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PEG-Based Clotrimzole Microparticles Improved Ischemia-Reperfusion Injury in Rat Testicular Torsion and Detorsion Model: Evaluation of Tissue Oxidative Stress

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ABSTRACT

A rat testis model was used to assess effects of polyethylene glycol (PEG) clotrimazole microparticles on ischemia-reperfusion injury. Forty-eight healthy male Wistar rats were included and randomized into six investigational groups (n = 8): Group SHAM: Merely laparotomy was implemented. Group ISCHEMIA: Merely a 3-hour interval ischemia was done. Group I/R: A 3-hour interval ischemia, three-hour reperfusion for left testis, and one-week reperfusion for right testis were done and 20 µL normal saline was administered intraperitoneally (IP) 30 min before termination of ischemia. Group I/R/PEG: The same as group I/R as well as 20 µL PEG (IP) 30 min before termination of ischemia. Group I/R/CLTMZLMP: The same as group I/R and 20 µl (10 mg/kg) of clotrimazole microparticles (IP) 30 min before termination of ischemia. Group I/R/PEG-CLTMZLMP: The same as group I/R and 20 µL PEG-based clotrimazole microparticles (IP) 30 min before termination of ischemia. Evaluations were based on analyses of biochemical assays. PEG-based clotrimazole microparticles improved enhanced antioxidant activity ($p < 0.05$). PEG-based clotrimazole microparticles 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 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,

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the existence of endogenous antioxidant defense systems help preserve tissues from the detrimental consequences of the free oxygen radicals.[8]

Microparticulate drug delivery system is one of the processes to provide the sustained and controlled delivery of drug to long periods of time. They are small particles of solids or small droplets of liquids surrounded by walls of natural and synthetic polymer films of varying thickness and degree of permeability acting as a release rate controlling substance.[9]

Among different kinds of polymers, poly-(lactic-co-glycolic acid) (PLGA) is one of the most accepted materials for this purpose, because of its biodegradability (due to the presence of ester linkages that are degraded by hydrolysis in aqueous environments) and safety (PLGA is a Food and Drug Administration (FDA)-approved compound).[9] Most studies have attempted to prevent excessive calcium influx into the cells during hypoxia/ischemia. Calcium overload can result in the activation of calcium-dependent proteases, the destruction of mitochondria, the activation nitric oxide synthase, and the generation of free oxygen species.[10] As one of the most important free radical scavengers, clotrimazole is a potent inhibitor of the plasma membrane calcium channels that are activated by emptying the intracellular calcium stores.[10,11] Osmanağaoğlu *et al*. (2011) demonstrated that clotrimazole had some effects on ovarian damage scores and tissue MDA levels, which could be evidence of the ovarian-protective effects of clotrimazole on ovarian ischemia.[12]

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

Materials and Methods

Chemicals

Clotrimazole, Kollicoat IR® (Molecular weight = 45,000 Da), PEG 6000 and Silicon dioxide were obtained from Serva Fine Biochemical GmbH Co. (Heidelberg, Germany). Sodium lauryl sulfate was obtained from E-Merck (Darmstadt, Germany). All other chemicals were of reagent grade. The biochemical assay kits were purchased from Navand Salamat, Urmia, Iran.

Preparation of Binary Mixtures

Physical mixtures were prepared by mixing clotrimazole and each of the carriers in a mortar. The ratio of clotrimazole to the carrier used was 1:4 by weight. Spray-dried binary systems using Kollicoat IR®. As previously was describe by Fouad *et al*. (2011).[13] Appropriate mass ratio (1:4) of clotrimazole and Kollicoat IR® was prepared in a 2:1 v/v hydroalcoholic solution where clotrimazole was completely dissolved in ethanol and Kollicoat. The aqueous solution was added gradually to the ethanolic drug solution with subsequent vigorous stirring for 1 h to assure equilibrium. The resultant suspension was spray-dried in a Mini Spray-Dryer B-290 (Büchi Labortechnik AG, Flawil, Switzerland) with the following conditions: inlet temperature 130 °C, outlet temperature 60–65 °C, suspension flow rate 5 mL/ min, air flow rate 40–50 m 3/h, and atomizing air pressure 1.0–1.1 bar. The batch size of the prepared ratios was 10 g each.

Preparation of PEG Based Clotrimazole Microparticles via Spray-Dried Binary Systems

Based on the method of Fouad *et al*., (2011)[13] clotrimazole in combination with PEG 6000 in mass ratios of 1:4 was dissolved in 100 mL of dichloromethane. To these clear solutions, silicon dioxide (2% w/v) was slowly added to obtain uniform suspensions. The suspension was spray-dried in the Büchi mini spray-dryer with the following conditions: inlet temperature 50 °C, outlet temperature 30 °C, solution flow rate 5 mL/min, air flow rate 40–50 m 3/h, and atomizing air pressure 1.0–1.1 bar. The batch size was again 10 g.

Analytical and Characterization Methods of Microparticles and the Final Product

Transmission electron microscopy (TEM), Field emission scanning electron microscopy (FESEM) to assess size and the morphology of the samples. The FESEM instrument was operated at an accelerating voltage at 10 kV. The size distribution of the microparticles was detected through Zetasizer Nano ZS (Malvern Instruments Limited) particle analyzer. The synthesized nanoparticles were subjected to Fourier-transform infrared (FTIR; range 4000–400 cm−1) to investigate the occurrence of chemical bonds between the microparticles and the polymer.

Ethical Considerations

The procedures were approved by the Institutional Animal Care and Use Committee of the University. All procedures were performed under conditions to minimize any potential suffering of the animals.

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. Forty-eight healthy male Wistar rats were included and randomized into six investigational groups (n = 8): Group SHAM: Merely laparotomy was implemented. Group ISCHEMIA: Merely a 3- hour interval ischemia was done. Group I/R: 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 I/R/PEG: The same as group I/R as well as 20 µL PEG (IP) 30 min before termination of ischemia. Group I/R/CLTMZLMP: The same as group I/R and 20 µL (10 mg/kg) of clotrimazole microparticles (IP) 30 min before termination of ischemia. Group I/R/PEG-CLTMZLMP: The same as group I/R and 20 µL PEG based clotrimazole microparticles (IP) 30 min before termination of ischemia. In each group left testes were undergone 3 hours reperfusion and immediately removed for biochemical 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.[14] 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 and biochemical assays 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 Phosphate Buffered Saline. 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 enzymes. All assays were carried out at room temperature. Antioxidant activities including total antioxidant capacity (TAC), superoxide dismutase (SOD) activity, malondialdehyde (MDA), glutathione peroxidase (GPx) were performed based on a prevoiusly reported methods.[15] In brief, when a standard hydrogen peroxide solution was oxidized with free radicals, a yellow-brown color was produced. Therefore, antioxidants within the sample suppressed the oxidation and color formation. This reaction was monitored by spectrophotometry and thus TAC was indirectly measured and expressed as millimolar trolox equivalent per liter (mmol Eqv/l) for TAC. SOD was estimated by assessment of the generation of superoxide radicals produced by xanthine and the xanthine oxidase system, which reacts with nitroblue tetrazolium to form formazan dye using special kit (Navandsalamat, Iran). Superoxide dismutase activity was then measured at 560 nm wave length by the degree of inhibition of this reaction and is expressed as mmol per minute per mg of tissues.

MDA levels were measured using special kit (Navand Salamat, Urmia, Iran). The lipid peroxidation was determined by estimating MDA using the thiobarbituric acid test. The testicles were weighed digested and homogenized in 10 ml of 100 g/l KCl. The homogenate (0.5 ml) was added to a solution containing 2-thiobarbiturate (1.5 ml of 8 g/l), acetic acid (1.5 ml of 200 g/l), sodium lauryl sulfate (0.2 ml of 80 g/l), and distilled water (0.3 ml). The mixture was incubated at 98 °C for 1 hr. N-butanol:pyridine 5 ml (ratio: 15:l) was then added. The mixture was vortexed for 1 min and centrifuged for 30 min at 4000 rpm. The absorbance of the supernatant was measured at 532 nm wave length using a spectrophotometer. The standard curve was obtained by using 1,1,3,3-tetramethoxypropane. GPx activity within the tissues was determined according to the method of Lawrence and Burk using kit (Navand Salamat, Urmia, Iran). Following tissue homogenization, supernatant was used for GPx measurement. Following the addition of KH2PO4, EDTA, GSH, B-NADPH, NaN3, and GR, the mixture was incubated. As soon H2O2 was added the chronometer was turned on and the absorbance at 340 nm wave length was recorded for 5 min every 15 sec.

Statistical Analysis

Data were analyzed by a commercially available Statistical Package for Social Sciences (SPSS 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

Analytical Assessments

Microstructural and physicochemical characterizations. Light microscope image of

clotrimazole microparticles showed encapsulation of the particles by PEG (Figure 1A). The FESEM image of the microparticles (Figure 1B) evidently illustrated that the particles were typically sphere-shaped with a size range of less than 1000 nm. The Z-average (r.nm) value for sizes of the microparticles was about 835 ± 267 nm (% Intensity 100, Width: 91.11, Derived count rate (kcps): 999033.1287) based on the dynamic light scattering (DLS) technique (Figure 2). The clotrimazole microparticles presented uniformity in the PEG-based matrix. FTIR findings showed that there were no significant interactions between PEG and the microparticles (Figure 3).

Biochemical Findings

In the present study the TAC, SOD and GPO activities were significantly increased in I/R/PEG-CLTMZLMP group compared to those of other experimental groups ($p < 0.05$) and amount of MDA was significantly decreased in I/R/PEG-CLTMZLMP group in comparison with other groups ($p < 0.05$) (Table 1).

Discussion

In the present study, it was investigated whether intraperitoneal administration of polyethylene glycol based clotrimazole microparticles was useful or not in

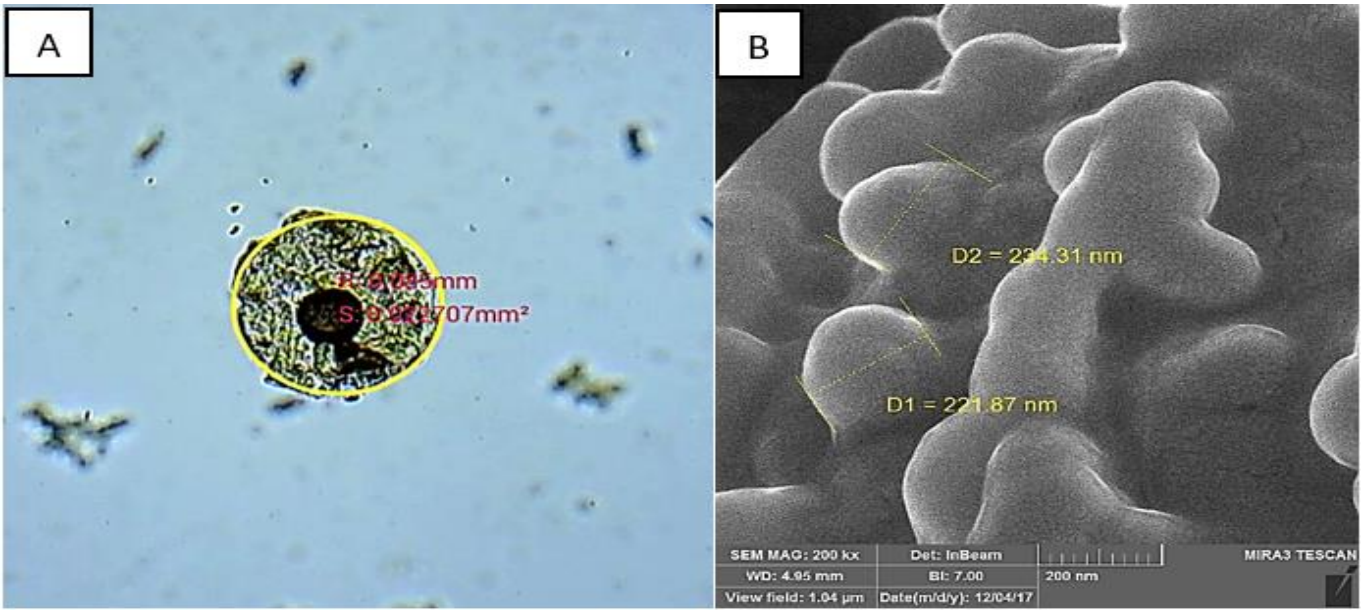

Figure 1. Microstructural characterization of PEG based clotrimazole microparticles. A) TEM micrograph of PEG based clotrimazole microparticles. B) FESEM micrograph of PEG based clotrimazole microparticles.

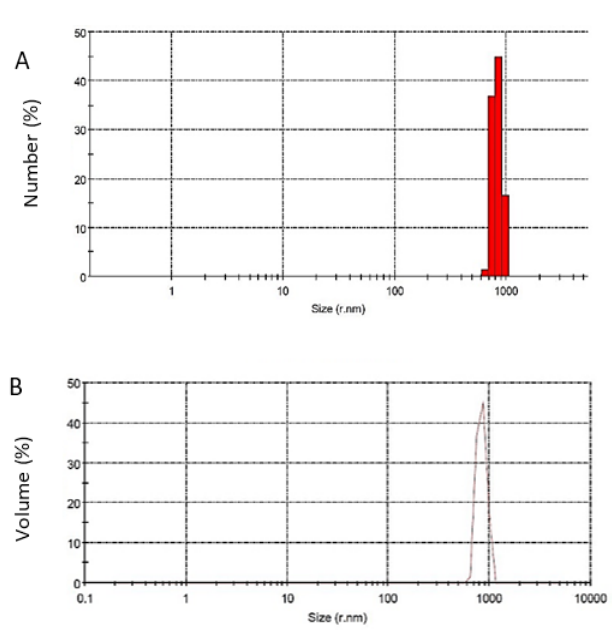

Figure 2. Size distribution of PEG based clotrimazole microparticles based on numbers (A) and volumes (B).

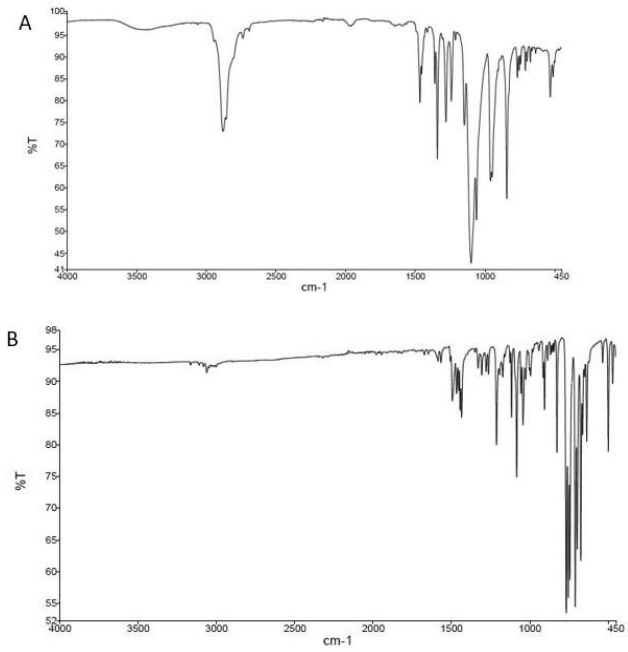

Figure 3. A) FTIR spectrum of clotrimazole microparticles and B) FTIR spectrum of PEG based clotrimazole microparticles.

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

Variables	SHAM	ISCHEMIA	I/R	I/R/PEG	I/R/CLTMZLMP	I/R/PEG-CLTMZLMP
TAC (mmol Eqv./l)	0.45 ± 0.09	0.08 ± 0.05	0.16 ± 0.07	0.15 ± 0.07	0.41 ± 0.05	0.53 ± 0.05*
SOD (mmol/min/mg)	42.36 ± 3.65	7.15 ± 0.31	14.61 ± 0.55	15.47 ± 1.82	29.17 ± 3.92	36.47 ± 0.76*
MDA (µmol/g protein)	0.24 ± 0.04	1.37 ± 0.17	0.95 ± 0.05	0.93 ± 0.04	0.65 ± 0.04	0.22 ± 0.03*
GPx (U/g protein)	21.40 ± 2.16	3.23 ± 0.15	9.37 ± 1.75	10.69 ± 1.25	14.45 ± 1.76	19.33 ± 2.49*

TAC: Total antioxidant capacity, SOD: Superoxide dismutase, MDA: Malondialdehyde and GPx: Glutathione peroxidase. *, $p < 0.0$ *vs.* other experimental groups.

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 I/R/PEG-CLTMZLMP group were significantly different from those of other groups showing that the polyethylene glycol based clotrimazole microparticles could improve damages induced by ischemia.

Testicular torsion is a urological emergency that induces biochemical and morphological changes.[16] Testicular torsion can affect males of any age, however, it occurs more often in neonates, boys and young men.[17] 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.[18] Therefore, beyond rapid diagnosis and treatment several methods have been developed to minimize the injury caused by testicular torsion.[19,20] 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.[21]

Several antioxidants have been investigated with promising results in rats subjected to testicular torsion.[22-25] It has been demonstrated that blood flow following ischemia starts further damage to the reperfused tissue.[26] 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.[27,28] 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.[29]

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.[30]

In our findings, mean values of SOD in testicular tissue were measured and compared in the experimental groups. Free radicals are neutralized by superoxide dismutase and endogenous antioxidant. This protects tissues from the detrimental effects of free radicals and active oxygen species.[31] In our findings in the I/R/PEG-CLTMZLMP treated animals mean values of TAC and superoxide dismutase activity were augmented in comparison with those in other groups and testicular tissue was secured against ischemia-reperfusion injury via intraperitoneal administration of PEG- CLTMZLMP. MDA, a lipid peroxidation product, is produced due to the peroxidation of fatty acids with three or more double bonds. Cross-linking of membrane components are ensued by MDA that ends up undesirable consequences such as alterations in ion permeability and enzyme activity via disturbing the ion exchange through the cell membranes.[32] MDA mean values in our findings were found to be far lesser in the PEG-CLTMZLMP treated animals compared to those in other groups. Activity of GPO is significantly diminished in tissues undergoing oxidative stress-related settings such as ischemia-reperfusion injury.[33] GPO depollutes the radicals of hydrogen peroxide that are produced in the cell via conversion to water and avoids the production of more toxic mediators from radicals of hydrogen peroxide.[34]

Osmanağaoğlu *et al.* (2012)[12] demonstrated that clotrimazole significantly decreased plasma levels of serum malondialdehyde, ischemia-modified albumin, and total oxidant status and concluded that clotrimazole had the protective effects on ovarian ischemia/reperfusion injury. We found that oxidative stress was minimized and the severe damage due to sudden reperfusion was prevented in PEG-CLTMZLMP treated animals more than those in curcumin treated animals.

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 administration of PEG-CLTMZLMP 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 administration of PEG based clotrimazole 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 PEG based clotrimazole on improving testicular function might be more complicated than our findings.

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Conflict of Interest

None to declare.

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