Protective Effect of Grape Juice on Testicular and Epididymis Damage in Rats Exposed to Cigarette Smoke

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ABSTRACT

Objective: Cigarette smoke is a major reason for inducing reactive oxygen species that result in oxidative damage of testicular tissues and epididymis. This study aimed at investigating the possible protective effect of grape juice on testicular and epididymis damage in rats exposed to cigarette smoke. Methods: Forty adult male rats were used and divided into four groups for this study: control (group I), grape juice (group II), rats exposed to cigarette smoking (group III), and that exposed to cigarette smoking, followed by treatment with grape juice (group IV). After one month, the rats were weighed and sacrificed. Then, tissues were removed, processed for routine paraffin embedding, stained, and examined for histopathological changes in all groups, and their testosterone levels and antioxidant markers were assessed. The statistical significance among different groups was determined using one-way ANOVA. Results: Histological evaluation of testes showed degenerative alterations, perturbation, and atrophy of spermatogenesis in various seminiferous tubules, along with a reduced number of Leydig cells and less of sperm in epididymis interstitial spaces, as well as reduced activities of antioxidants superoxide dismutase, catalase, and testosterone levels in group III than in control groups I and II. Histopathological changes were reduced while testosterone levels and activities of antioxidants were improved in animals from group IV. In conclusion, these results may suggest that grape juice has a protective effect against the adverse effects of cigarette smoke in rats via inhibition of oxidative stress and testicular damage.

Key Words: *Antioxidants, Cigarette smoke, Epididymis, Grape, Testis, Testosterone.*

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INTRODUCTION

Infertility is a global issue, affecting 10% to 15% of childbearing couples. Amongst infertility patients, male infertility accounts for approximately 30%~50% [1, 2]. Although male infertility occurs for various reasons, infertility caused by the high concentrations of active oxygen in semen prompted a decrease in sperm viability and density and oxidative DNA damage [3, 4]. It has been reported that many factors, among which smoking was one of the most common reasons, could induce the production of reactive oxygen species. Excessive generation of ROS, such as super-oxide free radical and oxygen ions, may disturb the oxidant-antioxidant balance, consequently causing oxidative damage to tissues [5]. Several studies have shown that smoking could cause DNA damage of germ cells and reduction of the level of sex hormones, despite the fact that antioxidant level decreases in smokers' body, the adverse effects in testes could be improved by oral administration of antioxidants [6]. Cigarette contains many components, including nicotine, which is a few liquid alkaloid. Nicotine is related to cardiovascular disorder, congenital anomalies, and toxicity [7, 8]. Furthermore, nicotine reduces the antioxidant level in rats [9]. Fats within the cellular membrane are prone to be damaged by those radicals and oxidation. Antioxidants suppress this damage by removing free radicals and inhibiting different oxidative reactions [10].

Grape (Vitis vinifera) is a substantially consumed fruit worldwide. It has many active products, including procyanidins, proanthocyanidins, anthocyanins, polyphenols, and flavonoids [11]. Proanthocyanidin acts as

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an antioxidant and radical scavenger [12]. Furthermore, grape modifies many metabolic reactions and has cytoprotective and anti-inflammatory properties [7]. It has been reported that organic grape juice contains higher polyphenol levels than their counterparts [13]. Polyphenols exhibit a crucial antioxidant effect, which may additionally protect the cells from oxidative damage [14]. Administration of grape flavonoids exhibits antioxidant effect and reduces thrombus formation and inflammation; there have been some studies on the role of grape juice in multiplied serum antioxidant capability and protection of LDL against oxidation [15]. Grape juice has lately been used as an alternative natural food. As a result, and because of limited evidence, the present study aimed at investigating whether grape juice can modulate the testicular tissues, toxicity, and histology of male rats exposed to cigarette smoke.

MATERIALS AND METHODS

Grape juice

The grape samples used in this investigation were purchased from Agriculture Company, Saudi. Grape juice was then obtained using organic (free of genetic engineering and pesticides) and conventional (traditionally cultivated) techniques. **Table 1** displays the essential characteristics of grape juice [16]. Ascorbic acid, proteins, lipids, and carbohydrates determinations were done according to the Association of Official Analytical Chemists [17]. The quantity of polyphenol was calculated to be equivalent to 4 glasses of natural grape juice (200ml each) [18]. According to the American Dietetic Association, intake of about 200 – 500 ml grape juice gives mild to strong evidence of positive physiological effects [19]. The individual dose of purple grape juice concentrate was adjusted according to the animal weight, that is, during the experiment rats given 222 mg/d of grape juice/g body weight by stomach tube twice a day.

Table 1: Chemical characteristics of conventional and organic purple grape juices.

Parameter	Conventional juice	Organic juice
Energetic values (kcal)	81.63±0.55	82.85±0.57
Carbohydrate (%)	17.5±0.28	17.50±0.01
Lipid (%)	1.07±0.06	1.25±0.09
Protein (%)	0.50±0.01	0.38±0.03
Anthocyanins (mg/L)	255.03±0.35	340.76±0.47
Catechins (mg/L)	14.06±0.01	33.68±0.01
Gallic acid (mg/L)	8.27±0.01	5.30±0.01
Procyanidins (mg/L)	15.21±0.27	14.47±031
Resveratrol (mg/L)	0.15±0.01	0.22±0.41
Ascorbic acid (mg %)	26.71±1.65	45.34±1.64
Phenolic content (mg %)	125.76±1.71	146.32±1.01

Values are expressed as means ± SE of duplicate results.

Experimental animals

Forty male Wistar rats (weighing approximately 300 g, 3-month-old) from King Fahad Medical Research center were used in the experiments. The animals were handled under standard laboratory conditions consisting of a 12:12 light/dark cycle and maintained at 23 ± 2 °C. All animals acclimatized for 7 d before the experiment. Rats had free access to water and commercial diet. Experiments were performed from 8:00 AM till 3:00 PM in a noise-free room.

Treatments

Forty rats were randomly divided into 4 groups (10 animals per group). Group I control animals were administered with saline via a stomach tube for 1 month. Group II animals received purple grape juice daily (200 mg/g body weight/d) via a stomach tube for 1 month. Group III animals were exposed to cigarette smoke three times a day, thirty minutes per time for 1 month. Group IV animals were exposed to cigarette smoke three times a day, thirty minutes per time plus received purple grape juice via a stomach tube daily for 1 month. This experimental study was conducted under the approval of King Fahad Medical Research. All experimental protocols regarding animals conformed to the procedures described in the Guiding Principles for the Use of Laboratory Animals according to the guidelines specified by the institution of King Abdulaziz City for Science and Technology (KACST) **No.215221.**

Conditions for cigarette smoke exposure

The test animals were put in a stainless steel cage, which was then placed in transparent acrylic cages (60 × 30 × 25 cm) with a small opening with a width of 6 cm. The cigarette smoke was administered using three lit cigarettes for 10 min each to fill the acrylic cage with smoke for 30 min. Group III (exposed to cigarette smoke group) rats were exposed to a nose-only system to second-hand smoke of cigarette twice a day for five consecutive days a week, for 1 month and each time six cigarettes were used with a declared content of 6 mg tar, 0.5 mg nicotine, and 7 mg tobacco. Each puff contained 20 mL of cigarette smoke and each cigarette was smoked in 16 puffs, with intervals of 25 s. Nonsmoker animals underwent the same procedure but the cigarette was not lit.

Biochemical analyses

Before sacrifice, the blood of the animals was collected and kept in a heparin-coated tube. The testis and epididymis were carefully segregated, washed in normal saline solution (0.9%), blotted, and separately weighed, and the

average weights were recorded. The collected blood was centrifuged at 25 °C for 10 min at 4000 rpm to obtain the serum. The serum samples were then stored in the deep freezer (-18 °C). The blood testosterone levels were assayed using ELISA (Abcam 108666, USA) [20]. Superoxide dismutase activity was analyzed using a commercially available kit, Superoxide Dismutase Assay Kit, which utilizes a tetrazolium salts to detect superoxide radicals generated by hypoxanthine and xanthine oxidase. Catalase activity was determined according to Goth's method [21] using hydrogen peroxide as a substrate.

Histological study

The rats were sacrificed at the end of the experiment. The testes were fixed in Boun while cauda epididymis was fixed in 10% buffered formalin for 24h, and then, embedded in paraffin blocks. The paraffin sections of 5 µm thickness were cut and placed on glass microscope slides, which were incubated at 60 °C in an oven for 2h, followed by xylene dewaxing for two times, and washing serially with anhydrous ethanol, 95% ethanol, 85% ethanol, and distilled water. The nuclei were hematoxylin stained for 15 min, treated with 5% hydrochloric acid alcohol, washed with 0.1% ammonia water, dyed with 15% eosin, subjected to all levels of alcohol dehydration, baked, xylene treated, subjected to gum resin sheet, and finally, observed under a microscope [22].

Statistical analysis

Weight of body, testis, and testosterone hormone was evaluated using the SD Student's *t*-test, and calculated as a mean value. P <0.05 was considered significant [23]. One-way ANOVA was used to determine the statistical significance among different groups using SPSS software.

RESULTS

Weight of body and testis

In this study, exposure to cigarette smoke led to significantly decreased body weight and relative weight of testis in rats in comparison with the control and grape juice group. The body and testis weights were increased in animals treated with grape juice in comparison with cigarette smoke and control animals (Table 2).

Table 2: Body, testis weights, serum testosterone levels, and antioxidant markers in rats from all groups experimental.

Parameters	Control group(I)	Grape group (II)	Cigarette smoke group (III)	cigarette smoke with grape juice group (IV)
Body weight (g)	434.3±0.02	407.1±0.03	332.2±0.04*a, b	396.1±0.01*b, c
Testis weight (g)	3.10±1.01	3.09±0.31	2.42±0.23* a, b	3.02±1.01*c
Testosterone (ng/dl)	350.3±0.6	343.7±0.5	194.6±0.2* a, b	300.4±0.4*c
SOD(unit/mg protein)	1.20±0.05	1.21±1.03	0.79±2.11* a, b	1.08±1.02*c
CAT(unit/mg protein)	22.45±0.22	23.53±0.15	15.40±0.23*a, b	20.21±1.01*b, c

Values are as mean ± SD. A comparison was made using the One Way ANOVA test Significant at ($P < 0.05$). $^{a}p < 0.05$ compared with control group, $^{b}p < 0.05$ compared with grape group and $^{c}p < 0.05$ compared with cigarette smoke group.

Testosterone hormone and antioxidant markers

Rats exposed to cigarette smoke showed a significantly decreased level of testosterone with reduced superoxide dismutase (SOD), catalase (CAT) in rats from the cigarette smoke group as compared with control and grape juice group (**Table 2**). However, in groups exposed to cigarette smoke and co-treated with grape juice exhibited improvement parameters than the control and III groups in testosterone levels and antioxidant levels significantly increased than other experimental groups (**Table 2**). Testosterone is not only responsible for the development of male secondary sexual characteristics during puberty but also essential for maintaining the normal function of the seminiferous epithelium.

Histological results

The seminiferous tubule in control group animals I (Positive) and II (Negative) consists of a central lumen lined with a specialized seminiferous epithelium containing two distinct cell populations: the somatic cells and the spermatogenic cells (spermatids, spermatocytes, and spermatogonia) **(Fig. 1a, b)**. The Sertoli cells are columnar cells extending from the basal lamina to the lumen of the seminiferous tubule. The seminiferous epithelium is encircled by a basement membrane and a wall formed by collagenous contractile cells, fibroblasts, and fibers. A more detailed view of seminiferous epithelium is shown in **Fig. 1b, c**. The nuclei of spermatogonia and Sertoli cells are closely associated with the seminiferous tubular wall. Overlying the spermatogonial cell population are the primary spermatocytes, their nuclei are larger and clumps of chromatin represent the meiotic chromosomes. Close to the lumen are the early spermatids with a round light nucleus and the late spermatids with elongated-shaped condensed nuclei **(Fig. 1c)**.

The space between the seminiferous tubules is occupied by abundant blood vessels (arterioles, capillaries, and venues) and endocrine cells called Leydig cells **(Fig. 1b)**. They

occur in clumps or singly and are embedded in the rich plexus of lymph and blood capillaries that surround the seminiferous tubules, the nucleus is round with dispersed chromatin and one or two nucleoli at the periphery. The extensive eosinophilic cytoplasm contains variable numbers of lipid vacuoles. Testosterone is the main hormone secreted by Leydig cells. The epididymis is a tube of smooth muscle lined by a pseudostratified epithelium. The smooth muscle displays rhythmic slow contractility that gently moves spermatozoa towards the ductus deferens. The epithelium lining of the epididymis exhibits a gradual transition from a pseudostratified columnar form as seen in the micrograph **(Fig. 1d)**.

The group III animals showed alterations in the body and testis weights and varying degrees of congestion in the blood vessels and structural damage to the seminiferous tubules, with the absence of mature spermatids, vacuolization, disorganized, and loss of germinal cells in the basement membrane **(Fig. 2a)**. The results indicated that exposed cigarette smoke caused degenerative alterations in the seminiferous tubules, revealed by thickened basal lamina, Sertoli cell vacuolation, decreased layer of spermatogenic cell masses, and altered general tubular architecture **(Fig. 2b)**. These alterations were related to the levels of exposed cigarette smoke. They also exhibited perturbation of spermatogenesis, degenerative alterations, and atrophy in several seminiferous tubules, as well as a reduced number of Leydig cells and increased interstitial spaces. Marked testicular epithelial damage and sperm gathering were observed in rats suffering from passive smoking alone **(Fig. 2c)**. The epididymis histology showed decreased sperm density and empty in some tubules from spermatozoa. In addition, residual bodies and damaged spermatocytes were seen in the tubular lumen **(Fig. 2d)** compared with the control.

Group IV rats showed improvement in the changes of the seminiferous tubules and exhibited large sperm count and smaller sperm deformation rate. Histopathological analyses in testis slices of that group revealed striking differences than those observed in testis histology of rats of other groups **(Fig. 3a)**. The somatic cells, called Sertoli cells, are found within the seminiferous tubules **(Fig. 3b, c)**, which support, protect, and nourish developing spermatogenic cells. Phagocytic cell portions, i.e. residual bodies, discarded by spermatids at the end of spermatogenesis facilitate releasing mature spermatids into the seminiferous tubule lumen. Androgens, acting through Sertoli cells, stimulate spermatogenesis. Myoid cells are responsible for the rhythmic contractile activity that propels the non-motile sperm to the rete testis. Sperms acquire forward motility after they have passed through the epididymal duct. Sertoli cells are closely associated with the seminiferous tubular wall. Primary and secondary spermatocytes are located close to the lumen **(Fig. 3c)**. In addition, Leydig cells in the interstitial supporting tissue between the seminiferous tubules synthesize and secrete male hormones. The major function of the epididymis is the accumulation, storage, and maturation of spermatozoa in the epididymis. The spermatozoa develop motility **(Fig. 3d)**. The principal cells of the epididymis epithelium bear tufts of very long microvilli called stereocilia, which are speculated to be involved in the absorption of excessive fluid accompanying the spermatozoa from the testis.

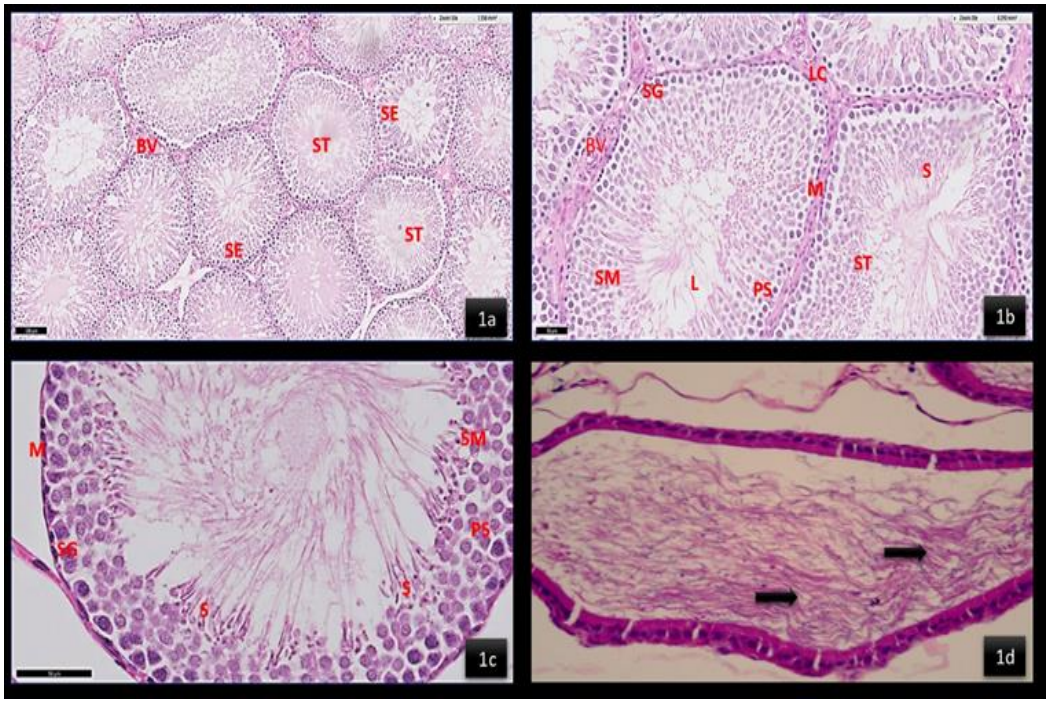

Figure (1a-d): Light micrograph of control rat's testis and epididymis (positive & negative) transverse section with cross-sectional. (a-c): the normal histological structure of the seminiferous tubules (ST) populated by seminiferous

epithelium (SE) surrounded the tubular lumen (V) Profiles of blood vessels (BV). In these crosses of seminiferous tubules, most of their cell types can see. Outside the tubules are myofibroblast (M). Inside near the basement membrane are many spermatogonia (SG), the primary spermatocytes (PS) are the largest spermatogenic cells and usually abundant at all levels between the basement membrane and lumen. Also, note spermatid (SM) and finally highly sperm cells (S). All stages of spermiogenesis and spermatogenesis occur with the cells closely associated with the surface of the adjacent Sertoli cell (SC). Leydig cell (LC) the principal cell type in the interstitial supporting tissue between the seminiferous tubules. (d): showing epithelium cell of the epididymis and wide lumen full of spermatozoa (arrow).

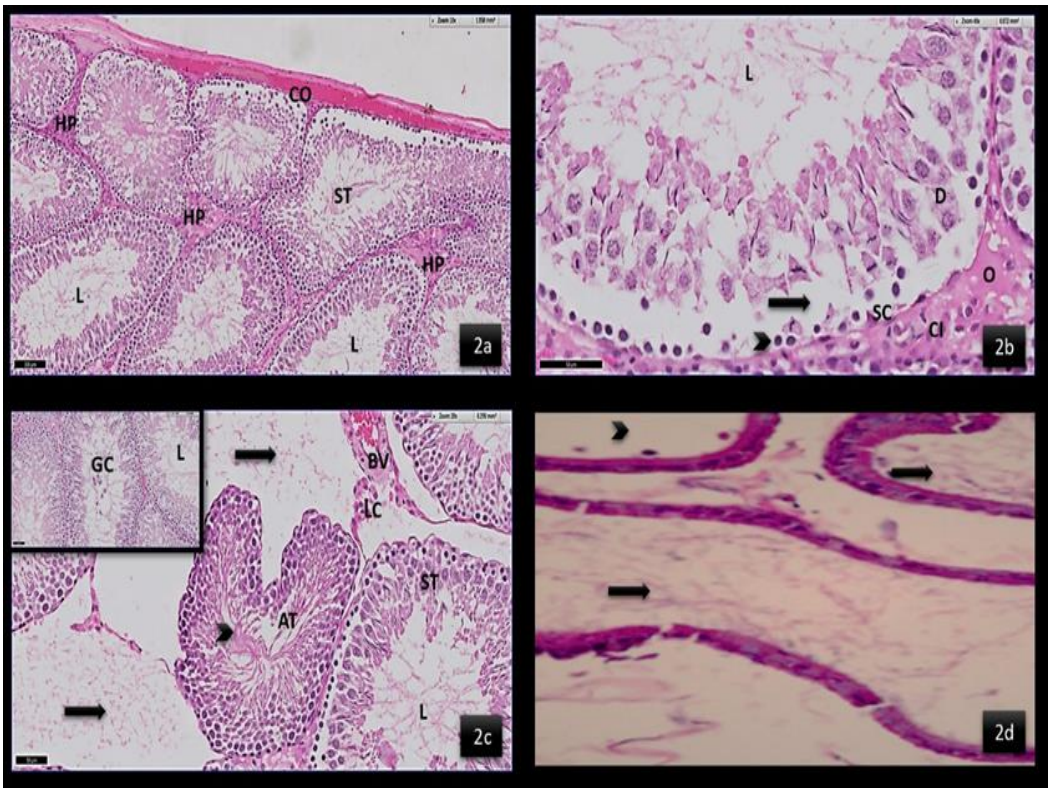

Figure (2a-d): sections of male rats testicular tissue Expose of cigarette smoke group (II): (a): shows fibrosis in the capsule of testis and hyperplasia in the intertubular tissue (HP), congestion in the blood vessel (CO), and enlarged seminiferous tubules (ST) populated by spermatocytes and spermatids. Note the decrease of sperm inside the lumen (L) of some tubules; x400. (b): higher magnification showing a disturbance in the germinal epithelium of the seminiferous tubules, reduced number of spermatogenic cells, and separated from allayers (arrow) as well as necrosis and detachment from the basement membrane (head arrow). Also, note degenerative of spermatocytes (D) and no sperm lined by Sertoli cell (SC) in the tubule, oedematous interstitium (O) associated with cellular infiltration (CI) and eosinophilic droplets; x1000. (c): showing marked tubular disorder and deformed of seminiferous tubules (ST) atrophic tubules (AT) filled with cellular debris and immature spermatids (head arrow), irregular basement membrane. Note damaged and necrotic Leydig cells (LC). Also, note the extension and expansion of interstitial and lysis of connective tissues (arrows), congestion in the blood vessel (BV). Higher magnification shows multinucleated giant cells (GC) and residual bodies in some tubular lumen, whereas appeared empty of some tubules (L); x400. (d): showing epididymis reduce of spermatozoa inside the lumen (arrows) and empty in some tubule (head arrow); x1000.

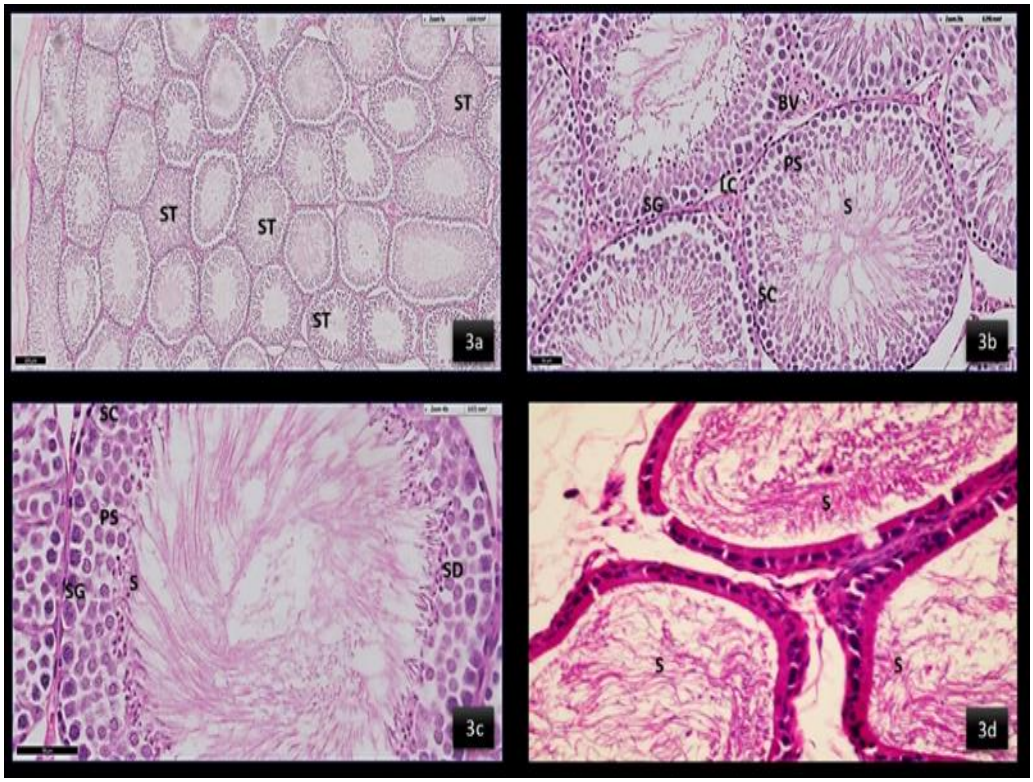

Figure (3a-d): sections of male rats testicular tissue expose of cigarette smoke and treated with grape juice group (III): (a): showing the normal distribution of collagenous fibers in the around the testicular tubules, improvement the histological structure of the seminiferous tubules (ST) and populated by many stages of spermatocytes in the tubular lumen.;x400. (b,c): High power from the previous section showing spermatogonium (SG)with many mitotic stages of division, primary spermatocytes (PS), spermatids (SD), tubular basal lamina with Sertoli cell (SC). Note myoid cell (M) and narrow intertubular space contain interstitial Leydig's cells (LC) and less congestion in blood vesicle (BV); x1000. (c): showing epithelial cell of epididymis and wide lumen full of spermatozoa (S); x1000.

DISCUSSION

Nicotine is one of the dangerous components of a cigarette. Its consequences are great and extend to all of the body systems, such as renal, respiratory, cardiovascular, and reproductive systems. In several studies, nicotine is oxidized into cotinine in the liver, produces free radicals, and induces oxidative damage in tissues [24]. Moreover, nicotine can also increase the levels of neurotransmitters, including dopamine and serotonin, which might be suppressors of appetite and decrease relative weights of testis possibly due to a reduction in body weight. In addition, the growth of testis weight might be related to nicotine in cigarette smoke [25].

Antioxidant enzymes are an essential part of the cellular defense. The activities of antioxidant enzymes including CAT (scavenges or reduces hydrogen peroxide to form oxygen and water) and SOD (converts superoxide anion to oxygen and hydrogen peroxide) [26], significantly decreased in rats from the cigarette smoke group. The imbalance between scavenging activities and ROS generation may induce oxidative stress, which in turn may induce oxidative damage to the cellular component and altered many cellular functions including loss of enzymatic activities [27]. So, the decreased CAT and SOD activities may be due to decreased enzyme synthesis by the inactivation of the enzymes or damage of testicular cells.

Testicular epithelial damage is a vital occurrence in testis oxidative injury [28]. We found a significant inhibition of cell damage, consistent with previous investigations. Oxidation may ultimately result in structural damage of the cell and due to excessive ROS; mutations in the mitochondrial genome could possibly disturb the formation of functionally and morphologically mature spermatozoa [29]. High concentrations of unsaturated fatty acids resulting from ROS in the spermatogenic cells give rise to several dual links in the plasma membrane, which might decrease cytoplasmic antioxidants, making spermatogenic cells susceptible to oxidative damage. Consequently, oxidation of the membrane fatty acids could result in the lack of membrane fluidity and could decrease of channels and activity of enzymes of sperm cells [30]. Sankako *et al.* [31] proved residual damage after exposure to cigarette smoke on sperm morphology, motility, and concentration [32]. Loss of germ cells in numerous seminiferous tubules and epididymal histology showed decreased sperm density in nicotine exposed individuals [27]. Kolawole *et al.* [33] stated that serum LH, FSH, and

testosterone levels decreased compared to the nicotine-exposed control group [33]. In addition, animals exposed to cigarette smoke showed various degrees of damage to seminiferous tubules, absence, and shrinkage of mature spermatids, vacuolar degeneration, and loss of germinal cells in the basement membrane [32]. In addition, Nesseim *et al.* [34] reported that nicotine caused structural damage in the seminiferous tubules, decreased thickness of spermatogenic cell masses, and Sertoli cells vacuolation [34].

Nowadays, medicinal plants have many applications and reproductive organs, including sperm and testis parameters are of the target tissues for these plants [24]. Ahmed, *et al*., [35] found that grape intake protects the liver tissue against hepatotoxins by scavenging the free radicals [35]. In addition, grape juice is a powerful antioxidant and ameliorates renal fibrosis and dysfunction. Grape juice contains several phenolic compounds that increase intracellular vitamin C, lower capillary permeability, act as free radical scavengers, and inhibit lipid peroxidation [11]. In addition, Nesseim *et. al.* [34] reported that the histological damage in seminiferous tubules of rats was, in part, recovered after nicotine withdrawal, specifically at a small dose [34]. Similarly, these findings have been in agreement with the results of Sankako, *et al.* [33] who stated that termination of nicotine administration leads to partial amelioration of ovarian and uterine damages and complete healing may also occur if nicotine exposure termination is accompanied with the use of an antioxidant [33].

Nicotine reduces the antioxidant level in rats. Antioxidants in grape suppress this damage by removing free radicals and inhibiting different oxidative reactions [14]. Therefore, grape increases CAT and SOD bioavailability probably by decreasing the damage to the testicular cells as proved in the present investigation. The reduced activity of SOD may subsequently scavenge excess superoxide anion in the rat testis following exposure to CS. Moreover, the elevated activity of CAT can be a compensatory mechanism of scavenging high hydrogen peroxide formed by the concomitant increased activity of SOD in rats of the CS group. Grape juices have a high level of total polyphenols, anthocyanins, catechins, and resveratrol [17], and exhibit vital antioxidant activity [18]. These effects are because of a high level of polyphenols in grape juices. In previous studies, grape juice exhibited a similar protective effect against oxidative damages induced by exposure to cigarette smoke in both the testis and serum of rats as shown in this study.

Previous evidence suggests that antioxidants represent the most potent defensive mechanism that ameliorates the toxicity related to free radicals [36]. Therefore, these antioxidants could be used for the alleviation of cell-damaging effects triggered by several pathological changes. Results of some studies confirmed that polyphenolic compounds in grape juice protect lymphocytes from injury, decreasing oxidative DNA damage probably by reducing free radicals stages [31]. Grape juice contains high levels of resveratrol that exhibit antioxidative, anti-inflammatory properties in mammals [33]. Since nicotine is also responsible for the generation of ROS, it appears that amelioration of oxidative effect by flavonoids in purple grape juice can assist in the cure and protection of sperm cells.

CONCLUSION

In conclusion, grape juice treatment in this study had a protective effect against cigarette smoke in male Wister rats, especially histological changes in epididymis and testis, through inhibiting oxidative damage.

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