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Ultra-Structural Study on the Effect of *Salvadora persica* (Miswak) on Testes of Albino Rats

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Abstract: *Salvadora persica* (Miswak chewing sticks) has been widely used in the traditional medicine, but little is known about its toxic effect especially on the male reproductive organs. The present study aims to investigate the induced effect of watery extract of *Salvadora persica* on the testicular weight, histochemical and ultra-structural parameters. Adult male albino rats (n=18) were divided equally into group A (control rats) received saline solution and group B (experimental rats) orally administrated with *S. persica* extract (350 mg/ kg Body Weigh) daily for a month. The administration of *S. persica* watery extract had no effect on the body weight gain compared to group A, whereas, significant ($P < 0.05$) reduction was exhibited in the sperm count, diameter of seminiferous tubules, testicular weight and testicular weight per body weight of group B compared to group A. Also, the administration of *S. persica* induced ultrastructural and histochemical changes including: signs of damage in Sertoli cells, pyknotic nuclei of the germ cells, deformed or misshape spermatozoa and clumps of Leydig cells. The cytoplasm of Leydig cells was filled with abnormal number of steroid secretary granules, RBCs and leucocytes. Significant ($P < 0.05$) increase in the percentage of collagen fibers exhibited in the seminiferous tubules of rats of group B. The results suggested that *Salvadora persica* has potential toxic effect in male Albino rats.

Key words: *Salvadora persica* · Miswak · Testes · Toxicity · Ultrastructure · Histochemistry

INTRODUCTION

The use of plants in the traditional medicine to maintain good heath and treat different illness is widespread in many countries. Flavonoid, saponins and chloride are the principle contents of most medical plants. Recently, research has focused on how the phytochemical plants could induce adverse and toxic effects especially on the male reproductive organs [1, 2]. Meerwal and Jain [3] reviewed a large number of medical plants with toxic and anti-fertility effects in male rodents.

The common medical plant species of *Salvadora persica* Lnn. family Salvadoraceae is found in the deserts of Egypt, Saudi Arabia, Sudan, India and Asia. The tree of *S. persica* is also known by the name of Miswak, Mustard and Arak tree [4]. In the Middle East, all different parts of *S. persica* trees are used for medical purposes for both oral and systemic conditions. The stems of *S. persica* tree are cut into short sticks and used as a natural tooth brush [5]. Extracts released from *S. persica* stems contain biological properties with anti-plaque and anti-bacterial effects [6-8]. Also, extracts from *S. persica* stems have been used as a complementary therapy due to their anti-diabetes [9, 10], anti-hyperlipidemic [11] and anti-stress effects [12]. Different phytochemical contents are found in the aqueous extract of *S persica* stems including fluorides, tannins, vitamin C, salicylic acid, volatile oils-sinigrin, trimethylamine and salvadoricine related alkaloids, organic sulpher compounds**,** saponins, β-sitosterol, lignin glycosides and flavonoids [13-15].

Although *S. persica* Miswak is considered to be an important medical plant, certain disadvantages are associated with its uses. Darmani *et al.* [16] evaluated the toxic potential of *S. persica* plant on male and female reproductive systems and fertility. They showed that the direct administration of a high dose (800 mg/kg) of *S. persica* Miswak extract to mice revealed abnormalities in the reproductive weight and reduction in female fertility. Reddy *et al.* [17] showed that the administration of high doses of *S. persica* extract to male mice induced toxic effects especially on sperm morphology. The effect of *S. persica* stem extract on the histopathology of male

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reproductive system has not been previously studied; therefore, it seems of interest to further investigate the adverse effect of prolonged administration at a lower dose of *S. persica* (Miswak) stem extract on rats' testes using ultra-structural and histochemical techniques.

MATERIALS AND METHODS

Preparation of *S. persica* Extract: *Salvadora persica* (Miswak chewing sticks) were purchased from herbal drugs market at Madina El-Monawarah, K.S.A. The chewing sticks were cut into small pieces and allowed to dry. The plant stems were grind into powder. The extract was freshly prepared according to the method of Ramadan and Alshamrani [12] by adding the plant powder (20 g) into 400 ml of distilled water. The mixture was boiled for 30 min, allowed to cool in room temperature and filtrated using Whatman (size 2) filter paper. The watery extract was administrated to rats at a dose of 350 mg/ kg.

Experimental Animals: Eighteen Wister albino male rats with mean body weight 186±7.6 (g) were obtained from the animal house, Helwan Agricultural University, Egypt. Before the experiment, all rats were accommodated for a week in metal cages under controlled conditions of light (12h: 12h light dark cycle), temperature (25±20C). The animals were fed on a standard pellet diet and water was provided *ad libitium*. The experimental animals were cared according with the Institutional Animal Ethic committee of Al-Azhar University. The 18 adult male rats were divided equally into group A (control rats) received saline solution and group B (experimental rats) intragastrical administrated using intubation needle with *S. persica* (Miswak) extract (at a dose of 350 mg/ kg body weight) daily for a month.

Organ Weights and Sperm Count: Initial and final body weights of each rat were recorded. The testes was dissected out, freed from adherent tissues and weighted to the nearest milligram. Sperms of both control and Miswak treated rats were released out of the vas deferens by putting pressure on the epididymis, diluted twice in water and counted using a hemocytometer.

Histological and Histochemical Analysis: Testes of both groups were fixed in 10% formol saline for twenty four hours. Washing was done in tap water then serial dilutions of ethyl alcohol and absolute ethyl were used for dehydration. Specimens were cleared in xylene and embedded in paraffin. Blocks of paraffin wax tissue were sectioning at 5 µm in thickness. For general histology, sections were stained with Harris' haematoxylin and eosin [18]. Periodic-acid Schiff (PAS) staining was carried out for detecting of glycogen and Mallory's trichrom stain was carried out for detecting collagen fibers [18,19]. The optical density of PAS^{+ve} stained materials and percentage of reaction of Mallory's trichrome stained fibers were analyzed by using software Leica Qwin Live Analyzer Program for microscopy.

Ultra-structural Analysis: Testes of the control and treated rats were immediately removed, cut to small pieces (0.5 cm) and fixed in 4% glutaraldehyde in 0.2M cacodylate buffer (pH 7.2) for 24h at 4°C. Specimens were post fixed in 1% OsO_4 in cacodylate buffer and embedded in epon [20]. Semi-thin sections taken by LKB ultra-microtome (about 0.5µm in thickness) were stained with toluidine blue and photographed by sc 30 Olympus Camera. Ultra-thin sections (about 100 A° in thickness) using Leica AG Ultra-microtome were stained with uranyle acetate and lead citrate and examined by TEM 100 CXII (at 80 kv).

Statistical Analysis: Statistical analyses were performed using analyses of significant differences between means of *S. persica* treated and control groups by using one-way ANOVA test. Data were presented as mean±SD and *P* value of ≤ 0.05 was considered statistically significant.

RESULTS

Effect of *S. persica* Extract on Rat's Testes Weight, Body Weight and Testes/body Weight Ratio: There is a significant ($P < 0.05$) decrease in the testes weight of rats treated with *S. persica* extract (at a dose of 350mg/ kg B.W) daily for 30 days compared to the control rats, whereas, the rats' body weight exhibited a significant ($P < 0.05$) increase when compared to the control rats. The rats' testes weight/body weight ratio is 0.47 % in the *S. persica* treated rats and 0.59 % in the control rats. This was found statistically significant (*P* value < 0.05) (Table 1).

Effect of *S. persica* Extract on Rats' Diameter of Seminiferous Tubules and Sperm Count: There is a significant (*P* value < 0.05) decrease in the diameter of the seminiferous tubules of rats' testes treated with *S. persica* extract when compared to the control group. Also, the sperm count was 200.7 million/ml in the control rats, while it was 68.3 million/ml in rats treated with *S. presica* extract (Table 2).

Table 1: Effect of *S. perisca* watery extract on body and testis weights

Groups	T.W (g)	Final B.W (g)	B.W gain (%)	TW/B.W (%)
A: Control	1.32±0.08	221.22±6.39	35.22g = (19%)	0.59±0.02
B: Treated	1.07±0.03	227.55±4.85	41.55 g = (22%)	0.47±0.009
P-value	0.000*	0.031*	0.000*	0.000*

B.W: body weight, T.W: testes weight. Values are expressed as mean±SD for n=9 male rats in each group.

Table 2: Effect of *S. perisca* watery extract on fertility of male rat

Groups	Diameter of S.T (µm)	Sperm count (million/ml)
Control	288.88±9.28	200.77±5.23
Treated	196.66±7.90	68.33±6.57
P-value	0.000*	0.000*

S.T: Seminiferous tubules. Values are expressed as mean±SD for n=9 male rats in each group.

Histopathological Effect of *S. persica* on Rats' Testes

Light Microscope: Examination of cross sections stained with H&E of testes of the control animals (received physiological saline) illustrated the normal histological structure of the mature seminiferous tubules and the normal organization of spermatogenic cells (Fig. 1A). Examination of semi-thin sections stained with toluidine blue presented clearly the germ cells at various stages of spermatogenesis composing of spermatogonia, spermatocytes, spermatids and mature spermatozoa (Fig. 1B). Mature spermatozoa are in the lumen of the tubule and the developing spermatozoa are in contact with Sertoli cells. Also, the interstitial cells of Leydig located between the seminiferous tubules showed normal

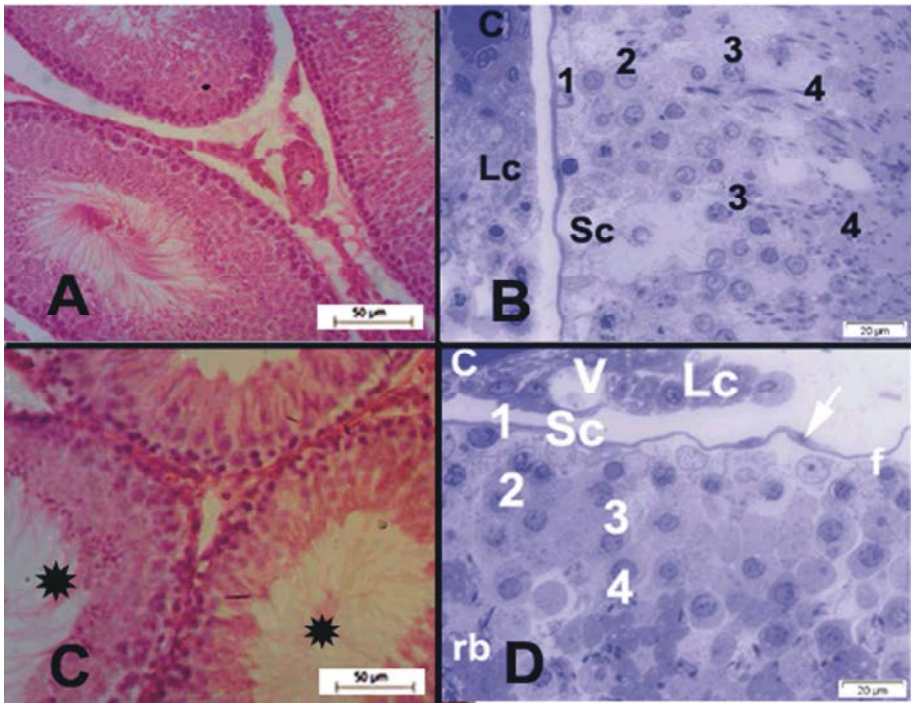

Fig. 1(A-D): Micrographs of sections of testes belonging to rats of group A (control) and group B (treated with *S. persica* extract). H&E

A, testes of rats of group A with normal morphological appearance and normal spermatogenesis process (Hx & E stain). B, a seminiferous tubule of rats of group A with normal spermatogenesis and interstitial cells (Toluidine blue stain). C, testes of rats of group B with abnormal reduction in germ cells and spermatozoa (Hx & E stain).

D, a seminiferous tubule of rats of group B with abnormalities including: Leydig cells with large vacuoles and blood capillary, corrugated basement membrane (arrow), miss-shaped nucleus in the spermatogenic cells and not-distinguished acrosomal cap and granules of the spermated cells. Spermatozoa appear variable in shape and size with presence of abundant residual bodies. Sertoli cells appear numerous (Toluidine blue stain).

Leydig cells (Lc), Blood capillaries (c), vacuoles (V). Seminiferous tubules lined with basement membrane (arrow), Sertoli cells (Sc), the spermatogenic cells in form of Spermatogonia (1), Spermatocyte (2), Spermated (3) and Spermatozoon (4), fat globule (f), residual bodies (rb).

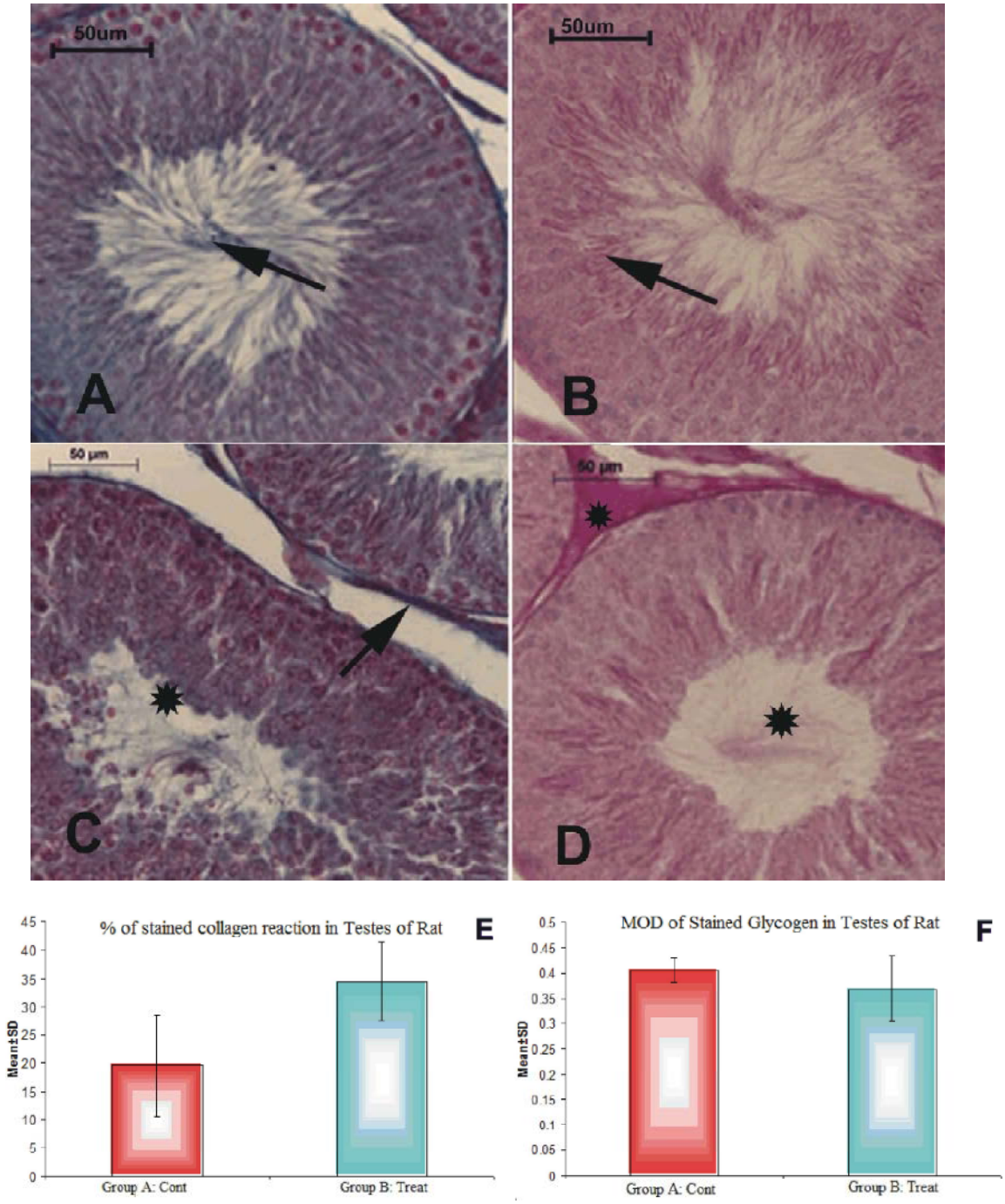

Fig. 2(A -F): Effect of *S. perisca* watery extract on collagen and glycogen content in the testes of rats:

A, showing normal distribution of collagen fibers in the spermatozoa (Arrow) and interstitial cells of testes of the control rats (Mallory's trichrome stain). B, showing the normal distribution of glycogen in the spermatozoa (Arrow) inside the testes of control rats (PAS stain). C, testes of treated rats showing abnormal increase in collagen fibers (Arrow) and reduction in the number of mature sperm (star). D, testes of treated rats showing abnormal distribution of glycogen in the interstitial area (Star) and reduction in the number of stained spermatozoa (star). E, F, Showing the mean optical density (MOD) intensity of glycogen in control and treated rats.

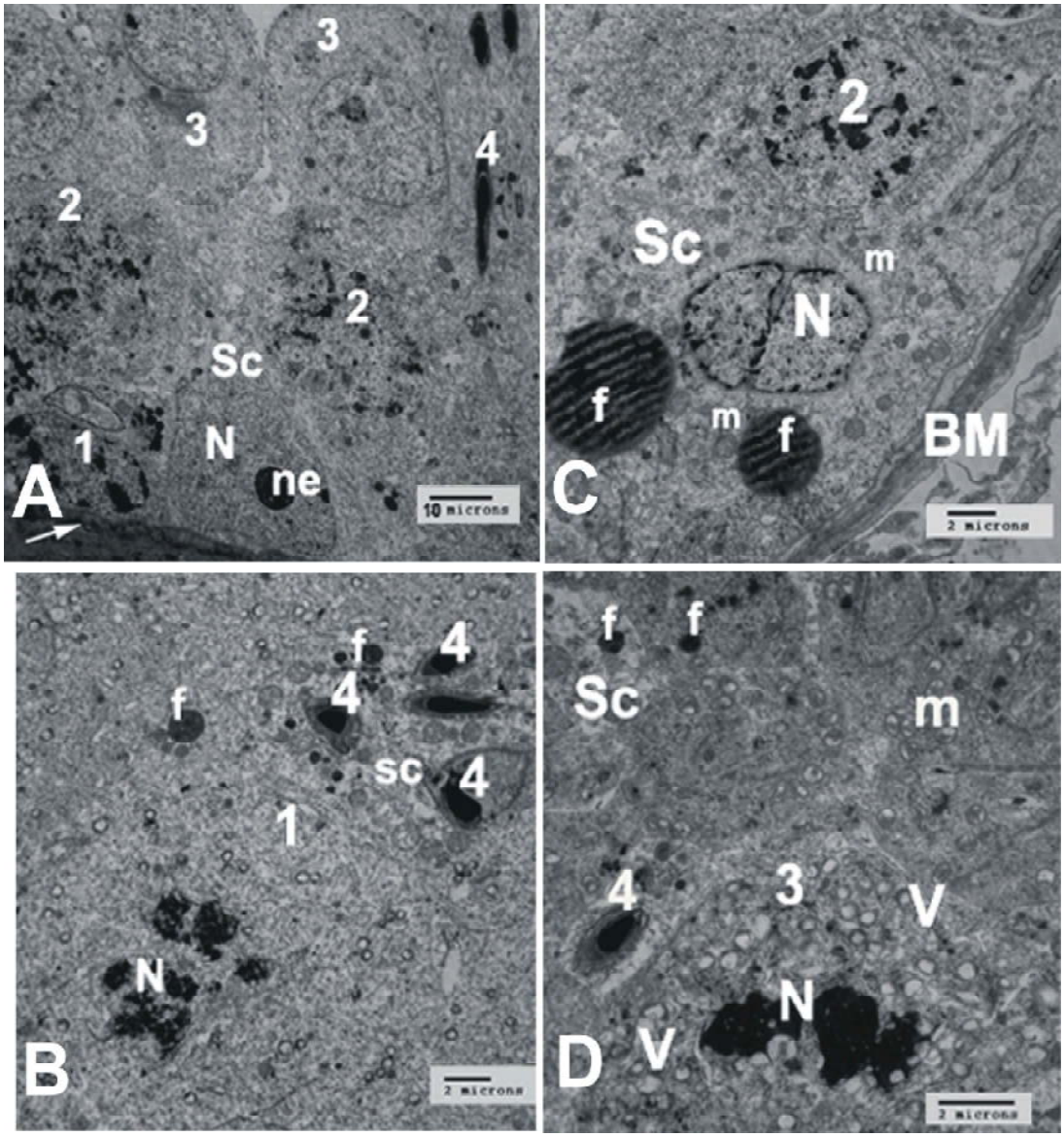

Fig. 3(A- D): T.E. Micrographs of the seminiferous tubules belonging to group A (control rats) and group B (*S. perisca* treated rats).

A, showing the seminiferous tubules of group A formed by basement membrane (arrow), Sertoli cell (Sc) containing large vesicular nucleus (N) with prominent nucleolus (ne), Spermatogonia (1),Spermatocyte (2),Spermated (3) and Spermatozoon (4) and all of normal morphological appearance.

B, spermatogenic cells in stat of pyknosis as well as deformity of the nucleus (N) with presence of membrane bounded vesicle in its cytoplasm. Notice presence of miss shaped or deformed Spermatozoon (4) imbedded in Sertoli cell (Sc) process which contain numerous fat globules (f).

C, thickening of the wall and the basement membrane (BM) of the seminefrous tubule. The sertoli cell (Sc) contain large fat globules (f), numerous mitochondria (m) in its cytoplasm and the nucleus (N) dysplastic. Notice presence of Spermatocyte (2).

D, spermated cell (3) in state of necrosis where the nucleus (N) shrinking and condensed with presence of numerous vacuoles (v) in its cytoplasm. Notice presence of Sertoli cell (Sc) processes contain numerous mitochondria (m) and fat globules (f).

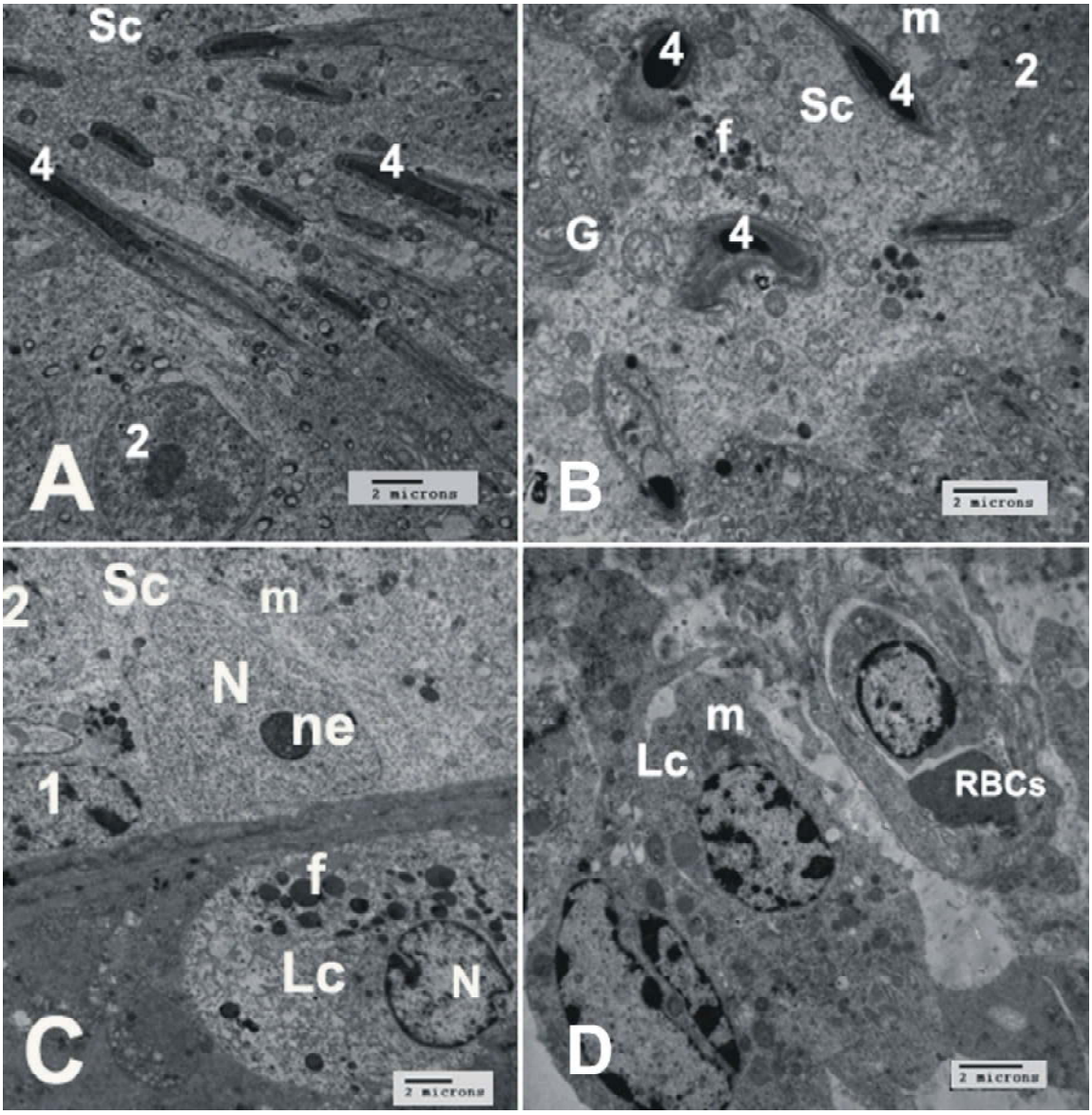

Fig. 4(A- D): T.E. Micrograph of seminiferous tubule belonging to control (A and C) and S. perisca treated groups (B and D).

A: The control group showing spermatocyte (2) and numerous spermatozoon (4) of normal morphological appearance embedded in the Sertoli cell processes (Sc). B. the treated group shows numerous deformed Spermatozoon (4) imbedded in the sertoli cell (Sc) processes containing numerous mitochondria (m), Gologi (G) and fat globule (f).

C. Interstitial tissue belonging to control group showing Leydig cell (Lc) containing numerous fat globules (f),mitochondria (m) endoplasmic reticulum and dentated nucleus (N). The seminefrous tubule contain Sertoli cell (Sc)laying on the basement membrane and having large nucleus (N) with prominent nucleolus (ne)and abundant cytoplasm rich with cell organelles such as mitochondria (m).Notice presence of part from spermatocyte (2).

D. Interstitial tissue belonging to the treated group showing clumps of Leydig cells (Lc), their cytoplasm filled with numerous mitochondria (m), electron dens granules, blood vesicle with abnormal number of RBCs and leukocyte.

presentation (Fig. 1B). Leydig cells are embedded in the rich plexus of blood and lymph capillaries which surround the seminiferous tubules. Normal distribution of collagen fibers was located in the interstitial area, basement membrane circulating each seminiferous tubule and spermatozoa (Fig. 2A). PAS positive materials were localized mainly in the basement membrane of the seminiferous tubules, head of spermatozoa and the connective tissue containing the interstitial cells (Fig. 2B).

Examination of sections stained with H&E (Fig. 1C) and semi-thin sections stained with toluidine blue (Fig. 1D) of rats' testes treated with watery extract of *S. persica* revealed several histopathological alterations. The seminiferous tubules appeared with a few mature sperms compared to these of the control group (Fig. 1C). The tubules appeared with corrugated wall of basement membrane. The spermatogenic cells appeared with miss-shaped nucleus and the acrosomal cap and granules were not distinguished (Fig. 1D). Sertoli cells appeared numerous and contained fat globule. Interstitial tissue containing Leydig cells had large vacuoles and congested blood capillary (Fig. 1D). There was a significant (P value < 0.05) increase of collagen fibers observed inside and around the seminiferous tubules compared to the control group (Fig. 2C, 2E). PAS positive materials showed reduction in the germ cells and spermatozoa (Fig. 2D). The reduction in stain affinity of PAS^{+ve} materials was not significant in the treated group when compared to the control group (Fig. 2F).

Electron Microscope: The normal ultra-structure of the mature active seminiferous tubule with complete spermatogenic series was recorded in the control group (Fig. 3A). Each seminiferous tubule is surrounded by a thin basal lamina followed by myoid cells and a thin fibrous layer. Spermatogonia and Sertoli cells rest on the basal lamina of the seminiferous tubule (Figs. 3A and 4C). Sertoli cells are irregular in shape and their cytoplasm extends to the lumen of the tubule, filling the narrow spaces between the cells of the spermatogenic series (Fig. 3A). The cytoplasm is rich with cell organelles such as mitochondria, endoplasmic reticulum and free ribosomes. The nucleus is polymorphous with a deep indentation and possesses a prominent nucleolus (Figs. 3A and 4C). Normal appearance of spermatogonia and spermatocytes with cytoplasm contained normal organelles and spherical nuclei (Figs. 3A and 4C). The spermatids and spermatozoa lie close to the lumen of the seminiferous tubules (Fig. 3A). Spermatids are small cells with spherical nuclei and their cytoplasm possesses flattened cisternae of endoplasmic reticulum, ribosomes and mitochondria with vacuolated appearance (Fig. 3A). The spermatids undergo metamorphosis into spermatozoa which found in contact with Sertoli cells (Fig. 4A). The interstitial cells of Leydig are relatively large in size and possess spherical nuclei with dentition (Fig. 4C). The cytoplasm of Leydig cells contains numerous lipid droplets, abundant mitochondria in variable size and shape and cisternae of rough endoplasmic reticulum.

Ultra-structural examination of the tissue sections of testis treated with watery extract of *S. persica* confirmed the previous observations and added more striking signs of pathogenesis. Some spermatogonia cells appeared in the stat of pyknosis as well as deformity of the nucleus with presence of numerous membrane bounded vesicle in its cytoplasm (Fig 3B). The basement membrane of seminiferous tubules exhibited marked changes; the inner non-cellular layer was thickened by increasing the collagenous fibres while the outer cellular layer, of smooth muscle cells, was folded (Fig. 3C). Some spermated cells appeared in state of necrosis, where, the nucleus shrinking and condensed with presence of numerous vacuoles in its cytoplasm (Fig. 3D). The cytoplasm contain numerous membrane bounded vesicles and numerous electron dens granules (Fig. 3D). Sertoli cells appeared with dentated nuclear envelop and pale and dispersed heterochromatin instead of the normal characteristic clumped form of chromatin. The cytoplasmic organelles revealed signs of damage; the mitochondria were swollen with ill-defined internal structure; large fat globules and numerous electron dense granules (Fig. 3C). Spermatozoa which found in contact with Sertoli cells were deformed or misshaped (Fig. 4B). The interstitial tissues showed clumps of Leydig cells that fused together (Fig. 4D). The cytoplasm of of Leydig cells is filled with abnormal number of steroid secretary granules, RBCs and leucocytes. No obvious damage was observed in the cellular organelles and cell membrane of Leydig cells.

DISCUSSION

Salvadora persica (Miswak chewing sticks) is a phytochemical plant used in the traditional medicine. The present study aimed to evaluate whether *S. persica* watery extract administration induced toxic effect on rats' testes. The main finding of the present study revealed that *S. persica* oral administration for a month caused ultrastructural and histochemical abnormalities in the testes of Albino rats. Also, significant reduction in the

testes weight, diameter of seminiferous tubules and epidermal sperm count were observed in *S. persica* treated rats when compared to the control rats. The administration of *S. persica* for 30 days didn't affect the body weight gain of the treated rats when compared to the control rats. This result is in line with previous observations reported in El-Dein *et al*. [21] and El-Kholy *et al*. [22] that showed improvement in the weight gain when adding S. *persica* extract in the animal diet.

Previous studies on the structural effect of *S. persica* on testes are rare. The present study was unique in demonstrating the marked histo-pathological changes in the testes of albino rats induced after the administration of *S. persica* extract. The changes include: signs of damage in Sertoli cells; reduced cells of the spermatogenic layers, clumped Leydig cells with abnormal accumulation of RBC and leucocyte in their cytoplasm. Some seminiferous tubules showed degeneration and atrophy with complete lose of spermatogenic series and this was accompanied with the significant decreased number of mature sperms inside the testis. Helal and Yousef [23] reported that *S. persica* extract has a negative feed back mechanism on the hypothalamic-pituitary-gonadal (HPG) axis leading to imbalance in the ovarian hormones and phases of estrus cycle in the treated female rats. The disruption in the HPG axis could explain the causes of the histo-pathological changes induced in the testes of male rats after *S. persica* administration. A parallel to this explanation is the results of Ibrahim and El- Gengaibi [24] who recorded reduction in the testosterone level and increased in the estrogen level and prostate hyperplasia in male rats treated with 4g/kg Miswak aqueous alcoholic extract.

The present study showed that there is a significant decrease in the mean value of testes weight, diameter of the seminiferous tubules and epididymis sperm count in rats administrated with *S. persica* extract. The significant reduction in the testes weight and the tubular diameter were generally associated with disruption of the spermatogenesis process and the increase in the testicular atrophy. Comparison between the two ratios of testes weight to body weight confirmed the loss of testes weight after treatment with *S. persica* extract. This also may explain the finding of Darmani *et al*. [16] who reported the abnormal increase in the weight of testes and perpetual gland and a 72% reduction in pregnancies in untreated females impregnated by testes males, reducing the risk of egg fertilization and prevent pregnancy after treatment with *S. persica* extract (at a dose of 800mg/k g of B.W).

Sperm count is considered the most sensitive test for spermatogenesis process. The significant reduction in sperm count in *S. persica* extract treated rats may be due to adverse effect of the extract on spermatogenesis. This result is in line with previous results where extract of *S. persica* has been reported to cause decline in the sperm quality and to increase morphologicl abnormalities in spermatozoa of rats [17]. In Reddy *et al*. [17] study, *S. persica* leaves extract at high dose (2.0%) exhibited sperms with abnormalities such as circular head and short tail.

The PAS (+) staining was carried out in the present study to show glycogen distribution in the testes. Glycogen in the testes determined that the number of mature sperms were lower in *S. persica* (Miswak) treated rats in comparison with the control rats. The reduction in glycogen distribution in the testes of Miswak treated rats may indicate toxicity *of S. persica* extract on glycogen synthesis due to the activation of the enzymes responsible for glycogen degradation [25].

Collagen in the testes is important to regulate the adhesion of spermatogonia to the basement membrane and the detachment of late spermatide to the lumen [26]. Germ cells including Sertoli cells undergo apoptosis when prevented from attachment to the basement membrane [26]. The significance increase of the percentage of collagen reaction in the testes of rats administrated with *S. persica* extract indicates basement membrane or cellular damage and atrophy in the germ cells. Collagen was increased in proportional to the number of affected cells. In earlier study, collagen synthesis was reported to have increased in mice as a result of being fed high dose of Curcumin that contains phenolic compound similar to that found in *S. persica* extract [27]. It seems that *S. persica* extract contains a substance that has toxic effects on male reproductive system and reduces the quality of the male fertility of rats as observed in the present study. Studies on *S. persica* reported the presence of phytochemicals such as alkaloids, tannins, salvadorine, flavonoids, steroids, trimethylaine and salvadoricine [6, 7]. Many studies reported that oral ingestion of medical plants with phyto chemicals could induce changes to the male reproductive system ranges to either positive or negative [1-3].

CONCLUSION

Testicular apoptosis with disruption in spermatogenesis following *S. presica* administration

could be correlated with the possible theory that phytochemicals have anti-fertility effect. Finally, data on safety and efficacy is need for proper understanding in the use of *S. presica* as herbal medicines.

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