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Research Article

Immunohistochemical and Ultrastructural Changes Related To Methylphenidate In Rat Pituitary and Pineal Glands

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Summary

Objective: The presence of a dopaminergic function in pituitary and pineal glands is well known. Methylphenidate (MPH), a widely prescribed psychostimulant for attention deficit/hyperactivity disorder, is an indirect dopamine agonist which could have the capacity of influencing the hypothalamo-neurohypophysial system with the pineal gland. Our aim is to investigate dose-dependent immunohistochemical dopamine 2 expression, possible cell apoptosis and ultrastructural changes of the rat pituitary and pineal gland tissue, to demonstrate possible toxicity of the chronic extended use of the MPH.
Material and Methods: In this study, 27 female prepubertal Wistar albino rats, divided into three different dose groups (5, 10 and 20 mg/kg), were treated orally with MPH dissolved in saline solution for 5 days per week during three months. At the end of the third month, after perfusion fixation, pituitary and pineal glands were removed and sections were collected for immunohistochemical, TUNEL assay and ultrastructural studies.
Results: We observed that methylphenidate induced dose-related ultrastructural changes in pituitary and pineal glands.
Conclusion: High dose administration of this drug could influence the functions of these glands. Thus, we suggest that the therapeutic dose of methylphenidate must be kept in minimum level.

Key words: Methylphenidate, dopamine D2 receptor, pituitary gland, pineal gland, in situ nick-end labelling, ultrastructure

Rat Pineal ve Hipofiz Bezlerinde Metilfenidata Bağlı Olarak Oluşan İmmünohistokimyasal ve İnce Yapısal Değişiklikler: Doz Bağımlı Çalışma

Özet

Amaç: Metilfenidat (MPH), dikkat eksikliği/hiperaktivite bozukluğu için oldukça sık reçetelenen psikostimulan bir ilaç olup dolaylı dopamine agonist etkisi üzerinden, pineal ve hipofiz bezlerindeki varlığı bilinen dopaminerjik fonksiyonlar nedeni ile hipothalamo-nörohipophysial sistemi ve pineal bezi etkileyebilecek kapasiteye sahip görülmektedir. Metilfenidatın kronik uzun süreli kullanımı nedeniyle oluşabilecek toksisiteyi gösterebilmek amacıyla, doz bağımlı dopamine 2 ekspresyonunda gelişebilecek değişiklikler, olası hücre ölümü ve ince yapısal değişiklikler araştırılmıştır.
Materyal ve Metod: Çalışmada Wistar albino tipi 24 adet prepubertal sıçana (5,10 ve 20 mg/kg) üç ay boyunca, haftada 5 gün oral yolla serum fizyolojik içersinde çözünmüş Metilfenidat uygulanıldı. Kontrol grubuna serum fizyolojik verildi. Uygulamanın üçüncü ayında perfüzyon tespiti sonrasında pineal ve hipofiz dokuları alındı ve kesitler immünohistokimyasal, TUNEL ve elektron mikroskopik incelemeler için toplandı.

Bulgular: Metilfenidat, doza bağımlı olarak hipofiz ve pineal bez dokusunda ince yapısal değişikliklere neden olmaktadır.
Sonuç: Bu ilacın yüksek dozlarda kullanımı, bu bezlerin fonksiyonlarını etkileyebilir. Dolayısıyla, metilfenidatın tedavi edici dozlarının minimum düzeylerde tutulmasının daha iyi olacağı kanısındayız.

Anahtar Kelimeler: Metilfenidat, dopamine D2 reseptörü, hipofiz bezi, pineal bez, in situ nick-end labelling, ultrastrüktür

INTRODUCTION

Methylphenidate therapeutically used for the management of attention-deficit hyperactivity disorder in children most commonly nowadays. The therapeutic use of drug increase, but its common and high/over dose usage causes some severe side effects[2,15,22,24,28,54]. Its toxicity has been shown in many article[2,15,22,24,28,54].

Attention-deficit hyperactivity disorder (ADHD), characterized by inattention, hyperactivity and impulsivity, is a common neurodevelopmental disorder of childhood and about 30-60% of these ADHD cases persisted into adulthood[2,28]. Methylphenidate hydrochloride (MPH) is a widely prescribed psychostimulant for ADHD, with a pharmacological profile similar to amphetamine, cocaine and all these drugs are known as indirect dopamine agonists while the dopaminergic system of the brain plays a central role in reward and motivation[3,24,54]. Dopamine is an important neurotransmitter of the central nervous system controlling the various organ activities depending on the activation of different receptors[18]. Methylphenidate blocks dopamine reuptake transporter (DAT), thereby elevating extracellular dopamine levels in various limbic, striatal, cortical, cerebellar terminal fields, and increases dopamine signalling and dopamine response duration[32,45,47,49,51,53].

The great concern about the treatment of MPH is that, in a critical time of development and maturation of the brain such as adolescence, the exposure to this psychostimulant can change the gene expressions, neuronal morphology and activity[7,14,15,17,20,21,22,25,26,35,37,38,39,48,50].

The expanded use of methylphenidate stimulates the need to expend researches on the capacity of influencing the hypothalamo-neurohypophysial system with its interaction with the pineal gland and potential toxicity of this drug[4,5]. Honma and Honma[29] reported that MPH can induce a circadian locomotor rhythm in suprachiasmatic nuclei-lesioned rats. The relation between the behavioural effects of MPH with time of application during the light-dark cycle was demonstrated by several researchers[23,24] Also, Appenrodt and Schwarzberg[5] reported that the behavioural stimulation of MPH was modulated by both the pineal gland with its hormone Melatonin (Mel) as well as the neuropeptide vasopressin (AVP).

Although the toxicity of methylphenidate has been shown in many article its dose dependent effects on pituitary and pineal glands and also the relationship of this degenerative effect with D2 receptor is limited. With an idea to give different dosage of methylphenidate to rats and take their pituitary and pineal glands, we decided to investigate degenerative effects on both glands with ultrastructural method and to show cell death by TUNEL. Additionally, because of the increasing expression of D2 receptor on toxicity of methylphenidate we decided to show dose depended differences on D2 receptor expression on both glands.

MATERIAL AND METHODS

Animals and treatment

The experimental protocol was approved by the local Ethical Committee for animal studies. In the experimental protocol, twenty-four female Wistar albino rats with a weight of 110 g (±20), divided into four different groups (3 different dose; 5, 10 and 20 mg/kg per day and a control group) and six animal in each group were prepared. The doses of the methylphenidate (MPH) were chosen according to the studies which indicate that, in rats, the metabolism rate of this drug was faster than humans[13,34,46]. Female rats were selected for this study according to the previous studies reporting that female rats are more anxious than male rats and their nervous systems may be more susceptible to alteration by psychomotor stimulants[12,16,53]. Prepubertal (25 day-old) rats were treated orally with MPH dissolved in saline solution for 5 days per week during three months. We gave MPH orally since this is the route of administration used therapeutically for ADHD. The animals were synchronized to a light-dark cycle (lights on from 08:00 h to 20:00 h) beginning at least 2 weeks before the commencement of experiments. These conditions were maintained for 12 weeks during March-May to avoid the possibility of seasonal rhythms affecting the findings.

Tissue sampling

At the end of the third month, all the animals were anaesthetized by ketamine hydrochloride (Ketalar, Parke-Davis, Istanbul, Turkey) 30 mg/kg intramuscularly. For muscle relaxation, 2% xylazine hydrochloride (Rompun, Bayer, Istanbul, Turkey) 6 mg/kg was used. Then, they were perfused with 1.25% glutaraldehyde and 1% paraformaldehyde solutions.

Following perfusion, the pineal body was separated from the skull and pituitary gland was removed from the sella turcica. Pituitary and pineal glands from each group were fixed in neutral formalin for 72 hours and processed for m thickness were processed for polylysinμparaffin embedding. Sections of 4-5 microscope slides.

Immunohistochemistry

For immunohistochemical examination of Dopamine 2 Receptor (D2DR), Zymed Histostain-Plus Broad Spectrum kit used 85-9043, Sanfrancisco, CA, USA). Endogenous peroxidase#50681666, Cot#(Lot TA-125-HP,#AHP40114, Cot#activity was blocked in 3% hydrogen peroxide (Lot LabVision, Fremont, CA, USA). Epitopes were stabilized by application of serum 85-9043, Sanfrancisco, USA). Sections were#50681666, Cot#blocking solution (Lot F2204,#incubated with Dopamine 2 Receptor (D2DR, mouse polyclonal antibody Lot sc-5303, Santa Cruz, CA, USA) diluted in PBS (phosphate buffer saline pH#Cot 00-3000, Zymed, San Francisco, CA, USA) overnight at#50381417, Cot#7.4, Lot 85-9043,#50681666, Cot#+40C. After that, the biotinated secondary antibody (Lot biotinated secondary antibody) was applied. Then, Streptavidin peroxidase 85-9043) was applied to the slides. 3-amino-9-ethylcarbazole#50681666, Cot#(Lot TA-125-HA, Lab Vision, USA) was used as chromogen.#AH41013, Cot#(Lot

Afterwards, all slides were counterstained with Mayer's haematoxylin. Slides were examined with Photo-light microscope (DM4000B Image Analyze System and, Leica, Germany) and Leica DFC280 plus camera. The number of immune positive cells are measured manually by using QWin software programme in consecutive areas for serial cutaways taken from each subject[1].

Electron microscopic study

For electron microscopic studies, pituitary and pineal glands of all groups were fixed in 0.1M phosphate-buffer containing 2.5% glutaraldehyde for 2-3 hours; then they were post fixed in 1% osmium tetra oxide

(OsO4) and dehydrated in a series of graded alcohols (50, 60, 70, 80, 90, 96 and 100% ethanol). After passing through propylene oxide, the specimens were embedded in Araldite CY 212, DDSA (2-dodecenyl succinic anhydride), BDMA (benzyldimethyl amine) and dibutylphitalate. Semithin sections were cut and stained with toluidin blue and examined with a BH2 Olympus light microscope. Ultra-thin sections were stained with uranyl-acetate and lead-citrate and examined with a Carl Zeiss EM 900 transmission electron microscope (TEM).

Terminal Transferase mediated Dig-DUTP Nick end labeling method (TUNEL)

Apoptosis in the pituitary and pineal glands was demonstrated in situ by the TUNEL (terminal deoxynucleotidyl-transferase-mediated dUTP-biotin nick end labeling staining) assay. In order to determine the number of apoptotic and oncotic SGCs, sections were stained with in situ cell death detection kit (Apop Tag Plus Peroxidase In Situ Apoptosis Detection Kit, S7101, Lot: 25050483, Chemicon). Slices were incubated with 20 µg/ml proteinase K. Washing with PBS was performed in every stage. Endogenous peroxidase activity was blocked with 3% H2O2. After washing with PBS, sections were incubated with equilibration buffer and TdT enzyme (77 µl reaction buffer + 33 µl TdT enzyme mix, 1 µl TdT enzyme) at 37°C. Working strength stop/wash buffer (1:10) was applied at room temperature and slices were incubated with anti-digoxigenin conjugate. After washing with PBS the sections were stained with DAB components to detect TUNEL positive cells and then counter stained with methyl green. To assess the distribution of TUNEL positive cells in both glands, we subdivided tissues 1 central and 5 peripheral area in each slice. For each of the tissues TUNEL positive cells were counted and noted[27].

In our study, three different dose groups and their control groups; apoptotic cell number was evaluated statistically. Results of the groups were analyzed by Kruskal-wallis test. All groups were analyzed pairly by Mann-Whitney test to determine the group making the difference. Correlation coefficients are significant when they are lower than 0.05 ($P<0.05$), whereas they are not significant when the values are higher than 0.05 ($P>0.05$).

RESULTS

Dopamine 2 receptor reactivity

Pituitary gland

In the pituitary gland, the histological evaluation of the control group showed a weak positive reaction for D2 in widespread tissue (Figure 1A). In the adenohypophysis, some cells displayed a weak membranous and cytoplasmic immunoreactivity in some area while a group of cells showed no reactivity at all (Figure 1B). In the neurohypophysis, D2 immunoreactivity was negative in most pituicytes but some displayed a weak reactivity. Also, a moderate immunoreactivity was observed in some unmyelinated axons (Figure 1C).

In the low dose treated group (5mg/kg/day), D2 immunoreactivity was similar to the control group (Figure 2A). In the adenohypophysis, some cells displayed a weak membranous and a weak cytoplasmic immunoreactivity in some area while a group of cells showed no reactivity at all (Figure 2B). In the neurohypophysis, D2 immunoreactivity was negative in most pituicytes and some unmyelinated axons showed a moderate reactivity (Figure 2C). No significant difference for D2 reactivity was detected among these two groups.

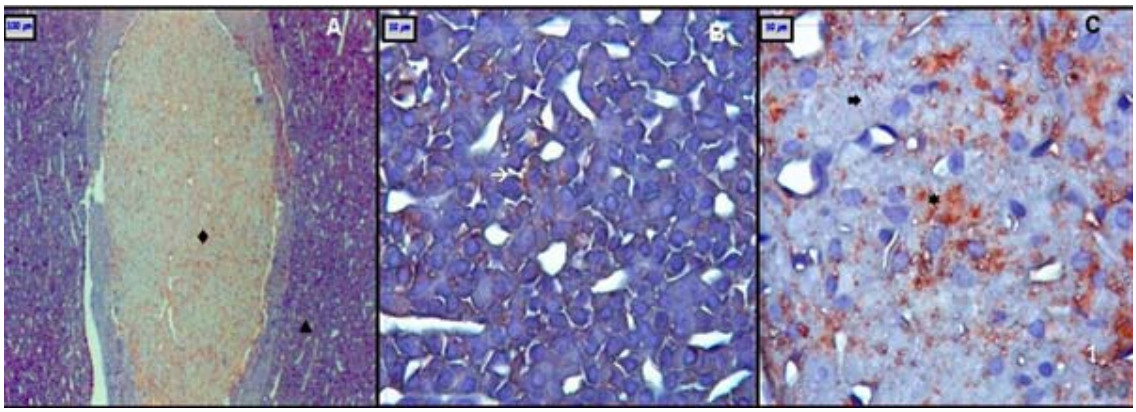


Figure 1: *(A) A weak Dopamine 2 receptor immunoreactivity in the control group of the adenohypophysis. X100. ♦, ▲: immunoreactive areas. These areas shown in B and C with higher magnification, (B) a membranous immunoreactivity in some adenohypophysis cells X400, (C) in neurohypophysis, a weak reactivity in some pituicytes, a moderate reactivity in some unmyelinated axons X400. →: a cell displaying a membranous reactivity, ♦: pituicytes *: axons*

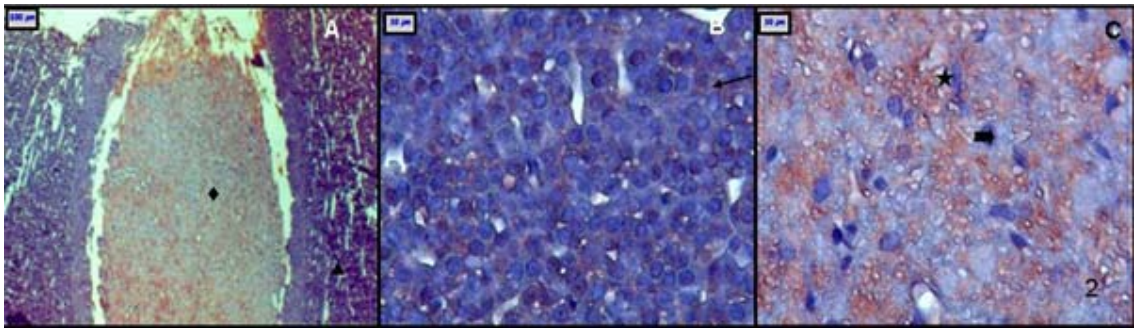


Figure 2: *(A) In the low dose treated group, a weak D2 immunoreactivity in general X100. ♦, ▲: immunoreactive areas. These areas shown in B and C with higher magnification, (B) a weak membranous immunoreactivity in some adenohypophysis cells X400, (C) in neurohypophysis, a weak reactivity in pituicytes, a moderate reactivity in unmyelinated axons X400. →: a cell displaying a membranous reactivity, ⇒: pituicytes *: axons*

In the curative dose treated group (10mg/kg/day), D2 immunoreactivity was slightly increased compared to the low dose treated group (Figure 3A). In the adenohypophysis, the cells nuclei displayed no reactivity while the membranous and cytoplasmic immunoreactivity were observed varying from moderate to strong (Figure 3B). In the neurohypophysis, pituicytes showed a weak staining in some cells but generally there was a negative reactivity while an increased immunoreactivity was observed from moderate to strong in some unmyelinated axons compared to the low dose treated group (Figure 3C).

In the high dose treated group (20mg/kg/day), a widespread and intense D2 receptor immunoreactivity was noticed compared to the curative dose treated groups (Figure 4A). In the adenohypophysis, a strong membranous reactivity was observed in most cells while some cells also exhibited a strong nuclear and cytoplasmic reactivity (Figure 4B). In the neurohypophysis, a strong cytoplasmic D2 immunoreactivity was observed in some pituicytes while others showed no staining. Also, Herring bodies displayed a strong granular form staining in this group (Figure 4C) (Table 1).

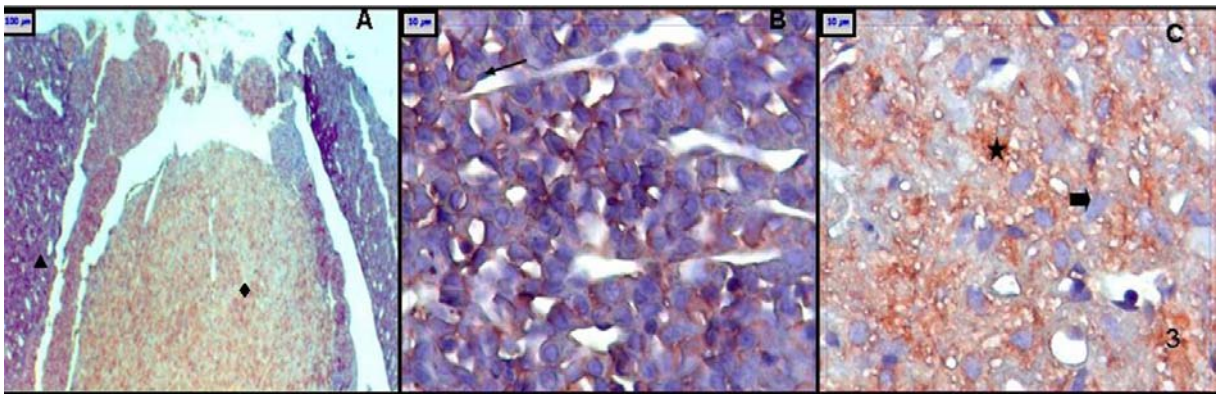


Figure 3: *(A) In the curative dose treated group, a slightly increased D2 immunoreactivity compared to the low dose treated group X100. ♦, ▲: immunoreactive areas. These areas shown in B and C with higher magnification, (B) a moderate to strong membranous immunoreactivity in some adenohypophysis cells X400, (C) in the neurohypophysis, a weak reactivity in pituicytes and a moderate reactivity in unmyelinated axons. X400. ': a cell displaying a membranous reactivity, ⇒: pituicytes ⁎: axons*

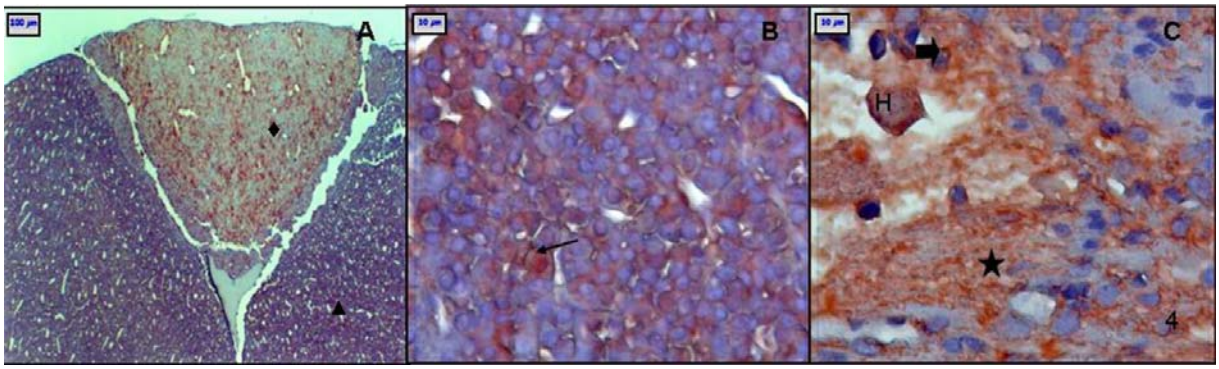


Figure 4: *(A) In the high dose treated group, a widespread and intense D2 receptor immunoreactivity X100. ♦, ▲: immunoreactive areas. These areas shown in B and C with higher magnification, (B) a strong nuclear and cytoplasmic reactivity in some adenohypophysis cell. X400, (C) in neurohypophysis, a strong immunoreactivity in some pituicytes, axons and Herring bodies X400. →: a cell displaying a membranous reactivity, ⇒: pituicytes ⁎: axons H: Herring body*

Table 1: Distribution of reactivity pattern of D2 receptor in pituitary gland

Pattern	**G1 (n=6)**	**G2 (n=6)**	**G3 (n=6)**	**G4 (n=6)**
Adenohypohysis cells	-/+	-/+	++	+++
Neurohypohysis cells	-/+	-/+	++	++
Unmyelinated axons	-/+	-/+	+++	+++
Herring bodies	-/+	-/+	++	+++

Distribution of reactivity pattern in pituitary gland. Negative (-) when the cells/structures were devoid of any detectable expression, slightly positive (+), moderately positive (++), strongly positive (+++). G1=Control group, G2= low dose treated group (5mg/kg/day), G3=curative dose treated group (10mg/kg/day), G4= high dose treated group (20mg/kg/day).

Pineal gland

In the pineal gland, the histological evaluation of the control group showed from weak to moderate D2 immunoreactivity of the gland (Figure 5A). The pinealocytes nuclei displayed no reactivity while some cytoplasm showed a weak D2 immunoreactivity. A moderate D2 reactivity was noticed in interstitial cells and sinusoids (Figure 5B).

In the low dose treated group, D2 receptor reactivity was almost similar to the control group with a minor increase (Figure 6A). The pinealocytes showed a weak to moderate immunoreactivity while some interstitial cells and sinusoids displayed a moderate to strong reactivity (Figure 6B).

In the curative dose treated group, the dopamine 2 reactivity was similar compared to the low dose treated group (Figure 7A). The D2 reactivity was observed moderate in pinealocyte cytoplasm while some cells also displayed a nuclear immunoreactivity. The interstitial cells and sinusoid endothelial cells were stained with an increased intensity compared to the low dose treated group and more like strong (Figure 7B).

All the same, in the high dose treated group, a diffuse and highly increased D2 reactivity was observed in all over the tissue (Figure 8A). Most pinealocytes displayed a strong cytoplasmic immunoreactivity while some cell nuclei also showed a strong reactivity. This immunoreactivity was highly increased compared to the curative dose treated group. Also, similar to these findings, the interstitial cells and sinusoids displayed a strong reactivity (Figure 8B) (Table 2).

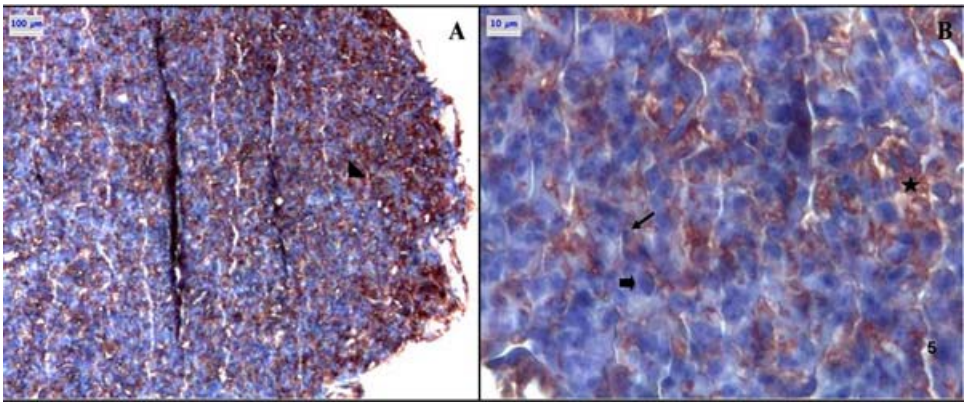


Figure 5: *(A) Dopamine 2 receptor immunoreactivity in the control group of the pineal gland. X100. Δ: immunoreactive area. This area shown in B with higher magnification, (B) a weak immunoreactivity in pinealocyte, a moderate reactivity in interstitiel cells and sinusoids. ⇒: pinealocyte →: interstitiel cell ∗: sinusoid X400.*

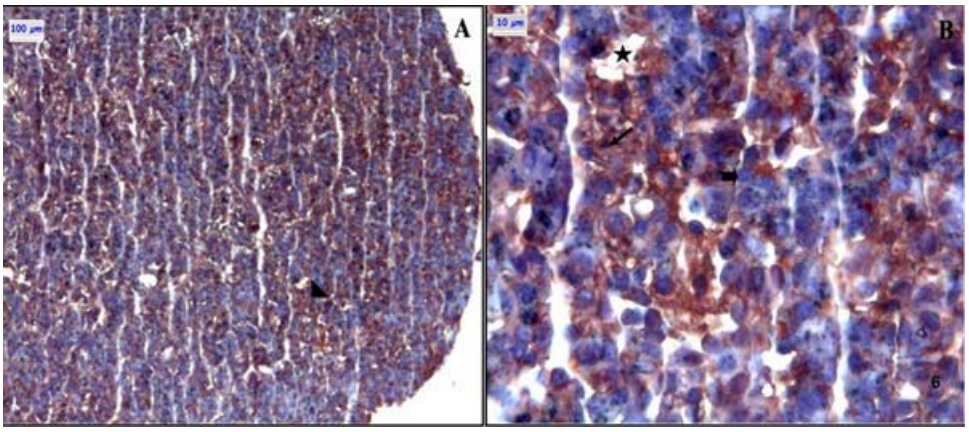


Figure 6: *(A) In the low dose treated group, a moderate D2 immunoreactivity in general X100. Δ: immunoreactive area. This area shown in B with higher magnification, (B) a weak immunoreactivity in pinealocyte, a moderate reactivity in some interstitiel cells and sinusoids compared to the control group. ⇒: pinealocyte →: interstitiel cell ∗: sinusoid X400.*

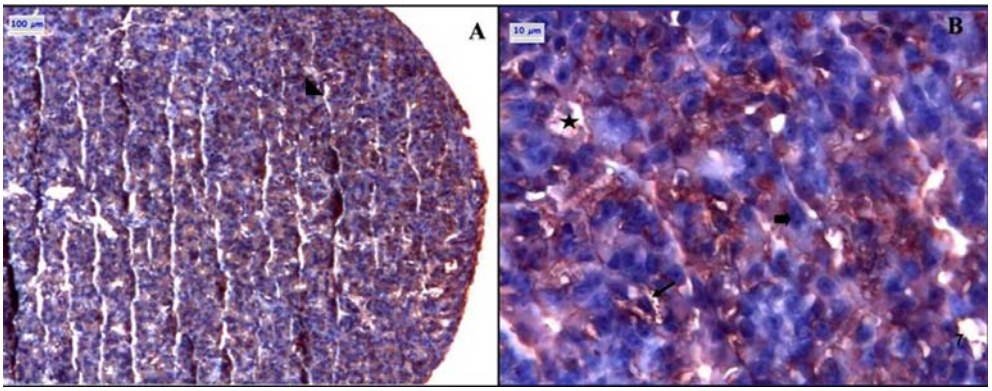


Figure 7: *(A) In the curative dose treated group of the pineal gland, a moderate D2 immunoreactivity in general X100. Δ: immunoreactive area. This area shown in B with higher magnification, (B) a moderate to strong immunoreactivity in pinealocyte, a moderate reactivity in interstitiel cells and sinusoids compared to the low dose treated group. ⇒: pinealocyte →: interstitiel cell ∗: sinusoid X400.*

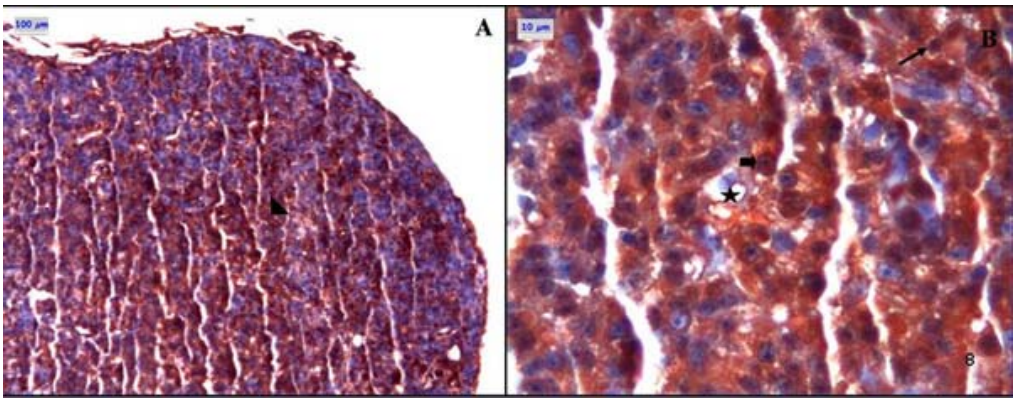


Figure 8: *(A) In the high dose treated group, a strong D2 immunoreactivity X100. Δ: immunoreactive area. This area shown in B with higher magnification, (B) a strong reactivity in pinealocytes, interstitiel cells and sinusoids ⇒: pinealocyte →: interstitiel cell ∗: sinusoid X400.*

Table 2: Distribution of reactivity pattern of D2 receptor in pineal gland

Electron microscopic criteria	G1 (n=6)	G2 (n=6)	G3 (n=6)	G4 (n=6)
Pinealocytes nuclei	-	-	-/+	++
Pinealocytes cytoplasm	+	+	+	++
Interstitial cells	+/++	+/++	++	+++
Sinusoids	+/++	+/++	++	+++

Distribution of reactivity pattern in pituitary gland. Negative (-) when the cells/structures were devoid of any detectable expression, slightly positive (+), moderately positive (++), strongly positive (+++). G1=Control group, G2= low dose treated group (5mg/kg/day), G3=curative dose treated group (10mg/kg/day), G4= high dose treated group (20mg/kg/day).

Ultrastructural findings

Pituitary gland

In the adenohypophysis, the ultrastructural evaluation of the control group revealed a large amount of chromophil cells, active rough endoplasmic reticulum cisternaes (rER), mitochondrions in cross sections and both large and small electron dense secretory granules. The chromophil cells nuclei were located in the center while their granules were dispersed under the membrane. A small number of the chromophobe cells were dispersed between other cells. These cells were observed with eucromatic nuclei and electrone lucent granules. Their mitochondrions were very prominent but other organelles were observed to be less developed. Sinusoidal structure was observed to be normal (Figure 9A).

In the low dose treated group, in the chromophil cells a slight loss of nuclear chromatin and in some chromophobe cell degranulation was detected. Related to the degranulation, active rough endoplasmic reticulum cisternae dilatation was observed. Also, a minimal oedema was observed in chromophobe cells and in pericapillary area (Figure 9B).

In the curative dose treated group, in partially degranulated chromophil cells, dilatation of the rough endoplasmic reticulum cisternae was obvious. Mitochondrions were in normal ultrastructure. The chromophil cells were more electron dense with active rough endoplasmic reticulum cisternaes compared to the control group. In the adenohypophysis, membranous structures were detected in the intercellular areas. Sinusoids were minimally collapsed with large vacuoles in a group of endothelial cells. A slight increase in pericapillary oedema was observed (Figure 9C).

A most prominent degenerative finding was observed in high dose treated group and mostly in chromophil cells. In these cells, rough endoplasmic reticulum cisternaes were highly dilateted with a vacuolary appearance. In some areas, wrapped rough endoplasmic reticulum cisternaes were includes otophagic structures in their centers. Because of this appearance, the process of apoptosis was thought to begin. Lysosomes were increased. Pericellulary oedema with membranous structures was prominent. In this group, myelin figures were observed in the sinusoidal lumens (Figure 9D) (Table 3).

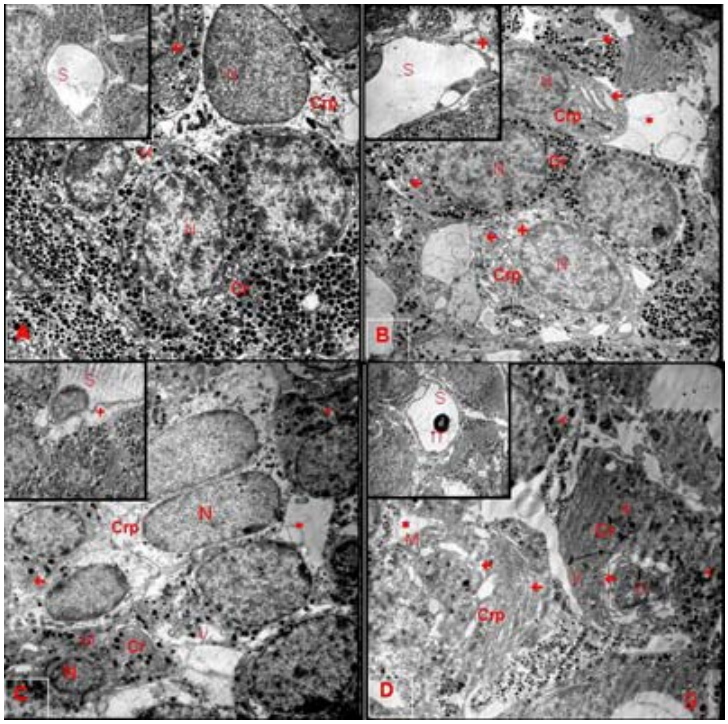


Figure 9: *In the adenohypophysis, (A) control (B) low dose treated group, (C) curative dose treated group, (D) high dose treated group. Sinusoid (S), mitochondrion (M), rough endoplasmic reticulum (←), nucleus (N), chromophobe cell (Crp), chromophil cell (Cr), pericapillary oedema (+), membranous structure in intercellular space (*), intracytoplasmic vacuolary formation (V), myelin figure in sinusoid (↑↑), otophagic structure (O), lysosome (∠), (Uranyl acetate – lead citrate X3000)*

Table 3: Summary of electron microscopic criteria in the pituitary gland

Electron microscopic criteria	**G1 (n=6)**	**G2 (n=6)**	**G3 (n=6)**	**G4 (n=6)**
Loss of nuclear chromatin	0	1	2	3
Degranulation	1	1	4	4
Rough endoplasmic Reticulum cisternaes dilataion	1	2	3	5
Oedema in chromophobe	0	2	3	5
Oedema in pericapillary area	0	2	4	5
Mitochondrial degeneration/cristalizis	0	1	3	5
Presence of myelin and membranous structure	0	1	2	5

Degenerative criteria scoring: '0' indicates no degeneration, '25' indicates maximum degenerative findings and 'n' indicates number of rats. G1=Control group, G2= low dose treated group (5mg/kg/day), G3=curative dose treated group (10mg/kg/day), G4= high dose treated group (20mg/kg/day).

Pineal gland

In the pineal gland, the ultrastructural evaluation of the control group showed the pinealocytes with electron dense cytoplasm and interstitial cells with electron lucent cytoplasm. These cells showed a normal nuclear and organelle ultrastructure. In some interstitial cells lipid droplets were prominent. Sinusoids were normal (Figure 10A).

In the low dose treated group, most prominent finding was the increase of the lipid droplets in interstitial cells. Intercellular dilatation and related to this, vacuolary formations were observed. Also, sinusoidal dilatation was prominent with the presence of the eosinophils in some of these lumens (Figure 10B).

In the curative dose treated group, some pinealocytes had a picnotic nucleus but their organelles were normal. The interstitial cells had an increased number of lipid droplets. Intracytoplasmic oedema and vacuolary formation were prominent. Sinusoidal appearance was similar to the low dose treated group (Figure 10C).

In the high dose treated group, a severe ultrastructural degeneration was observed compared to the other groups. In some areas, apoptotic pinealocytes were detected with otophagic vacuoles and picnotic nuclei. Intercellular oedema with vacuolary formation was also prominent in this group. Intracytoplasmic oedema was also prominent like the curative dose treated group. In this group, sinusoids were highly degenerated with severe pericapillary oedema (Figure 10D) (Table 4).

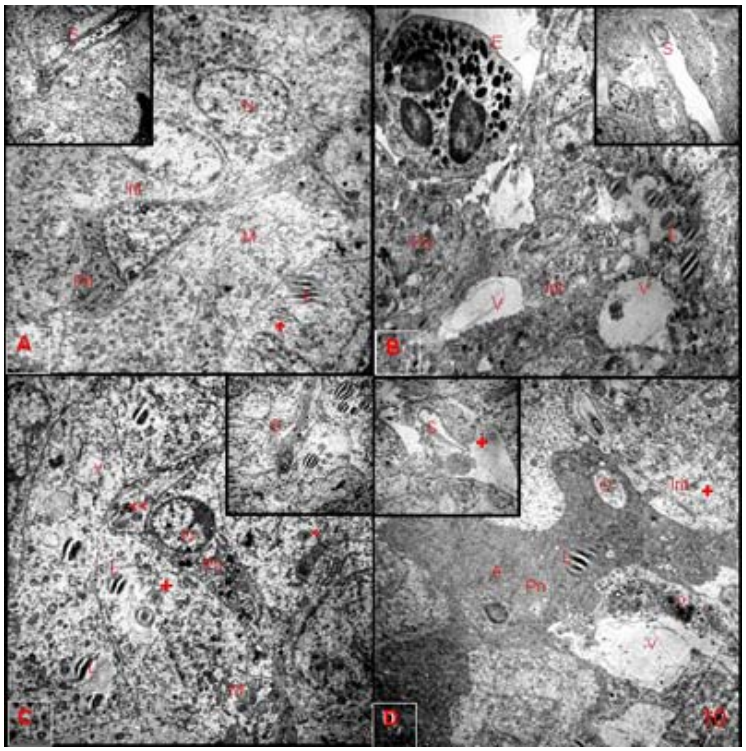


Figure 10: *In the pineal gland, (A) control group; sinusoid (S), mitochondrion (M), rough endoplasmic reticulum (←), nucleus (N), lipid droplet (L), pinealocyte (Pn), interstitiel cell (Int), (B) low dose treated group; eosinophil (E), sinusoid (S), lipid droplet (L), vacuolary formation (V), pinealocyte (Pn), interstitiel cell (Int), (C) curative dose treated group; sinusoid (S), vacuolary formation (V), primary lysosome (∠), secondary lysosome (∠∠), lipid droplet (L), nucleus (N), mitochondrion (M), pinealocyte (Pn), interstitiel cell (Int), intracytoplasmic oedema (+), (D) high dose treated group; apoptotic pinealocyte (A), lipid (L), vacuolary formation with membranous structure (V), pinealocyte (Pn), interstitiel cell (Int), intracytoplasmic oedema (+), otophagic vacuole (O), sinusoid (S), pericapillary oedema (*) (Uranyl acetate – lead citrate X3000).*

Table 4: Summary of electron microscopic criteria in the pineal gland

Electron microscopic criteria	G1 (n=6)	G2 (n=6)	G3 (n=6)	G4 (n=6)
Increase in lipid droplets	1	2	2	3
Intercelular dilatation	0	2	4	5
Vacuol formation	1	2	4	5
Sinusoidal dilatation	0	3	3	5
Presence of eosiniphils	0	2	4	5

Degenerative criteria scoring: '0' indicates no degeneration, '25' indicates maximum degenerative findings and 'n' indicates number of rats. G1=Control group, G2= low dose treated group (5mg/kg/day), G3=curative dose treated group (10mg/kg/day), G4= high dose treated group (20mg/kg/day).

TUNEL findings

In the control and low dose treated groups, adenohypophysis showed a small number of TUNEL (+) cell (Figure 11A,B). In the curative dose and high dose treated groups the number of apoptotic cells were slightly increased (Figure 11C,D). Besides, in all dose groups neurohypophysis was TUNEL (-) in general (Figure 12A-D). In the adenohypophysis, statistical analysis of the data's along these findings 0.05) (Table<show a meaningful difference of apoptotic cell between groups (p< 0.05) (Table 5).

In the pineal gland, all dose groups revealed an apoptotic cell number equal to the control group. Especially, the curative dose and high dose treated group do not show a prominent increase of TUNEL (+) cell (Figure 13A-D). Statistical analysis of the data's along these findings do not show a meaningful difference between groups (p=0,837, p>0) (Table 6).

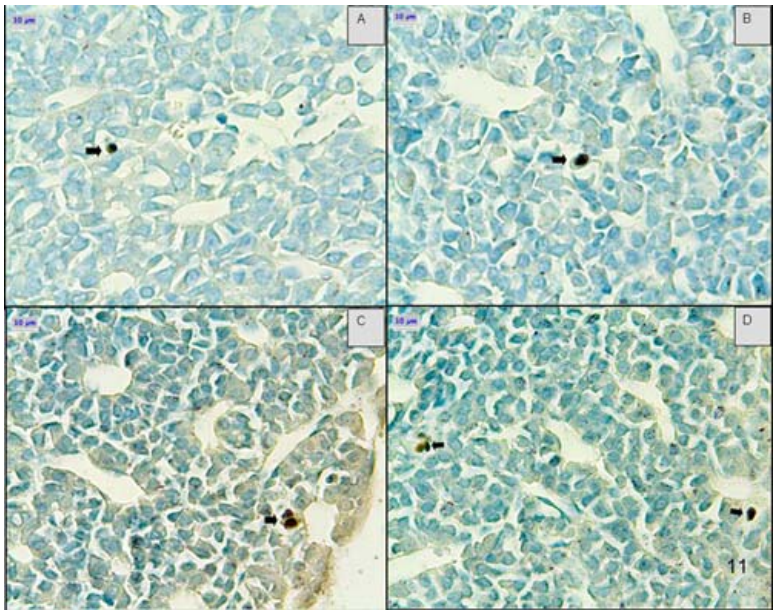


Figure 11: *A small number of TUNEL (+) cell in adenohypophysis (A) control group (B) low dose treated group, (C) curative dose treated group, (D) high dose treated group. ⇒: TUNEL (+) cell*

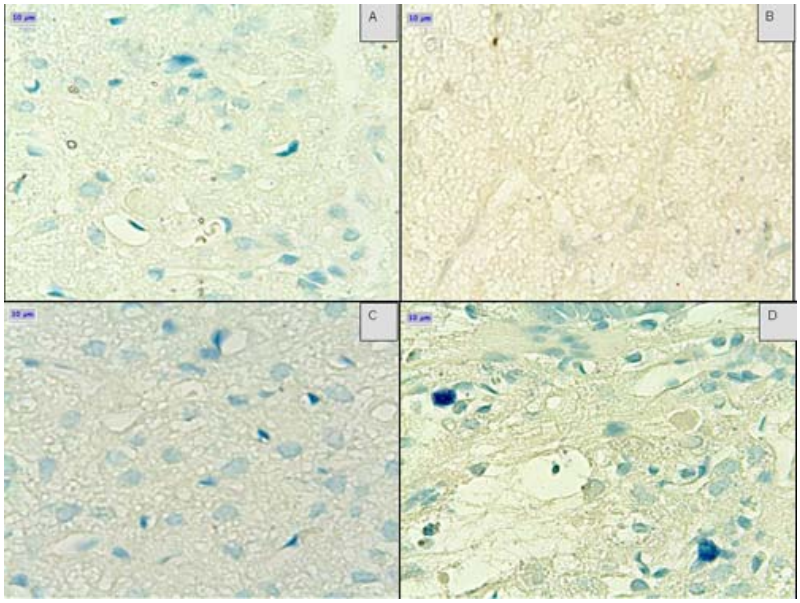


Figure 12: *TUNEL (+) cells were not detected in neurohypophysis (A) control group (B) low dose treated group, (C) curative dose treated group, (D) high dose treated group.*

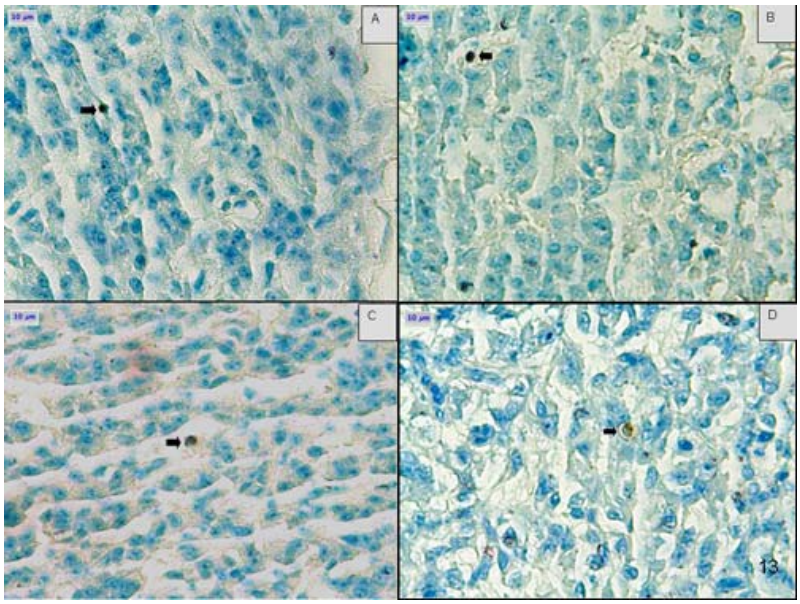


Figure 13: *Some TUNEL (+) cell in pineal gland (A) control group (B) low dose treated group, (C) curative dose treated group, (D) high dose treated group. ⇒: TUNEL (+) cell*

Table 5: Analysis of the apoptotic cell number in the pituitary gland

Groups	**Apoptotic cell number**
Control (n=6)	1,17 ± 0,408
Low dose (5 mg/kg) (n=6)	1,33 ± 0,516
Curative dose (10 mg/kg) (n=6)	2,50 ± 0,548
High dose (20 mg/kg) (n=6)	3,83 ± 0,753
p* < 0,005	

Table 6: Analysis of the apoptotic cell number in the pineal gland

Groups	**Apoptotic cell number**
Control	11,50
Low dose (5 mg/kg) (n=6)	11,50
Curative dose (10 mg/kg) (n=6)	13,50
High dose (20 mg/kg) (n=6)	13,50
p* < 0,837 (p>0,05)	

DISCUSSION

Many biochemical studies have shown the dose-dependent side effects of methylphenidate. The aim of the study was to investigate its possible degenerative effects on pituitary and pineal glands on the misroscopic level by using different methods. Therefore TEM was used to show changes in the ultrastructure, TUNEL was used to show cell death and since D2 receptor was known to show increased expression in cellular toxicity, we performed immunostaining with D2 receptor. Depending on the increasing doses of methylphenidate, increased degeneration was observed in both ultrastructure of glands and also correlated with this result increased number of apoptotic cells were observed with TUNEL method. In high dose methylphenidate treated groups, strong D2 receptor immunreactivity was observed in both glands. Treatment of high dose methylphenidate caused cellular degeneration and toxicity also increased apoptosis was observed in both glands.

The presence of a dopaminergic function in pituitary and pineal glands is well demonstrated by several researchers[9,40,43,44]. Ben-Jonathan[9] reported two major dopamine (DA) pathways of the hypothalamus, the incertohypothalamic and the tuberoinfundibular (TIDA) which also receives projections from periventricular DA neurons. The TIDA system is directly participating in the regulation of prolactin secretion and the perikarya of their neurons are located in the arcuate and periventricular nuclei of the medial basal hypothalamus (MBH)[10,11]. These neurons are divided into two subgroups such as the TIDA, with terminals in the median eminence and pituitary stalk, and the tuberohypophysial with terminal in the neural and intermediate lobes of the pituitary but the anterior pituitary is not innervated[9]. He also revealed that the DA projections to the intermediate and neural lobes are originating from the anterior and central portions of the arcuate nucleus while in the neural lobe, they are in close proximity to pituicytes, magnocellular axon terminals, and precapillary spaces[9]. Also, they are making close contacts with melanocytes in the avascular intermediate lobe. Besides, the portal vasculature is serving as a link between hypothalamic hormones and the anterior pituitary regarding the lack of direct innervation[9]. Meanwhile, Barden et al.[8] reported diurnal variations in the concentrations of DA and norepinephrine (NE), with the highest levels during daylight hours.

The noradrenergic sympathetic system is the major neuronal innervation in the mammalian pineal gland controlling melatonin synthesis but also the dopaminergic system is involved controlling the gland[40]. The presence of dopamine in pineal gland was first described in 1969[6] but since then many researcher have reported the biphasic effects of the dopamine on melatonin production[40,43,44]. Santanavanich et al.[44] reported the distinct effect of the dopamine receptors on pineal function. They demonstrated that, in a concentration-dependent manner, a selective D1-agonist was enhancing N-acetyltransferase (NAT) activity and increasing melatonin level, whereas, a selective D2-agonist was inhibiting NAT activity and decreasing melatonin level[43,44]. Phansuwan-Pujito et al.[40] reported that the perikarya origin of dopamine fibers might be located outside the pineal gland and they are sending their central pinealopetal projection into the gland apart from noradrenergic nerve fibers.

Researchers reported that MPH influences DA content in the brain[33,52] but also the hypothalamic-pituitary-adrenal axis in that it increases the plasma ACTH and

corticosterone levels[31,41,42]. In our study findings were also seen in our high dose treated group in both glands.

Chatterjee-Chakrabarty et al.[19] reported that the chronic MPH administration during adolescence is perturbing pubertal onset, adversely affecting maturation of the female reproductive axis by retarding pituitary LH release and adversely affecting ovarian folliculogenesis. Scaini et al.[45] suggested that acute or chronic exposure to MPH increased creatine kinase activity, an enzyme involved in energy homeostasis, in the brain of young and adult rats.

Reiter[41] has suggested that the pineal gland is involved in the regulation of synthesis and release of pituitary hormones. Researchers have reported that AVP is acting as an important ACTH secretagogue[30,36]. Appenrodt et al.,[4] demonstrated that MPH exerted marked effects on vasopressin and oxytocin levels in plasma, neurohypophysis and hypothalamus.

In our study, related to the increased dose of methylphenidate, dopamine 2 receptor expression was clearly increased in adenohypophysis and neurohypophysis. So, we suggested that the MPH increases the dopamine release in arcuate and periventricular nuclei, with a possible increase of synapses in neurohypophysis pituicytes. The most prominent ultrastructural degenerative finding was the degranulation in chromophil cells and related to this, the rough endoplasmic reticulum cisternaes were highly dilateted with a vacuolary appearance.

Meanwhile, in pineal gland, pinealocytes, interstitiel cell and sinusoids displayed an increased D2R expression with an increased dose of MPH. Thus, we suggest that elevated D2 receptor expression is decreasing melatonin synthesis.

CONCLUSION

We believe that methylphenidate is dose-related inducing ultrastructural changes in pituitary and pineal glands. High dose administration of this drug could influence the functions of these glands. Thus, we suggest that the therapeutic dose of methylphenidate must be kept in minimum level.

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