Melatonin ameliorates testicular damages induced by nicotine in mice

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A R T I C L E I N F O	A B S T R A C T
	Objective(s): Nicotine as a toxic substance leads to impairment of the reproductive system function. The aim of this study was to evaluate effects of melatonin on testicular alterations, sperm nuclear integrity, and epididymal sperm parameters in mice treated with nicotine. ***Materials and Methods:*** Male mice were divided into four groups. Group A received the vehicle, group B received nicotine 0.1 mg/100 g BW, group C received melatonin 10 mg/kg, group D received nicotine plus melatonin. Evaluations were made by histology and Johnson's score for study of spermatogenesis, immunostaining for study of male germ cells apoptosis, sperm chromatin dispersion (SCD) test for assaying sperm chromatin integrity, enzyme-linked immunosorbent assay (ELISA) for assessment of serum levels of testosterone and luteinizing hormone (LH), and sperm parameters including morphology, motility, and count. ***Results:*** Nicotine caused a significant decrease in spermatogenesis quantity and Johnson's score, sperm parameters, and sex hormones. Melatonin in group D, increased sperm chromatin integrity, improved spermatogenesis, Johnson's score, and sperm parameters (*P*<.01) and reduced apoptosis (*P* <0.01) in comparison with the nicotine group. Melatonin significantly increased testosterone and halo sperms. However, its effect on the LH level was insignificant. The serum testosterone and LH levels were negatively correlated with the DNA fragmentation index (DFI) (r= -0.86, *P*<0.001) and (r= -0.78, *P*<0.001), respectively. ***Conclusion:*** this study showed administration of melatonin in nicotine-treated mice increases both quality and quantity of spermatogenesis and integrity of sperm's chromatin through reducing apoptosis and modifying the testosterone level.

Introduction

Infertility is an important problem and has adverse effects on various psychological, social, personal, and economic aspects. About 50% of infertility is due to male factors (1). There is a significant relationship between smoking and infertility in men (2).

Nicotine is the most toxic compound present in the cigarette smoke (3). It has adverse effects on the reproductive system in both women (4, 5) and men (6, 7). The use of nicotine reduces gametogenesis and steroidogenesis and inhibits secretion of gonadotropic hormones (8).

Different studies demonstrated that nicotine consumption reduces testosterone release (6, 9, 10), estradiol (4, 5), follicle-stimulating hormone (FSH), and luteinizing hormone (LH) (8). Nicotine reduces fertility in men by affecting the number, movement, survival, and reduction of normal morphology of sperms (11, 12). It also reduces the weight of the testes, body, and libido (10, 11). In addition, through changes in the pituitary-hypothalamic axis it stimulates the release of cortisol, vasopressin, and oxytocin, therefore it increases oxidative stress (11, 13). Meanwhile, it has been shown that nicotine usage in both women and men is associated with an increase in apoptosis in the reproductive system

(14-17).

Highly antioxidant compounds may be effective in reducing the effects of nicotine. Melatonin is considered as the main product of pineal gland with a powerful antioxidant activity (18). It can reduce the toxicity of drugs (18). Melatonin has a role in some physiological function such as sleeping, waking up, reproduction, aging, immune response, and tumor growth by itself (19). Melatonin in men is absorbed by the testicles, regulates the activity of the testis and also synthesis by the testes (20). Melatonin through binding receptors can regulate secretion of testosterone and increase the response of the Sertoli cells to FSH during the development of the testicle. It also regulates cell growth, mitosis, and secretion of some types of cells in the testes (20).

Protective effects of melatonin are shown in testis damaged by chemotherapy (21), diabetes (22), hyperlipidemia, and testicular torsion (20). However, there are some reports about effects of nicotine on the male reproductive system but little is known about the effect of nicotine plus melatonin on the testis. The purpose of this study was to investigate effects of exogenous melatonin on spermatogenesis, sperm parameters, and nuclear sperm integrity in adult mice

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treated with nicotine.

Materials and Methods

Animals

A total of 32 adult NMRI male mice aged 10–12 weeks and weighing 40–45 g were obtained from the Razi Institute, Tehran, Iran and divided into four different groups (8 per group). The animals were housed in small groups under standard lighting conditions (12 hr light-dark cycles) at 25±2 °C with free access to water and food. They were allowed to adapt for at least 1 week in the animal room before they were subjected to treatment. Animals were maintained and handled according to the protocols approved by the Animal Care and Use Committee of Guilan University of Medical Sciences. Animals were divided randomly into four groups:

Group A: Mice received normal saline intraperitoneally once daily for 30 days. Group B: Mice received 0.1 mg/100 g body weight nicotine (Sigma USA) intraperitoneally once daily for 30 days. Nicotine was diluted with normal saline (9,15). Group C: Mice received 10 mg/kg body weight, melatonin (Sigma USA) intraperitoneally once daily for 30 days. Group D: Mice received 0.1 mg/100 g body weight nicotine plus 10 mg/kg melatonin once daily for 30 days.

The dose of 0.1 mg/100g BW of nicotine, is equivalent to the human serum levels of nicotine in those who smoke 20 cigarettes per day (11, 15). Animals in all groups were killed by cervical dislocation and dissected on the 31st day. Testes and epididymis were removed from the mice. The right testis was fixed in Bouin's solution, dehydrated, and embedded in paraffin. Then, 5 μm sections were prepared and were stained with Haematoxylin and Eosin for assessment of quality and quantity of spermatogenesis. For detection of apoptosis immunohistochemical assessments by TUNEL was performed.

Spermatogenesis assessment

To evaluate spermatogenesis, an optical microscope was used. Using Johnson's scoring method, the quality and maturation of spermatogenesis in seminiferous tubules were scored from 1 to 10 (21). Also, the number of germ cells including spermatogonia, primary spermatocyte leptotene and pachytene cells, and elongated and round spermatids in the transverse section of 20 seminiferous tubules were counted. It should be noted that the tubules that were in stages VII & VIII were counted. Counting was done in the area of 1 mm ^{2} in each animal, three slides were evaluated.

Assessment of apoptosis

To evaluate apoptosis in germ cells, the TUNEL method was used. The principles of work are based on the guideline contained in the kit (Roche, Germany). In each animal, the number of apoptotic cells in the transverse section of 20 seminiferous tubules was counted and averaged.

Hormone measurement

Blood samples were collected from inferior vena cava immediately after sacrificing the mice. The serum was separated and stored at –80 °C. Testosterone and LH levels were measured using the ELISA kit (Monobind, USA).

Epididymal sperm parameters

For evaluation of the sperm parameters, the tail of epididymis was used, it was moved to a petri dish containing Ham's F10 media that was warmed to 37 °C previously. The Epididymis was cut into small pieces using a scalpel and then put in an incubator maintained at 37 °C and 5 % CO_2 for half an hour.

Following pipetting, 10 μl of the solution was placed on a microscopic slide to evaluate motility. In each animal at least 5 microscopic fields with 400× magnification were studied and percent of motile sperms was specified. Motile sperms were classified into rapid and slow progressive and nonprogressive sperms.

Using a Neubauer slide and 10 μl sperm solution, the number of sperms in 5 cells was counted and expressed as per milliliter. For the study of sperm's morphology, 10 μl sperm suspension was smeared and dried in air and fixed in 96% ethanol and then stained with Haematoxylin-Eosin. Using a light microscope morphology was evaluated and abnormalities in head and tail was expressed as percentage. In each animal 200 cells were observed with 1000× magnification.

Sperm nuclear DNA integrity

Using sperm chromatin dispersion test (SCD), the integrity of sperm chromatin and fragmentation of sperm's DNA was measured. At first, the processed sperm solution was diluted with PBS (1:3). Then 30 μl sperm solution and 70 μl low melted agarose were mixed at 37 °C. 10 μl of the above mixed solution was put on a slide covered by 0.65% agarose. After that, a lamella was put on the slide and transferred into the refrigerator and left for 4 min. Then the lamella was removed slowly in a horizontal position. Slides were put in HCL 0.12 N for a duration of 7 min and left in the dark. They were then immersed in the acid denaturation solution containing 2-mercaptoethanol l0.8 m, acidic Tris 0.4 m sodium dodecyl sulfate (SDS) 1%, ethylenediaminetetraacetic acid (EDTA) 50 mM, and sodium chloride (NaCl) 2 m in room temperature for 25 min. Then slides were washed in distilled water two times. They were stained with PBS-Wright (1:1) for 10 min. Observations were done with a light microscope (Olympus, Japan) with 400× magnification. In each animal 200 cells were studied. The fragmented cells had no hallo however, the normal cells with normal DNA strands had a big or small pink hallo around their nucleus. To measure the integrity of sperm chromatin, the percentage of total big and small hallo cells was calculated. DNA fragmentation index (DFI) was measured by counting the ratio of halo-free cells to total cells and was expressed as a percentage.

Statistical analysis

For statistical analysis, the SPSS software package was used. The data were analyzed by analysis of variance (ANOVA) and Tukey's *post hoc* tests. Pearson correlation coefficients were used for assaying the relationship between variables. $P < 0.05$ was considered statistically significant.

Results

Nicotine in group B caused a significant decrease in germ cell count, spermatogenesis maturation (Johnsen's score), and germ cell number in comparison with the controls (*P*<0.001).

However, in all nicotine-treated animals, all types of male germ cells and Sertoli cells could be observed (Tables 1, 2). An insignificant decrease was observed in spermatogonia cells (Table 1).

Melatonin in a dose of 10 mg/kg had no adverse effects on spermatogenesis quality or quantity in comparison with the controls. However, administration of nicotine plus melatonin in group D increased germ cells in stages of leptotene and pachytene primary spermatocytes, round and elongated spermatids when compared with the nicotine only treated group (Table 1) and (Figures 1 and 2).

In controls' seminiferous tubules the apoptotic cells were scattered. Administration of nicotine in doses of 0.1 mg/100 g weight once daily for 30 days significantly increased apoptotic cells (*P*<0.01). Apoptotic cells were

Table 1: Effects of nicotine and melatonin on male mouse germ cell count

Groups	Spermatogonia	Leptotene P.S.	Pachytene P.S	Round spermatid	Elongated spermatid
A	11.38±0.53	23.37±0.37	35.25±0.52	121.38±0.9	83.87±3.16
B	9.80±0.36	15.00±0.53[a]	18.62±0.67[a]	95.5±2.29[a]	67.5±1.87[a]
C	11.50±0.56	21.5±0.77[b]	33.5±0. 9[b]	118.50±1.32[b]	82.87±1.68[b]
D	10.15±0.48	20.12±0.63[ab]	27.37±1.01[ab]	109.71±2.27[ab]	79.12±2.72[b]

Data are expressed as (mean±standard error). A: controls, B: nicotine group, C: melatonin group D: nicotine+ melatonin. P.S.: Primary Spermatocyte. a:significant compared with control (*P*<0.001). b: significant compared with the nicotine group (*P*<0.01)

Table 2: Effects of nicotine and melatonin on male mouse germ cell parameters and sperm chromatin integrity

Groups	Apoptotic cells (n)	Johnsen's score	Halo sperm (%)	SDFI (%)
A	5.43±0.45	9.53 ±0.06	73.27±1.01	12.25±0.53b
B	13.87±0.89a	8.35±0.16a	55.88±1.27a	32.25±0.81a
C	7.25±0.53b	9.30 ±0.06b	73.62±1.54b	10.50±0.7b
D	7.78±0.44b	9.05±0.06ab	62.5±2.09ab	20.28±0.81b

Data are expressed as (mean±standard error). A: controls, B: nicotine group, C: melatonin group D: nicotine+ melatonin. a: significant in comparison with control. n: number (*P*<0.001). b: significant compared with the nicotine group (*P*<0.01). Sperm DNA fragmentation index (SDFI)

Figure 1. Photomicrograph of seminiferous tubules in mice. Bm: basement membrane, Sg: spermatogonia, Ps: pachytene primary spermatocyte, Ls: leptotene primary spermatocyte, Rs: Round spermatid, Es: elongated spermatid, L: lumen, Le: Leydig cell. S: Sertoli cell. The arrow shows the thickness of germinal epithelium (GE). Haematoxylin-Eosin. 400×
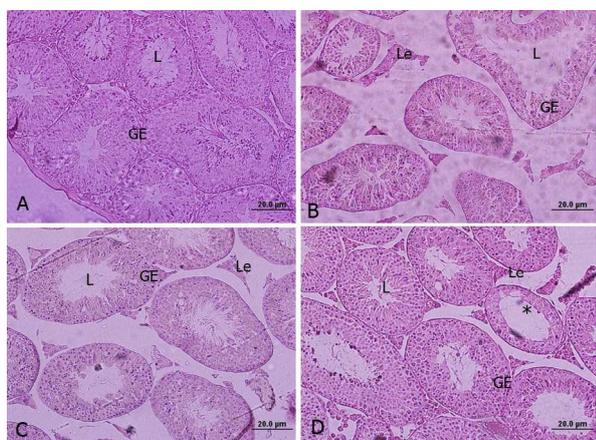

Figure 2. Histology of the testis in mice. A: controls, B: nicotine group, C: melatonin group, D: nicotine+ melatonin. GE: germinal epithelium, L: lumen, Le: Leydig cells in interstitial tissues. In picture B notice reduced Johnson's score and germ cells especially elongated spermatids
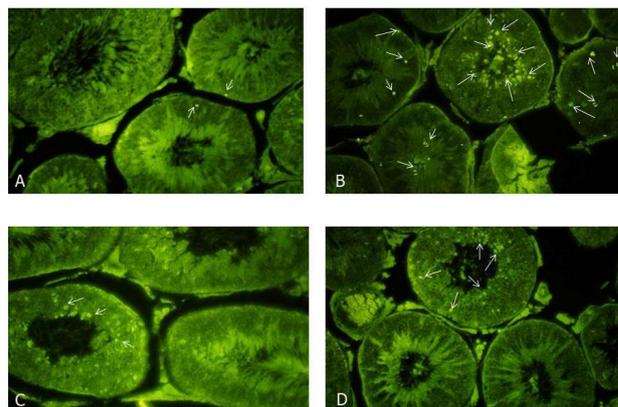

Figure 3: Immunostaining of seminiferous tubules. A: controls, B: nicotine group, C: melatonin group, D: nicotine+ melatonin group. White arrows show brilliant apoptotic germ cells. TUNEL. 400×

mostly observed in primary spermatocytes and also elongated spermatids. Melatonin in the last group reduced the apoptotic cells in comparison with the nicotine-treated group (Table 2) (Figure 3). Melatonin also increased germ cell count and Johnsen's score in comparison with the nicotine group (*P*<0.01) (Table 2) (Figure 3).

Nicotine reduced sperm count, motility and normal morphology in epididymal sperms when compared with controls (*P*<0.001). It also reduced halo cells (*P*<0.001). DNA fragmentation index (DFI) was increased in the nicotine group (*P*<0.001). Administration of melatonin in the last group increased the abovementioned parameters in epididymal sperms compared with the nicotine-treated group (*P*<0.01) (Tables 2, 3) and (Figure 4).

A significant decrease in serum LH and testosterone

Table 3. Effects of nicotine and melatonin on epididymis sperm parameters

Groups	Count (×10 6/ml)	Rapid progressive motility (%)	Slow progressive motility (%)	No progressive motility (%)	Total motility (%)	Abnormal morphology (%)
A	32.52±1.46	10.1±0.17	38.4±2.16	15.11±0.27	63.97±1.24	27.50±1.16
B	23.43±0.75[a]	2.4±0.2[a]	30.2±2.83	30.41±0.4[a]	41.07±1.37[a]	45.50±0.85[a]
C	33.08±0.88[b]	12.5±0.13	44.2±2.23	10.5±2.48	67.62±1.90[b]	25.87±0.5[b]
D	29.82±0.80[b]	8.6±0.06	35.4±2.58	18.61±2.9[ab]	63.75±2.68[b]	29.62±1.03[b]

Data are expressed as (mean±standard error). A: controls, B: nicotine group, C: melatonin group, D: nicotine+ melatonin. SDFI: Sperm DNA Fragmentation index. a: significant in comparison with controls (*P* <0.001). b: significant compared with the nicotine group (*P* <0.01)
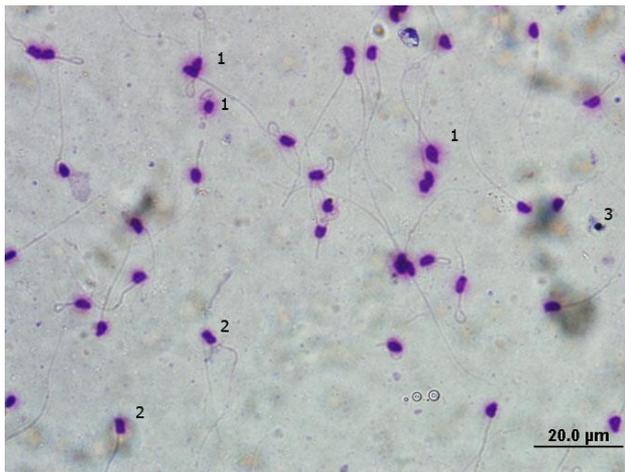

Figure 4: light photomicrograph from halo sperms in control mice. 1: big halo spermatozoa, 2: small halo spermatozoa, 3: degraded spermatozoa. Sperm chromatin dispersion test. 400×
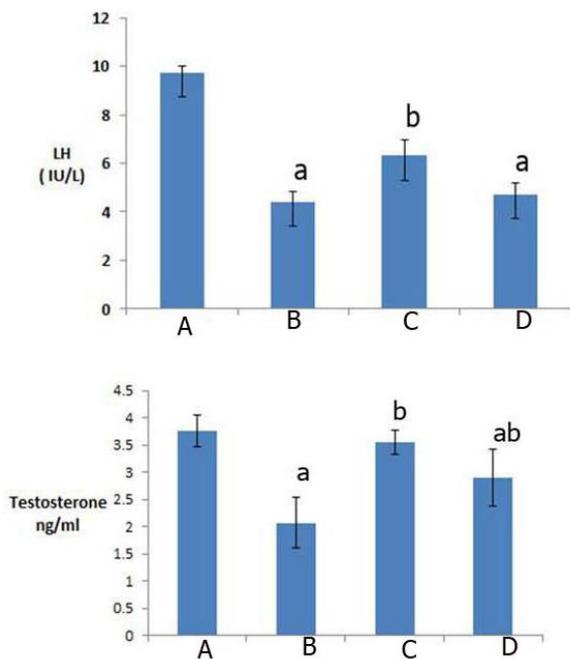

Figure 5: Effects of nicotine and melatonin on serum testosterone and LH levels. Data are expressed as (mean±standard error). A: controls, B: nicotine group, C: melatonin group, D: nicotine+ melatonin. a: significant compared with controls (*P* <0.001). b: significant compared with the nicotine group (*P* <0.001)

levels was observed in group B in comparison with controls (*P* <0.001). Administration of nicotine plus melatonin significantly increased the testosterone level compared with the nicotine-treated group (*P* <0.001). The amount of LH in serum in the last group was a little higher than in the nicotine-treated group but it was insignificant (*P* >0.05) (Figure 5).

The serum testosterone level was negatively correlated with the DFI (r= -0.86, *P* <0.001). There was a negative correlation between the LH level and DFI (r= -0.78, *P* <0.001). Abnormal sperm morphology showed a positive correlation with DFI (r=0.85, *P* <0.01). Male germ cell apoptosis positively correlated with DFI (r=0.78, *P* <0.01). Sperm motility showed a negative correlation with DFI (r=- 0.79 *P* <0.001).

Discussion

The present study showed administration of nicotine in a dose of 0.1 mg/100 g BW for 30 days in adult male mice caused adverse changes in spermatogenesis, apoptosis, sperm parameters, and sperm chromatin integrity. This effect is likely due to alterations in pituitary gonadotrophins essential for initiating and completing spermatogenesis and steroidogenesis in the testis. Because nicotine can suppress central nervous system, which can inhibit the neural stimulus essential for the release of gonadotrophins from hypophysis (23). Studies have implicated nicotine in infertility due to affecting sperm function by decreasing the sperm count motility, morphology, viability, and testicular weight (24, 25).

Spermatogenesis is a highly regulated process and is controlled by hormones especially testosterone and gonadotropins FSH and LH (8, 26). Our ELISA assay revealed a significant decrease in serum testosterone and LH levels in the nicotine group. Testosterone is a key hormone for the spermatogenesis process and is required for beginning and maintenance of spermatogenesis. LH is secreted from hypophysis and affects the Leydig cells and stimulates them for secretion of testosterone. Therefore it seems reduced LH causes reduced testosterone and our results confirm it.

Nicotine and cotinine are both 3α-hydroxysteroid dehydrogenase inhibitors, which is an important enzyme in the metabolism of testosterone and dihydrotestosterone and plays a role in changing the function of androgens in testosterone-dependent tissues such as prostate (6). In this study, contrary to the

fact that most germ cells were reduced after nicotine use, spermatogonia cells did not decrease significantly. Perhaps this difference is due to the presence of different receptors on the various types of germ cells in the testes (27).

Apoptosis is a programmed physiological death that can be created in different types of cells during different periods of life. Increased apoptosis in this study may be due to nicotine's own toxic agent (24, 25) or due to the reduction of testosterone and LH, which our study confirms. It may also be due to the higher levels of oxidants in nicotine-treated groups. In this regard, it has been shown that nicotine reduces the testicular antioxidant level and increases testicular lipid peroxidation (11).

In the present study, we performed the SCD test for evaluating sperm chromatin integrity. The traditional evaluation of sperm to study the fertility status of men is not enough and the evaluation of the sperm DNA yields more information about fertility (26). High levels of sperm DNA damage are associated with reduced fertility (26), and nowadays oxidative stress is considered as a strong factor for single- and double-strand deoxyribonucleic acid (DNA) breaks (27) effect (28).

Our study showed nicotine increased both abnormal sperms and fragmentation of sperm's DNA. Condorelli *et al.* in an *in vitro* study, showed that nicotine, depending on the dose and time, reduced sperm motility and live sperm, and increased sperm percentage by decreasing the mitochondrial membrane potential and the integrity of the chromatin DNA of the sperm (29).

Our study showed a negative correlation between testosterone and LH sex hormones with SCD. In this regard, a previous study on humans, has shown that there is a negative relationship between testosterone and estradiol hormones with sperm's DFI in fertile men (30). In confirmation of our findings regarding the existence of a negative link between motility and DFI, some studies have shown a significant negative relationship between sperm motility and sperm DNA damage (31, 32).

This study showed melatonin in the last group improved both spermatogenesis and sperm parameters. Previous studies have shown that melatonin reduces adverse effects of nicotine on the ovary and uterus (4, 5). It also increases spermatogenesis and sperm parameters in mice treated with chemotherapy (21) and acetylsalicylic acid (33) and in diabetic rats (34). It also modifies spermatogenesis in hyperlipidemic animals and following testicular torsion (20).

Melatonin is a hormone synthesized mainly in the pineal gland and also in the other organs such as retina and lacrimal glands (35). The concentration of melatonin in the testicular tissue is similar to that of other tissues in the body (26). Studies have shown that melatonin has high antioxidant properties and the potential to trap free radicals (26). Sperm exposure to melatonin *in vitro* results in better semen quality (36). Therefore these findings show the beneficial effects of melatonin on sperms.

The better spermatogenesis status in the last group may be due to anti-apoptotic and antioxidant effects of melatonin (4, 22) and our study confirmed it. Melatonin also affects the hypothalamic–pituitary–gonadal (HPG) axis and modifies the secretion of sex hormones such as estrogen, testosterone, FSH, and LH (19). In confirmation of these, in our study, melatonin also significantly increased testosterone and partly the LH hormone and improved DFI in the last group compared with the nicotine group. This is likely due to the direct effect of melatonin on Leydig cells and in this regard, the presence of melatonin receptors on the rat Leydig cells has been shown (37). Melatonin increased the integrity of sperm DNA in the nicotine-treated group. Casao *et al.* showed that there is a relation between melatonin and testosterone hormones and antioxidant enzymes in ram seminal plasma (38). Therefore these studies confirm that melatonin has an effect on sperm's DNA fragmentation index.

The exact mechanism of the effect of melatonin-nicotine on the quality of spermatogenesis is not clear and requires more studies.

Conclusion

This study showed administration of melatonin in nicotine-treated mice increases both quality and quantity of spermatogenesis and integrity of sperm chromatin through reducing apoptosis and modifying the testosterone level.

Ethical Statement

Animals were maintained and handled according to the protocols approved by the Animal Care and Use Committee of Guilan University of Medical Sciences, Rasht, Iran.

Acknowledgment

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