



Reactive Oxygen Species Levels in Seminal Plasma in a Sample of Iraqi Infertile Men using Advanced Stimulatory Method for Activation of Spermatozoa

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ABSTRACT

Background: Seminal oxidative stress (OS) is known as one of the important factors of male infertility through pathogenesis of sperm dysfunction and DNA damage. OS results from variation in reactive oxygen species (ROS) production and ROS scavenging by seminal antioxidants. **Objective:** The aim of this study was to detect the ROS level by Enzyme-Linked Sorbent Assay (ELISA) in seminal plasma of infertile men before and after activation using the Density Gradient Centrifugation (DGC) and glutathione combined with DGC method. **Patients and Methods:** This study involved 60 males; the recruited individuals were divided into 3 groups, (20 asthenozoospermic, 20 oligozoospermic and 20 normozoospermic subjects) during the period of attendance to the infertility clinic at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies, Al-Nahrain University. The collected semen samples were obtained, and seminal fluid analysis was assessed. Semen samples were divided into 3 parts. The first part prepared was in vitro sperm characterization before activation, the second part using Density Gradient Centrifugation (DGC) technique, while the last part was prepared using DGC combined with glutathione. **Results:** Reactive oxygen species concentration showed a significant decrease in the 3 groups of normozoospermia, asthenozoospermia, and oligozoospermia when using the DGC technique with glutathione, compared to both before sperm activation and DGC technique. It is important to refer that for the oligozoospermia the level of ROS significantly decreased only between the DGC and DGC with glutathione. **Conclusion:** Reactive Oxygen Species (ROS) in semen plasma of infertile men was significantly decreased after activation using DGC with glutathione.

Keywords: Reactive oxygen species (ROS), Density gradient centrifugation (DGC), Glutathione, Infertile men

INTRODUCTION

Reactive oxygen species have a central role in normal sperm functions and low levels were involved with capacitation, acrosome reaction, stimulating hyper-activation and binding to zona pellucida of the oocyte [1]. Immature spermatozoa and semen leukocytes are the major sources of ROS generation in semen. Aerobic metabolism is associated with the generation of ROS. It appears to be elevated in spermatozoa as a result of cellular necrosis, morphological errors and the following cryopreservation, and because spermatozoa have a high content of polyunsaturated fatty acids, become highly susceptible to free radicals and ROS [2].

The mechanism of ROS-induced damage to spermatozoa include an oxidative attack on spermatozoa plasma membrane lipids, that leads to initiation of lipid peroxidation cascade, and as a consequence, the spermatozoa lose their capacity for movement, acrosome reaction, and sperm-oocyte fusion.

The 3 major reactive oxygen species were involved in spermatozoon damage (superoxide anion, hydrogen peroxide and hydroxyl free radical), the peroxide anion is thought to be responsible for most nuclear and membrane injury that occurs during cryopreservation process [3]. Sperm plasma membrane contains an abundance of polyunsaturated fatty acids, and these fatty acids regulate the fluidity and permeability of sperm membrane, so oxidation of these polyunsaturated fatty acids affect the fluidity of the sperm membrane, the membrane fusion events such as acrosome reaction, binding capacity, sperm-egg interaction and sperm motility [4].

One of the important markers of oxidative stress is malondialdehyde (MDA), which is an end product of lipid peroxidation. High levels of MDA represent high lipid peroxidation rate which may cause changes in sperm and diminish fertility [5].

Reactive oxygen species can have beneficial or detrimental effects on spermatozoa functions depending on the nature and concentration of ROS, as well as the location and length of exposure to it [1]. During epididymal transit, sperm acquires the ability to move progressively. However, they acquire the ability to fertilize, in the female tract through a series of physiological changes called "capacitation". Under physiological conditions, spermatozoa produce small amounts of ROS, which are needed for capacitation and acrosome reaction. Superoxide anion appears to play a role in this process [1].

Glutathione is one of the antioxidants added to different semen specimen, it has a serious role in the antioxidant role of endogenous, exogenous compounds [6]. Glutathione a naturally occurring tri-peptide in semen play a serious role in scavenging reactive O₂ intermediates and other radicals with the help of the glutathione reductase/peroxidase cycle [7]. Glutathione has different functions with very important roles in the physiological and metabolism of the cell including the protection from oxidative stress, production of protein and DNA and fertilization of gamete cell. Glutathione effect cell metabolism by detoxication and block production of free radicals in spermatozoa [8]. Assisted reproductive technologies (ART's) were advanced through the earlier decades to produce high-yielding numbers of embryos, and have revealed the need for suitable and effective techniques of sperm treatment in the laboratory [9]. The density gradient centrifugation technique can be modified to treat the issues of each individual specimen, and it is the method of choice for preparation of the sperm in the majority of ART's and andrology laboratories [10].

PATIENTS AND METHODS

Total 60 infertile males were involved in this study, individuals were divided into 3 groups, (20: asthenozoospermic, 20: oligozoospermic, and 20: normozoospermic subjects) during their attendance to the infertility clinic at High Institute for Infertility Diagnosis and Assisted Reproductive Technologies; Al- Nahrain University. The seminal fluid analysis was assessed, and each semen sample was divided into 3 parts. The first part was prepared for sperm characterization and assessment of ROS before activation, the second part using DGC technique and assessment of ROS after activation, while the last part was prepared using DGC combined with glutathione with an assessment of ROS.

Discontinuous Density Gradient Centrifugation (DGC) Technique

Density gradient centrifugation technique used for the separation of spermatozoa has been a golden technique in a lot of ART's laboratories because of its performance that is easily and quickly resulting in high quality of sperm motility [11].

This technique is carefully done by adding 1 mL of 80% of Sil-Select Plus gradient as a first layer solution in a test tube followed by 1 mL of 40% of Sil-Select Plus gradient as a second layer solution, then 1 mL liquefied semen sample was added on the second layer. This test tube was carefully put in centrifuge at 3000 rpm for 15 minutes. Then the supernatant was discarded and 1 mL of FertiCult Flushing medium was added to the pellet and put in air incubator for 30 minutes at 37°C. A drop of 10 µL was aspirated and put on a slide with a coverslip and was examined under the microscope at the 400X objective to assess the sperm parameters.

Discontinuous Density Gradient Centrifugation (DGC) Technique with TAD 600 mg Glutathione

The experiment was repeated as mentioned above with the addition of 0.5 mL (15.4 mg/mL) glutathione to the last step of the procedure with the FertiCult Flushing medium and placed in air incubator for 30-45 minutes at 37°C [12].

Enzyme-Linked Immunosorbent Assay for Reactive Oxygen Species Evaluation

All study semen plasma samples were carried out for the measurement of ROS (before and after activation with and without Glutathione), with the aid of a commercially available ELISA kit, YH Biosearch Laboratory, China. According to the manufacture leaflet, the procedure was performed.

RESULTS

Assessment of *In vitro* Sperm Activation-Reactive Oxygen Species (ROS) Concentration

Reactive oxygen species concentration showed a significant decrease at ($p < 0.05$) in the 3 groups of normozoospermia, asthenozoospermia and oligozoospermia when using the DGC technique with glutathione (7.09 ± 0.38 , 6.61 ± 0.46

and 7.91 ± 0.31 ng/mL respectively, compared to both before sperm activation (13.49 ± 1.49 , 9.76 ± 1.25 and 6.1 ± 0.54) and DGC technique (11.72 ± 0.35 , 10.87 ± 0.54 and 10.55 ± 0.31) ng/ml respectively, as shown in Table 1 and Figure 1. It is important to refer that for the oligozoospermia the level of ROS was significantly decreased only between the DGC and DGC with glutathione.

Table 1 Before-and after *in vitro* sperm activation comparison in reactive oxygen species between groups

Treatment	Mean \pm SE of ROS ng/ml			LSD value
	Normozoospermia	Asthenozoospermia	Oligozoospermia	
Before activation	13.49 ± 1.49	9.76 ± 1.25	$6.1 \pm 0.54^{**}$	4.835
DGC	$11.72 \pm 0.35^*$	$10.87 \pm 0.54^*$	$10.55 \pm 0.31^*$	0.011 (N.S)
DGC+TAD	$7.09 \pm 0.38^*$	$6.61 \pm 0.46^*$	$7.91 \pm 0.31^{**}$	0.012 (N.S)
LSD value	5.482*	2.568*	5.765	-

*Significant at $p < 0.05$; **Highly significant $p < 0.01$; N.S: Non-significant; SE: Standard error; DGC: Density gradient centrifugation

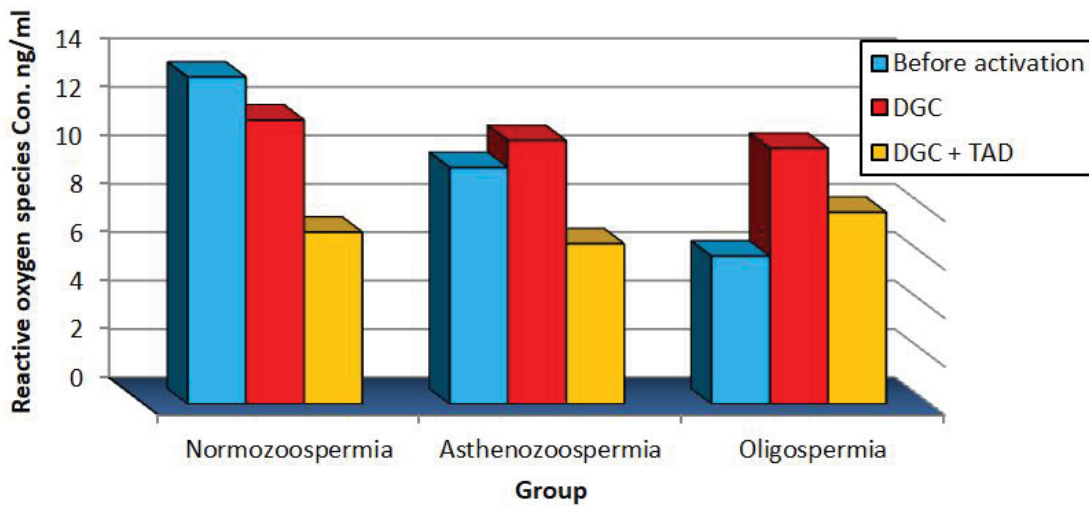


Figure 1 Differences before and after sperm activation on sperm ROS concentration

DISCUSSION

There is now important evidence to back up a link between OS and male infertility, ROS such as hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-) and superoxide anions (O_2^-) are produced by human sperm as a part of their normal metabolism [13]. The results of this study showed that ROS levels were decreased significantly when using DGC with glutathione compared to DGC alone, this supports the need for low ROS concentration for better sperm health and ability to fertilize the egg. At low levels, ROS increase sperm capacitation and hyperactivation, also directing the acrosome reaction and binding to the oocyte zona pellucida. ROS are preserved at low levels by effective anti-oxidant pathways, in the sperm cytoplasm and more significantly in the seminal plasma, where high levels of ROS scavengers were found. However, if the production of ROS is more than the capacity of these anti-oxidant pathways to stay appropriate at low levels, then oxidative stress occurs, which lead to pathological effects [10,14]. ROS initiates peroxidation of membrane lipids, proteins, and DNA, which leads to the formation of potentially genotoxic and mutagenic adducts, damaging membrane function, ion gradients, and receptor-mediated signal transduction as well as interfering with DNA methylation. This affects the fertilization process [15]. As well as causing DNA fragmentation and gene mutations [16]. Manifestations of oxidative stress include semen parameter impairment, particularly a reduction in motility and vitality. Retained cytoplasmic droplets on immature sperm are an origin of excess ROS production; leukocytes in semen are up to a thousand fold more effective at generating ROS [17].

The study has shown that during periods of examination stress, seminal plasma glutathione concentrations decline. There is also a corresponding reduction in sperm quality. During periods of stress, free radical activity increases. This may lead to a depletion of glutathione as it works to minimize the oxidative stress, leaving spermatozoa vulnerable to damage [18].

CONCLUSION

Reactive oxygen species (ROS) in semen plasma of infertile men were significantly decreased after activation using DGC with glutathione.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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