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EFFECTS OF METHYLPHENIDATE ON THE ONSET OF PUBERTY AND REPRODUCTIVE SYSTEM IN MALE AND FEMALE RAT MODELS

THE MASTER OF SCIENCE THESIS

FIRAS KHOUBBIEH, M.D.

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This thesis has been deemed by the jury in accordance with the relevant articles of Yeditepe University Graduate Education and Examinations Regulation and has been approved by Administrative Board of Institute with decision dated ..... and numbered .....

Prof. Dr. Bayram YILMAZ

Director of Institute of Health Sciences

DECLARATION

I hereby declare that this thesis is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which has been accepted for the award of any other degree except where due acknowledgment has been made in the text.

07/08/2021

Firas Khoubbieh

DEDICATION

To all the scientists, my family and all the beloved people in my life…

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Firas Khoubbieh, M.D.

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ABSTRACT

Khoubbieh, F., (2021) Effects of Methylphenidate on the Onset of Puberty and Reproductive System in Male and Female Rat Models, Yeditepe University, Institute of Health Sciences, Department of Physiology, MSc thesis, İstanbul.

Methylphenidate (MPH) is a central nervous system stimulant, which blocks the reuptake of dopamine and noradrenaline. It is widely used to treat attention deficient hyperactivity disorder (ADHD) in children and adolescents. This study aimed to investigate the effect of chronic administration of MPH on reproductive functions in both male and female rats and the reversibility of those effects. Sprague Dawley rats from both sexes were administered with 5 mg/kg of MPH or physiological saline via oral gavage from postnatal day (PND) 21 to PND 60 and from PND 21 to PND 90. In addition, recovery groups from both sexes in which stopped receiving MPH from PND 60 to PND 90 were included. In the end of the study, the results showed slightly but significantly early puberty in male models, slight decrease in testosterone levels in treated groups to increase again in the recovery group. The histological findings showed seminiferous tubule degeneration in the testes in MPH-treated groups. Leydig and Sertoli cells were affected in the MPH-treated groups, and all the findings were improved in the recovery group. In female animals, a significant delay in puberty onset was observed. Estradiol levels were decreased in MPH-treated group up to PND 90 compared to control group and a surge in luteinizing hormone levels was detected in recovery group. Histological findings showed morphological deterioration in the basement membrane of the ovaries of MPH-treated groups, more atretic follicles were detected and these findings were improved in the ovaries of recovery group. This study demonstrates that MPH could affect the reproductive functions in both male and female rats and those effects are transient.

Key words: Methylphenidate, Ovary, Puberty, Sex hormones, Testes

ÖZET

Khoubbieh, F., (2021). Metilfenidat'ın Erkek ve Dişi Sıçan Modellerinde Püberte Başlangıcı ve Üreme Fonksiyonları Üzerine Etkilerinin Araştırılması, Yeditepe Üniversitesi, Sağlık Bilimleri Enstitüsü, Fizyoloji Anabilim Dalı, Yüksek Lisans Tezi, İstanbul.

Metilfenidat (MPH), dopamin ve noradrenalinin geri alımını bloke eden bir merkezi sinir sistemi uyarıcısıdır. Çocuklarda ve ergenlerde dikkat eksikliği hiperaktivite bozukluğunu (DEHB) tedavi etmek için yaygın olarak kullanılmaktadır. Bu çalışma, MPH'nin kronik uygulamasının hem erkek hem de dişi sıçanlarda üreme işlevleri üzerindeki etkisini ve bu etkilerin tersine çevrilebilirliğini araştırmayı amaçlamıştır. Her iki cinsiyetten Sprague Dawley sıçanlarına postnatal gün (PNG) 21'den PNG 60'a ve PNG 21'den PNG 90'a kadar oral gavaj yoluyla 5 mg/kg MPH veya fizyolojik salin uygulandı. Ayrıca, PNG 60'tan PNG 90'a kadar MPH uygulamasının kesildiği (derlenme grubu) birer grup da dahil edildi. Çalışmanın sonunda, MPH uygulanan erkek modellerde az ama anlamlı olarak erken püberte, testosteron seviyelerinde hafif düşüş ve derlenme grubunda tekrar artış gözlemlendi. Histolojik bulgular ise MPH uygulanan gruplarda testislerde seminifer tübül dejenerasyonu gösterdi. MPH uygulanan gruplarda Leydig ve Sertoli hücreleri etkilenmişken ve derlenme grubunda tüm etkiler düzelmiştir. Dişi hayvanlarda ise püberte başlangıcı anlamlı şekilde gecikmiştir. MPH uygulanan grupta estradiol seviyeleri kontrol grubuna göre PNG 90'da düşmüşken, derlenme grubunda luteinleştirici hormon seviyelerinde bir artış tespit edilmiştir. MPH uygulanan grupların yumurtalıklarının bazal membranında morfolojik bozulma olduğu gözlemlenirken, daha fazla atretik folikül tespit edilmiş ve bu bulgular derlenme grubu hayvanların yumurtalıklarında düzelmiştir. Sonuç olarak bu çalışma, MPH'nin hem erkek hem de dişi sıçanlarda üreme fonksiyonlarını etkileyebileceğini ve bu etkilerin tersine çevrilebilir olduğunu göstermektedir.

Anahtar kelimeler: Cinsiyet hormonları, Metilfenidat, Over, Püberte, Testis

LIST OF SYMBOLS and ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
ANOVA	Analysis of variance
BMI	Body mass index
DAT1	Dopamine transporter gene
DLPFC	Dorsolateral prefrontal cortex
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
LH	Luteinizing hormone
MPH	Methylphenidate
NICE	National Institute for Clinical Excellence
PET	Positron emission tomography
PND	Postnatal day
SEM	Standard error of the mean
USA	United States of America

1. INTRODUCTION and PURPOSE

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders (1), it is diagnosed in childhood and could persist into adulthood. The prevalence of ADHD is believed to be up to 7.2% among children and 2.5% among adults (2), however, the rate of the disease is expanding overtime as more disease awareness has been developed especially in the western societies (3).

ADHD is defined by persistent symptoms of hyperactivity, inattention and motor restlessness/impulsivity that cause functional impairments (4). ADHD can be classified into three types: 1) predominantly inattentive 2) predominantly hyperactive-impulsive and 3) combined hyperactive-impulsive and inattentive type which considered to be the most common type, understanding the type of the disease is critical because the treatment plan depends on the type (5). Sleep problems could be another symptom as 50% of the patients have witnessed sleep disturbance episodes (6). The diagnosis of ADHD is still clinical and it has been performed by close observation by the parents at home or the teachers at school then the information must be gathered and added to family history, physical examination, and mental assessment to reach the right diagnosis (7).

The criteria were followed to diagnose ADHD (according to DSM5) are based on the symptoms and in order to meet these criteria "the patients with ADHD must show 6 or more symptoms of inattention for children up to 16 years old or 5 or more symptoms for adolescents 17 years and more and adults and these symptoms must persist for a period of 6 months or more plus the patients with ADHD must also show six or more symptoms of hyperactivity-impulsivity for children up to age 16 years, or five or more for adolescents age 17 years and older and adults, these symptoms must persist for at least 6 months or more" (4) no laboratory imaging tools have been approved as diagnostic methods for ADHD (7).

The pathophysiology of ADHD is still unclear, however, some studies have indicated the dopamine system as major cause of this disease where neuroimaging reports suggested direct effect to the brain areas regulated by dopamine and its receptors in ADHD patients and this matched with other neuroimaging reports showing higher amount of dopamine in some brain areas such as frontal cortex and basal ganglia where the circuits pathway responsible for behaviors such as attention, organization, motivation and planning (8). ADHD is a disease with multiple etiologies it includes genetic and

environmental factors and the interactions between them (9, 10). Hereditary pathway has been reported in this disease when studies have shown higher rates of ADHD between siblings (11). The dopaminergic genes have shown significant alteration in connection with ADHD (10). Because of its work in bringing the dopamine from synaptic cleft to the reuse state by neurons, the dopamine transporter gene (DAT1) is believed to have the major impact in ADHD cases (12). On the other hand, some environmental factors have been linked with ADHD such as alcohol, nicotine and lead exposure of the mother, and some diseases such as anemia and iron deficiency while some other infant related factors such as low birth weight and prematurity, cerebral trauma, infections and iron deficiency (13). According to the National Institute for Clinical Excellence (NICE) guidelines, combination of behavioral and pharmacological treatment should be followed for treatment of ADHD (14).

The pharmacological treatment includes methylphenidate (MPH) as the treatment of choice for ADHD (15). Various studies have reported positive effects of MPH and other psychostimulants on ADHD (15, 16). However, just like any other drug it can cause some side effects including sleep disturbance, nervousness, decreased appetite, headache, increased blood pressure and heart rate and growth-related effects in children (15, 17-19). Moreover, MPH may have adverse effect on puberty onset and reproductive functions (20).

The aim of present study was to investigate the effect of MPH on puberty and sexual development in male and female rats. For this purpose, animals were administered with 5 mg/kg MPH up to either postnatal day (PND) 60 or PND 90. Onset of the puberty were followed, and the animals were sacrificed on either PND 60 or PND 90. Serum samples were collected to investigate the levels of follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone. Moreover, reproductive organs were dissected for histopathological examinations.

2. LITERATURE REVIEW

2.1. Attention Deficit Hyperactivity Disorder (ADHD)

2.1.1. Definition and Prevalence

ADHD is one of the most common neurodevelopmental disorders. It is frequently diagnosed in children and adolescents (1). The prevalence ranges between 5.3% to 7.2% among children and adolescents, and 2.5 % of the adults around the World with more studies suggesting the increase in needs of health care related to ADHD among people aged 50 years and older (2). However, studies regarding the prevalence have been showing that the rates of the disease are expanding overtime with higher prevalence in the western societies like United States of America (USA), the reason behind this raise is believed to be due to the enhancement in the rates of the diagnosis and the awareness of the disease (3). However, in 2007, the first comprehensive review in which a total of 102 studies included regarding ADHD was published and important variabilities were associated in the estimate including the geographical origin of the studies which showed noteworthy differences only between North American and both Middle East and Africa but no significant differences between North America and Europe (21).

2.1.2. Symptoms and Diagnosis

ADHD is defined by persistent symptoms of hyperactivity, inattention and motor restlessness/impulsivity that cause functional impairments (4), however, this disease can be classified into subtypes according to the symptoms as follows: predominantly inattentive, predominantly hyperactive-impulsive, combined hyperactive-impulsive and inattentive type. Inattentive symptoms include difficulty in focusing – not obeying instructions – not being able to complete tasks – not seeming to listen or paying attention and this type is rated higher among girls. Hyperactive-impulsive symptoms include fidgeting – not being able to wait – higher tendency to move and talk and interrupting people while talking. The combined hyperactive-impulsive and inattentive type is the most common type in ADHD and it includes both inattentive and hyperactive symptoms, deciding the type is important as the treatment plan depends on the type (5). Sleep problems were reported in children and adolescents suffering from ADHD with 50% of the children with ADHD have experienced episodes of sleeping disorders (6). It has been reported that ADHD symptoms diagnosed during childhood could persist into adulthood (2).

Despite the fact that the criteria that were used to diagnose ADHD have been developing overtime, the tools for evaluation have lasted the same. Unfortunately, laboratory diagnosis that is based on laboratory reports and tools such as neuropsychological tests, electroencephalography (EEG) or neuroimaging tools are still limited to be used to diagnose ADHD (7). Although studies have been going on to clarify the connection between the neurotransmitter systems and ADHD, as the experiments on animal models showed some positive results, no assessment methods based on neurotransmitter systems have been verified to be used in the diagnosis of ADHD (7). However, the diagnosis of ADHD has been carried on being clinical and it relies on close observation of the child at home and school by both his parents and teachers and then gathering information as the part of comprehensive history taking to evaluate the performance of the child at home and school and this can be added to family history, physical examination, and mental assessment (7).

The criteria which have been used to diagnose ADHD were first included in 1968 in the second edition of the American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorder (DMS 2), then these criteria were evolved over time and the (DMS 5) has been the latest update to distinguish ADHD (22). "The (DMS 5) criteria were based on the symptoms and in order to meet these criteria the patients with ADHD must show 6 or more symptoms of inattention for children up to 16 years old or 5 or more symptoms for adolescents 17 years and more and adults and these symptoms must persist for a period of 6 months or more plus the patients with ADHD must also show six or more symptoms of hyperactivity-impulsivity for children up to age 16 years, or five or more for adolescents age 17 years and older and adults, these symptoms must persist for at least 6 months or more" in addition some circumstances must be found such as noticing some inattentive and hyperactivity-impulsivity symptoms before age 12 years, several symptoms must be appearing in two different environments (school-home-work), the symptoms must be affecting the daily functional life, while other mental disorders must be excluded (4).

2.1.3. Pathophysiology

The pathophysiology behind ADHD is not completely understood, however, some studies suggest that dopamine system plays major role. Dopamine is a neurotransmitter which happened to be found in higher rate in parts of the brain such as frontal cortex and

basal ganglia where studies referred that the circuits pathway is directly responsible for some behaviors such as attention, organization, motivation, and planning, nonetheless, reports from neuroimaging tools declare a direct effect to the areas and pathways regulated by dopamine and its receptors in ADHD patients (8).

Patients with ADHD who show cognitive or functional symptoms were connected to some irregularities in their brains, which were visible in the neuroimaging tools(23). One of the signs was a smaller diameter of the anterior cingulate gyrus and dorsolateral prefrontal cortex (DLPFC) in ADHD-related patients and it is believed to be the reason behind the goal-directed behavior abnormalities (24, 25). In addition, the frontostriatal region has been shown less active in the brains of ADHD patients compared to disease free individuals (25). These finding are crucial and used as guidelines by the scientists to invent the suitable treatment.

2.1.4. Etiology

ADHD is a disorder of combination of multiple etiologies, it includes genetic and environmental components and the interaction between these two causes (9, 10), statistical studies have shown the higher rates of ADHD between twins and siblings which strongly suggest the hereditary pathway of the disease (11). The genes that regulate the neurotransmitter systems and dopaminergic genes shown significant connections with ADHD and more studies correlate the effect of dopamine D4 receptor gene (*DRD4*), dopamine receptor gene, *DRD5,* the gene encoding catechol-O-methyltransferase (*COMT*), Solute carrier family 6 member (*SLC6A3*), synaptosomal-associated protein 25kDa (*SNAP-25*), 5-Hydroxytryptamine receptor 1B (*HTR1B*) (10). However, the dopamine transporter gene (*DAT1*) is believed to have the major significant effect on ADHD because of its work in bringing the dopamine from the synaptic cleft back into neurons for reuse (12). Moreover, studies showed that some environmental factors including the maternal exposure to alcohol, nicotine, and lead, having anemia or iodine deficiency, and some factors are associated with the infants such as low birth weight and prematurity, infections, cerebral trauma, and iron deficiency are linked with ADHD symptoms (13).

2.2. Methylphenidate (MPH)

2.2.1. MPH in ADHD Treatment

According to the NICE guidelines, combination of behavioral and pharmacological treatment should be followed for treatment of ADHD (14).

MPH is a central nervous system stimulant widely used for treatment of ADHD (15). MPH is a drug with the molecular formula of $C_{14}H_{20}ClNO_2$ and molecular the weight of 269.77 and are available under different brand names such as Ritalin, Ritalin SR, Concerta and Metadate (26), this drug was first formed by Swiss company (Ciba) in 1954 and was used in United States in 1955 (27). In 2018 it was ranked as the 45^{th} most prescribed drug with more than 17 million orders had been made by United States physicians (28). The most common form of MPH is oral tablets and it can affect in long-acting formulation or short-acting formulation (29). Another used form is the transdermal patches on the skin (30). There are several European, Australian, and North American guidelines based on studies around the world on how this medication should be prescribed (31). Several guidelines advise to start using it in children with severe symptoms or moderate cases which worsen with time, nonetheless, same goes for adults with severe and moderate ADHD problems (31). Some of those guideline's treatments suggest giving the patients minimum dose then increase it gradually to reach the target while others recommend giving the medication in a long-acting form, whereas some propose to treat every patient individually in accordance with the severity of the impairment (31). Several regulations strongly advise follow ups to patients to observe the results and manage any possible side effects (31). The studies suggested an average dose of 20/30 mg/day for children and 1/kg/day for adults while some patients might need more than that, however MPH overdose will result in toxicity and symptoms such as delirium, confusion, toxic psychosis, hallucinations and euphoria might develop (18). The MPH is considered to be safe when it is taken within the recommended doses orally however the abuse could be more likely when it is taken intravenously or snorted because by this route the brain utilizes the drug very fast and could reach the peak within 8-15 mins while it needs 60 to 90 mins after the oral administration (32).

2.2.2. Mechanism of Action of MPH

The mechanism of action of MPH is believed to be targeting the dopamine system in the brain by promoting the release of dopamine and preventing the reuptake which will

result in high concentration of dopamine in the synaptic cleft, this amount of dopamine is enough to stimulate parts of the brain such as prefrontal cortex and the limbic system related and inhibition of the motor system which will lead to behavioral adjustment and decreased impulsiveness, MPH is also found to be weak activator of serotonin receptor 5HT1A in the presynaptic and postsynaptic regions which contributes to more dopamine release (33, 34). There are studies to observe the effect of MPH on dopamine system, one of them was conducted on healthy adults who they were given different amounts of MPH and they were included in positron emission tomography (PET) scan 2 hours after the administrations, the results showed that the transporter locations in the cortex and basal ganglia were filled with a percentage of 12% in the individuals who were given 5 mg of MPH whereases 74% in the individuals who were given 60 mg of MPH. That supported the theory of the MPH function in blocking the dopamine transporters so excessive concentration of dopamine remains in the synaptic cleft (8).

2.2.3. Other usages and contraindications of MPH

MPH has been approved as a treatment for some other disorders including narcolepsy which defined as chronic sleeping disorder with excessive daytime drowsiness and sudden sleep attack (35). MPH has been approved in the US as one of the treatments of narcolepsy as its mechanism of stimulating the CNS will lead to increase in wakefulness and attentiveness (36). MPH has been found to be effective in enhancing the mood, memory and attention and it was proved to be useful in patients with stroke, HIV, cancer, bipolar and major depressive disorders, and some evidence suggested MPH in Alzheimer's Disease (37-39). MPH is not recommended to be prescribed to children under six years old as the studies are limited on its safety, MPH is contraindicated in several medical conditions such as serious hypertension, glaucoma, motor tics and Tourette syndrome (34). Allergy must be monitored in the patients whom taking MPH and if any sign is developed the medication must be stopped right away (34).

2.2.4. Adverse Effects of MPH

The use of MPH may bring about some adverse effects including sleep disturbance, nervousness, decreased appetite, headache, increased blood pressure and heart rate (15, 17). In addition, there have been discussions around the growth-related effects of its use in children with ADHD (18, 19), as there are some suggestions linking the usage the stimulant drugs and the delay in growth and other suggestions refer to the

possibility of the ADHD itself to have connection with weight and height deficit and since the studies have been showing delay in brain maturation so growth slowdown could be similar, however, more researches are needed as the etiology is yet to be understood (40). In a study on 342 ADHD patients aged between 6-18 years old and they were treated with MPH for 10 years, the reports showed significant decrease in weight and body mass index (BMI) and slight decrease in height when the age of 12 was the starting point of the treatment with the higher dose the higher impact on the height (40). In another study conducted in Guangzhou, P. R. China 146 school age children who were diagnosed with ADHD and received MPH were followed and compared to 29 drug naïve children and this study persisted for 2-4 years and it showed significant decrease in height in drug taker group and the longer period of treatment the higher impact on this significant decrease, whereas no significant change in body weight and BMI (41). Another study targeted the physical growth on MPH treated children across three years of follow up, total of 370 children were divided to medicated and non-medicated main groups and the medicated group children were divided into subgroups including newly administrated, consistently and inconsistently administrated, the results after three years assessment showed decrease in growth among the newly administrated children of 2 cm and 2.7 kg compared to drug free children (42). In a study to evaluate the effect of MPH on physical growth and fitness among male Korean children, three groups of study were inducted with 50 male children diagnosed with ADHD and were on MPH treatment, 69 children diagnosed with ADHD but MPH drug free and 60 healthy children as control group. The results showed improvement in intelligence quotient, attention and balance in MPH recipient children but no changes in weight and height between the three groups (43). Animal models have been also used to investigate the effect of MPH on body weight. In a study conducted on rats, different oral regimes of MPH administration were followed such as giving different doses 20, 30, or 60 mg/kg/day and dual dosages starting from low amount and higher over 8 hours interval a day, this experiment resulted in decreased body weight in animals which were under the effect of treatment, the results suggested different possible reasons such as the effect of MPH on speeding up the metabolism or decreasing the appetite as the food intake was less among the medicated rats (44). This finding could be significant to exploit the dopamine pathway in a new weight loss medications (45). Another study on Wistar Hannover rats which were treated with three different doses (5 mg/kg/day, 50mg/kg/day and 100mg/kg/day) from day seven until day 70 (46). The results indicated

decrease in body weight in 50mg/kg/day dose group and this decrease was compensated after the drug was stopped in the recovery period and rebound growth had occurred, but no significant changes in the 5mg/kg/day dose group were reported (46). Based on the studies the decrease in body weight in rats took place in higher doses such as more than 50mg/kg/day and 20mg/kg/day (46, 47).

There are relatively few studies on the effects of MPH on the neuroendocrine control of puberty and reproductive functions (20). The hypothalamic-pituitary-gonadal axis regulates the onset of puberty. Hypothalamic gonadotropin-releasing hormone (GnRH) controls the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary gland (48). The mechanism of MPH on puberty and reproductive function is still unclear, however, the mechanism of MPH in expanding the amount of dopamine and noradrenaline in the synaptic cleft could be crucial in the change of GnRH (49, 50). For the sake of understanding the link, the effect of dopamine on GnRH was tested on mut5 mice by recordings of gramicidin-perforated patch clamp from the neurons of GnRH which resulted in inhibitory effect of dopamine on the excitability of GnRH neurons by α_1-adrenergic receptors (51). In a study conducted on juvenile male monkeys, chronic treatment with high dose (12.5 mg/kg, oral) of MPH resulted in significantly delayed testicular descent, smaller sized testicle, low serum LH levels and low testosterone levels (52). Another study with a follow-up to 20 years old boy diagnosed with ADHD and received MPH for 17 years who witnessed delay in puberty and all the signs accompanied such as lack of erection, absence of body hair and low energy, on examination the genital organs were small as well (53). When the boy was tested and the sex hormones were analyzed, the results showed low serum FSH, LH, and testosterone levels (53). Testosterone supplements were given as a result and after four months, the patient started to show some improvements and puberty signs were demonstrated (53). Another longitudinal study investigated the pubertal development in boys aged between 12-15.99 that were treated with MPH for three years the results suggested delay in puberty and poor physical development during the period of puberty (54). Reversed outcomes came out of a study of seven children with ADHD had treated with MPH for a period of six months (0.5 mg/kg/dose for three times daily), where they suffered from signs of early puberty, however the children were tested and the results showed precocious puberty in the mean age of 8.17 years, however, testosterone, LH and FSH were all in the normal range (55). The study suggested that dopamine might be

working as excitatory stimulant on GnRH release and accelerating the puberty (55). The effect of MPH on rat testes was also evaluated. In a study conducted on 42 Wistar rats which were divided into 3 groups as control group, 5 mg/kg oral of MPH group and 10 mg/kg of MPH group (56). Testicular weight, body weight and histological examination were performed. The results showed no significant changes in body weight, but reduction in testicular weight and in round spermatids with higher rate of apoptotic death and p53 activation among the experimental groups with higher affection in the higher dose group, which indicate negative impact on spermatogenesis by MPH (56). Another study on 30-40 days old rats treated with MPH (2 mg/kg once a day) resulted in higher testicular weight and increased sperm quantity, but still decreased level of testosterone in testes (57). Liver examinations suggested higher hepatic testosterone catabolism as a result of the effect of MPH on the activity of testosterone hydroxylases (57). In summary, the study indicated that the hormonal and reproductive variations were common to be noticed as a result of MPH administration, however, those changes were described to be only transient (57). Moreover, another study on animal models occurred when 27 male mice were treated with 2 mg/kg and 10 mg/kg of MPH for 40 days resulted in reduction in body weight and decrease in Leydig cell counts, suggesting an impact of MPH on serum testosterone (58).

The studies on the effect of MPH on female rats are relatively few. However, an experiment investigated the effect of MPH on the reproductive functions of female rats where 20 of them aged 28 days old were divided into experimental and control groups (59). The experimental group of rats were treated with 450 μg/day for four weeks and they reached puberty, and the results showed higher levels of LH in the pituitary gland (59). On the hand, histological assessment indicated poor and incomplete ovarian follicles and physically, abnormal vaginal opening was observed without no significant differences in FSH and estradiol levels between the control and experimental groups(59). These results indicated the adverse of MPH on the pubertal onset and the impact on the reproductive axis, and the higher amount of LH in pituitary was due to the lack of release which might negatively affected the ovarian folliculogenesis (59). On the other hand, another study on female rats revealed that chronic administration of MPH (5 mg/kg/day and 10 mg/kg/day) showed no major effects on reproductive system as the tests resulted in no significant changes in sex hormones (60).

MPH has been proved to have histopathological characters on reproductive organs in male rats. However, a study on 54-week-old rats administrated with 2 and 10 mg/kg daily by oral gavage for 60 days resulted in significant changes on the testes (61, 62). It is believed that the negative impact of MPH on gonadotropin hormones might be the reason for testicular dysfunction and spermatogenesis impairment (61, 62). Postnatal (PND) 21 days old male Wistar rats were administered with MPH at doses of 2.5 mg/kg and 5 mg/kg (by oral gavage) until day 60. The results demonstrated that repeated – chronic administration MPH caused abnormal sperm morphology, but serum testosterone levels, sperm count and motility were not affected on PND 100, the interstitial tissue volume of testes was increased in the treated groups and the number of spermatogonia type A was higher in the 5 mg/kg group with no significant changes in body weight (63). There are relatively few studies to investigate the adverse effect after cessation of drug, one study on newly born rat treated with MPH (35 mg/kg) twice a day showed decreased body, pituitary and testes weights in the MPH groups compared to control group then another group was left to recover with no MPH administration for 30 days, the results showed no significant changes in same parameters compared to control group and growth rebound phenomenon had occurred (64). Moreover, several studies suggested that lower doses of MPH could minimize the adverse effects on the physical and reproductive growth (54).

3. MATERIALS and METHODS

3.1. Animal Husbandry

Seventy adult male and female Sprague-Dawley rats (n=35 for each sex) were included in this study. Rats were fed with standard rat chow and received water *ad libitum*. Animals were maintained in a controlled environment of 24 ± 2 °C, 50 ± 10% relative humidity and a 12-12 h light/dark cycle. All the experiments involving animals were performed following the guidelines established by Yeditepe University Medical School Experimental Research Centre (YÜDETAM).

3.2. Experimental Groups and Administration of the Drugs

The rats were separated from their mothers on the day of weaning (day 21) and they were divided into subgroups as the following:

Male model (21 days old male rats)

1. Control PND 60 (Saline) n=7
2. Control PND 90 (Saline) n=7
3. MPH PND 60 (5 mg/kg) n=7
4. MPH PND 90 (5 mg/kg), n=7
5. MPH (5 mg/kg until PND 60) + 30-day recovery until PND 90, n=7

Female model (21 days old female rats)

6. Control PND 60 (Saline) n=7
7. Control PND 90 (Saline) n=7
8. MPH PND 60 (5 mg/kg) n=7
9. MPH PND 90 (5 mg/kg), n=7
10. MPH (5 mg/kg until PND 60) + 30-day recovery until PND 90, n=7

The dose of MPH was decided according to a study on Sprague-Dawley rats showed that the dose of 5 mg/kg had the maximum effect on those rats by raising the

concentration of cellular dopamine 40 mins after the administration and according to same study it is considered to be low dose of MPH (65).

MPH (Ritalin® (10 mg/tablet); Novartis) was dissolved in physiological saline, centrifuged at 4000 rpm for 15 mins, aliquoted and kept at 4 ºC. The administration and general observation took place daily at (2-3 pm).

The animals in the experimental groups were administered with 5mg/kg of Ritalin via oral gavage from PND 21 to PND 60, while animals in control groups received the same amount of saline.

The termination of the experiment was divided into two phases, experimental male group and control male group of PND 60 were scarified at the end of PND 60 while experimental female group and control female group of PND 60 were monitored and oestrous cycle in female rats was followed by vaginal smear after the day 55, and their experiment was terminated on the day of diestrus between PND 60 and PND 63.

Then the other groups (Recovery and control PND 90) were allowed to "recover" (no drug administration) for 30 days and were sacrificed on PND 90. Oestrous cycle in female rats was followed by vaginal smear after the day 85, and their experiments was terminated on diestrus between PND 90 and PND 93 on the day of diestrus.

Body weight of the animals were measured weekly and animals were examined daily regarding the toxicity signs. The animals were scarified with high dose of anesthesia. Blood collection and reproductive organs dissection were followed.

3.3. Sexual Development and the Onset of Puberty

The onset of puberty was monitored by physical examination of preputial detachment starting on PND 35, while in female animals the onset of puberty was monitored by physical examination of vaginal opening starting on PND 35.

3.4. Serum Collection and Determination of Sex Hormone Levels

On the termination days blood was collected from abdominal aorta from all animals into 13mm x 75mm tubes which contains 3.2% of coagulation sodium citrate then immediately the blood was centrifuged then the serum was collected into 2 ml tubes which were prepared and labelled earlier, afterwards the samples were frozen and kept in –80ºC freezer until assay

Enzyme-linked immunosorbent assay (ELISA) kits for LH (E-EL-R0026, Elabscience®), FSH (E-EL-R0391, Elabscience®), testosterone (E-EL-0155, Elabscience®) and estradiol (E-EL-0152, Elabscience®) were purchased from Elabscience®. FSH and LH levels were tested in both sexes, Testosterone levels were tested only for male rats and estradiol levels only for female rats. All the tests took place at Yeditepe University laboratories where the protocols provided by the manufactures were followed.

3.5. Tissue Processing and Morphological Analysis

At the end of the study, decapitation was performed for tissue isolation from the experimental and control groups. Then, the abdominal region was dissected, and testes were isolated. Testes were fixed in Bouin's fixative (Histo Plus, #HCM-BOU-1000) for 36 hours. Testes were trimmed for tissue processing.

The fixative was thoroughly removed from the tissues. Afterwards, the testes were processed through graded alcohols, cleared in xylene, and embedded in liquid paraffin. Five μm sections were taken from the paraffin blocks on the microtome (Leica, #RM2245). Sections were incubated for 1 hour at 60°C to remove paraffin and left in xylene for 2x20 min to completely remove paraffin. Then the tissue sections were incubated in decreasing alcohol series for 10 minutes each (100%, 90%, 80% and 70%). The sections were washed with distilled water and stained with Hematoxylin Eosin staining (Bio Optica, Gill3 #05-06015), then washed with tap water and they were stained with eosin (Sigma, #HT110132). After washing with distilled water, the sections were incubated in increasing alcohol series (70%, 80%, 90%). After incubating in 100% alcohol, sections were placed 30 min in xylene. The slides were covered with coverslip using xylene-based mounting medium (Bio Optica, #05-BMHM508). Imaging was performed on a Leica DM 6000.

3.6. Statistical Analysis

The data were expressed as mean ± standard error of the mean (SEM). The distribution of the data was investigated by Shapiro-Wilk normality test. The outliers in the datasets were determined and removed by using ROUT method (Q=1%) (66). The differences between the body weights of the animals in different groups on different weeks were investigated by two-way analysis of variance (ANOVA) followed by Tukey's

multiple comparison test. The onset of the puberty was investigated by both Gehan-Breslow-Wilcoxon test and Student's unpaired t-test. For the investigation of the hormone levels, Student's unpaired t-test was used for the binary comparisons, while one-way ANOVA followed by Tukey's multiple comparison test was used for the comparison of multiple groups. A p value lower than 0.05 was accepted as statistically significant. Data was analyzed by using GraphPad Prism 7 (GraphPad Software, USA).

4. RESULTS

4.1. Body Weight

Body weight of both male and female animals did not significantly differ among the groups throughout the experimental period up to PND 60 (Figure 1a and b) and PND 90 (Figure 2a and b).

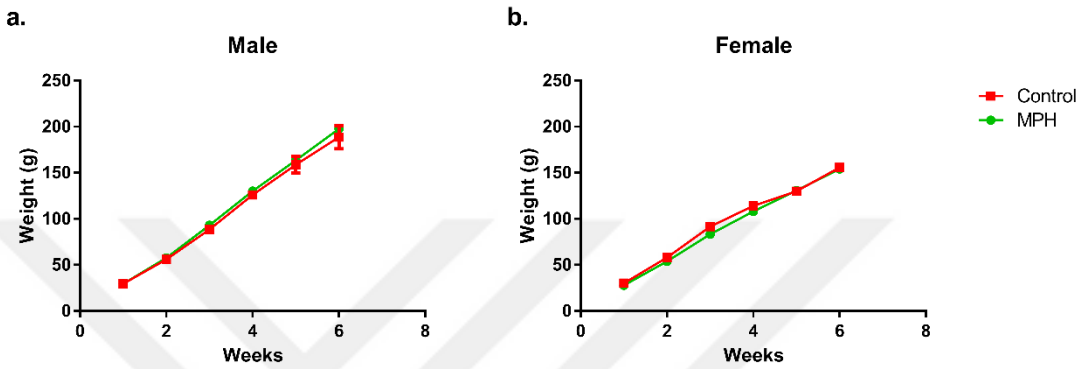

Figure 1. Body weight of **(a)** male and **(b)** female animals throughout up to PND 60.

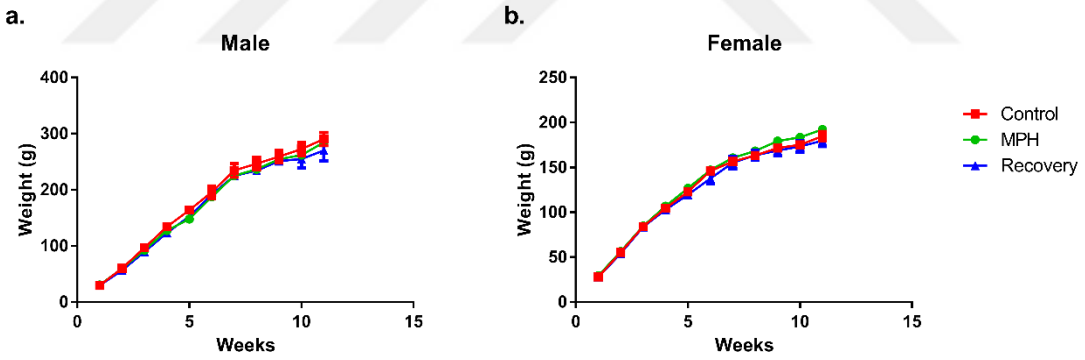

Figure 2. Body weight of **(a)** male and **(b)** female animals throughout up to PND 90.

4.2. Sexual Development and the Onset of Puberty

Preputial detachment was slightly but significantly lower in the MPH group ($p<0.01$; Table 1). On the other hand, vaginal opening was significantly delayed in MPH group ($p<0.05$; Table 1).

Table 1. Onset of puberty in the experimental groups.

Gender/Group	Control (days)	MPH (days)
Puberty male (days)	46.83 ± 0.29	46.0 ± 0.00
Puberty female (days)	38.50 ± 0.66	40.55 ± 0.74

4.3. Serum Hormone Levels

4.3.1. Male Animals

MPH treatment did not cause any significant changes in FSH between the treated groups and the control groups, however the concentrations in recovery group increased slightly but yet no significant changes (Figure 3a). Moreover, MPH treatment did not cause any significant changes in LH neither between the treated groups and the control groups, nor between recovery and control groups (Figure 3b). On the other hand, daily administration of MPH slightly decreased the testosterone in the treated group 60 insignificantly as well as group 90 compared to control groups, however, the testosterone in recovery group tended to increase slightly (Figure 3c).

4.3.2. Female Animals

MPH treatment did not cause any significant changes in FSH neither between the treated groups and the control groups, nor between recovery and control groups (Figure 4a). MPH administrations significantly increased the levels of LH in recovery group compared to other groups, while no significant changes occurred between MPH treated groups compared to control groups (Figure 4b). MPH treatment significantly reduced the estradiol levels on PND90 compared to control group ($p<0.05$), while there was no difference between the recovery and control groups (Figure 4c). Moreover, although there was a trend to normalization of estradiol levels in the recovery group, there were no significant differences between the MPH and recovery groups (Figure 4c).

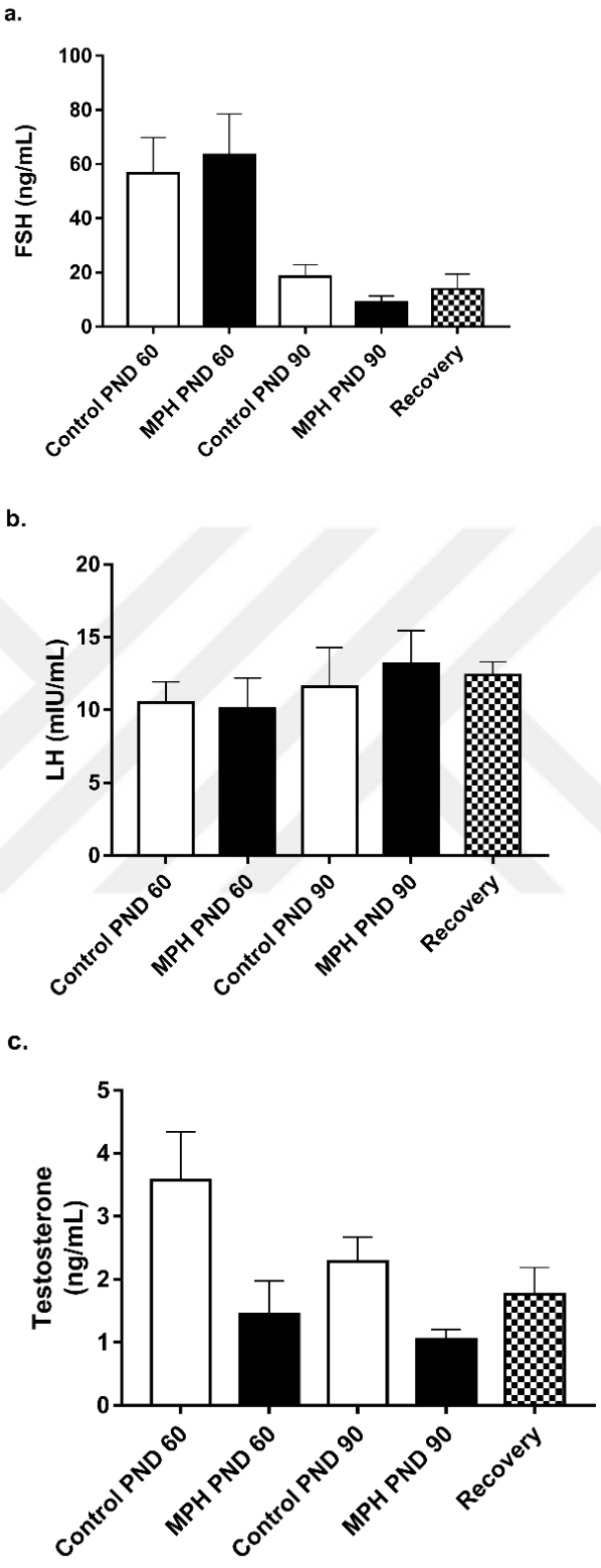

Figure 3. Serum **(a)** FSH **(b)** LH and **(c)** testosterone levels of the male animals throughout the experimental period.

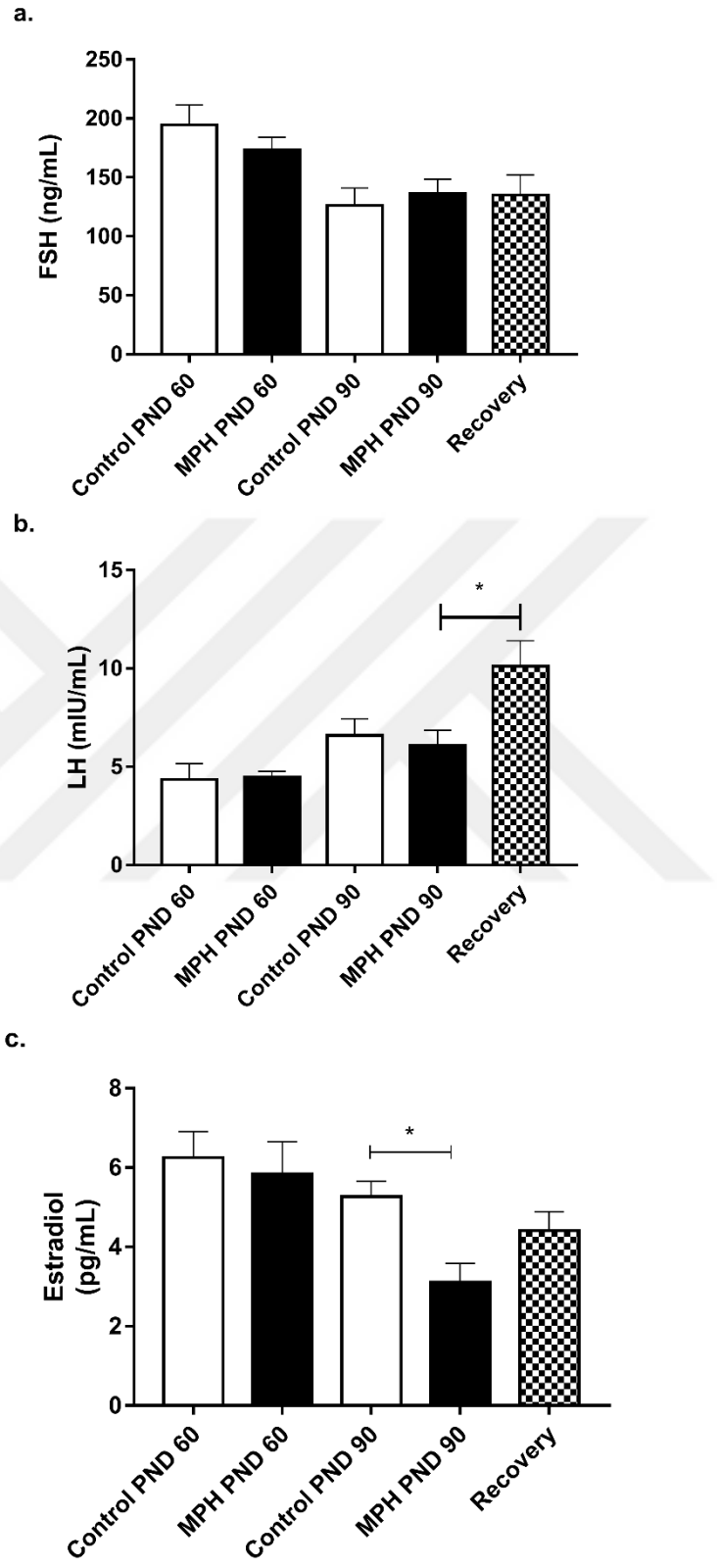

Figure 4. Serum **(a)** FSH **(b)** LH and **(c)** testosterone levels of the animals throughout the experimental period (*$p<0.05$ and ***$p<0.001$).

4.4. Morphological Evaluation of Rat Testis and Ovarian Tissue between PND 60 Control and PND 60 MPH group

Testis and ovarian morphology from PND 60 and 90 MPH group and control group were examined under light microscope with Hematoxylin-Eosin staining.

4.4.1. Male Animals

In sections taken from testicular tissue of control group; seminiferous tubules, Leydig cells and basement membrane of seminiferous tubules were observed in normal structure. Interstitial connective tissue was observed in regular morphology. It was observed that the seminiferous tubules were lined with stratified cuboidal epithelium, and the tubule epithelium was composed of spermatogenic cell lines and Sertoli cells. A large number of spermatogenic series cells with normal morphology were observed, located between the lateral folds of Sertoli cells resting on the basal lamina. It was observed that secondary spermatocytes and spermatids were in higher ranks depending on the developmental stages. Early spermatids were distinguished by their cap-shaped PAS-positive acrosome structure sitting on the nucleus, and late spermatids that would turn into spermatozoa with their tails extending into the lumen and dark long heads. Sperm cells were found in many seminiferous tubule lumens (Figure 5A-A’’).

In the testis from MPH group, seminiferous tubule degeneration and tubular atrophy, which is remarkable with the decrease in tubule diameters, were clearly observed with vacuolization of different sizes in the tubules. Thick interstitial connective tissue was detected around the seminiferous tubules. It was observed that most of the cells of the spermatogenic series disappeared in the tubules, and only a few spermatogonia and Sertoli cells remained at the basal compartment. It has been shown that the nuclei of Sertoli cells became irregular and sometimes they were located far away from the basement membrane and close to the tubule lumen. A few and clustered Leydig cells and blood cells in the intratubular area were observed. Although the distance between the seminiferous tubules was not very wide, infiltration of tubular cells towards the interstitial area was observed (Figure 5B-B’’).

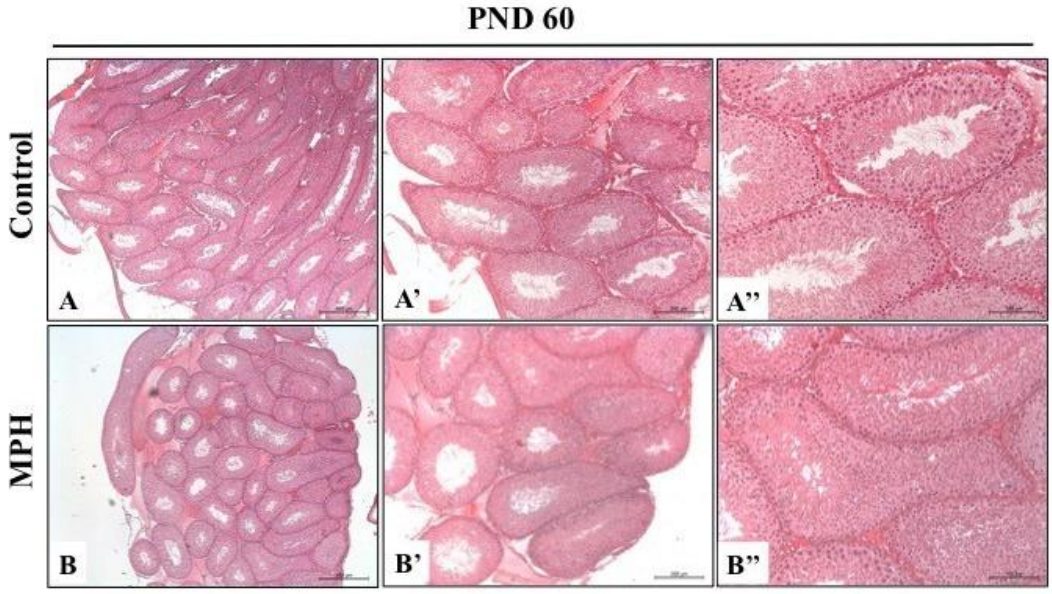

Figure 5. Control testis tissue section (5X (A), 10X (A'), 20X (A'') magnification) and MPH group testis tissue section (5X (B), 10X (B'), 20X (B'') magnification).

In the testis of MPH group; compared to the control group (Figure 6A-A''), it was observed that the seminiferous tubule basement membrane structure was disrupted and tubular atrophy was observed in some testicular sections of the experimental group (Figure 6B-B''). The intertubular area is more irregular, and fewer Leydig cells and blood cells were found compared to the control. In most seminiferous tubules, little or no luminal area has been detected. It was observed that most of the cells of the spermatogenic series disappeared in the tubules, and only a few spermatogonia and Sertoli cells remained at the basal compartment. It was shown that the nuclei of Sertoli cells became irregular and sometimes they were located far away from the basement membrane and close to the tubule lumen (Figure 6B-B'').

It was shown that the basement membrane of the seminiferous tubule was improved in the recovery group, similar to the control group. It was detected that the interstitial space and the distribution of cells in this area were similar to the control group. The spermatogenic cell lines and the Sertoli cells that supported them uniformly distributed in the compartments within the seminiferous tubule. It was observed that the lumen area was seen with normal structure which was similar to the control group (Figure 6C-C'').

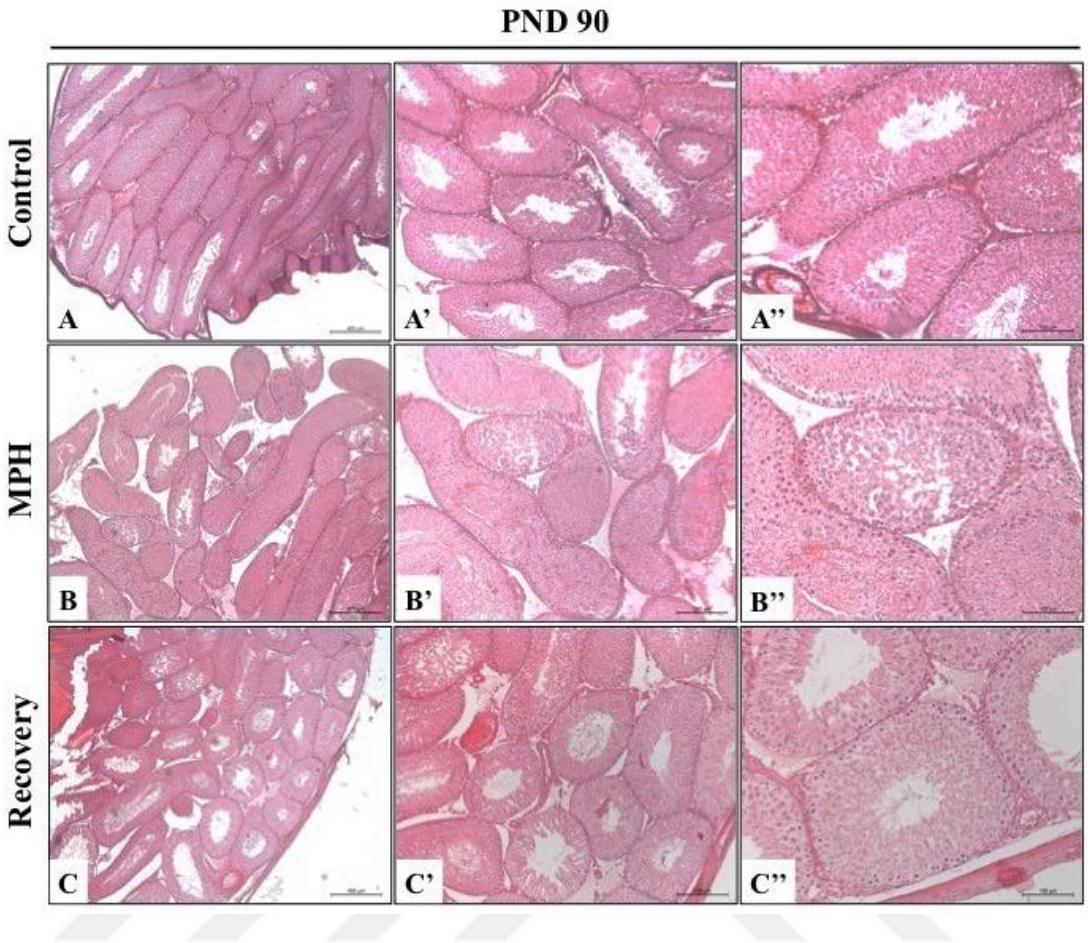

Figure 6. Control testis tissue section (5X (A), 10X (A'), 20X (A'') magnification), MPH group testis tissue section (5X (B), 10X (B'), 20X (B'') magnification) and recovery group testis tissue section (5X (C), 10X (C'), 20X (C'') magnification).

4.4.2. Female Animals

In the sections taken from the ovarian tissue of the control group; the follicles at different stages of follicular development were determined in the ovarian cortical tissue. When we evaluated oocyte morphology in the developing follicles, we determined that oocyte cytoplasm and zona pellucida were normal. We also identified atretic follicles in the control group ovaries that could not complete their development as a requirement of a normal follicular process (Figure 7A-A'').

In the ovaries of MPH group, we identified follicles at different stages of follicular development in ovarian cortex. However, we observed that the number of developing follicles is higher than primordial and primary follicles that are waiting dormant in early development. When we examined developing follicles, we observed disruptions in the

cytoplasm and zona pellucida of oocytes in the follicle compared to control ovary. Also, the atretic follicles seen in the MPH group ovaries were quite striking (Figure 7B-B’’).

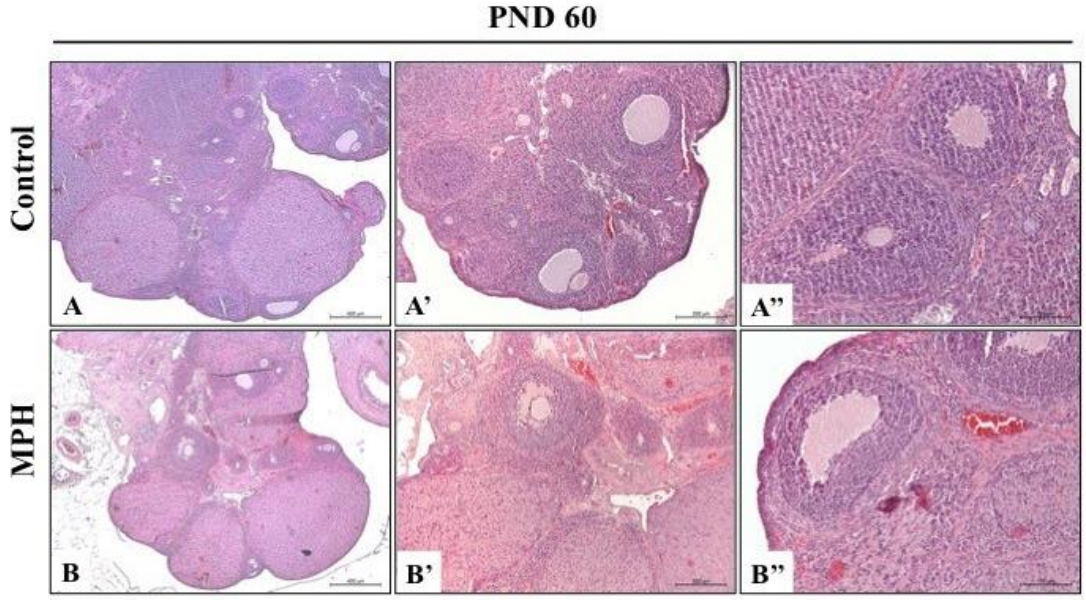

Figure 7. Control ovarian tissue section (5X (A), 10X (A’), 20X (A’’) magnification) and MPH group ovarian tissue section (5X (B), 10X (B’), 20X (B’’) magnification).

In the ovaries, more atretic follicles were observed in the ovarian sections of the experimental group (Figure 8B-B’’) compared to the control group (Figure 8A-A’’). A high percentage of blood vessels and blood cells were seen in the ovary (Figure 8B-B’’). Morphological deterioration was observed in the basement membrane structure of the follicles at different stages (Figure 8B-B’’).

It was observed that there were many follicles in different developmental stages in the recovery group, similar to the control group. The basement membrane of the follicles presented normal morphology. When we evaluated oocytes morphology in the developing follicles, we determined that oocyte cytoplasm and zona pellucida were normal. Fewer atretic follicles were observed compared to the experimental group (Figure 8C-C’’).

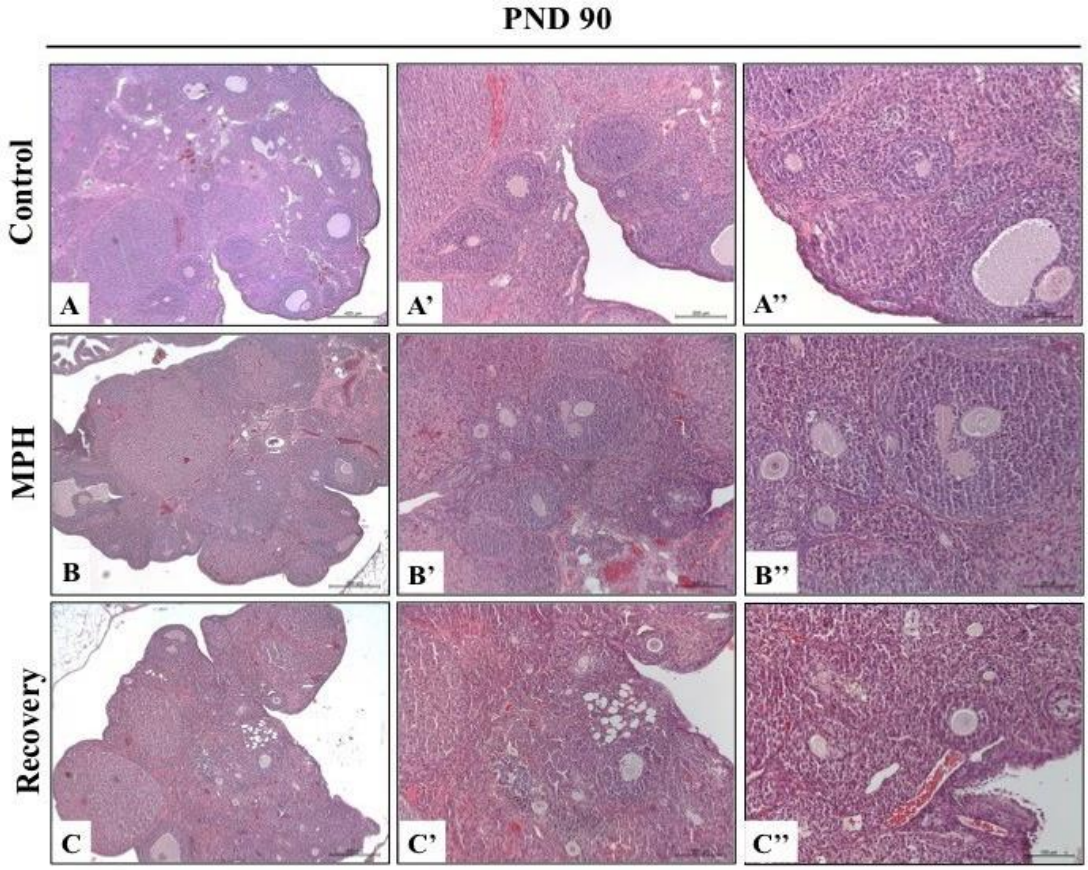

Figure 8. Control ovarian tissue section (5X (A), 10X (A'), 20X (A'') magnification), MPH group ovarian tissue section (5X (B), 10X (B'), 20X (B'') magnification) and recovery group ovarian tissue section (5X (C), 10X (C'), 20X (C'') magnification).

5. DISCUSSION and CONCLUSION

Methylphenidate is the treatment of choice of ADHD which occurs in childhood and could persist into adulthood, which indicates the usage of MPH in crucial period of the development in children (2). The studies have indicated growth related effects as well as concerns regarding pubertal development and reproductive functions (18, 19), those factors showed the need for the preclinical studies to understand the mechanism(s) behind those side effects which would better assess the number of prescriptions made around the World. Studies have been submitted on model animals like rats and mice (63), as well as primates (52), the studies on humans are so limited and yet many outcomes are still to be understood (20, 53-55).

Studies on humans concluded body weight loss as main side effect, as well as the decrease in height and physical appearance (40-42). In rats, some studies have been showing decrease in body weight in the MPH treated groups (44, 45). It was noticed that those body weight changes have been observed in high MPH doses (20 mg/kg/day and more (47), and 50mg/kg/day and more (46)), however, this was explained as the high doses of MPH could act as appetite suppressant or enhance the metabolism or raise the energy expenditure in the animals in long term, which would result in weight loss. On the other hand, and as mentioned in the literature, the dose of 5 mg/kg/day did not have significant impact on body weight (46, 63). In the present study, no significant changes were observed in neither of the groups, however, this stability meant that the results of reproductive functions were not caused by the body weight changes.

The stage of puberty is a complicated period of time where dramatic changes usually occur, however, any consistent usage of drug around that period could influence the process, in the present study MPH was administrated through the onset of puberty. The hypothalamus-pituitary-gonadal axis controls the onset of puberty, and it is activated by the pulsatile release of GnRH from hypothalamus which stimulates the secretion of gonadotropin hormones FSH and LH which in turn initiate the production of sex steroids (32, 48, 50). In our study, we observed significant changes in puberty onset in both sexes. Preputial detachment was slightly but significantly earlier in the MPH group. On the other hand, vaginal opening was significantly delayed in MPH group. The dopaminergic system is believed to affect the puberty onset, the mechanism of MPH on puberty and

reproductive function is still unclear, however, the mechanism of MPH in expanding the amount of dopamine and noradrenaline in the synaptic cleft could be crucial in the change of GnRH (32, 50). The effect of dopamine on GnRH was tested on mut5 mice by recordings of gramicidin-perforated patch clamp from the GnRH neurons. They demonstrated that inhibitory effect of dopamine on the excitability of GnRH neurons by acting on α1-adrenergic receptors would result in delayed puberty (51), which corresponds with our findings in female rats. Moreover, a study conducted on juvenile male monkeys, chronic treatment with high dose (12.5 mg/kg, orally) of MPH resulted in significantly delayed testicular descent (52). However, other studies suggested otherwise, indicating that the excitatory nature of dopamine could induce puberty by affecting and stimulating its own receptors and since the mechanism of MPH is gathering dopamine in synaptic cleft, this would induce precocious puberty (55). The precocious puberty reports mostly reported in the males upon MPH use (55, 67). There are few data in the literature about the relation between MPH and precocious puberty. Seven children with ADHD were treated with MPH for a period of 6 months (0.5 mg/kg/dose for 3 times daily), then they suffered from signs of early puberty (55). Another case report of 12 years old male who had been receiving MPH showed recurrent erections in the prepubertal period [66]. Further investigations are needed to differentiate whether the effect of dopamine on GnRH is gender related.

As explained above, the interference of MPH in the puberty time could disturb the sex hormones either by the direct effect on reproductive organs or by affecting the release of GnRH (32, 48, 50). Testosterone levels were slightly altered in the present study, this alteration was observed in male animals treated with MPH on PND 60 and PND 90 compared to control groups. However, the testosterone levels were similar to the levels in control group of animals and were compensated after 30 days of drug cessation. Our findings match few studies in the literature where serum testosterone were decreased (52, 53). This could be a result of direct effect of MPH on hypothalamus may disturb the release of GnRH, higher hepatic testosterone catabolism by the effect of MPH on the activity of testosterone hydroxylases or the impairment of testes and the function of Leydig and Sertoli cells by MPH (52, 53, 57). However, these changes were transient and adaptive physiological response was likely to happen after the drug was discontinued (57). Studies on sex hormones were included in many reports showed lower levels of FSH and LH after MPH treatment (52, 53), while other studies showed higher levels of LH,

decreased testosterone and slight FSH changes (62). Sex hormones are regulated by GnRH (32, 48, 50) and FSH could have direct impact on spermatogenesis process as it has major role in the primary stage of the process (68, 69), however, in our study, no significant changes were observed in the levels of FSH and LH.

In testes naturally the spermatogenesis takes place in the seminiferous tubules area which is major component in the testes beside the interstitial tubules (70, 71). The seminiferous tubules consist of spermatogenic cell and Sertoli cells (72). Sertoli cells have major influence on spermatogenesis, and they regulate the testosterone by releasing androgen-binding protein with help of FSH and regulates the release of FSH through inhibin B (73). Leydig cells are found in the interstitial cells and they are responsible for the secretion of testosterone under the LH regulation from the pituitary (72). In our study MPH caused seminiferous tubule degeneration and tubular atrophy. Thick interstitial connective tissue was detected and spermatogenic series disappeared in the tubules, and only a few spermatogonia and Sertoli cells remained at the basal compartment with irregular nuclei and those changes occurred in both MPH-treated groups up to PND 60 and PND 90. However, in the recovery, group basement membrane of the seminiferous tubule was improved, and it was detected that the interstitial space and the distribution of cells in this area were similar to the control group which suggest recovery process in some parts of testes after the cessation of the drug. However, longer recovery period time would be significant to examine how much improvement we would witness in the testes.

In female mammalians, the hypothalamo-pituitary-gonadal axis regulates estrous cycle (60). Gonadal steroids in turn have negative feedback effects on the hypothalamic GnRH neurons, and also gonadotrophin (LH and FSH) release from the anterior pituitary gland (32, 48, 50). In a study conducted on female rats, chronic administration of MPH (5 mg/kg/day and 10 mg/kg/day) resulted in no significant changes in estradiol and other sex hormones and suggested no harmful effects of MPH on reproductive system (60). While another study showed higher levels of LH in the pituitary gland and normal estradiol and FSH levels in female rats after receiving MPH for 4 weeks (59). In the present study, there were no significant changes in FSH, but estradiol levels decreased in MPH-treated group up to PND 90 compared to control group. These results suggest that chronic MPH treatment could either affect the ovary or the pituitary gland, however, the short-term treatment of MPH (up to PND 60) did not cause any significant changes. One

possible reason of it might be that the decrease in the conversion from testosterone to estradiol, which would higher the testosterone levels (74), however, in our study, testosterone levels were not measured in female groups. Another possible reason could be that lower estradiol levels might be due to an increase in the metabolism of estrogen (74), however, the liver pathology was not monitored in our study.

On the other hand, LH levels surged in the recovery with no significant changes in MPH-treated groups up to PND 60 and PND 90 compared to control groups. In several studies, that tested the effect of the central noradrenergic medications on rat models showed that the higher levels of noradrenaline activity in hypothalamus could trigger the surge of LH and GnRH, however the release of LH might be suppressed by some components such as opioids (75-80). Histology results showed negative effects of MPH on the treated groups. Disruptions in the cytoplasm and zona pellucida of oocytes and atretic follicles were seen in the MPH group ovaries that corresponds to a study that represented poor and incomplete ovarian follicles with signs of rupture and poor development of oocytes (59). Negative impact of MPH on folliculogenesis with the higher rate of atretic follicles could be as a result of hormonal disturbance (81). Nevertheless, in our study, the histological effects of MPH were transient, and the recovery group showed that oocyte cytoplasm and zona pellucida were normal and atretic follicles were fewer suggesting reversible effects and improvement after stopping the treatment and that lines with a study suggesting the impairment of estrous cycle by MPH was transient and did not persist after cessation of the treatment (82).

In summary, the chronic administration of MPH affected the puberty in both males and female rats and that is in line with the hypothesis of the major role of dopaminergic system on the onset of puberty. In addition, MPH may have impact on testes by causing seminiferous tubule degeneration and tubular atrophy, harming Leydig and Sertoli cells, and on ovaries by causing disruptions in the cytoplasm and zona pellucida of oocytes and atretic follicles that might have led to sex hormone disturbance. However, those impacts on both testes and ovaries were transient as the improvement occurred after the drug being discontinued. Longer recovery period would be useful to observe further changes. The findings of the present study could allocate the impact of MPH on hypothalamic-pituitary-gonadal axis and directly on reproductive functions. We conclude that 5 mg/kg dose of

MPH after chronic administrations could have adverse effect on reproductive organs, however these effects are transient and reversible.

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7. APPENDICES

7.1. Ethical Approval

T.C. YEDİTEPE ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu (HADYEK)

ETİK KURUL KARARI

Protokol No	Toplantı Tarihi	Toplantı Sayısı	Karar No	Proje Yürütücüsü
2021-012	20.01.2021	2021/01	2021/01-12	Prof. Dr. Bayram Yılmaz
'Methylphenidat'ın Erkek ve Dişi Sıçan Modellerinde Püberte Başlangıcı ve Üreme Fonksiyonları Üzerine Etkilerinin Araştırılması' isimli proje oy birliğiyle etik açıdan uygun görülmüştür.				

Hayvan Türü / Irkı	Toplam Hayvan Sayısı	Hayvanın Cinsiyeti
Sıçan / Spraque Dawley	70	Erkek ve Dişi

Görevi	Adı Soyadı	Katılım Durumu
Başkan	Prof. Dr. Bayram YILMAZ	
Başkan Vekili	Prof. Dr. Erdem YEŞİLADA	
Üye	Dr. Veteriner Hekim Engin SÜMER	
Üye	Prof. Dr. M. Ece GENÇ	
Üye	Prof. Dr. Rukset ATTAR	
Üye	Prof. Dr. Gamze TORUN KÖSE	
Üye	Doç. Dr. Ediz DENİZ	
Üye	Doç. Dr. Aylin YABA UÇAR	
Üye	Doç. Dr. Burcu GEMİCİ BAŞOL	
Üye	Hakan GÖKSEL	
Üye	Ahmet ŞENKARDEŞLER	

7.2. Curriculum Vitae

Kişisel Bilgiler

Adı	Firas	**Soyadı**	Khoubbieh

Öğrenim Durumu

Derece	Alan	Mezun Olduğu Kurumun Adı	Mezuniyet Yılı
Doktora			
Yüksek Lisans			
Lisans	Faculty of Medicine	Near East University	2015
Lise	BSc degree (scientific phase)	Al Motafaweken	2008

Başarılmış birden fazla sınav varsa (KPDS, ÜDS, TOEFL; EELTS vs), tüm sonuçlar yazılmalıdır

Bildiği Yabancı Dilleri	Yabancı Dil Sınav Notu (**#**)
English	
Arabic	
Greek	
German	Goethe A1-A2
Turkish	TOMER A1-A2

İş Deneyimi (Sondan geçmişe doğru sıralayın)

Görevi	Kurum	Süre (Yıl - Yıl)
Assistant doctor	Internal medicine department (Nicosia)	2013-2014
Assistant doctor	Surgery department (Nicosis)	2014-2014
Assistant doctor	Emergency department (Nicosia)	2015-2015
Assistant doctor	Gynecology department (Nicosia)	2015-2015
Doctor	Emergency department (Nicosia)	2015-2016
Doctor	Emergency department (Cairo)	2016-2017

Bilgisayar Bilgisi

Program	Kullanma becerisi
Microsoft programs	İyi

*****Çok iyi, iyi, orta, zayıf olarak değerlendirin

Bilimsel Çalışmaları

SCI, SSCI, AHCI indekslerine giren dergilerde yayınlanan makaleler

Diğer dergilerde yayınlanan makaleler

Uluslararası bilimsel toplantılarda sunulan ve bildiri kitabında (*Proceedings*) basılan bildiriler

Hakemli konferans/sempozyumların bildiri kitaplarında yer alan yayınlar

Diğer (Görev Aldığı Projeler/Sertifikaları/Ödülleri)

Degree of Doctor of Medicine
Certificate in Laboratory Animal Science