermatozoa under varying experimental conditions.

## 5.2 Materials and methods

*Sample collection*

Our study was approved by the institutional review board of Cleveland Clinic Hospital. Normospermic men were selected and analyzed based on normal semen analysis according to the World Health Organization (WHO) (1999) guidelines (section 2.2.1). Fresh semen samples were used to standardize the measurement of intracellular NO in sperm using DAF-2DA by means of flow cytometry.

Samples were collected by masturbation following a minimum of 48 to 72 hours of sexual abstinence. Following liquefaction at 37 ºC for 20 minutes, semen specimens were evaluated for volume, sperm concentration, total cell count, motility and morphology. A total amount of 5µL of the sample was used for evaluation of

concentration and motility using a microcell slide chamber (Conception Technologies, San Diego, CA).

*Preparation of motile sperm samples*

Motile sperm were retrieved from semen samples using direct swim-up technique. This procedure was adapted from the one described by World Health Organization (1999) (section 2.2.1). Briefly, spermatozoa are selected based on the ability to swim out of seminal plasma into the human tubal fluid (HTF). We use only untreated, fresh semen in this process. 0.8 ml of homogenized semen was pipetted carefully under 1 ml of HTF placed in a 15 ml conical centrifuge tube while avoiding the mixing between the semen and the supplemented medium. Afterward, the tube was left at 90º angle for 1 hr at 37ºC. The uppermost ($\sim$ 0.6 – 0.8 µL) medium containing only the highly motile sperm is transferred to another test tube, and centrifuged for 4 min at 1600 rpm. Then, the top layer was discarded, and the sperm in the bottom resuspended in 0.5 mL HTF. The resulting motile sperm are washed and their concentration adjusted as needed.

*DAF-2DA preparation*

DAF-2DA was dissolved in dimethyl sulfoxide (DMSO) because it has a very low solubility in water. Although DMSO is a very suitable solvent for DAF-2DA, it is considered toxic to human sperm when added at higher concentrations [24]. Therefore, in our experimental design, we added DAF-2DA/DMSO solution to sperm medium at a concentration of $\leq$ 5% (v/v). We did not see a statistical difference in sperm viability after adding DMSO of $\leq$ 5% (v/v) compared to sperm without DMSO (Figure 6-5). We

run our experiments in the dark conditions in order to avoid the exposure of DAF-2DA to the light, because fluorescein can be easily photobleached, a photochemical destruction of the fluorophore [25] .

*Measurement of DAF-2T fluorescence at various sperm concentrations*

The concentration of washed motile sperm is adjusted with HTF to within 0.5- 8.0 x $10^6$/ mL range. The samples (n=6) are then supplemented with 20 µM of DAF-2DA, incubated for 90 min at 37 ºC in dark, and analyzed with flow cytometry. Identical samples without addition of DAF-2DA are used as controls.

*Measurement of DAF-2T fluorescence at various DAF-2DA concentrations*

Samples (n=6) of 2 x $10^6$/mL sperm concentration are incubated with various concentrations (0.5 nM, 5 µM, 50 µM, 0.5 mM and 1 mM) of DAF-2DA for 90 min at 37 ºC in dark. After incubation, the fluorescence of DAF-2T was measured using flow cytometry along with control samples.

*Measurement of sperm viability*

Sperm viability was measured by the Eosin-Nigrosin staining procedure (see page 25, chapter II)

*NO measurement in sperm after NOS inhibition*

Sperm samples (n=8) at 2 x $10^6$/mL concentration are incubated at 37ºC with various concentrations (100, 400 and 800 µM) of the NOS inhibitor $N^w$ –nitro -L-arginine

methyl ester (L-NAME) for 120 min in the dark. 15 min after the addition of L-NAME, DAF-2DA is added to the samples to a final concentration of 20 µM. The fluorescence of DAF-2T is measured immediately after incubation using flow cytometry. Samples without incubation with L-NAME are taken as controls for this series of experiments.

*NO measurement in the presence of an exogenous NO donor.*

Sperm samples (n=6) at $2 \times 10^6$/mL concentration were incubated at 37ºC with various concentrations (10, 100, 300, 500 and 800 µM) of the NO donor 2-(*N,N*-diethylamino)-diazenolate-2-oxide, sodium salt (DEA-NONOate) for 45 min in dark. DAF-2DA at 20 µM is added to the samples 45 min prior to the addition of the NO donor. The fluorescence of DAF-2T was measured immediately after incubation using flow cytometry. Samples in absence of NONOate are taken as controls.

*Flow cytometry*

Flow cytometry data acquisition is completed within 10-15 min from sample preparation. The flow cytometer (FACScan; Becton Dickinson, San Jose, CA) is equipped with a 515-nm argon laser as a light source. Samples are adjusted to have 20,000 cells examined for each assay at a flow rate of 100 cells/second. Data analysis is performed using FlowJo v4.4.4 software (Tree Star Inc., Ashland, OR).

In our flow cytometry data, we used 20,000 count from the $2 \times 10^6$/mL total sperm in each sample to obtain consistent measurements. The threshold value of the forward scatter (FSC) was set < 40 FSC to exclude small cells, cell fragments, and debris from the analyzed sperm. The sperm window was established after running multiple

unstained sperm samples (instrumental control) submitted to swim up and followed by washing with HTF.

*Statistical analysis*

All values were recorded as mean ± S.D. Differences between samples and their control were considered significant at $P < 0.05$. Statistical analyses were performed by one-way analysis of variance (ANOVA) and an unpaired Student's $t$-test using the SPSS/PC computer software (SPSS 10.0.7, SPSS Inc.)

## 5.3 Results and discussion

The work we presented here is a modification of what Strijdom *et al.* (2004) and S. du Plessis *et al.* (2006) have described [26; 27]. The fluorescent NO-indicators diaminofluoresceins (DAFs) were first designed and synthesized by Kojima *et al* (1998) [25]. DAF-2DA, a non-fluorescent dye, is able to cross the membrane of live cells and is hydrolyzed to 4,5-diaminofluorescein (DAF-2) by intracellular esterases [28]. DAF-2 reacts rapidly with NO (in the presence of $O_2$; the reaction is actually with an $NO^+$ equivalent likely from a nitrogen oxide derivative of NO) to form the fluorescent dye triazolofluorescein (DAF-2T) (Figure 5-1). DAF-2T fluoresces at 515 nm upon excitation at 495 nm. Because of the specificity of the reaction of DAF-2 with NO, the intensity of the fluorescence is proportional to the amount of intracellular NO generated.
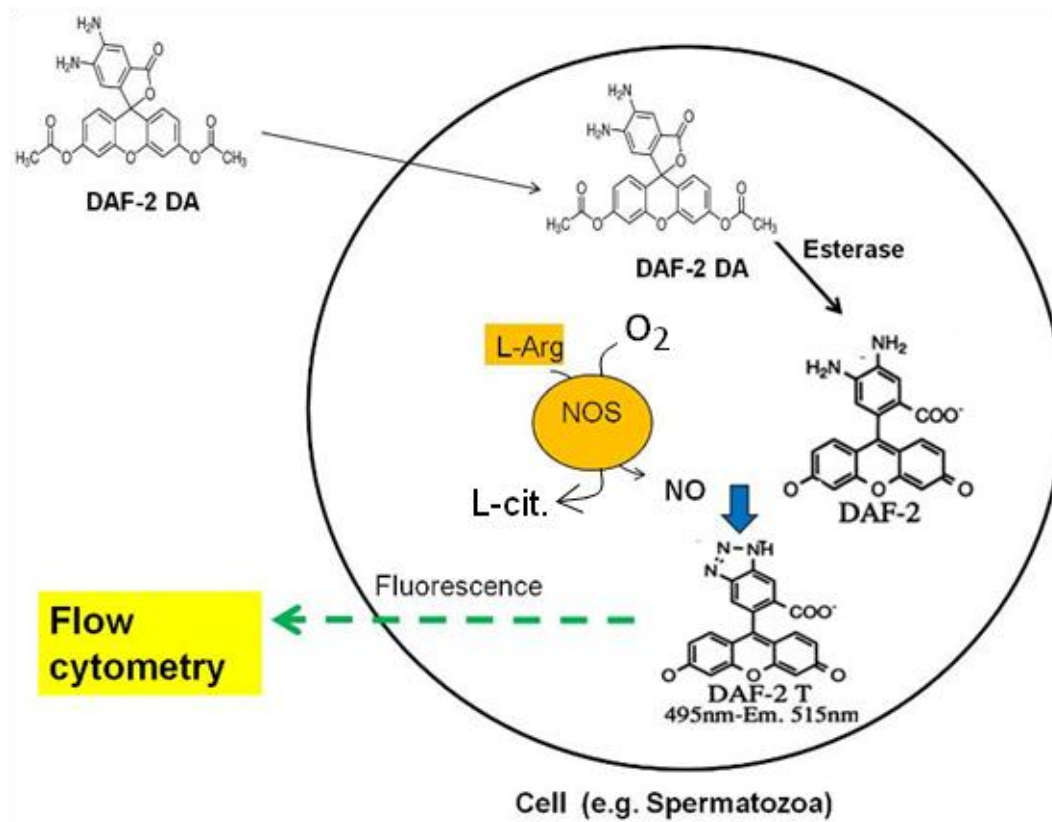
**Figure 5-1:** Schematic representation of fluorometric NO measurement procedure for human spermatozoa using the DAF-2DA precursor.

*Effect of sperm concentrations on DAF-2T fluorescence at 20 µM DAF-2DA*

To determine the optimum sperm concentration of samples used in our flow cytometry measurements, we monitored DAF-2T fluorescence as a function sperm concentration for a set concentration of the dye DAF-2DA at 20 µM (Figure 5-2). No significant difference ($P > 0.05$; *t*-test) in average fluorescence is seen between sample with sperm concentrations 0.5, 1.0 and 2.0 x $10^6$/mL. On the other hand, a significant difference ($P < 0.05$; *t*-test) in average fluorescence is observed at higher concentrations (4 and 8 x $10^6$/mL). Unstained control samples (i.e. 0.0 µM DAF-2DA) exhibit very negligible fluorescence.

Our experiments indicate that concentrations in the range of 0.5-2.0 x $10^6$/mL could all be used for a set DAF-2DA concentration of 20 µM. Sample with higher sperm concentrations such as 4x or 8x$10^6$/mL showed lower fluorescence, likely a direct result of a lower ratio of DAF-2DA molecules per sperm cell.
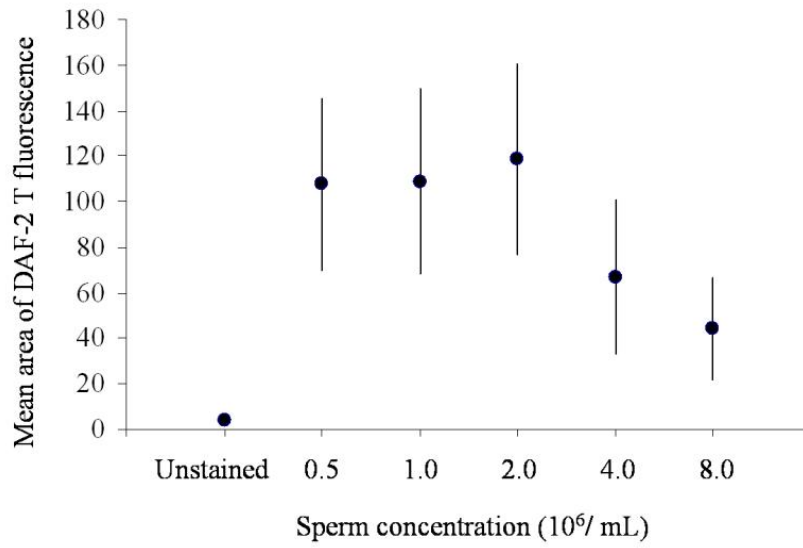
**Figure 5-2**: Observed fluorescence as a function of sperm concentration with 20 µM dye concentration. The values reported are averages ± standard deviations; n=6. The control is the unstained sample.

*Effect of DAF-2DA concentrations on observed fluorescence*

Figure 5-3 shows flow cytometry histograms of DAF-2DT fluorescence (A) as well as the mean areas for these histograms (B) at various concentrations of DAF-2DA (0.5 nM -1 mM). All DAF-2DA concentrations used show a statistical increase ($P < 0.05$; *t*-test) in fluorescence compared to unstained control samples (without DAF-2DA). Sperm samples incubated with 50 µM of DAF-2DA show the highest intensity of DAF-2T fluorescence, while the lowest fluorescence is seen for 0.5 nM dye concentration. The intensity of the fluorescence decreased at higher doses (above 0.5 mM) of DAF-2DA used.

Our results showed a statistically significant increase in fluorescence even at a low concentration of DAF-2DA of ~ 0.5 nM compared to the control sample (without DAF-2DA). Because concentrations of 0.5 mM and 1 mM of DAF-2DA exhibited lower fluorescence compared to 50 µM, we examined if sperm viability is compromised at these higher concentrations. Indeed, we find that the millimolar range of dye concentration significantly affects sperm viability, which explains the observed decrease in fluorescence (Figure 5-4).
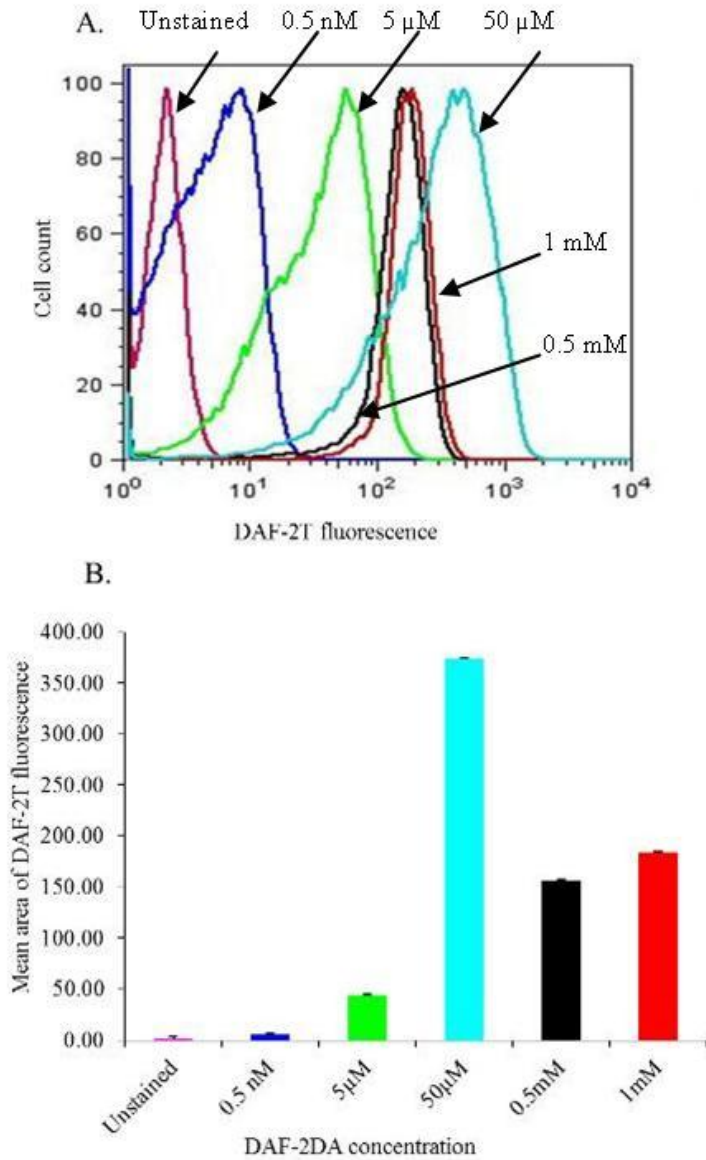
**Figure 5-3**: (A) Flow cytometry histograms of DAF-2T fluorescence as a function of concentrations of the DAF-2DA dye (0.5nM, 5µM, 50µM, 0.5mM, 1mM); (B) Mean areas of DAF-2T fluorescence at the various DAF-2DA doses; data are means (for n=6) ± standard deviations; the control is the unstained sample (i.e. no DAF-2DA). Sperm concentration used in the sample was $2 \times 10^6$/mL with 20,000 cells acquired in flow cytometry.
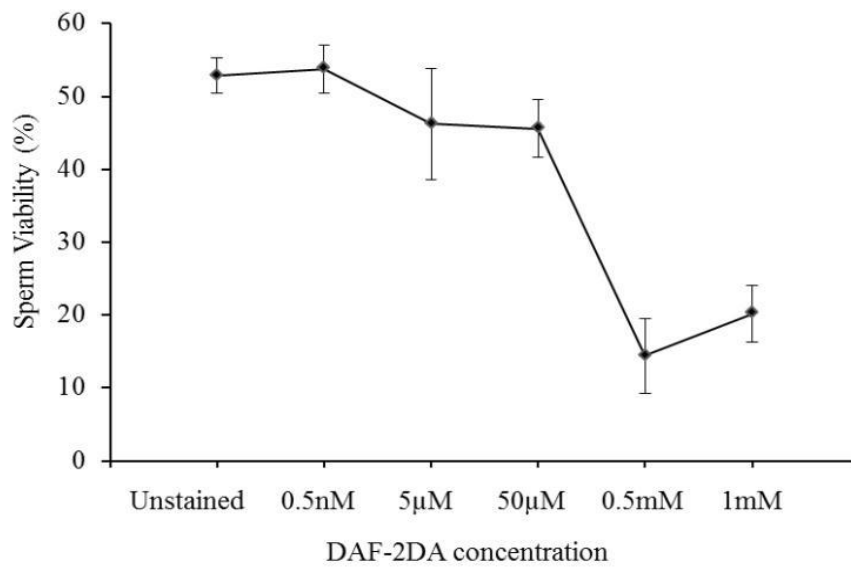
**Figure 5-4**: Effect of various DAF-2DA concentrations on sperm viability as evaluated by the Eosin-Nigrosin test. The evaluation of sperm viability performed immediately after the flow cytometry measurements. Data are means (n=6) ± standard deviations.

*Dynamic NO sensitivity of DAF-2DA in the presence of NOS inhibitor and an NO donor.*

To determine the dynamic sensitivity of this fluorometric method to sudden variations of intracellular NO concentration in spermatozoa, we monitored fluorescence changes in response to an NO donor as well as in the presence of a NOS-inhibitor.

a. *Effect of the NOS inhibitor L-NAME on observed fluorescence*

Figure 5-5 shows the effect of various concentrations (100, 400, and 800 µM) of L-NAME on the observed DAF-2T fluorescence. Data represented are mean areas of fluorescence ± standard deviations. The figure shows a significant decrease ($P < 0.05$) in the intensity of fluorescence of DAF-2T in the presence of L-NAME in a dose dependent manner. The fluorescence decrease of DAF-2T did not significantly change for L-NAME concentrations between 100 and 400 µM ($P > 0.05$; *t*-test).

b. *DAF-2T fluorescence in response to varying concentration of the NO donor*

The specificity of DAF-2DA to measure NO and to respond to increases of intracellular NO concentration was validated using incubation with DEA-NONOate, a known NO donor.

Figure 5-6 shows the effect of various concentrations of DEA-NONOate, the NO releasing compound, on observed fluorescence in human sperm. We observed significant increase in fluorescence in samples with 100 µM NO donor concentration while the change was not significant with 10 µM. Also, the mean area of DAF-2T fluorescence did not increase significantly above the level measured with 100 µM NONOate for concentrations above 300 µM.
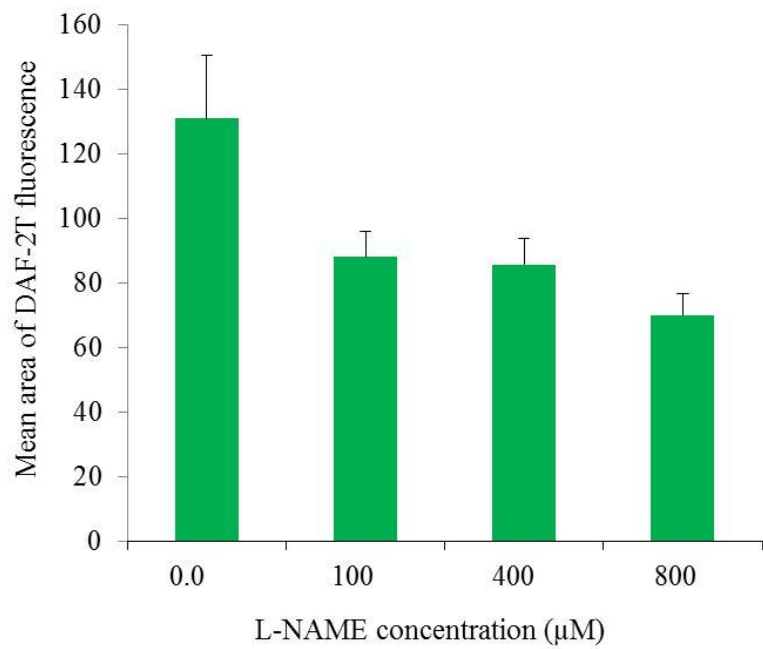
**Figure 5-5**: DAF-2T flow cytometry fluorescence in human sperm as a function of L-NAME concentration. Data are means (n=10) ± standard deviation.
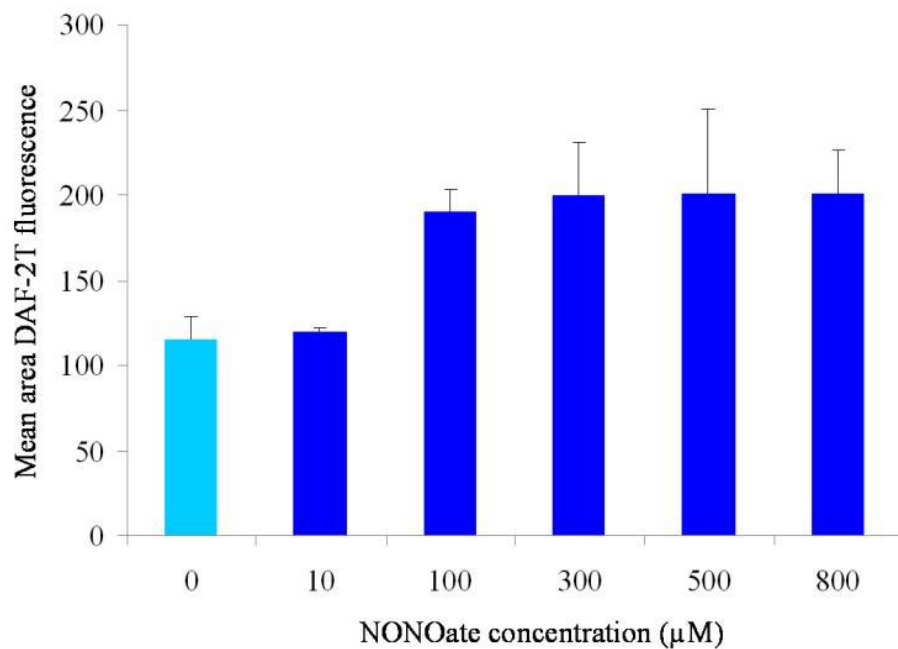
**Figure 5-6**: DAF-2T flow cytometry fluorescence in human sperm as a function of

NONOate concentration. Data are means (n=6) ± standard deviations.

Our results show that the fluorescence of DAF-2T increases after adding the NO donor, indicating that the method follows dynamic increases of intracellular NO. However, the method shows that the fluorescence did not increase significantly beyond the level seen for 100 μM of the NO donor. It is known that concentrations of NONOates (in the range of 0.3- 1.0 mM) affect sperm motility [17]. In addition to sperm motility, we suspected that sperm viability might be behind the sluggish increase (for 300 μM) and then decrease of DAF-2 fluorescence (for 500-800 μM). To confirm this, we measured the viability of sperm immediately after the flow cytometry analysis (Figure 5-7). As expected, the figure shows a significant decrease in sperm viability after 100 μM in NO donor. This is not a surprise since literature reports established that NO donors can have both a protective role (at low concentration) but can also induce cell death in a variety of cell types at high concentrations [29; 30]. The cytotoxic effects of NO are mainly due to higher oxides that derive from NO and that are more reactive. Peroxynitrite is one of them and has many reactivity facets under physiologic conditions which contribute to cell dysfunction, including the disruption of the cell's ability to sustain healthy levels of endogenous NO, as well as other vital functions.
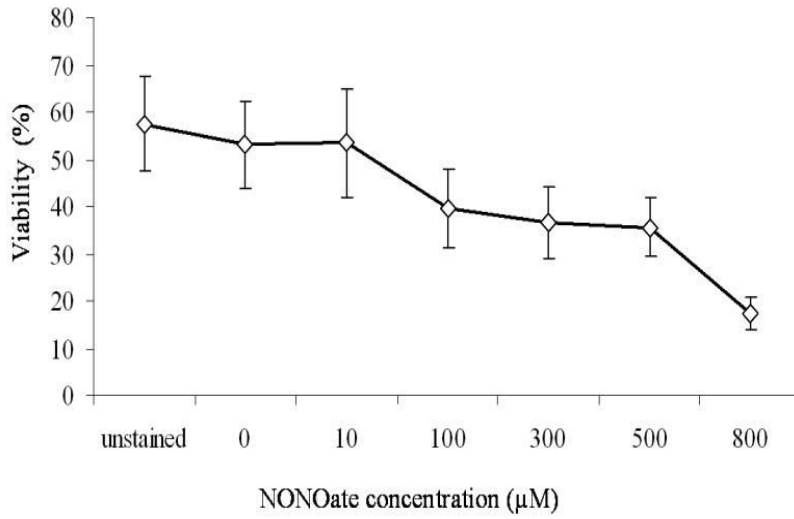
**Figure 5-7**: Effect of varying concentration of NONOate on sperm viability as evaluated by the Eosin-Nigrosin test. The viability test is conducted immediately after the flow cytometry analysis. Sperm concentration was $2 \times 10^6$/mL. Data are means (n=6) $\pm$ standard deviations.

**5.4 Conclusion**

Collectively, these findings show that measurement of intracellular nitric oxide in human sperm can be performed using the DAF-2DA fluorometric method coupled to flow cytometry. The method has now been standardized for human spermatozoa and will be of assistance to elucidate the NO mechanisms of action in human sperm as well as its role in maintaining sperm quality or in inducing cytotoxicity.

**5.5 Effect of L-Carnitine (LC) on NO production in human spermatozoa assessed by the DAF-2DA fluorometric method coupled with flow cytometry**

In this section, we measured the effect of L-carnitine (LC) on NO production in human sperm using the method that we have standardized as described in the previous section of this chapter. As we have seen in chapter III, supplementation of sperm with LC at 0.5 mg/mL increased sperm motility. On the other hand, as we described previously, nitric oxide has been implicated for adequate sperm function including motility. Our goal here is to test whether LC supplementation causes the levels of endogenous NO to increase in sperm. This may be a mechanism by which LC stimulates sperm motility.

*Methodology and experimental design*

Highly motile sperm were prepared using direct swim up and adjusted to 2 x $10^6$/mL concentration with HTF. After that, Semen Samples (n=4) were supplemented with 0.1 mg/mL LC, followed by addition of DAF-2DA at 20μM concentration. Samples were then incubated for 90 min at 37°C in the dark. After incubation, the fluorescence of DAF-2T formed was measured immediately using flow cytometry as described in above.

*Results and discussion*

In chapter 2, we have shown that 0.5 mg/mL LC concentration is beneficial to sperm at 5 million/mL. Here we supplemented the sperm with LC at 0.1 mg/mL because we treated lower sperm number (2 million/mL). Figure 6-7 shows the effect of 0.1 mg/mL LC on DAF-2T fluorescence in human spermatozoa. The figure shows an increase in DAF-2T fluorescence, and thus an increase in the intracellular NO produced in the sperm supplemented with LC. Control samples assayed without LC supplementation do not show any significant increase in fluorescence. Data in the figure are representative of four independent experiments. As reported in chapter 4, NOS in all of its isoforms is present in human spermatozoa. NO has a major role in the physiology and function of the spermatozoa [10; 31]. A number of studies showed that NO is very important for adequate motility of human spermatozoa [12; 17; 32]. Our results from the fluorometric determination of intracellular NO production in spermatozoa upon incubation with LC show a causal relationship between LC supplementation and NO production. Whether the LC-induced NO production is direct or via an indirect pathway cannot be concluded and awaits further investigation. For now, the evidence shows that LC induces intracellular NO production, and this may explain the enhanced motility of spermatozoa incubated with L-Carnitine.
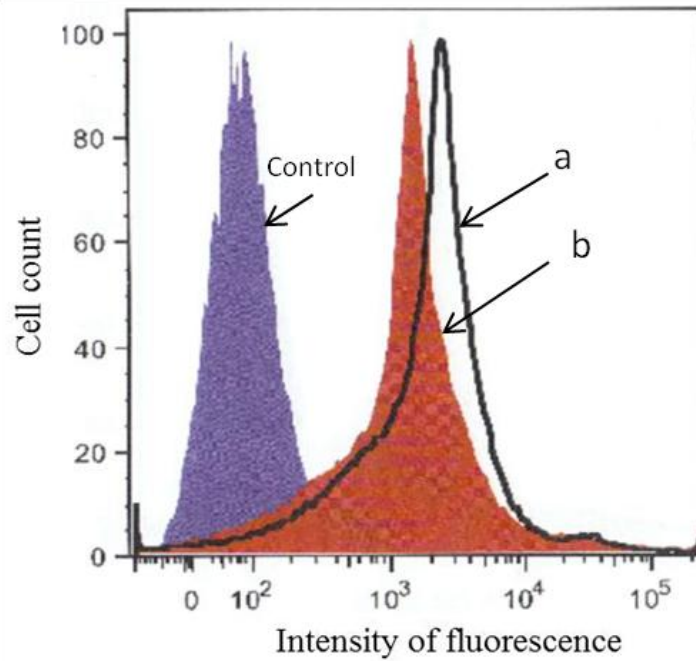
**Figure 5-8**: Effect of LC on DAF-2T fluorescence in human spermatozoa. Sperm (2 x $10^6$/mL) incubated 90 min with 20 μM DAF-2DA in the presence of 0.1 mg/mL LC (a), and without LC (b). The control histogram is for sperm population without DAF-2DA, and without LC. Data are representative of four independent experiments.

## 5.6 References

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