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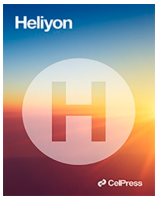

Research article

The impact of N-acetylcysteine on hypoxia-induced testicular apoptosis in male rats: TUNEL and IHC findings

Majid Shokoohi [a,b,d], Amir Afshin Khaki [b], Leila Roshangar [b], Mohammad Hossein Nasr Esfahani [c], Gilda Ghazi Soltani [a], Alireza Alihemmati [b,d,*]

[a] Clinical Research development unit of Tabriz Valiasr hospital, Tabriz University of Medical Sciences, Tabriz, Iran
[b] Department of Anatomical sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
[c] Department of Animal Biotechnology, Cell Science Research Center, Royan Institute for Biotechnology, ACECR, Isfahan, Iran
[d] Molecular Medicine Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

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ABSTRACT

The present study aimed to evaluate the impact of *N-acetylcysteine* (NAC) on testicular hypoxia caused by varicocele, focusing specifically on the regulation of genes related to apoptosis and oxidative stress in the testes of mature Wistar rats.

Thirty-two rats were divided into four groups: Control (Sham), hypoxia, testicular hypoxia treated with NAC (Hypoxia + NAC), and healthy animals treated with NAC. After the 8-week treatment period, testicular histopathology and the levels of oxidative stress markers—superoxide dismutase (SOD), glutathione peroxidase (GPx), and malondialdehyde (MDA)—in serum were examined. The expression of Bax and Bcl-2 mRNA was analyzed using immunocytochemistry and RT-qPCR assays, while the apoptosis rate was determined using the TUNEL method.

Histopathological evaluations showed that parameters such as Johnsen's score, epithelium width, and seminiferous tubule diameter indicated significant improvement in the Hypoxia + NAC group compared to the Hypoxia group. NAC administration resulted in elevated serum levels of GPx and SOD, accompanied by a reduction in MDA levels ($p < 0.003$). Furthermore, the study revealed that NAC decreased Bax expression and enhanced Bcl-2 gene and protein expression compared to the varicocele group ($p < 0.05$). Additionally, NAC administration significantly decreased the rate of apoptosis in germ cells ($p < 0.05$).

These findings suggest that NAC administration can mitigate testicular damage induced by hypoxia from varicocele in rats, primarily due to its antioxidant properties.

1. Introduction

Testicular hypoxia, characterized by insufficient oxygen supply to testicular tissue, is a significant contributor to male infertility and reproductive dysfunction. Various pathological conditions, such as varicocele—a common vascular disorder marked by abnormal

Abbreviations: **NAC**, N-acetylcysteine; **SOD**, Superoxide Dismutase; **GPx**, Glutathione Peroxidase; **MDA**, Malondialdehyde; **TUNEL**, Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling; **ROS**, Reactive Oxygen Species; **MSTD**, Mean of Seminiferous Tubule Diameter; **MJS**, Mean Johnson's Score.

* Corresponding author. Department of Anatomical sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
E-mail addresses: dr.hemmatti.a@gmail.com, Hemmatti@yahoo.com (A. Alihemmati).

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dilation of the testicular veins—can lead to compromised blood flow and subsequent hypoxia within the testicular microenvironment. This hypoxic state triggers a cascade of molecular events, including oxidative stress, dysregulated apoptosis, and impaired spermatogenesis, ultimately culminating in testicular injury [1–3].

N-acetylcysteine (NAC), a derivative of the amino acid cysteine, has garnered considerable attention for its potent antioxidant properties and diverse therapeutic applications. Beyond its well-established role as a mucolytic agent in respiratory conditions, NAC has demonstrated promising effects in mitigating oxidative stress and apoptosis in various tissues, including the testes. Its ability to replenish intracellular glutathione levels, scavenge reactive oxygen species (ROS), and modulate apoptotic pathways makes NAC a compelling candidate for managing testicular hypoxia-induced injury [4–6].

The rationale for investigating the therapeutic potential of NAC in the context of testicular hypoxia stems from its multifaceted mechanisms of action. By neutralizing ROS and reducing oxidative damage to biomolecules, NAC exerts cytoprotective effects against hypoxia-induced oxidative stress in the testicular microenvironment. Furthermore, NAC has been shown to regulate apoptotic signaling pathways, including those involving Bcl-2 family proteins, thereby promoting cell survival and preserving testicular function [7,8].

This experimental study aims to provide some details that how NAC administration can attenuate testicular hypoxia-induced injury and highlights avenues for future research aimed at optimizing its clinical utility in the field of male reproductive medicine.

2. Materials and methods

2.1. Animals

The experiment utilized 32 male Wistar rats, weighing between 200 and 250 g. The rats were housed in standard cages under controlled environmental conditions for a 2-week acclimatization period, with free access to food and water and a 12-h light/dark cycle. Ethical approval for the study was obtained from the Laboratory Animal Ethics Committee of Tabriz University of Medical Sciences (Ethical code: IR.TBZMED.AEC.1402.011).

The rats were randomly assigned to 4 groups: a control group undergoing laparotomy surgery without varicocele induction (sham), a varicocele-induced group receiving normal saline orally for 8 weeks, and a varicocele-induced group treated with *N-acetylcysteine* (Pharmachemie, Iran) at a dose of 150 mg/kg for 8 weeks. Additionally, there was a healthy group that received *N-acetylcysteine* at the same dose (150 mg/kg) through oral gavage for 8 weeks.

2.2. Induction of testicular hypoxia by varicocele

For the surgical procedures, the animals were given general anesthesia via an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (50 mg/kg). They were then carefully positioned in a supine position on a specialized rodent board. The abdominal area was completely shaved and sterilized using betadine solution.

Varicocele induction was performed by making a 2-cm incision in the mid-abdomen. The left renal vein was isolated from surrounding tissues, and a silk thread was passed beneath it. Two 5-0 double-armed suture needles were looped around the vein to form a partial ligature. The needles were then carefully withdrawn from the ligature. By loosening the ligature and compressing the vein's outer diameter, blood flow through the vein was constricted, resulting in the development of varicocele [9]. Four weeks after the surgery, the abdominal incision was reopened in all animals from both the experimental and control (sham) groups to assess typical signs of varicocele, such as vein hyperemia, testicular swelling, and inflammation of the affected testicle. After confirming the induction of varicocele, the incision was closed using 6/0 sutures. Treatment was initiated and continued for 8 weeks following the induction of varicocele [9].

Following the 8-week treatment period, all animals in the study groups were anesthetized using xylazine and ketamine. Their testes were carefully dissected, and blood samples were collected from the left ventricle of the heart to assess the levels of antioxidant enzymes present in the serum.

2.3. Histopathological assessments

In testicular tissue samples (right testis), histopathological changes were assessed by examining 50 seminiferous tubules using Johnson's score [10]. Each tubule was graded on a scale from one to ten based on its histological appearance.

Additionally, the width of 50 cross-sectioned seminiferous tubules and the height of the seminiferous epithelium—from the basement membrane to the lumen of the tubule—were measured to evaluate morphological alterations. Morphometric analysis of the seminiferous tubules was conducted using a light microscope at 400x magnification. The morphological parameters were quantified using image analysis software, specifically ImageJ (ImageJ, NIH, USA).

2.4. Evaluation of oxidative stress markers, SOD, GPx, and MDA

Malondialdehyde (MDA) concentrations in serum were determined by measuring the spectrophotometric absorbance of the pink-colored solution formed from the reaction with thiobarbituric acid-reactive substances, following the method described in a previous study [11]. Superoxide dismutase (SOD) and glutathione peroxidase (GPX) enzyme concentrations in serum were quantified using an ELISA reader (BioTek ELx800, USA) according to the instructions provided with the kits (Randox kit and Ransod kit, UK).

2.5. RNA isolation, cDNA synthesis, and RT-qPCR

RNA isolation was carried out using Trizol Reagent (Invitrogen, Paisley, UK), and the concentration was assessed using spectrophotometry. Following this, RNA samples were adjusted to a concentration of 0.5 μg/ml. Subsequently, complementary DNA (cDNA) was synthesized from the whole RNA using a commercial kit (from Thermo Scientific, Waltham, MA) in a total volume of 20 μl, utilizing 5 μg of the extracted RNA. The sequences of primers used for gene expression analysis presented in Table 1. The GAPDH gene was used as an internal control.

Real-time quantitative PCR (qPCR) reactions were performed according to methods described in prior research [12]. Numerical analysis was conducted using the Pfaffl formula, representing ratios ($2^{-\Delta CT}$ target: $2^{-\Delta CT}$ reference) [13–15].

2.6. Immunohistochemistry (IHC) staining

The expression of Bcl-2 and Bax proteins was assessed using a commercial immunoperoxidase kit from Santa Cruz Biotechnology, Inc. Tissue sections of 5 μm thickness were prepared, with three sections per slide, and 20 seminiferous tubules measured for analysis [9,12].

In the first step, sections were de-paraffinized by immersing them in xylene, followed by 100 % ethanol. Gradual hydration was achieved through successive immersions in 96 %, 90 %, and 80 % ethanol, concluding with a rinse in deionized water. Then, antigen retrieval was performed by placing the slides in citrate buffer (pH 7.4) and heating in a microwave. After cooling to room temperature, the slides were washed. In addition, following two washes with TBS containing 0.03 % Triton X-100, blocking was conducted using 10 % normal serum or 1 % BSA in TBS for 2 h at room temperature. Slides were then incubated with primary antibodies against Bcl-2, Bax, and GAPDH, diluted at 1:100, for 4 h at 37 °C or overnight at 4 °C. After additional washes, slides were incubated with FITC-conjugated IgG as the secondary antibody (1:150 dilution) in the dark for 90 min, followed by DAPI staining. In the final step, the slides were washed again, then dehydrated through a series of ethanol solutions, and mounted with an appropriate medium and coverslips.

2.7. TUNEL staining

Testis tissue samples were deparaffinized and subsequently dehydrated, followed by washing with phosphate-buffered saline (PBS, Sigma-Aldrich, Germany). The sections were then incubated with proteinase K (15 μg/ml) at 37 °C for 20 min. After this incubation, a permeabilization solution was applied for 10 min, followed by another wash with PBS. Following these preparations, the tissue slides were incubated with 50 μl of TUNEL dye solution at 37 °C for 1 h. After multiple washes with PBS, the samples were examined using an Olympus fluorescence microscope (BX51, Japan). The nuclei of the testicular cells were counterstained with DAPI. The number of TUNEL-positive cells in the testicular tissue slides was determined in randomly selected microscope fields. The apoptosis index was calculated as the percentage of TUNEL-positive cells relative to the total number of cells.

2.8. Statistical analysis

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

3. Results

3.1. Oxidative stress markers

In the present study, both GPx and SOD levels were significantly decreased ($p < 0.003$) in the Hypoxia and Hypoxia + NAC groups compared to the control group. Notably, the Hypoxia + NAC group exhibited significantly higher levels of GPx and SOD ($p < 0.005$) compared to the Hypoxia group, as detailed in Table 2.

Additionally, serum MDA concentrations were markedly elevated ($p < 0.005$) in the Hypoxia group relative to the control group. In contrast, MDA levels were significantly lower ($p < 0.005$) in the Hypoxia + NAC group compared to the Hypoxia group. Importantly, no significant differences were observed between the control and NAC-treated healthy animals for all oxidative parameters ($p > 0.05$).

Table 1
Primer sequences.

Genes	Primer sequences
Bcl-2	F: 5′-CTTTGCAGAGATGTCCAGTCAG-3′ R: 5′-GAACTCAAAGAAGGCCACAATC-3′
Bax	F: 5′-GGCGAATTGGAGATGAACTG-3′ R: 5′-TTCTTCCAGATGGTGAGCGA-3′
GAPDH	F: 5′-GCAGCTCCTTCGTTGCCGGT-3′ R: 5′- CCCGCCCATGGTGTCCGTTC-3′

3.2. Histopathological assessment of testicular tissues

Histopathological assessment revealed that both the mean Johnson's score (MJS), the seminiferous tubule diameter (STD), and were significantly reduced in the Hypoxia and Hypoxia + NAC groups compared to the control group ($p < 0.005$). Treatment with NAC significantly improved both MJS and STD in the Hypoxia + NAC group compared to the Hypoxia group ($p < 0.005$). No significant differences were noted between the NAC and control groups regarding STD and MJS (Fig. 1A–D and Table 3). Moreover, the highest of the seminiferous tubule epithelium (HE) was significantly lower ($p < 0.005$) in both the Hypoxia and Hypoxia + NAC groups compared to the control. However, NAC administration significantly improved the highest of the seminiferous epithelium in the Hypoxia + NAC group compared to the Hypoxia group ($p < 0.005$).

3.3. Apoptotic-related gene expression, Bax and Bcl-2, in testicular tissues

The expression of the Bax/Bcl-2 ratio and the GAPDH gene is summarized in Table 4.

Bax mRNA expression was significantly increased ($p < 0.005$) in both the Hypoxia and Hypoxia + NAC groups compared to the control. Furthermore, Bax expression was notably reduced ($p < 0.005$) in the Hypoxia + NAC group compared to the Hypoxia group. Conversely, Bcl-2 mRNA expression was significantly decreased ($p < 0.005$) in both the Hypoxia and Hypoxia + NAC groups compared to the control, with a significant increase in Bcl-2 mRNA expression in the Hypoxia + NAC group compared to the Hypoxia group ($p < 0.005$). No significant differences were observed between the control and NAC-treated healthy group for both genes ($p > 0.05$). Additionally, the Bax/Bcl-2 ratio was significantly elevated in both the Hypoxia and Hypoxia + NAC groups compared to the control group ($p < 0.005$), although it was significantly reduced in the Hypoxia + NAC group compared to the Hypoxia group ($p < 0.005$). No significant differences were observed between the control and NAC groups.

3.4. Protein expression of Bcl-2 and Bax in testicular tissues

Immunofluorescence staining was conducted to evaluate the expression of Bax and Bcl-2 proteins in testicular tissues across all experimental groups (Figs. 2 and 3). Analysis revealed immunoreactivity of seminiferous tubule cells to both Bax and Bcl-2 proteins.

The immunofluorescence assay demonstrated significantly higher expression of Bax (Fig. 2) in the testicular samples of the Hypoxia group compared to the control ($p < 0.05$), indicating an elevated level of apoptosis in the varicocele-induced testes. Conversely, Bax protein expression was significantly reduced in the Hypoxia + NAC group compared to the Hypoxia group ($p < 0.05$). No significant differences were noted between the control and NAC groups regarding Bax expression. Similarly, Bcl-2 protein levels (Fig. 3) were significantly decreased in the Hypoxia group compared to the control ($p < 0.05$), suggesting a higher rate of apoptosis. In contrast, the Hypoxia + NAC group exhibited increased fluorescence intensity for Bcl-2 compared to the Hypoxia group ($p < 0.05$). No significant differences were observed between the NAC and control groups regarding Bcl-2 expression ($p > 0.05$).

3.5. Germ cell apoptosis in testicular tissues

The impact of *N-acetylcysteine* on apoptosis levels in the testicular germ cells of rats was evaluated using the TUNEL assay. As illustrated in Fig. 4, the number of TUNEL-positive cells was significantly higher ($p < 0.01$) in the testicular samples of the Hypoxia group compared to the control. Notably, there was a significant decline ($p < 0.05$) in the count of TUNEL-positive cells in the Hypoxia + NAC group compared to the Hypoxia group. Furthermore, no significant differences were observed in the number of TUNEL-positive cells between the control and NAC groups (Fig. 4).

4. Discussion

The cellular and molecular insights from this study elucidate the mechanisms by which *N-acetylcysteine* exerts protective effects against testicular injury induced by hypoxia, particularly within the context of varicocele-related testicular hypoxia.

Oxidative stress is a critical factor in the pathogenesis of testicular dysfunction, especially in conditions such as varicocele, where

Table 2
Serum levels of oxidative stress markers.

Groups	SOD (U/ml) ± SD	MDA (nM/ml) ± SD	GPX (U/ml) ± SD
Sham	1.7 ± 0.035	0.68 ± 0.035	2.05 ± 0.025
Hypoxia	0.75 ± 0.060 *	2.20 ± 0.030 *	1.05 ± 0.046 *
Hypoxia + NAC	1.30 ± 0.043 *#	1.55 ± 0.050 *#	1.65 ± 0.064* #
NAC	1.85 ± 0.034	0.65 ± 0.020	2.15 ± 0.022

In the second column (SOD: Superoxide Dismutase), *P < 0.003 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the third column (GPx: Glutathione Peroxidase), (*P < 0.003) indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the fourth column (MDA: Malondialdehyde), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. SD: Standard deviation.

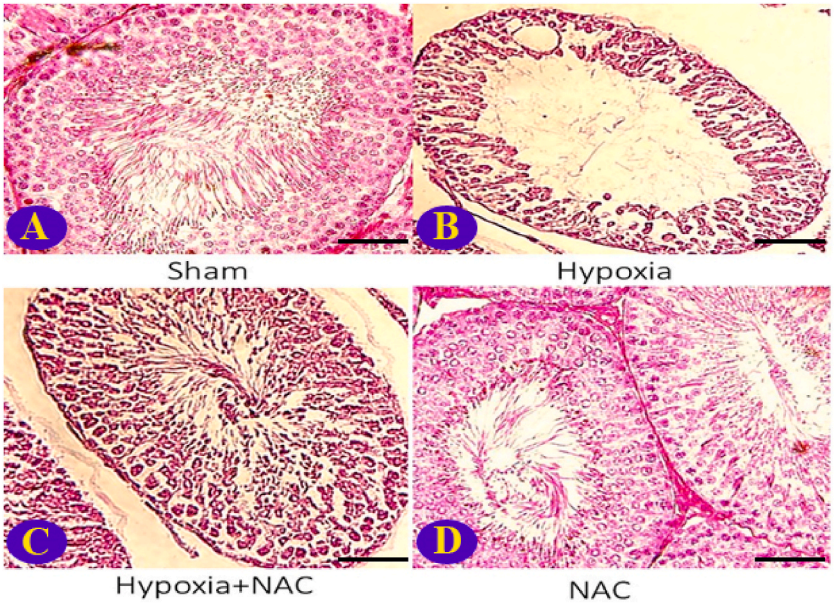

Fig. 1. Histological Assessment of Testis Tissue Stained with Hematoxylin and Eosin (H&E). (A) control (sham) group showing typical testicular structure. (B) Hypoxia group displaying severe testicular damage. (C) Hypoxia + N-acetylcysteine group indicating improvement in seminiferous tubule structure. (D) N-acetylcysteine group exhibiting normal testicular structure. Scale bar: 50 μm.

Table 3
Histopathological assessment in testicular tissues.

Groups	MJS ±SD	STD ±SD	HE ± SD
Sham	9.50 ± 0.15	260.40 ± 4.70	70.0 ± 3.19
Hypoxia	4.60 ± 0.25*	160.30 ± 3.25*	36.5 ± 1.30*
Hypoxia + NAC	6.50 ± 0.20*#	190.15 ± 4.10*#	52.45 ± 2.98*#
NAC	9.60 ± 0.15	265.30 ± 3.25	70.5 ± 1.94

In the second column (MJS: Mean Johnson's Score), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the third column (STD: Seminiferous Tubule Diameter), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the fourth column HT: Height of epithelium *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. SD: Standard deviation.
HE: Height of epithelium.

Table 4
mRNA expression of Bcl-2 and Bax analyzed by RT-qPCR.

Groups	Bax	Bcl-2	Bax/Bcl-2
Sham	0.42 ± 0.02	1.00 ± 0.02	0.42 ± 0.05
Hypoxia	1.00 ± 0.03 *	0.45 ± 0.037 *	2.22 ± 0.02 *
Hypoxia + NAC	0.68 ± 0.03 *#	0.70 ± 0.023 *#	0.97 ± 0.03 *#
NAC	0.40 ± 0.023	1.08 ± 0.042	0.37 ± 0.01

In the second column (Bax), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the third column (Bcl-2), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. In the fourth column (Bax/Bcl-2 ratio), *P < 0.005 indicate significant differences between the sham and Hypoxia groups, #P < 0.005 indicate significant differences between hypoxic group and Hypoxia + N-acetylcysteine group. SD: Standard deviation.

impaired blood flow results in tissue hypoxia [16]. At the cellular level, hypoxia disrupts the delicate equilibrium between the production of ROS and the efficacy of antioxidant defense mechanisms. This disruption leads to oxidative damage to various cellular components, including lipids, proteins, and DNA, ultimately contributing to testicular injury [17–19]. As a precursor to glutathione, NAC serves as a potent antioxidant by replenishing intracellular glutathione levels and scavenging ROS. At the molecular level, NAC alleviates oxidative stress through direct interactions with ROS, restoring the activity of antioxidant enzymes such as GPx and SOD,

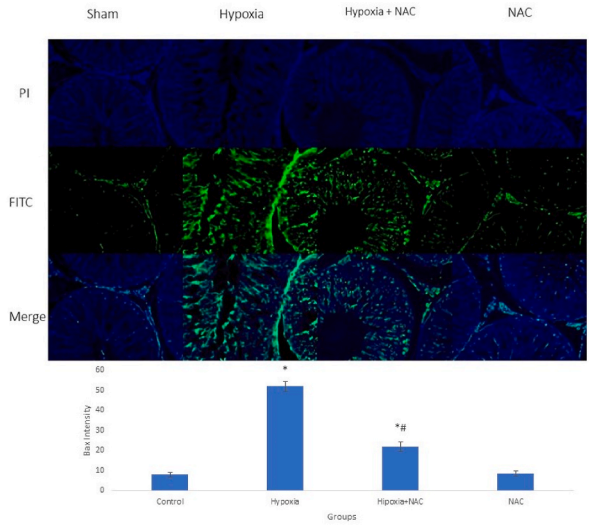

Fig. 2. Immunofluorescence Staining of Bax Protein in Rat Testicles. In the Hypoxia group, there was a significant increase in the intensity of Bax protein compared to the sham group. However, in hypoxic rats treated with N-acetylcysteine, the elevated Bax intensity was significantly reduced in comparison to the untreated hypoxic group. Notably, no significant differences were observed between the two healthy control groups ($p > 0.05$). (*, $P < 0.05$) indicates significant difference compared to the sham group and (#, $P < 0.05$) denotes significant difference compared to the Hypoxia group.

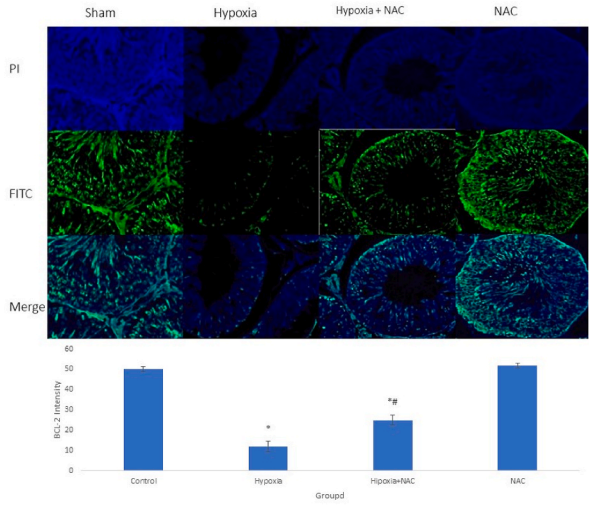

Fig. 3. Immunofluorescence Staining of Bcl-2 Protein in Rat Testicles. In the Hypoxia group, there was a significant decrease in the intensity of Bcl-2 protein compared to the sham group. However, in hypoxic rats treated with N-acetylcysteine, the downregulated Bcl-2 intensity was significantly increased in comparison to the untreated hypoxic group. Notably, no significant differences were observed between the two healthy control groups ($p > 0.05$). (*, $P < 0.05$) indicates significant difference compared to the sham group and (#, $P > 0.05$) denotes significant difference compared to the Hypoxia group.

while also modulating redox-sensitive signaling pathways. By enhancing antioxidant defenses, NAC protects testicular cells from oxidative damage and helps preserve their functionality [20–22].

In this study, NAC treatment significantly improved oxidative stress markers, as indicated by the restoration of GPx and SOD levels in the serum of hypoxia-induced rats. These findings suggest that NAC operates at the cellular level to bolster antioxidant defenses and mitigate oxidative damage in the context of varicocele-induced testicular hypoxia.

In this regard, Shokoohi et al. demonstrated that the induction of varicocele in rats leads to increased levels of malondialdehyde and decreased activity of antioxidant enzymes, such as SOD and GPx. This finding indicates an imbalance between the production of oxidants and antioxidants [9,12]. Furthermore, additional studies corroborate that varicocele induction in rats resulted in oxidative stress and disrupts the balance of oxidative stress markers [23,24]. Conversely, the application of antioxidant compounds mitigates the excessive activity of ROS and free radicals, thereby preventing oxidative damage. Numerous studies have reported that NAC enhances

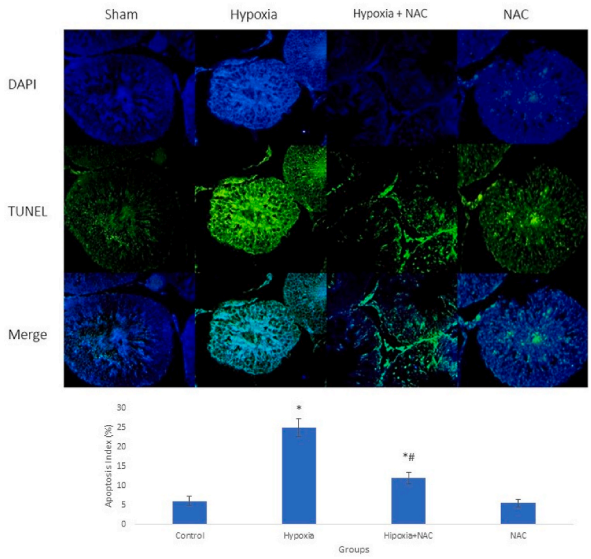

Fig. 4. TUNEL-Positive Cells in Rat Testicular Tissues. In the column belonging to the Hypoxic group, increased TUNEL positive cells can be observed. The number of TUNEL positive cells was significantly decreased in the Hypoxic group treated with N-acetylcysteine compared with the untreated Hypoxic group. (*, $P < 0.05$) indicates significant difference compared to the sham group and (#, $P < 0.05$) denotes significant difference compared to the Hypoxia group.

the activity of antioxidant enzymes, thereby restoring equilibrium between oxidant and antioxidant production [4,8].

Histological evaluations in this study revealed that hypoxia induced by varicocele resulted in significant testicular tissue damage, characterized by reduced cell counts in seminiferous tubules, as well as decreases in the Johnson score and the diameter and height of the tubules. These findings can be attributed to the detrimental effects of diminished oxygen supply to testicular cells and the resultant activity of free radicals. Previous research has consistently shown that testicular hypoxia leads to reductions in the Johnson score, tubule diameter, and thickness of seminiferous tubules [25,26].

Moreover, excessive generation of ROS can activate the intrinsic pathway of apoptosis by inducing the release of cytochrome *c* from the mitochondria into the cytoplasm. This cascade leads to the formation of an apoptosome, which subsequently activates pro-caspase 9. The intrinsic apoptosis pathway is regulated by two primary groups of proteins: anti-apoptotic proteins (e.g., Bcl-2 and Bcl-xL), which inhibit apoptosis, and pro-apoptotic proteins (e.g., Bax, Bad, and Bid), which promote apoptosis. Research indicates that oxidative stress caused by ROS production enhances the phosphorylation of Bcl-2 family proteins, disrupting the balance between pro-apoptotic and anti-apoptotic factors and favoring the activation of pro-apoptotic signals [9,27,28].

Apoptosis, or programmed cell death, plays a critical role in testicular dysfunction. At the molecular level, apoptosis is regulated by a complex interplay between pro-apoptotic and anti-apoptotic proteins, including Bax and Bcl-2. Bax promotes apoptosis by permeabilizing the mitochondrial outer membrane, while Bcl-2 inhibits apoptosis by preventing this permeabilization [29–31]. In the context of varicocele-induced testicular hypoxia, oxidative stress and mitochondrial dysfunction can activate apoptotic signaling pathways, leading to germ cell apoptosis and subsequent testicular injury. This study demonstrated that hypoxia significantly upregulated Bax expression and downregulated Bcl-2 expression, resulting in an imbalance between pro-apoptotic and anti-apoptotic proteins and an increase in apoptotic cell death. However, NAC treatment effectively restored the balance between Bax and Bcl-2 expression, thereby reducing apoptotic cell death and preserving testicular function.

Immunofluorescence staining further confirmed the modulation of apoptosis-related proteins by NAC, revealing decreased Bax expression and increased Bcl-2 expression in NAC-treated rats compared to those subjected to hypoxia. These findings suggest that NAC exerts anti-apoptotic effects at the molecular level by regulating the expression of Bax and Bcl-2 proteins and modulating apoptotic signaling pathways. Moreover, the TUNEL assay provided additional insights into the impact of NAC on apoptotic cell death in testicular tissues. Hypoxia significantly increased the number of TUNEL-positive cells, indicative of apoptotic cell death, whereas NAC treatment significantly reduced this number, underscoring the anti-apoptotic effects of NAC and its potential as a therapeutic intervention for testicular dysfunction [32].

In a previous study, we demonstrated that varicocele increased the expression of pro-apoptotic proteins such as Bax and elevated the number of apoptotic cells in the testicular tissue of affected rats. Correspondingly, the expression of Bcl-2 protein and gene was found to be decreased in varicocele groups, a finding that aligns with the results of the current study [9,12]. At both cellular and molecular levels, NAC appears to protect against testicular injury induced by hypoxia through multiple mechanisms, including the enhancement of antioxidant defenses, modulation of apoptotic signaling pathways, and reduction of apoptotic cell death. These findings have significant implications for the management of testicular disorders, such as varicocele, where oxidative stress and apoptosis play key pathogenic roles [33,34]. However, various studies have indicated that NAC prevents the expression of Bax and Bcl-2 due to its antioxidant properties, thereby protecting testicular tissue cells from apoptosis induced by hypoxia [34,35].

Nonetheless, it is important to acknowledge the limitations of this study. The specific molecular mechanisms underlying the protective effects of NAC were not fully elucidated, and future research should focus on exploring the downstream signaling pathways and molecular targets involved in NAC-mediated protection against testicular injury. Additionally, the translational potential of NAC as a therapeutic intervention for testicular disorders warrants further investigation in clinical trials.

5. Conclusion, study limitations, and Suggestions

The findings of this study provide significant insights into the cellular and molecular mechanisms through which *N-acetylcysteine* exerts its protective effects against testicular injury induced by hypoxia. By enhancing antioxidant defenses, modulating apoptotic signaling pathways, and reducing apoptotic cell death, NAC emerges as a promising therapeutic intervention for managing testicular disorders. These results underscore the potential of NAC in preserving testicular function, particularly in the context of varicocele-induced hypoxia.

However, this study is not without limitations. Utilizing a rat model of varicocele-induced testicular hypoxia, the research provides valuable insights into biological processes; nonetheless, animal models may not fully capture the complexity of human testicular disorders, including varicocele. Therefore, caution is warranted when extrapolating these findings to human populations, as they may not be directly applicable to all cases of testicular dysfunction.

Moreover, while varicocele is a recognized cause of testicular hypoxia and oxidative stress, it represents only one of many potential etiologies contributing to testicular dysfunction. Consequently, the efficacy of NAC treatment in other forms of testicular dysfunction remains to be determined. Future research should aim to elucidate the specific molecular targets of NAC and optimize its therapeutic efficacy in various contexts of testicular disorders. Investigating the translational potential of NAC through clinical trials will be essential for establishing its role in the clinical management of testicular dysfunction.

CRediT authorship contribution statement

Majid Shokoohi: Writing – review & editing, Writing – original draft, Visualization, Validation, Investigation, Data curation, Conceptualization. **Amir Afshin Khaki:** Visualization, Validation, Supervision, Software, Resources, Funding acquisition. **Leila Roshangar:** Visualization, Validation, Resources, Investigation. **Mohammad Hossein Nasr Esfahani:** Writing – review & editing, Writing – original draft, Methodology, Data curation. **Gilda Ghazi Soltani:** Writing – review & editing, Writing – original draft, Visualization, Methodology. **Alireza Alihemmati:** Supervision, Software, Project administration, Methodology, Data curation, Conceptualization.

Data Availability Statement

N/A.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Alireza Alihemmati reports financial support, administrative support, statistical analysis, and writing assistance were provided by Tabriz Valiasr Hospital. Alireza Alihemmati reports a relationship with Tabriz University of Medical Sciences that includes: board membership. The authors affirm that they have no conflicts of interest to declare. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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