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Histomorphometric and Histopathological Alterations of Rat Testis Following Exposure to Hydrogen Peroxide: Protective Role of Resveratrol Supplement

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A B S T R A C T

Resveratrol (RS) is widely used in medical fields as an antioxidant. Current research investigated the protective role of RS supplement on histomorphometric and histopathological alterations in testes were caused by hydrogen peroxide (H_2O_2) of rats as an animal model. Thirty-two adult rats were utilized in this study, divided randomly into 4 equal groups as follows. The group C was given tap water only and served as control, the 2nd group (G1) was given 0.5% of H_2O_2 of tap water, the 3rd group (G2) was given tap water containing 0.5% of H_2O_2 and intubated RS supplement at 87 mg/kg BW, and 4th group intubated RS supplement 87 mg/kg BW. after 56 days of treatment, rats were euthanized, dissected then, specimens of testes tissue were collected for histomorphometric and histopathological evaluation. Our results showed that administration of H_2O_2 caused a significant histomorphometric with histopathological changes in the form of a thickness of fibrous tunica albuginea, disarrangement of germ cells, necrosis of spermatogonia, edema, and loss of sperms as compared to other groups. Meanwhile, these histological alterations were partially attenuated in the G2 group that intubated resveratrol. Thus, the current study concluded that resveratrol may have therapeutic value in the treatment of induced testicular injury by H_2O_2 due to its antioxidant activity and attenuation of harmful effects of oxidative stress through a mechanism that should be elucidated in future studies.

Keywords: resveratrol, hydrogen peroxide, histological changes, histomorphometry, testis

INTRODUCTION

Stress refers to the reaction of the organism with different causes responses to adapt daily life challenges (1), include, the stressor of different environmental events pressures of work, family, and others consequently bodily reaction to the source of stress that can result into increases in negative behaviors and many of pathophysiological problems (2).

Oxygen (O_2) is a stable product and when there is gain or loss of an electron, it becomes reactive and reacts with metabolic compounds to form free radicals (FRs) which cause biological damage (3). Mitochondrial dysfunction, environmental pollution, abnormal metabolism, phagocytic cells and exposure rays, seminal fluid from cytoplasmic glucose-6-phosphate dehydrogenase, varicocele, high temperature of the scrotum, and different pathological conditions are the most important source of reactive oxygen species (ROS) generation and further the oxidative stress that occurred (4) through reaction of ROS with macromolecules which are considered as the most causative factor for etiology of infertility (5). Besides, ROS

has a role in activation of cell signaling cascades, gene expression and apoptosis (3). Moreover, activation of glucocorticoids receptors by stress-induced impairment of spermatogenesis accompanied with cell cycle arrest apoptosis in germ cells, anoikis, and autophagy (6, 7).

Hydrogen peroxide (H_2O_2) does not consider a free radical because it is produced via different enzymes including oxidase and monooxygenase (8) and it is involved in the regulation of cellular metabolism (9). H_2O_2 acts as an oxidizing agent, through inactivating a few enzymes by oxidizing their thiol groups to form hydroxyl radical, which easily diffuse into the cell membrane ultimately resulting in its damaging effects, as well as the most deleterious effect of H_2O_2 comes from its reaction with transition metals like iron and copper ions by the Fenton reaction (10, 11). In the male genital tract, low concentrations of ROS are necessary to complete the process of spermatogenesis, capacitation (12) and for the fusion of spermatozoa with female ova (13).

Under physiological conditions, seminal plasma can protect sperm from ROS by neutralize free radical activity and then control lipid peroxidation by its total antioxidant capacity (14). But under some pathological conditions, such as abnormally increase of macrophage, leukocytes and immature germ cells that consider playing a role in increased generation of ROS associated with diversity of male infertility complications, including dysfunctional spermatozoa, oligospermia (5, 12, 15),loss of motility and viability of spermatozoa (16).

Antioxidants are chemical substances that protect cells from the damage caused by ROS (17). Resveratrol (RS) is a polyphenolic phytoalexin and it has been classified as a stilbeniod, a type of natural phenolic compound (18). Many reports suggested that RS as a nutritional supplement possesses has many beneficial health effects, such as prolonging of life-span (19), ameliorating Alzheimer's disease (20), antiamyloidogenic properties (21), furthermore, anti-inflammatory, anti-obesity, ant-diabetic, and antioxidant activities have been reported (22, 18, 23) and attenuated acute respiratory distress (24). Therefore, this work was undertaken to explore the possible protecting effects of RS supplement by examining the histomorphometric and histopathological changes in hydrogen peroxide-induced sub-chronic testis damage of rats as a model.

MATERIALS AND METHODS

Animals and Experimental Design

A total of thirty-two healthy adult male Wistar rats weighing 190±20 g at 11–12 weeks of age were used in this experiment. They were housed at the animal house under control laboratory condition with well-ventilated room in standard plastic cages at room temperature 25±2°C with a natural light-dark cycle in the College of Veterinary Medicine-University of Baghdad. The animals were quarantined for two weeks to acclimation prior to the experiment and fed on a commercial pellet and drinking water ad libitum.

All rats were divided randomly into 4 groups of 8 rats each and treated for 56 days as follows: Group (C) were dosed distilled water as well received spigot water (in bottle) and served as control, the second group (G1) were received spigot water contain 0.5% of H_2O_2 (35% Merck, Germany) (25), the animals of the third group (G2) have drunk spigot water containing 0.5% of hydrogen peroxide and intubated resveratrol (Now Company, America) at a dosage rate 87 mg/kg. BW through the experimental period, while group G3 were intubated resveratrol only as in group G2. In addition, tap water contain 0.5% H_2O_2 was changed every 24 h. At the last day of the experiment, all rats were anesthetized with ketamine 90 mg/kg BW and xylazine 40 mg/kg BW as initial and supplement doses-Alfasan Company (26).

Sample Collection

At the last day of the experiment rats were anesthetized (by overdose of ketamine and xylazine), sacrificed then right testes were cleaned from connective tissues, dissected and small specimen were fixed by placing them in 10% neutral buffered formaldehyde for preparation of microscopic sections with a thickness of 5-6 µ and stained with haematoxylin-eosin stain (H&E) to study histopathological changes of the testes as mentioned by Suvarna et al. (27). For histomorphometric assessment of hight of epithelial cells, diameter of seminiferous tubules and number of Leydig cells a total of 20 microscopic fields were measured for each sample and 5 areas were measured within each microscopic field for histological measurements by using low power field lens (10×) and high power (40×) lens. Light microscope had been used for examination tissue sections. Microphotography has been made through Future Win Joe microscopic camera, the images have been analyzed and scored by using Fiji image analyzer system (28).

Statistical Analysis

The collected data were analyzed using IBM SPSS Statistics (Version 26.0) software. All data were subjected to one-way analysis of variance (ANOVA). Significant differences among group means were tested using the least significant differences (LSD) test at $P \leq 0.05$ (29).

RESULTS

Histomorphometric analysis of testes sections obtained from rats in group G1 revealed a significant ($P<0.05$) decrease in the number of Leydig's cells comparing with control and G2 treated group (Table1). Rats that dosed

resveratrol concurrently with hydrogen peroxide in group G2 revealed an increase in the number of these cells (2.80±0.37) without reaching a significant (P>0.05) level compared to group G1 (2.2±0.37). There was no statistically significant difference (P>0.05) between group G3 and the control group for this component; the mean values were 3.40±0.24 and 3.40±0.24, respectively. Furthermore, a significant (P<0.05) decrease in high of epithelial cells and diameter of seminiferous tubules of G1 in comparison with the control, G2, and G3 groups was also observed. While group G2 showed a significant (P<0.05) elevation in high of epithelial cells and diameter of seminiferous tubules when compared with group G1. Conversely, the results pointed to non-significant (P>0.05) differences in high of epithelial cells in G3 as compared to control. But a significant (P<0.05) increase in diameter of seminiferous tubules in same group was noticed as compared with control, G1, and G2 groups (Table 1).

Table 1. Morphometric results in the testis of four experimental groups

Groups	Number of Leydig cells (cell/μ^2)	High of epithelial cells (µm)	Diameter of seminiferous tubules (µm)
C	3.40 ± 0.24 ^{a}	68.33 ± 2.63 ^{a}	153.7 ± 5.19 ^{b}
G1	2.20 ± 0.37 ^{b}	21.62 ± 1.61 ^{c}	76.33 ± 2.63 ^{d}
G2	2.80 ± 0.37 ^{ab}	42.48 ± 2.56 ^{b}	119.4 ± 8.02 ^{c}
G3	3.40 ± 0.24 ^{a}	62.04 ± 4.14 ^{a}	189.2 ± 4.58 ^{a}

Values are expressed as mean ± SE (n=8). Control: rats received tap water .G1: rats received 0.5% hydrogen peroxide in drinking water. G2: rats received 0.5% H_2O_2 in drinking water plus resveratrol (87 mg/Kg BW) orally G3: rats given 87 mg/Kg BW resveratrol orally. Means with different lowercase superscripts denote significantly different (P≤0.05) in the same column

Histological examination of testes of the control group showed normal spermatogenesis, with thin basement membrane. The seminiferous tubules contained spermatogonial, primary, and secondary spermatocytes, spermatid, and spermatozoa, besides Leydig and Sertoli cells were presented in the interstitial space (Figure 1a, b). The sections obtained from testis of rats exposed to 0.5% H_2O_2 (G1) revealed fibrous thickening of tunica albuginea, marked incomplete spermatogenesis and atrophy of seminiferous tubules which ranged from degenerative changes of spermatogonial cells to necrosis and dissolution of spermatids (Figure 2a, b), as well as dissolution of spermatogonial cells, appeared as eosinophilic rounded bodies in the cavity of seminiferous tubules and loss of sperm, in addition, hypertrophy of blood vessels with few in inflammatory cells (Figure 3a, b). A marked focal pyknotic necrotic of spermatogonial cells, a diminished number of Leydig's cells and edema in interstitial tissue results from congested blood vessels was observed (Figure 4a, b) in comparison to the control group. Sections of testes of rats in group G2, however, revealed mild loss of sperms, a proliferation of sperms, mild edema between tubules and subscapular congestion of blood vessels (Figure 5a, b) when comparing to the histopathological sections of group G1 (Figures 1-4). Testis of resveratrol treated rats showed normal histological architecture of seminiferous tubules, normal active spermatogenesis, absences of differences between seminiferous tubules (Figure 6a, b), presence of many Leydig's cells, and an increase in the diameter of seminiferous tubules in comparison with control group.

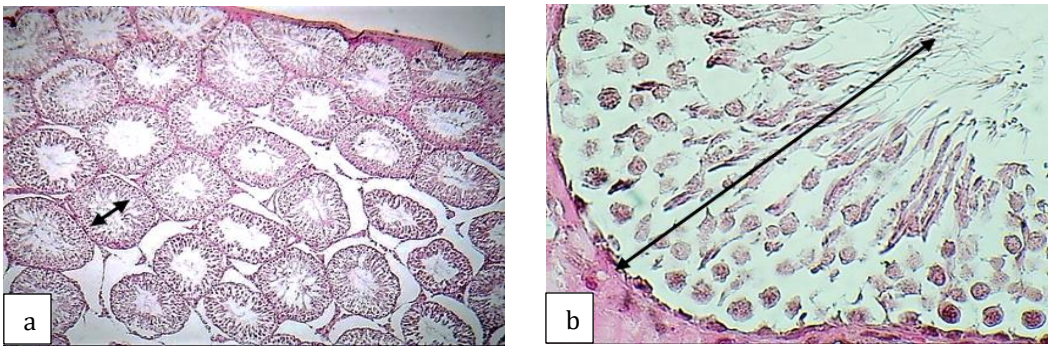

Figure 1. A photomicrograph of the right testis of an adult male Wistar rat in control group showing **a)** normal architecture of seminiferous tubules with no lesions (arrow), **b)** typical spermatogenesis phases (arrow) including normal spermatogonia, spermatocytes, spermatids, and spermatozoa (H&E, 40×)

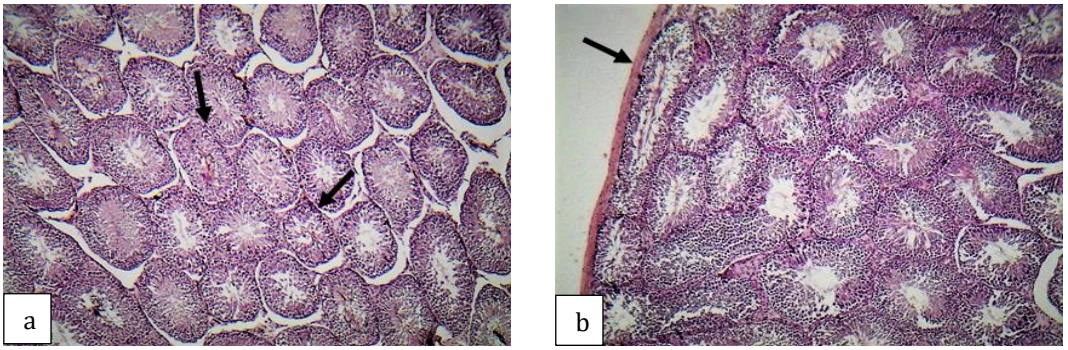

Figure2. A photomicrograph of the right testis of an adult male Wistar rat received H_2O_2 (G1): showing **a)** dissolution of sperms (increased eosinophilia) atrophy and loss of spermatids (arrow), **b)** thick fibrous tunica albuginea (arrow) and disruption of seminiferous tubules (H&E, 40×)

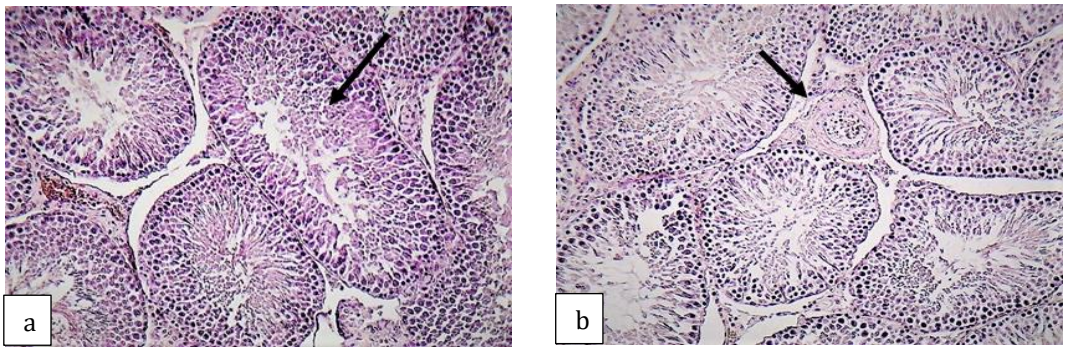

Figure 3. A photomicrograph of the right testis of an adult male Wistar rat received H_2O_2 (G1) showing **a)** dissolution of spermatogonial cells as eosinophilia (arrow) and loss of sperms (H&E, 100×), **b)** hypertrophy of blood vessel (arrow) and contain few inflammatory cells and in interstitial (H&E, 200×)

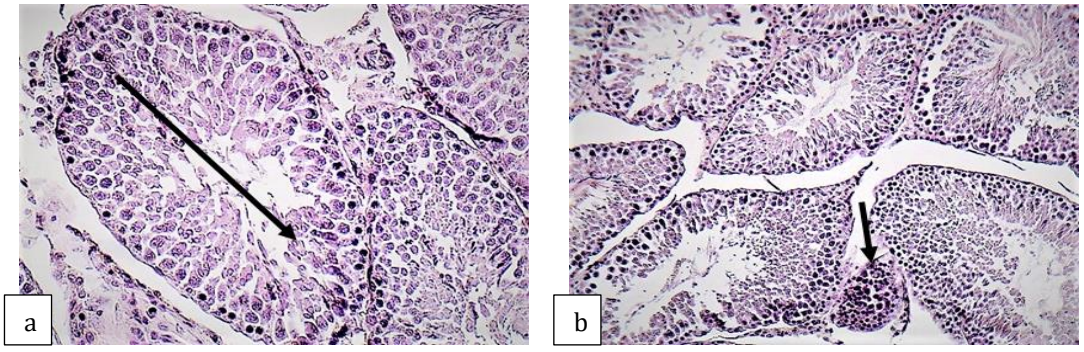

Figure 4. A photomicrograph of the right testis of an adult male Wistar rat received H_2O_2 (G1): showing **a)** necrosis of spermatogonial (arrow) spermatids and loss of sperms (H&E, 400×), **b)** severe necrosis of tubules and marked focal pyknotic necrotic spermatogonial cells (arrow) (H&E, 100×)

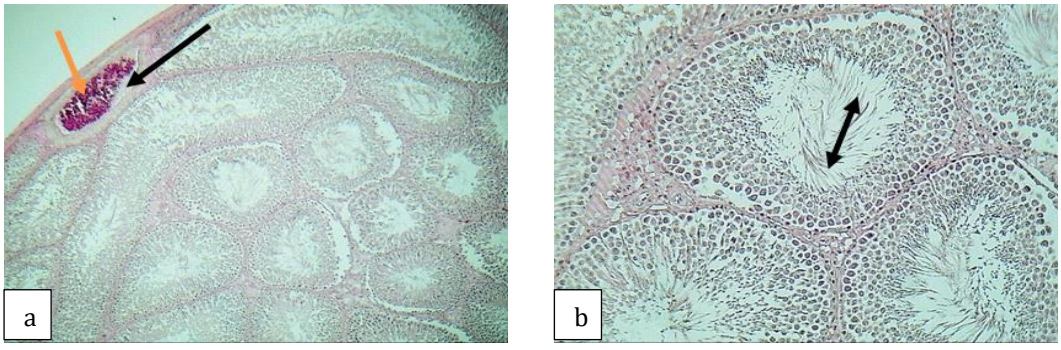

Figure 5. A photomicrograph of the right testis of an adult male Wistar rat received H_2O_2 plus resveratrol (G2) showing **a)** edema (black arrow) and subscapular congestion (red arrow). (H&E, 100×), **b)** active sperms in the tubules (arrow) (H&E, 200×)

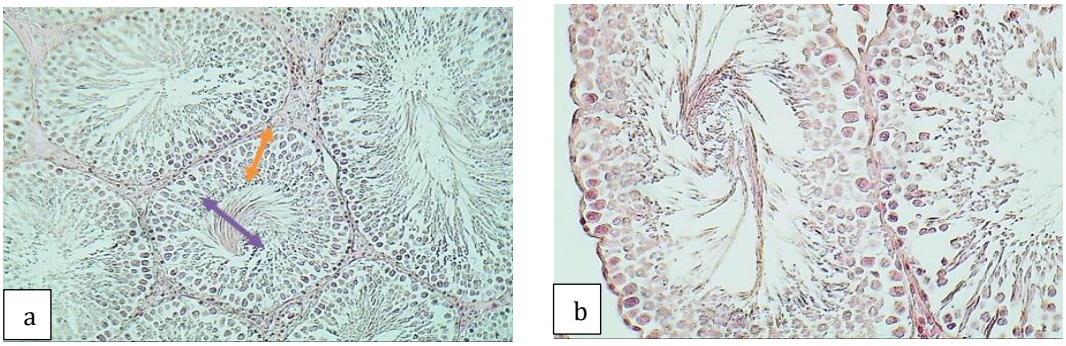

Figure 6. A photomicrograph of the right testis of an adult male Wistar rat intubated resveratrol (G3) showing **a)** normal architecture of germinal layers of seminiferous tubules (red arrow) filled with spermatozoa (black arrow) (H&E, 200×), **b)** normal appearance of seminiferous tubules with active spermatozoa (arrow) (H&E, 400×)

DISCUSSION

A decrease in diameter of seminiferous tubules, high of epithelial cells, as well as number of Leydig cells in group G1 might be resulted from testicular damage induced by ROS, which enhances disrupts of structural and functional integrity. These results are consistent with the previous findings (30, 32, 33). It has been shown that the balance of oxidant/antioxidant status in testis is necessary for male fertility, and hence testicular oxidative stress causes adverse effects on male reproductive performance (33, 34). In this regard, testicular tissue microenvironment characterized with low O_2 tension may be the main mechanism by which the testes protect themselves from FRs, in addition, the testis contains many antioxidant enzymes which protect the testicular functions from oxidative stress, mutation of genes, and apoptosis of germ cells (35). The main damaging effects of H_2O_2 on tissues are caused by its ability to induce lipid peroxidation in the cell membrane. This process has the potential to affect the fluidity and permeability of cell membranes (36, 37) with depletion of mitochondrial ATP, increases intracellular calcium, and activates membrane phospholipid proteases (33, 38). In addition, lipid peroxidation triggers mitochondrial membrane hyperpolarization, which in turn leads to the translocation of Bax and Bad, the discharge of cytochrome C from mitochondria (39), and the activation of the caspase cascade, which ultimately results in apoptosis and causes a significant amount of germ cell death via both the Fast and Fasil systems (40).

Normally, autophagy is an intracellular self-digesting mechanism by lysosomes degradation pathway (41) and removal of damaged organelles and tissues for regulating diverse cellular functions in response to oxidative stress, for example, hyperglycemia (42) and hypoxia (43). Many researchers have been emphasizing that the mechanism of male infertility is caused by the effect of oxidative stress inducesautophagy in the testis via significantly downregulation or inhibits the PI3K/Akt mTOR signaling pathway and disruption of nuclear factor erythroid 2-like 2 (Nrf2) and p62 signaling cause an impairment of Leydig's cellsfunction and promotes oxidative insult of the testes (44,45,46). Moreover, an accumulation of autophagosomes, such as disrupts the integrity of blood-testis-barrier (BTB), spermatogenesis disturbance, accumulation of damaged mitochondria which play a pivotal role in male infertility (47).

According to the findings in this study, histomorphometric and histopathological testicular alterations-induced by H_2O_2 were rescued partially or completely neutralized by administration of RS in group G2 represented by ameliorating testicular tissue damage via preserving the integrity of the cellular membrane, presumably through its antioxidant properties. Similar findings have been reported by (48) and (49). Many researchers suggested that RS may be promising for protection against oxidative stress-mediated by significantly upregulated of p85, PI3K/pAkt pathway, thus it can be used as an antioxidant (50-52) leading to decrease autophagy. Khanzadehand his colleagues., (53) confirmed the synergistic effect of a mixture of resveratrol and prednisolone cause decreased the expression of BCL2 and overexpression of BAXin a dose- and time-dependent manners via regulation of anti-apoptotic and pro-apoptotic proteins of the BCL-2 family. As well, Sirtuin-1 inactivates the proapoptotic P53 protein through an NAD+ dependent pathway, while resveratrol stimulates the poly-ADP-ribose polymerase-1(PARP1) protein expression and ultimately increases NAD+ concentration, therefore resveratrol caused an increase in sirtuin-1 activity indirectly(54).

Outcomes of present study suggest that resveratrol may be an another option as a protective agent in ameliorating the testicular injury -induced by hydrogen peroxide experimentally to a certain limits. Thus our results could be suggested that resveratrol to be used as protective supplement in attenuating testicular tissues damage due to ROS-mediated hydrogen peroxide experimentally.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR'S CONTRIBUTIONS

ABN, designed and performed the experiments, analyzed, and interpreted the data; AJM, provided technical support and conceptual advice, wrote the first draft. Both authors read and approved the final manuscript draft of the.

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التغيرات الشكلية القياسية والنسيجة المرضية للخصية بعد التعرض لبيروكسيد الهيدروجين :الدور الوقائي لمكمل ريسفيراترول

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الخلاصة

يستخدم الريسفيراترول (RS) على نطاق واسع في المجالات الطبية كمضاد للأكسدة. أستقصى البحث الحالي تأثيرمكمل الريسفيراترول على التغيرات القياسية النسجية والنسيجة المرضية في الخصيتين الت يتم تكوينها بواسطة بيروكسيد الهيدروجين (H_2O_2) في الفئران كنموذج حيواني. تم استخدام أربعين فأراً بالغاً في هذه الدراسة، قسمت عشوائياً إلى أربع مجموعات متساوية وتمت معاملتها لمدة 56 يوماً على النحوالتالي، المجموعة الضابطة (C) أعطيت ماء الصنبورفقط، المجموعة الثانية (G1) أعطيت 0.5٪ بيروكسيد الهيدروجين مع ماء الصنبور، المجموعة الثالثة (G2) أعطيت 0.5٪ بيروكسيد الهيدروجين مع ماء الصنبور ومكمل ريسفيراترول بجرعة 87 ملغم/كغم من وزن الجسم، والمجموعة الرابعة أعطيت مكمل ريسفيراترول بجرعة 87 ملغم/كغم من وزن الجسم. في نهاية التجربة تم القتل الرحيم للجرذان وتم تشريحها، وجمعت عينات من أنسجة الخصيتين لغرض التقييم النسيجي والمرضي. أظهرت النتائج أن إعطاء بيروكسيد الهيدروجين تسبب في تحليل نسج شكلي مع تغيرات نسيجية مرضية في شكل سمك الغلالة الليفية، واختلال الترتيب. من الخلايا الجرثومية، نخرالحيوانات المنوية، وذمة وفقدان الحيوانات المنوية مقارنة بالمجموعات الأخرى. في الوقت نفسه، تم تخفيف هذه التغيرات جزئيًا في مجموعة G2 التي قامت بتنبيب ريسفيراترول. وهكذا، خلصت الدراسة الحالية إلى أن ريسفيراترول قد يكون له قيمة علاجية في علاج إصابة الخصية المستحثة بواسطة بيروكسيد الهيدروجين بسبب نشاطه المضاد للأكسدة والتخفيف من الآثار الضارة للإجهاد التأكسدي من خلال آليات يجب توضيحها في الدراسات المستقبلية.

الكلمات المفتاحية: بيروكسيد الهيدروجين ، التغيرات النسيجية ، القياسات النسجية ، ريسفيراترول ، الخصية