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Journal of Antioxidant Activity

ISSN: 2471-2140

DOI: 10.14302/issn.2471-2140.jaa-21-3910

Research Article | Freely Available Online

Antioxidative Protective Effect of Ozone Therapy on Isolated Mitochondria from Human Sperm

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Running title:

Ozone Therapy protected sperm cell

Keywords:

Ozone/oxygen therapy, mitochondrial dysfunction, oxidative stress, Human spermatozoa, motility, anti-oxidative protection.

Received: Jul 26, 2021

Accepted: Aug 10, 2021

Published: Aug 19, 2021

Editor: Abdelmonem Awad Mustafa Hegazy, Professor and Former Chairman of Anatomy and Embryology Department, Faculty of Medicine, Zagazig University, Egypt

Abstract

Oxidative stress is implicated in male infertility and significantly higher reactive oxygen Species (ROS) are detected in 25% of infertile males. We showed that Ozone oxygen therapy (O_2/O_3) induces protective effects for oxidative stress factors and its consequences on isolated mitochondria obtained sperm which may provide insight into the role of Ozone oxygen in human infertility. The present study was carried out to further characterize and compare protective effect of Ozone oxygen on isolated mitochondria obtained from sperm. Semen was collected from human normal donors. We gained human sperm mitochondria by differential centrifugation and isolated mitochondria incubated with different concentrations (5μg/ml, 30 μg/ml, 80 μg/ml) of O_2/O_3. O_2/O_3 prevent significant decrease in reactive oxygen species formation and mitochondrial membrane potential collapse on isolated Human sperm mitochondria. Ozone oxygen

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therapy induced increase in ATP concentration on isolated mitochondria. Our findings showed that O_2/O_3 prevent toxicity in sperm, effect on mitochondrial respiratory chain and avoid to cytochrome c release and apoptosis signaling.

Introduction

Spermatogenesis is an intricate cellular function involving the differentiation and proliferation of male germ cells, the role of spermatozoa is to fertilize eggs. The entire process takes 35 and 76 days in mice and humans , in that order, and needs a particular microenvironment produced by testicular somatic cells, Sertoli [28, 18] . Sperm cells contain a significant number of mitochondria relative to their cytoplasm, making them susceptible to oxidative relative damages. Oxidative stress damages the human sperm acrosome and causes major loss of sperm motility [3, 22] and enzymatic activity in mouse spermatozoa [8] Moreover, many risk factors can stimulate various reactive oxygen species (ROS) creation in human sperms maturation and may be detrimental because of the production of a burst of ROS [2]. For example, Varicocele is defined as a dilatation of the pampiniform plexus veins. It is the most common cause of male infertility affecting about 15%–20% of the general population and 35%–40% of men presenting for an infertility evaluation (replace with numbers). Oxidative stress and elevated sperm DNA fragmentation have been associated with varicocele mediated infertility [14] Ozone therapy acts by regulating oxidative stress mainly through stimulating the antioxidant system of the cell [9]. The cell structure of spermatozoa, the plasma membrane, a large number of mitochondria, low cytoplasm, and low antioxidant in sperm cytoplasm make them potentially susceptible to damage from free radicals [13] ROS which is produced on cold-shock and osmotic stress of this procedure affect sperm organelles. Exposure to high ROS concentrations can result in the disruption of mitochondrial and plasma membranes, cause chromosomal and DNA fragmentation and lead to a reduction in sperm motility [32]. It is recognized that human sperm generate ROS in physiologic amounts, which play a role in sperm functions during sperm capacitation, acrosome reaction, and oocyte fusion [1]. Notably, repeated rectal administration of ozone is able to induce an adaptation to oxidative stress and promote an oxidative preconditioning preventing hepatocellular damage mediated by free radical [9] . In addition to ROS evaluation, changes in MMP (mitochondrial membrane potential) could be a good display of a functional damage because sperm mitochondria side piece generate energy to support motility [13] However, the special effects of several stress factors (mechanical stresses and environmental conditions) on mitochondria obtained from humans sperm (reactive oxygen species and mitochondrial membrane potential) have not been studied. There is still a lack of information regarding the mechanism by which rat spermatozoa are affected by physical interventions. Motility is strongly related to the ability of spermatozoa to manage its energy status. Flagella of sperm movement is the product of dynein ATPase activity that is restricted into the axoneme along the entire length of the flagellum and depends on the supply of ATP [5] One of the reasons for sperm immobility may be impairment of the function of sperm mitochondria. In the past decade a growing number of tests have been developed to explore different functions of sperm cells [22]. Imaging and flow cytometer have been implied to increase the accuracy, selectively speed, power, and suitability of semen analysis in the clinic and basic research. Ozone therapy or more specifically, O3-AHT, has been used for almost 40 years. The first report on ozone ozone/oxygen therapy was published by Wolff in 1974 [17, 28] .Though ozone therapy is now used all over the world, it has not been accepted as orthodox medicine in all countries. ozone/oxygen therapy has been used in the treatment of ischemic disorders, although there are no data from clinical trials. .It is suggested that ozone could enhance antioxidant systems and regulate inflammatory response, improve vascular rheology, and increase blood flow in cerebral arteries and tissue oxygenation in hypoxic tissues [13] Several authors have clearly demonstrated the

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therapeutic efficacy of ozone/oxygen is related to controlled and moderate oxidative stress produced by the reactions of ozone/oxygen with biological components and moderate exercise could reverse this moderate side effect [12, 32] The adverse effects of oxidation can be reduced by antioxidants that are present as an element of seminal plasma. An equilibrium between ROS production and seminal antioxidants normally exists; however, special effects of endogenous antioxidants in poor semen samples are often diminished while the concentration of ROS is abnormally high [17] . Ozone/Oxygen therapy acts by regulating oxidative stress mainly through stimulating the antioxidant system of the cell [17] The aim of this study was to determine antioxidant effect of Ozone/ Oxygen therapy on isolated mitochondria from semen specimen.

Materials and Method

Materials

All chemicals and reagents were purchased from Sigma-Aldrich (Taufkrichen, Germany) in the best commercial grade.

Experimental Design

Fractions were classified into__three groups as follows: group 1: control group (without ozone=C), group 2: varicocele group (C.V) and varicocele group (incubation with 5µg/ml ozone/Oxygen; group 3: incubation with 30µg/ml ozone/Oxygen, group 4: incubation with 80µg/ml ozone/Oxygen) and choose 5µg/ml ozone/Oxygen.

Medozon

The Medozon serves for medical application (HAB company 2015, ozone generator, UMDNS-Nr.12899) was used for the generation of an oxygen/ozone mixture (Ozone/ Oxygen ratio: 99.95%/0.05%). The ozone / oxygen concentration can be adjusted from 5 to 80 µg/ml (ozone / oxygen concentration was regulated by spectrophotometry.

Semen collection

Semen samples were produced by masturbation into sterile containers from voricosperm, (N=5) human volunteers with proved varicocele. The mean age was between 20-25years and all of them were non-smoker. They were referred to the Hilal Ahmar infertility Clinic in Tehran. We give of ethics committee. The full description of the subject with participants, we collected semen. all of the participants accepted detail sentence. We give ethical code of Baqiyatallah University (Ir.bmsu.rec.1395.2) .

Semen Analysis

Sample of Semen were collected by masturbation after 3–5 days of moderation. All samples were allowed to liquefy at 37°C for 60 minutes and were then measured according to World Health Organization guidelines (WHO 2010) ([11] , [26] Following incubation for 1 hour at 37 ° C in air, 10 ml of sperm suspension was placed on a Makler chamber and sperm motility parameters were analyzed by Computer Assisted Sperm Analysis (CASA). The semen analyzer used was the Hamilton Thorne Research semen analyzer (IVOS, Version 10.8x. Hamilton Thorne, Beverly, USA). The resulting variables were taken into reflection: ejaculate volume (mL), sperm concentration (10^6/mL), total sperm number (10^6/ ejaculate), motility (%), and morphology (% abnormal forms). In addition to raw data on the percentage of motility, we also reflected absolute values in terms of motile sperm of millions per ejaculate (obtained by multiplying the total sperm per ejaculate by the percentage of sperm motility). Automated computer analysis of sperm motility (CASA System; Hamilton Thorne) was carried out on all Semen samples and included a heated stage at 37°C. The variables taken into consideration were curvilinear velocity (VCL m/s) , Beat frequency (BCF), Straightness= VSL/VAP×100) STR(,velocity of average nice if you have it path) VAP(, amplitude lateral head) ALH(,velocity of straight line)VSL [21] .

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Cellular Toxicity Assay

Cell Viability Assay

Cell viability was assessed by3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) staining as described by Mosmann [6] The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells .The dehydrogenases using NADH or NADPH as co enzyme can convert the yellow form of the MTT salt to in soluble, purple formazan crystals [33] Formazan solution is read spectrophotometrically after the crystals are dissolved DMSO). Spermatozoa (1×10^4 cells/well) were incubated in 96-wellplates in the presence or absence of O_2/O_3 for 48h in a final volume of 100 µl. At the end of the treatment, 20 µl of MTT (5mg/ ml in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple blue MTT formazan precipitate was dissolved in100 ml of DMSO and the absorbance was measured at 570nm using a ELISA reader (Tecan, Rainbow Thermo, Austria). Each concentration was tested in five different experiments run in five replicates for each sample. The concentrate of Ozone /oxygen (O_2/O_3) used were 5, 30 and 80 µg/ml and mitochondrial fractions were incubated in Tris buffer to we give optimum dose.

Mitochondrial Preparation

Sperm samples were directly used after two washes in PBS and centrifugation at 800g for 5min. Finally, mitochondrial pellets (0.5 mg/ml) were suspended in Tris buffer at room temperature for all other experiments. Except for mitochondria used to assess ROS production, in which MMP were suspended in respiration buffer, MMP assay buffer. Protein concentrations were determined through the Coomassie blue protein-binding method as explained by Bradford [10]. For in vitro experiments, Ozone oxygen (O_2/O_3) was dissolved in distilled water.

Dehydrogenase (Complex II) Activity

Mitochondrial succinate dehydrogenase (complex II) activity was measured by the reduction of MTT to formazan at 570 nm as described in previous studies [33]

Quantification of Mitochondrial ROS Level

The mitochondrial ROS measurement was performed by flow cytometry using DCFH-DA. Briefly, isolated sperm mitochondria were incubated with, Ozone/ oxygen (5µg/ml) in respiration buffer. In the interval times of 5 and 60 min following the Ozone oxygen addition, a sample was taken and DCFH-DA was added (final concentration, 10 µm) to mitochondria and was then incubated for 10 min. O_2/O_3 prevented ROS generation in isolated sperm mitochondria were determined through the flow cytometry (BD) equipped with a 488-nm argon ion laser and supplied with the softwaring 1.2.5 and the signals were obtained using a 530-nm band pass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 10,000 counts [7]

Determination of the MMP

Mitochondrial uptake of the cationic fluorescent dye, rhodamine 123, has been used for the estimation of mitochondrial membrane potential. The mitochondrial fractions (0.5 mg protein/ml) were incubated with various concentrations of O_2/O_3 and then 10 µm of rhodamine 123 was added to mitochondrial solution in MMP assay buffer. The fluorescence was monitored using determined through the flow cytometry (BD) equipped with a 488-nm argon ion laser and supplied with the softwaring 1.2.5 and the signals were obtained using a 530-nm band pass filter (FL-1 channel). Each determination is based on the mean fluorescence intensity of 10,000 counts[7], [34]

Assay of ATP

The ATP level was measured by luciferase enzyme as described by [30] Bioluminescence intensity was measured using Sirius tube luminometer (Berthold Detection System, Germany).

Statistical Analysis

Results are presented as means ± SD. All

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statistical analyses were performed using the SPSS software, version 17. Assays were performed in triplicate and the mean was used for the statistical analysis. Statistical significance was determined using the one-way ANOVA test, followed by the post-hoc Tukey test. Statistical significance was set at $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$.

Results

Determination of Semen Collection

Semen were collected by masturbation after 3–5 days of moderation. We showed for All Samples generally characterize: Sperm number($\times 10^6$/ejaculate) , Volume (ml), Sperm viability (%),Sperm concentration(M/ml),Normal sperm morphology(%).there are generally characterize on the standard semen collection.(Table.1) There are not significant differences between all of the treatment groups (varicocele) and health groups.

Determination of Sperm Motility and Kinetic Parameters

The post swim-up sperm motility parameters and kinetic parameters are shown in Table 2,3and 4. There are significant differences between the treatment group with varicocele groups +5µg/ml O_2/O_3 (5µg/ml) and the control group (C) and varicocele groups (C.V).

Cell Viability Assay

For determination of O_2/O_3 we used MTT assay. First, we measured the probable cytotoxicity of O2/O3on sperm. Our results on sperm showed that O_2/O_3 was cytotoxic at the 30 and 80 µg/mL concentration. The results with MTT assay showed that only at the lowest concentration (5 µg/ml) O_2/O_3 has nontoxic effect toward sperm. As shown, O_2/O_3 at concentrations of 30 ($p<0.01$) and 80 µg/mL, significantly ($p<0.001$) reduced cell viability. When mitochondria have normal activity in 5µg/ml concentration of O2/O3 [7] then we chose 5µg/ml concentration of for step 2. (Fig.1)

Effects of O2/O3 (5µg/ml) on Mitochondrial Succinate Dehydrogenase Activity

In varicocele groups +5µg/ml O_2/O_3 (5µg/ml) group, significantly decrease ($p<0.01$)succinate dehydrogenase (complex II) activity after 5 min but we show did not any reduce in enzyme activity compared with control group (C) after 60 min .varicocele groups (C.V) significantly decreased ($p<0.001$) enzyme activity compare with control group (C). (Fig. 2)

Effects of O2/O3 (5µg/mL) on Mitochondrial ROS Production

As shown in (Fig.3), in varicocele groups +5µg/ml O_2/O_3 (5µg/ml), the rate of ROS formation in isolated sperm mitochondria low level increase compared to control group (C) after 5 but we show did not any different ROS production between varicocele groups +5µg/ml O_2/O_3 (5µg/ml) and control group (C) after 60 min. we did not a difference in Ratio of DCF fluorescence (H1/H2)/all event) intensity between the varicocele groups +5µg/ml O_2/O_3 (5µg/ml) and the control group (C). (Table.5)

Effects of O2/O3 (5µg/ml) on Mitochondrial Membrane Potential (MMP)

As shown in (Fig.4), in varicocele groups +5µg/ml O2/O3 (5µg/ml), the rate of ROS formation in isolated sperm mitochondria low level increase compared to control group (C) after 5 but we show did not any different ROS production between varicocele groups +5µg/ml O_2/O_3 (5µg/ml) and control group (C) after 60 min. we did not a difference in Ratio of DCF fluorescence (H1/H2)/all event) intensity between the varicocele groups +5µg/ml O_2/O_3 (5µg/ml) and the control group (C). (Table.6)

Effects of O2/O3 (5µg/ml) on Mitochondrial ATP Level

Mitochondrial electron transfer chain is required for mitochondrial ATP production. In varicocele groups +5µg/ml O_2/O_3 (5µg/ml)group, significantly decrease

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Table 1. Semen collection and semen characteristic. Values represented as mean ± SD (n=5). **P > 0.01compared to control health group (C).

Parameters	C	T
Sperm number(×10^6/ejaculate)	22±1	25±2.1
Volume(ml)	±1.11	1.5±2
Sperm viability (%)	±1001	94.2±2
Sperm concentration(M/ml)	±47.110.4	42.16±21.12
Normal sperm morphology (%)	1.2±100	87±1**

Table 2. CASA result for the semen parameters and sperm vitality of the motility classes of diseases group (C. V) and 5 µg/ml of O2/O3 compared control health group(C). Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001

Groups	Fields	Quantity	Concentration (M/ml)	Class: A+B (PR)%	Class: C(NP) %	Class: D(IM) %	Class: A+B (PR)+C %
C	4±1	282±19.11	15±1.2	60±6.22	21±10.21	10±4.2	81±10.11
C.V	3.5±0.57*	134.5±10.34***	34.4175±30**	37.5±19.2**	18.25±8.8*	38±30.1**	54.25±28.1***
5 µg/ ml	4±1.1	288±19.16	24.085±19.5	62±6.44	25±12.21	18±6.66	80±11.23

Table 3. Comparison between mean percentages kinematic parameters in human sperm in diseases group (C.V) and 5 µg/ml of O2/O3 compared control health group (C). Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001.

Parameters	Class Name	VSL, µm/s	VCL, µm/s	VAP, µm/s	ALH, µm	STR, rel. units	BCF, Hz
C	A+B(PR)	92.47±2.1	111.95±2	104.67±1.1	1.68±1	88.34±1.3	3.44±1.4
C.V	D(IM)	31.22±1.1	129±2.2	55.1±2.45	1±0.5	55.66±1	1.1±0.7
5 µg/ml	A+B(PR)	90.21±0.1**	111.95±2*	100.00±2.1***	1.68±1*	90.21±1.3***	3.44±1.4**

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Table 4. Comparison between mean percentages motion parameters in human sperm in diseases group (C. V) and 5 μg/ml of O2/O3 compared control health group (C) Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001, compared to control group

Groups	C	C.V	5 μg/ml
Progressive (%)	39±8	11±3**	35±8.2*
Non-progressive (%)	35±8	17.1±3**	35±10
Immotile (%)	57.4±18	77.68±11***	20.12±26.60 **
Total motility (%)	39.5±14.5	20.18±2***	±**5529.2

Table 5. Ratio of DCF fluorescence (H1/H2)/all event) intensity, after incubation time. ROS formation in diseases group (C. V) and 5 μg/ml of O2/O3 compared control health (C). mitochondria. ROS formation after the addition of various concentrations of O2/O3 (5 μgr/ml) at intervals of 5 ,60 min after the addition. ROS formation was determined through flow cytometry using DCF-DA as described in Materials and methods. Values represented as mean ± SD (n=5).

ROS		
Time /Groups	5min	60min
C	0.83±2.3	0.83±2.3
C.V	14.16±1	18.10±1
5μg/ml	1.28±0.34	1.08±1.7

Table 6. Ratio of MMP fluorescence (H1/H2)/all event) intensity, after incubation time. MMP in diseases group (C.V) and 5 μg/ml of O2/O3 compared control health (C) mitochondria at intervals of 5 ,60 min after the addition, ROS formation was determined through flow cytometry using Rh123 as described in Materials and methods. Values represented as mean ± SD (n=5). **P > 0.01; *** P> 0.001.

MMP		
Time /Groups	5min	60min
C	1.1±1	1.1±1
C.V	35.11±1	35.11±1
5μg/ml	9.21±1***	11.1±1.2**

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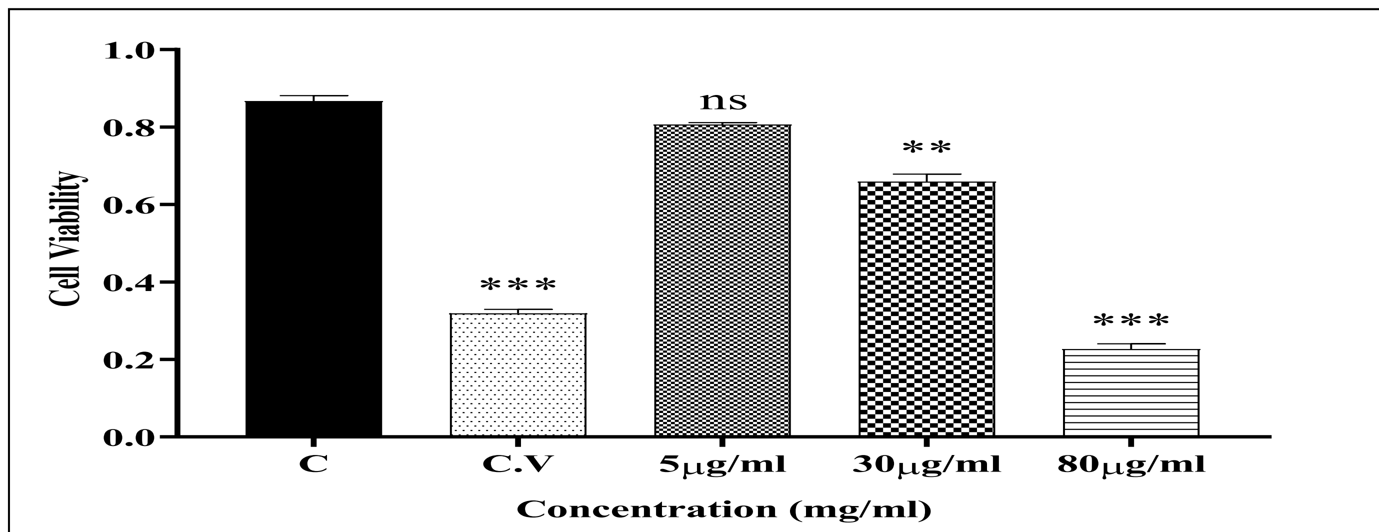

Figure 1. Effect O2/O3on cell viability. Succinate dehydrogenase activity was measured using MTT dye as described in Materials and methods. Isolated mitochondria (0.5 mg/ml) were incubated for 1h with various concentrations of O2/O3 (5,30, 80μg/ml). Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001, **** P> 0.0001compared to control mitochondria.

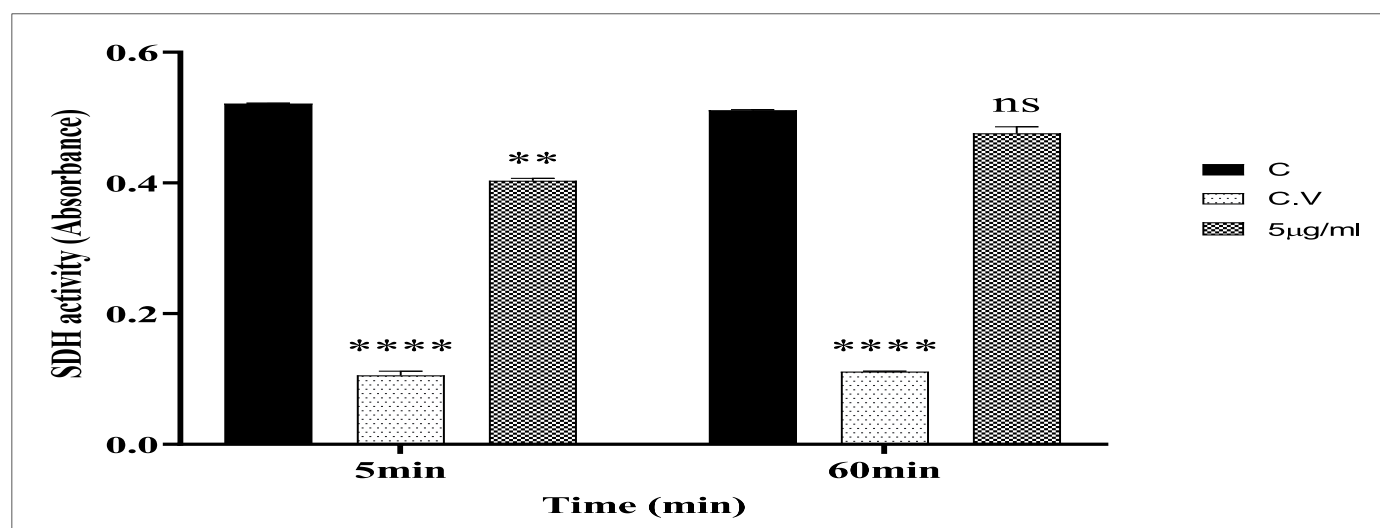

Figure 2. Effect O2/O3 on Succinate dehydrogenase activity. Succinate dehydrogenase activity was measured using MTT dye as described in Materials and methods. Isolated mitochondria (0.5 mg/ml) were incubated for 5, 60min with concentrations of O2/O3 (5μg/ml). Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001, **** P> 0.0001 compared to control mitochondria.

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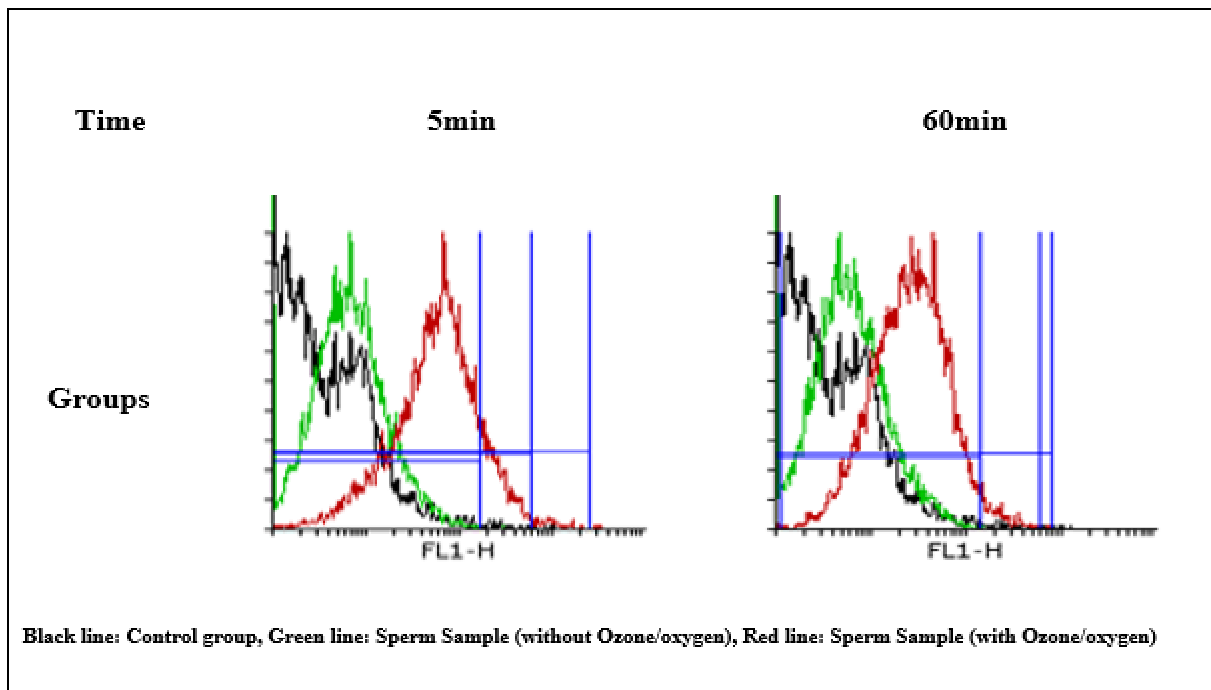

Figure 3A and B. ROS formation in O2/O3-treated mitochondria. ROS formation after the addition of various concentrations of O2/O3 (5 µg/ml) at intervals of A) 5 min after the addition, B) 60 min after the addition. C) ROS formation was determined through flow cytometry using DCF-DA as described in Materials and methods. FL1: the fluorescence intensity of DCF.

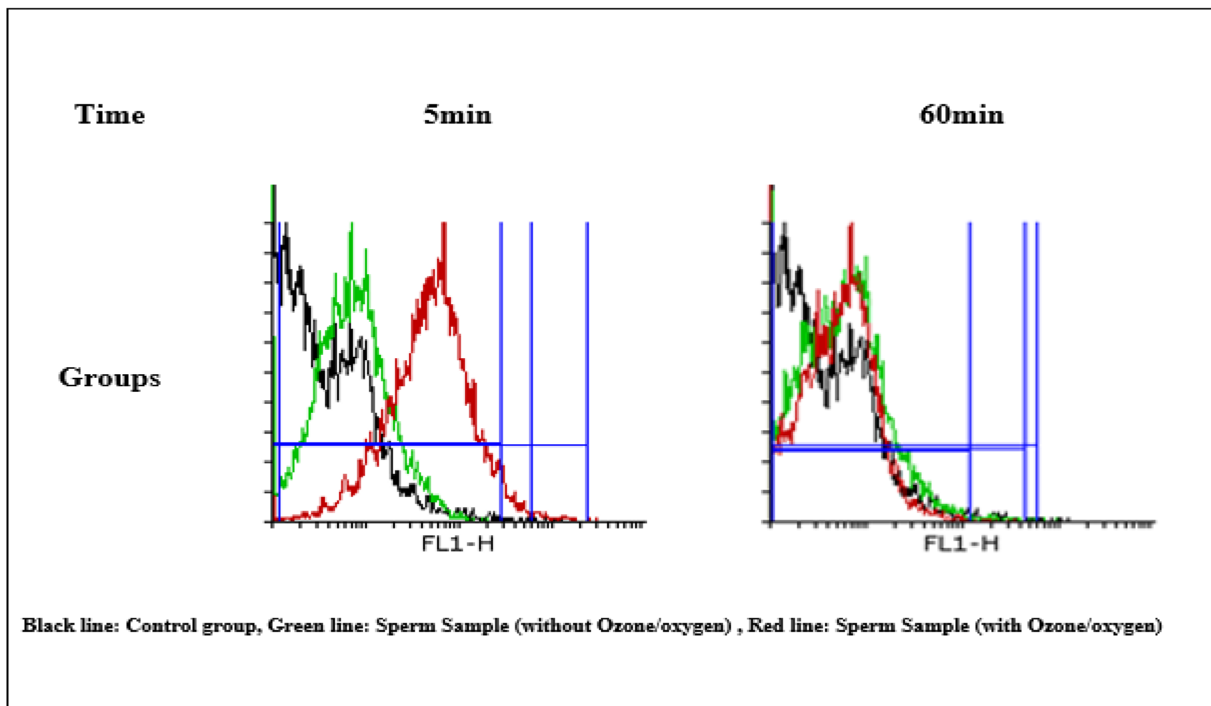

Figure 4. The effect of O2/O3 on the mitochondrial membrane potential (MMP) in sperm mitochondria. MMP was measured by rhodamine 123 as described in Materials and methods. A) The effect of O2/O3 (5µg/ml) on the mitochondrial membrane potential in Sperm mitochondria. Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001, **** P> 0.0001compared to control mitochondria MMP was determined through flow cytometry using Rh123 as described in Materials and methods. FL1: the fluorescence intensity of Rh123.

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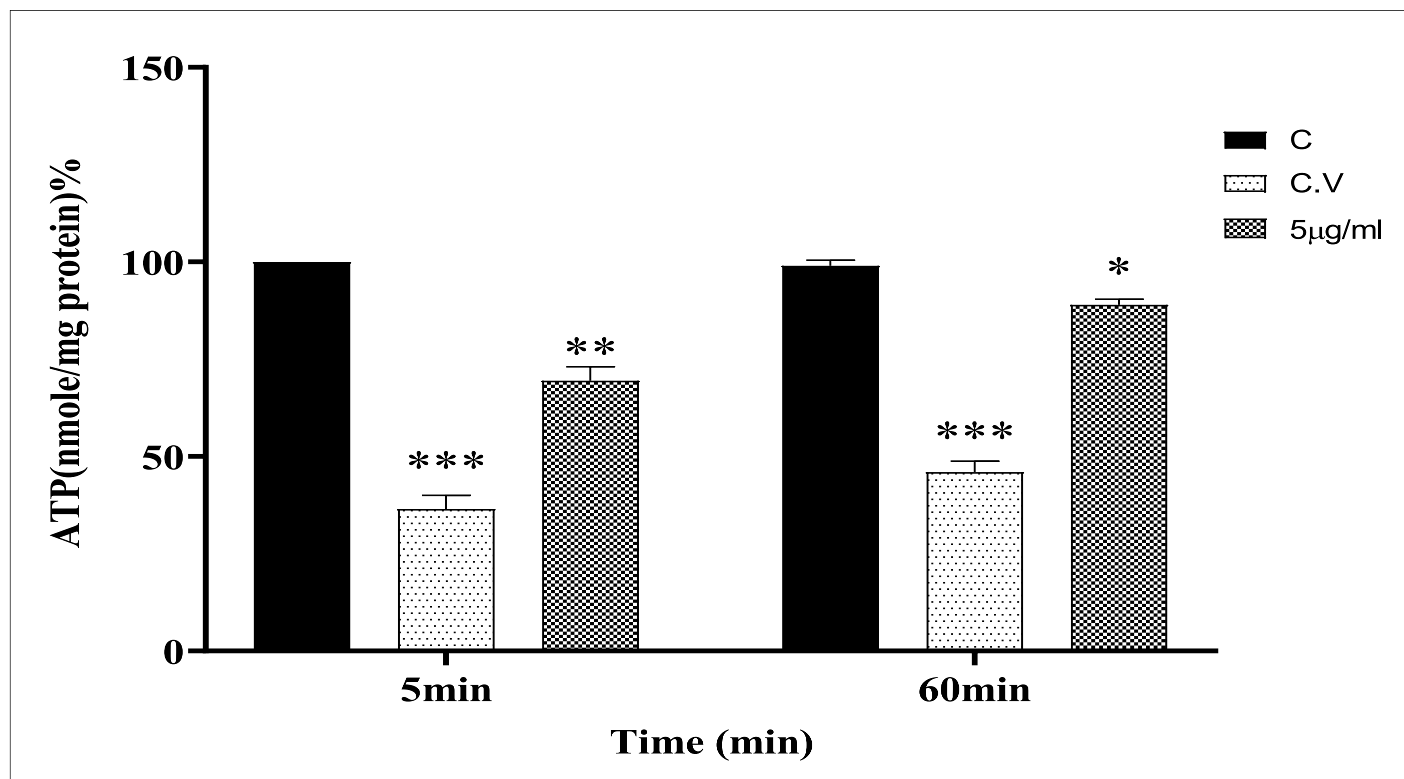

Figure 5. Effect of O2/O3 on mitochondrial ATP level. Sperm mitochondria (0.5 mg/ml) were incubated with of O2/O3 (5µg/ml) and A ATP level were determined using Luciferin/Luciferase Enzyme System as described in Materials and methods. Values represented as mean ± SD (n=5). *P > 0.05; **P > 0.01; *** P> 0.001, **** P> 0.0001compared to control mitochondria.

(p<0.01)ATP after 5 min but we show low level decreased in ATP compared with control group (C) after 60 min varicocele groups (C.V) significantly decreased (p<0.001) enzyme activity compare with control group (C). (Fig. 5). [30]

Discussion

Previous studies showed that low-level ROS play a critical role in normal sperm physiology, such as fertilizing ability (acrosome reaction, hyper activation, capacitation, and chemotaxis) and sperm motility, while increased ROS generation and/or decreased antioxidant capacity upsets the balance between oxidation and reduction in sperm physiology, a state dubbed sperm oxidative stress [14] This condition is widely considered to be a significant contributor to sperm DNA damage/apoptosis, lipid peroxidation, and reduced motility, which, in turn, increases the risk of male infertility/sub-fertility and birth defects. So far, numerous studies have focused on antioxidant therapy. Although a great many studies have experimentally and clinically documented that the therapeutic strategy could significantly improve sperm function and motility, the overall effectiveness remains controversial, principally due to non-standardized assay used to measure the level of ROS and sperm DNA damage, various antioxidant supplementation strategies, and inadequate data on fertilization and pregnancy after clinical treatment [15, 19, 21, 31] Therapeutic effect of ozone on Adriamycin (ADR) induced testicular toxicity in an experimental rat model showed that treatment with rut and/or ozone, however, improved the aforementioned parameters. Ozone therapy alone almost completely reversed the toxic effects of ADR and restored all parameters to normal levels[4, 29] .According to some epidemiological studies, environmental and genetical factors plays a role in the development of Infertility and it is a major health hazard. Environmental factors are components with toxicological properties. Infertility is an

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imperative clinical problem, affecting people psychosocially and medically. Oxidative stress has been recognized as one factor that affects fertility status. Researchers are trying to prevent the oxidative damage on human body but antioxidants. O_2/O_3 is one of the special antioxidant materials used in the clinic. O_2/O_3 therapy has been used in the treatment of ischemic disorders, although there are no data from clinical trials yet and it has not yet been accepted as orthodox medicine. Hoping to make it possible for the treatment of urological diseases such as O_2/O_3 therapy on testicular torsion as a urological emergency disorder in which one testicle gets twisted in the scrotum, subsequently cutting off its blood supply. An affected testicle tends toward ischemia and reproductive system dysfunction [8, 27] It is suggested that O_2/O_3 could enhance antioxidant systems and regulate inflammatory response, improve vascular rheology, and increase blood flow in cerebral arteries and tissue oxygenation in hypoxic tissues [13] Several authors have clearly demonstrated the therapeutic efficacy of O_2/O_3 is related to the reactions of O_2/O_3 with biological components [32, 12] Spermatogenesis is an intricate cellular function involving the differentiation and proliferation of male germ cells result in production of spermatozoa with capacity to fertilize egg. The importance of sperm motility during the progression of fertilization has established considerable attention over the past decades. Iguer-Ouada and Verstegen (2001) showed Computer Assisted Sperm Analysis (CASA) enables the observation of numerous factors of sperm motility and provides repeatable and accurate results with specific and standardized settings. Motility is one of the most important criteria in assessing sperm quality in normal and abnormal semen specimen. CASA is an automated method that can provide specific information on the kinetic of sperm cells with standard manual semen analysis of World Health Organization (WHO). "CASA has been developed to decrease the amount of time spend in sperm observation, reduce intra-observer differences, and improve the accuracy of final results.[25] This method is objective, accurate, and enables quantification of physical components of sperm movement. Researchers showed that ROS-induced oxidative damage could be minimized by a wide variety of antioxidants in mixture with basic common cry protectants in fish [1], bull [12], ram [26] goat [11], boar [23], canine [17] and human sperm cryopreservation[32] . Several researchers have reported the relationship of potential fertility in vitro and sperm motility parameters measured with CASA. [28] Objective analysis of sperm motility parameters resulted in significant correlations between the value of VCL m/s [18] and LIN % and the in vitro fertilization rates. a subsequent morphological change known as the acrosome reaction.[7] Mitochondria is stationary cellular energy producer which can be considered as the source of the ATP production and metabolic center. It is also reported that beyond interacting with other organelles, mitochondria interact with microtubules and is in direct contact with the ER (endoplasmic reticulum) (6) the physical association and dynamic cellular distribution of mitochondria in networks and with organelles are reflective of the dynamic functions of mitochondria. The thermal regulation, the ROS containment, the apoptotic regulation, the assists with extracellular and intracellular trafficking as well as cell movement, the central to inflammation, immunity, the stress response, and the cell hazard response. Energy is stored in the mitochondria as a proton concentration gradient and an electric potential gradient across the membrane. These different gradients are generated by electron transport maintained by the inner mitochondrial membrane and drive the synthesis of ATP. O_2/O_3 (5µg /ml) was intended to protect the mitochondrial inner membrane from lipid peroxidation instead of mitigation ROS production in general. Rhodamine 123 is a cationic color, have been used to show the mitochondria membrane potential. Any changes in mitochondrial membrane potential could be indirectly pointer of sperm motility. Mitochondria membrane potential of spermatozoa has been evaluated with Rhodamine 123 in a variety of species [16, 32] we showed, Rhodamine 123 was used and flow cytometry, (BD) fluorescence spectrophotometer singly to assess the

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sperm mitochondrial functional status. Our results show that there are not significant differences in mitochondrial membrane potential between the treatment groups and the control groups (Fig.4, Table 6). ROS generated from electron "leakage" of the mitochondrial respiratory chain, which can be evaluated by the production of DCF, the highly fluorescent oxidized derivative of DCFH-DA. The reliability of the DCF method is proved by using control, which is well known to induce a ROS increase in isolated mitochondria. Using ROS probe and flow cytometry, (BD) [24, 25] it is shown that untreated isolated mitochondria displayed substantial fluorescence, but exposing them to O_2/O_3 (5µg/ml) concentration caused a decrease in ROS formation (fig.3 and Table.5). After incubation time we also deliberately tested the effect of hydrogen peroxide on production of ROS and sperm viability, succinate dehydrogenase activity in sperm samples [7] Indeed, H_2O_2-induced ROS production and lipid peroxidation were inversely correlated with sperm viability. In the present study, we found that addition of mitochondrial-targeted antioxidant O_2/O_3 (5µg/ml) and mitochondrial reduced the production of ROS and ATP production increased significantly when treatment groups were compared with the control sperm samples. Previous studies suggested that the Krebs cycle and electron flow in the mitochondrial respiratory chain provide the proton motive force for the transformation of ADP to ATP in the F0F1 ATP synthesis complex [7, 20] Our results showed that O_2/O_3 (5µg/ml) prepared the electron transfer in complex (V) of the mitochondrial respiratory chain via leading to ATP production (Fig.5). We also observed that O_2/O_3 (5µg/ml) significantly increased ATP in the treated group (Fig.5). ATP acts as a molecular motor generating and force for sperm flagella, thus sperm motility is the result of a complex molecular process, comprising oxidation of energy substrate, phosphorylation of the proteins involved in signal transduction through the plasma membrane, and the conversion of chemical energy into mechanical energy in the axoneme. It is supposed that increased ROS formation and oxidation of membrane protein thiol groups caused mitochondrial permeability transition (MPT) pore opening as an event in mitochondrial dysfunction, followed by un-limited proton movement across the inner mitochondrial membrane and induction of mitochondrial swelling, MMP collapse and uncoupling of oxidative phosphorylation [7] . Also, we suggested that oxidation of thiol groups in the inner mitochondrial membrane could promote the MPTinduction and release of cytochrome c from mitochondria as an endpoint of cell death signaling [7] . However, O_2/O_3 at low concentration (5µg/ml), did not induce release of cytochrome c, and did not produce death signaling (apoptosis or necrosis). In addition, we will also explore whether these mitochondrial specific approaches could have enhanced benefits for individuals or species that are highly sensitive to oxidative damage. Future studies are needed to explore whether the improvement in sperm viability, as well as reduced ROS and lipid peroxidation could result in a better fertilization.

Funding

This research did not receive any specific grant from any funding agency in the public, commercial or not for profit sector.

Acknowledgements

The data provided in this article was extracted from the project of Parvaneh Naserzadeh. The project was conducted under supervision of Prof. Morteza Izadi at Ozone Complementary Research Center, Tehran, Iran.

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