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Preservation of Testicular Tissue and Alleviation of Oxidative Stress by Carvacrol Following Torsion/Detorsion in Adult Male Rats

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Abstract

Objectives: Testicular torsion is a critical medical condition necessitating prompt diagnosis and intervention. This study aimed to explore the potential safeguarding effect of carvacrol against histological damage and oxidative stress resulting from torsion/detorsion (T/D) in rat testes.

Materials and Methods: A total of 32 adult male rats were randomly divided into four groups. The sham group did not undergo any intervention. The second group received an intraperitoneal injection of 75 mg/kg carvacrol half an hour before detorsion application. The third group was administered 80 mg/kg carvacrol intraperitoneally without detorsion. The fourth group (control) experienced (T/D) through the administration of saline. Following a 5-hour reperfusion period, the left testis was excised for histological slide preparation. Blood serum was used to measure antioxidant enzyme levels. Data analysis was performed using the SPSS version 19 software and analysis of variance tests.

Results: Significant histological alterations were observed between the sham and other three groups. The levels of superoxide dismutase (SOD), glutathione peroxidase (GPx), and testosterone significantly decreased in all treatment groups compared to the control group ($P<0.05$). Conversely, malondialdehyde (MDA) levels increased in the torsion control group in contrast to the sham group ($P<0.05$). Carvacrol administration mitigated MDA levels in the treatment groups. Also, there were significant differences in tissue parameters between the sham and the other groups ($P<0.05$).

Conclusions: According to the results of this study, carvacrol possesses the potential to mitigate testicular tissue damage, enhance testicular function, and ameliorate oxidative stress consequential to testicular rotation.

Keywords: Torsion, Detorsion, Testis, Rat, Carvacrol

Introduction

Testicular torsion, characterized by the rotation of the testis around its axis, followed by subsequent detorsion, is a urological emergency that can lead to testicular damage and impaired fertility in males (1). Torsion/detorsion (T/D) injury triggers a cascade of pathological events, including ischemia-reperfusion injury, oxidative stress, inflammation, and histological alterations, which collectively contribute to testicular dysfunction. Therefore, exploring potential therapeutic interventions to mitigate the detrimental effects of T/D injury on testicular tissue is of utmost importance (2,3).

Oxidative stress, arising from an imbalance between reactive oxygen species (ROS) production and antioxidant defense mechanisms, is a critical mediator of tissue injury in various pathological conditions, including T/D injury (4). The testis is particularly vulnerable to oxidative stress due to its high polyunsaturated fatty acid content and low antioxidant capacity. Excessive ROS production during T/D injury leads to lipid peroxidation, protein oxidation, DNA damage, and activation of pro-inflammatory pathways, ultimately resulting in testicular dysfunction (5,6).

Histological alterations, another hallmark of T/D injury, involve disruption of the seminiferous epithelium, germ cell apoptosis, interstitial edema, and infiltration of inflammatory cells. These changes can impair spermatogenesis and adversely affect testicular function. Therefore, preserving the structural integrity of testicular tissue is crucial for maintaining reproductive health (1).

Carvacrol, a monoterpenoid phenol found in the essential oils of various aromatic plants, has gained attention in recent years due to its diverse pharmacological properties, including antioxidant, anti-inflammatory, and cytoprotective effects. Studies have demonstrated the beneficial effects of carvacrol in different pathological conditions, highlighting its potential as a therapeutic agent. However, the protective effects of carvacrol against testicular damage induced by T/D injury have not been extensively investigated (7-9).

Understanding the protective mechanisms of carvacrol against T/D-induced testicular injury can provide valuable

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Key Messages

- Testicular torsion/detorsion led to tissue damage in adult rats.
- Carvacrol can protect the testis tissue against oxidative damage.

insights into its therapeutic potential in urological emergencies. If proven effective, carvacrol could serve as a novel adjunct therapy for mitigating testicular damage caused by T/D injury, ultimately improving fertility outcomes in affected individuals. Accordingly, this study aimed to investigate the potential protective effect of carvacrol on T/D-induced testicular injury in adult male rats. We hypothesized that carvacrol administration could attenuate histological damage and oxidative stress in the testis, thereby improving testicular function following T/D injury.

Materials and Methods

Study Design

A total of 32 male Wistar rats weighing approximately 200 g were procured from the animal house of Tabriz University, Tabriz, Iran. Prior to the experimental phase, a one-week acclimation period was provided for the animals in the animal facility. During this time, the rats were housed in a controlled environment with a temperature of 25 °C and a standard 12-hour light-dark cycle to allow for adaptation (3). Based on the following formula, eight rats were considered for each group:

$$n = 1 + 2C\left(\frac{S}{d}\right)^2 = 1 + 2 * 7.85\left(\frac{0.212}{0.556}\right)^2 \approx 4$$

$$\acute{n}=n\sqrt{g-1}=4\sqrt{5-1}=8$$

The animals were allocated randomly into four groups, each containing eight rats. Group 1 (Sham), experienced a scrotal incision procedure devoid of any torsion or detorsion induction. The incision area was promptly sutured, and no T/D manipulation was executed. This particular group was designated as the control group for subsequent comparisons (n=8). Group 2, denoted as the torsion/detorsion group (TD), underwent a surgical intervention aimed at instigating testicular torsion. The left testis of each rat underwent a 720-degree anticlockwise rotation and was retained in this torsion state for a duration of 4 hours. Following the 4-hour torsion interval, the left testis was meticulously rotated back to its original orientation, simulating the detorsion procedure. Group 3, denoted as the T/D with carvacrol (TDC), underwent the same torsion process as the TD group. However, in this group, the animals were treated with carvacrol as an intervention. Carvacrol, a natural compound with known antioxidant and anti-inflammatory properties, was administered at a dose of 75 mg/kg, 30 minutes prior to the detorsion procedure. Subsequently, the rats received daily administrations of carvacrol for a period of two weeks (n=8). Group 4, known as the carvacrol group, consisted of healthy rats that did not undergo the T/D procedure. Instead, they were solely treated with carvacrol at a dose of 75 mg/kg for two weeks (8, 9). This group served as a control to evaluate the effects of carvacrol in the absence of testicular injury (n=8).

Throughout the experimental period, close monitoring of the rats was conducted to ensure their well-being and to detect any signs of distress or complications that may arise. After the two-week treatment period, the animals were euthanized to assess the effects of carvacrol on testicular tissue.

Surgery Procedure

To induce testicular T/D injury in the animal model, a combination of ketamine and xylazine was utilized for anesthesia at dosages of 50 and 10 mg/kg, respectively (6). The animals were carefully prepared under sterile conditions, and an abdominal incision was made to gain access to the left testis. The left testis was then rotated 720 degrees in a counterclockwise direction. Suturing of the scrotum ensured that the left testis remained in the torsion position for a duration of 4 hours (3,10).

Following the 4-hour torsion period, the sutures were removed and the left testis was gently rotated back into its normal position, simulating the detorsion process. The rats were then subjected to a treatment period for two weeks. Throughout this period, the animals were closely monitored and any necessary interventions or care were provided.

After the two-week treatment period, the animals were sedated and blood samples were collected for further analysis. The blood samples were obtained to assess various parameters, such as hormone levels, antioxidant enzyme activity, or other relevant markers (3,10).

Subsequently, testis tissue samples were harvested for histological and biochemical analyses. These tissue samples were obtained to evaluate the structural and cellular changes that occurred as a result of the T/D injury, as well as to investigate the potential effects of the administered treatment.

The utilization of this experimental model allowed for the examination of the therapeutic efficacy of the intervention during the treatment period. We also conducted a comprehensive evaluation of the physiological, biochemical, and histological changes induced by testicular T/D and the subsequent effects of the treatment by assessing both blood samples and testis tissue samples (3,10).

Histological Evaluation

After collection, the testicular specimens were immersed within Bouin's solution for a duration of two days to guarantee optimal fixation. The fixed samples were then subjected to a series of dehydration steps using graded

concentrations of ethanol. Subsequently, the dehydrated samples were embedded in paraffin wax, allowing for the preparation of thin sections (10).

The paraffin-embedded testicular samples were cut into 5 µm-thick sections using a microtome. These sections were then mounted on glass slides and subjected to hematoxylin and eosin (H&E) staining. The H&E staining procedure imparted distinct coloration to different cellular structures, facilitating their visual analysis under a light microscope (Olympus CX310, Japan).

The images of the stained sections were captured using a digital camera (Olympus, Japan) connected to the light microscope. The captured images provided a detailed view of the cellular architecture and morphology of the testicular tissues.

To ascertain the average diameter of the seminiferous tubules (STD) and the depth of the germinal epithelium (HE), 10 randomly selected seminiferous tubules were measured per slide. The measurements were conducted using the calibrated scales available on the eyepiece of the light microscope at an appropriate magnification.

Furthermore, spermatogenesis in the seminiferous tubules was assessed based on Johnson's scoring system. For this assessment, a total of 50 seminiferous tubules were observed and analyzed per section. Each individual tubule was subjected to meticulous examination under a light microscope, and a numerical score, ranging from 1 to 10, was assigned in accordance with the attributes exhibited by the epithelial structures. This scoring system provided a quantitative measure of spermatogenic activity and served as a means to evaluate the impact of the experimental conditions on testicular function and spermatogenesis.

Determination of Serum Oxidative Stress Markers

To determine the levels of malondialdehyde (MDA) in serum samples, a series of procedures were performed. Firstly, 0.20 cm^3 of serum samples was combined with 3.0 cm^3 of glacial acetic acid in a microtube. Subsequently, a solution containing 1% thiobarbituric acid (TBA) in 2% sodium hydroxide (NaOH) was added to the microtube. The microtube was then subjected to boiling water for 15 minutes. After cooling the samples, the absorbance of the resulting pink-colored solution was measured at a wavelength of 532 nm using a spectrophotometer (Biospect, USA). To establish a calibration curve, MDA tetrabutylammonium salt obtained from the Sigma Aldrich, USA was used as standard solutions for MDA.

The levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) were determined using commercially available kits from the Sigma, USA. The ELISA method was employed to read the measurements using Ransod and Randox kits, following the manufacturer's instructions. To analyze oxidative stress markers in testicular tissues, the tissues were homogenized using a homogenizer. A 20% w/v homogenate was prepared using ice-cold potassium phosphate buffer. The homogenate was then centrifuged at 4000 rpm for 15 minutes, and the resulting supernatant was collected for further analysis of SOD, GPx, and MDA.

Measurement of Serum Hormones Levels

Following blood collection, the samples were subjected to centrifugation at 5000 rpm for 5 minutes. To assess the levels of testosterone in the serum samples, ELISA technique was utilized. For this study, we employed a specific ELISA kit obtained from the Demeditec Diagnostics, Germany (11).

Statistical Analysis

Statistical analysis was conducted using the SPSS software, version 19. All the obtained results were presented as mean ± standard deviation (SD), providing a measure of the variability within the data. To evaluate the differences among the 4 groups, one-way analysis of variance (ANOVA) was employed. Following the ANOVA analysis, a Tukey test was applied as a post hoc test to further examine pairwise differences between the groups. In this study, a significance level of $P<0.05$ was chosen as the threshold for statistical significance.

Results

Histopathological Evaluations

Histopathological assessment was conducted to evaluate the tissue changes in different experimental groups. Johnson's scoring system was utilized to quantify the severity of histopathological alterations. The findings revealed a noteworthy reduction in Johnson's scores within the TD group compared to the sham group ($P=0.001$), indicating more pronounced pathological changes in the testes of the TD group. Consistent with this, the TD group exhibited reduced STD and compromised HE compared to the sham group (Figure 1).

Additionally, histological samples extracted from the TD group exhibited more pronounced and severe abnormalities within the seminiferous tubules, providing further evidence of the detrimental impact of T/D on testicular tissue. In contrast, the TDC group, which received carvacrol treatment prior to detorsion, exhibited a significant improvement in pathological changes compared to the TD group ($P=0.001$).

The summarized results of Johnson's scores, STD, and HE levels in different groups are presented in Table 1. Importantly, the healthy groups treated with carvacrol at a dose of 75 mg/kg did not display significant differences in Johnson's scores, STD, and HE levels compared to the sham group ($P>0.05$); this indicates that carvacrol alone could not induce notable histological changes in the testicular tissue (Figure 1).

The Oxidative Stress Markers Levels in Serum

The serum levels of MDA in the TD group were notably higher compared to the sham group ($P<0.01$),

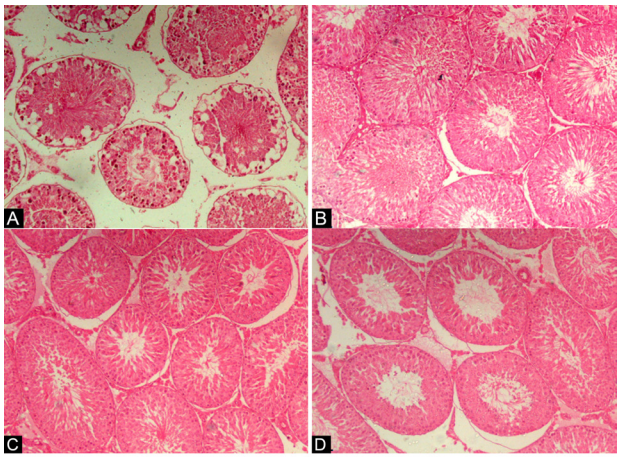

Figure 1. Testicular tissue histopathology in different study groups. (A) Control torsion/detorsion group, (B) Sham group, (C) Healthy group receiving carvacrol, (D) torsion/detorsion group receiving carvacrol

indicating increased oxidative stress in the testicular tissue. Conversely, the levels of SOD and GPx showed a markable decrease in the TD group compared to the sham group ($P<0.05$), suggesting impaired antioxidant defense mechanisms. In contrast, in the TDC group, the serum levels of MDA decreased, indicating a reduction in oxidative stress, while the levels of SOD and GPx significantly increased ($P<0.05$), indicating enhanced antioxidant capacity.

Additionally, the analysis of serum MDA levels between the TDC group and the TD group revealed a substantial reduction in MDA levels within the TDC group ($P<0.01$), indicating a systemic reduction in oxidative stress due to carvacrol treatment. The serum level of SOD in the TDC group was significantly higher compared to both the TD and sham groups ($P<0.05$), indicating an improved antioxidant status. Conversely, the serum concentrations of GPx were significantly diminished in all TD groups in contrast to the sham group, indicating a notable decline in antioxidant capacity (Table 2). Notably, the levels of GPx were elevated in the TDC group compared to the TD group ($P<0.01$), indicating a beneficial effect of carvacrol in restoring GPx levels.

Serum Testosterone Levels

The serum testosterone level in the TD group was significantly lower compared to the sham group ($P<0.01$), indicating a negative impact of testicular T/D on testosterone production. However, administration of carvacrol in the TDC group resulted in a substantial elevation of serum testosterone levels ($P<0.05$) (Figure 2).

Discussion

Testicular T/D is a urological emergency that can lead to testicular ischemia-reperfusion injury and subsequent testicular dysfunction. In this study, we investigated the potential protective effects of carvacrol on testicular injury induced by T/D in a rat model. Our findings revealed significant alterations in various parameters, including histopathological changes, oxidative stress markers, and serum testosterone levels, highlighting the potential therapeutic role of carvacrol in mitigating testicular

Table 1. Comparison of Mean Testis Johnson's Score, Tumor Tube Diameter, and Epithelium Height in the Study Groups

Groups	MJS Mean ± SD	STD Mean ± SD	HE Mean ± SD
Sham	9.75 ± 0.025	158.42 ± 1.41	70.02 ± 2.21
TD	4.25 ± 0.025[a]	142.80 ± 1.63[a]	32.12 ± 2.32[a]
TDC	7.32 ± 0.015[a,b]	212.18 ± 1.30[a,b]	59.18 ± 1.6†[a]
Carvacrol	9.87 ± 0.19[b]	167.32 ± 2.35[b]	70.30 ± 3.45[b]

MJS: mean Johnson's score; STD: seminiferous tubule diameter; HE: the height of seminiferous epithelium; SD: standard deviation.
TD: group subjected to testicular torsion/detorsion; TDC: torsion/detorsion + carvacrol group, Carvacrol: a group of healthy rats without torsion/detorsion that received carvacrol
[a] shows a significant difference with sham group and [b] shows a significant difference with TD group.

Table 2. Comparison of Oxidative Stress Markers in the Study Groups

Groups	SOD Mean ± SD	MDA Mean ± SD	GPx Mean ± SD
Sham	0.70 ± 0.015	1.89 ± 0.041	1.70 ± 0.21
TD	2.20 ± 0.018[a]	0.65 ± 0.063[a]	0.82 ± 0.02[a]
TDC	1.48 ± 0.015[a,b]	1.12 ± 0.050[a,b]	1.25 ± 0.06[a,b]
Carvacrol	0.68 ± 0.019[b]	1.86 ± 0.035[b]	1.75 ± 0.045[b]

SOD: superoxide dismutase; GPx: glutathione peroxidase; MDA: malondialdehyde; SD: standard deviation.
TD: group subjected to testicular torsion/detorsion; TDC: torsion/detorsion + carvacrol group, Carvacrol: a group of healthy rats without torsion/detorsion that received carvacrol
[a] shows a significant difference with sham group and [b] shows a significant difference with TD group.

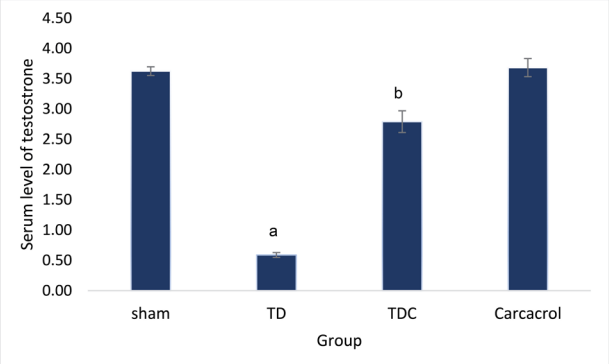

Figure 2. The Testosterone Serum Levels in The Study Groups. TD: group subjected to testicular torsion/detorsion; TDC: torsion/detorsion + carvacrol group, Carvacrol: a group of healthy rats without torsion/detorsion that received carvacrol. [a] shows a significant difference with sham group and [b] shows a significant difference with TD group.

damage (12,13).

Histopathological analysis plays a crucial role in assessing tissue damage and pathological changes (14). In our study, the Johnson's score, which reflects the severity of testicular injury, was significantly diminished in the TD group compared to the sham group. This observation suggests that testicular T/D induces substantial histopathological alterations, including STD and impaired spermatogenesis. These findings are consistent with previous studies documenting the detrimental effects of testicular T/D on testicular morphology (14). However, treatment with carvacrol in the TDC group resulted in less severe pathological changes compared to the TD group, as indicated by an improvement in the Johnson's score. These results suggest that carvacrol administration may exert a protective effect against testicular injury induced by T/D.

Oxidative stress is a key contributor to tissue damage during testicular T/D. The ROS generated during ischemia-reperfusion can lead to lipid peroxidation and cellular oxidative damage (15). In our study, we assessed oxidative stress markers, including MDA, SOD, and GPx to evaluate the antioxidant potential of carvacrol (8,9). The testicular MDA levels were significantly increased in TD group compared to the sham group, indicating enhanced lipid peroxidation and oxidative stress in response to T/D. Conversely, treatment with carvacrol in the TDC group led to a decrease in testicular MDA levels, suggesting a reduction in lipid peroxidation and oxidative stress. These findings highlight the antioxidant properties of carvacrol and its ability to mitigate oxidative damage in testicular tissue subjected to T/D.

Moreover, the levels of SOD and GPx, which are important enzymatic antioxidants involved in scavenging ROS, were significantly decreased in the TD group compared to the sham group. This decrease in enzymatic antioxidants further indicates the impaired antioxidant defense system in response to testicular T/D. However, treatment with carvacrol in the TDC group resulted in a significant increase in SOD levels and a decrease in GPx levels compared to the TD group (13,16). These findings suggest that carvacrol may enhance the antioxidant defense system by promoting the activity of enzymatic antioxidants, thus reducing oxidative stress in the testicular tissue.

Another important parameter affected by testicular T/D is the serum testosterone level (3,4,14). Testosterone is a crucial hormone for male reproductive function, and any disruption in its production can lead to adverse effects on spermatogenesis and fertility. In our study, the TD group exhibited a significantly lower serum testosterone level compared to the sham group, indicating testicular dysfunction resulting from T/D (17). However, treatment with carvacrol in the TDC group resulted in a significant increase in serum testosterone levels compared to the TD group (18,19). These findings suggest that carvacrol may have a positive effect on testosterone production, potentially preserving or enhancing testicular function in the context of T/D.

The mechanisms underlying the protective effects of carvacrol in testicular T/D are likely multifactorial. Carvacrol possesses potent antioxidant properties and can scavenge free radicals, thereby reducing oxidative stress and lipid peroxidation. Additionally, carvacrol has been reported to exhibit anti-inflammatory effects, which may further contribute to its protective actions in testicular injury. Further studies are needed to elucidate the precise molecular mechanisms involved in the protective effects of carvacrol and its potential targets in testicular tissue (20,21).

Limitations of the Study

In this study, we did not investigate the reproductive power of rats and the expression of proteins and genes related to apoptosis in the testicular tissue due to financial deficiencies.

Conclusions

The results of this study demonstrated the potential therapeutic benefits of carvacrol in mitigating testicular injury induced by T/D. Carvacrol administration effectively reduced histopathological changes, attenuated oxidative stress, and improved serum testosterone levels in the experimental model. These findings highlight the promising protective effects of carvacrol and its potential as a therapeutic agent for the management of testicular T/D. Further investigations are warranted to explore the precise mechanisms of action and to evaluate the long-term effects of carvacrol treatment in preserving testicular function and fertility in a clinical setting.

Authors' Contribution

Conceptualization: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.

Data curation: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.

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Investigation: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Methodology: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Project administration: Arash Khaki.
Resources: Arash Khaki.
Software: Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Supervision: Arash Khaki.
Validation: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Visualization: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Writing–original draft: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.
Writing–review & editing: Arash Khaki, Ghazal Rahmanifar, Afshin Teymoori, Arman Khatami, Ali Rezaii.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

This study was approved by the Animal Ethics Committee of Islamic Azad University of Tabriz, Iran (No. IR.IAU.TABRIZ.REC.14014.220).

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