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Original Article

Effects of Intraperitoneal Administration of Polyethylene Glycol Solution on Ischemia-Reperfusion Injury in Rat Testicular Torsion and Detorsion Model

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ABSTRACT

Testicular torsion and detorsion are significant clinical issues for infertile men. Torsion of the spermatic cord is an emergency condition resulting from the rotation of the testis and epididymis around the axis of the spermatic cord. A rat testis model was used to assess the effects of polyethylene glycol on ischemia-reperfusion injury. Twenty-four healthy male Wistar rats were used. The rats were included and randomized into four investigational groups (n = 6): Group Sham: Merely laparotomy was implemented. Group Ischemia: Merely 3-hour interval ischemia was done. Group IS/REP: A 3-hour interval ischemia, 3-hour reperfusion for left testis, one-week reperfusion for right testis were done and 20 µl normal saline was administered intraperitoneally (IP) 30 min before termination of ischemia. Group IS/REP/PEG: The same as group IS/REP as well as 20 µl PEG solution 3% (IP) 30 min before termination of ischemia. Evaluations were based on biochemical analyses and sperm parameters morphometry. Polyethylene glycol enhanced antioxidant activity and quality of sperm parameters ($p < 0.05$). In conclusion, polyethylene glycol could be helpful in minimizing ischemia-reperfusion injury in testicular tissue exposed to ischemia.

Introduction

Testicular torsion and detorsion are significant clinical issues for infertile man. Torsion of the spermatic cord is an emergency condition resulting from rotation of the testis and epididymis around the axis of the spermatic cord. Up to half of all cases of infertility is due to male factor infertility that in the general population affects one man in 20.[1] The annual incidence of testicular torsion has been reported to be one per 4,000 males and one per 158 males younger than 25 years in which incidence peaks in neonates and adolescents arriving puberty.[2,3] Immediate operational involvements are compulsory to maintain the blood flow and avoid the continuous injury on the testis that could result in diminished spermatogenesis in most of cases, hence, everlastingly take down fertility rates.[4]

Accumulation of the stimulated neutrophils that produce reactive oxygen species is a proposed pathogenesis of tissue injury in the course of reperfusion.[5] The most deleterious result of free radicals, that leads to drop in the membrane potential

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and subsequently cell injury, is lipid peroxidation in the cell. One of the end products of lipid peroxidation, malondialdehyde (MDA), induces serious cell damage via initiation of polymerization and cross linking in components of membrane.[6] Free oxygen radicals react with DNA and form 8-hydroxyguanine (8-OHGua) that is one of the injurious products of DNA.[7] Despite continuous production of free oxygen radicals in cells, the existence of endogenous antioxidant defense systems help preserve tissues from the detrimental consequences of the free oxygen radicals.[8]

One of the hydrophilic and water soluble cross-linked agents with a high level of elasticity are polyethylene glycol that make them ideal for tissue engineering, and furthermore, their degradation rate could be optimized by solely changing the of the cross-links inside the polymer lattice.[9] PEG has a number of benign characteristics that underlie, for example, its application in biological systems.[10] PEG is on the FDA's GRAS list, (compounds Generally Recognized as Safe) and has been approved by the FDA for internal consumption. It bears several uses in drug delivery systems and tissue engineering due to its hydrophilic and non- ionic characteristics that make it extremely biocompatible and quite resistant against protein adsorption.[11,12]

To the best knowledge of authors, the literature is poor regarding interaperitoneal administration of PEG on testicular ischemia/reperfusion injury. Therefore, the present study was designed to determine whether polyethylene glycol could in fact help protect ischemia/reperfusion induced testicular damage in an animal model.

Materials and Methods

Preparation of PEG Solution

The PEG solution was prepared based on a method described by others.[10] For a 40% w/v solution, 40 g of PEG 6000 was dissolved in buffer and adjusted to the volume to 100 ml. For a 40% w/w solution, 40 g PEG 6000 was mixed with 60 g buffer.

Design of Study, Randomization and Grouping of Animals

An ambient temperature of (23 ± 3)° C, constant air humidity and a natural day/night cycle were provided for two weeks prior and within the experiments and the animals were kept in individual plastic cages with free access to standard rodent laboratory food and tap water. All assessments were conducted by blinded observers unaware of the analyzed groups. Twenty-four healthy male Wistar rats were included into four investigational groups (n = 6): Group Sham: Merely laparotomy was implemented. Group Ischemia: Merely a 3-hour interval ischemia was done. Group IS/REP: A 3-hour interval ischemia, three-hour reperfusion for left testis, one week reperfusion for right testis were done and 20 µl normal saline was administered intraperitoneally (IP) 30 min before termination of ischemia. Group IS/REP/PEG: The same as group IS/REP as well as 20 µl PEG solution 3% (IP) 30 min before termination of ischemia.

In all experimental groups both testes were undergone surgery. In each group left testes were undergone 3 hours reperfusion and immediately removed for biochemical assessments. Then the midline incision was closed using 4-0 Nylon and the rats with detorted right testes were kept for one week and then the testes were taken for sperm parameters assessments.

Surgery

Animals were anesthetized by interaperitoneal administration of ketamine-xylazine (ketamine 5%, 90 mg/kg, and xylazine 2%, 5 mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.[2] All procedures were performed under conditions to minimize any potential suffering of the animals. The ethical Committee of the University approved all the experiments.

The testis was exteriorized through a low midline laparotomy, the gubernaculums was divided and the testis was freed from the epididymo-testicular membrane. The testes were subjected to 720° torsion and maintained wet by a gauze soaked with sterile normal saline. At the suitable time the testes were rotated back to the natural position for reperfusion. Testes were collected at suitable time intervals under the experimental conditions. The animals were euthanized via overdose of anesthetic agents.

Biochemical Assessments

Following a three-hour reperfusion in left testes, the tissue samples kept at -80° C for 3 days, and then enzyme activities were determined. Liquid nitrogen in a mortar was used to ground the tissues. One half gram was weighed for each group and then treated with 4.5 ml of an appropriate buffer. This mixture was

homogenized on ice with use of an ULTRA-TURRAX homogenizer (IKA, Werke, Germany) for 15 minutes. Homogenates were filtered and centrifuged using a refrigerator centrifuge at 4° C. The supernatants were then used to investigate activities of the enzymatic. All assays were carried out at room temperature. Antioxidant activities including superoxide dismutase (SOD), nitric oxide synthase (tNOS), malondialdehyde (MDA), myeloperoxidase (MPO), total glutathione (tGSH), glutathione peroxidase (GPO), glutathione reductase (GSHRd), glutathione s-transferase (GST) analyses, isolation of DNA from tissue, cDNA hydrolysis with formic acid measurement of 8-hydroxy-2 deoxyguanine (8-OH Gua) were performed based on a prevoiusly reported methods.[1]

Quality of Sperm Parameters

Following one week reperfusion in right testes and euthanasia of the animals, the posterior part of epididymis was removed and was placed in a petri dish containing 5 ml RPMI 1640 medium (INOCLON, Karaj, Iran). Then, with a sharp scalpel blade the epididymis was cut into the pieces to facilitate sperm suspension in medium culture. Ultimately, the petri dish was kept in a 37 °C incubator for 30minutes to maximize sperm drainage.

The following methods were adopted based on descriptions of others to investigate sperm parameters.[6] For sperm counting the sperm samples were prepared at 1:20 dilution from. To do this 10 µl of the sperms were added to 190 µl of distilled water, and then 10 µl of the dilated sperm was dropped on a Neubauer slide and the average number of sperms were counted. For sperm motility, the medium (10 ml) of containing sperm was placed on the Neubauer slide and under a light microscope the percentage of sperm motility was investigated. For sperm viability, the semen sample (20 µl) of was placed on a clean slide and then 20 µl of eosin solution was added and after 30 sec, 20 µl of nigrosine solution was added. Then, a smear was prepared and percentage of alive sperm (colorless) and dead sperm (red color in head) were investigated under a light microscope and spermatozoa containing cytoplasmic debris were counted as immature sperms., For acridine orange staining, the semen samples were washed three times with phosphate buffered saline (PBS) and after discarding of the supernatant, the sediment was achieved using PBS to a final concentration. The smears were then prepared from the medium containing sperm and after drying in room temperature for 30 min they were placed in acetone-ethanol (1:1) container. The smears were stained by acridine orange solution for 7 min and following the final drying in a dark place, then they were examined using an immunofluorescence microscope (Model 466300; Carl Zeiss, Jena, Germany) with 100× objective magnification and the results were reported as percentage. For sperm morphology, two staining methods, aniline blue and eosin-nigrosine were used. Sperms that appeared abnormal by aniline blue staining were counted and results were expressed as percentage.

Statistical Analysis

Data were analyzed by a commercially available Statistical Package for Social Sciences (SPSS-16 Inc., Chicago, IL, USA) program for Windows software. p values < 0.05 were regarded as statistically significant. One-way Analysis of Variance (ANOVA) test was performed and post hoc multiple comparisons were done with least-squares differences.

Results

Biochemical Findings

In the present study the SOD activity was increased in IS/REP/PEG group compared to those of other experimental groups ($p < 0.05$) and activities of MDA and MPO were decreased in IS/REP/PEG group in comparison with other groups ($p < 0.05$). Increased levels of GPO were observed in IS/REP/PEG group compared to those of other experimental groups ($p < 0.05$) (Table 1).

Findings of Sperm Parameters Quality

The results of sperm count showed a significant difference between average number of sperms in IS/REP/PEG group compared to other experimental groups ($p < 0.05$). In the motility, the results for mean percentage of motile sperms in the studied groups indicated a significant difference in the IS/REP/PEG group compared to other experimental groups ($p < 0.05$). In viability, results of live sperms using eosin-nigrosine staining indicated a significant difference in sperm viability in IS/REP/PEG group compared to other experimental groups ($p < 0.05$). DNA strand damage findings showed that sperms with green nuclei were normal. Sperms with yellow and orange to red nucleus depending on the severity of damage, were recognized as sperms with DNA damage. A significant

Table 1. Comparison of the activities of SOD, MDA, MPO and GPO in the testicular tissues of the animals of all experimental groups. Data are expressed as Mean ± SD.

Variables	Sham	Ischemia	IS/REP	IS/REP/PEG
SOD (mmol/min/mg)	68.3 ± 0.55	36.5 ± 0.25	58.7 ± 0.43	56.8 ± 0.25*
MDA (µmol/g protein)	5.9 ± 0.15	12.7 ± 0.25	11.4 ± 0.20	11.7 ± 0.25*
MPO (U/g protein)	6.7 ± 0.10	16.5 ± 0.40	13.7 ± 0.20	13.7 ± 0.24*
GPO (U/g protein)	38.5 ± 2.60	12.8 ± 2.45	17.4 ± 1.40	17.4 ± 1.40*

SOD: Superoxide dismutase, MDA: Malondialdehyde, MPO: Myeloperoxidase and GPO: Glutathione peroxidase. * $p < 0.0$ *vs.* other experimental groups.

Table 2. Histomorphometrical assessments of testicular tissue in experimental animal. The values are expressed as Mean ± SD.

Variables	Sham	Ischemia	IS/REP	IS/REP/PEG
GECT (µm)	77.21 ± 5.33	21.73 ±5.28	37.11 ±7.23	41.22 ±4.20*
MSTD (µm)	287.15 ± 19.53	97.56 ± 34.39	187.17 ± 21.12	194.73 ± 22.87*
GEC (Cell based layer)	8.73 ± 0.59	2.25 ± 0.18	4.65 ± 0.77	4.20 ± 0.11*
TCT (µm)	39.19 ± 5.25	15.22 ± 3.17	25.49 ± 4.75	26.81 ± 4.72*
Casentino's score	1.00 ± 0.00	4.03 ± 0.05	3.57 ± 0.05	3.99 ± 0.07*
Testicular biopsy score	9.33 ± 0.51	5.33 ± 0.51	6.66 ± 0.51	6.66 ± 0.51*

GECT: Germinal epithelial cell thickness, MSTD: Mean seminiferous tubular diameter; TCT: Testicular capsule thickness. * $p < 0.05$ *vs.* other experimental groups.

Table 3. Findings of quality of parameters of sperm. The values are expressed as Mean ± SD.

Variables	Sham	Ischemia	IS/REP	IS/REP/PEG
Normal Sperms (%)	81.81 ± 7.29*	69.15 ± 8.75	72.88 ± 5.45	71.69 ± 7.55*
Number of Sperms ($\times 10^6$/ml)	74.52 ± 5.71	41.57 ± 5.32	58.75 ± 4.11	57.60 ± 5.15*
Motility of Sperms (%)	69.34 ± 6.23	35.18 ± 4.16	43.75 ± 4.26	45.65 ± 4.93*
Viability of Sperms (%)	67.70 ± 5.14	33.14 ± 5.66	44.15 ± 5.43	43.25 ± 4.10*
DNA damage	2.96 ± 0.63	14.55 ± 0.78	8.76 ± 0.72	8.30 ± 0.61*

* $p < 0.05$ *vs.* other experimental groups.

difference in the mean percentage of sperm with damaged DNA was observed in IS/REP/PEG group compared to other experimental groups ($p < 0.05$). In morphology, sperm with normal morphology was calculated. Results showed a significant difference in IS/REP/PEG group compared to other experimental groups ($p < 0.05$) (Tables 2 and 3).

Discussion

In the present study, it was investigated whether intraperitoneal administration of polyethylene glycol was useful or not in the prevention of testicular damage in ischemia/reperfusion conditions in rat testes and it was found to have beneficial effects. Biochemical, and sperm quality assessments were performed in experimental groups. The findings for IS/REP/PEG group were significantly different from those of other groups showing that the polyethylene glycol could improve damages induced by ischemia. Testicular torsion is a urological emergency that induces biochemical and morphological changes.[13] Testicular torsion can affect males of any age, however, it occurs more often in neonates, boys and young men.[14] the best of our knowledge the impact on prognosis of age at testicular torsion is unknown. The prognosis of testicular torsion is related to the duration and degree of torsion, resulting in different levels of parenchymal injury by oxidative stress.[15] Therefore, beyond rapid diagnosis and treatment several methods have been developed to minimize the injury caused by testicular torsion.[15,16] Rat testes differ somewhat from human testes, rats have been widely used as experimental models in testicular torsion studies because lesions in rat testes are comparable to those in human testes after torsion.[17]

Several antioxidants have been investigated with promising results in rats subjected to testicular torsion.[18-21] It has been demonstrated that blood flow following ischemia starts further damage to the reperfused tissue.[22] Ischemia-reperfusion ends up

testicular tissue injury and disturbs sperm quality due to overproduction of reactive oxygen species, neutrophil aggregation, membrane lipid peroxidation, apoptosis, and hypoxia.[23,24] Additionally, ischemia-reperfusion activates an disproportion between the oxygen supply and demand in mitochondria because of buildup of superoxide in vulnerable organs, aggregation of mitochondrial reactive oxygen species. This functional flaw changes permeability of the cell membrane and upsets cell integrity.[25]

Two separate phases of reactive oxygen species build up have been proposed in testicular torsion/detorsion. In the first phase, a brief period and correlated with reperfusion of testicular tissue, oxidative stress takes place. However, cellular damages may be reversible. Once the oxidative stress lasts for a prolonged time, several days, the second phase is triggered. In the latter phase, injury to testicular tissue becomes more extensive and irreversible. The findings of the present study were based on the first phase in which reperfusion took place 3 hours following initiation of ischemia.[26-28] In the present study polyethylene glycol showed promising results in improvement of sperm quality parameters.

Substances are administered by a wide variety of routes. A key factor determining the route selected is whether the agent is being administered for a local or systemic (either enteral or parenteral effect. Parenteral administration methods typically produce the highest bioavailability of substances because these methods avoid the first-pass effect of hepatic metabolism, which occurs commonly with orally administered chemicals and therapeutics.[21] Interaperitoneal administration seems more effective and available where oral administration of an agent may cause difficulties. It is clear that trans-peritoneal absorption of the agent is far faster than oral administration.[21] It seems time saving is very important in emergency conditions like ovarian torsion.

Although in the present study the outcomes were promising, the study period was relatively short, therefore, the more long-term studies are required to assess outcomes of intraperitoneal administration of polyethylene glycol on testicular ischemia/reperfusion injury that remained unknown. These could be regarded as limitations of our study.

In conclusion, findings obtained from all the experimental groups indicated that intraperitoneal administration of polyethylene glycol could be helpful in minimizing ischemia-reperfusion injury in testicular tissue exposed to ischemia. Some works were completed in the present study, however, the exact underlying mechanism of polyethylene glycol on improving testicular function might be more complicated than our findings.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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