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Alterations in Rat's Testicular Structure and Function following Simultaneous Administration of Nicotine and Methylphenidate: An *in Vitro* Dose-Response Study

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ABSTRACT

Methylphenidate (MPH) and nicotine (NCT) have deleterious effects on testicular tissue, individually. Methylphenidate intake could increase the tendency to use NCT. In this study, the possible synergistic effects of MPH and NCTon the intensity of structural and functional changes of testicular tissue were evaluated using experimental animal study. Eighty male mature Wistar rats were used. MPH (5 and 10 mg/kg, IP) and NCT (2 and 4 mg/kg, IP) were administrated individually (MPH or NCT) or simultaneously (MPH + NCT) to eight treated groups of adult rats (n = 10/group) once a day for eight weeks. After the recording of body and testicular weight, the plasma level of pituitary gonadotropins, testosterone, and malondialdehyde (MDA) as lipid peroxidation index were measured. Also, testes samples were prepared for tissue lipid peroxidation assay, histomorphology, immunohistochemistry of p53 protein, and electron microscopy. Moreover, sperm analysis was performed on cauda epididymis. There was not any significant difference in initial and final body weight between groups. Testicular weight and testicular to body weight ratio were reduced in treated groups. LH, Testosterone, and plasma and tissue MDA were increased and FSH decreased dose-dependently in the most treated groups. Most of the histomorphometric, cell population, and sperm analysis parameters were decreased in treated groups in a dose-dependent manner. The light and electron micrographs showed various alterations in testicular tissue which were more obvious in higher doses of NCT or MPH and NCT + MPH groups. Immunostaining of testicular tissue revealed upregulation of p53 in treated groups, particularly in NCT + MPH groups. The results of this study showed that simultaneous administration of NCT and MPH could induce more alterations in the structure and function of testicular tissue compared to their individual administration.

Introduction

Spermatogenesis is regulated by complex cellular and endocrine/paracrine events.[1] Testicular tissue, and consequently spermatogenesis and androgenesis are susceptible to a wide range of exogenous and environmental stressors.[2] Exposure to different xenobiotics can affect the male reproductive system by

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making the structural and functional changes in testicular tissue.[3,4] Therefore, testicular toxicity has become a serious concern due to the industrialization of the societies and the ever-increasing use of chemicals and drugs.[4]

Methylphenidate (MPH) is a drug, primarily used to treat the attention-deficit/hyperactivity disorder (ADHD).[5] Also, growth retardation, weight loss and changes in the weight of the brain, spleen, heart, and prostate have been reported as adverse effects of MPH on various body organs in human and experimental animals.[6] Misuse of prescription stimulants like MPH, has become a serious problem, especially among college students who may use MPH to help them for all-night study sessions. Because of its high potential for being abused and bring addiction, MPH is classified as Schedule II medications by the United States drug enforcement administration (DEA).[7] Nicotine (NCT) is an alkaloid, which is exposed to humans through different routes including tobacco products and insecticides.[8] The alterations in spermatogenesis, reduction of semen quality, and malfunction of the pituitary-testis axis have been reported following administration of NCT, as a gonadotoxic agent, in several experimental and clinical studies.[9,10]

There is a lot of information indicating the negative effects of MPH or NCT on the male reproductive system. Nevertheless, it has been reported that, the patients who have been prescribed MPH as well as MPH abusers, may also smoke or be exposed to NCT through other ways.[2] On the other hand, some smokers may also take MPH at the same time, which raises a question on the concomitant effects of NCT and MPH on the male reproductive system and fertility.

In present dose-response study, the effects of long-term concurrent exposure to MPH and NCT on the structure and the function of testicular tissue were evaluated among adult rats.

Materials and Methods

Chemicals

Nicotine and methylphenidate were purchased from Sigma-Aldrich, St Louis, MO 63178, USA.

Animal's Procedures and Experimental Design

All animal's procedures used in the present study, were approved by the University of Tabriz standards for the care and use of laboratory animals (Approval ID: IR.TABRIZU.REC.1398.025), in accordance with the animal ethical committee (AEC) of the ministry of health and medical education of Iran (adopted in April 17, 2006) in terms of the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

After environmental adaptation, 90 healthy adult male Wistar rats (70-80 days old) were randomly divided into nine experimental groups (n = 10/group). Animals were housed in plastic cages under photoperiod of 12-h in light/12-h in darkness, and were provided with rat pellets and water ad libitum.

Nicotine was administrated once a day at dose of 2 and 4 mg/kg to the groups I and II, while the rats of groups III and IV received MPH at dose of 5 and 10 mg/kg daily, respectively.[11] Groups V-VIII received both the NCT and MPH two hours apart at the earlier-mentioned doses and frequencies. Normal saline was given to the control group. Normal saline, NCT, and MPH were intraperitoneally administrated for eight weeks.

Weight Recording and Sample Preparation

Body weight was recorded for each animal at the beginning of the study, and then twice a week during the study. At the end of the study, animals were anesthetized by intraperitoneal injection of 80 mg/kg ketamine and 5 mg/kg xylazine and blood samples were collected through a cardiac puncture in EDTA-coated tubes and were then centrifuged. The obtained plasma samples were stored at -20° C until the time of performing the analysis.

After blood collection, animals were euthanized by sodium thiopental (100 mg/kg, IP) and the weights of the right and the left testis for each animal were recorded. The cauda epididymis was separated for sperm analysis. Small pieces of the left testes were fixed in 3% glutaraldehyde in phosphate buffer (pH = 7.2) for 2-4 hours and post-fixed in 1% osmium tetroxide for electron microscopy. The remnants of the left testis were immediately fixed in 10% buffered formaldehyde solution for performing the histological studies, and the right testis was frozen for tissue lipid peroxidation assay.

Hormonal Assay

Plasma levels of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were quantified using ELISA method (DRG Instruments GmbH, Germany). The ELISA method (Monobind Inc. USA) was also used to measure the plasma testosterone level.

Plasma and Tissue Lipid Peroxidation Assay

Malondialdehyde (MDA), as lipid peroxidation index, was quantified based on the thiobarbituric acid reactive substances (TBARS) assay both in plasma and testicular tissue lysate. [12]

Tissue Preparation and Morphometric Analysis

Five sections (5 µm thickness) of each testicular sample were prepared and stained with hematoxylin and eosin (H&E). For performing morphometric assessments, 10 microscopic fields (200×) and at least 20 seminiferous tubules (STs) were studied in each section. For quantitative calculating of the populations of the cells, the number of cells was counted in every tubule and this process repeated for at least 10 tubules. Then the whole number of counted cells for 10 tubules presented as the mean of the cell population.[13] The analyses were performed on the images obtained and digitalized by Dino-Eye Eyepiece Camera AM7023B (Dino-Lite Digital Microscope). The images were processed by Dino-Lite image analysis software. To obtain extra precise results, the STs transversely sectioned were studied and the shortest diameter of STs was considered for the measurement.

Evaluation of Spermatogenesis in Testicular Tissue

To assess the spermatogenesis in testicular tissue, the number of seminiferous tubules with more than three layers of germinal cells derived from type-A spermatogonia (tubular differentiation index: TDI) was calculated; along with the ratio of active spermatogonia to inactive cells (repopulation index: RI) and the ratio of seminiferous tubules with spermatozoids to the empty tubules (spermiogenesis index: SPI).[13]

Sperm Analysis

Sperm analysis was performed on the cauda epididymis. Briefly, the cauda epididymis was separated from the testis, and then cut into small pieces in one milliliter of Ham's F10 culture medium. Sperms were counted in terms of the standard hemocytometric method. The sperm samples were diluted 1:8 in Ham's F10medium and 20 µl was used for examining the sperm motility. Only the motile sperms that were moving forward were counted within 10 boxes. Sperm viability assay was performed using the eosin-nigrosin staining method. In brief, 50 µl of epididymal sperm was mixed with 20 µl of eosin. After 5 seconds, 50 µl of nigrosin was added to that and mixed. The mixture was smeared on the slide and examined under a bright field microscope. The colorless sperms were considered as live and red-stained sperm were marked as dead. A hundred of spermatozoa was counted from each animal.[14]

Electron Microscopy

The prepared samples were dehydrated and embedded in Spurr resin embedding medium. Semithin and ultrathin sections were obtained using ultramicrotome (Leica Ultracut R, Austria). After monitoring, semithin sections were stained with toluidine blue. The ultrathin sections were mounted on copper grids and then stained with uranyl acetate and lead citrate.[15] The obtained sections were observed using a Philips C-100 Bio transmission electron microscope at 80 kV.

Immunohistochemical (IHC) Analysis

Paraffin-embedded testicular tissue sections were prepared for immunostaining of p53 protein [16]. Briefly, antigen retrieval was conducted on deparaffinized and rehydrated slides kept in 10 mM sodium citrate solution (pH = 6.0) at 95° C in a water bath for 40 minutes. Immunohistochemical staining was performed in terms of the manufacturer's protocol (St John's Laboratory Ltd, UK). Briefly, endogenous peroxidase activity was blocked with 0.3% H_2O_2. Tissue slides were washed with PBS (pH = 7.2) and then incubated with rabbit polyclonal anti p53 antibody (as primary antibody) (1:500) at 4° C overnight. Sections were treated with horseradish peroxidase (HRP) conjugated goat anti-rabbit IgG (as secondary antibody) (Agrisera Antibodies, SE-911 21 Vännäs, Sweden) in a humidified chamber for 1 hour. Diaminobenzidine (DAB) chromogen was added to the tissue sections and incubated for 5 minutes. Also, the tissue slides were dehydrated and cover-slipped after hematoxylin counterstaining.

Statistical Analysis

Statistical analyses were performed using the GraphPad PRISM® software (version 5.04, USA). Also, One-way ANOVA was used to examine the differences between groups in tested quantitative parameters. All results were expressed as mean ± SD. p values of < 0.05 were considered as statistically significant.

Results

Body Weight, Testicular Weight, and Organ Relative Weight

There was no significant difference in the initial and

Table 1. The body and testicular weight (Mean ± SD) in experimental groups.

	IBW (g)	FBW (g)	RTW (g)	LTW (g)	TTW (g)	TW / BW (%)
Control	138.0 ± 19.54	163.6 ± 18.19	1.16 ± 0.12	1.17 ± 0.12	2.34 ± 0.24	1.43 ± 0.11
NCT2	122.0 ± 11.66	160.8 ± 19.68	0.98 ± 0.23	0.99 ± 0.23	2.11 ± 0.10	1.36 ± 0.16
NCT4	122.0 ± 14.49	148.0 ± 20.74	0.75 ± 0.22	0.78 ± 0.21	1.54 ± 0.43	1.02 ± 0.15
MPH5	126.2 ± 10.11	157.3 ± 10.56	1.05 ± 0.13	0.94 ± 0.11	1.81 ± 0.25	1.18 ± 0.20
MPH10	119.6 ± 11.95	154.4 ± 18.41	0.82 ± 0.33	0.85 ± 0.34	1.68 ± 0.67	1.06 ± 0.32
NCT2 + MPH5	123.0 ± 3.31	148.3 ± 9.91	1.04 ± 0.09	0.98 ± 0.15	1.98 ± 0.47	1.22 ± 0.22
NCT2 + MPH10	127.4 ± 4.33	152.8 ± 12.42	0.94 ± 0.08	1.03 ± 0.14	2.08 ± 0.12	1.37 ± 0.17
NCT4 + MPH5	123.8 ± 6.83	149.5 ± 10.28	0.97 ± 0.12	0.97 ± 0.17	1.79 ± 0.22	1.20 ± 0.15
NCT4 + MPH10	124.4 ± 3.64	152.0 ± 12.00	0.93 ± 0.13	0.96 ± 0.12	1.90 ± 0.25	1.25 ± 0.12

All statistical comparisons were performed within each column. IBW: Initial body weight; FBW: Final body weight; RTW: right testicular weight; LTW: left testicular weight; TTW: total testicular weight; TW/BW: testicular weight/body weight ratio. The presence of different superscripts in each column indicates the significant difference between the groups.

Table 2. The level of pituitary gonadotropins, testosterone and lipid peroxidation indices (Mean ± SD).

	FSH (IU/ml)	LH (µIU/L)	Testosterone (ng/ml)	Serum MDA (nmol/mg protein)	Tissue MDA (nmol/mg protein)
Control	0.37 ± 0.12	20.0 ± 0.95	0.25 ± 0.17	0.63 ± 0.23	0.76 ± 0.42
NCT2	0.47 ± 0.03	21.2 ± 1.31	0.18 ± 0.11	0.91 ± 0.28	0.85 ± 0.07
NCT4	0.45 ± 0.05	22.5 ± 1.07	0.19 ± 0.005	1.04 ± 0.46	1.05 ± 0.64
MPH5	0.50 ± 0.17	19.4 ± 1.16^{c}	0.24 ± 0.09	0.93 ± 0.08	0.97 ± 0.13^{d}
MPH10	0.35 ± 0.05	20.4 ± 0.97	0.30 ± 0.27	1.16 ± 0.62	2.55 ± 1.11^{ab}
NCT2 + MPH5	0.78 ± 0.02^{abcde}	19.0 ± 1.66^{c}	0.32 ± 0.09	0.70 ± 0.08	1.09 ± 0.26^{d}
NCT2 + MPH10	0.70 ± 0.14^{ade}	19.1 ± 0.69^{c}	0.17 ± 0.07^{e}	0.78 ± 0.24	1.16 ± 0.18
NCT4 + MPH5	0.54 ± 0.05	19.4 ± 1.19^{c}	0.17 ± 0.06	0.97 ± 0.20	1.15 ± 0.37
NCT4 + MPH10	0.30 ± 0.005	21.2 ± 1.82	0.70 ± 043^{c}	1.03 ± 0.32	1.94 ± 0.89

All statistical comparisons were performed within each column. Notes: FSH: follicle stimulating hormone; LH: luteinizing hormone; MDA: Malondialdehyde. Letters a, b, c, d, and e show significant difference compared to the control, NCT2, NCT4, MPH10, and NCT4 + MPH10 groups, respectively.

final body weights between the experimental groups (Table 1). The weight gains of NCT4 and all NCT + MPH receiving groups were similar to the control group. NCT2, MPH5, and MPH 10 receiving groups had more weight gain compared to the control group (daily weighting data is not shown).

According to Table 1 the weights of right and left testis and total testicular weight did not changed significantly in the treated groups compared to the control group. NCT and MPH dose-dependently decreased the mean of total testicular weight. In this regard, the most reduction in total testicular weight occurred in NCT4 + MPH10 group. Accordingly, the mean of the testicular weight/body weight ratio was reduced in all the treated groups compared to the control group. Likewise, this reduction was higher in MPH + NCT groups compared to their individual administration.

Hormonal Analysis

Assessment of plasma levels FSH, LH, and testosterone showed some alterations between the treated groups (Table 2). FSH increased in the NCT2 + MPH5 and NCT2 + MPH10 groups ($p < 0.0001$). Also, NCT and MPH showed dose-dependent effects on the FSH levels. NCT4 + MPH10 had more reductive effect on

FSH compared to their combination in lower doses (NCT2 + MPH5). Also, there were no significant differences in LH and testosterone between the experimental groups. However, individual administration of NCT and MPH dose-dependently raised the LH and testosterone levels. NCT4 + MPH10 had more additive effect on the LH and testosterone levels compared to their combination in lower doses.

Plasma and Tissue MDA

According to table 2, the mean of plasma and tissue MDA levels none-significantly increased in all the treated groups compared to the control group. NCT and MPH raised the plasma MDA level in a dose-dependent manner. Also, the plasma MDA was higher in the NCT4 + MPH10 group compared to the NCT2 + MPH5 group.

The mean of tissue MDA level increased in all the experimental groups compared to the control group (Table 2). However, this change was statistically significant in the MPH10 group (p = 0.008). NCT or MPH had dose-dependent effects on tissue MDA. Co-administration of NCT and MPH in higher doses induced more elevation in tissue MDA levels compared to their lower doses.

Testicular Histomorphometry

The mean of STs diameter reduced in all the treated groups in comparison to the control group (Table 3). This reduction was significant in the NCT4, MPH10, NCT2 + MPH5, NCT4 + MPH5, and NCT4 + MPH10 groups (p < 0.0001). Combination of NCT and MPH with higher doses had more effects on the STs diameter. Also, the mean of germinal epithelium height decreased in all the experimental groups compared to the control group (Table 3). This change was significant in the NCT2, NCT4, and NCT4 + MPH10 groups (p < 0.0001). The height of germinal epithelium significantly decreased in the NCT4 + MPH10 group compared to theNCT2 + MPH5 group. In all the experimental groups, the mean of testicular connective capsule thickness increased in comparison to the control group. However, these changes were not statistically significant. Co-administration of NCT and MPH with higher doses induced more thickening of testicular capsule in comparison to their combination in lower doses.

The Populations of Sertoli Cells and Germ Cells Lineage

The mean of Sertoli cells count decreased (p < 0.0001) in all the treated groups, except for the NCT2, in comparison to the control group (Table 3). Higher doses of NCT and MPH had more decreasing effects on the Sertoli cells count. The mean of spermatogonia cells count decreased in all the experimental groups in comparison to the control group; however, this reduction was only significant in the MPH10 group (p = 0.0001). NCT and MPH had dose-dependent effects on spermatogonia cells count, and their combination with higher doses was more effective.

The mean of spermatocytes count significantly decreased in the MPH10, NCT2 + MPH5, NCT2 + MPH10, NCT4 + MPH5, and NCT4 + MPH10 groups (p < 0.001). Spermatocytes count was lower in the NCT2 + MPH5 group compared to the NCT4 + MPH10 group.

Microscopic Indices of Spermatogenesis

As shown in table 4, TDI, SPI, and RI decreased in the treated groups in comparison to the control group (p < 0.0001). These changes were more obvious following the individual administration of NCT compared to MPH. Moreover, the most reductions of TDI, SPI, and RI were observed in the NCT + MPH groups compared to their individual administration.

Sperm Analysis

The results of epididymal sperm analysis showed a reduction in sperm count, sperm motility, and sperm viability in all the treated groups in comparison to the control group (Table 4). Sperm count significantly decreased in the NCT4, NCT4 + MPH5, and NCT4 + MPH10 receiving groups (p < 0.0001). NCT and MPH had dose-dependent effects on Sperm count. Sperm count decreased more in the NCT4 + MPH10 group compared to the NCT2 + MPH5 group.

Sperm motility was lower (p < 0.0001) in all the experimental groups, except for MPH5, in comparison to the control group. The NCT4 + MPH10 had more reductive effects on sperm motility compared to the NCT2 + MPH5 receiving group.

Sperm viability significantly decreased (p < 0.0001) in all the treated groups in comparison to the control group. NCT had dose-dependent reductive effect on sperm viability. Combination of NCT and MPH with higher doses had more reductive effects on sperm viability.

The summary of the dose dependent effects of individual or simultaneous administration of nicotine or methylphenidate on the structural and functional parameters of testicular tissue is presented in Table 5.

Table 3. Testicular histomorphometry and cellular population of seminiferous tubules (Mean ± SD).

	STD (µm)	GEH (µm)	CT (µm)	Srt (#/20 tubules)	Sptg (#/20 tubules)	Sptc (#/20 tubules)
Control	292.5 ± 14.37	81.2 ± 5.73	21.64 ± 1.41	22.9 ± 2.82	66.5 ± 9.99	63.1 ± 21.2
NCT2	237.6 ± 60.55	68.70 ± 8.55[a]	21.06 ± 1.29	23.0 ± 2.58	62.6 ± 12.5	52.2 ± 19.5
NCT4	209.2 ± 44.57[a]	63.05 ± 8.79[a]	22.42 ± 1.86	15.6 ± 2.06[ab]	58.0 ± 14.5	51.4 ± 14.3
MPH5	246.3 ± 48.22	73.99 ± 9.46	21.13 ± 1.31	18.8 ± 3.13[abce]	60.0 ± 6.83[d]	50.3 ± 7.91
MPH10	224.3 ± 47.39[a]	71.27 ± 9.03	21.88 ± 2.84	18.6 ± 2.25[abce]	47.3 ± 13.4[ab]	43.3. ± 11.7[a]
NCT2 + MPH5	267.9 ± 26.29[e]	75.93 ± 7.07[ce]	22.19 ± 2.01	17.1 ± 3.57[ab]	62.9 ± 7.98[d]	44.0 ± 8.24[a]
NCT2 + MPH10	256.4 ± 34.19	75.3 ± 7.47[c]	22.58 ± 1.78	16.7 ± 2.85[ab]	61.7 ± 7.13[d]	40.6 ± 10.1[a]
NCT4 + MPH5	227.7 ± 45.40[a]	72.32 ± 7.76	22.28 ± 2.05	16.4 ± 3.56[ab]	60.6 ± 7.62[d]	47.8 ± 11.9[a]
NCT4 + MPH10	203.6 ± 45.23[a]	64.37 ± 7.73[a]	22.34 ± 1.89	15.5 ± 2.18[ab]	57.5 ± 13.5	51.0 ± 15.3[a]

All statistical comparisons were performed within each column. Notes: STD: Seminiferous tubules diameter; GEH: Germinal epithelium height; CT: capsular thickness; Srt: Sertoli cells; Sptg: Spermatogonia cells; Sptc: Spermatocytes. Letters a, b, c, d, and e show significant difference compared to the control, NCT2, NCT4, MPH10, and NCT4+MPH10 groups, respectively.

Table 4. Microscopic indices of spermatogenesis and sperm analysis (Mean ± SD).

	TDI (%)	SPI (%)	RI (%)	Sperm count (10^6/ml)	Sperm motility (%)	Sperm viability (%)
Control	81.17 ± 4.43	82.8 ± 6.48	81.1 ± 5.51	18.4 ± 1.99	81.7 ± 9.62	80.1 ± 7.52
NCT2	67.53 ± 6.87[a]	68.2 ± 7.18[a]	70.9 ± 7.16	17.8 ± 1.46	62.6 ± 5.59[a]	56.8 ± 6.75[a]
NCT4	66.86 ± 5.52[a]	67.0 ± 7.07[a]	68.9 ± 11.1[a]	15.2 ± 1.36[a]	55.2 ± 9.07[a]	53.0 ± 8.37[a]
MPH5	60.93 ± 9.85[a]	74.9 ± 5.61	77.0 ± 5.99	17.1 ± 1.34	66.0 ± 8.49	57.2 ± 6.72[a]
MPH10	70.74 ± 10.50	74.2 ± 5.38	69.7 ± 6.72[a]	16.2 ± 1.56	63.6 ± 6.88[a]	63.5 ± 6.66[a]
NCT2 + MPH5	73.58 ± 5.58[f]	75.5 ± 8.61	74.5 ± 5.94	16.8 ± 0.49	58.8 ± 9.00[a]	56.2 ± 8.64[a]
NCT2 + MPH10	59.09 ± 10.08[adg]	68.0 ± 8.73[a]	70.7 ± 8.48	15.8 ± 1.74	56.0 ± 10.30[a]	58.0 ± 6.84[a]
NCT4 + MPH5	67.53 ± 6.87[a]	63.6 ± 9.21[afg]	61.2 ± 8.37[afg]	15.4 ± 1.09[a]	49.2 ± 5.67[a]	47.8 ± 7.55[a]
NCT4 + MPH10	61.52 ± 9.20[ag]	65.0 ± 10.5[a]	66.1 ± 7.48[af]	13.7 ± 2.32[abfg]	48.8 ± 8.41[a]	51.7 ± 14.2[a]

MPH: methylphenidate; NCT: nicotine; TDI: tubular differentiation index; SPI: spermiogenesis index; RI: repopulation index. All statistical comparisons were performed within each column. Letters a, b, c, d, e, f and g show significant difference compared to the control, NCT2, NCT4, MPH10, NCT4 + MPH10, MPH5, and NCT2 + MPH5 groups, respectively.

Histology and Immunohistochemistry of Testicular Tissue

Testicular tissue of the treated groups showed various histological alterations (Figure 1). Accordingly, these structural changes consisted of the atrophy of STs, decrease in germinal epithelium height, nuclear changes such as hyperchromasia, coarse chromatin, and changes in nuclear size and shape in developing spermatids, thickening of interstitial connective tissue, and germinal epithelium dissociation. These alterations were more obvious in four groups that received NCT + MPH simultaneously. Immunostaining of testicular tissue for p53 revealed faint positive areas in the control group (Figure 2). More intensive positive areas were observed in the treated groups compared to the control group (Figure 2). Simultaneous administration of NCT and MPH had the most inductive effect on p53 expression compared to the other treated groups. Moreover, the overexpression of p53 was evident in round spermatids and developing spermatozoids. The extent of positive reaction areas per every seminiferous tubule was observed in more degrees in the NCT2 + MPH10, NCT4 + MPH5, and NCT4 + MPH10 receiving groups, respectively (Figure 3).

Table 5. The summary of the dose dependent effects of individual or simultaneous administration of nicotine or methylphenidate on the structural and functional parameters of testicular tissue.

	(NCT) N2 / N4	(MPH) M5 / M10	(NCT + MPH) N2 + M5 / N4 + M10	(NCT + MPH) N2M10 / N4 + M5
	Individual	**Individual**	**Simultaneous**	**Simultaneous**
FBW	N2 (-) N4 (-)	M5 (-) M10 (-)	N2 + M5 (-) N4 + M10 (-)	N2 + M10(-) N4 + M5 (-)
TTW	N2 (-) N4 (-)	M5 (-) M10 (-)	N2 + M5 (-) N4 + M10 (-)	N2 + M10(-) N4 + M5 (-)
TW / BW	N2 (-) N4 (-)	M5 (-) M10 (-)	N2 + M5 (-) N4 + M10 (-)	N2 + M10(-) N4 + M5 (-)
FSH	N2 (+) N4 (+)	M5 (+) M10 (-)	N2 + M5 (+)*dd N4 + M10 (-)dd	N2 + M10(+)* N4 + M5 (+)
LH	N2 (+) N4 (+)	M5 (-) M10 (+)	N2 + M5 (-) N4 + M10 (+)	N2 + M10(-) N4 + M5 (-)
Testosterone	N2 (-) N4 (-)	M5 (-) M10 (+)	N2 + M5 (+) N4 + M10 (+)	N2 + M10(-) N4 + M5 (-)
Serum MDA	N2 (+) N4 (+)	M5 (+) M10 (+)	N2 + M5 (+) N4 + M10 (+)	N2 + M10(+) N4 + M5 (+)
Tissue MDA	N2 (+) N4 (+)	M5 (+)dd M10 (+)*dd	N2 + M5 (+) N4 + M10 (+)	N2 + M10(+) N4 + M5 (+)
STD	N2 (-) N4 (-)*	M5 (-) M10 (-)*	N2 + M5 (-)dd N4 + M10 (-)*dd	N2 + M10(-) N4 + M5 (-)*
GEH	N2 (-)* N4 (-)*	M5 (-) M10 (-)	N2 + M5 (-)dd N4 + M10 (-)*dd	N2 + M10(-) N4 + M5 (-)
CT	N2 (-) N4 (+)	M5 (-) M10 (+)	N2 + M5 (+) N4 + M10 (+)	N2 + M10(+) N4 + M5 (+)
Sertoli	N2 (+)dd N4 (-)*dd	M5 (-)* M10 (-)*	N2 + M5 (-)* N4 + M10 (-)*	N2 + M10(-)* N4 + M5 (-)*
Spermatogonia	N2 (-) N4 (-)	M5 (-)dd M10 (-)*dd	N2 + M5 (-) N4 + M10 (-)	N2 + M10(-) N4 + M5 (-)
Spermatocyte	N2 (-) N4 (-)	M5 (-) M10 (-)*	N2 + M5 (-)* N4 + M10 (-)*	N2 + M10(-)* N4 + M5 (-)*
TDI	N2 (-)* N4 (-)*	M5 (-)* M10 (-)	N2 + M5 (-)dd N4 + M10 (-)*dd	N2 + M10(-)* N4 + M5 (-)*
SPI	N2 (-)* N4 (-)*	M5 (-) M10 (-)*	N2 + M5 (-) N4 + M10 (-)*	N2 + M10(-)* N4 + M5 (-)*
RI	N2 (-) N4 (-)*	M5 (-) M10 (-)*	N2 + M5 (-) N4 + M10 (-)*	N2 + M10(-) N4 + M5 (-)*
Sperm count	N2 (-) N4 (-)*	M5 (-) M10 (-)	N2 + M5 (-)dd N4 + M10 (-)*dd	N2 + M10(-) N4 + M5 (-)*
Sperm motility	N2 (-)* N4 (-)*	M5 (-) M10 (-)*	N2 + M5 (-)* N4 + M10 (-)*	N2 + M10(-)* N4 + M5 (-)*
Sperm viability	N2 (-)* N4 (-)*	M5 (-)* M10 (-)*	N2 + M5 (-)* N4 + M10 (-)*	N2 + M10(-)* N4 + M5 (-)*

FBW: Final body weight; TTW: total testicular weight; TW/BW: testicular weight/body weight ratio;FSH: follicle stimulating hormone; LH: luteinizing hormone; MDA: Malondialdehyde; STD: Seminiferous tubules diameter; GEH: Germinal epithelium height; CT: capsular thickness; TDI: tubular differentiation index; SPI: spermiogenesis index; RI: repopulation index. (-) and (+) symbol represent the decrease or increase of parameter in comparison to the control group, respectively. * Significant difference compared to the control group. dd Dose-dependent effect between lower and higher doses administrated.

Electron Microscopy

The ultrastructural study of testicular tissue revealed some structural alterations in the cells of STs (Figure 4). The alteration of the cellular structure and junctions in basal compartment of STs, folding of the wall of seminiferous tubule and the crumple of basement membrane, increase of connective tissue fibers, nuclear shrinkage of Sertoli cells, abnormal vesicular mitochondria in the cytoplasm of round spermatids, and the giant mitochondria in the cytoplasm of Sertoli cells were the notable changes observed amongthe experimental groups

Discussion

Our study showed that the simultaneous administration of NCT and MPH in adult rats has a

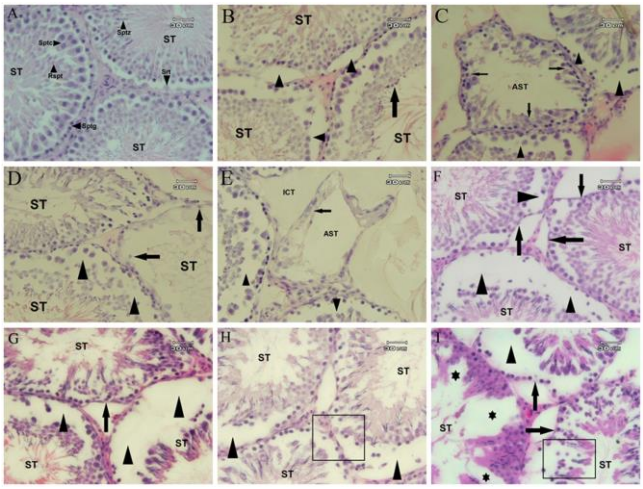

Figure 1. Histological structure of seminiferous tubules in cross sections of testicular tissue samples in various experimental groups. **A**, Control group: seminiferous tubules (ST) with normal cell architecture. Tubular epithelium with consistent arrangement of the Sertoli cells (Srt) and different germ cells including spermatogonia (Sptg), spermatocytes (Sptc), round spermatids (Rspt) and spermatozoids (Sptz). **B**, MPH5 group: Atrophic seminiferous tubules with basal compartment detachment (arrowheads). Nuclear changes (arrow) in developing spermatids are visible. **C**, MPH10 group: Atrophic seminiferous tubule (AST) with decline in spermatocytes' population (arrow). Alteration of intercellular junctions (arrowheads) is observed. **D**, NCT2 group: Disruption of cell-cell junctions (arrowheads) along with prominent reduction of the cellular population (arrows) is observed. **E**, NCT4 group: Atrophic seminiferous tubule (AST) with noteworthy thick interstitial connective tissue (ICT). Decline of the height of germinal epithelium (arrow) and disruption of intercellular junctions (arrowheads) are visible. **F**, NCT2 + MPH5 group: Detachment and folding of tubular wall (arrows) along with interruption of intercellular junctions (arrowheads) are visible. **G**, NCT2 + MPH10 group: Prominent disruption of cell-cell junctions with depleted epithelium (arrowheads) with tubular wall wrinkles (arrow). **H**, NCT4 + MPH5 group: Depletion of germinal epithelium (arrowheads) with the alteration (Cell detachment) of cellular junctions (inside square) is visible. **I**, NCT4 + MPH10 group: Atrophy of tubular wall (arrows), germinal epithelium dissociation (asterisks) and depletion (arrowhead) along with disruption of cell junctions (inside square) are visible notably. H&E; 400×.

notable negative effect on the microscopic structure and the function of testicular tissue. The consumption of MPH could induce the increasing desire for smoking, as one of the most available resources of NCT.[16] Therefore, despite performing various studies, which demonstrate the negative effects of NCT or MPH on testicular tissue; this study was a new report that described the effects of co-administration of these compounds on the conception of fertility problems through conducting an animal experimental study. Oxidative stress-related cellular and tissue damages

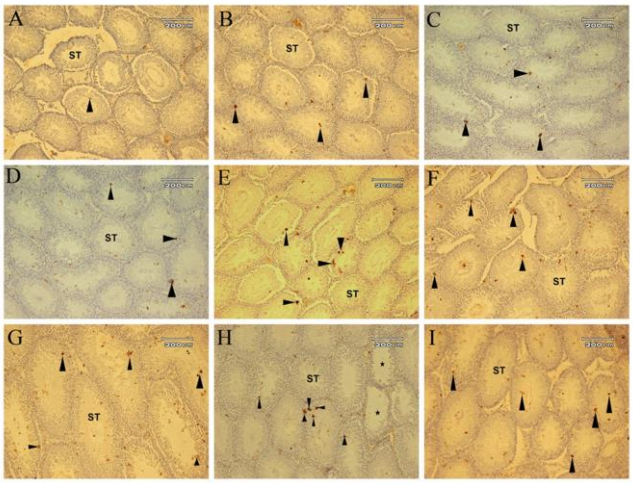

Figure 2. Immunohistochemical labeling of p53 in cross sections of seminiferous tubules in experimental groups. **A**, Control group: seminiferous tubules (ST) with faint p53 immunopositivity (arrowhead). **B**, **C**, MPH receiving groups: increase in areas with positive reaction to p53 expression (arrowheads). **D**, **E**, NCT receiving groups: the positive reaction to p53 is detectable in most tubules. **F**, **I**, MPH + NCT receiving groups: the immunoreactions to p53 expression are visible with higher intensity. Note: F: NCT2 + MPH5; G: NCT2 + MPH10; H: NCT4 + MPH5; I: NCT4 + MPH10. Asterisks represent the seminiferous tubules with depleted germ cells. IHC; 100×.

play a major role in male infertility problems.[17] Methylphenidate is one of the most common medications used for the treatment of ADHD disorder, and also other diseases such as depression and obesity.[5] Methylphenidate works through dopamine transporter and D2 receptors. The rat testicular tissue and spermatozoa have D2 and several adrenergic receptors.[18,19] The presence of dopamine receptors in testicular tissue and germ cells suggests that, the MPH treatment has a direct action on the reproductive system. Despite changes in spermatogenesis, which has been reported in some studies,[5,20] the exact mechanism of action of MPH on the testicular tissue function has not been recognized yet. Dopaminergic pathways work in the modulation of GnRH release,[21] Our previous study revealed a non-significant increase in pituitary gonadotropins and a decrease in testosterone levels in blood plasma of the rats treated with MPH,[22] The levels of plasma testosterone, FSH, and LH showed mild alterations between the experimental groups in this study. There are some reports specifying a disturbance in the pituitary-testicular axis in the MPH treated rats.

It was shown that, the MPH can induce some elevation in plasma levels of FSH and LH and also a reduction in testosterone levels.[22-24] However, our results showed the elevation in testosterone and FSH

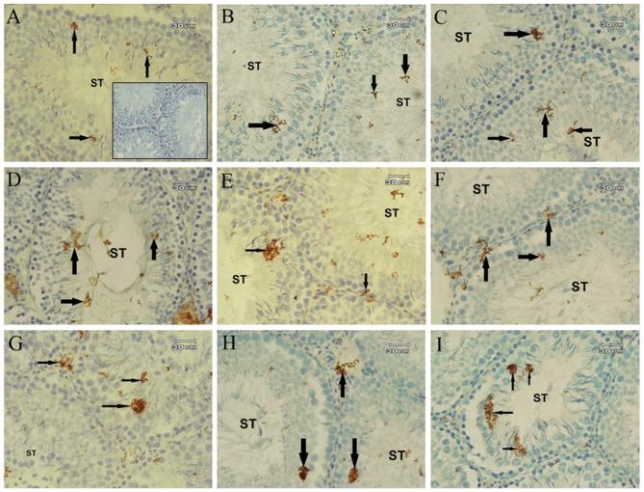

Figure 3. Immunolabeling of p53 in the seminiferous tubules in higher magnification. The positive immunoreaction to p53 is observed in all types of spermatogenic cells and in the interstitial endocrine cells (Leydig cells) as well. The overexpression of p53 is evident in round spermatids and developing spermatozoids. The extent of positive reaction areas per every seminiferous tubule was observed in more degrees in the NCT2 + MPH10, NCT4 + MPH5, and NCT4 + MPH10 groups respectively. Note: A: Control group. With no p53 immunopositivity (inside square); B, C: MPH receiving groups; D, E: NCT receiving groups; F: NCT2 + MPH5 group; G: NCT2 + MPH10 group; H: NCT4 + MPH5 group; I: NCT4 + MPH10 group. IHC; 400×.

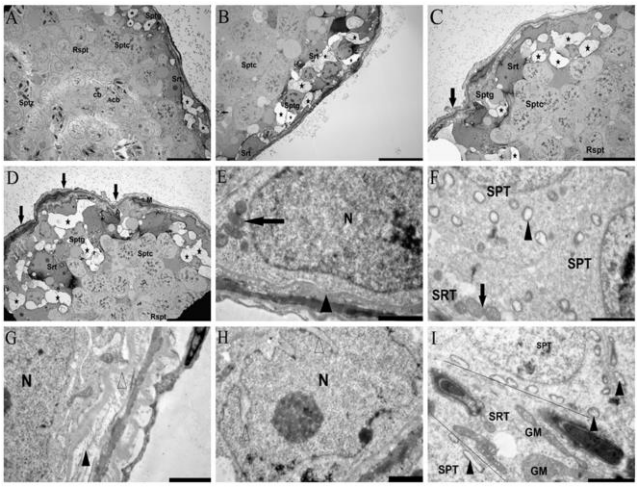

Figure 4. An electron micrographs of testicular tissue in experimental groups. **A**, MPH receiving group: low magnification of cellular architecture of seminiferous tubule. Sertoli cell (Srt), spermatogonia (Sptg), spermatocytes (Sptc), round spermatids (Rspt) and spermatozoids (Sptz) are observed. Wide spaces (asterisks) indicating the alteration of the cellular structure and junctions are observed in basal compartment. Chromatoid bodies (cb) are visible in the cytoplasm of round spermatids. **B**, NCT receiving group: low magnification of seminiferous tubule's cellular architecture. Wide separations (asterisks) are observed between spermatogonia cells and Sertoli cell. Round spermatid with abnormal nucleus (N) containing electron dense chromatin (C) is visible (arrow) in middle part of seminiferous tubule. **C**, NCT2 + MPH10 receiving group: low magnification of seminiferous tubule's cellular architecture. Wide separations (asterisks) are observed in basal compartment of tubule. Folding and thickening of the basement membrane (arrow) is visible. **D**, NCT4 + MPH10 receiving group: Wide separations (asterisks) between adjacent cells in basal compartment of tubule are increased markedly. Folding of the wall of seminiferous tubule (arrows) represents the tubular atrophy. Myoid cell nucleus (M) is visible. **E**, Control group: Sertoli cell structure oval nucleus (N) with normal basement membrane (arrow head) and round mitochondria (arrow). **F**, Control group: Sertoli cell cytoplasm (SRT) with neighboring two spermatids (SPT). Normal mitochondria of Sertoli cell (arrow) and normal appearance of mitochondria of spermatids (arrow head) is visible. **G**, NCT receiving group: the nucleus of Sertoli cell (N) is visible. Crumple of basement membrane (white arrow head) with notable increase of connective tissue fibers (black arrow head) is observed. **H**, MPH receiving group: Nuclear shrinkage (white arrow heads) of Sertoli cell is visible. **I**, MPH + NCT receiving group: Apical cytoplasm of Sertoli cell (SRT) between two spermatids (SPT). Abnormal vesicular mitochondria (black arrow heads) in cytoplasm of spermatids and the giant mitochondria (GM) in cytoplasm of Sertoli cell are visible (TEM, A-D 1400×; H 5800×; E, F, G, I 9700×).

levels in the MPH receiving groups. Also, these changes were observed in all the NCT + MPH receiving groups. One of the possible mechanisms, which involves in the reduction of testosterone levels following the MPH administration, is the raise in the activity of testosterone hydroxylases that results in the increased hepatic catabolism of testosterone.[20] The differences in testosterone levels in this study from previous studies may be due to hepatic dysfunction, which could be seen in the form of increased plasma MDA levels. Also, a significant reduction in the blood levels of FSH, LH, and testosterone has been reported in the NCT administrated rats.[23] However, our study revealed an increase in FSH and LH levels and a decrease in testosterone levels of both administrated doses of NCT. Moreover, the levels of LH and testosterone dose-dependently increased in the NCT receiving groups. These changes may be related to a decrease in the population of target cells of LH (Leydig cells) or could be related to the decrement in the population of testosterone target cells in seminiferous tubules such as Sertoli cells.[25] So, in this study, the increase in the plasma testosterone levels could be related to the alteration in the activity of above-mentioned hepatic enzymes (were not measured in this study). Our results showed that, in the NCT + MPH receiving groups, the changes in FSH, LH, and testosterone levels are different compared to the separate administration of MPH or NCT.

Our results showed that, the simultaneous administration of NCT and MPH had no prominent effect on body weight. The measurement of absolute and relative organ weight is considered as the important indices in toxicological studies.[24] Also, a decrease in testicular weight has been reported after the administration of cocaine hydrochloride, which is structurally similar to the NCT.[26] Our previous study revealed that, the administration of MPH individually or in combination with other compound like monosodium glutamate induces testicular weight loss in rats.[22] In the present study, testicular weight loss was also observed in the MPH receiving or in the NCT + MPH receiving groups. It has been demonstrated that, the weight of testes depends on the mass of differentiated spermatogenic cells.[27] Accordingly, it seems that the reduction of testes weight and as well the testes/body weight ratio in the present study may be due to the changes in the structure of testis such as the loss of cellular population and atrophy of the STs. Also, the generation of reactive oxygen species (ROS) is an important factor in infertility complications, and the NCT has a direct effect on the production of ROS, lipid peroxidation, and cellular damages of testicular tissue.[28] In this study, the plasma and tissue levels of MDA increased in all the treated groups. Compared to other germ cells, the spermatozoids are more susceptible to cellular damages induced by oxidative stress due to the high levels of lipids in their structure.[29] Hence, it could be suggested that, the increase in lipid peroxidation predominantly in testicular tissue may involve in the reduction of sperm population, motility, and viability, especially following the co-administration of the MPH and NCT.[29] A decrease of round spermatids has been reported in the MPH treated rats.[22] As well, NCT induces a significant reduction in the sperm count and sperm motility, and also a non-significant reduction in the sperm viability.[5,8] In the present study, sperm count, motility, and viability reduced in the rats that received NCT or MPH individually or simultaneously. The most reduction in sperm quality was observed in the NCT + MPH receiving groups, which may indicate a greater negative effect of this type of administration compared to the individual consumption of NCT or MPH. p53 protein is involved in apoptosis, and the exogenous factors trigger the up-regulation of the expression of this protein due to DNA damage.[30-32] In this study, immunostaining of testicular tissue showed the up-regulation of p53 in the treated groups compared to the control group. These changes were observed in more degrees following the simultaneous administration of the MPH and NCT.

The small extent expression of p53 protein indicates mild damage to the nuclear DNA of the cell, in which the DNA repair mechanisms are activated. However, the over-expression of this protein indicates severe damage of nuclear DNA, in which the mechanisms associated with apoptosis can be activated. Consequently, an increase in the expression of this protein may indicate an increase in the number of cells involved in the apoptosis process. Moreover, increased apoptosis and its related cell population decline may disrupt the cellular arrangement and the decrease in the microscopic indices of spermatogenesis.[30-32]Therefore, upregulation of p53 may explain the loss of germ cells and the reduction of microscopic indices observed in the current study due to the p53-dependent apoptosis.

Nicotine-associated ultrastructural changes such as the increase of STs' basement membrane thickness, polymorphic mitochondria in Sertoli cells, and mitochondrial damages of spermatids have been reported.[33] However, there are little data on the effect of MPH on the ultrastructure of testicular tissue. Also, some studies have pointed out ultrastructural changes in capillaries of cerebrum and cerebellum such as basal membrane thickening after oral administration of MPH in rats.[34] The present study showed various changes in the ultrastructure of testicular tissue such as increased amount of collagen fibers within the lamina propria, as an important indicator of tubular hyalinization, which is in consistence with previous reports.[35] In this regard, the formation of elongated giant mitochondria (As observed in our study) reflects some metabolic abnormalities like oxygen deficiency related to an insufficiency of gas exchange due to thickening of STs' basement membrane.[36,37] Accordingly, these ultrastructural changes can lead to changes in the process of spermatogenesis.

As Table 5 summarizes, both NTC and MPH had dose-dependent adverse effects on the structure and function of testis. Accordingly, negative effects of NTC were more than MPH, and simultaneous administration of NCT and MPH had more negative effects compared to their individual administration. Moreover, co-administration of NCT and MPH with high doses (NCT4 + MPH10), had more adverse effects compared to their co-administration with low doses, which may confirm the dose-dependent effects of NCT and MPH.

In conclusion, the results of the current study showed that, simultaneous administration of NTC and

MPH could induce more alterations in the structure and function of testicular tissue in comparison to their separate administration in a dose-dependent manner.

Acknowledgments

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Conflict of Interests

The Authors declare there is no conflict of interest.

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