

Role of Oxidative Stress in Semen Quality of Infertile Men

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ABSTRACT

ROS are highly toxic to all types of biological molecules including DNA, lipids, protein and carbohydrates, and ROS considered as a cause of much disease including genital diseases. The study consists of 72 infertile with different categories (Oligozoospermia, Asthenozoospermia and Normozoospermia) and 24 healthy men as control in the period from March to November 2012. Patients were selected and analyzed according to WHO 2010. The following biochemical parameters have been examined for each sample (net semen ROS (Reactive oxygen species), washed Sperm ROS Lipid Peroxidation and DNA damage level). Result show a significant increase in ROS, Lipid peroxidation, DNA damage in different groups infertile men compared to fertile, while no significant difference in washed spermatozoa ROS level. Low sperm count in Oligozoospermia group may be due to apoptosis process in the testes activated by high level of ROS. The link between ROS and reduced motility may be due to the decrease in axonemal protein phosphorylation and sperm immobilization. Lipid peroxidation in Normozoospermia infertility occurs in a high level in the sperm acrosome, leading to loss of the ability to penetrate the ovum.

Introduction

Infertility affecting approximately 80 million (1:7) couples worldwide. A male factor is involved in 50% of cases and is the sole cause of infertility in 30% (1). A large proportion of infertile men fail to impregnate their female counterpart because of lack of sperm azoospermia(AZO) or too little sperm Oligozoospermia(OLZ); infertility may also be due to abnormal sperm morphology Tetratozoospermia (TZO); insufficient sperm motility Athenozoospermia (ASZ) and may have normal semen parameters but no explained causes Normozoospermia (NOZ) (2).

It is very important to identify the factors/conditions which affect normal sperm functions. Among various causes, oxidative stress (OS) has been attributed to affect the fertility status and physiology of spermatozoa (SPZ) (3). High ROS levels have been detected in the semen samples of 25% to 40% of infertile men (4). Excessive production of free radicals or reactive oxygen

species (ROS) can damage sperm and ROS has been extensively studied as one of the major mechanism of infertility (5).

Reactive oxygen species (ROS)

ROS represent a broad category of molecules that indicate the collection of radicals (hydroxyl ion, superoxide, nitric oxide, peroxy, etc.) and nonradicals (ozone, single oxygen, hydrogen peroxide) and oxygen derivatives (3).

From a chemical point of view, any form of oxygen containing a non-bound external electron is highly unstable and capable to react with a variety of organic substrates. These forms of oxygen are free chemical radicals and, as their chemical behavior suggests, they are a kind of ROS (6). The step-wise reduction of molecular oxygen via one-electron transfers shown in the figure 1:

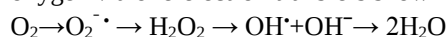


Figure1. The step-wise reduction of oxygen via one electron transfers

Pathogenic Role of ROS in Male Infertility

Human semen is known to contain different types of cells, such as mature and immature SPZ, round cells from different stages of spermatogenesis, leukocytes, and epithelial cells, from these different cell types, leukocytes and sperm have been shown to be the two main sources of ROS (7).

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The site of tissue damage by free radicals is dependent on the tissue and the reactive species involved. When a cell membrane or an organelle membrane is damaged by free radicals, it loses its protective properties. Extensive damage can lead to death of the cell; this may be by necrosis or apoptosis depending on the type of cellular damage. This puts the health of the entire cell at risk (8).

Proteins can undergo direct and indirect damage following interaction with ROS, including peroxidation, damage to specific amino-acid residues, changes in their tertiary structure degradation, fragmentation (9) denaturation, loss of function, cross-linking, aggregation and fragmentation of connective tissues as collagen (10). Also ROS attacks poly-unsaturated fatty acids (PUFA) in the cell membrane, leading to a cascade of chemical reactions called lipid peroxidation (7). The onset of lipid peroxidation (LPO) within biological membranes is associated with changes in their physicochemical properties and with alteration of biological function of lipids and proteins (10).

ROS can induce sperm DNA damage *in vitro*, confirming that ROS may play a role in the etiology of sperm DNA damage in infertile men (11). DNA bases are susceptible to oxidative damage resulting in base modification, strand breaks, and chromatin crosslinking (4). Apoptosis occurs because of the DNA fragmentation which is seen in the sperm of infertile men, Therefore, sperm apoptosis is a strong and beneficial index for the state of male fertility (12).

Disruption in homeostasis can result in oxidative stress and tissue injury (13). OS results from an imbalance between production and removal of ROS leading to concentration of reactive intermediates higher than normal (14). There is growing evidence to suggest that seminal OS is involved in many aspects of male infertility (15). OS has been reported as one of the most important factors contributing to poor quality semen. Since SPZ are unable to repair the damage induced by oxidative stress, because sperm lack the cytoplasmic enzyme systems required to accomplish the repair (16). In the past few years, the role of OS in pathogenesis of male infertility has been emphasized (17). It was reported that high levels of seminal ROS are detected in 25–40% of infertile men. The generation of ROS has become a real concern because of their potential toxic effects at high levels on sperm quality and function (18).

Method and Materials

The study population consists of 72 infertile and 24 healthy men as control, The patients were selected and analyzed according to (WHO 2010) (19) manual of semen analysis during the period from March to November 2012. All the biochemical tests have been done in the laboratories of Biology and Chemistry departments in the Faculty of Educational sciences / univ. of Garmian.

Semen Analysis

After liquefaction semen sample taken for microscopic examinations which includes: sperm concentration, count were calculated using a Hematocytometer, and the final concentration described as millions sperm/ml. Live SPZ tested by Hypo-Osmotic Swelling (HOS) test using a phase contrast microscopy. Sperm activity has been examined directly after liquefaction on a pre-warmed slide, the result described as, Rapid progressive, non progressive and Sluggish motility. Sperm morphology has been examined using India ink, the slides seen by oil emersion lens. The abnormality, the abnormality category included abnormal head, midpiece and tail.

Leucocyte has been determined in semen by O-toluidine test described by (WHO, 2010) (19), peroxides produces $O_2^{\bullet-}$ which is reacts with O-toluidine to form a brown color. Semen sample contains less than 1×10^7 Leucocyte/ml were considered normal.

Measurement of Semen Reactive Oxygen Species (ROS)

Levels of ROS were estimated by chemiluminescence assay using a method described by (Agarwal, 2008) (20). Method depend on the reaction of ROS with Luminol and the Levels of ROS were determined by measuring chemiluminescence and luminescent signal was read for 15 min and calculated to the level of sperm concentration of 10^7 SPZ.

Measurement of (ROS) in Washed Sperm Cells

A modified colorimetric Nitro Blue Tetrazolium (NBT) test was used to evaluate ROS production. After washing in Phosphate Buffer Saline (PBS), sperms has been incubated with an equal volume of NBT working reagent at 37° for 45 min. the resulting intracellular formazan was solubilized in 60 μ L of 2 M KOH and dimethyl sulphoxide (DMSO), and the resulting color reaction was measured spectrophotometrically on a microplate reader at 630 nm, ROS production was expressed as μ g of formazan per 10^7 sperm.

Sperm DNA Damage Assay

Sperm DNA damage determined by comet assay developed by N.P. Singh (21) using a research kit (cell biolab, USA). Semen samples were prepared by suspending the cells at 1×10^5 cells/ml in the agarose and applied on a slide. The samples are electrophoresed for 10 min. Following electrophoresis, the samples are dried, stained with Vista Green Dye. After drying slides viewed by epi-fluorescence microscopy using a FITC filter, Sperm DNA damage calculated using a computer program (CometScore) and the results expressed as percent % DNA damage.

Estimation of Lipid Peroxidation

LPO in SPZ and seminal plasma was measured by reaction of thiobarbituric acid (TBA) with MDA according to Roa *et al.* (22). In the presence of heat and

acid, MDA reacts with TBA to produce a pink colored end product. The absorbance of the resulting pink color read at 535 nm, MDA in (nmol/ml) level was determined from this equation:

Where: Ab=absorbance of the sample; Vs=Sample volume (0.1 ml); Vt=total volume (1.0 ml) and 1.56×10^5 = molar absorption coefficient for MDA-TBA complex

Statistical analysis

The statistical significance of difference in mean was assessed by ANOVA. Values less than 0.05 was considered statistically significant.

Results and Discussion

Role of OS in Oligozoospermia

Data in (table 1) show the evidence signifies the presence of ROS in OLZ infertile group. (Mean $\pm$ SD) of semen ROS for patients and control groups were (3951 $\pm$ 4320 and 12 $\pm$ 9 RLU/ 108 sperm) respectively. No significant difference in washed SPZ seen (13.70 $\pm$ 4.95, 11.65 $\pm$ 3.64 formazan/ 10^7 sperm) respectively.

Data also showed a significance in the presence of LPO and DNA damage in OLZ infertile men, mean (Mean $\pm$ SD) of seminal plasma MDA and sperm DNA damage for OLZ infertile and control groups MDA were (117.30 $\pm$ 36.88 and 44.13 $\pm$ 13.07 nmol/ml) and DNA damage (24.19 $\pm$ 4.82, 8.12 $\pm$ 3.53 %) respectively, this represents a significant elevation ($P \geq 0.01$) in MDA and DNA damage of OLZ infertile group compared with control groups.

Usually, the over production of ROS is balanced by antioxidant factors. High level of ROS may be due to low AOs level in semen samples of infertile men as shown in our previous article (23) that showed significant decrease in AOs level in infertile men. Low semen TAC in infertile group was due to the nutrition. Nutrition is the main cause low semen TAC in infertile group because of low people culture in nutrition, since they consume limited food types (23).

Low sperm count in OLZ may be due to apoptosis process in the testes activated by high level of ROS in semen samples. Apoptosis plays an important role in regulating spermatogenesis. It has been extensively investigated in spermatogonia, spermatocytes and spermatids in the testis (24). G.J.E. Oostrhuis, I.Vermes reported that the percentage apoptotic SPZ is inversely correlated with sperm concentration (25). OS may play a fundamental role in the regulation of apoptosis, recently, and OS itself was a direct inducer of apoptosis in testicular cells (26). High levels of ROS disrupt the inner and outer mitochondrial membranes resulting in release of cytochrome-C protein from the mitochondria that activates the caspases and induces apoptosis. (18).

A ROS dependent pathway for apoptosis was suggested based on the finding that H_2O_2 induces

apoptosis (18). Thus, exposure of human SPZ to H_2O_2 can readily trigger an apoptotic cascade characterized by the activation of caspase 3 and the appearance of annexin-V binding positivity (27). High concentrations of H_2O_2 induce LPO and result in cell death (28). In case of

$$(\text{MDA}) \text{ conc.} = \frac{\text{Ab} \times 106 \times 103 \times V_s}{(1.56 \times 10^5) \times V_t}$$

decrease AOs sperm are susceptible to different oxidative mediated damage. LPO degradation of sperm membrane integrity may thus be held responsible for abnormal sperm form, this oxidative damage is a probable cause of idiopathic male infertility involving disruption of spermatogenesis (29).

When spermatogenesis is impaired, the cytoplasmic extrusion mechanisms are defective. SPZ released from the germinal epithelium carrying surplus residual cytoplasm are thought to be immature and functionally defective. Retention of residual cytoplasm by SPZ is positively correlated with ROS generation via mechanisms that may be mediated by the cytosolic enzyme glucose-6-phosphate-dehydrogenase (G6PD). This enzyme controls the rate of glucose flux through the hexose monophosphate shunt, which, in turn, controls the intracellular availability of nicotinamide adenine dinucleotide phosphate (NADPH). The latter is used as a source of electrons by SPZ to fuel the generation of ROS by an enzyme system known as NADPH-oxidase (16).

Excess ROS can decrease sperm motility presumably by a rapid loss of intracellular ATP leading to axonemal damage, decrease sperm viability and increased mid-piece morphological defects with deleterious effects on sperm capacitation and acrosome reaction (30). Therefore it's expected to observe a decrease in sperm activity, our results show a significant decrease ($p=0.001$) in sperm motility of OLZ infertile group compared to control, mean $\pm$ SEM of sperm activity (38.13 $\pm$ 17.27 and 57.44 $\pm$ 11.11 %) respectively.

Different pathological condition associated with high lipid peroxidation can cause damage to the cell membrane, loss of cytosolic components and cell death (30). Therefore we found a significant decrease ($p=0.002$) in live SPZ percent in OLZ compared to control group (117.30 $\pm$ 36.88 and 44.13 $\pm$ 13.07) respectively.

It's known that the excessive generation of ROS in the reproductive tract not only affects the fluidity of the sperm plasma membrane, but also the integrity of DNA in the sperm nucleus (4). In fact, significantly elevated levels of ROS have been found causing DNA damage and apoptosis of germ cells during spermatogenesis, as well as DNA fragmentation and death of ejaculated SPZ, usually resulting in oligozoospermia of patients subjected to sperm analysis (6).

Role of OS in Asthenozoospermia (ASZ)

The results represents in (table 2) an elevation in ROS level of asthenozoospermia group compared with

fertile group, (Mean $\pm$ SD) was (2217 $\pm$ 1595 and 12 $\pm$ 9 RLU/10⁸ sperm), while there was no significant difference in washed SPZ (14.64 $\pm$ 5.28 and 11.65 $\pm$ 3.64 μ g Formazan /10⁷ sperm) respectively. These results agree with Tarish findings, who suggested higher ROS production, evidenced by increased MDA levels, support to the oxidative stress in ASZ (31). Also (table 2) show the evidence signifies the presence of ROS in ASZ infertile group. (Mean $\pm$ SD) of semen MDA for patients and control groups were (131.45 $\pm$ 119.61 and 44.13 $\pm$ 13.07 nmol/ml) and DNA damage was (21.79 $\pm$ 5.748.12 $\pm$ 3.53) respectively.

In ASZ the production of ROS is associated with loss of motility and a decreased capacity for sperm-oocyte fusion (32). One possible mechanism by which motility is inhibited by ROS formation is through decreased ATP production (33). ROS decreases in axonemal protein phosphorylation and sperm immobilization, both of which were associated with a reduction in membrane fluidity that was necessary for sperm-oocyte fusion (18), (34).

Mammalian SPZ are rich in polyunsaturated fatty acids and, thus, are very susceptible to ROS attack which results in a decreased sperm motility, presumably by a rapid loss of intracellular ATP leading to axonemal damage, decreased sperm viability, and increased midpiece morphology defects with deleterious effects on sperm capacitation and acrosome reaction (35). A beating flagellum is the predominant mechanism of sperm motility, and an axoneme is the predominant 'motor' by which the flagellum is propelled (36). Flagellar axonemes are made of tubulin, dynein and many associated proteins are the structures that allow motility for sperm (36). H₂O₂ has the ability to diffuse across the membranes into the cells and inhibit the activity of enzymes such as G6PDH, leading to a decrease in the availability of NADPH. These changes can cause a decrease in the antioxidant defenses of the SPZ, which ultimately leads to the peroxidation of membrane phospholipids (16).

Lipid peroxidation causes ATP to deplete rapidly resulting in decreased phosphorylation of axonemal proteins and causes transient impairment of motility (37).

Role of OS in Normozoospermia (NOZ)

The results represent (table 3) an elevation in ROS level of NOZ group compared with fertile group, (Mean $\pm$ SD) was (2217 $\pm$ 1595 and 12 $\pm$ 9 RLU/10⁸ sperm) respectively. These results agree with results by Pasqualotto *et al.*, they reported that ROS levels were higher in the idiopathic infertility compared to fertile group (15). No significant difference seen in washed sperm of NOZ group compared to control, results was (14.64 $\pm$ 5.28), (11.65 $\pm$ 3.64) respectively.

Mean $\pm$ SEM of MDA in the seminal plasma in infertile group is (113.82 $\pm$ 21.90 nmol/ml) which is higher than in control (44.13 $\pm$ 13.07 nmol/ml) and a significant

increase in DNA damage (20.07 $\pm$ 5.32%) compared to control group (8.12 $\pm$ 3.53%).

These results agree with previous reports where premature acrosomal reaction and the inability of the spermatozoa to release the proper stimuli have been associated with idiopathic male infertility. Acrosomal dysfunction can be considered as one of the important causes of male infertility. (38).

Studies have found that incubating SPZ with low concentrations of (H₂O₂) stimulates sperm capacitation, hyperactivation, and the ability of the spermatozoa to undergo then acrosome reaction and oocyte fusion (34).

Elevation of ROS in semen possibly affects the fertile capacity of patients with these different etiologies as well as the idiopathic infertility (39).

ROS impair the fertilization process by preventing the initiation of sperm-oocyte fusion events (37). H₂O₂, and not superoxide anions, was detrimental to the SPZ, inhibiting fusion with the oocytes (40), this is because of high ROS (H₂O₂) levels may lead to alteration of sperm membrane permeability and fluidity (17).

The excessive production of ROS (superoxide anions and hydrogen peroxide) by the SPZ themselves may result in peroxidation (PUFAs) of the plasma membrane. As a result, the fluidity of the spermatozoa membrane assured by the complex network of PUFAs is compromised by the ROS and inhibits proper membrane fusion with the oocytes (40).

LPO may damage membrane integrity with increased cell membrane permeability, thus leading to enzyme inactivation (26). Increased lipid peroxidation and altered membrane function can render sperm dysfunctional through impaired metabolism, motility, acrosome reaction reactivity and fusogenic capacity as well as oxidative damage to sperm DNA (41). LPO in NOZ occurs in a high level in the sperm acrosome, leading to loss of the ability to penetrate the female ovum as shown in the figure 2. LPO process results in loss of membrane fluidity due to disorganization of membrane architecture and reduction in the activity of membrane enzymes and ion channels. As a result, SPZ are unable to initiate the necessary biochemical reactions associated with acrosome reaction, zona pellucida binding and oocyte penetration (37).

It is concluded that:

1. There are relationships between the different Biomarker (ROS, LPO and DNA damage) and dimension of sperm normal function.
2. Elevation of ROS in the major cause of dimension of sperm normal function (motility, activity, viability and morphology) via lipid peroxidation of sperm membrane.

Male infertility can be expressed by Biochemical markers such as ROS, MDA, DNA damage level.

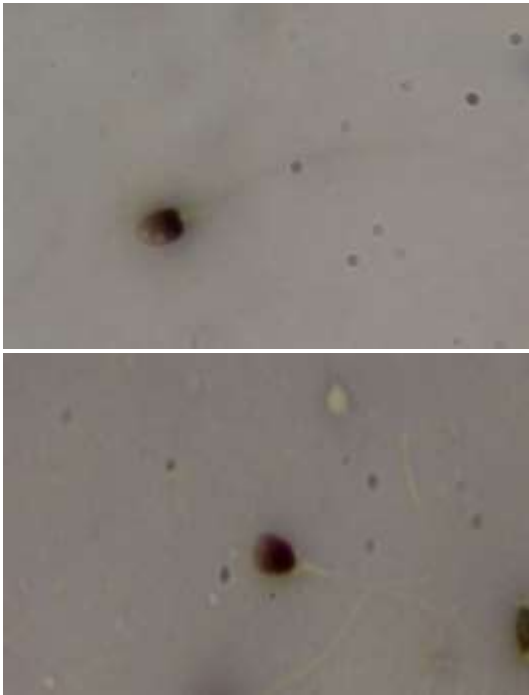


Figure 2: acrosomal LPO in the Asthenozoospermia (left), Normal acrosome (right).

Table 1: Mean of ROS parameters of Fertile and OLZ Infertile groups

Parameters	Count millions/ml	Live %	Active %	Progressive %
Oligozoospermia (n=20)	3.96±4.19	63.65±19.46	38.13±17.27	22.18±12.75
Fertile (n=24)	58.31±30.70	74.50±10.01	57.44±11.11	43.88±14.96
P value	0.001	0.002	0.001	0.001

ROS (RLU/10 ⁸ sperm)	Normal morph. %	Sluggish %	Non prog. %
3951±4320	50.65±16.65	25.12±15.72	15.94±12.83
12±9	61.04±11.79	17.06±7.84	13.56±11.92
0.001	0.063	0.015	0.003

ROS(µg Formazan /10 ⁷	LPO (MDA nmol/ml)	DNA damage
13.70±4.95	117.30±36.88	24.19±4.82
11.65±3.64	44.13±13.07	8.12±3.53
0.039	0.01	0.01

Table 2: Mean of ROS parameters of Fertile and ASZ Infertile groups

Parameters ASZ Infertile	Count millions/ml
Asuetoospermia (n=31)	78.04±46.81
Fertile (n=24)	58.31±30.70
P value	0.001

DNA damage %	LPO (MDA nmol/ml)	ROS(µg Formazan /10 ⁷ cells)	ROS (RLU/10 ⁸ sperm)	Normal morph. %	Sluggish %	Non prog. %	Progressive %	Active %	Live %
21.79±5.74	131.45±119.61	14.64±5.28	2217±1595	50.95±14.48	26.10±12.70	25.68±14.65	17.97±8.06	43.66±16.58	69.76±13.84
8.12±3.53	44.13±13.07	11.65±3.64	12±9	61.04±11.79	17.06±7.84	13.56±11.92	43.88±14.96	57.44±11.11	74.50±10.01
0.01	0.01	0.039	0.001	0.063	0.015	0.003	0.001	0.001	0.002

Table 3: Mean of OS parameters of Fertile and NOZ Groups				
Parameters	Normozoospermia (n=27)	Fertile (n=24)	P value	
Count millions/ml	75.91±43.79	58.31±30.70	0.001	
Live %	78.40±10.06	74.50±10.01	0.002	
Active %	43.66±16.58	57.44±11.11	0.001	
Progressive %	39.39±6.09	43.88±14.96	0.001	
Non prog. %	17.91±8.91	13.56±11.92	0.003	
Sluggish %	21.11±5.09	17.06±7.84	0.015	
Normal morph. %	52.98±17.21	61.04±11.79	0.063	
ROS (RLU/10 ⁸ sperm)	2217±1595	12±9	0.001	

DNA damage n/2	LPO (MDA nmol/ml)	ROS(µg formazan/10 ⁸ cells)
20.07±5.3	113.82±21.9	14.64±5.28
8.12±3.53	44.13±13.07	11.65±3.64
0.01	0.01	0.039

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دور الاجهاد التاكسدي في نوعية السائل المنوي في الرجال العقيمين

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الخلاصة

إن لأصناف الاوكسجين الفعالة تأثيراً سلبياً على جميع المركبات الحيوية مثل الحامض النووي، الدهون، البروتينات والسكريات، وتعتبر سبباً لكثير من الامراض ومن ضمنها الامراض التناسلية. تضمن البحث 72 حالة مرضية ضمن مجاميع مختلفة وهي (قلة الحيامن ، ضعف حركة الحيامن والمجهولة السبب)، و 24 حالة طبيعية كمجموعة سيطرة، جمعت النماذج في الفترة من مارس-تشرين الثاني لسنة 2013 ، وتم اختيار المرضى وجمع النماذج وتحليلها تبعاً لطرق العمل المعتمدة من قبل منظمة الصحة العالمية لسنة 2010، بعد ذلك اجريت القياسات الكيموحيوية التالية للنماذج (اصناف للمني ، للحيامن المغسولة، تأكسد الدهون وتحطم الحامض النووي)، اظهرت النتائج ارتفاعاً معنوياً في مستويات وتأكسد الدهون وتحطم الحامض النووي للمجاميع المرضية المختلفة مقارنة بمجموعة السيطرة فيما لم تظهر فروقاً معنوية في مستويات للحيامن المغسولة. ان سبب انخفاض الحيامن في مجموعة قلبي الحيامن ربما يعود لعملية الابتناء الناتجة من زيادة، وضعف الحركة في المجموعة المرضية الثانية يعود لانخفاض عملية فسفرة البروتين في محور الذيل، ويسبب تأكسد الدهون في غشاء رأس الحيمن بفقدان قدرة الحيمن على اختراق البيضة.