Induced Mild Oxidative Stress by Ozone/Oxygen Therapy Enhances Therapy Antioxidant Capacities and Protects Testicular Ischemia/Reperfusion Injury in Rats

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Research article

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Abstract

Background Testicular ischemia-reperfusion injury is an urgent situation which needs a timely and precise diagnosis for prevention of testis damages.

Methods In here we investigated the preventive effect of combination ozone/oxygen therapy in testicular ischemia induced by reperfusion injury. For this purpose, animal was divided to four groups; control, torsion/detortion, torsion/detortion + ozone/oxygen (30 μg/ml) and only ozone/oxygen. Four hours after detorsion, in all groups orchiectomy was done on the animal at for measurement of oxidative stress and mitochondrial toxicity parameters. Also, we preformed analysis of testicular spermatogenesis after three months.

Results Our data indicated that testicular torsion-detorsion induced a remarkable decline in the enzymes such ad catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD), activities and also a significant raise in mitochondrial toxicity and decrease of spermatogenesis, malondialdehyde and GSSG levels were shown. The rats treated with ozone/oxygen therapy indicated a significant raise in GPx, SOD and CAT activities. Also, spermatogenesis, a remarkable decrease in malondialdehyde GSSG levels and mitochondrial toxicities were observed when compared with torsion-detorsion group.

Conclusion Obtained results for this research proved that mild ozone/oxygen therapy enhance antioxidant properties in the spermatogenic cells and protects testes from ischemia-reperfusion injury.

Background

In pediatric urology, testicular ischemia-reperfusion injury is an urgent situation which needs a timely and precise diagnosis for prevention of testis damages. This situation is also common event in young men population [1]. Testicular torsion interrupts the blood flow transfer to the testicular tissues and results in ischemia in testis. If it is not remedied during 4–6 hours, necrosis will happen. The only treatment is currently surgical detorsion that lead to blood reperfusion into testes. However, even in patients who have undergone surgery, the both testis often becomes dysfunctional, for all time [2]. Ischemia-reperfusion injury is the important concern of testicular torsion detorsion. It has been suggested that overproduction of free reactive oxygen species (ROS) is the main mechanism in the testicular injury. Previous studies have showed that overproduction of ROS after this condition leads to damage in spermatogenesis process. Injury generates ROS, apoptosis and anoxia, pro-inflammatory cytokines, possibly causing subsequent infertility [3]. Apoptosis plays main role during homeostasis and development [4]. Apoptosis are triggered by mechanisms such as ROS formation and inflammation, resulting to germ-cell death in ischemia reperfusion injury [5]. During testicular ischemia reperfusion injury, apoptosis-associated molecules increase such as FasL, Bcl-XL, Bax and cytochrome c that are related to cell death through apoptosis, which happens through activation of caspase 9 and 8 pathways [4]. An upregulation in the overexpression of both proinflammatory cytokines like interleukin−1β (IL−1β) and tumor necrosis factor (TNF-α) is seen in the murine testis after 0.5 h of ischemia reperfusion in the testis [6]. Additionally,

ischemia reperfusion injury is associated with mitochondria damages in testes. Previous studies demonstrated that extreme over production of superoxide in the testicular cells through mitochondria pathway, as an outcome of the disbalance between the mitochondrial respiratory function and reinstatement of oxygen supply is related with testicular damages [4, 7]. Free radical of oxygen as well as oxidizes lipids in the mitochondrial membranes and cell, which modifies membrane permeance and interrupts the totality of cells. During the last four decades ozone/oxygen therapy have been carried out in Europe without any problem [8]. It must be emphasized that the treatment with ozone/oxygen therapy is perfectly tolerated [8]. Previous studies indicated that ozone therapy is more helpful in ischemia. Ozone-oxygen therapy has a disinfectant property which motivates the protective mechanisms of cells and organs increasing the efficacy of endogenous oxygen free radicals' scavenging properties [9]. Ozone-oxygen treatment causes a decrease in nicotinamide adenine dinucleotide (NADH) and assist to cytochrome C oxidization. There is a stimulus of genesis of prostacyclin and enzymes which behave as cell protectors and free radical scavengers such as glutathione peroxidase, superoxide dismutase and catalase [10]. In here, we intend to assay the ameliorative effect of ozone-oxygen therapy on testicular ischemia-reperfusion damage in a rat after induction of testicular torsion-detorsion.

Methods

Animals

In this work, we used of animal and all protocols by Animal Experimentation Committee of Baquatollah University of Medical Sciences. The 40 male Sprague-Dawley rats with weighing 250–300 g and 8 weeks old were purchased from Institute Pasteur (Tehran, Iran). Animals were homed in cages with a 12 h light: 12 h dark cycle at 25 ± 3°C under normal environmental conditions and had free availability to tap water and pellet diet.

Animal Model

Animals anesthetization was performed by intra-peritoneal injection of 100 mg/kg ketamine hydrochloride combined with 20 mg/kg xylazine. Full experiments were done under aseptic environment. In the torsion/detorsion group, via a left-sided ilioinguinal cutting the left testicles were scooped. The left testicles were whirled 720° in an anticlockwise direction and retained in this position through fixation by 11–0 silk suture to the scrotum and was reversed to the scrotum, and the cutting point was sealed. Testicular ischemia reperfusion injury is caused testicular torsion-detorsion. In the treated group (control), the left testis was subjected with the same cutting mentioned above. The incised point was opened after 2 hours, and the testicles were rotated to the normal position. The testicles were still viable and was inserted into the scrotum again [2]. Orchiectomy operation in both testes was done on half of the animals after the recovery of torsion at 4 hours in each group. After one month, the animals were euthanized by cervical decapitation for collection of tissues.

Study Design

Animals were at random grouped into 4 groups, including control, torsion/detortion, torsion/detortion + ozone/oxygen (30 µg/ml) and only ozone/oxygen, with the same surgical method was done as in the torsion-detorsion group. Ozone-oxygen therapy (30 µg/ml) was syringed intravenously (IV) via the tail vein. The animal number in each group were 10.

Obtaining Ozone in Different Concentrations by Medozon

Medical ozone therapy was received to rats using the major autohemotherapy procedure. In this method, under sterile conditions, 99.5% oxygen and 0.5% of an ozone are mixed using an ozone generator (HAB company, Ozone generator, UMDNS-Nr.12899, Germany). The ozone concentration within the mixture = 30 µg/mL). This therapy was intravenously performed.

Histological Evaluation

The tissues of testis were gathered for histological examinations. The sample were put in 10% formalin and inserted in paraffin then slice into 4 microns sections, and dyed with hematoxylin and eosin (H&E). The tissues were blindly evaluated under light microscope by a pathologist. Using Johnsen score, spermatogenesis and testicular injury were graded. All tissue sections are consistently analyzed and each is imputed a score from 1 to 10 [11].

Testicular tissue preparation

All testicular tissue was separately located in a 1.5 mL centrifuge tube. Add 250 µL of RIPA buffer (20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 150 mM NaCl, 1% NP–40, 1 mM Na_2EDTA, 1 µg/ml leupeptin, 1 mM βglycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4 and 1% sodium deoxycholate) with protease inhibitors. Lysates were then centrifuged at 11000 ×g at 4◦C for 10 min. The supernatant was applied for the measurement activities of GPx, SOD, CAT, MDA, GSH and GSSG.

Measurement of Malondialdehyde

Evaluation of MDA in testicular tissue were measured by measuring the level of MDA produced during the disintegration of lipid hydro peroxides by monitoring the absorbance at 532 nm by Beckman DU–7 spectrophotometer [12].

Catalase activity

Catalase enzyme activity was measured by spectrophotometer method at controlled temperature. The disappearance of H_2O_2 in a reaction medium including 10 mM potassium phosphate buffer, pH 7.0, 0.1–0.3 mg protein/mL, 0.1% Triton X–100, and 20 mM H_2O_2 is the base of this procedure at 240 nm were detected. The specific activity is expressed as units/mg protein and one catalase unit is described as 1 µmol of hydrogen peroxide consumed per minute [13].

Superoxide dismutase activity

Capacity of pyrogallol to autoxidize is used for the evaluation of SOD activity in the samples. The inhibition of oxidation of pyrogallol happens in the attendance of SOD, the enzyme activity then indirectly measured at 420 nm, by a spectrophotometer. Using different concentrations of SOD, a standard calibration curve was drawn, in order to estimate the activity of SOD in the samples. The findings were presented as units of SOD/mg protein [13].

Glutathione peroxidase activity

GPx activity was assayed using substrate of tertbutyl hydroperoxide. Disappearance of NADPH was measured at 340 nm by a spectrophotometer. The medium included 0.15 U/mL glutathione reductase, 0.1 mM NADPH, 0.5 mM tert-butyl-hydroperoxide, 2 mM glutathione and 0.4 mM azide. The specific activity is expressed as units/mg protein and one GPx unit is described as one µmol of NADPH consumed per minute [13].

GSSG and GSH assay

GSSG and GSH were assayed pursuant to the spectrofluorometric method. Using different concentrations of GSSG and GSH, a standard calibration curve was drawn for each form of glutathione and samples were assayed by a fluorimeter set at 350 nm excitation and 420 nm emission wavelengths [14].

ADP/ATP ratio Assay

The alterations of the ADP and ATP contents have been measured to differentiate modes of viability and cell death in testicular tissue by luminometer using of ADP and ATP Assay kit (MAK135 sigma, USA). ADP and ATP was done pursuant to the producer's instructions [15].

Mitochondria isolation

The testicular tissue was homogenate and spun down for at 2,000 g at 4 °C 10 min to throw away the pellet. Then supernatant was removed and suspended in HEPES buffer and 0.75 M sucrose and centrifuged at 10,000 g for 30 min. The supernatant was throwed away and the mitochondria pellets were collected in HEPES buffer and centrifuged at 10,000 g again for 10 min. After this stage, supernatant was also throwed away and the final pellet containing mitochondria was suspended again in phosphate buffered saline (PBS). It was stored at - 80 °C until use [16].

Determination of Reactive Oxygen Species

Briefly obtained mitochondria was suspended in respiratory buffer (0.1 mM KH_2PO_4, 10 mM Tris, 20 mM Mops, 0.5 mM $MgCl_2$, 50 µM EGTA, 5 mM sodium succinate and 0.32 mM sucrose). For determination of ROS formation, dichlorofluorescein diacetate (DCFH-DA, 1.6 µM) was added to the isolated mitochondria which this probe reacts with ROS and produces the highly fluorescent dichlorofluorescein (DCF). Fluorescence intensity was determined by flow cytometry. The results were reported as fluorescent intensity per 100 µg/protein of mitochondria [12].

Mitochondrial Membrane Potential Assay

Rhodamine 123 as a fluorescent cationic dye has been utilized for the measurement of mitochondrial membrane potential collapse. The mitochondria pellet (0.5 mg protein/mL) were suspended mitochondrial membrane potential assay buffer (10 mM HEPES, 10 mM KCl, 68 mM D-mannitol, 2 mM $MgCl_2$, 5 mM KH_2PO_4, 50 µM EGTA, 220 mM sucrose, 5 mM sodium succinate, 2 µM Rotenone and 10 µM of rhodamine 123). With using of flow cytometry, the fluorescence intensity of rhodamine 123 was assayed [12].

Mitochondrial Swelling Assay

Mitochondria were incubated in 96-well plates (100 µg protein per well) at 25 ºC in swelling buffer (0.5 mmol/L EGTA, 10 mmol/L NaCl, 140 mmol/L KCl, 0.5 mmol/L KH_2PO_4, 20 mmol/L HEPES, 2 mmol/L $MgCl_2$; KOH used for adjusting pH to 7.2) supplemented with 10 mmol/L succinate and1 mg/mL rotenone at 540 nm. Mitochondrial swelling was assayed spectrophotometrically in duration 60 minutes [14].

Cytochrome C Release Assay

Using the Quantikine Rat/Mouse Cytochrome C Immunoassay kit (Minneapolis, Minn) the concentration of cytochrome C was measured. Cytochrome C amounts was done pursuant to the producer’s

instructions [12].

Sperm isolation

At the end of the term, the animals were sacrificed and sperm were isolated from the left epididymis to analysis the kinematic parameters, vitality and characterization (Adamkovicova et al., 2016).

Sperm analysis

To evaluate sperm analysis a computer aided semen analysis SpermVision™ CASA System (Hamilton Thorne, USA).) with Olympus BX 51 phase contrast microscope (Olympus, Tokyo, Japan) was utilized. The samples of sperm were diluted with 10 µl of physiological solution and transferred into a Makler Counting Chamber and immediately analyzed. In each measurement, 1000 sperms were analyzed for each sample. Semen characterization, kinematic parameters and vitality were analyzed [17].

Statistical analysis

Findings are expressed as mean ± SED. Each experiment was done in triple, and the mean was utilized for statistical analysis. Statistical significance was defined using the one-way ANOVA tests, followed by the post hoc Tukey. Statistical significance was set at $P < 0.05$.

Results

Histopathological Evaluation

The results of the histopathological examination for each group are displayed in Figure 1. The presence of uniform seminiferous tubular morphology and was normal testicular structure seen in thesham and ozone/oxygen groups. In torsion/detorsion group, there was a severe distortion of tubules and a significant decrease in the diameter of the seminiferous tubular. Administration of ozone/oxygen protected the seminiferous tubular from damage after torsion/detorsion.

Malondialdehyde Assay

Based on the results of Figure 2 A, MDA level of testicular tissue in torsion/detorsion group has significant increase comparing to control group ($p<0.001$). MDA level of testicular tissue has a significant decrease ozone/oxygen-torsion/detorsion group comparing to torsion/detorsion group.

GHS and GSSG of Testicular Tissue

GHS level of testicular tissue has a significant decrease in 4 hours torsion/detorsion group comparing to control group ($p<0.001$). Also, GSSG level of testicular tissue has a significant increase in torsion/detorsion group comparing to control group. These effects inhibited by oxygen/ozone therapy for both measured parameters (Figure 2 B and C).

ATP Level

As shown in Figure 2 D, ischemia reperfusion injury in torsion/detorsion group significantly decreased ATP content in testicular tissue. Animals treated with oxygen/ozone and torsion/detorsion showed a significant increase in the ATP content in comparison with torsion/detorsion group.

Antioxidant Enzymes

The antioxidant enzymes activities of GPx, SOD and CAT were measured in 4 groups and the results were presented in Table 1. The ischemia and ozone/oxygen therapy increased the activity of GPx, SOD and CAT in compared to control group. Interestingly the activity of CAT, SOD and GPx in ischemia + ozone/oxygen increased in compared to torsion/detorsion group.

Mitochondrial ROS Level

Figure 3 A showed that the rate of ROS formation in testicular mitochondria in torsion/detorsion group significantly raised compared to untreated control group. Treatment of animals with ozone/oxygen showed reduction in ROS formation in isolated mitochondria compared to torsion/detorsion group.

Mitochondrial Membrane Potential Collapse

The redistribution of the rhodamine 123 into the cytosol has been utilized for assessment of the MMP collapse as the known indicator of mitochondrial damage which subsequent leads to mitochondrial membrane permeability transition (MPT). As shown in Figure 3 B, there was a remarkable increase in the rhodamine 123 redistributions in torsion/detorsion group compared to control group. Moreover, it was seen that MMP collapse significantly were reduced in mitochondria in treated with torsion/detorsion + ozone/oxygen in testicular mitochondria.

Cytochrome c Release

We examined cytochrome c release in the isolated mitochondria obtained from testes in each group. Cytochrome c release was significantly raised in the isolated mitochondria in torsion/detorsion group in compared to control group. Moreover, cytochrome c release was inhibited with oxygen/ozone therapy (Figure 4 A).

Mitochondrial Swelling

Monitoring of decrease of absorbance at 540 nm of mitochondria suspension was considered as mitochondrial swelling, which is another index of mitochondrial membrane permeability transition (MPT). As shown in Figure 4 B, our results show that ischemia reperfusion injury led to mitochondrial swelling in isolated mitochondria obtained from testes that this effect significantly attenuated by ozone/oxygen therapy.

Determination of Semen Collection

We isolated the sperm from the left epididymis and analyzed. We showed that semen characteristic parameters such as sperm number (×106/ejaculate), volume (ml), sperm concentration(M/ml) showed no changes in all groups but sperm viability (%) and normal sperm morphology (%) parameters significantly decreased in compared to control and torsion/detorsion+ozone/oxygen group. The results are presented in Table 2.

Determination of Sperm Motility and Kinetic Parameters

Sperm motility and kinetic parameter results are shown in Table 3. The results indicated remarkable changes in sperm motility and kinetic parameter in torsion/detorsion group compared to control and torsion/detorsion+ozone/oxygen group. There was a significant ($P < 0.001$) decrease in Linearity (LIN) = VSL/VCL × 100, Velocity of Straight Line (VSL), Velocity of Curved Line (VCL), Velocity of Average Path (VAP) and Straightness (STR) = VSL/VAP x 100. Compared with the control and torsion/detorsion+ozone/oxygen group, there was no significant statistical difference in Beat Frequency (BCF) and Lateral Amplitude (ALH) in torsion/detorsion group.

Discussion

Testicular injury estimates about 13–54% of cases of acute pediatric scrotal disease, and happens each year 1 in 4,000 males aged under 25 years and 1 in 160 males over 25 years [18]. If detorsion of the spermatic cord is carried out under 6 h, the testicular reclamation rate will be 90%. Although, this reclamation rate declines to 50%, after 12 h, and to <10% after 24 h [19]. Changed hormone formation,

infertility are outcomes of testicular torsion, which interrupt the blood supply to the testis, and of further torsion, which results in a blast of oxygen free radicals that afford extra damages in the testicular tissues [4]. Ischemia-reperfusion injury involves formation of ROS that can repress the protective antioxidant capacity. Upon reperfusion, additional formation of $O_2^{\bullet}$ in the testicular tissues through mitochondria, as an outcome of the disbalance among the mitochondrial respiratory function and restoration of oxygen supply, leading to mitochondrial membrane permeability, peroxidase lipids, and more production of ROS disrupts the integrity of mitochondria [4]. A published paper have proved, that reperfusion enhance expression of inducible NOS (iNOS) gene, which enhances nitric oxide (NO) levels [20]. Notwithstanding its main effect in fundamental cellular activities, NO can be adverse at great concentration; it can react with $O_2^{\bullet}$ to generate highly reactive peroxy-nitrite, which leads to destruction of RNA, DNA, proteins and lipids and result in the discharge of endoplasmic reticulum calcium storehouses [21]. The main controller of transcriptional responses at hypoxia conditions are hypoxia-inducible factors (HIFs). HIF–1α is a known transcription factor that controls hypoxia-sensitive genes involved in metabolic apoptotic processes [22]. Studies indicated that HIF–1α is made in the testis by Leydig cells, and probably be effective in the antiapoptotic defence [23]. Our findings in this study showed that torsion/detorsion of testes induced oxidative stress with raising of malondialdehyde as a final indictor of lipid peroxidation created by free radical of oxygen and the oxidative stress indicator, which was associated with decreasing of enzymatic and non-enzymatic antioxidants (Figure 2). These findings demonstrated that oxidative stress has a major role in ischemia reperfusion injuries after torsion/detorsion that is in accordance with the abovementioned studies. Efficient remedy could safeguard against the serious outcomes of ischemia reperfusion after testicular torsion. Several therapeutic chemicals have been experimented in animal investigations for their potential as auxiliary treatments of testicular torsion after surgical repair. These agents usually have ROS-scavenging or antioxidant and anti-inflammatory properties [4].These agents and interventions such as vitamins, selenium, hormones, phosphodiesterase inhibitors, plant extracts, anesthetics, NSAIDs and hyperbaric oxygen have been searched experimentally in animal studies for testicular torsion, with prosperous outcomes. However, there is no clinical trial to investigate the advantage of these agents and effectiveness has not been carried out in humans [4]. Previous studies proposed that hyper-baric oxygen can decrease damages to testes probably through diminishing oxidative damages, suppressing inflammation and reducing NO generation. Previous studies have demonstrated the therapeutic role ozone therapy. The therapeutic potential of ozone therapy likely is due to induction of the mild oxidative stress produced by ozone [8]. The border between toxicity and effectiveness of ozone is associate to the intensity of the oxidative stress. Serious oxidative stress triggers nuclear transcriptional factor kappa B (NF B) activation, leading to an inflammatory response and tissue damage through the production of PGE2, COX2, and cytokines. However, nuclear factor-erythroid 2-related factor 2 (Nrf2) is activated by mild oxidative stress [8]. Nrf2 then stimulate the transcription of antioxidant response genes (ARE). Transcription of these genes results in the overexpression of antioxidant enzymes [8]. Both anti-oxidative enzymes and free antioxidants safeguards cells from inflammation and oxidative damages and also, they are capable to invert the oxidative stress [8]. The present study showed involvement of mitochondria in the protection exerted by mild ozone/oxygen therapy in testis ischemia.

Conclusions

Our results here showed that elevated enzymatic and non-enzymatic antioxidants capacity of the testicular tissue and protected them from damages induced by ischemia reperfusion injury after torsion/detorsion of testes.

Abbreviations

ROS: Reactive Oxygen Species; DCFH-DA: Dichlorofluorescein Diacetate; DCF: Dichlorofluorescein; MDA: Malondialdehyde; MMP: Mitochondrial Membrane Potential; LIN: Decrease in Linearity; VSL: Velocity of Straight Line; VCL: Velocity of Curved Line; VAP: Velocity of Average Path; STR: Straightness; SOD: Superoxide Dismutase; GPx: Glutathione Peroxidase; CAT Catalase; H&E: Hematoxylin and Eosin; NF□ B: Nuclear Transcriptional Factor Kappa B; ARE: Antioxidant Response Genes; HIFs: Hypoxia-Inducible Factors; NO: Nitric Oxide; BCF: Beat Frequency; ALH: Lateral Amplitude; GHS: Glutathione; GSSG: Oxidized Glutathione

Declarations

Ethics approval and consent to participate

All volunteers signed an informed consent form and this study was authorized by Animal Experimentation Committee of Baquatollah University of Medical Sciences.

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Availability of data and materials

All data and materials related to the study can be obtained in Materials and Methods Section.

Authors’ contributions

‘PN’ participated in collected the data; ‘ZJ’ and ‘HC’ participated in data support; ‘MI’ designed and supervised the study; ‘AS’ participated in designing of the study, analysis of the data and drafting of the manuscript. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

All authors report no competing interest relevant to this study. We declare that Dr. Ahmad Salimi is a member of Editorial board in BMC Pharmacology and Toxicology.

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Tables

Table 1. Effect of torsion/detorsion and ozone/oxygen therapy on catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities in testes after reperfusion ischemia.

Enzyme activities (units/mg protein)	Control	TD	TD+ O2/O3	O2/O3
CAT	16.1 ± 0.3	21.31 ± 1.1[a]	26.21 ± 1.21[a, b]	24.1 ± 1.2[a]
SOD	8.6 ± 0.1	10 ± 0.3[a]	15.2 ± 0.3[a, b]	13.2 ± 0.9[a]
GPx	19.4 ± 0.4	23.4 ± 0.4[a]	27 ± 0.6[a, b]	26.3 ± 0.6[a]

a Enzyme activity of CAT, SOD and GPx significantly (P 0< 0.05) increased compared with control group.

b Enzyme activities of CAT, SOD and GPx significantly (P 0< 0.05) increased compared with TD group.

Table 2. Analysis of semen collection

Group	Control	TD	TD+ O2/O3	O2/O3
Parameters	Mean + SD	Mean + SD	Mean + SD	Mean + SD
Volume (ml)	1.1 ± 0.2	1.2 ± 0.3	1.3 ±0.1	1 ±0.1
Sperm viability (%)	100 ±0.0	48 ±2.4[a]	100 ± 0.0[b]	98 ± 2.1
Sperm morphology (%)	100 ± 0.0	76 ±3[a]	95± 4.2[b]	98 ± 1.9
Sperm concentration(M/ml)	40.1± 1.2	35.66 ± 1.4[a]	40.33± 0.9[b]	41.12 ± .8

a Parameters significantly (P 0< 0.05) increased compared with control group.

b Parameters significantly (P 0< 0.05) increased compared with TD group.

Table 3. Analysis of sperm motility and kinetic parameters

Group Parameters	Control Mean + SD	TD Mean + SD	TD+ O2/O3 Mean + SD	O2/O3 Mean + SD
LIN	85.47 ± 3.3	50.91 ± 4.1	85 ± 2.4	82± 1.1
VSL	98 ±1.2	50 ± 3.2[a]	78±3.1[b]	97 ± 2.3
VCL	117 ±1.3	95 ± 2.1[a]	121 ± 2.2[b]	115 ± 2.0
VAP	110 ± 1.4	45.4 ± 1.7[a]	96.3 ± 2.4[b]	112. 2 ± 1.3
ALH	1.3 ± 0.4	1.5 ±0.3	1 ±0.0	1 ± 0.0
STR	0.95 ± 0.1	0.55 ± 0.1[a]	0.84 ± 0.2[b]	0.85 ± 0.1
BCF	4.11 ±0.7	4 ± 0.3	4 ± 0.0	4 ± 0.0

a Parameters significantly (P 0< 0.05) increased compared with control group.

b Parameters significantly (P 0< 0.05) increased compared with TD group.

Figures

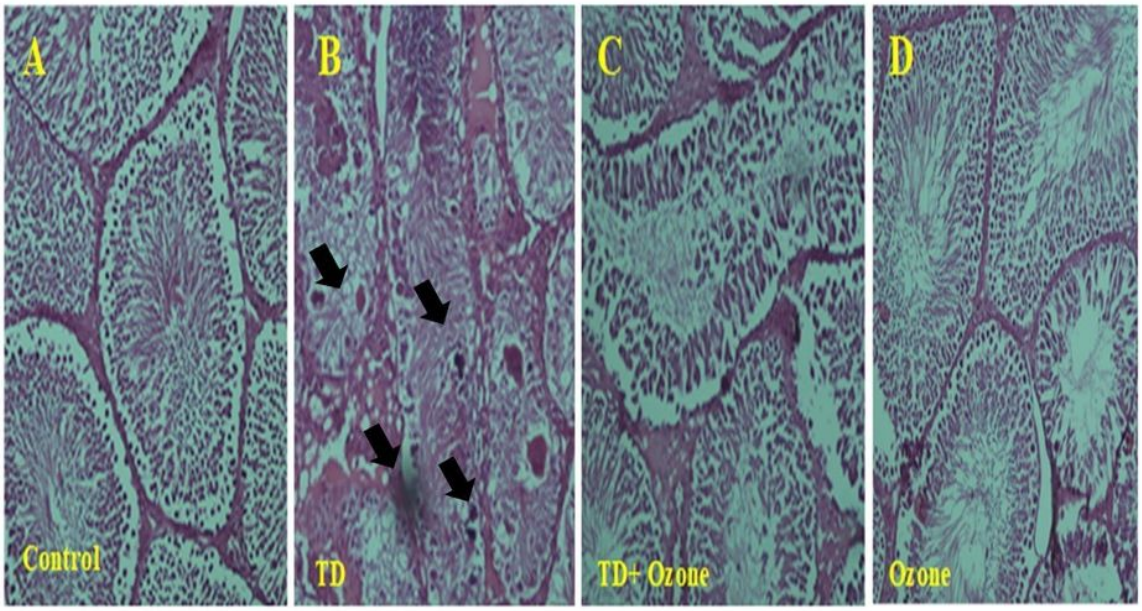

Figure 1

Light microscope observations of H&E stained sections (×100). (A) Sham group: normal testicular architecture was seen. (B) Torsion /detorsion group, (C) torsion/detorsion (T/D) + ozone/oxygen therapy group and (D) ozone/oxygen group: severe damage to testis was noted in Torsion /detorsion group which decreased in torsion/detorsion (T/D) + ozone/oxygen therapy group

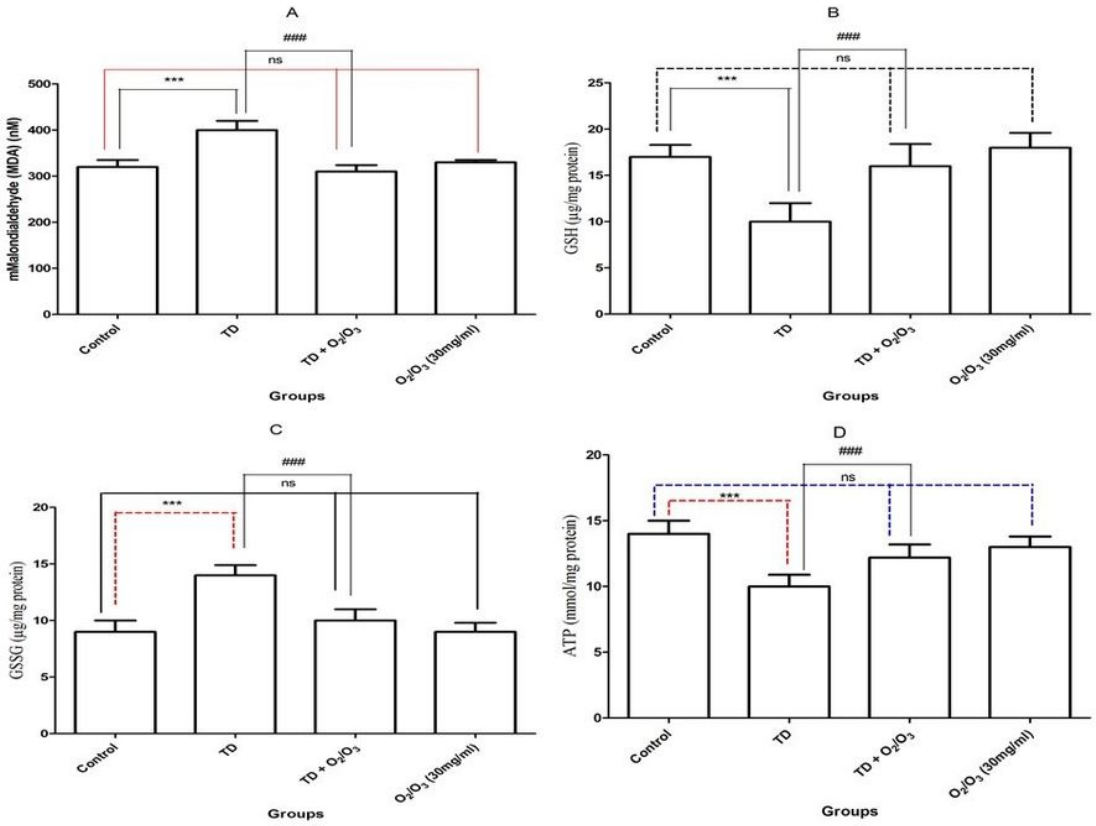

Figure 2

Effect of Torsion /detorsion and ozone/oxygen on MDA, GSH, GSSG and ATP in rat testes (A, B, C and D). MDA, GSH, GSSG and ATP were measured in testicular tissues after treatment with Torsion /detorsion and ozone/oxygen. Graphs A shows that lipid peroxidation significantly increased in TD group in compared to control group while this effect inhibited in TD + ozone/oxygen group. Graph B shows that content of GSH significantly ($p < 0.001$) decreased in TD group in compared to control group. The content of GSH not show any significant changes in TD + ozone/oxygen and ozone/oxygen treated groups in compared to control group. Also, C graph indicates that content of GSSG significantly increased in TD group in compared to control group. While there are not any significate changes in the content of GSSG in TD + ozone/oxygen and ozone/oxygen treated groups in compared to control group. Similarly, ATP content decrease significantly in TD group in compared to control group while this effect reflected in TD + ozone/oxygen group. Ozone/oxygen alone did not show a significant effect. Ns indicates not significant difference with control group. *** indicates significant difference with control group. ### indicates significant difference with TD group. Sample size= 5

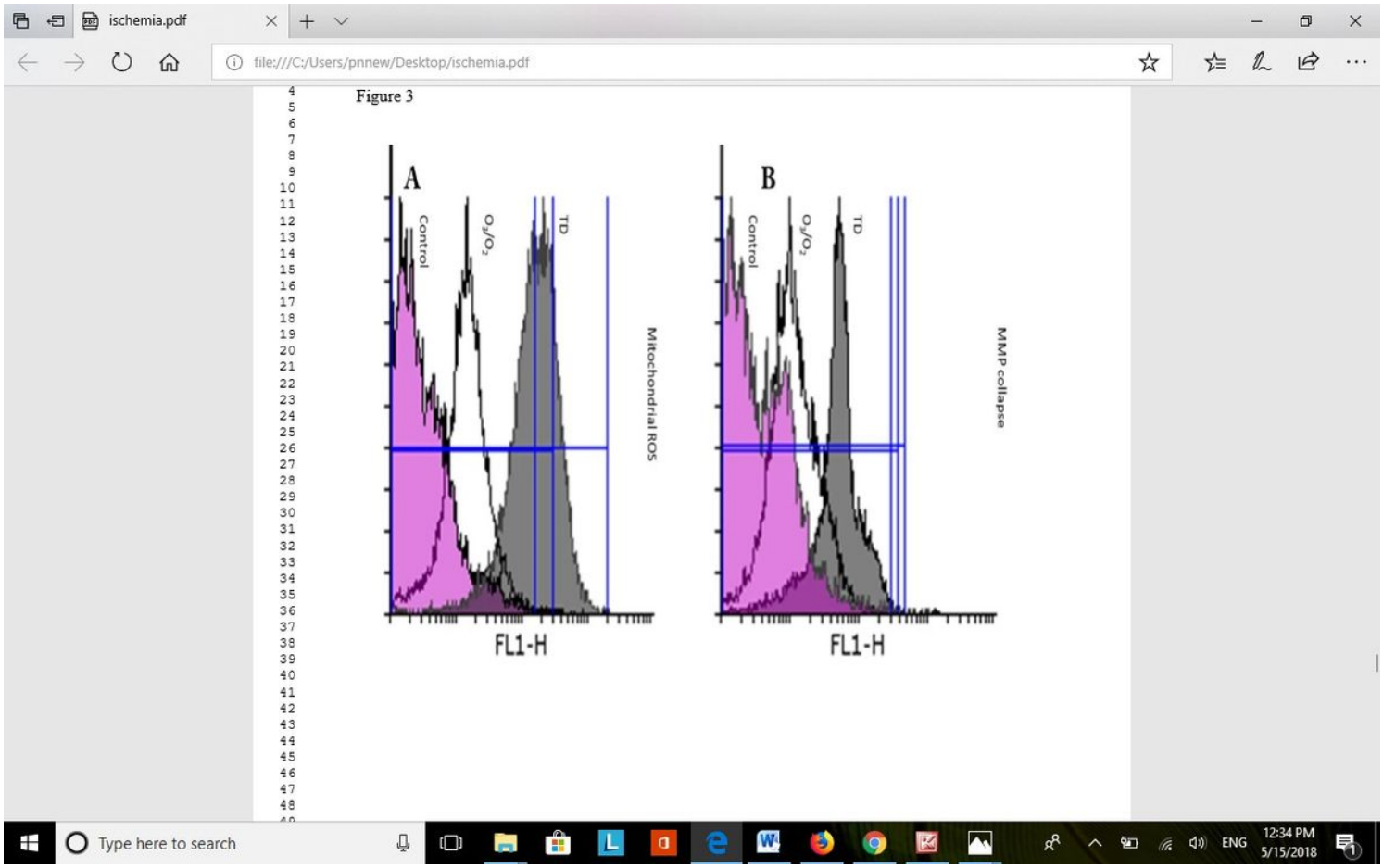

Figure 3

Flowcytometric analysis of ROS formation (A) and MMP collapse (B) in testes isolated mitochondria. Analysis were measured using BD flow cytometry. Fluoresce intensity mean of DA-DCF significantly increased in TD group in compared to control group that led to transporting pick from left to right of the histogram. Fluoresce intensity mean of DA-DCF. significantly decreased in TD + ozone/oxygen group treated group in compared to TD group that led to transporting pick from right to left of the histogram. Mitochondrial damage led to redistribution of rhodamine 123 from mitochondria to suspension buffer and increased fluoresce intensity mean of rhodamine 123 in TD group in compared to control group that led to transporting pick from left to right of the histogram. fluoresce intensity mean of rhodamine 123 significantly inhibited by ozone/oxygen in testes isolated mitochondria (B). Ns indicates not significant difference with control group. *** indicates significant difference with control group. ### indicates significant difference with TD group. Sample size= 5

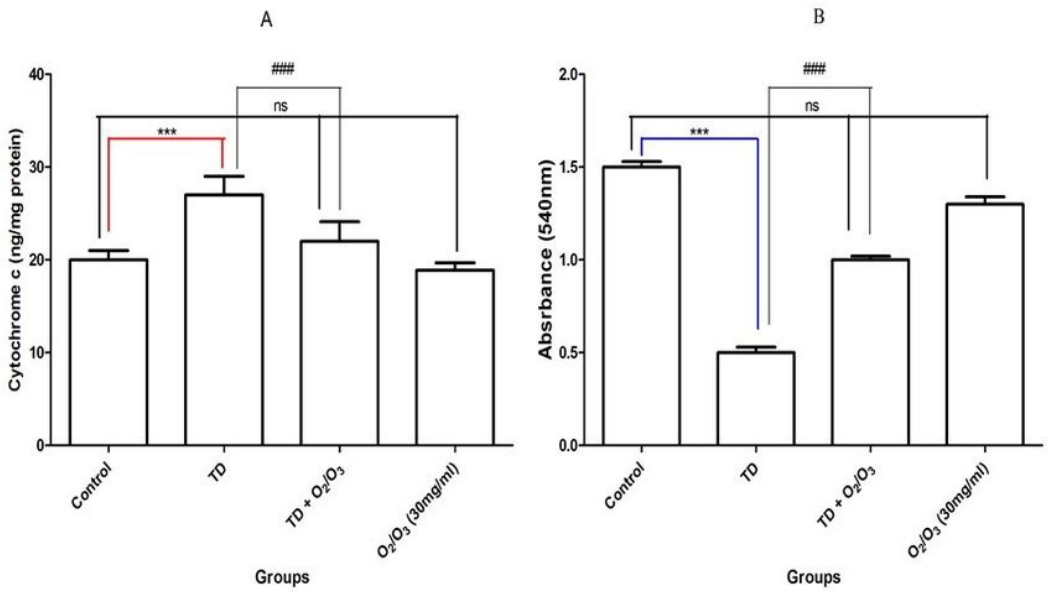

Figure 4

Cytochrome c release (A) and mitochondrial swelling (B) in testes isolated mitochondria. As shown in graph A TD induced cytochrome c release and ozone/oxygen therapy significantly reflected this endpoint in isolated mitochondria. Graph B indicates that mitochondrial swelling occurred in TD group in compared to control group. Intestinally mitochondrial swelling attenuated in TD + ozone/oxygen group in compared to TD group. Ns indicates not significant difference with control group. *** indicates significant difference with control group. ### indicates significant difference with TD group. Sample size= 5