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Investigating the Therapeutic Potential of Clove Extract in Mitigating Testicular Injury Associated With Testicular Hypoxia in Male Rats

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Abstract

Objectives: This study aims to comprehensively assess the therapeutic potential of clove extract in mitigating testicular damage and preserving male reproductive function under hypoxic conditions in male rats.

Materials and Methods: We randomly divided 24 male rats into three groups: a hypoxia group that just received normal saline, a hypoxia + clove extract (4 mg/kg) group, and a sham operation group without treatment. After eight weeks, the serum level of oxidative stress parameters was examined, and the testicle histopathological examination, such as seminiferous tubule diameters and epithelium thickness, was assessed.

Results: The serum malondialdehyde (MDA) concentration was significantly enhanced in the hypoxic group, and the levels of superoxide dismutase (SOD) and glutathione peroxidase (GPx) declined ($P<0.05$). The diameters and thickness of the seminiferous tubule were diminished notably ($P<0.05$). Johnson's score decreased in the hypoxic group. Treatment with clove extract also led to improvements in this parameter.

Conclusions: Our study provides compelling evidence for the therapeutic potential of clove extract in mitigating testicular injury associated with varicocele-induced testicular hypoxia in male rats.

Keywords: Testis, Hypoxia, Clove, Oxidative stress, Male fertility

Introduction

Testicular hypoxia, characterized by a deficiency in oxygen supply to the testicular tissue, poses a significant threat to male reproductive health by instigating various pathological conditions, including testicular apoptosis. Testicular apoptosis, or programmed cell death in the testes, is a critical mechanism underlying infertility and reproductive dysfunction in males. Despite advancements in understanding the etiology of testicular hypoxia, effective therapeutic interventions remain elusive. However, emerging evidence suggests that natural compounds, such as clove extract, hold promise in attenuating testicular apoptosis associated with hypoxic conditions (1-3).

Clove (*Syzygium aromaticum*) is a well-known spice derived from the dried flower buds of the clove tree, indigenous to Indonesia and other parts of Southeast Asia. Beyond its culinary applications, clove has a long history of use in traditional medicine, particularly for its analgesic, anti-inflammatory, and antioxidant properties. The bioactive constituents of clove, including eugenol, eugenyl acetate, and β-caryophyllene, exhibit potent antioxidant and cytoprotective effects, which could be harnessed for mitigating testicular injury induced by hypoxia (4-7).

Testicular hypoxia triggers a cascade of events leading to oxidative stress, inflammation, and, ultimately, apoptosis of germ cells and Sertoli cells within the testes. Apoptotic cell death in the testicular microenvironment disrupts spermatogenesis and compromises male fertility. Clove extract, through its antioxidative and anti-inflammatory actions, has been postulated to counteract these deleterious processes by scavenging reactive oxygen species (ROS), inhibiting lipid peroxidation, and modulating pro-inflammatory cytokines (7-9).

Moreover, recent preclinical studies have provided compelling evidence supporting the efficacy of clove extract in ameliorating testicular injury under hypoxic conditions. These studies have demonstrated a reduction in apoptotic cell death, testicular architecture preservation, and spermatogenesis restoration following clove extract supplementation in hypoxic animal models. Mechanistically, clove extract appears to regulate key signaling pathways involved in oxidative stress and apoptosis, including the nuclear factor erythroid 2-related factor 2 (Nrf2) pathway and the mitochondrial apoptotic pathway (9,10).

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Despite these promising findings, further investigations are warranted to elucidate the precise molecular mechanisms underlying the protective effects of clove extract against testicular apoptosis induced by hypoxia. Additionally, translational research is needed to evaluate clove extract's safety, efficacy, and dosage regimen as a potential therapeutic agent for managing testicular dysfunction associated with hypoxic insults in clinical settings. Therefore, this study aims to comprehensively assess the therapeutic potential of clove extract in mitigating testicular damage and preserving male reproductive function under hypoxic conditions in male rats.

Materials and Methods

Animal Model and Ethical Considerations

Male Sprague-Dawley rats weighing 200-250 g were procured from the "Tabriz University of Medical Sciences" animal facility and acclimatized for one week before experimentation. The rats were housed under standard laboratory conditions with a 12-hour light-dark cycle and ad libitum access to food and water. All experimental procedures followed the guidelines outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Ethics Committee of "Tabriz University of Medical Sciences" (IR.TBZMED.AEC.1402.039).

Induction of Varicocele

Rats were anesthetized using a combination of ketamine (80 mg/kg) and xylazine (10 mg/kg) administered intraperitoneally. Adequate depth of anesthesia was ensured by monitoring the pedal withdrawal reflex. The scrotal region was shaved and sterilized with 70% ethanol. A midline abdominal incision (approximately 2 cm) was made to expose the left spermatic vein and artery. Using a surgical microscope for magnification, the left renal vein was identified, and a small segment of the vein was carefully dissected free from surrounding tissue. A microvascular clip (size: 0.5 mm) was placed across the left renal vein just below the junction of the left spermatic vein, causing partial occlusion of venous outflow and subsequent development of varicocele. The clip was left in place for 30 minutes to induce venous hypertension in the left testis (11,12).

Sham operation (control group): In the sham-operated group, rats underwent an identical surgical procedure without the placement of a microvascular clip on the left renal vein (12).

Closure and postoperative care: Following varicocele induction or sham operation, the abdominal incision was closed in layers using absorbable sutures (4-0 Vicryl) for the peritoneum and non-absorbable sutures (4-0 Nylon) for the skin. Postoperatively, rats were allowed to recover on a warming pad and closely monitored for signs of pain or distress. Buprenorphine (0.05 mg/kg) was administered subcutaneously for postoperative analgesia, and rats were housed individually for the duration of the study.

Validation of Varicocele

Confirmation of varicocele: The presence of varicocele was confirmed by visual inspection and palpation of the left testis for the presence of dilated veins and an alteration in testicular texture (12).

Assessment of testicular hypoxia: Testicular hypoxia was assessed by measuring the histological evaluation of testicular tissue samples stained with hematoxylin, and eosin was performed to assess morphological changes associated with hypoxia.

Experimental Design

Rats were randomly assigned to three groups: the varicocele group, the sham-operated control group, and the varicocele group treated with clove extract (4 mg/kg) (n=8 per group) (13).

Tissue Collection and Processing

Sacrifice and Tissue Harvesting

Rats were euthanized at the designated time points following varicocele induction or sham operation. Deep anesthesia was induced using an overdose of pentobarbital sodium (150 mg/kg) administered intraperitoneally. Confirmation of euthanasia was ensured by the absence of reflexes, including pedal withdrawal and corneal reflex.

Testicular Excision

A midline incision was made in the abdomen, and both testes were carefully exposed and excised en bloc. The testicular tissues were immediately immersed in 10% neutral buffered formalin for fixation.

Histological Processing

Testicular tissues were fixed in 10% neutrally buffered formalin. After dehydration and embedding, paraffin-embedded testicular tissues were dissected into 5-µm-thick slices using a microtome.

Testicular sections were mounted onto glass slides, deparaffinized, and rehydrated through graded alcohol solutions. To visualize cellular morphology and tissue architecture, sections were stained with hematoxylin and eosin.

Microscopic Examination

Stained testicular sections were examined under a light microscope equipped with a digital camera. Multiple fields of view were captured at various magnifications (e.g., 100x, 200x, and 400x) to assess the histological features of the testicular parenchyma. Morphometric analysis of the "seminiferous tubules" was conducted using a light microscope at 400X. The morphological parameters were

quantified using image analysis software, specifically ImageJ.

Histomorphometric Measurements

Digital images of testicular sections were analyzed using image analysis software (Image J). Histomorphometric parameters including seminiferous tubule diameter from the "basement membrane" to the lumen of the tubule were assessed to examine morphological alterations. Germinal epithelial thickness, and Johnson's score per seminiferous tubule, were quantified. Measurements were performed in a blinded manner to minimize observer bias.

Oxidative stress analysis

Blood Collection

Following the euthanasia of rats at designated time points post-varicocele induction or sham operation, blood samples were collected via cardiac puncture using sterile syringes and needles. Care was taken to minimize hemolysis during blood collection.

Serum Separation

Whole blood samples were allowed to clot at room temperature for 30 minutes and then centrifuged at 1500 × g for 10 minutes at 4 °C to obtain serum. The supernatant (serum) was carefully transferred to sterile microcentrifuge tubes using a Pasteur pipette and stored at -80 °C until further analysis.

Assessment of Oxidative Stress Parameters

a. Measurement of Malondialdehyde (MDA) Levels

Serum MDA levels, an indicator of lipid peroxidation, were determined using a commercially available colorimetric assay kit following the manufacturer's instructions. In brief, serum samples were incubated with thiobarbituric acid (TBA) reagent at a high temperature to form a pink chromogen, which was quantified spectrophotometrically at 532 nm. MDA concentrations were calculated based on a standard curve generated using 1,1,3,3-tetraethoxypropane as a standard.

b. Assessment of Superoxide Dismutase (SOD) Activity

Serum SOD activity, an enzymatic antioxidant defense mechanism, was measured using a spectrophotometric assay based on the inhibition of pyrogallol autoxidation. Briefly, serum samples were incubated with pyrogallol substrate, and the rate of inhibition of pyrogallol autoxidation was monitored spectrophotometrically at 420 nm. SOD activity was expressed as units per milliliter (U/mL) of serum, where one unit of SOD activity is defined as the amount of enzyme required to inhibit 50% of pyrogallol autoxidation.

c. Evaluation of Glutathione (GSH) Levels

Serum GSH levels, a crucial non-enzymatic antioxidant, were determined using a colorimetric assay based on the reaction between GSH and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), forming a yellow chromophore. The absorbance of the chromophore was measured spectrophotometrically at 412 nm, and GSH concentrations were calculated based on a standard curve generated using GSH standards.

Statistical Analysis

All data are presented as mean values ± standard deviation (SD). One-way analysis of variance (ANOVA) was employed to assess differences between the studied groups, followed by Tukey's post hoc test for pairwise comparisons. Statistical significance was set at $P<0.05$. Data analyses were conducted using SPSS software version 19 (SPSS Inc., Chicago, IL).

Results

Histomorphometry Results

Varicocele induction alters testicular morphology and germ cell dynamics.

Seminiferous Tubule Diameter

Rats with varicocele significantly reduced seminiferous tubule diameter compared to sham-operated controls ($P<0.05$). Varicocele induction led to tubular atrophy, characterized by a decrease in the diameter of seminiferous tubules, indicative of testicular structural damage. Treatment with clove extract led to a significant preservation of seminiferous tubule diameter compared to untreated varicocele rats ($P<0.05$). Clove extract supplementation mitigated tubular atrophy, resulting in a maintenance of tubular diameter comparable to sham-operated controls (Table 1, Figure 1).

Germinal Epithelial Thickness

The germinal epithelial thickness in the seminiferous tubules was markedly reduced in rats with varicocele compared to the sham-operated group ($P<0.05$). Varicocele induction resulted in thinning of the germinal epithelium, reflecting impaired spermatogenesis and disruption of germ cell development. Treatment with clove extract prevented the thinning of the germinal epithelium observed in untreated varicocele rats, leading to a significant preservation of epithelial thickness ($P<0.05$). Clove extract supplementation restored the integrity of

Table 1. Histopathological Findings of Testis Tissue in Study Groups

Groups	Mean Johnson's Score ± SD	STD ± SD	HE ± SD
Varicocele	4.30± 0.25[a]	155.40 ± 4.25[a]	68.0 ± 3.19[a]
Sham	9.40 ± 0.3	250.25 ± 4.25	35.5 ± 2.30
Varicocele+clove	6.50 ±0.2[b]	190.30± 6.25[b]	55.5 ± 3.25[b]

STD, seminiferous tubule diameter; HE, highest of epithelium.

[a] $P < 0.05$ compared to the sham group.

[b] $P < 0.05$ compared to the varicocele group.

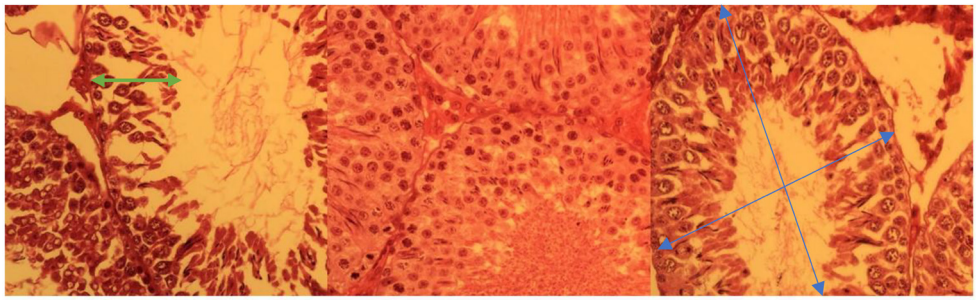

Figure 1. Histological Findings in Research Groups. Blue arrow shows the diameters of seminiferous tubules. Green arrow shows the thickness of seminiferous tubules.

the germinal epithelium, facilitating spermatogenesis and germ cell development (Table 1, Figure 1).

Johnsen Score

Rats with varicocele significantly decreased Johnsen score compared to sham-operated controls ($P<0.05$). Varicocele induction led to a depletion of the germ cell population within the seminiferous tubules, indicating impaired spermatogenic activity and germ cell loss.

Rats with varicocele treated with clove extract exhibited a significant increase in Johnsen score compared to untreated varicocele rats ($P<0.05$). Clove extract supplementation effectively preserved the germ cell population within the seminiferous tubules, promoting spermatogenic activity and germ cell survival (Table 1, Figure 1).

These histomorphometric findings indicate that varicocele induction results in testicular architectural alterations and disruption of germ cell dynamics, contributing to impaired spermatogenesis. However, treatment with clove extract effectively preserves testicular morphology and germ cell population in varicocele rats, highlighting its potential therapeutic efficacy in mitigating testicular dysfunction associated with varicocele-induced testicular hypoxia.

Oxidative Stress Parameters

Malondialdehyde Levels

Serum MDA levels, indicative of lipid peroxidation, were significantly elevated in rats with varicocele compared to the sham-operated control group ($P<0.05$). Varicocele induction led to a notable increase in MDA concentrations, reflecting enhanced oxidative damage to lipid membranes in the serum. Rats with varicocele treated with clove extract exhibited a significant decrease in serum MDA levels compared to untreated varicocele rats ($P<0.05$). Clove extract supplementation effectively mitigated lipid peroxidation in the serum, reflecting its antioxidative properties (Figure 2).

Superoxide Dismutase Activity

SOD activity in serum, an enzymatic antioxidant defense mechanism, was significantly reduced in rats with varicocele compared to sham-operated controls ($P<0.05$).

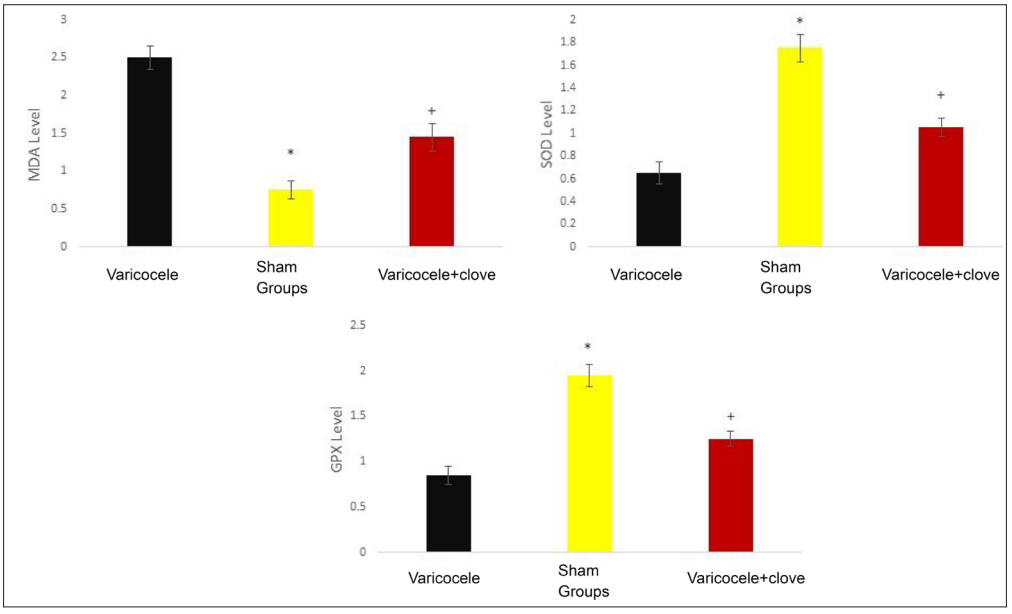

Figure 2. The Serum Level of Oxidative Stress Markers. *Sham in contrast with varicocele, + treated group in contrast with varicocele.

Varicocele induction resulted in a decline in SOD activity, suggesting impaired antioxidant capacity to neutralize superoxide radicals. Treatment with clove extract restored SOD activity in the serum of rats with varicocele to levels comparable to sham-operated controls ($P > 0.05$). Clove extract supplementation effectively enhanced SOD activity, indicating the restoration of antioxidant defense mechanisms (Figure 2).

Glutathione Levels

Serum GSH levels, a critical non-enzymatic antioxidant, were significantly decreased in rats with varicocele compared to the sham-operated group ($P<0.05$). Varicocele induction led to a depletion of GSH reserves in the serum, compromising the cellular antioxidant defense against oxidative stress. Clove extract-treated varicocele rats showed a significant increase in serum GSH levels compared to untreated rats ($P<0.05$). Clove extract supplementation replenished GSH reserves in the serum, enhancing the cellular antioxidant capacity (Figure 2).

These findings suggest that varicocele induction leads to oxidative stress in rat serum, characterized by elevated lipid peroxidation, reduced antioxidant enzyme activity, and depleted GSH levels. However, treatment with clove extract effectively attenuates oxidative stress parameters, restoring antioxidant defense mechanisms and mitigating systemic oxidative damage associated with varicocele.

Discussion

Varicocele is a common vascular abnormality characterized by dilating the pampiniform plexus veins within the scrotum, affecting approximately 15% of the male population. Despite being prevalent, varicocele's pathophysiological mechanisms leading to testicular dysfunction, including oxidative stress and hypoxia, remain incompletely understood (14,15). Our study aimed to investigate the therapeutic potential of clove extract in mitigating testicular apoptosis associated with testicular hypoxia induced by varicocele in male rats. Through comprehensive evaluations of oxidative stress parameters in serum and histomorphometric analysis of testicular architecture, our findings shed light on the protective effects of clove extract against varicocele-induced testicular injury.

Our research has shown that varicocele induction in rats results in systemic oxidative stress. This is supported by the elevated levels of MDA, a marker of lipid peroxidation, as well as the decreased activities of SOD and the reduced levels of GSH, which are vital enzymatic and non-enzymatic antioxidants, respectively, in serum. These findings are consistent with previous studies implicating oxidative stress as a pivotal mediator of testicular dysfunction in varicocele (11,16,17). The increased production of ROS and impaired antioxidant defense mechanisms contribute to cellular damage and apoptosis within the testicular microenvironment, ultimately disrupting spermatogenesis and compromising male fertility (18,19).

Importantly, our study demonstrates that treatment with clove extract effectively mitigates oxidative stress associated with varicocele, restoring antioxidant enzyme activities and GSH levels in serum. Clove extract, enriched with bioactive compounds such as eugenol and β-caryophyllene, possesses potent antioxidative properties attributed to its ability to scavenge free radicals and modulate antioxidant enzyme systems. These findings align with previous research highlighting the antioxidant potential of clove extract in ameliorating oxidative stress-induced tissue damage in various pathological conditions (20-22).

Histomorphometric analysis of testicular tissue further corroborates the protective effects of clove extract against varicocele-induced testicular injury. Varicocele rats exhibited significant alterations in testicular morphology, including reduced seminiferous tubule diameter, thinning of the germinal epithelium, and decreased germ cell population within the seminiferous tubules, indicative of impaired spermatogenesis and germ cell loss. These histological changes are consistent with the pathological features of testicular dysfunction observed in varicocele patients, emphasizing the translational relevance of our findings (23,24).

In contrast, treatment with clove extract preserved testicular architecture and germ cell dynamics in varicocele rats, mitigating tubular atrophy, preserving germinal epithelial thickness, and maintaining germ cell population within the seminiferous tubules. These histomorphometric improvements suggest a protective role of clove extract against varicocele-induced testicular apoptosis and germ cell loss, possibly through its antioxidative and anti-apoptotic properties. Clove extract supplementation may mitigate hypoxia-induced cellular damage and apoptosis within the testicular microenvironment, thereby preserving spermatogenic function and male fertility.

The observed beneficial effects of clove extract on testicular morphology and oxidative stress parameters underscore its potential as a natural therapeutic agent for managing varicocele-associated testicular dysfunction (13). The multi-targeted antioxidative and cytoprotective mechanisms of clove extract offer promising avenues for intervention in varicocele patients, particularly those experiencing infertility or impaired spermatogenesis(13,25). Moreover, the safety profile and accessibility of clove extract make it an attractive candidate for clinical translation, warranting further investigation in human studies (25,26).

However, several limitations of our study should be acknowledged. Firstly, the precise molecular mechanisms underlying the protective effects of clove extract against varicocele-induced testicular injury remain to be elucidated. Future studies should explore the signaling

pathways involved in clove extract-mediated antioxidative and anti-apoptotic actions within the testicular microenvironment. Additionally, the optimal dosage and duration of clove extract supplementation require further optimization to maximize therapeutic efficacy while minimizing potential adverse effects.

Conclusions

Our study provides compelling evidence for the therapeutic potential of clove extract in mitigating testicular injury associated with varicocele-induced testicular hypoxia in male rats. Clove extract offers a promising adjunctive therapeutic strategy for managing varicocele-associated testicular dysfunction and male infertility by alleviating oxidative stress, preserving testicular morphology, and enhancing germ cell dynamics. Further clinical studies are warranted to validate the efficacy and safety of clove extract supplementation in varicocele patients, paving the way for developing novel treatment modalities in reproductive medicine.

Authors' Contribution

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Project administration: Alireza Rahimi Mamaghani.
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Validation: Alireza Rahimi Mamaghani.
Visualization: Alireza Rahimi Mamaghani.
Writing–original draft: Majid Sokoohi, Somaye Salehpoor, Maryam Sadr Ameli, Leila Majdi.
Writing–review & editing: Majid Sokoohi, Somaye Salehpoor, Maryam Sadr Ameli.

Conflict of Interests

None declared.

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