

The Protective Effects of Exogenous Melatonin on Nicotine-induced Changes in Mouse Ovarian Follicles

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Abstract

Background: Nicotine exposure causes impaired fertility and ovarian dysfunction. The aim of this study was to investigate the possible protective role of melatonin, which is known as an antioxidant agent on altered ovarian functions upon nicotine exposure.

Methods: A total of 32 female adult NMRI mice were divided randomly into four groups (n=8). The control group received vehicle, while group 2 received nicotine (40 $\mu\text{g}/\text{kg}$) for 15 days and group 3 melatonin (10 mg/kg) for 5 days. Group 4 received both nicotine (40 $\mu\text{g}/\text{kg}$) and melatonin (10 mg/kg) for the same periods. All animals were treated intraperitoneally. After autopsy on the 16th day, histopathological and morphometrical examinations were performed and serum estradiol concentrations were measured. The data were analyzed using ANOVA and Tukey post hoc test. A value of $p < 0.05$ was considered significant.

Results: Nicotine significantly reduced the number of pre-antral and antral follicles, as well as estradiol concentration compared to the control group ($p < 0.05$). However, the decrease in the number of primordial follicles was not significant in the nicotine treated group. A significant increase in the atretic follicles were observed in group 2 compared to the control group ($p < 0.05$). Moreover, melatonin caused a marked normalization in the number of ovarian follicles and estradiol levels in group 4 compared to group 2.

Conclusion: The results from this study suggest that melatonin may have a protective effect against nicotine-induced ovarian changes on the number of different stages of follicle growth.

Keywords: Melatonin, Mouse, Nicotine, Ovarian follicle, Ovary, Protection.

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Introduction

Cigarette smoke contains a mixture of 4000 toxic chemicals, including nicotine, addictive components, carbone monoxide, and several recognized carcinogens and mutagens (1). Smoking has deleterious effects on cardiovascular, pulmonary physiology and reproductive system (1). In women, smoking is associated with infertility, spontaneous abortion, menstrual abnor-

malities, ectopic pregnancies and early onset of menopause (2, 3).

Nicotine is a highly toxic substance and it is quickly absorbed through the respiratory tract, mouth mucosa and skin (1). Nicotine is extensively metabolized to a number of metabolites by the liver. Quantitatively, the most important metabolite of nicotine in mammalian species and humans

is the lactam derivative cotinine. In humans, about 70 to 80% of nicotine is converted to cotinine (4). In adult humans, cotinine has been detected in the follicular fluid of women who smoke, demonstrating that nicotine reaches the ovary and developing follicles. Cotinine, has also been detected in granulosa lutein cells (1).

Treatment of rats with nicotine is associated with a decrease in estrogen dependent parameters, including uterine weight, myometrial and endometrial diameter and thickness (1). Moreover, Nicotine causes a significant decrease in the concentrations of Na, K, and Cl in the uterine fluid and endometrial cells in situ. Nicotine also reduces uterine blood flow by an average of 30 to 49% (5).

There are different forms of nicotine administration for evaluating its absorption pharmacokinetics such as smoking, nasal spray, gum, inhaler, sublingual tablets, tooth patch, transdermal patch, intravenous and subcutaneous injections, oral capsule, oral solution and enema. In animal models, nicotine is usually administered subcutaneously, intraperitoneally or orally. Intraperitoneal and subcutaneous administrations of nicotine are more effective than the oral route. This may be due to the fact that intraperitoneal or subcutaneous routes facilitate the rapid absorption of the substance. The oral bioavailability of nicotine is about 20 to 45%; however, in intraperitoneal or subcutaneous routes it is much more (about 100%). Oral bioavailability of nicotine is incomplete because of the hepatic first-pass metabolism (4).

All of this evidence indicates that nicotine can affect gamete cell function. *In vitro* studies in luteal cells have shown that nicotine causes luteal insufficiency by inhibiting progesterone release (3). Investigations on nicotine have indicated that nicotine, inhibits the release of gonadotropins from the pituitary gland by affecting the central nervous system (6).

Exposure of the uterus to nicotine causes impaired fertility, altered ovarian steroid hormone and protein concentrations and increased numbers of atretic follicles in the offspring of adult female rats (3). Nicotine has a direct effect on the ovaries and morphology of treated adult female rats (6). Administration of nicotine in adult female rats has resulted in the increase in the number of atretic follicles in the ovary, irregularities in the estrous cycle, impaired ovulation, altered steroid hormone concentration, decrease in the number and size of Graafian follicles and corpora lutea (1, 3).

Nicotine has been shown to induce apoptosis in multiple tissues (7). Nicotine exposure during fetal, neonatal (8) and adult hood of female rats (6, 8) induces apoptosis in the ovaries.

Generally, there are few options to protect the ovarian function in females against side-effects of drugs, chemotherapy or radiotherapy. These include transposition of the ovaries outside the fields of radiation, administration of gonadotrophin releasing hormone (GnRH) analogs and cryopreservation of parts of the ovarian cortical tissue. Most of these options are either ineffective or still belong to the field of research (9). Drugs that could protect the oocyte and its surrounding feeder cells from such damages would be very useful. Melatonin could be considered as a drug with such effect.

As a neurohormone and highly conserved molecule, melatonin is produced in all vertebrate species. It is the chief secretory product of the pineal gland and a powerful free radical scavenger and antioxidant (10). Melatonin facilitates various physiological functions as follows: circadian rhythm functions, such as sleeping and waking up; sexual activity and reproductive functions; tumor growth; immune response; and aging (11). Furthermore, due to its low toxicity, melatonin is used as a pharmacological substance for treating sleep disorders (12). Melatonin receptors have been detected in several organs, including brain, retina, cardiovascular system, gastrointestinal tract, kidney, immune cells, adipocytes, prostate and breast epithelial cells, ovary granulosa cells, myometrium and skin (13).

A better understanding of the effects of nicotine is critical for women who are unable to quit smoking use nicotine replacement therapy (NRT) for cessation of smoking and its related side-effects. In comparison with smoking, NRT reduces exposure to thousands of toxic chemicals in the cigarette smoke (4). There are no reports about supportive effects of melatonin on ovaries exposed to nicotine. The purpose of this study was to investigate the protective effects of melatonin on mouse ovary and folliculogenesis following exposure to nicotine.

Methods

Animals and treatment: A total of 32 adult NMRI female mice were obtained from the Razi Institute in Karaj, Iran and they were randomly divided into four groups (n=8). The animals were housed in small groups under standard lighting conditions

with free access to water and food. They were allowed to adapt for at least one week in the animal room before they were subjected to treatment. Animals were maintained and handled according to the protocols approved by the Guilan University of Medical Sciences Animal Care and Use Committee. The assigned groups were as follows:

- Group 1 (controls): the mice received 1% ethanol (0.3 ml) in normal saline intraperitoneally for 15 consecutive days.

- Group 2 (Nicotine): the mice received 40 µg/kg body weight nicotine (Sigma Chemical Company, St. Louis, USA) intraperitoneally for 15 days.

- Group 3 (Melatonin): the mice received melatonin (Sigma, USA) that had been dissolved in ethanol and further diluted in saline to give a final concentration of 1% ethanol; a dose of 10 mg/kg was administered intraperitoneally for 5 consecutive days.

- Group 4 (Nicotine and Melatonin): the mice received 40 µg/kg body weight nicotine (Sigma, USA) and 10 mg/kg melatonin for 5 days. After that they received only nicotine from 6th day to 16th day.

We chose 40 µg/kg nicotine for a duration of 15 days based on the previous studies on mouse and rat (6, 14); in addition, we used different doses of nicotine (0.1, 0.2, 0.4 and 0.6 mg/kg) on both ovary and testis in our pilot study (15). A dose of 0.6 mg/kg nicotine killed most of the animals, while a dose of 0.1 mg/kg did not have any effects on ovaries. The most effective dose was 0.4 mg/kg. In humans it has been shown that shortly after smoking several cigarettes, the mean peak of nicotine in blood is about 50 ng/ml which is equivalent to 20 cigarette/day based on the dose of 40 µg/kg nicotine (14).

Another study showed that the role of melatonin on the cells is both time- and dose-dependent (16). The dose and timing of melatonin administration were selected according to previous studies in which the antioxidant action of this agent was apparent (17, 18).

All animals were dissected on the 16th day after initiation of the treatment. Following ether anesthesia, blood samples were collected for serum estradiol assay. Ovaries were removed, fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Later, 5 µm sections were prepared followed by staining for histological and morphometrical assessments.

Hormone measurement: Blood samples were collected through the inferior vena cava, immediately after sacrificing the mice. The serum was separated and stored at -80 °C. Serum estradiol concentrations were measured using ELISA kits (Demeditec Diagnostics GmbH, Germany).

Assessment of folliculogenesis:

For this purpose, 5 µm sections were stained by Periodic Acid Schiff (PAS) and observed using a standard light microscope. Follicles were classified based on their morphological characteristics as: primordial, primary, secondary and Graafian follicles.

Primordial follicles: They were identified by the flattened granulosa cells surrounding the oocyte (Figure 1).

Primary follicles: They were identified by one layer of cuboidal granulosa cells surrounding the oocyte (Figure 1).

Secondary follicles: They were characterized by two or more layers of cuboidal granulosa cells or with presence of one or more cavity inside the granulosa cells with no visible antrum (19–21) (Figure 1).

Graafian (antral follicles): They were identified by the presence of a large antral cavity filled with secretory fluid (19–21), (Figure 1).

In each sample, all types of follicles were counted and measured using a graded ocular lense calibrated on an Olympus light microscopy (Japan) with ×400 magnification.

Statistical evaluations: The data were analyzed by the analysis of variance (ANOVA) and Tukey

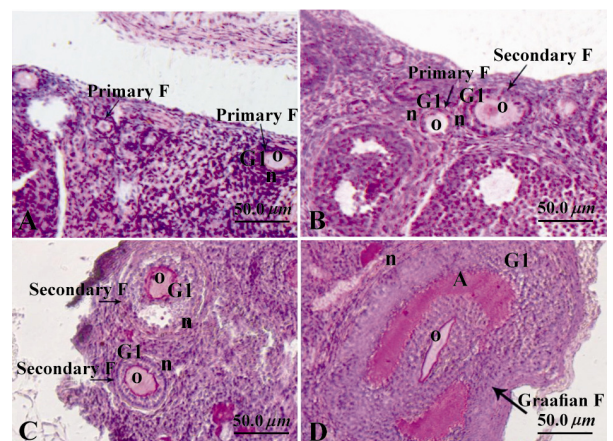


Figure 1. Photomicrographs of mouse ovary; A: Primordial and primary follicles; B: A primary and secondary follicle; C: Two secondary follicles; D: A Graafian follicle with a large antral cavity; O: Oocyte; G1: Granulosa layer; TI: Techa layer and A: antral cavity. Hematoxiline and PAS staining. Magnification 400X. Bar: 50 micron

post Hoc tests. The $p < 0.05$ was considered statistically significant.

Results

In control ovaries, all types of follicles were present. Ovaries were surrounded by a layer of simple squamous germinal epithelium. In the ovarian cortex, there were different types of follicles with various sizes. Medulla was occupied by many blood and lymph vessels and a regular zona pellucida was observed around the oocytes in primary follicles onward. Serum estradiol concentration in the control group was 60.26 ± 10.07 pg/ml (Figure 2).

Upon microscopic examination, degeneration of follicles and desquamation of granulosa cells inside the antral cavity and irregular zona pellucida around the oocytes were observed in the nicotine treated group (Figure 2). Degenerative symptoms mostly were observed in secondary and Graafian follicles. In some follicles there were vacuoles inside the granulosa layers and shedding of picnotic granulosa cells inside the antral cavity was evident.

Visibly, administration of nicotine for 15 days caused a significant decrease in the number of secondary and Graafian follicles ($p < 0.05$) in comparison with the controls. The number of corpus lutea decreased significantly in nicotine treated group in comparison with the controls ($p < 0.05$). Additionally, nicotine reduced serum estradiol concentration in comparison with the controls (44.21 ± 16.49 vs. 60.26 ± 10.07 pg/ml, $p < 0.05$), (Table 1).

In contrast to nicotine, melatonin had no significant effects on estradiol concentrations (53.30 ± 7.12 vs. 60.26 ± 10.07 pg/ml) or ovarian follicles (Table 1 and Figure 2).

Co-administration of nicotine and melatonin significantly increased number of secondary and Graafian follicles and corpus luteum in compare-

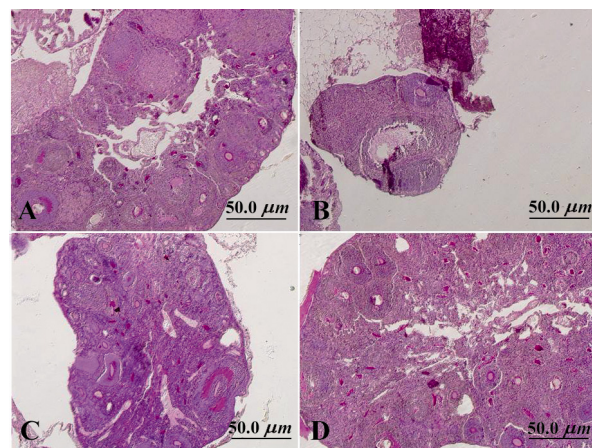


Figure 2. Photomicrographs of mouse ovary in different groups; A: control mouse; B: nicotine treated mouse; C: melatonin-treated mouse and D: nicotine+melatonin treated mouse. Notice regression of ovary and decrease in number and size of follicles in nicotine-treated ovary (B). In D, melatonin has improved the folliculogenesis in the ovary in comparison with nicotine-treated mouse. Hematoxiline and PAS staining. Magnification 100X. Bar: 50 micron

son with the nicotine only treated mice (Table 1). Serum estradiol concentrations significantly increased in group 4 (55.22 ± 12.43 vs. 44.21 ± 16.49 pg/ml) in comparison with group 2 (Table 1 and Figure 2).

Discussion

The present study showed that nicotine causes histopathological changes in the ovaries of mice, however, co-administration of melatonin and nicotine improved these alterations by providing protection against the impairment of folliculogenesis partly through effect on hypothalamo-pituitary gonadal axis.

Results from in vivo and in vitro studies have clearly demonstrated that nicotine alone can have adverse effects on ovarian function (22). Similar to our study, it has been shown that nicotine exposure has a direct effect on the ovarian morphology of the treated female mice (6).

Table 1. The effects of nicotine and melatonin on the number of mouse ovarian follicles and serum Estradiol level

| Ovarian follicles | Controls | Nicotine | Melatonin | Nicotine+Melatonin |
|-------------------------------|-------------|--------------------------|------------|---------------------------|
| Primordial | 17.85±2.74 | 7.50±2.61 | 18.12±2.90 | 18.50±2.56 |
| Primary | 7.41±2.1 | 5.18±1.4 ^a | 6.80±1.4 | 7.31±0.6 ^b |
| Secondary | 14.32±2.0 | 8.17±3.4 ^a | 12.24±1.41 | 12.04±2.1 ^b |
| Graafian | 4.15±0.78 | 2.30±1.10 ^a | 4.32±0.6 | 3.81±0.4 ^{ab} |
| Corpus luteum | 7.2±0.04 | 4.2±0.03 ^a | 6.9±0.03 | 5.6±0.04 ^{ab} |
| Serum Estradiol level (pg/ml) | 60.26±10.07 | 44.21±16.49 ^a | 53.30±7.12 | 55.22±12.43 ^{ab} |

The values are expressed as (mean±SD). The values are comparable in the same row, a: significant from control group ($p < 0.05$); b: significant from nicotine-treated group ($p < 0.05$); F (follicle)

In the nicotine treated group, all follicles were affected except primordial follicles. Pre-antral and antral follicles were more affected than other types of follicles. This condition may be due to the increased effect of nicotine on granulosa cells. The number of granulosa cells in large follicles is much more than in small follicles. In this regard, nicotine acetylcholine receptors (nAChR-2) have been identified on granulosa cells (8). It has been shown that nicotine can alter ratio of bcl2: bax and activate caspase 3 in different tissues through its nAChR-2 and -7 receptors (23, 14). Probably, nicotine has induced apoptosis in granulosa cells in this study. Therefore, through this probable mechanism and by nAChR-2, nicotine can have adverse effects mostly on growing or Graafian follicles (14).

The above mentioned alterations in ovary might be due to induction of apoptosis by nicotine. An increase in apoptotic granulosa cells was found in rats following fetal and neonatal exposure to nicotine (8). Through its interaction with specific nAChRs, nicotine has been shown to induce apoptosis in multiple tissues (25–28). Expression of nAChRs-2 and nAChRs-7 have been identified in both fixed ovarian tissue and in isolated granulosa cells (8). Therefore, one mechanism by which nicotine may have initiated a change in ovarian morphology and function is by directly causing ovarian cell apoptosis via the nAChRs. Through this mechanism, nicotine would affect growing and developing follicles but would also likely negatively affect the pool of primary follicles, reducing subsequent ovulation events (8).

Cigarette smoke likely causes follicle loss in mice ovaries without affecting ovulation (3). In contrast Blackburn et al. demonstrated that nicotine causes LH-independent inhibition of ovulation *in vivo* and *in vitro* in PMSG-primed immature female rats (29). Decreased estrogen level in nicotine treated group can probably be explained by its toxic effects on granulosa cell function or aromatase activity. This observation correlates well with our histological findings that granulosa cells were mostly affected. Moreover, nicotine has been shown to inhibit the induction of progesterone synthesis in cumulus cells by FSH and the production of other androgens by theca interna cells (14). In contrast to our study, nicotine did not affect estradiol production by human granulosa cells (1). However, Gocze et al. (30) and Bodies et al. (31) observed a slight increase in estradiol production by human granulosa cells treated

with nicotine, while Barbieri et al. (32) reported decreased aromatase activity in these cells treated with aqueous tobacco smoke extract and nicotine alone. One reason for these conflicting results may be the variation in the form of used nicotine preparations. Another reason could be the source of granulosa cells and the length of culture period and also species differences and dose of nicotine (1).

Melatonin in dose of 10 mg/kg body weight daily for 5 days had no adverse effects on adult mouse ovary morphology. However, it caused significant increase in estradiol concentration in group 4 in comparison with the nicotine only treated group. Melatonin exerts its primary reproductive action at the level of the brain and pituitary gland. However, the presence of high melatonin concentration in follicular fluid and the presence of receptors in granulosa cells suggest a potential beneficial property of melatonin on the ovarian function (5). Generally, melatonin toxicity is extremely low and no adverse effects have been found when 10 to 250 mg/kg melatonin has been fed to mice; 100 to 250 mg/kg to rats or even 800 mg/kg to rabbits, dogs or cats. In human volunteers, no side effects were observed by the oral administration of melatonin in a dose of 1 to 300 mg or even 1 g daily for 30 days (33).

Low doses of melatonin does not affect estrogen level *in vitro*, however, in higher doses melatonin reduces production of estrogen (9). In other words, effect of melatonin on steroidogenesis is dose-dependent. Probably, melatonin exerts its effect via a receptor-mediated action at the level of the ovary to modulate steroidogenesis (34), and possibly luteolysis because it has been shown that melatonin Mt₁ and Mt₂ receptors are expressed in human granulosa lutein cells too. Melatonin also regulates luteinizing hormone receptors (LHR), gonadotropin releasing hormone (GnRH) and gonadotropin releasing hormone receptor (GnRHR) (34). Melatonin increases the number of atretic follicles in mice (35). Contrary to our expectation, melatonin was not effective on the number of follicles.

In hamsters, melatonin causes decreased number of Graafian follicles and corpora lutea and a proliferation of interstitial tissue in the ovary. Hence, the exogenous melatonin may have an inhibitory, a stimulatory or no effect on the reproductive system of rodents depending on the model system and species used (35) and in humans, the effect of exogenous melatonin is different in the menstrual

phase (36). Indeed, studies about the effects of melatonin on the ovary are also contradictory. Woo et al. showed melatonin could increase LH receptors and secretion of progesterone in human luteal granulosa cells in cell culture (34), however, Bodies et al. showed reduction in the secretion of Estradiol and progesterone in human luteal granulosa cells in cell culture (37). In webley's study, melatonin did not alter estradiol concentration (38). It seems these conflicts depend on the cell type (theca or granulosa cells), duration of treatment, experimental model (cell or follicle cultures), species and dose of melatonin (39).

We showed that administration of melatonin in nicotine-treated mice protects folliculogenesis in the ovary. Melatonin may also protect ovarian tissue against γ -irradiation (35). It may also protect testicular tissue against busulfan, cisplatin and X-ray, cardiac muscle cells from damage induced by doxorubicin, and intestinal organ injury following mesenteric ischemia/reperfusion (40).

The other protective mechanisms of melatonin on ovary in nicotine-treated mice may be due to antioxidant activity of melatonin; although, we did not measure the antioxidant property of melatonin.

Estrogen stimulates the proliferative activity of granulosa cells via FSH receptors. In the present study, nicotine decreased estrogen levels. Proliferative activity of granulosa cells may change in nicotine treated groups. However Petric et al. did not find antiproliferative activity of nicotine on granulosa cells (8). On the other hand, it has been shown in previous studies that melatonin has antiproliferative activity on male germ cells (40). Antiproliferative effects of melatonin using MCF7 cells as a model to study the anti-estrogenic effect of this hormone has been shown in several studies (41, 42).

Conclusion

In conclusion, this study indicates that melatonin improves the ovarian functions in nicotine-treated mice through alterations in estrogen concentration. Our results also suggest that melatonin may have a significant beneficial effect for clinical applications and subfertility or infertility induced by smoking in women.

Conflict of Interest

Authors declare no conflict of interest.

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