

## Evaluation of histopathologic and histomorphometric changes of testicular tissue and gonadotropin levels following consumption of methylphenidate in male mice

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**Background/aim:** One of the most common psychiatric disorders in children is attention-deficit/hyperactivity disorder, which is treated extensively by methylphenidate. This study investigates the assessment of the effects of methylphenidate on histopathologic and histomorphometric changes of the testes and serum levels of gonadotropin in mice.

**Materials and methods:** In this study, 36 adult male mice were used. After determining their body weights, the animals were divided randomly into 2 experimental groups and 1 control group. The experimental groups received methylphenidate (2 and 10 mg kg<sup>-1</sup> day<sup>-1</sup>) via gavage for a period of 40 days. After evaluation of body weight, general anesthesia was used for taking blood samples from the heart in order to measure testosterone and levels of gonadotropin in serum. For the purpose of weighing the bodies and measuring the thickness of the germinal epithelium, the testes were removed and the possibility of any pathological changes was considered.

**Results:** The results showed that methylphenidate could decrease the thickness of the germinal epithelium and body weight significantly, and could increase the levels of spermatogonia and serum gonadotropins and testosterone. Histopathological changes were also seen for vascular dilatation and congestion in interstitial tissue.

**Conclusion:** Our findings demonstrated that administration of methylphenidate in adulthood may have an effect on spermatogenesis due to the influence of gonadotropin hormones on testis function.

**Key words:** Methylphenidate, testis, morphometric, gonadotropin, histopathology

### 1. Introduction

Attention-deficit/hyperactivity disorder (ADHD) is a neuropsychiatric disorder commonly diagnosed during childhood, characterized by excessive levels of inattentiveness, impulsivity, and hyperactivity (1). Ritalin hydrochloride (methylphenidate hydrochloride), as a mild central nervous system (CNS) stimulant, is available in the form of 5, 10, and 20 mg tablets for oral administration; Ritalin-SR is available as sustained-release tablets of 20 mg for oral administration. Although reported figures vary considerably depending on diagnosis criteria, socioeconomic status, geographic source of sample, and sex, it is estimated that up to 3%–5% of elementary school-age children meet the criteria for diagnosis of ADHD (2)

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with a reported population prevalence of up to 12% (3). Stimulant medications have been effective for the treatment of ADHD (4). Methylphenidate (MPH) is the most commonly prescribed therapeutic agent (5), accounting for more than 90% of stimulants used for the management of ADHD in the United States (6). Treatment duration for the disorder can last for years (7), because longitudinal studies report that ADHD symptoms can continue into adulthood at a stimulated rate of persistence in up to 75% of diagnosed children (8) and because stimulants remain the preferred treatment for the management of adult ADHD (9). Due to the extensive usage of MPH in ADHD treatment in order to control unfavorable behaviors, many investigations have been performed, including studies on

change and disorder in hormone secretion (10). Another study indicated that the body weight of male mice receiving MPH was significantly decreased compared to the control group (11). In another report, disorder in animal growth following long-term administration of MPH was observed (12). Another similar chemical substance, amphetamine, also caused body weight reduction (13). It has also been shown that cocaine, with a similar structure to MPH, can induce body weight reduction and have harmful effects on fertility (14). Moreover, the weight of the testis and seminal vesicle in these mice decreased significantly. Some changes in the weight of organs such as the brain, heart, ovary, spleen, and prostate are also indicative of its effect on different body organs (15). Little is known, however, about the long-term effects of this drug on the reproductive axis and thickness of the germinal epithelium.

Thus, regarding the high usage of MPH in children and adults and the importance of genital glands in gamete production, an evaluation of chronic usage of MPH on morphometric changes in the testes of mice was the subject of this study. The primary objective of our study was to examine the effects of MPH on the male reproductive axis, which undergoes many dynamic changes during puberty. Moreover, the evaluation of histopathologic and histomorphometric changes of testicular tissue following consumption of MPH in male mice seemed to be necessary.

## 2. Materials and methods

### 2.1. Animals and experimental design

Thirty-six male mice of 90 days old were purchased from the Raazi Institute in Iran. All animals were kept under a 14/10-h light-dark cycle with a room temperature of 22–24 °C and relative humidity of 50%–65%. The animals were acclimated to the laboratory for 1 week prior to the commencement of the experiment and were fed ad libitum with food and water throughout the experiment. The male mice were randomly divided into 2 treatment and 1 control groups ( $n = 12$ ). The mice were initially weighed. The treatment groups received 2 or 10 mg/kg of hydrochloride methylphenidate solution (produced by the Novartis Company) daily by gavage method, using an animal feeding incubation needle. The control group received only normal saline. The animals were treated for 40 consecutive days. This administration period was necessary to determine the effect of MPH on sperm production because mice need a period of 35–40 days for a complete spermatogenic cycle including spermatocytogenesis, meiosis, and spermiogenesis (16).

### 2.2. Sample collection

After treatment and being weighed again, the mice were sacrificed using ether anesthesia at the end of 40 days. Blood samples were collected from the heart via a sterile injector containing heparin and centrifuged at 3000 rpm

for 5 min. Plasma was separated and then stored at  $-70\text{ }^{\circ}\text{C}$  until biochemical and hormonal analyses. This study was reviewed and approved by the Institutional Animal Care and Use Committee and was conducted in a facility that is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

### 2.3. Testosterone and gonadotropin levels

The plasma testosterone and gonadotropin levels were measured by the ELISA method using a DRG ELISA testosterone and gonadotropin kit according to the kit manufacturer's instructions.

### 2.4. Histological examination

By opening the abdominal cavity, the testes were removed in order to determine histomorphometric and histopathologic changes. For determining the changes in spermatogenic cell density, testis tissues were fixed in Bouin's solution for 48 h after washing in physiologic serum. They were dehydrated through graded concentrations of ethanol, embedded in paraffin wax, sectioned at  $5\text{ }\mu\text{m}$  of thicknesses, and stained with Mayer's hematoxylin and eosin (H&E). Twenty seminiferous tubules (STs) were randomly examined per section, their diameters and germinal cell layer thicknesses (from the basal membrane towards the lumen of the tubule) were measured using an ocular micrometer in a light microscope, and the mean sizes of STs and germinal cell layer thicknesses were calculated. Finally, photographs were taken with a calibrated photomicroscope connected to computer and equipped with Axiovision 4.8 software and studied. A morphometric study of the diameter of 20 STs in each section was made. All of the sections were studied by means of an Olympus light microscope with multiple magnifications ( $400\times$ ). The thickness of the germinal epithelium was measured and the number of spermatogonia was counted.

### 2.5. Histopathological changes

For observation of the probable pathological changes in tissue samples in the control group and the treatment groups that received 2 or 10 mg/kg of MPH, a histopathological study was carried out using the following parameters: vacuolization of Sertoli cells, thickening of basement membrane, hyperplasia of Leydig cells, fibrosis of interstitial cells, and shedding of immature cells inside the lumen of STs.

### 2.6. Statistical analysis

Data are presented as mean  $\pm$  standard error of the mean (SEM). The degree of significance was set at  $P < 0.05$ . One-way analysis of variance and the post-hoc Tukey honestly significant difference test were used to determine the differences among the groups in terms of all the sperm characteristics, biochemical parameters, and histological findings. All the analyses were carried out using SPSS for PC.

### 3. Results

In the present study, the results on some aspects of histological features, such as histomorphometry of testes, and the biochemical factors of serum level of gonadotropins and germinal cell counts in adolescence have been considered. The results obtained from primary and secondary weighing of the mice showed that the difference between primary and secondary weights of the treatment groups reflected a significant reduction in comparison with the control group ( $P < 0.05$ ; Table).

#### 3.1. Histological and histomorphometric findings

Spermatogonia are composed of 2 types of dark and light cells, which were both counted in this study. Concerning the effect of MPH on the number of spermatogonia in this study, there was a significant difference between the groups receiving MPH and the control group. The group that received 10 mg/kg of body weight showed a significant increase, and the group that received 2 mg/kg of body weight also showed an increase, although not significant, when compared with the control group (Table). Another aspect of this study was the measurement of the thickness of the germinal epithelium, which is defined as the height from the base of the spermatogonia to the nuclei of spermatozoa in STs. In this study, in the experimental groups, there was a significant decrease in the thickness of the germinal epithelium compared to the control group (Table; Figures 1–3). Another factor in this study was the measurement of the diameter of STs, in which no significant difference was observed between the control and experimental groups.

#### 3.2. Biochemical findings

Comparing the average level of testosterone in the experimental groups with the control group, a reduction was observed, which was significant in the group receiving

2 mg/kg of body weight compared with the control group (Table). Concerning the level of luteinizing hormone (LH), a significant increase was shown between the groups that received 2 and 10 mg/kg of MPH compared with the control group ( $P < 0.05$ ; Table). Follicle-stimulating hormone (FSH) was also measured and no significant difference was observed between the control and experimental groups.

#### 3.3. Histopathological findings

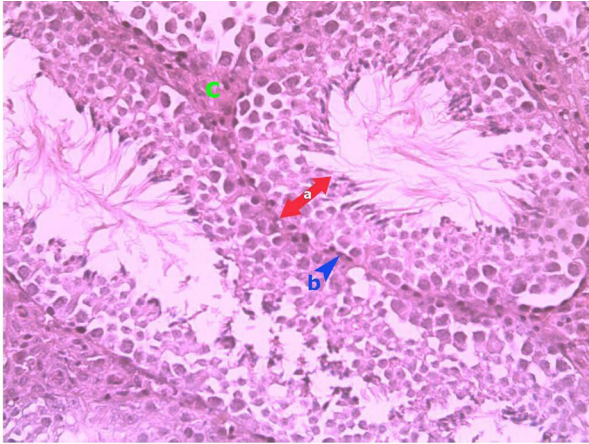
No significant histopathological changes were seen for vacuolization of Sertoli cells, mature germ cell shedding, or changes of Leydig cells, but we observed vascular dilatation and congestion in interstitial tissue in the experimental specimens compared with the control specimens.

### 4. Discussion

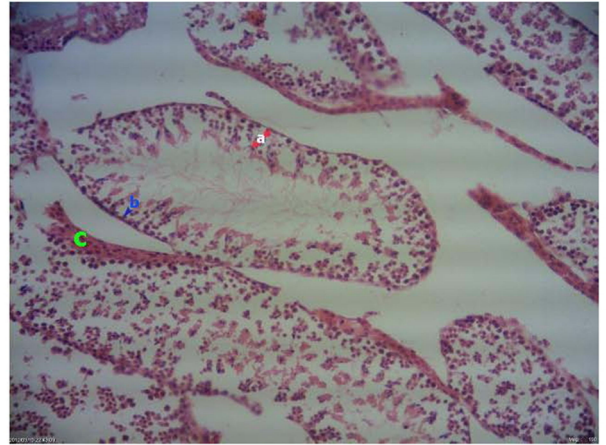
Methylphenidate hydrochloride is a stimulant for CNS with a structure close to dextroamphetamine (17,18). MPH is an isomer of amphetamine (19). The neuropharmacological profile of MPH is also similar to that of cocaine (20). This drug was first synthesized in 1944 and was used initially as an analeptic for several types of barbiturate-induced coma, and also in treatment of ADHD, but later it was used as a drug to improve memory in depressed elderly patients, for concentration in adults, and for patients with brain tumors. Most of the studies using systemic MPH application reported that it exhibits reinforcing effects. Humans who were administered MPH systemically reported similarities in the reinforcing effects of MPH to those of cocaine or amphetamine. Moreover, MPH has a higher mortality rate than cocaine and amphetamine when taken systemically (21–26). In a study carried out on the effect of MPH on the fertility of male mice, it was indicated that MPH could significantly decrease fertility (16).

**Table.** Comparison of the average difference of body weight, diameter of STs, thickness of germinal epithelium, number of spermatogonia, and levels of FSH, LH, and testosterone hormones in the groups receiving MPH and the control group at the end of the experiment. The measurements from 12 animals are expressed as mean  $\pm$  SEM values. Different superscript letters (a, b, c) in each row indicate a significant difference at the level of  $P < 0.05$ .

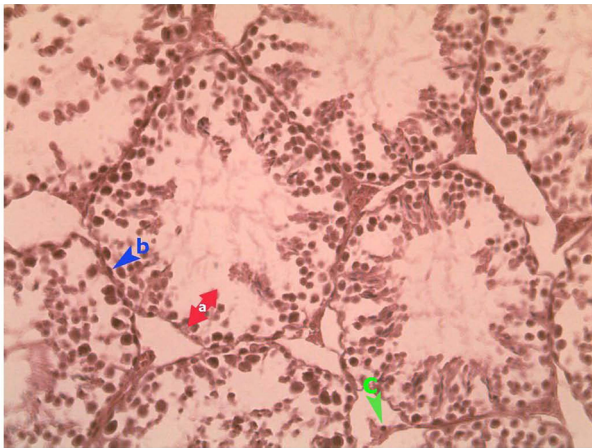
Parameters	Groups		
	Control group	Experimental I, 2 mg/kg	Experimental II, 10 mg/kg
Body weight differences (g)	11.70 $\pm$ 0.80 <sup>a</sup>	3.66 $\pm$ 1.0003 <sup>b</sup>	4.26 $\pm$ 0.81 <sup>b</sup>
Number of spermatogonia (n)	40.92 $\pm$ 1.33 <sup>a</sup>	47.25 $\pm$ 2.60 <sup>a</sup>	58.25 $\pm$ 1.60 <sup>b</sup>
Thickness of germinal epithelium ( $\mu$ m)	219.33 $\pm$ 19.15 <sup>a</sup>	154.13 $\pm$ 5.13 <sup>ab</sup>	184.60 $\pm$ 8.07 <sup>a</sup>
Diameter of STs ( $\mu$ m)	1333.54 $\pm$ 69.91 <sup>a</sup>	1377 $\pm$ 14.61 <sup>a</sup>	1448.89 $\pm$ 17.66 <sup>a</sup>
FSH	1.608 $\pm$ 0.1764 <sup>a</sup>	2.058 $\pm$ 0.1616 <sup>a</sup>	1.472 $\pm$ 0.1944 <sup>a</sup>
LH	0.1250 $\pm$ 0.0066 <sup>a</sup>	0.6583 $\pm$ 0.04369 <sup>b</sup>	1.1758 $\pm$ 0.26096 <sup>c</sup>
Testosterone	69.81 $\pm$ 5.78 <sup>a</sup>	2.32 $\pm$ 0.32 <sup>b</sup>	14.33 $\pm$ 0.40 <sup>c</sup>



**Figure 1.** A photograph of STs in the control group that shows a- the thickness of germinal epithelium indicated by arrows in 2 tubules, b- spermatogonia, c- Leydig cells (H&E, 100×).



**Figure 3.** A photograph of several STs in the experimental groups that received MPH that shows a- the thickness of germinal epithelium, b- spermatogonia, c- Leydig cells (H&E, 100×).



**Figure 2.** A photograph of several STs in the experimental groups that received MPH that shows a- the thickness of germinal epithelium in some tubules, b- spermatogonia, c- Leydig cells (H&E, 100×).

Thus, regarding the importance of the genital system in gamete production, and also the relatively small number of studies on the effects of MPH on some parameters related to the male genital system, more studies of its effects on histomorphometric and histopathological aspects of the testes and serum levels of the hormones related to testicular function seem to be necessary. The animals used for this experiment were adult male mice and the doses of methylphenidate hydrochloride were 2 and 10 mg/kg of body weight, orally administered by gavage method. The effect of MPH on body weight was such that a significant decrease occurred in the groups that received MPH at doses of 2 or 10 mg/kg of body weight daily for 40 days in comparison with the control group. Another

survey on mice claimed that chronic usage of MPH can induce a significant decrease in body weight (13). Other research, on the weight of male mice of the breed B6C3F1 that received MPH chronically, demonstrated that there was a significant decrease in the weight of the treatment group compared with the control group, and a similar result was deduced for the weights of testes and seminal vesicles (11). In various research it has been shown that reduction in body weight following administration of MPH could be due to decreased appetite and lower food consumption, or reduced absorption, which is directly related to the body weight and affects animal growth (23). In this study, it was seen that MPH could affect the number of spermatogonia in STs by inducing a significant increase in the experimental group receiving MPH at a dose of 10 mg/kg of body weight. The group that received MPH at the dose of 2 mg/kg of body weight showed an increase in the number of spermatogonia compared with the control group. A survey on mice aged from 30 to 44 days old revealed that using MPH as an intraperitoneal injection with a dose of 2 mg kg<sup>-1</sup> day<sup>-1</sup> could have a positive effect on testicular growth (10). Another result of this investigation was a significant decreasing in the thickness of the germinal epithelium in the group that received MPH chronically at a dose of 2 mg/kg of body weight. In the group that received MPH at a dose of 10 mg/kg of body weight, there was also a decrease compared with the control group. In a similar study of the effect of MPH on spermatogenesis, it was claimed that following the usage of MPH chronically by gavage method at doses of 5 and 10 mg/kg of body weight daily, the number of spermatids showed a significant reduction,

which could be due to the increasing of apoptosis and P52 gene expression through the use of MPH (27). Considering the structural and functional similarities of cocaine and MPH, a study showed that consumption of cocaine hydrochloride at a dose of 15 mg/kg of body weight at the ages of 3, 6, and 12 weeks for 28 days induced apoptosis in germinal cells in Sprague-Dawley rats. Investigations into activity of caspase 3 in germinal cells under influence of cocaine have been done and showed induced apoptosis (28). In this study, considering the thickness of the germinal epithelium and apoptosis of germinal cells by consumption of MPH or cocaine, it might be concluded that MPH could have an effect on the germinal cells via an influence on their inheritance and inhibiting of cell division, thus inducing cessation in the cell life cycle. A histomorphometric finding of this study was the measurement of the diameter of STs, in which no significant difference between the control and experimental groups was observed, whereas some other investigations claimed an effect of drugs and other chemicals on the diameter of these tubules. In this regard, the significant decreasing effect on the diameter of STs of busulphan in the treatment of cancer should be mentioned (29). In the present study, the testosterone serum levels in experimental groups receiving MPH showed a significant decrease compared with the control group. Such a decrease with other chemicals has also been reported. In relation to this, some studies on the effect of opium drugs on testosterone serum level have shown a significant decrease in experimental groups compared with the control group (30). In a survey on testosterone secretion in mice, it was shown that using diethylcarbamazine daily at the dose of 200 mg/kg of body weight can cause a significant decrease in testosterone secretion (31). In this survey, LH serum level in experimental groups increased compared with the control group. Another study of the effect of MPH on LH serum level in female mice showed no significant difference between the experimental groups and the control group (23). Regarding the results of studies of LH and testosterone serum levels and the changes in these hormones, it can be deduced that there might be a close relationship between the changes, and so when considering a significant decrease in testosterone serum level and a corresponding significant increase in LH serum level, it might be concluded that LH serum levels increase in order to compensate for decreasing testosterone by promoting testosterone secretion through their influence on the surface of Leydig cells, which possess the receptors for this hormone.

On the other hand, surveying the structure of Leydig cells and noting a lack of any changes in their cytoplasm for eosinophilic and granule specifications in some specimens, it seems that, with regards to the previous studies suggesting that the enzyme effective for testosterone production is metabolized in the liver, perhaps MPH inducing an increase in LH does not have any negative effect on the rate of testosterone but, due to metabolization of the enzyme, causes a reduction in testosterone production. Additionally, a scattering of the Leydig cells in some specimens of the experimental groups could induce such a reduction. Thus, regarding the results of this study, it might be deduced that a decrease in testosterone production is not the result of LH reduction, but is rather due to some factors such as the metabolization of its producing enzyme in the liver and a scattering of the Leydig cells. Confirming this is the level of FSH, which in this study did not show any significant changes in the experimental groups compared with the control group. It can thus be claimed that perhaps MPH does not have any negative effect on the pituitary gland and consequently on gonadotropes. Accordingly, there was a study of the effect of this drug on gonadotropes of female mice in which the level of LH in pituitary glands showed a significant increase (23). The effect of this drug chronically on testis structure induces a significant increase in spermatogonia but, due to its effect on their development, including cell division and differentiation, it negatively impacts spermatogenesis and spermiogenesis. Moreover, some studies have shown that consumption of MPH for a short period of time in adult mice could have an influence on the enzyme inducing testosterone production, lead to it to being metabolized, and thus decrease testosterone (10). In this study, no pathological changes were observed between the control and experimental groups in the observation of histopathological changes in microscopic specimens with regards to some parameters including vacuolization of Sertoli cells, thickening of basement membrane, hyperplasia of Leydig cells, fibrosis of interstitial cells, and shedding of immature cells inside the lumen of STs. Some degree of vascular congestion in the specimens of the group receiving MPH at doses of 2 and 10 mg kg<sup>-1</sup> daily<sup>-1</sup> was observed.

By surveying testicular tissue in the experimental groups, it was concluded that due to the use of this drug in patients with ADHD and also in adults as an aid to concentration, for which it is used by students, it should be controlled carefully and it should only be available on prescription. Psychiatrists should be advised, if possible, to replace it with another medication.



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