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The Effect of β-Cryptoxanthin on Testicular Ischemia-Reperfusion Injury in a Rat Model: Evidence from Testicular Histology

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ABSTRACT

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. A rat testis model was used to assess effects of β-cryptoxanthin on ischemia-reperfusion injury. Twenty healthy male Wistar rats were included and randomized into four investigational groups (n = 5): Group SHAM: In this group, midline incision of the scrotum was performed and the testicles were taken out for 2 hours with a 720-degree rotation. Group ISCHEMIA: In this group, midline incision of the scrotum was performed and the testicles were taken out and undergone ischemia for 2 hours with a 720-degree rotation. Group IS/REP/Oil: In this group, a midline scrotum cut was performed and the testicles were taken out and ischemia was created for 2 hours with a 720-degree rotation and at the end of ischemia 100 µl of corn oil (β-cryptoxanthin solvent) was injected intraperitoneally. Group IS/REP/CRPTXNTN 2.5: The same as group IS/REP/Oil as well as intraperitoneal administration of 100 µl of β-cryptoxanthin (2.5 µg/kg) at the end of ischemia. In all groups, the testes were returned back to scrotum and after 60 days were dissected out and removed for histopathological analyses. β-cryptoxanthin at the dose of 2.5 µg/kg significantly improved histologic indices compared to other treatment groups ($p < 0.05$). β-cryptoxanthin could be helpful in minimizing ischemia-reperfusion injury in testicular tissue exposed to ischemia.

Introduction

In infertile man, testicular torsion and detorsion are significant clinical issues and 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

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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]

Usage of potent antioxidants has been reported by others. Protective effects of rosmarinic acid on testicular torsion-detorsion has been demonstrated.[9] It has been reported that L-carnitine had beneficial effects on ischemia-reperfusion injuries following testicular torsion in a rat model.[10] It has been shown that betaine could protect testicular tissue damage induced by testicular torsion in rat.[11]

β-Cryptoxanthin is an oxygenated carotenoid with a chemical structure similar to, but more polar than, β-carotene. Although β-carotene is present in large amounts in numerous fruits and vegetables, β-cryptoxanthin is found at high concentrations in only a small number of foods. β-Cryptoxanthin can accept energy from singlet oxygen. The evidence that β-cryptoxanthin is an antioxidant in vitro at most physiological concentrations is persuasive. However, a recent review suggested that there is a difference between nonvitamin A–forming carotenoids (such as lycopene) and vitamin A–forming carotenoids (such as β-cryptoxanthin). Nonvitamin A–forming carotenoids protected against DNA damage in almost all circumstances, but vitamin A–forming carotenoids protected against DNA damage in some cases and enhanced DNA damage in others, especially when they were present at high concentrations.[12,13]

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

Materials and Methods

Ethical Considerations

The procedures were approved by the Institutional Animal Care and Use Committee of the University under code number# IR-UU-AEC-3/13 dated 14/-5/2024. All procedures were performed under conditions to minimize any potential suffering of the animals. Ketamine (500 mg/kg; IP) and xylazine (50.00 mg/kg; IP) were administered to euthanize the animals.[14]

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 healthy male Wistar rats were included and randomized into four investigational groups (n = 5): Group SHAM: In this group, midline incision of the scrotum was performed and the testicles were taken out for 2 hours with a 720-degree count clock wise rotation. Group ISCHEMIA: In this group, midline incision of the scrotum was performed and the testicles were taken out and undergone ischemia for 2 hours with a 720-degree count clock wise rotation. Group IS/REP/Oil: In this group, a midline scrotum cut was performed and the testicles were taken out and ischemia was created for 2 hours with a 720-degree rotation and at the end of ischemia 100 µl of corn oil (β-cryptoxanthin solvent) was injected intraperitoneally. Group IS/REP/CRPTXNTN 2.5: The same as group IS/REP/Oil as well as intraperitoneal administration of 100 µl of β-cryptoxanthin (2.5 µg/kg) at the end of ischemia. In all groups, the testes were returned back to scrotum and after 60 days the left testis was dissected out and removed for histopathological analyses and right testis was dissected out and removed for sperm parameters evaluations.

Surgical Procedures

Animals were anesthetized by intraperitoneal 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.[15] The ethical Committee of the University approved all the experiments.

The testis was exteriorized through scrotum, the gubernaculum 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. One testis was removed 60 days post operation and evaluations were based on analyses of histopathologic studies.

Histological Assessments

Following 60 days reperfusion and euthanasia of the animals, 10% buffered formalin was used to fix the testes for 24 hours. The tissue samples were then processed, embedded in paraffin and a 5-µm semi-thin sections were prepared. The samples were then stained routinely with hematoxylin-eosin and Masson's trichrome. The sections were then observed under a light microscope and the following parameters were assessed: Testis weight,

epididymis weight, Testis/body weight, Johnsen score, Cosentino score, seminiferous tubule diameter, Sertoli cell index, repopulation index, miotic index, Leydig cell nuclear, tubular differentiation index, spermiogenesis index.

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

The impact of ischemia/reperfusion on spermatogenesis in testicular tissue is shown in Figure 1. The level of damage caused by testicular torsion/detorsion compared to the SHAM and testicular biopsy scores for the testicular sections from experimental groups are also presented in Table 1. Cross sections from testes of ISCHEMIA group showed seminiferous tubules with severe sloughing and disorganization. Damage was at the spermatozoa, spermatids and some spermatocytes level. The interstitial was disorganized and contained some blood. In, seminiferous tubules showed normal spermatogenesis, exhibiting all stages of spermatogenic cells including abundant spermatozoa. Some sloughing and tissue disorganization were observed ($p < 0.05$). Testis weight, epididymis weight, testis/body weight, Johnsen score, Cosentino score, seminiferous tubule diameter, Sertoli cell index, repopulation index, miotic index, Leydig cell nuclear, tubular differentiation index, and spermiogenesis index in IS/REP/CRPTXNTN 2.5 group showed significant improvements compared to other groups ($p < 0.05$).

Discussion

In the present study, it was investigated whether intraperitoneal administration of various concentrations of β-cryptoxanthin was useful in the prevention of testicular damage in ischemia/reperfusion conditions in rat testes and it was found that the concentration of 2.5 µg/kg showed the most beneficial effects. Histopathologic assessments were performed in experimental groups. The findings for IS/REP/CRPTXNTN 2.5 group were significantly different from those of other groups showing that β-cryptoxanthin at the concentration of 2.5 µg/kg could improve damages induced by ischemia.

Testicular torsion is a urological emergency that induces biochemical and morphological changes.[16] Testicular torsions 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

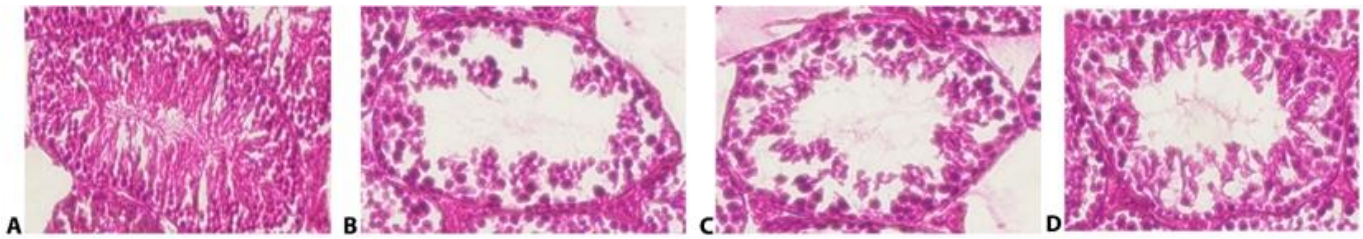

Figure 1. Representative histologic micrograph in different experimental groups. A) SHAM, B) ISCHEMIA group; C) IS/REP/Oil group and D) IS/REP/CRPTXNTN 2.5 group (hematoxylin and eosin staining, 400×).

Table 1. Histological parameters. Values are expressed as mean ± SEM.

Parameters	SHAM	ISCHEMIA	IS/REP/Oil	IS/REP/CRPTXNTN 2.5
Testis weight (g)	1.529±0.06 ^{a}	0.507±0.04 ^{c}	0.504±0.06 ^{c}	0.593±0.02 ^{d}
Epididymis weight (g)	0.783±0.04 ^{a}	0.261±0.05 ^{d}	0.267±0.02 ^{d}	0.324±0.04 ^{d}
Testis/body weight (%)	0.38±0.21 ^{a}	0.20±0.11 ^{d}	0.20±0.13 ^{d}	0.23±0.16 ^{d}
Johnsen score	8.39±0.19 ^{a}	3.88±0.36 ^{d}	3.95±0.29 ^{d}	4.65±0.32 ^{d}
Cosentino score	1.07±0.03 ^{d}	3.83±0.19 ^{a}	3.96±0.31 ^{a}	3.25±0.24 ^{b}
Seminiferous tubule diameter (STsD) (µm)	254.41±13.49 ^{a}	114.49±17.61 ^{d}	115.83±13.46 ^{d}	130.19±16.02 ^{d}
Sertoli cell index (SCI)	25.31±1.36 ^{a}	3.01±0.07 ^{e}	2.84±0.09 ^{e}	4.35±0.25 ^{d}
Repopulation index (RI)	79.21±2.18 ^{a}	35.93±1.44 ^{d}	36.89±1.61 ^{d}	37.31±1.78 ^{d}
Miotic index (MI)	3.80±0.43 ^{a}	0.79±0.25 ^{d}	0.83±0.49 ^{d}	1.05±0.04 ^{d}
Leydig cell nuclear (LCND) (cell/mm²)	9.46±0.31 ^{a}	2.88±0.15 ^{d}	2.85±0.46 ^{d}	3.19±0.63 ^{d}
Tubular differentiation index (TDI) (%)	80.05±2.69 ^{a}	45.78±1.63 ^{e}	47.50±1.39 ^{e}	51.62±1.73 ^{d}
Spermiogenesis index (SPI) (%)	75.13±3.84 ^{a}	45.21±1.40 ^{de}	44.04±1.60 ^{e}	48.62±1.45 ^{d}

$^{a\text{-}e}$ Different superscripts within the same row demonstrate significant differences ($p < 0.05$).

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] It has been demonstrated that blood flow following ischemia starts further damage to the reperfused tissue.[23] 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.[24,25] 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.[26]

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.[27-29]

Testicular I/R injury can significantly deteriorate spermatogenesis and may cause infertility. Spermatogenesis was evaluated by the Johnsen tubular score and seminiferous tubule diameter measurements in all experimental groups and found to be significantly damaged in the IS/REP group. However, the Johnsen tubular score and seminiferous tubule diameter were significantly higher in IS/REP/CRPTXNTN 2.5 group in comparison with other treatment groups. This finding indicated that β-cryptoxanthin (2.5 µg/kg) administration before detorsion decreased spermatogenesis damage and also could prevent testicular I/R injury induced infertility.

Changes in histological parameters (Testis weight, epididymis weight, Testis/body weight, Johnsen score, Cosentino score, seminiferous tubule diameter, Sertoli cell index, repopulation index, meiotic index, Leydig cell nuclear, tubular differentiation index, spermiogenesis index) have been used in the evaluation of the cell condition.[30] In consistent with the findings of the present study, it was reported that β-cryptoxanthin alleviated myocardial ischemia/reperfusion in rats.[31] In a rat model of hepatic I/R injury, supplementation with β-carotene has been shown to increase the level of antioxidants.[32] Moreover, β -carotene was demonstrated to protect the protects the gastric mucosa against ischemia-reperfusion injury in rats by inhibiting the peroxidation of lipids, improving the activity of antioxidant enzymes and preventing the infiltration of neutrophils.[33] Taken together, the findings suggested that β -carotene had multiple beneficial roles as a gastro protective agent, and might be responsible for the reduction in pro-in amatory cytokine release and the increase in anti-inflammatory cytokines.[34]

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. Intraperitoneal 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.[22] It seems time saving is very important in emergency conditions like ovarian torsion.

Although in the present study the outcomes were promising, however, extensive molecular assessments are required to evaluate outcomes of intraperitoneal administration of β-cryptoxanthin 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 β-cryptoxanthin at concentration of 2.5 µg/kg 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 β-cryptoxanthin on improving testicular function might be more complicated than our findings.

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Conflict of Interest

None.

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