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Effects of Syzygium Aromaticum (Clove) Extract on Male Fertility Factors and Oxidative Stress After Torsion/Detorsion in Adult Male Rats Using Intrauterine Insemination Method

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Abstract

Objectives: The current study was conducted on adult male models to assess the impact of the Syzygium aromaticum (clove) extract on male fertility factors and oxidative stress after torsion/detorsion using the intrauterine insemination (IUI) method.

Materials and Methods: This experimental study was performed on 56 adult male Wistar rats including 28 males and 28 females. The male subjects were randomly assigned to four groups of sham (G1), 4 hours of testicular torsion following a surgical torsion/detorsion (TD/G2), TD treated with the clove extract (4 mg/kg, orally/G3) 30 minutes before detorsion, and healthy subjects treated with the clove extract (4 mg/kg/G4). The levels of blood testosterone and some oxidative stress indices were investigated in the testis tissue. In addition, some sperm parameters were evaluated, including the concentration, motility, and morphology of the sperm. Finally, the fertilization potential of adult female rats was assessed through the IUI method.

Results: The histological evaluation revealed considerable adverse changes in the G2 in comparison with the sham group. The serum levels of testosterone, and glutathione peroxidase, and superoxide dismutase meaningfully reduced in the testis of rats in the G2. In addition, the malondialdehyde level was significantly higher during the ischemia although all the mentioned changes improved in the treated groups. Nonetheless, the sperm quality and fertility power considerably reduced in the G2 compared to the sham group.

Conclusions: The results of the current experimental study demonstrated that the testicular torsion/detorsion has an adverse impact on the testis function and decreases the fertilization potential, and finally, treatment with the clove extract can improve these adverse changes.

Keywords: Oxidative stress, Clove, Testis, Fertility, Intrauterine insemination

Introduction

Testicular torsion is one of the urological emergencies, commonly presenting with acute scrotal or abdominal pain and vomiting (1-3). The incidence of testicular torsion is estimated to be 1 per 4000 males, and it can affect males of any age. Nevertheless, it is known to be more prevalent in younger individuals (4-8). Testicular torsions are resulted from spermatic cord rotation, leading to the arterial and vain blockage of the testis, and consequently, the discoloration of the testis and necrosis. In this regard, the prognosis of testicular torsion depends on the timely diagnosis and surgical management of the disease (9-11). Accordingly, late interventions may result in the ischemic infarction and necrosis of the twisted testicle, and infertility (11,12). In addition, low sperm counts and poor sperm motility are reported to be the probable consequences of testicular torsions in spite of persevering the twisted testicle (10,13). Furthermore, ensuing the restoration of blood flow, lipid peroxidation, excessive production of reactive oxygen species (ROS), and activation of the enzymatic antioxidant defense system associated with ischemic reperfusion may lead to DNA and endothelial injury and apoptosis. According to some studies, these injuries may decrease through the administration of exogenous antioxidants (10,11).

The *Syzygium aromaticum* (L., clove extract), which is derived from the clove tree of the Myrtaceae family, is known for antibacterial, anti-inflammatory, antifungal, anesthetic activity, and more importantly, the antioxidant influence due to the presence of phenolic compounds such as eugenol, thymol, and eugenol acetate (6,14-17). Previous research reported the strong potential of the clove oil for ROS scavenging activity (18). In this regard, it is estimated that the clove essential oil has ten times greater antiradical activity than butylated hydroxyanisole. According to a previous study, clove buds can elevate the testosterone

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Key Messages

- Testicular torsion/detorsion decreases the quality of sperm.
- *Syzygium aromaticum* can improve the sperm quality.

level, motility of sperms, and the secretory activities of the epididymis and seminal vesicle (6). Considering the negative impact of torsion on sperm function and the role of oxidative stress in testicular injuries associated with torsion, the current study aimed to investigate the effect of the clove extract on male fertility factors and oxidative stress after torsion/detorsion (TD) in adult male rats by the intrauterine insemination (IUI) method.

Materials and Methods

Study Design

The current experimental study was conducted on 56 adult male Wistar rats (including 28 males and 28 females) with an average weight of 250-300 g. All the experimental rats were kept in the animal house of Gonabad University of Medical Sciences under the standard conditions (at the temperature of 23 ± 2°C, humidity of 60%-70%, and a 12/12-hour light/dark cycle) with free access to sufficient food and water. Male rats were categorized into four groups (n = 7) of sham (G1), testicular torsion for four hours followed by surgical TD (G2), TD receiving the clove extract (4 mg/kg, orally) 30 minutes before the detorsion (TDOJ/G3), and healthy rats that only received (4 mg/kg) the clove extract (OJ/G4). The study protocol was approved by the Ethics Committee of Gonabad University of Medical Sciences.

Surgical Procedure

All the surgical interventions were initiated after anesthesia with ketamine/xylazine (5/1 mg/kg). First, the left testis of male rats was exposed and rotated 720° counterclockwise through an incision on the scrotum. Then, the tunica albuginea of the twisted testicle was sutured to the dartos muscle with three 6/0 silk sutures, and finally, the scrotum was closed using 5/0 silk sutures. The testicles were kept twisted for four hours and then detorsion surgery was performed as well. After fourteen days, the blood sample was gathered from the superior vena cava vein under anesthesia. Blood samples were centrifuged at 3000 rpm (for 10 minutes) and were kept at -70 °C for the assessment of the testosterone hormone level after separating the serum. On day fourteen, bilateral orchiectomy was performed on male rats, and some of their left testicles were fixed in Bouin's fluid and some of them were used for the assessment of the oxidative stress marker.

IUI Procedure

Sperm Extraction from Epididymis: After the reperfusion period, the epididymis tail of male rats was dissected and placed into the 5.0 mL of Hams F10 solution. Several cuttings were made in the epididymis with scissors so that the sperm could get out and float for five minutes. Then, some suspensions were investigated for morphology, motility, and count of the sperm. The suspension of the sperm was maintained in the incubator at 37°C for 20 minutes until insemination.

Insemination: After initial anesthesia, the caudal part of the uterus was exposed by an incision in the low midline of the abdomen of the female rats. Then, the sperm suspension (0.1 mL) was injected into each horn of the uterine lumen and the incision was closed with 5/0 silk sutures (19).

Counting of Embryos

After three days, the fallopian tubes, the rostral portion of uterine horns, and both ovaries of female rats were dissected and placed in a plate. Then, a 27-gauge needle was put into the infundibulum of the uterine horn, and then the preimplantation embryos were collected by phosphate-buffered saline (PBS) flushing and the number of embryos was counted under a stereomicroscope. Female rats were considered to be not pregnant when there were unfertilized oocytes although there was at least one embryo in the pregnant rats (19).

Tissue Preparation

Dissected testicles were put into falcon tubes containing Bouin's solution for 48 hours. Afterward, the steps of the tissue passage were conducted, and paraffin-embedded testicles were cut into 5 µM thickness slides. Each slide was stained with hematoxylin and eosin (H&E) and then investigated under an optical microscope (Nikon, Japan) at the magnification of 400X (20,21).

Histopathological Evaluations

The testicles of male rats were dehydrated with an ascending ethanol sequence, cleared with xylene, and embedded in paraffin in a bid to examine histological changes in the seminiferous tubules of fixed testicles. The spermatogenic cells were counted in each tubule, and the slides were examined in terms of pathology (20). For the morphometric evaluation of sperms, thirty round or nearly round seminiferous tubules were randomly selected from each slide. Furthermore, the morphology was evaluated using a microscope with linear eyepiece grids at the magnification of 400X. (20). Finally, the counting of the germ cell was performed by ImageJ software.

Measurement of Malondialdehyde, Superoxide Dismutase, and Glutathione Peroxidase Levels in the Testis Tissue

Testis tissues were homogenized to assess oxidative stress indices. The level of lipid peroxidation was investigated through assessing the malondialdehyde (MDA) level. The thiobarbituric acid-trichloroacetic acid-hydrochloric acid (TBA-TCA-HCL) solution was made by the following

procedure.

A 375 mg of TBA was liquefied in 2 mL of HCL, and then the attained solution was added into 100 mL of 15% TCA. The water bath with a temperature of 50°C was administered to resolve the sediment. The tissue samples were weighed and homogenized instantly with a solution of potassium chloride 5.1% to achieve a 10% homogenized-mixed solution. Next, 1 mL of the homogenized tissue mixture was resolved in 2 mL of TBA-TCA-HCl solution, and it was warmed in boiling water for 45 minutes (pink-orange solution). The solution was centrifuged for 10 minutes at 1000 rpm after cooling. The solution absorption (A) was assessed through a spectrophotometer (Biospect) at 532 nm. Then, the activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) enzymes of the testis tissue was evaluated by an enzyme-linked immunosorbent assay (ELISA) reader device (Antus) following the instruction of the kits (Randox, and Ransod, UK).

Serum Testosterone Measurement

The serum level of testosterone was assessed according to the instruction of the kit of the testosterone ELISA (Demeditec Diagnostics, Germany), and the absorbance of the product was evaluated at 405 nm.

Sperm Counting

The left epididymis was pounded in 5 mL PBS (pH = 7.2), and a new solution was made by dissolving 100 λ of sperm solution in 900 λ of PBS. Then, one drop of the solution was poured into the Neubauer chamber and the count of the sperm was investigated based on the World Health Organization (WHO) protocol (9, 22).

Morphology of Sperm

Once sperm smears were prepared for morphology evaluations, the slides were dried through air exposure, fixed with alcohol 96%, and stained with H&E. Afterward, 150 sperms in each slide were randomly counted, and the percentage of normal and abnormal sperms were reported based on the WHO protocol (9,22).

Statistical Analysis

Statistical analysis was performed by SPSS software, version 18 (IBM, USA). The normality of data was determined using the Kolmogorov-Smirnov test. Moreover, a one-way ANOVA test, followed by a post-hoc Tukey's HSD test was used for comparison purposes. All data are presented as the mean ± standard error (SE), and $P < 0.05$ was considered as the level of statistical significance.

Results

Histopathological Parameters of Testis

Table 1 and Figure 1 indicate the histopathological parameters of the testis. The count of spermatogonial cells, primary spermatocytes, and round-shape spermatid meaningfully reduced in the TD group in comparison to the sham group. All the mentioned parameters elevated in the group receiving the clove extract ($P < 0.05$). On the other hand, the pathological examination showed the hemorrhagic and inregulation in the seminiferous tubule of the TD group although treatment with the clove extract replaced this damage.

Testosterone

The testosterone serum level notably decreased in the TD group compared to the sham group ($P < 0.05$). Nevertheless, its level was significantly higher in the treated groups in comparison with the TD group (Figure 2).

Oxidative Stress Markers

The levels of GPx and SOD meaningfully decreased in the TD group compared to the sham group ($P<0.05$). Conversely, the levels of SOD and GPx in the testis tissue raised significantly in the treated groups with clove extract ($P<0.05$). Eventually, the MDA level reduced ($P\leq0.01$) in the treated groups in spite of the meaningful elevation of the MDA serum in G2 compared to G1 (Table 2).

Parameters of the Sperm

A significant reduction was demonstrated regarding the sperm count in the semen analysis of the G2 group in comparison with the G1 group. Contrarily, the sperm count significantly elevated in the therapeutic groups as compared to G2 ($P<0.02$). Moreover, the morphology assessment of the sperm in different groups revealed a higher percentage of abnormal sperms in the TD group compared to the sham group. Conversely, the percentage of the abnormal sperm decreased in TDCE and CE groups in comparison to the TD group ($P\leq0.001$). The investigated sperm motility showed a notably lower sperm motility rate in G2 than G1. However, the sperm motility increased ($P\leq0.001$) in the treated groups (Table 3, Figure 3).

Table 1. The Count of the Germ Cell in Study Groups

Groups	Round Spermatid	Primary Spermatocytes	Spermatogonia Cells	Sertoli Cells
Sham	200.35±2.85	105.65±2.25	45.10±1.05	35.04±2.07
TD	90.01±2.07	48.25±2.60	13.55±2.15	20.12±1.05
TD + Clove	148.20±1.90*	78.54±4.36*	28.54±2.08	25.41±1.25
Clove	215.10±4.85*	111.41±2.25	50.35±1.05	35.15±2.07

Note. TD: Torsion/Detorsion.

* shows a significant difference between the control and TD group and + indicates a significant difference between the TD and TD treatment groups ($P<0.05$).

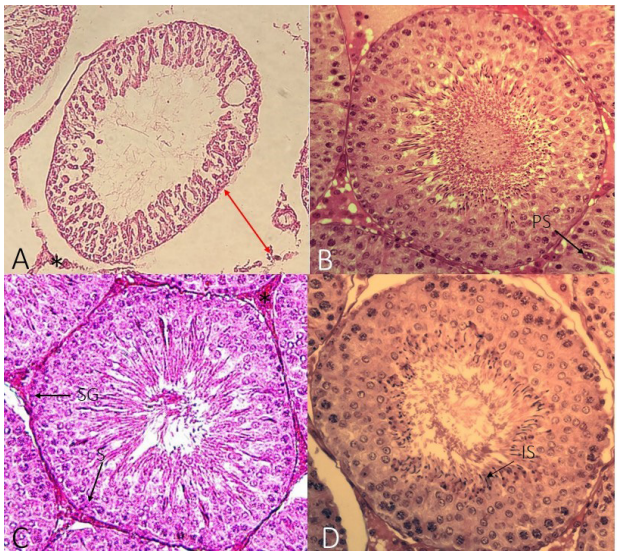

Figure 1. Histopathological Changes in the Study Group (×400). Note. TD: Torsion/Detorsion; A: TD control group without ant treatment, B: Sham group, C: TD group treated with Clove, D: Health group that received the clove extract. SG: Spermatogonia cells; PS: Primary spermatocytes; IS: Immature Spermatid, S: Sertoli cells. The asterisk * shows the hemorrhage in the intraluminal space in TD groups. The red arrow displays the enhancement in the intratubular space in the TD group.

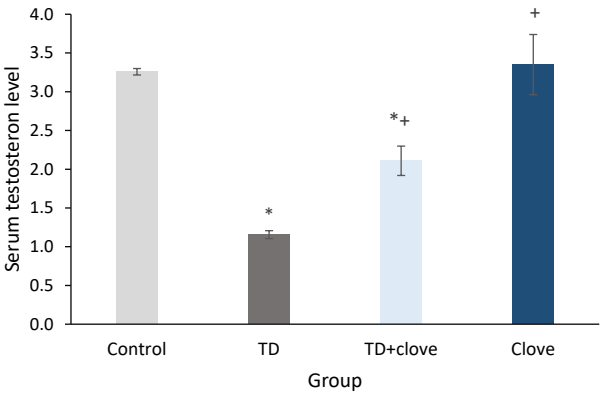

Figure 2. A Comparison of Testosterone Levels in the Study Groups. Note. TD: Torsion/Detorsion.

The Rate of Pregnancy and the Number of Embryos

The evaluated pregnancy rate by the IUI method demonstrated that all rats in the sham group were pregnant whereas only one rat was pregnant in the TD group, and consequently, a notable difference was observed between the groups in this regard (P<0.02). Additionally, a significant difference was found in the TD group in comparison with the sham group (P<0.01). In the TDCE group, 5 (n = 7) out of all rates were pregnant whereas in all rats (n = 7) were pregnant the CE group. In this regard, the rate of pregnancy in CEOJ and CE groups was notably higher (P<0.02) than that of the TD group (Table 4).

Discussion

Torsion is one of the main urological emergencies, specifically at young ages. Moreover, early diagnosis and the intervention of torsion play a crucial role in further associated complications such as infertility (19,20). It is noteworthy that later therapeutic interventions lead to a poorer prognosis of the twisted testicles (12). More precisely, the golden time of surgery is within 4-6 hours, and the rate of testis survival reduces to 50% and 10% after 12 and 24 hours, respectively (21). In this regard, the reperfusion followed by detorsion brings about excessive hazardous metabolites such as ROS and inflammatory cytokines, are were produced under the ischemic circumstances (10). Consequently, these toxic substances may lead to further injuries of germ cells. The increased levels of oxidative markers after reperfusion, which is well-documented in the literature, indicate the importance of oxidative stress in torsion-related injuries

Table 2. The Concentration of Oxidative Stress Markers in the Testis Tissues of the Rats of Four Groups

Groups	MDA (Mean ± SE)	GPX (Mean ± SE)	SOD (Mean ± SE)
Sham	55.08 ±1.13	3.65 ± 0.057	25.08 ± 1.38
TD	170.05 ± 2.04[a]	0.88 ± 0.048[a]	12.15 ± 1.33[a]
TD + Clove	90.57 ± 1.14[b]	2.72 ± 0.032[b]	19.25 ± 1.31[b]
Clove	52.03 ± 1.02[b]	3.88 ± 0.027[b]	26.40 ± 1.22[b]

Note. TD: Torsion/Detorsion; MDA: Malondialdehyde; GPx: glutathione peroxidase; SOD: superoxide dismutase; SE: Standard error.
[a] in comparison with the sham group (P=0.001) and [b] in comparison with the TD group (P=0.001).

Table 3. The Comparison of Sperm Parameters in Study Groups

Groups	Concentration, Mean ± SEM (%)	Normal Morphology, Mean ± SEM (%)	Normal Motility, Mean ± SEM (%)
Sham	35.18 ± 2.11	72.0 ± 5.08	70.0 ± 2.31
TD	10.15 ± 3.24[a]	15.0 ± 2.35[a]	13.62 ± 3.49[a]
TD + Clove	24.25 ± 2.17[b]	54.34 ± 4.21[b]	50.21 ± 2.21[b]
Clove	36.36 ± 1.08[b]	75.16 ± 2.08[b]	72.34 ± 3.14[b]

Note. TD: Torsion/Detorsion; SEM: standard error of mean. The letter **a** shows a significant difference with the sham group and the letter **b** represents a significant difference with the TD group (P≤0.05).

Table 4. The Comparison Results of IUI in Study Groups

Groups	Pregnancy Rate (%)	Number of Embryo Left Horne	Number of Embryo Right Horne
Sham	100	5 ± .725	4 ±0 .816
TD	16.28	0.12 ± 0.37*	0 ± 0*
TD + Clove	70.55[a]	1.50 ± 1.46[a]	1.85 ± 1.34[a]
Clove	100[a]	4 ± 1.27[a]	5 ± 0.75[a]

Note. IUI: Intrauterine insemination; TD: Torsion/Detorsion. The asterisk * denotes a significant difference with the sham group and the symbol a shows a significant difference with the TD group ($P \leq 0.05$).

(3,22,23). Furthermore, research has shown the positive impact of antioxidant administration on the function of preserved testis in the setting of TD (3). Furthermore, the clove extract is a well-known antioxidant herb and its antioxidant activity has been observed in numerous studies (18). In this regard, the current study sought to investigate the effect of the clove extract on male fertility factors and oxidative stress after TD in adult male rats by the IUI method.

In our study, the serum level of testosterone notably decreased after TD. The decreased level of testosterone after TD represents a testicular injury probably due to the excessive production of ROS, nitric oxide, lipid peroxidation, and inflammatory factors. Some studies have reported that inflammatory factors and cytokines can trigger the apoptosis of caspase cascade enzymes in germinal cells through reducing the blood flow (10,24,25). Interestingly, the level of testosterone was justified significantly in the group treated with the clove extract. Moreover, the levels of GPx and SOD meaningfully decreased in the treated group with the clove extract compared with the sham group. Based on the mentioned evidence, this extract could notably improve TD injuries through its scavenging properties.

Consistent with previous studies (3,9), the motility and sperm count decreased after torsion/detorsion in the present study. Similar to the level of testosterone, the motility and sperm count may be associated with germ cell apoptosis and excessive production of ROS. Likewise, the decreased level of testosterone can affect sperm production quality. Accordingly, the ovaries of female rats fertilized through the IUI method by the sperms of TD male rats were examined to assess the fertility rate (10). Based on our findings, the rate of pregnancy and the count of embryos significantly reduced in the TD group. However, a decreased pregnancy rate was expected due to the mentioned adverse effect of TD on sperm function and testosterone level. Similar findings were observed in previous studies (6,10).

As previously discussed, the level of MDA represented a notable increase after TD, suggesting the possible role of lipid peroxidation in the TD-associated injuries. Nevertheless, the level of MDA reduced significantly after treatment with the clove extract. In addition, the serum level of antioxidant enzymes (e.g., SOD and GPx) decreased meaningfully after TD. Correspondingly, the mentioned enzymes significantly increased after treatment with the clove extract.

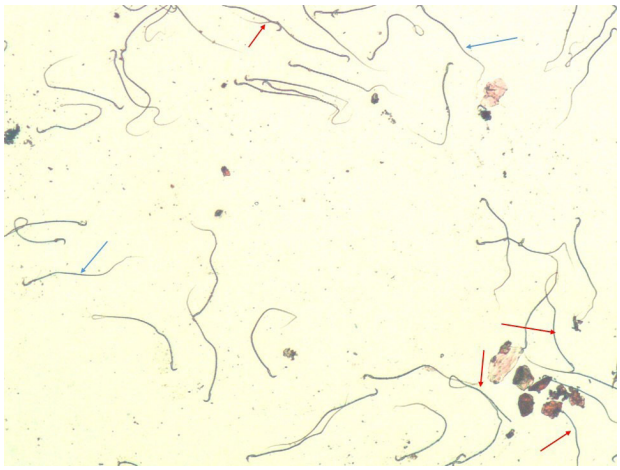

Figure 3. The Morphology of the Sperm. Note. The red and blue arrow depict abnormal and normal sperms, respectively.

The results of the current study showed that the clove extract can improve the TD injury and thus the fertility rate. The antioxidant properties of this extract were associated with phenolic compounds such as eugenol, thymol, and eugenol acetate (26).

Conclusions

In our study, clove extract administration improved the motility of sperms, the sperm count, and the serum level of testosterone. Further, the pregnancy rate increased following treatment with the clove extract. Our study results revealed that this extract reduces lipid peroxidation while increasing the level of antioxidant enzymes.

Authors' Contribution

MS, MH, TP, and MM planned and designed the experiments. MS performed the experiments. MS and HN analyzed the data. MS and BB and HK wrote the manuscript. MS and HN reviewed and discussed the data.

Conflict of Interests

Authors declare that they have no conflict of interests.

Ethical Issues

The current study was approved by the Ethics Committee of Gonabad University of Medical Sciences.

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