

Beneficial effects of melatonin and BQ-123 on the rat testis damage caused by cigarette smoke

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Background/aim: Several studies have demonstrated that cigarette smoke has detrimental effects on testicular function. However, it is unknown whether melatonin or BQ-123 has beneficial effects on the rat testis damage caused by cigarette smoke. The aim of the present study was to investigate the beneficial effects of melatonin or BQ-123 on the testicular damage caused by cigarette smoke.

Materials and methods: Twenty Wistar rats were randomly divided into 4 equal groups: control group (n = 5), cigarette smoke group (n = 5), melatonin group (n = 5), and BQ-123 group (n = 5). At the end of 4 weeks, all the rats were sacrificed for histopathological evaluation and subsequent stereological analysis. The optical fractionator counting method, the most efficient and unbiased method, was used to estimate the total number of spermatogonia and spermatocytes.

Results: All the control testes demonstrated complete spermatogenesis. There was a significant decrease in the germ cells of rats exposed to cigarette smoke for 4 weeks. After the application of melatonin or BQ-123, the total number of spermatogonia and spermatocytes in the testes was significantly higher.

Conclusion: Based on these findings, melatonin and BQ-123 are able to minimize the degenerative effects of cigarette smoke by increasing the germ cell count.

Key words: Cigarette smoke, rat, testis, stereology, melatonin, BQ-123

1. Introduction

Smoking causes serious health problems throughout the world, including Turkey, and cannot be adequately overcome (1–3). Today it is very important to examine and document the harmful effects of cigarette smoke on the urogenital system, especially in the young population (4,5). Tissues with rapid turnover including epidermis and sperm cells may be particularly sensitive to the mutagenic and carcinogenic materials found in cigarette smoke (6). More than 3000 different chemical compounds, such as nicotine, carbon monoxide, nitrosamine, polycyclic aromatic hydrocarbons, arsenic, and cadmium, are found in cigarettes. Some of these chemicals get into the blood circulation of the testes and can have a direct cytotoxic effect on spermatozoa by damaging the DNA of the sperm (7,8). These chemical products affect the urogenital system

either by changing hormone levels or by biotransformation into reactive products (9–11).

Exposure to cigarette smoke causes a lower proportion of motile sperm in human testicular ejaculate when compared to nonsmokers (12–14). The weight of the testis and the number of spermatocytes and spermatids are reduced by nicotine exposure, probably through a lack of pituitary gonadotrophins essential for initiating and completing spermatogenesis and steroidogenesis in the testis (15). Many studies have been done on smoking and the different methods of exposure to smoke, with animal models and also on the period of exposure, the results of which show apoptosis in the progenitor cells of the testis, reduction in the number and epithelial height of the germ cells, problems in the function of the germ cell mitochondria, and increments in the oxygen-free radicals (6,16–25).

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Melatonin, secreted by the pineal gland, has a well-known role in the circadian rhythm, and a very strong antioxidant activity. Receptor-mediated effects stimulate the expression of antioxidant and detoxification genes via intracellular signal transduction. Melatonin, in cells and tissues, possesses a scavenging ability and provides antioxidant properties against free radical damage (26). It is also a neuroprotector in the development of the central nervous system (27) and the regeneration of the peripheral nervous system (28,29).

Endothelin-1 (ET-1), secreted by vascular endothelial cells, is a potent endogenous vasoactive peptide that participates in the regulation of vascular tone (30). ET-1 has a strong affinity to the endothelin-A (ETA) and endothelin-B (ETB) receptors. ETA receptors, located in the vascular smooth muscle cells, mediate most of the vasoconstrictor and proliferative effects of ET-1. However, ETB receptors, located in the endothelial cell membrane, release nitric oxide (NO) and prostacyclin (PGI₂), which causes vasodilatation (31).

BQ-123 is a firstly described cyclic pentapeptide endothelin antagonist with high ETA selectivity (32), and especially has antioxidant and protective effects on ischemia and reperfusion injury (33,34). Cigarette smoke, a powerful vasoconstrictor agent that increases the circulating levels of endothelin-1 related to tissue damage, is known to lead to impaired function (35). In this study, we used rats as the animal model and evaluated the harmful effects of cigarette smoke on testes and the beneficial effects of the antioxidant melatonin, and the endothelin receptor antagonist BQ-123, separately.

2. Materials and methods

2.1. Animals

Twenty Wistar rats, aged 3–4 months and weighing 250–300 g, were exposed to cigarette smoke (3 × 30 min/day) for 4 weeks. Cigarette smoke was released into a large glass chamber by use of an electrical pump (36). After exposure to cigarette smoke, the first group (n = 5) was administered melatonin (25 mg/kg/day, ip) 5 days per week for 4 weeks and the second group (n = 5) was administered BQ-123 (1 mg/kg, iv) 1 day per week for 4 weeks. The third group (n = 5) was exposed to only cigarette smoke for 4 weeks. The control rats (n = 5) were exposed to room air.

At the end of day 28, the rats were sacrificed under deep anesthesia with 50 mg/kg ketamine and 5 mg/kg xylazine given intraperitoneally. The abdomens of the rats were incised widely and the testes were excised. The left testes were fixed in 10% formalin for 24 h immediately upon collection, dehydrated, and embedded in paraffin for histologic and stereological studies. For stereological

investigation, 30- μ m-thick sections were taken using the rotary microtome (Leica RM 2135, Leica Instruments, Nussloch, Germany) and sampled at a 1:12 ratio in a systematic random manner. The sections were stained with hematoxylin-eosin stain and the total numbers of spermatogonia and spermatocytes were estimated within the testes using a stereology workstation with an optical fractionator.

2.2. Stereological equipment

The stereological analyses were performed at a stereology workstation in the Department of Histology and Embryology, Gaziosmanpaşa University School of Medicine, Tokat, Turkey. It consisted of a modified trinocular microscope (Leica DM 2500, Leica Instruments, Nussloch, Germany), a motorized specimen stage for automatic sampling (Biopoint II motorized table, Ludl electronics, Hawthorne, NY, USA), a digital microcator (Heidenhain, Traunreut, Germany), a CCD video camera (QImaging; Hitachi, Tokyo, Japan), and stereoinvestigator software (MBF Biosciences, Williston, VT, USA).

2.3. Stereological analysis

Stereological analysis is the most effective and unbiased cell counting method and is a combination of the optical disector method, which is a cell counting method, and the fractionator, which is a sampling strategy (37). The principal of the fractionator is systematic random sampling. It uses regular sampling at a known interval that begins with a randomized start. As a result, every point of the structure has the same chance of being sampled while the particles are counted. The particle count is multiplied by the reverse of the sampling ratio and the total particle number is estimated according to the formula given below:

$$N = \sum Q^- \cdot \frac{1}{ssf} \cdot \frac{1}{asf} \cdot \frac{1}{tsf}$$

where N is the estimated total particle number, $\sum Q^-$ is the total particle number counted by the optical disector, ssf is the section-sampling fraction, which is a sampling of the sections taken from the tissue at a certain ratio, asf is the area-sampling fraction, which is a sampling of the sectional area of the interested tissue in a known area (step area) by the counting frame (frame area/step area), and tsf is the thickness-sampling fraction (h/t = height of the probe/section thickness).

2.4. Optical disector

Particle counting was redefined in a detailed way by Sterio in 1984 by reviewing the disector counting rule that says particle profiles are seen in one section but not seen in the successive one (38). After a few years, it became clear

that it was possible to take thin optical sections in thick histological sections. Focal plane or optical section at a certain distance of the section thickness can be moved from the upper surface of the section to the lower surface or from the lower surface to the upper surface and particle counting is performed at successive optical section serials by using the disector counting rules. This method is known in the literature as the optical disector (39,40).

2.5. Cell counting by stereoinvestigator

Stereoinvestigator software is widely used in computer-assisted stereology. Values for section sampling interval, step area, area of the counting frame upper guard zone, and probe height must be input for every subject at the beginning of the counting process. Then the same parameters are used automatically for the whole series of sections.

Beginning from the first of the selected testis sections, the boundary of the tissue, encircled using a mouse, step area, and the area of the counting frame were input. The program located the counting frames in the encircled area in a systematic and randomized manner. In this study step interval was determined as 1500 μm (step area of 2,250,000 μm^2) and the side of the counting frame was determined as 50 μm (frame area of 2500 μm^2). The upper guard zone was determined to be 5 μm . The probe height of the optical disector was limited to 15 μm . Then cell counting was performed at 100 \times magnification. The upper plane of the section was determined by seeing the first cell or few cells

clearly within the unbiased counting frame on the visual field. Then, by using the micro screw of the microscope, the visual field progressed downward optically and the level of the last clear vision was determined as the lower plane of the section. The stereoinvestigator program determined the upper guard zone automatically and placed the height of the disector at the level that the counting started in the section. Starting from a depth of 5 μm in the upper level of the section and using the micro screw, we progressed downwards in increments of 20 μm and took optical sections. According to the counting rules of the unbiased counting frame, the cells that were in the counting frame or intersected with the free lines but did not intersect with the forbidden lines or with their continuations were all counted by marking with the mouse. After the counting was completed for all the sections, the results were exported to Excel and the total number of spermatogonia and spermatocytes were estimated for each subject.

3. Results

The number of spermatocytes was decreased in all groups when compared with the control group (Table; Figures 1–5). There was a significant increase in the numbers of spermatocytes in the cigarette+melatonin and cigarette+BQ-123 groups compared with the cigarette smoke group. There were no significant differences between the cigarette+melatonin and cigarette+BQ-123 groups for the number of spermatocytes (Table).

Table. Total number of spermatocytes and spermatogonia of testes in all groups, given as mean \pm standard error of mean. N.S. = nonsignificant

| | Number of spermatocytes | Number of spermatogonia |
|-------------------------|------------------------------|------------------------------|
| 1 = control | 1,7101,166.8 \pm 636,058.0 | 14,116,333.2 \pm 354,349.2 |
| 2 = cigarette smoke | 10,061,000.0 \pm 398,052.8 | 9,998,000.0 \pm 346,875.5 |
| 3 = cigarette+melatonin | 13,987,666.6 \pm 522,257.6 | 12,465,000.0 \pm 431,826.7 |
| 4 = cigarette+BQ-123 | 14,084,666.6 \pm 567,060.0 | 13,039,666.8 \pm 567,774.0 |
| | P | P |
| 1–2 | 0.001 | 0.001 |
| 1–3 | 0.004 | N.S. |
| 1–4 | 0.005 | N.S. |
| 2–3 | 0.001 | 0.005 |
| 2–4 | 0.001 | 0.001 |
| 3–4 | N.S. | N.S. |

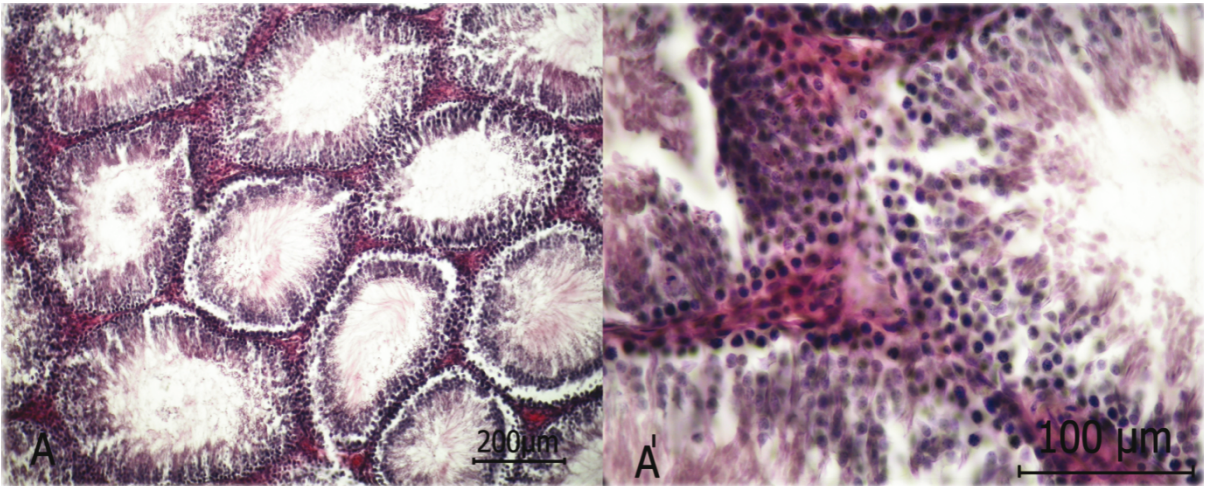


Figure 1. Photomicrograph of a testis from the control group rats. A at 10× and A' at 40× magnification (hematoxylin-eosin staining).

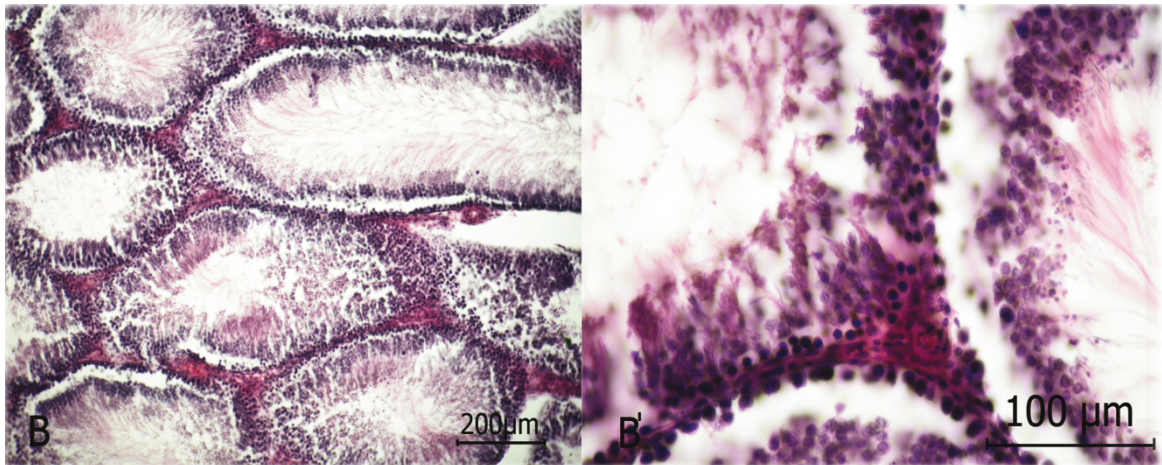


Figure 2. Photomicrograph of a testis from the BQ-123 group rats. B at 10× and B' at 40× magnification (hematoxylin-eosin staining).

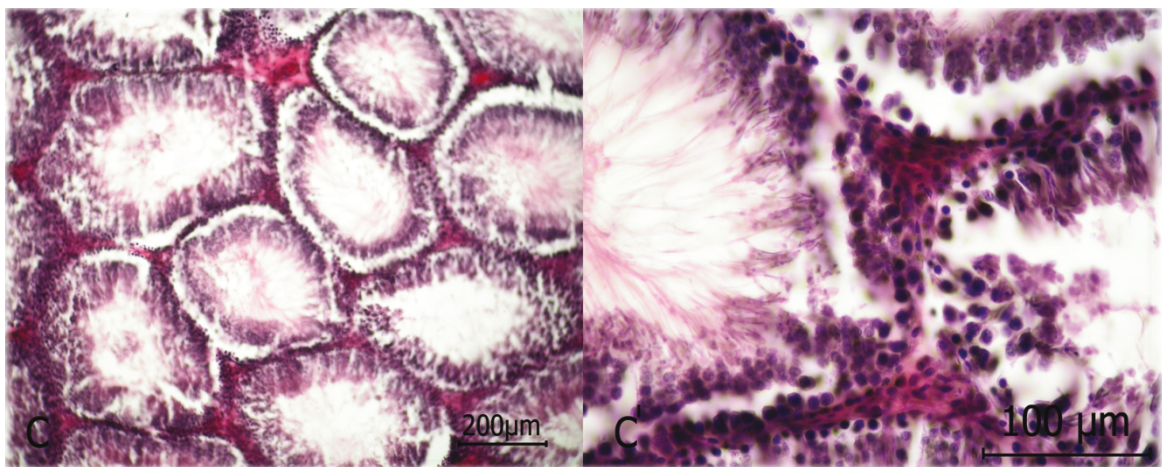


Figure 3. Photomicrograph of a testis from the melatonin group rats. C at 10× and C' at 40× magnification (hematoxylin-eosin staining).

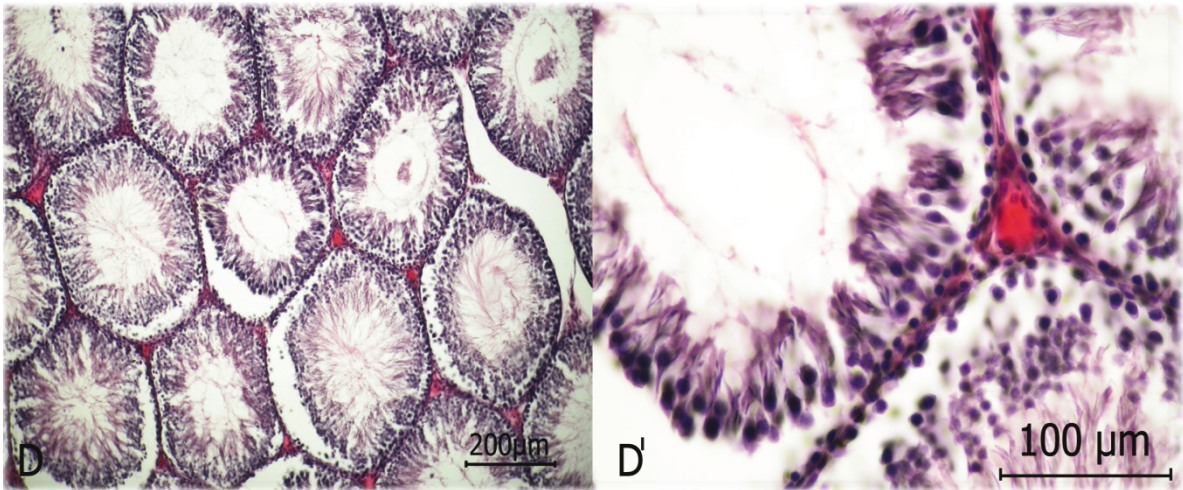


Figure 4, Photomicrograph of a testis from the cigarette smoke group rats. D at 10× and D' at 40× magnification (hematoxylin-eosin staining).

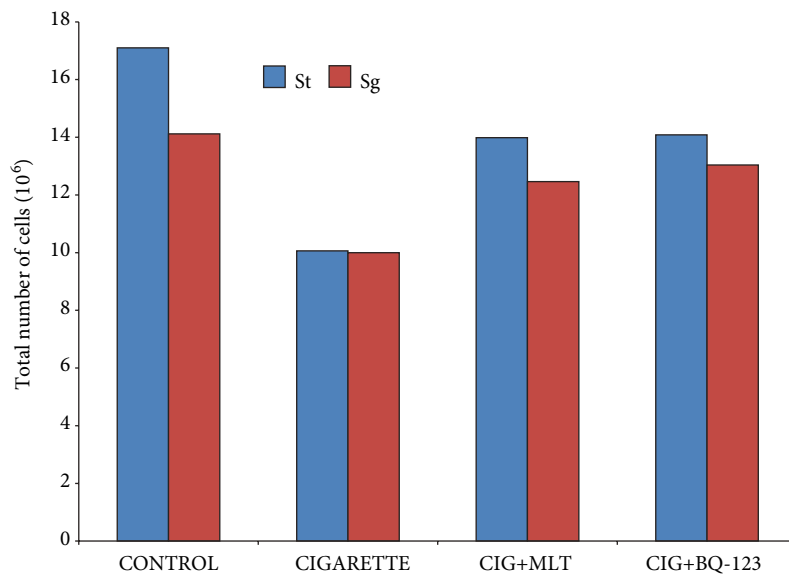


Figure 5. Total number of spermatocytes and spermatogonia in the testis of all rat groups. CIG = cigarette smoke, St = spermatocyte, Sg = spermatogonium, MLT = melatonin.

The number of spermatogonia was decreased significantly in the cigarette smoke group when compared with the control group; however, a significant increase was found in the cigarette+melatonin and cigarette+BQ-123 groups when compared with the cigarette smoke group (Table; Figures 1–5). Interestingly, the level of the increase in those groups almost reached the level of control group. Additionally, no significant differences were observed between the cigarette+melatonin and cigarette+BQ-123 groups for the number of spermatogonia (Table).

In rats exposed to cigarette smoke, the number of spermatocytes was significantly increased by the administration of melatonin and BQ-123 but did not reach the level of the control group (Figure 5).

4. Discussion

Cigarette smokers have been found to have a lower proportion of motile sperm when compared to nonsmokers (12,13), although some other studies (41,42) reported that smokers had a higher ratio of motile sperm compared

with nonsmokers. Additionally, it has been shown that smokers have an increased proportion of abnormal sperm (13), but Rodriguez-Rigau et al. found that smokers had a lower percentage of abnormally shaped spermatozoa (42). In addition to sperm morphology, it is also well known that cigarette smoke has an effect on germ cell numbers. Rajpurkar et al. showed that cigarette smoke exposure for periods of 15, 30, and 45 days caused a decrease in the number of germ cells, the height of the germinal epithelium, and the diameter of the seminiferous tubules. Moreover, apoptosis had been induced in the germ cells within the testes (23,24).

In a study done by Ahmadnia et al. that evaluated the effect of cigarette smoke on spermatogenesis in rats, they found a mild reduction in the number of germ cells (5). In another study, done by Güven et al. (43), it was reported that cigarette smoke caused atrophy of normal testicular components and further reduced the number of spermatogenic cells. Because of the rapid rate of cell division, spermatogenic cells of the germinal epithelium are more sensitive to gonadotoxins than Sertoli or Leydig cells (44). After exposure to a cytotoxic agent, spermatogenesis was disrupted due to lethal damage to the differentiated spermatocytes (45). Evidence suggests that exposure to nicotine, cigarette smoke, and/or polycyclic aromatic hydrocarbons produces testicular atrophy,

spermatogenic disruption, and changes in germ cell morphology (46).

In the present study, compatible with the majority of the literature, it has been quantitatively shown that there was a decrease in the number of germ cells after exposure to cigarette smoke and that decrease was focused in the number of spermatocytes that had higher metabolic activity. By the administration of melatonin and BQ-123 with the cigarette smoke, the decreased number of germ cells recovered.

As a result of these findings, it can be reported that exposure to cigarette smoke causes serious damage to germ cells and decreases their numbers. Moreover, both melatonin and BQ-123 have separately protective effects against oxidative stress, although their signaling mechanisms are not the same. There is strong evidence that they can be used as protective drugs by people who continue smoking.

Furthermore, because of BQ-123 being an antioxidant similar to melatonin but easier to use, it may be suggested to be used as an antioxidant control in oxidative stress studies.

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