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Protective Effects of Nano-Propolis Liposomes on Iischemia-Reperfusion Injury: A Rat Model of Testicular Torsion and Detorsion

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ABSTRACT

A rat testis model was used to assess the effects of nano-propolis liposomes on ischemia-reperfusion injury. Twenty-four healthy male Wistar rats were included and randomised into six investigational groups (n = 6): Group SHAM: only laparotomy was implemented. Group ISCHEMIA: only a 3-hour ischemia was induced. Group IS/REP: A 3-hour ischemia followed by 3 hours reperfusion for the left testis and one week reperfusion for the right testis was performed with 1 ml normal saline that was gavaged 1 hour before the onset of ischemia. Group IS/REP/NPL: As in group IS/REP, but with 1 ml nano-propolis liposomes gavaged 1 hour before the onset of ischemia. Evaluations were based on biochemical analyses and sperm parameters. Propolis enhanced antioxidant activity and quality of sperm parameters ($p < 0.05$). Nano-propolis liposomes could help minimize 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, the existence of endogenous antioxidant defense systems help preserve tissues from the detrimental consequences of the free oxygen radicals.[8]

Propolis is a natural product collected by bees from the poplar and conifer trees. Bees use propolis as an antibiotic against foreign organisms and also to repair the cracks of their hives.[9] It has vast majority of biological activities such as anti-inflammatory, anti-fungal, antioxidant, and immune-stimulating activity.[10]

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Most of these effects have been related to the remarkable *in vitro* antioxidant activity and free radical scavenging ability of propolis.[11] The major components of propolis are polyphenolics including aldehydes, caffeic acid, and caffeic acid phenethyl ester which plays a critical role in neurological disorders, cardiovascular diseases, pathophysiology of cancer, and diabetes.[12-15] Effects of propolis on ovarian ischemia-reperfusion injury (I/RI) were studied and reported to enrich regulatory T cells to exert anti-inflammatory effects.[16] There is also evidence, indicating that Propolis inhibited apoptosis by preventing phosphorylation of proapoptotic proteins.[17] Encapsulation of propolis with the support of liposomes is favorable for their biological and chemical purposes, as stated by Abdelnour *et al.* (2023), who observed that nano-propolis-liposomes (NPL) improved the sperm quality and freezing ability in buffalo.[18]

Recent studies have reported that propolis can influence key processes in the development of cancer including cell proliferation, angiogenesis, inhibition of apoptosis, and metastasis.[19] Although propolis has various therapeutic activities, its resinous nature, low bioavailability, poor solubility, and physical instability limit the use of this compound.[20] Due to the limitation of using free propolis, the use of nano carriers as a drug delivery system is a promising strategy. Various types of natural or synthetic nano carriers have been used to treat melanoma cancer such as carbon nanotubes, gold nanoparticles, liposomes, niosomes, micelles, polymeric nanoparticles, and dendrimers.[21] Among all nano carriers mentioned, liposomes have been widely studied as targeted drug delivery systems in cancer treatment.[22] Liposomes are spherical lipid-based vesicles composed of phospholipid bilayers that surround an aqueous core. Liposomes are useful for targeted drug delivery because they can localize the drug to the site of action, which reduces the concentration of the drug in other parts of the body.[23] Liposomes represent a wide range of advantages including biocompatibility, biodegradability, entrapping both hydrophobic and hydrophilic drugs, low toxicity, high encapsulation capacity, and controlled drug release.[24] To the best knowledge of authors, the literature is poor regarding oral administration of nano-propolis liposomes on testicular ischemia/reperfusion injury. Therefore, the present study was designed to determine whether nano-propolis liposomes could in fact help protect ischemia/reperfusion induced testicular damage in an animal model.

Materials and Methods

Preparation of Nano-Propolis Liposomes

The water-soluble propolis extract was purchased from Aksuvital Natural Products Food Industry Trade Inc. (Istanbul, Turkey). The NPL was prepared via the conventional thin-film hydration technique with slight modifications based on a method described by others.[25] In brief, propolis, cholesterol, and soybean lecithin were mixed and dissolved in the organic solvent mixture (methanol and chloroform, 1:2 v/v). Then, the mixture was exposed to a rotary evaporator until the organic solvent mixture was fully evaporated. After that, the nano liposomes of propolis were obtained.

Ethical Considerations

Our study protocol was reviewed and approved by Department of Surgery and Diagnostic Imaging, Urmia University. All animals received humane care in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication No. 85–23, revised 1985).

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 and randomized into six investigational groups (n = 6): Group SHAM: Only laparotomy was implemented. Group ISCHEMIA: Only 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 1 ml normal saline was gavaged 1 h before onset of ischemia. Group IS/REP/NPL: The same as group IS/REP as well as 1 mL the nano propolis liposomes that gavaged 1 h before onset of ischemia. The exact amount of nano-propolis liposomes was chosen based on works of others.[12] In all experimental groups both testes were undergone surgery. In each group left testes were undergone 3 hours reperfusion and immediately removed for biochemical and histopathological 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%, 5mg/kg). The procedure was carried out based on the guidelines of the Ethics Committee of the International Association for the Study of Pain.[26] 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 ºC. 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.[27]

Histological Assessments

Histological assessments were based on the others as follow. Following one week reperfusion in right testes and euthanasia of the animals using Ketamine (500 mg/kg; IP) and xylazine (50 mg/kg; IP), 10% buffered formalin was used to fix the testes. The samples were then stained routinely with hematoxylin-eosin and Masson's trichrome. The sections were then observed under a light microscope. The histopathological features of the tubules and the degree of damage were estimated as others.[28] Furthermore, mean diameter of seminiferous tubular MSTD, testicular capsule thickness TCT, and the thickness of germinal epithelial cell GECT were determined. To investigate substantial histopathological alterations in the tubules and germinal cells, Casentino's histopathological score was adopted Cosentino, Nishida, Rabinowitz, 1986. Seminiferous tubule profiles based on Johnsen's scoring system were also evaluated (Table 1).[29]

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). The epididymis was cut into the pieces with a sharp scalpel blade to facilitate sperm suspension in medium culture. Ultimately, the petri dish was kept in a 37 °C incubator for 15 minutes to maximize sperm drainage.

The following methods were adopted based on descriptions of others to investigate sperm parameters.[30] For sperm counting the sperm samples were prepared at 1:20 dilution from. To do this 10 µl of the sperms were added to 190µL 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 DNA strand damage, 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

Table 1. Scoring system for testicular biopsy for the testicular sections based on others.[22]

Score	Explanations
1	There are no cells in the tubule.
2	Only Sertoli cells are observed
3	The only germ cells observed are spermatogonia
4	Few spermatocytes (less than 5-10 per tubular cross-section) are observed, however, no spermatids or spermatozoa are observed
5	Many spermatocytes are observed, however, no spermatids or spermatozoa are observed
6	Few spermatids (less than 5-10 per tubular cross-section) are observed, however, many spermatocytes are observed.
7	No spermatozoa are observed, however, many round and elongated spermatids are observed.
8	Few spermatozoa (less than 5-10 per tubular cross-section) are observed, however, many round and elongated spermatids are observed.
9	Many spermatozoa, however, the germinal epithelium is disorganized with marked sloughing.
10	Complete spermatogenesis with many mature and shed spermatozoa is observed. The germinal epithelium are originated normally and an open lumen is observed.

acridine orange solution for 7 min and following the final drying in a dark place, 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 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/NPL group compared to those of other experimental groups (p = 0.001) and activities of tNOS, MDA, MPO and 8-OHGual/Gua, (a DNA damage product) were decreased in IS/REP/NPL group in comparison with other groups (p = 0.001). Increased levels of GSH, GPO, GSHRd and GST were observed in IS/REP/NPL group compared to those of other experimental groups (p = 0.001) (Table 2).

Histomorphometry

Histomorphometrical assessments of testicular tissue and scores for testicular biopsy are presented in Table 3. Ischemia significantly reduced GECT, MSTD, cell based layer of GECT, and TCT in comparison with those in the SHAM animals (p < 0.05). GECT, GECT cell layer, and MSTD and TCT were considerably higher in the IS/REP/NPL group in comparison with those of other groups (p < 0.05). The comparison for the Cosentino's score in the SHAM animals and other animals was significant (p < 0.05), however, Cosentino's score was significantly reduced in IS/REP/NPL group in comparison with other groups (p < 0.05). We did not observe noticeable damage on the testis of the SHAM animals and seminiferous tubule were in normal morphology that was confirmed by the high testicular biopsy score of 9.33 ± 0.51. In ISCHEMIA group we observed severe swelling and disorganization in seminiferous tubules and we confirmed that there was a damage at levels of spermatozoa, spermatids and some spermatocytes. We observed disorganized interstitial containing some blood. The testicular biopsy score of 5.33 ± 0.51 was observed. In IS/REP/NPL, seminiferous tubules showed normal spermatogenesis, exhibiting all stages of spermatogenic cells including abundant spermatozoa. Some swelling and tissue disorganization were observed. The testicular biopsy score of 7.44 ± 0.31 was observed that was significantly different from those of other experimental animals (p < 0.05).

Findings of Quality of Sperm Parameters

The results of sperm count showed a significant difference between average number of sperms in IS/REP/NPL group compared to other experimental groups (p = 0.001). In the motility, the results for mean percentage of motile sperms in the studied groups indicated a significant difference in the IS/REP/NPL group compared to other experimental groups (p = 0.001). 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 difference in the mean percentage of sperm with damaged DNA was observed in IS/REP/NPL group compared to other experimental groups (p = 0.001). In viability, results of live sperms using eosin-nigrosine

Table 2. Comparison of the activities of SOD, NOS, MDA, MPO, GSH, GPO, GST, GSHRd and a DNA damage product of 8-OHGua/Gua in the testicular tissues of the animals of all experimental groups. Data are expressed as mean ± SD.

Variables	SHAM	ISCHEMIA	IS/REP	IS/REP/NPL
SOD (mmol/min/mg)	67.3 ± 0.50	38.7 ± 0.33	60.7 ± 2.56	78.4 ± 0.37*
NOS (nmol/min/mg)	3.7 ± 0.15	3.5 ± 0.10	3.7 ± 0.55	4.1 ± 0.15*
MDA (µmol/g protein)	5.8 ± 0.10	13.3 ± 0.61	10.7 ± 0.71	5.9 ± 0.03*
MPO (U/g protein)	6.4 ± 0.11	17.6 ± 0.23	12.8 ± 0.90	6.4 ± 0.09*
tGSH (nmol/g protein)	9.6 ± 0.31	2.5 ± 0.56	5.8 ± 0.54	8.8 ± 0.25*
GPO (U/g protein)	37.3 ± 2.88	11.4 ± 2.07	19.9 ± 2.08	35.08 ± 1.92*
GSHRd (U/g protein)	30.5 ± 3.25	10.5 ± 2.06	17.5 ± 1.64	30.3 ± 1.85*
GST (U/g protein)	23.5 ± 2.33	9.4 ± 1.55	15.7 ± 1.34	20.2± 1.39*
8-OHGua/Gua (pmol/l)	1.1 ± 0.25	2.8 ± 0.38	2.2± 0.09	1.2 ± 0.02*

SOD: superoxide dismutase, NOS: nitric oxide synthase, MDA: malondialdehyde, MPO: myeloperoxidase, tGSH: total glutathione, GPO: glutathione peroxidase, GSHRd: glutathione reductase, GST: Glutathione S-transferase and 8-OHGua/Gua: 8-hydroxy-2 deoxyguanine.
*: p < 0.0 *vs.* other experimental groups.

Table 3. Histomorphometrical assessments of testicular tissue in experimental animal. The values are expressed as mean ± SD.

Parameters	SHAM	ISCHEMIA	IS/REP	IS/REP/NPL
GECT (μm)	76.31 ± 4.54	22.67 ± 4.02	36.78 ± 5.90	57.72 ± 8.78*
MSTD (μm)	276.13 ± 15.73	95.79 ± 30.14	179.97 ± 19.55	257.23 ± 16.52*
GEC (Cell based layer)	8.75 ± 0.54	2.41 ± 0.58	4.05 ± 0.11	7.09 ± 0.08*
TCT (μm)	36.76 ± 2.72	16.88 ± 3.29	26.25 ± 4.72**	31.07 ± 2.08*
Casentino's score	1.00 ± 0.00	4.07 ± 0.08	3.42 ± 0.08	1.33 ± 0.05*
Testicular biopsy score	9.33 ± 0.51	5.33 ± 0.51	6.66 ± 0.51**	7.44 ± 0.31*

GECT: germinal epithelial cell thickness, MSTD: mean seminiferous tubular diameter; TCT: testicular capsule thickness. *, **: $p < 0.05$ *vs.* other experimental groups.

Table 4. Findings of quality of parameters of sperm. The values are expressed as mean ± SD.

Parameters	SHAM	ISCHEMIA	IS/REP	IS/REP/NPL
Normal Sperms (%)	83.22 ± 5.69*	79.09 ± 3.65	75.08 ± 2.45	79.05 ± 3.10
Number of Sperms ($\times 10^6$/ml)	75.12 ± 3.31	40.88 ± 4.55	55.11 ± 2.20	69.13 ± 2.02*
Motility of Sperms (%)	68.72 ± 5.53	36.47 ± 4.87	44.19 ± 3.78	63.71 ± 2.60*
Viability of Sperms (%)	69.08 ± 4.64	35.89 ± 4.36	45.09 ± 4.18	63.02 ± 2.09*
DNA damage	2.05 ± 0.08	15.11 ± 0.14	9.01 ± 0.33	3.02 ± 0.07*

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

staining indicated a significant difference in sperm viability in IS/REP/NPL group compared to other experimental groups ($p = 0.001$). In morphology, sperm with normal morphology was calculated. Results showed a significant difference in IS/REP/NPL group compared to other experimental groups ($p = 0.001$) (Table 4).

Discussion

To date, the treatment strategy for protecting the testis following torsion/detorsion is surgical, however, exploring additional therapeutic alternatives is necessary.[31] Thus, the present study investigated whether nano-propolis liposomes could be useful or not in the prevention of testicular damage in ischemia/reperfusion (I/R) conditions in rat testes and it was found to have beneficial effects.

The process of I/R is multifactorial and there are several mechanisms involved in the pathogenesis.[31] We showed that this complication in testes exhibited significantly aggravated oxidative stress, compared to control rats. I/R increased the levels of biochemical indices of oxidative stress suggesting that oxidative stress was induced during I/R and caused oxidative damage in cellular DNA, protein, and lipids in testes. Excessive intracellular reactive oxygen species (ROS) generation is thought to trigger extensive mitochondrial oxidative damage.[31] Tissue cells undergoing ischemic insult produce excess amounts of ROS, which attack mitochondrial DNA (mtDNA) due to its special structural characteristics, resulting in aggregation of 8-OHdG, a sensitive marker of oxidative DNA damage, and excessive intracellular ROS generation is thought to trigger extensive mitochondrial oxidative damage.[32] The cells undergoing ischemic insult produce excess amounts of ROS, which attack mtDNA due to its special structural characteristics, resulting in aggregation of 8-OHdG, a sensitive marker of oxidative DNA damage, and mitochondria dysfunction.[33] Hence, it will be interesting to explore whether nano-propolis liposomes plays a role in alleviating mitochondrial oxidative damage after testicular ischemia in rats. In the present study, we found that nano-propolis liposomes administration significantly increased SOD, GSH, GPO, GSHRd and GST, and decreased NOS, MDA, MPO, a DNA damage product of 8-OHGua/Gua. Turkez *et al.* in 2010,[34] evaluated the effects of treatment with propolis on aluminum induced micronucleated hepatocytes and oxidative stress in rat liver. In another study, Tohamy *et al.* in 2014,[35] evaluated the effects of treatment with propolis against cisplatin-induced hepatic, renal, testicular genotoxicity by the bone marrow chromosomal aberration assay in male albino mice. Therefore, it is clear from these observations that nano-propolis liposomes may act not only as direct antioxidant, inactivating free radicals, but also as an attractive tool for prevention of DNA-instability. In the present study the findings for IS/REP/NPL group were significantly different from those of other groups showing that nano-propolis liposomes could improve damages induced by ischemia.

Testicular torsion is a urological emergency that induces biochemical and morphological changes.[36] Testicular torsions can affect males of any age, however, it occurs more often in neonates, boys and young men.[37] The prognosis of testicular torsion is related to the duration and degree of torsion, resulting in different levels of parenchymal injury by oxidative stress.[38] Therefore, beyond rapid diagnosis and treatment several methods have been developed to minimize the injury caused by testicular torsion.[39,40] 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.[41]

Several agents have been investigated with promising results in rats subjected to testicular torsion.[42,43] It has been demonstrated that blood flow following ischemia starts further damage to the reperfused tissue.[44] 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.[45] 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.[46]

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.[47-49]

In view of the susceptibility of sperm cells to ischemia/reperfusion injury, numerous studies have evaluated sperm parameters following testicular torsion/detorsion. In the present study, it was found that nano-propolis liposomes administration considerably increased GECT, MSTD, GECT and number, motility and viability of sperms. DNA damage was also decrease.

Our findings were in agreement with the findings of a study suggesting that testicular torsion/detorsion could damage spermatozoa, while co-administration of verapamil and salvia miltiorrhiza hydroalcoholic extract hydroalcoholic extract increased sperm motility and viability compared to that in the torsion/detorsion group.[50]

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 nano-propolis liposomes 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 nano-propolis liposomes 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 nano-propolis liposomes on improving testicular function might be more complicated than our findings.

Conflict of Interest

None.

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