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Ameliorating effects of curcumin on nicotine-induced mice testes

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Background/aim: The aim of this study was to determine the antioxidative effect of curcumin on nicotine-induced mice testis.

Materials and methods: Sixty Swiss albino male mice were divided into five groups, each containing 12 mice. The first group was used as a control. To induce toxicity in the second and third group, nicotine (0.4 mg/kg/day) was injected intraperitoneally into mice for 14 and 28 days, respectively. The mice in the fourth and fifth group were injected with nicotine (0.4 mg/kg/day) and orally treated with curcumin (200 mg/kg) for 14 and 28 days, respectively. Testosterone levels were measured from blood samples and testis tissues were examined under light and electron microscopes.

Results: Light and electron microscopic examinations of the nicotine-induced groups showed evident degenerations in spermatogenic cells, Sertoli cells, and Leydig cells. The groups treated with curcumin had less testicular alterations. The mice that were sacrificed after 28 days in the groups treated with curcumin showed minor degenerations. Furthermore, the median levels of testosterone significantly decreased in the nicotine-induced groups in comparison with those in the control group.

Conclusion: The results indicated that curcumin might be a potential therapeutic agent for testicular injury caused by nicotine addiction.

Key words: Testis, curcumin, nicotine, electron microscope, testosterone

1. Introduction

Tobacco addiction is the leading preventable cause of death, but the number of smokers is still rapidly increasing, particularly in undeveloped and developing countries (1,2).

Cigarette smoking kills people by causing a wide range of diseases such as vascular disease and chronic lung disease. Principally, it increases the risk of mouth, oral cavity, pharynx, larynx, esophagus, pancreas, lung, cervix, bladder, and kidney cancers (2–5). Moreover, maternal smoking is the greatest identified cause of fetal weight deficiency, premature birth, miscarriage, perinatal/neonatal death, neural tube defects, and congenital anomalies (6–11). Furthermore, it adversely affects the development of organs and tissues serving several functions in cardiovascular and skeletal systems (12).

Nicotine is a major toxic component of cigarette smoke. Moreover, it is a strong alkaloid, which is isolated from tobacco leaves (13,14). It has been reported that nicotine passes through biological membranes, including the blood–brain barrier, and induces apoptosis and malformations (15). In addition to its pathogenic toxic effects on tissues and organs, nicotine has an impact on gonadal functions and inhibits ovulation, estradiol production, and fertilization (16–18). The production of free radicals (ROS), which impair the antioxidant system, is increased by nicotine (19,20). Therefore, an imbalance between ROS production and ROS elimination by the antioxidant system can cause oxidative stress in tissues (19–22). As a cause of oxidative stress, it is well known that nicotine has detrimental effects on sperm count, motility, and morphology, and it impairs testicular structure and function, reducing the level of serum testosterone and estradiol (23–28). Antioxidant capacity against oxidative damage is extremely important for organs such as the testes, which have high metabolic activity and cell replication (29,30).

Turmeric (*Curcuma longa*) is a perennial plant that has been used as a spice and medicine in Asian and African countries for thousands of years (31,32). Curcumin, which is obtained from turmeric roots, is used as an antiinflammatory, antiseptic, antioxidant, anticarcinogenic, and hypocholesterolemic agent in traditional medicine (33–36). It is known that curcumin,

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as a potential antioxidant of ROS, neutralizes nitric oxide, provides continuity to antioxidant enzymes such as superoxide dismutase and catalase, and lowers lipid peroxidation in order to protect tissues and organs from oxidative damage caused by nicotine (36,37). Furthermore, curcumin appeared to be effective in enhancing male hormones, sperm count, motility, and morphology, and in reducing reproductive toxicity (38–55). The results suggested that curcumin could be useful in inhibiting the harmful effects of nicotine on the testes. Thus, the objective of the present study was to investigate the therapeutic effects of curcumin on testes of mice that were exposed to nicotine administration, using light and electron microscopes.

2. Materials and methods

2.1. Chemicals

Nicotine sulphate 40% aqueous solution (Cas no: 415655000) was obtained from Across Organics, Merck. Curcumin was obtained from Sigma (C7727) and dissolved in normal saline.

2.2. Animals

In our study, 60 adult Swiss albino male mice weighting 30–40 g were obtained from The Medical Sciences and Experimental Research and Application Center of Çukurova University (Adana, Turkey). The animals were housed in well-ventilated polypropylene cages with food and tap water ad libitum. They were kept under controlled laboratory conditions of a normal light/dark cycle and normal temperature (25 ± 2 °C) and were allowed to acclimatize for 1 week. All experimental procedures were carried out according to the Universal Declaration of International Animal Rights, after receiving the approval of Çukurova University's Experimental Animal Ethics Committee (Dated 30.09.2009).

2.3. Experimental design

Sixty male mice were divided into five groups, each containing 12 mice. The groups were as follows:

Group 1 (n = 12) received normal saline for 14 days.

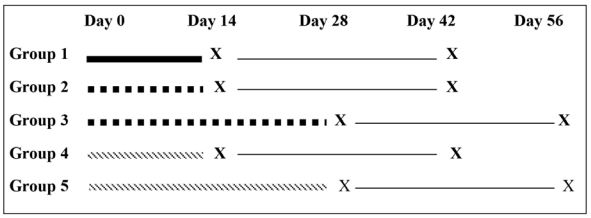
Group 2 (n = 12) received 0.4 mg/kg nicotine daily for 14 days.

Group 3 (n = 12) received 0.4 mg/kg nicotine daily for 28 days.

Group 4 (n = 12) received 0.4 mg/kg nicotine and 200 mg/kg curcumin daily for 14 days.

Group 5 (n = 12) received 0.4 mg/kg nicotine and 200 mg/kg curcumin daily for 28 days.

Each group was divided into 2 subgroups according to analyses on the days at the end of the treatment application and at the end of 28 days after the last treatment application. Testosterone levels were measured from the serum of blood samples and testis tissues were processed for light and electron microscopy.



2.4. Light and electron microscopy

At the end of the application period, the testes in each group were removed for investigation after cervical dislocation. In order to perform light microscopic examinations, one of the testes of each animal was fixed in Bouin's solution, dehydrated and embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. The sections were examined and photographed with an Olympus BX50 photomicroscope. For electron microscopy, the other testis of each animal was fixed in 5% glutaraldehyde in a phosphate buffer (pH 7.2) for 24 h. Then, tissue pieces were postfixed in 1% osmium tetroxide. Thereafter, tissue was dehydrated in graded ethanol, embedded in araldite, and processed for electron microscopy. The stained sections were examined with a JEOL-JEM 1400 transmission electron microscope.

2.5. Morphometric analysis

Testis sections from each group were evaluated for structural changes according to seminiferous tubule thickness measurements and Johnsen scores. The thickness of the seminiferous tubular wall was measured quantitatively by light microscopy (20× magnification). In each section, 10 randomly chosen circular cross sections of seminiferous tubules were analyzed and the average thickness of the tubular walls was measured by an Image J program.

Johnsen's tubular biopsy score (JTBS) was used for semiquantitative evaluation of spermatogenesis in 20 seminiferous tubules from each testicular section (47). JTBS was calculated by dividing the sum of all scores in each group to the total number of seminiferous tubules. A decrease in the average value was evaluated as an impairment of spermatogenesis. The scoring grades were as follows:

Score 10: complete spermatogenesis with regular tubules;

Score 9: many sperms, irregular germinal epithelium;

Score 8: few sperms;

Score 7: no sperms, many spermatids;

Score 6: few spermatids;

Score 5: no sperm or spermatids;

Score 4: few spermatocytes;

Score 3: presence of spermatogonia;

Score 2: presence of Sertoli cells;
Score 1: no cells.

All tubular parameters were analyzed by SPSS Version 18 and Student's t-test to assess the significance of changes between the control and experimental groups. $P < 0.05$ was considered significant.

2.6. Serum assays

Intracardiac blood samples were centrifuged at 3600 rpm for 5 min to separate the serum and plasma. Serum was kept in Eppendorf tubes at –20 °C. Serum testosterone levels were measured by the E170 immunoassay (Roche) method at Balcalı Hospital Central Laboratories of Çukurova University's Medical Faculty. Data analyses were performed by SPSS. Differences between serum testosterone levels were compared using Kruskal–Wallis and Mann–Whitney U tests. $P < 0.05$ was considered significant.

3. Results

3.1. Biochemical results

The analysis results indicated that nicotine and nicotine + curcumin applications had a significant influence (**$P < 0.05$) on serum testosterone levels. The differences between application times among the groups had no significant effect on testosterone levels (Figure 1).

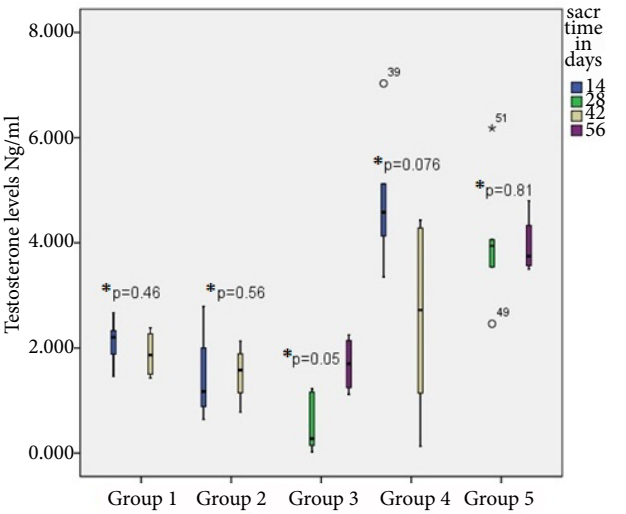


Figure 1. Identifier graphic of testosterone levels in the different groups.
*P: 0.33, when compared intragroup.
**$P < 0.05$, when compared with each group.

3.2. Morphometric results

The seminiferous tubule thicknesses and Johnsen's score values of each group are shown in Table 1. During morphometric analysis of the testes, the nicotine groups had significantly lower ($P < 0.05$) seminiferous tubules thicknesses and Johnsen's scores when compared with the control group. However, there were no significant differences (P: 0.33) between the control group and the nicotine + curcumin groups.

3.3. Light microscopic results

Light microscopic results are summarized in Table 2.

Normal testis morphology was observed in the control group (Figure 2A). The most prominent degenerative findings in the nicotine-induced groups (2 and 3) were disorganization of the germinal epithelium, desquamation of immature cells into the seminiferous tubule lumen, vacuolization in the seminiferous epithelium, and interstitial edema (Figures 2B, 2C, 2D, and 2E).

The degenerative changes in the nicotine groups were similar to each other. However, the degenerations in mice induced with nicotine for 28 days were more severe than those in mice induced with nicotine for 14 days. In the nicotine and curcumin induced groups (4 and 5) normal seminiferous tubule epithelium and interstitium, similar to those in the control group, were observed, except for some minor degeneration (Figures 2F, 2G, 2H, and 2I).

3.4. Electron microscopic results

Electron microscopic results are summarized in Table 3. Electron microscopic examination revealed

Table 1. Values of the morphological parameters compared among the mice groups according to the sacrifice time of mice.

Analyzed parameters (mean ± SD)	Group 1		Group 2		Group 3		Group 4		Group 5	
	14th day	42nd day	14th day	42nd day	28th day	56th day	14th day	42nd day	28th day	56th day
Thickness of seminiferous epithelium (µm)	65.87 ± 1.7	65.87 ± 0.88	43.8 ± 1.8	55.5 ± 0.7[a]	33.5 ± 1.28[a]	52.5 ± 1.08[a]	66.53 ± 1.5[b]	62.2 ± 1.2[b]	63.0 ± 1.7[b]	60.5 ± 1.2[b]
Johnsen's score	9.4 ± 0.2	9.74 ± 0.2	6.2 ± 0.22[a]	6.82 ± 1.28[a]	5.38 ± 0.39[a]	6.23 ± 1.2[a]	9.58 ± 0.2[b]	9.22 ± 0.2[b]	9.32 ± 0.2[b]	9.16 ± 0.2[b]

[a]: $P < 0.05$, when compared with control and nicotine + curcumin groups.
[b]: P: 0.33, when compared with control and nicotine groups.

Table 2. Light microscopic results summarized according to sacrifice time.

Severity of degeneration*	Group 1	Group 2		Group 3		Group 4		Group 5	
		14th day	42nd day	28th day	56th day	14th day	42nd day	28th day	56th day
Reduction in thickness of ST epithelium	Normal	++	+	+++	++	Normal	+	Normal	+
Desquamation of immature cells in the ST lumen	Normal	++	+	+++	+	Normal	Normal	Normal	+
Vacuolization in ST	Normal	++	+++	+++	++	Normal	Normal	Normal	Normal
Interstitial edema	Normal	+	Normal	++	Normal	Normal	Normal	Normal	Normal

* +: less, ++: moderate, +++: severe.
ST: seminiferous tubules.

a normal ultrastructure of the testes of mice in the control group (Figures 3A and 3B). However, electron photomicrographs of testicular sections from groups 2 and 3 revealed severe and extensive histological abnormalities. In the nicotine induced groups (2 and 3), degenerative mitochondria were gathered in the cytoplasm of Sertoli cells and Leydig cells, which had normal nuclei (Figures 3C, 3D, and 3E). Moreover, smooth endoplasmic reticulum vacuolization (Figure 3C) and too many large phagosomal bodies were obvious in the cytoplasm of Sertoli cells (Figure 3F). The integrity of the tight junctional complexes between Sertoli cells was maintained, but disassociation and irregular spaces between the degenerated spermatogenic cells were common (Figures 3C and 3G). Leydig cells in the interstitium had smooth endoplasmic reticulum (SER) vacuolization and an increased number of lipid droplets in their cytoplasm, except for degenerative swollen tubular mitochondria (Figures 3D and 3H). There were abnormal mitotic forms of spermatocytes with an enlargement and irregular chromatin accumulations in the nuclei, and spermatids with a double nucleus were observed (Figures 3G and 3I). Most of the tubules also discharged round and elongated spermatids, and many of them were seen in the tubule lumen (Figure 3J). The cells and nuclear membranes of the spermatocytes and spermatids had distinctly irregular contours (Figures 3C, 3I, and 3K). Moreover, spermatids embedded in the Sertoli cell cytoplasm had abnormal acrosomal vesicles (Figure 3L). Additionally, thickening and irregularity in the membrana propria were seen in some tubules from these groups (Figure 3I). The degenerative changes in the nicotine groups were similar to each other. However, degenerations in mice that were induced with nicotine for 28 days were more severe than those in mice that were induced with nicotine for 14 days.

In the nicotine and curcumin induced groups (4 and 5) normal membrana propria, spermatogenic cells, Sertoli cells, and Leydig cells were observed, similar to those in the control group, except for some minimal degenerations (Figures 3M, 3N, 3O, 3P, 3R, and 3S). The integrity of the tight junctional complexes between Sertoli cells was maintained (Figure 3N). However, there were few irregular spaces between the spermatogenic cells, and SER vacuolization was observed in the cytoplasm of Sertoli cells in some tubules (Figures 3O, 3P, and 3R). The cytoplasmic bridges between spermatogenic cells were normal, but thickening and irregularity in the membrana propria of some tubules were observed (Figures 3R and 3T). Moreover, some spermatogenic cells with distinctly irregular nuclear membrane contours and abnormal acrosomal vesicles were seen in the seminiferous tubules in these groups (Figures 3P, 3T, and 3U).

4. Discussion

It is known that toxic agents increase the production of ROS and cause oxidative stress by reducing antioxidant enzymes in all tissues and organs (19–22). The balance between ROS production and their elimination by antioxidant system enzymes is extremely important to organs such as the testes, which have high metabolic activity and cell replication (48–50). The influence of oxidative stress on the male fertility system became obvious in many studies on oxidative stress induced by toxic agents, but the mechanism of activity has remained unclear. It is well known that smoking has harmful effects on the male sexual health and reproductive system; however, in developing countries cigarette consumption is rapidly increasing (51,52). It was shown in some studies that smoking-caused oxidative damage in the testes was eliminated by antioxidant enzymes (51,52).

Curcumin is a powerful antioxidant that is widely used in Asia and Africa. Studies on the effects of curcumin on male infertility have increased in recent years (38–46). The antioxidant activity of curcumin in opposition to nicotine toxicity was investigated in the lung, liver, and

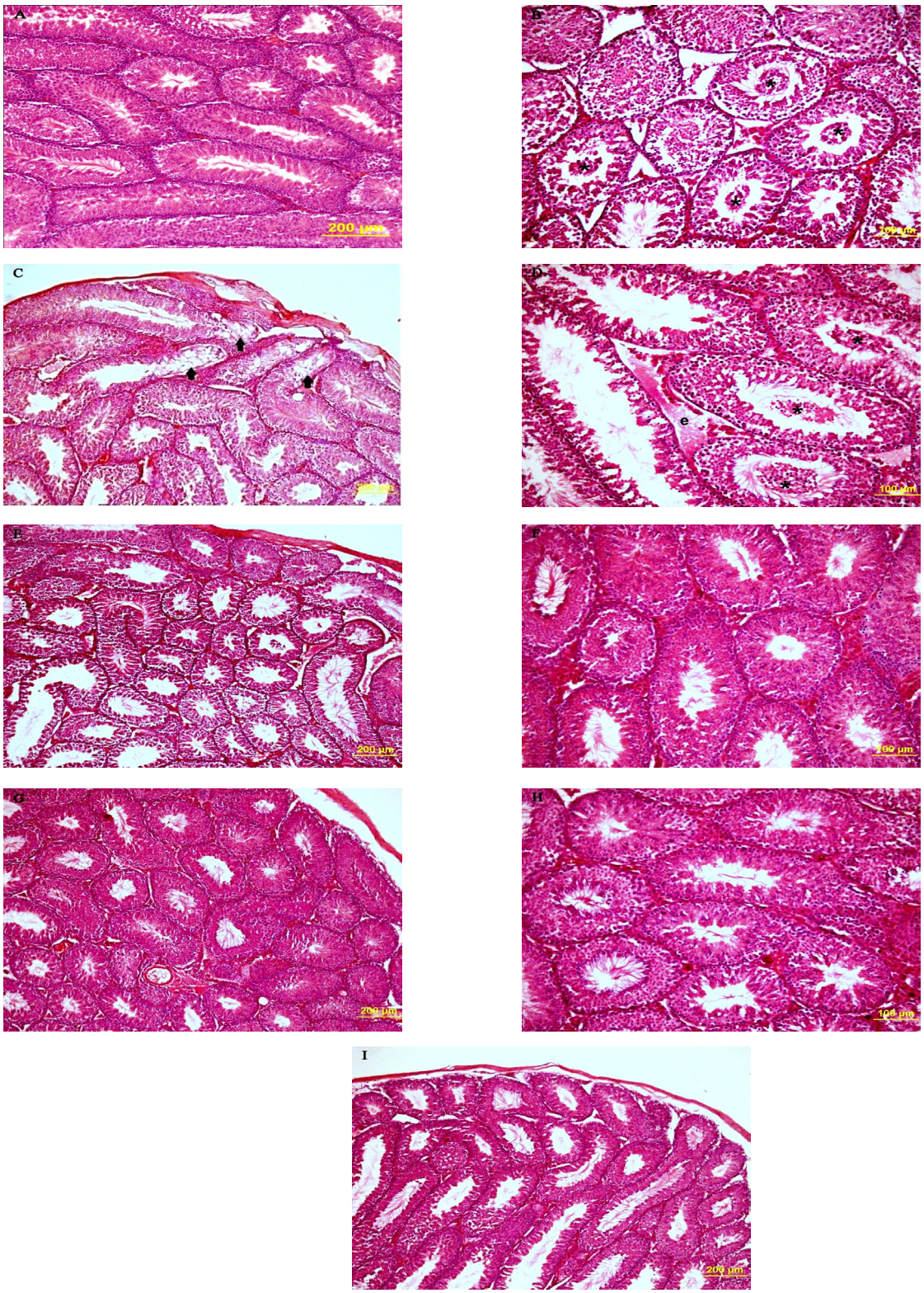


Figure 2. Photomicrographs of mice testes. (A) Normal seminiferous tubules and interstitium. (B) Desquamation of immature spermatogenic cells in the seminiferous tubule lumen (*) of mice at the end of the treatment application in group 2. (C) Vacuolization in the seminiferous tubules (arrows) of mice kept alive in group 2. (D) Severe reduction in the thickness of the seminiferous tubule epithelium, desquamation of immature spermatogenic cells (*), and interstitial edema (e) of the testes at the end of the treatment application in group 3. (E) Reduction in the thickness of the seminiferous tubule epithelium in mice kept alive in group 3. (F) Normal seminiferous tubules and interstitium in mice at the end of the treatment application in group 4. (G) Normal seminiferous tubules and interstitium in mice kept alive in group 4. (H) Normal seminiferous tubules and interstitium in mice at the end of the treatment application in group 5. (I) Normal seminiferous tubules and interstitium in mice kept alive in group 4 (H&E).

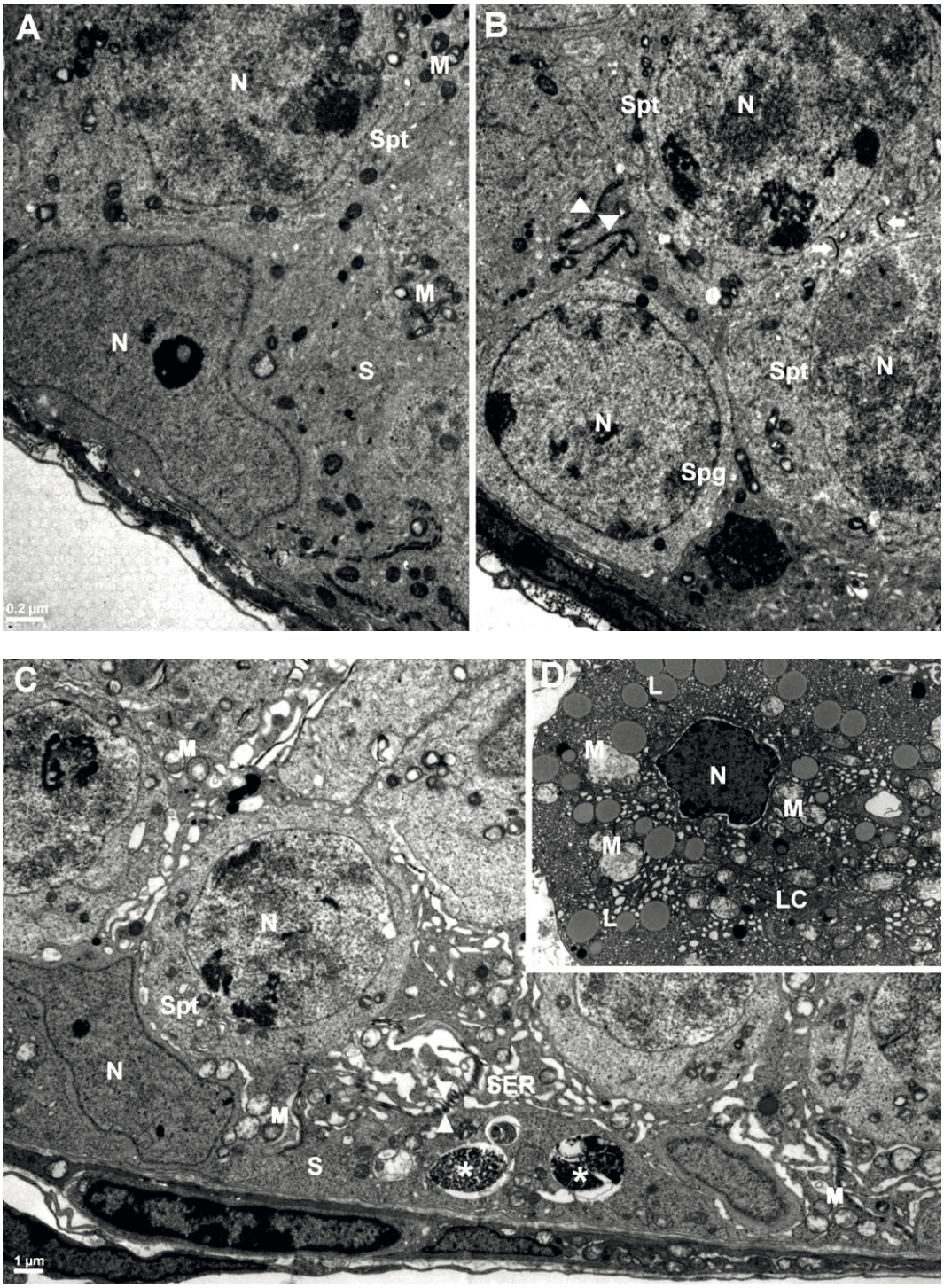


Figure 3. Electromicrographs of mice testes. (A and B) The nuclei (N) of spermatogonia (Spg), spermatocytes (Spt), and Sertoli cells (S) were normal in the testes of rats in the control group. Moreover, Sertoli–Sertoli cell junctions (arrow heads) and cytoplasmic bridges (arrows) between spermatogenic cells were normal. (C and D) Degenerative mitochondria (M), smooth endoplasmic reticulum (SER) vacuolization, and phagosomal bodies (*) in the Sertoli cell (S) cytoplasm; degenerative swollen mitochondria (M) and increased lipid droplets (L) in the Leydig cell cytoplasm (LC); and normal Sertoli-Sertoli cell junctions (arrow heads) were observed at the end of the treatment application in group 2. (E and F) Too many large phagosomal bodies (*) in the cytoplasm of Sertoli cells (S) were observed in mice kept alive in group 2. (G and H) Spermatids (Spd) with a double nucleus and disassociation (*) in the seminiferous tubule epithelium (*) were observed in the testes of mice at the end of the treatment application time in group 3. Moreover, too many degenerative swollen mitochondria (M) and lipid droplets (L) in the cytoplasm of Leydig cells (LC) were seen in this group. (I and J) Normal Sertoli–Sertoli cell junctions (arrow heads), thickening and irregularity in the membrana propria (MP), abnormal mitotic forms of spermatocytes (Spt), and discharge of round spermatids (Spd) into the tubule lumen (Lu) were seen in group 3 mice at the end of the treatment application. (K and L) Normal Sertoli–Sertoli cell junctions (arrow heads), irregular cell nuclear membrane contours of the spermatocytes (Spt), and spermatids (Spd) with abnormal acrosomal vesicles (A) were seen in group 3 mice that were kept alive. (M and N)

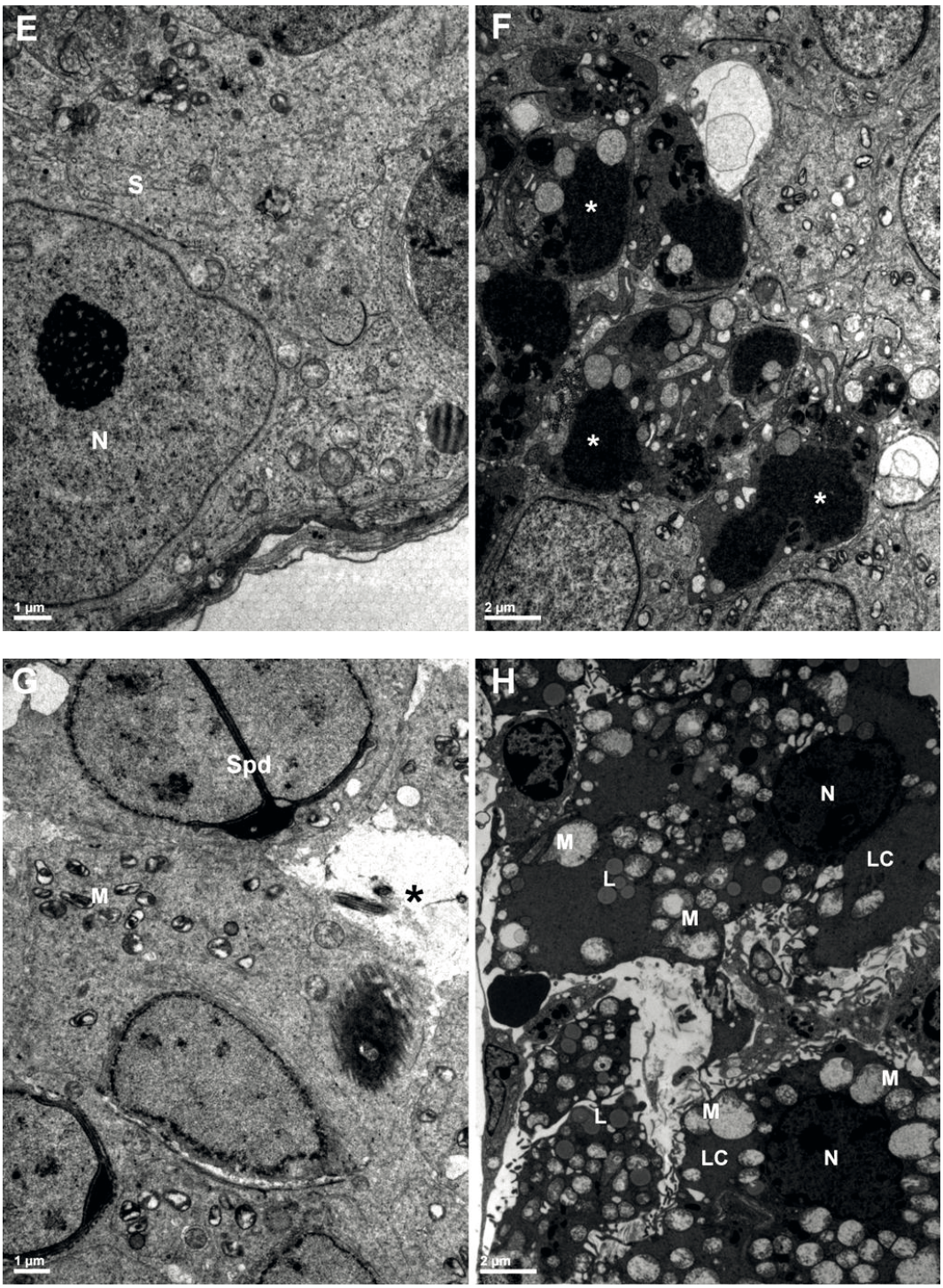


Figure 3. (Continued). Leydig cells (LC), Sertoli–Sertoli cell junctions (arrow heads), and spermatocytes (Spt) were normal in the testes of group 4 mice at the end of the treatment application. (O and P) Smooth endoplasmic reticulum (SER) vacuolization in the Sertoli cell (S) cytoplasm, irregular nuclear membrane contours of the spermatids (Spd), and irregular spaces (*) between spermatogenic cells were seen in the testes of group 4 mice that were kept alive. (R and S) Normal cytoplasmic bridges (arrow heads) between spermatids (Spd) and few irregular spaces (*) between spermatogenic cells were seen in the seminiferous tubules of group 5 mice at the end of the treatment application. Normal lipid droplets (L) in the Leydig cell (LC) cytoplasm were seen in the same group. (T and U) Thickening and irregularity in the membrana propria (MP), normal cytoplasmic bridges (arrow heads) between spermatocytes (Spt), and spermatids (Spd) with abnormal acrosomal vesicles (A) were observed in some tubules of group 5 mice that were kept alive.

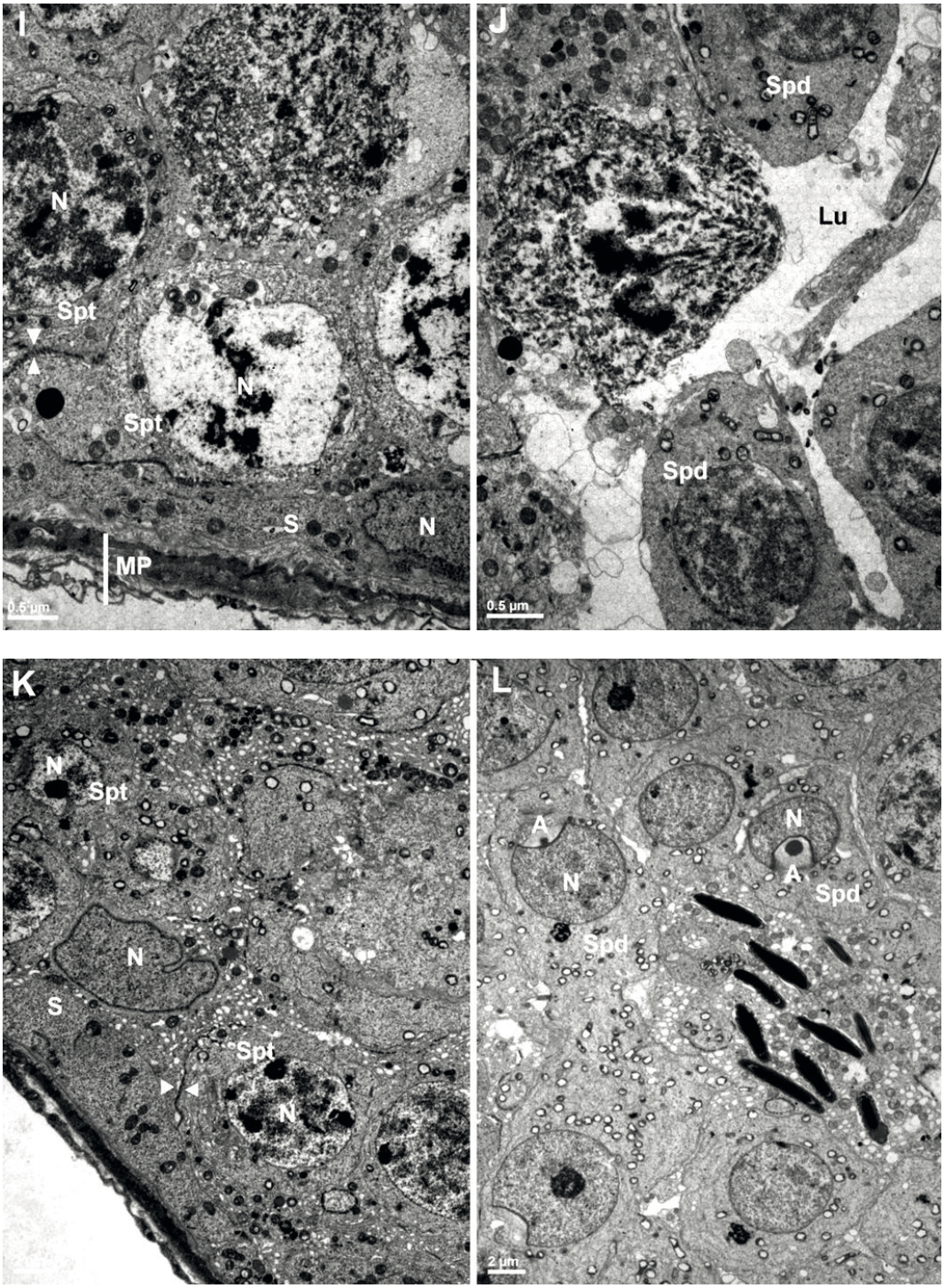


Figure 3. (Continued).

kidney. These studies concluded that ROS and oxidative enzymes were inhibited by curcumin (48–55). However, we did not encounter any studies on the effects of curcumin on nicotine-induced testes in the literature. Therefore, we analyzed the changes in serum testosterone levels (Figure 1) and investigated the protective effects of curcumin on nicotine-induced testes of mice under light and electron microscopes (Figures 2 and 3). Based on our light microscopic results, seminiferous tubular thickness parameters, and Johnsen's scores, we found a significant

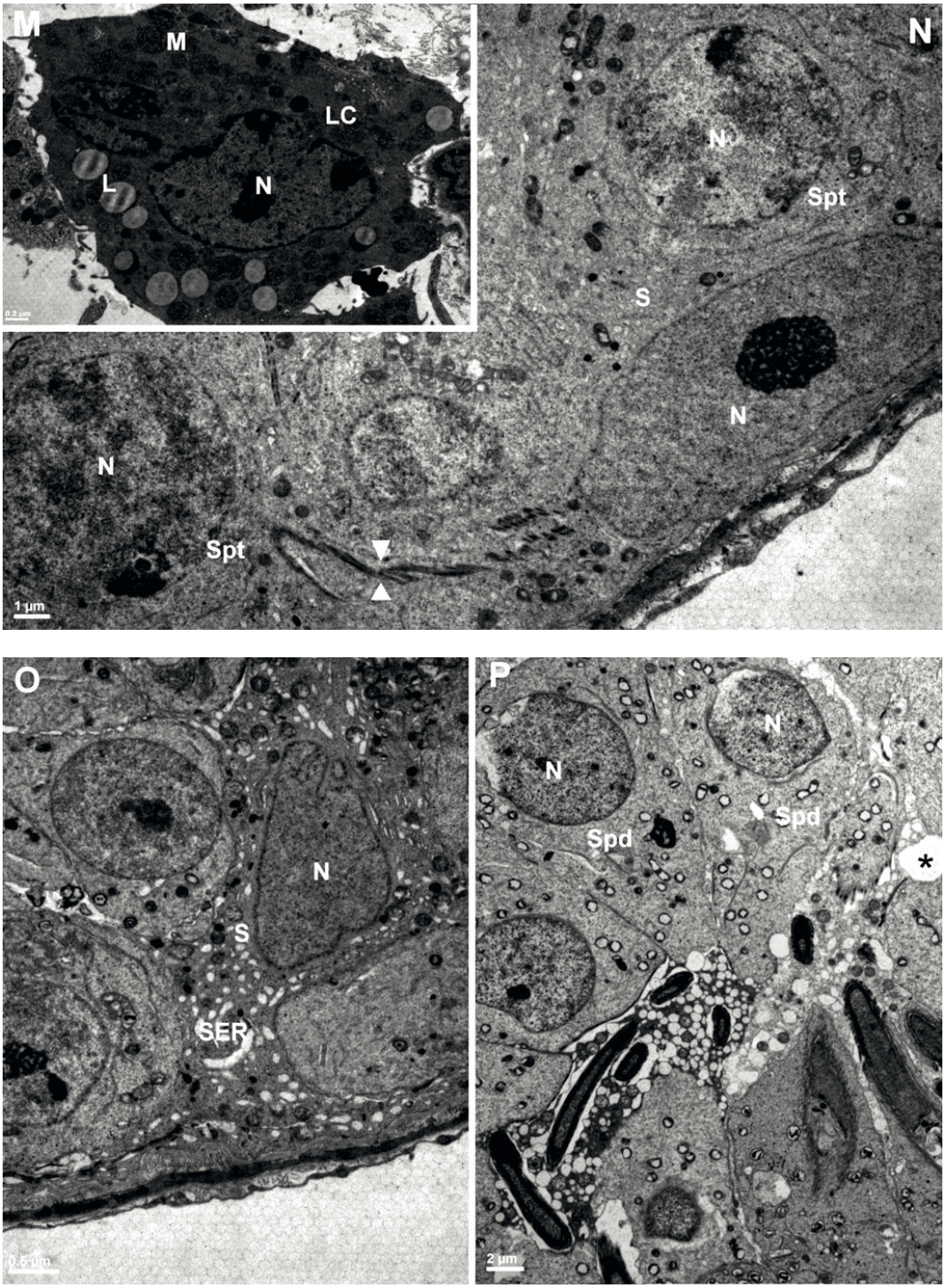


Figure 3. (Continued).

difference in the nicotine groups when compared with the control and the nicotine + curcumin groups (Tables 1 and 2). A reduction in the thickness of the seminiferous epithelium (Figures 2D and 2E), due to the desquamation of immature spermatogenic cells in the tubule lumen (Figures 2B and 2D), and vacuolization in the seminiferous epithelium (Figure 2C) were generally observed by light microscopic examination in the nicotine groups. Nicotine toxicity is known to cause vascular insufficiency (hypoxia) in the seminiferous tubules and changes in the membrana propria (27). A slight increase in the thickness of the membrana propria and invaginations and irregularity in

Table 3. Electron microscopic results summarized according to sacrifice time.

Severity of degeneration*	Group 1	Group 2		Group 3		Group 4		Group 5	
		14th day	42nd day	28th day	56th day	14th day	42nd day	28th day	56th day
Thickening and irregularity in MP	Normal	++	+	+++	++	+	+	Normal	++
The integrity of the tight junctional complexes between Sertoli cells and adjacent germ cells	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
SER vacuolization in Sertoli cells	Normal	+++	++	+++	+++	Normal	+	Normal	Normal
Degenerative mitochondria in Sertoli cells	Normal	++	+	++	+	Normal	Normal	Normal	Normal
Increased phagosomal bodies in Sertoli cells	Normal	Normal	+++	++	+++	Normal	Normal	Normal	Normal
Dissociation and irregular spaces between spermatogenic cells	Normal	+++	++	+++	++	Normal	+	Normal	+
Irregular cell or nuclear membrane contours of spermatogenic cells	Normal	++	++	+++	+++	Normal	++	Normal	+
Abnormal mitotic forms of spermatogenic cells	Normal	Normal	Normal	++	++	Normal	Normal	Normal	Normal
Spermatids with abnormal acrosomal vesicles	Normal	++	++	+++	+++	Normal	+	Normal	+
Swollen mitochondria in Leydig cells	Normal	+++	++	+++	++	Normal	Normal	Normal	Normal
Increased lipid droplets in Leydig cells	Normal	+++	++	+++	++	Normal	Normal	Normal	Normal

* +: less, ++: moderate, +++: severe.

MP: membrana propria.

the basal lamina were observed in the nicotine groups by electron microscopic examination (Figure 3I). Since the membrana propria is a physiological barrier for toxic agents, it was thought that the thickening in the membrana propria occurred to prevent the passage of toxic matter to the seminiferous tubule lumen. This thickening blocked not only toxic agents, but also substances needed for the spermatogenic cells. At the same time, it was reported that increased collagen fibrils and changes in myoid cell contractility caused exfoliation of immature cells in the seminiferous tubule lumen (27). A disruption in the excretion of residual bodies (Figure 3F) was caused by impairment in Sertoli cell functions, and this disruption had an impact on the improvement of the seminiferous tubule epithelium. Despite the fact that impairments in the tight junctions between Sertoli cells and between Sertoli and spermatogenic cells were reported in some studies, we observed generally normal tight junctions and cytoplasmic bridges in the nicotine groups (Figures 3C, 3I, and 3K). It has been reported that nicotine, which passes through the blood–testis barrier to reach the seminiferous tubules, has direct cytotoxic effects on spermatogenic cells (27,56). Spermatogenic cells are more sensitive to toxic agents than Sertoli and Leydig cells because of their rapid cell division (57). Accordingly, shrinkage and lysis in spermatogenic cells in the nicotine groups were shown by electron microscopic examination in our study (Figure 3K). The presence of a double nucleus, mitochondrial degeneration, and abnormal acrosomal structures in spermatids due to nicotine toxicity were frequently observed (Figures 3C, 3G, and 3L). The degeneration of mitochondria, which are more sensitive to ROS, is the reason for imbalances in ion transport, respiration, and oxidative phosphorylation. Mitochondrial enlargement was considered a cellular adaptation in order to reduce ROS. Mitochondria are also central organelles in steroidogenesis, and therefore the enlargement of the mitochondria in Leydig cells was associated with an increase in ROS (Figures 3D and 3H). All these degenerations were observed to be more severe at the end of the nicotine application period. We thought that the testes might regenerate in the rats that were kept alive under normal conditions following nicotine application.

Other studies have reported that curcumin provides support to the antioxidant enzyme systems, which reduce oxidative stress and lipid production in order to protect steroidogenesis and spermatogenesis against toxic agents (37–46). In our light microscopic examinations of the nicotine + curcumin groups, we observed generally normal seminiferous tubules and interstitium (Figures 2F, 2G, 2H, and 2I). Concordantly, our electron microscopic examinations of the nicotine + curcumin groups showed that the membrana propria and the seminiferous epithelial cells had less degenerative changes than those in the nicotine groups. The mitochondrial enlargement and the increase in lipid droplets in the nicotine groups were reduced in the nicotine + curcumin groups (Figures

3M and 3S). The minor degenerations in the testes of mice kept alive under normal conditions for 28 days in groups 4 and 5 made us think that curcumin had an effective role during the application period. Testicular oxidative stress induced by alcohol revealed an increase in megamitochondria formation in Leydig cells (39) and a decrease in testosterone levels, which were normalized during curcumin intake (39–44). The results of the present study also showed that the testosterone levels in the nicotine + curcumin groups were higher than those in the nicotine groups (Figure 1). Hence, we determined that curcumin has protective roles not only in morphological disorders, but also on biochemical parameters.

In conclusion, our experiments have proved that the toxic effects of nicotine were reduced with curcumin intake. Knowledge about the antioxidant defense mechanism against testicular oxidative stress will be helpful for clinical surgery and further experimental studies are recommended.

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