# Effects of methylphenidate on the mice adrenal glands and lymphoid organs: Results of histochemical, histometrical and histopathological investigations

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## Abstract:

**BACKGROUND:** Considering the wide administration of methylphenidate and also its immunosuppressive effects on different organs, the importance of related microscopic studies is obvious. **OBJECTIVES:** Determining histological effects of methylphenidate on adrenal glands and lymphatic organs in mice. **METHODS:** A total number of 30 adult male Balb/C mice were provided, weighed and divided into one control and two experimental groups. The control group received water by gavages once a day, for 40 days. The experimental groups were orally administered MPH hydrochloride (2mg/kg and 10mg/kg body weight,) respectively. Animals were anesthetized and blood samples were collected through cardiac puncture for analysis of blood cells. Spleen, thymus, lymph nodes and adrenal glands were removed and processed for microscopic studies through hematoxylin and eosin staining. Spleen samples were processed for plasma cell count and staining (label antibody CD138*). The data were analyzed using analysis of variance (ANOVA) and $p<0.05$ was considered significant. **RESULTS:** The changes in lymphoid organs provided morphological evidence for MPH induced immune suppression. Our findings showed increase in the number of megakaryocytes in spleen, neutrophils of peripheral blood and thickness in capsule of thymus and lymph node. Also, thickening of the adrenal cortex and medulla, decrease in the reticularis layer of adrenal cortex and medulla of thymus and decreasing lymphocytes in peripheral blood were significantly observed in experimental groups. Moreover, there were significant changes in serum cortisol. **CONCLUSIONS:** Regarding the resulted data indicating some pathologic, inhibitory and suppressive roles of methylphenidate on immune system and the studied organs, it is suggested that caution should be considered in prescription of this medication.

## Introduction

Methylphenidate hydrochloride is one of the most frequently prescribed pediatric drugs for the treatment of attention deficit hyperactivity disorder. Besides its well-known addictive properties, amphetamine (AMPH) was found to influence the immune functions as a potent immunosuppressor. AMPH and its derivatives (eg. 3, 4- metylendodioxymethamphetamine and fenfluramine) cause a decrease in leukocyte and lymphocyte numbers in the peripheral blood (Freire-Garabal et al., 1991; Connor, 2004; Pacific et al 2001).

Amphetamines are found to suppress cytokine and antibody production, lymph proliferative responses, as well as to decrease in natural killer cells cytotoxicity and induction of cytotoxic T lymphocytes (Freire-Garabal et al., 1991; Connor, 2004; Nunez –Iglesias et al 1996; Richards et al., 2014).

It has been suggested that AMPH can act either directly on peripheral cells or indirectly by affecting neuroendocrine pathway. Acute and chronic AMPH administration also could cause a marked stimulation of the hypothalamic– pituitary – adrenal axis and sympathetic nervous system resulting in the elevation of glucocorticoids (cortisol) and catecholamines (epinephrine and norepinephrine) levels (Wrona et al., 2005; Seiden et al., 1993; Ruginsk et al., 2011; Zuloaga et al., 2015). Glucocorticoids and catecholamine are known to have strong immunomodulating properties (McEwen et al., 1997; Friedman and Irwin 1997).

It has been shown that in vitro effects of glucocorticoids are immunosuppressive, however, it is becoming increasingly evident that the in vivo effects of glucocorticoids are frequently different from in vitro treatment or treatment with synthetic glucocorticoids such as dexamethasone (Fleshner et al., 2001).

Cytogenetic effects have been observed in peripheral lymphocytes in children treated with MPH for 3 months raising questions about the genetic toxicity of this compound. MPH has been found negative in most genetox studies performed; however, no in vitro chromosome aberration data in human lymphocytes has been reported. A chromosomal aberration study in cultured human peripheral lymphocytes has shown that d, 1-methylphenidate (MPH, Ritalin) in concentrations up to 10 Mm neither induced structural nor numerical chromosome abnormalities (Suter et al., 2006).

Bryant et al. implanted 75 mg morphine pellets into mice and observed marked atrophy and reduced cellularity of the spleen and thymus, and an attenuated lymphocyte proliferative response to T-and B-cell mitogens, concavalin-A and bacterial lipopolysaccharide (LPS), respectively (Bryant et al., 1987).

AMPH injection has produced differential effects on leukocyte populations in the peripheral blood. It has also induced a marked lymphopenia together with an increase in LGL- NK cell number, (lymphocyte subset) as well as an increase in granulocyte and monocyte numbers. The total number of WBC remained unaffected. In the spleen, AMPH- induced changes were more uniform – all the leukocyte populations were decreased. Parallel to LGL number increase, NKCC was enhanced in the peripheral blood after injection of AMPH (Schedlowski et al., 1993; Gagnon et al., 1992; Witt et al., 2008).

Proliferation of human peripheral blood

mononuclear leukocytes (PBML) is measured in the presence or absence of amphetamines, and has revealed that the abuse of amphetamines, especially the designer drugs, may adversely affect the activity of immunoregulatory cells and might lead to a compromised immune system in amphetamine abuser (Gagnon et al., 1992).

Opioids and opioid agents are known to have profound immune suppressive effects. Results of the functional assays have shown that acute morphine administration inhibits peripheral blood lymphocyte activity and causes a definite decline in the peripheral blood lymphocyte counts in the rat (Flores et al., 1995; Liang et al., 2016).

The aim of the present study was to determine overall effect of methylphenidate on immune system. The assessments were based on histological and histochemical evaluation in the mice lymphoid organs. In order to investigate the indirect pathway related to the effects of methylphenidate on the adrenal cortex, its histological structure was investigated histometrically.

## Materials and Methods

**Animals and experimental design:** Thirty male adult Balb/C mice, at three months of age and weighing 25-30 g, were randomly divided into 3 groups of 10 animals each. The animals were housed individually in the cages located in a pathogen free, temperature and humidity-controlled colony room which was maintained under a 12 hours day–night illuminating cycle with free access to food and water. Prior to the experiment, the animals were prepared for manipulations during a 1 week acclimatization in the Experimental Medical Research and Application Center of the Faculty of Medicine, Islamic Azad University.

The animals in the first group served as controls and received water without drug. Experimental groups received 2mg/kg and 10mg/kg body weight MPH respectively, as gavages for 40 days (Fazelipour et al., 2014).

**WBC and leukocyte subsets:** At the end of the administration period, blood samples from the hearts of the animals were taken into heparinized (100 IU heparin/ml blood) tubes. From each sample, four blood films were prepared, air dried and stained with Gimsa. Total WBC counts were determined using the hematology analyzer. The percentages of lymphocytes, granulocytes, and monocytes were determined by counting 200 WBC with a microscope Gimsa staining. The number of each leukocyte subset was calculated as WBC number × percentage of individual leukocyte subset.

**Histological and Histochemical studies:** The animals were sacrificed by cervical dislocation and spleens, thymuses, adrenal glands and lymph nodes were removed. Each sample was placed in 10% buffered formalin. Following the fixation, the tissue samples were processed using routine histological techniques. The spleen samples were fixed in alcoholic formalin and processed for plasma cell staining (label antibody CD 138*) (Dako-Denmark) and then splenic plasma cell counting.

For immunohistochemistry procedure, paraffin blocks were sectioned at 3 microns and after several hydration steps by H2O2 and alcohol, usage of antibody including secondary antibody as administered was implemented. In this method the dilution rate of 1:50 has been used.

Splenic plasma cells in unit area (1.44x104µm2 tissue area) were deter-

mined by counting in 10 randomly selected subcapsular white pulp regions using an ocular square micrometer and the results were expressed as cell count/ unit area (pc/UA). Histometrical measurements on the spleens, adrenal glands and thymuses were done with the aid of an ocular linear micrometer. For this purpose, 10 tissue semi- thin sections, 5µm, were taken from each animal and the values were expressed as Mean ± SD.

**Hormone analysis:** Blood samples were collected from the hearts of all animals on day 40 after treatment and then serum specimens collected from these samples were frozen at -20 °C. After collection of all specimens, serum levels of cortisol were measured using ELFA (Enzyme Linked Fluorescent Assay) technique (Fardavard Company-France).

**Statistical analysis:** The results are presented as mean±SD. One way analysis of variance (ANOVA) followed by the post-hoc Tukey test were used for the statistical analysis and a value of $p<0.05$ was considered significant.

## Results

**Spleen:** Mean splenic plasma cell count of the MPH treated animals was lower than those of the controls ($p<0.05$) (Table 1) (Fig. 1). Splenic megakaryocyte counts were relatively increased in the treatment group (Table 1) (Fig. 2).

**Thymus:** Lymphoid tissue of the thymus is organized as a dense cellular cortex with lesser cellular medulla. The thickness of the capsules was increased significantly ($p<0.05$), though for the medulla it was decreased significantly ($p<0.05$). Hassal corpuscles were rarely seen in the thymic medulla (Table 1) (Fig. 3).

**Mesenteric lymphatic nodes:** Mesenteric lymphatic nodes of the control group had larger cortical areas which were occupied by lymphoid follicles, paracortical zones formed by lymphatic cords and medullary areas containing large lymphatic sinuses. Some of the sinuses were with lymphocytes (Fig. 4). The thickness of the capsules of lymph nodes was significantly increased in treated groups ($p<0.05$) (Table1).

**Peripheral Blood:** Percentage of the peripheral blood lymphocytes was declined significantly ($p<0.05$). Significant ($p<0.05$)) increases were also observed in neutrophil cell ratio of the methylphenidate treatment group (Table 2).

**Adrenal glands:** Adrenal glands of the control animals showed typical morphology with a larger cortical area and a centrally located medulla region. Overall thickness of adrenal cortex was increased in the experimental group compared to those of the controls. Statistical analysis showed that methylphenidate with different doses could increase thickness of the glomerulosa and fasciculate layers of the adrenal cortex, and decrease the reticularis layer. On the other hand, the thickness of capsule and also the medullary layer were increased significantly in the experimental groups (10 mg/kg) compared to those of the control group ($p<0.05$). Significant changes in serum cortisol were observed in the MPH treated animals, however, no significant histopathological changes were seen in control specimens ($p<0.05$) (Table 3).

## Discussion

Methylphenidate hydrochloride is one of the most frequently prescribed pediatric drugs for the treatment of attention deficit

Table 1. The histometric characteristics of lymphoid organs in different groups. Group 1 mice received 2 mg/kg water. Groups 2 and 3 were treated with 2 mg/kg and 10 mg/kg MPH. respectively. The values from seven animals are expressed as mean ± SD. Different superscript letters in the same rows indicate a significant difference, $p<0.05$. PC/UA: plasma cell count/unit (1.44.104µm2) tissue area; MC/UA: Megakaryocyte count/unit (1.44.104 µm2) tissue area; TCT, Thymic capsule thickness; TM: Thymic Medulla; CD: capsule diameter.

| Organs/parameters | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Spleen | | | |
| PC/UA | 23.08±9.90[a] | 9.67±4.43[b] | 1.23±0.38[c] |
| MC/UA | 0.42±0.27[a] | 0.63±0.22[a] | 2.46±1.30[b] |
| Thymus | | | |
| TCT(µm) | 115.49±47.19[a] | 281.39 ±77.70[b] | 317.98±105.019[c] |
| TM (µm) | 21969.83±86[a] | 16342.41[b] | 18479.58[c] |
| Lymph node | | | |
| CD (C/UA) | 129,6±23.56[a] | 363.41±86.18[b] | 267.18±59.621[c] |

Table 2. The Peripheral blood cells. Group 1: mice received water without drug. Group 2 and 3 were treated with 2 and 10 mg/kg MPH, respectively. The values from seven animals are expressed as mean ±SD. Different superscript letters in the same rows indicate a significant difference, $p<0.05$.

| Organs/parameters | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Neutrophil (count/unit) | 9.33± 4.13[a] | 25.33± 10.25[b] | 13.33 ± 3.20[a] |
| Lymphocyte (count/unit) | 88.33± 4.80[a] | 74.33 ± 9.99[b] | 83.33 ± 3.88[a] |
| Monocyte (count/unit) | 1± 0.89[a] | 0[a] | 0.67± 1.63[a] |
| Eosinophil (count/unit) | 1.5± 1.22[a] | 0.33± 0.81[a] | 1.67± 1.96[a] |

Table 3. The histometric characteristics of adrenal gland and cortisol in different groups. Group 1: mice received water without drug. Group 2 and 3 were treated with 2 and 10 mg/kg MPH, respectively. The values from seven animals are expressed as mean ±SD values. Different superscript letters in the same rows indicate a significant difference, $p<0.05$. ZGL: zona glomerulosa layer, ZFL: zona fasciculata layer, ZRL: zona reticularis layer, ML: Medullary layer.

| Organs/parameters | Group 1 | Group 2 | Group 3 |
|---|---|---|---|
| Capsule (µm) | 15.42±2.43[a] | 9.06±2.70[b] | 14.64±5.50[a] |
| ZGL (µm) | 28.28±7.70[a] | 31.70±6.36[a] | 47.76±10.23[b] |
| ZFL (µm) | 109.84±26.99[a] | 194.26±82.26[b] | 210.08±40.99[b] |
| ZRL (µm) | 172.97±52.43[a] | 83.44±17.20[b] | 114.78±32.12[b] |
| ML (µm) | 918.45±123.99[a] | 924.22±286.56[a] | 996.60±243.47[b] |
| Cortisol (µg/dl) | 2.95 ±1.12[a] | 5.23 ±2.14[b] | 7.1 ±2.94[c] |

hyperactivity disorders.

From the results of the study it could be deduced that amphetamines as methylphenidate, can influence the peripheral blood and make significant changes in the number of leukocytes compared to the control group. Our study indicated that the number of lymphocytes, involved in immune functions, showed a significant decrease and the number of neutrophils was increased significantly compared to the control group, whereas the total percentage of leukocytes remained unaffected.

In a study by Gagnon et al. the effect of amphetamines on proliferation of leuko-

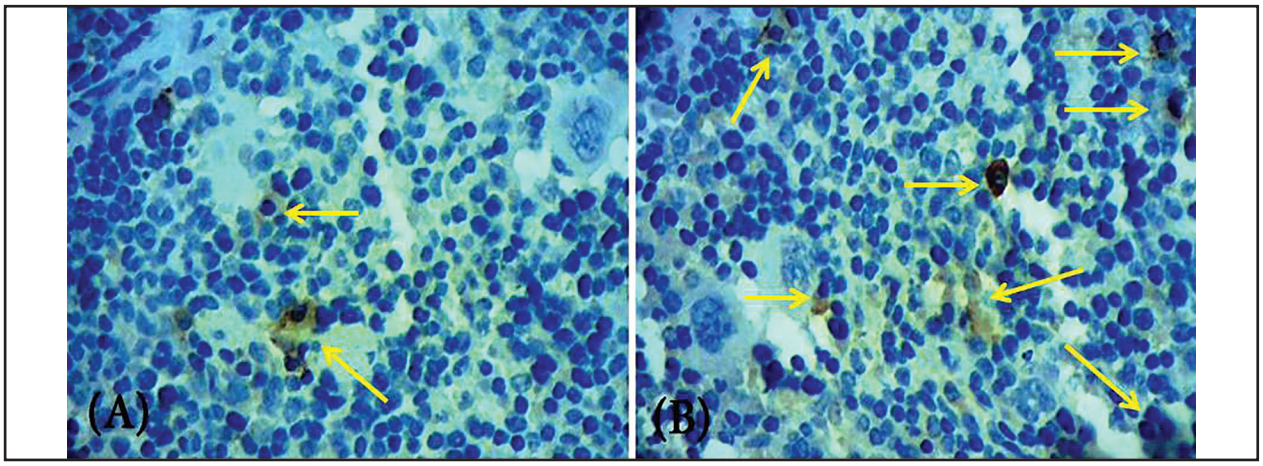


Figure 1. Photomicrograph of spleen (H&E, ×400); (A): Plasma cells, shown and stained with CD 138 antibody (arrows), in control group (B): Increased plasma cells, stained with CD 138 antibody (arrows), in experimental groups.

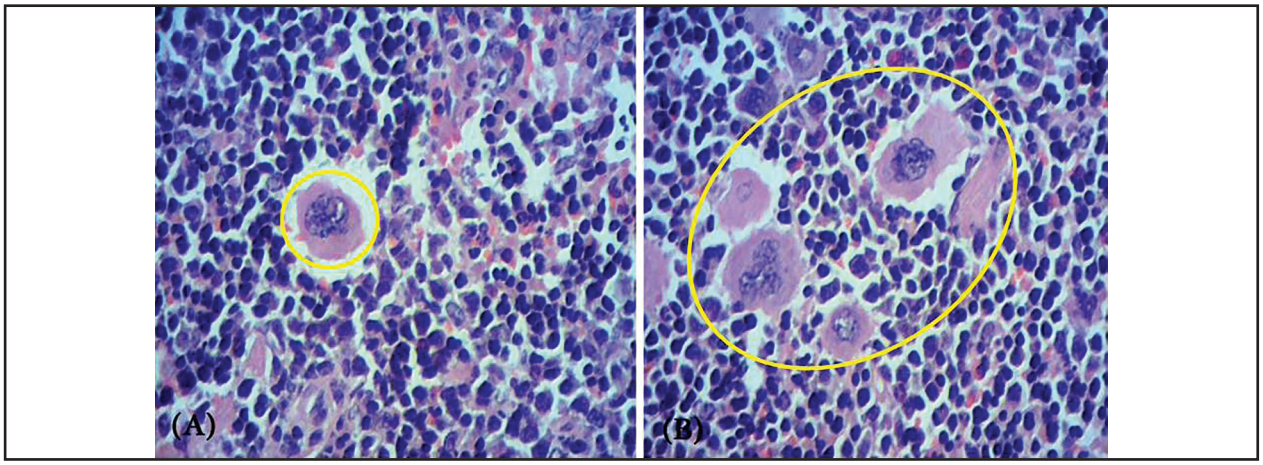


Figure 2. (A): Photomicrograph of a megakaryocyte (circle), in control group (H&E, ×400). (B): Photomicrograph of more megakaryocytes (ellipsoid), in experimental groups (H&E, ×400).

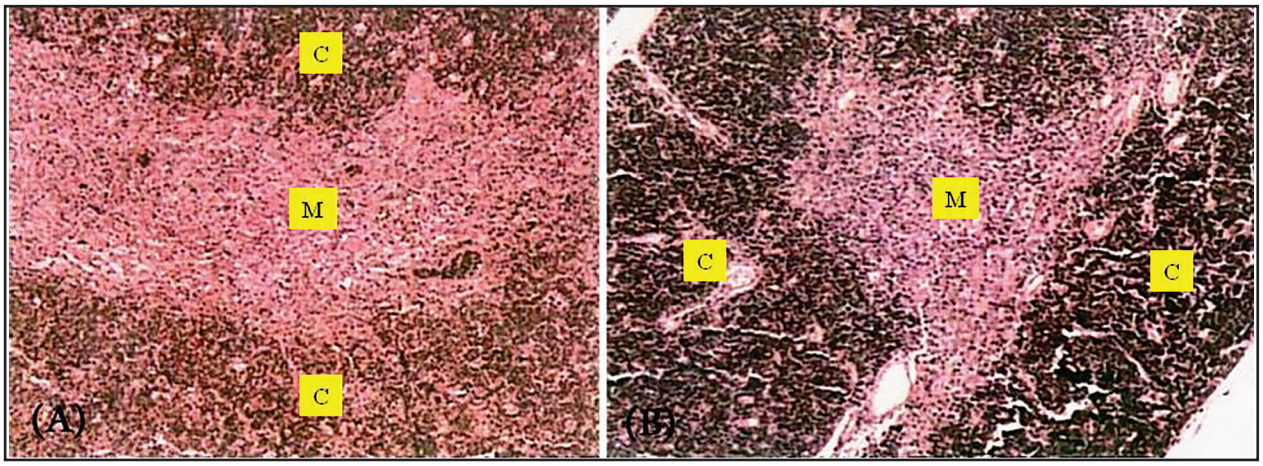


Figure 3. Photomicrograph of a thymic lobule (H&E, ×100); (A): Medullary thymus in control group with large medullary region (M); cortex (C); (B): More limited medullary region (M) in experimental groups; cortex (C).

cytes has been shown. Also different studies show a variety of effects on leukocytes of the peripheral blood and similar results have been reported by other investigators. Many studies have revealed that amphetamines can affect proliferation of immune

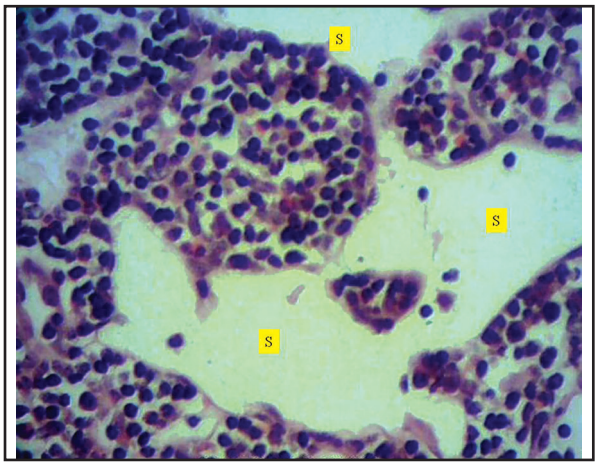


Figure 4. Photomicrograph of lymph node (H&E, ×400); Dilatation in medullary sinuses of lymph node (S) in experimental groups.

cells in other organs (Gagnon et al.,1992; Bredholt et al., 2013).

It has been stated that methylphenidate structurally resembles cocaine, a narcotic, and it is expected to have a preventive effect on immune system (Rofael et al., 2003).

Immunosuppression could be done by a variety of mechanisms which, besides the decrease of leukocytes, it could be through increasing catecholamines (Schedlowski et al.,1996).

Our study indicated that methylphenidate could influence the adrenal gland inducing thickness of zona glumerolosa and zona fasciculata in the adrenal cortex. The significant increase in the thickness of zona fasciculata indicated increase in the corticosteroids and subsequently significant increase in the cortisol, and subsequent suppression of the immune system.

Moreover, the thickness of medulla of the adrenal gland, responsible for synthesis and secretion of catecholamines showed a significant increase compared to the control group, which it was in turn another factor in suppression of the immune system in the present study. The serum cortisol level in experimental groups showed a significant increase which could be an indicator of suppression in the immune system due to its inhibitory effect on proliferation of lymphocytes. This was in agreement with another study that showed effects of cocaine, with a similar structure to methylphenidate, on the adrenal gland and increase in the cortisol that induced suppression of the immune system (Rofael et al., 2003).

In the present study a significant decrease in the number of splenic plasma cells with a pattern of greater decrease in higher doses was seen. On the other hand, a significant increase in the number of megakaryocytes in spleen was observed. This increase showed that hematopoiesis could take place outside the bone marrow.

From the results of this study, preventive effect of methylphenidate on immunity could be deduced by decrease in plasma cells and also decrease in lymphocytes in different lymphatic organs.

Also, a study on the effect of morphine on the adrenal gland with similar findings to methylphenidate has been reported (Salback et al., 2001; Vinson and Brennan., 2013).

Investigations have shown that the effects of different drugs, opioids and methylphenidate on the immune system could be induced through three mechanisms including: Direct effect of these substances on immune cells in peripheral blood, effect on hypothalamic pituitary adrenal axis which indirectly induces cortisol increase and 3 activating sympathetic nervous system which causes circulating levels of epinephrine from the adrenal medulla as well as norepinephrine from sympathetic nerve terminal leading to increase in the catecholamines (Wang et al., 2002).

In other studies increase in the catecholamines due to usage of amphetamines has

been observed. Methylphenidate could also affect immune cells in different organs by preventing their proliferation.16 Therefore, the present study indicated that methylphenidate influenced the spleen by significant decrease in plasma cells. Considering the spleen as the largest lymphatic organ and the only responsible organ for blood filtration, and so providing defense against blood antigens and microorganisms, reduction of plasma cells might represent an immunosuppression.

On the other hand, significant increase in megakaryocytes in spleen, indicating a pathologic condition, implies inhibitory role of methylphenidate on immune system function. Also, increased catecholamine release has been associated with suppression of natural killer cell function and altered lymphocyte function (Schedlowski et al., 1993).

An analysis of the effects of amphetamine on proenkephalin-derived peptides in brain areas and immune cells in rats showed that acute as well as a repeated amphetamine treatment decreased the concanavalin-A-induced lymphocyte proliferation, concomitantly with an increase of free metenkephalin in nucleus accumbens, prefrontal cortex, spleen, thymus and splenic macrophages (Assis et al., 2006).

The possibility that the adrenergic system may stimulate the NK cells activity in the peripheral blood was confirmed by the studies of Schedlowski et al. (1993) and Benshop et al. (1997). Moreover, treatment with β-adrenoreceptor antagonist inhibits an increase in both circulating NK cells and NKCC elicited by stress or cathecolamine infusion (Glac et al., 2006).

The present study showed that lymphoid tissue of the cortex in thymus was denser with fewer cellular portions than medulla. Besides, the thickness was increased in capsule while decreased in medullar region in the experimental groups due to effect of amphetamines on thymus. In lymph nodes also the thickness of capsule in experimental groups compared to the control group due to the effect of amphetamines was observed. In conclusion, it could be suggested that caution should be considered in prescription of amphetamines due to their effects on proliferation of immune cells in various organs, peripheral blood and also on the most defensive organ against blood pathogens i.e. spleen and possibly substitution with other medications.

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# اثرات متیل فنیدیت بر روی غدد فوق کلیوی و اندام‌های لنفاوی؛ نتایج بررسی‌های هیستوشیمیایی، هیستومتریک و هیستوپاتولوژیک

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## چکیده

**زمینه مطالعه:** اســتفاده گسترده از متیل فنیدیت و اثرات تضعیف کننده ایمنی آن بر روی اندام‌های مختلف، اهمیت مطالعات میکروسکوپیک در این رابطه را ضروری می‌سازد. **هدف:** تعیین اثرات متیل فنیدیت بر روی غدد فوق کلیوی و اندام‌های لنفاوی در موش می‌باشــد. **روش کار:** ۳۰ ســر موش نر بالغ از نژاد Balb/C تهیه و به یک گروه شاهد و دو گروه تجربی تقسیم گردیدند. گروه شاهد تنها آب آشامیدنی و به گروه های تجربی متیل فنیدیت هیدروکلراید به میزان ترتیبی ۲ و ۱۰ میلی گرم به ازای هر کیلوگرم وزن بدن روزانه بمدت چهل روز خورانده شد. سپس حیوانات را بیهوش و از قلب آنها نمونه‌های خون تهیه گردید. طحال، تیموس، عقده‌های لنفاوی و غدد فوق کلیوی برای مطالعه میکروسکوپی بروش هماتوکسیلین و ائوزین جدا گردیدند. جهت شمارش پلاسما ســل‌های طحال از label antibody CD۱۳۸× اســتفاده شــد. داده ها به روش آنالیز واریانس مورد تجزیه و تحلیل قرار گرفته و $p<۰/۰۵$ بعنوان سطح معنی داری تلقی گردید. **نتایج:** تغییرات در اندام‌های لنفاوی بیانگر اثرات تضعیف کننده ایمنی ایجاد شده می‌باشند. افزایش تعداد مگاکاریوسیت ها در طحال، افزایش نوتروفیل‌های خون محیطی و ضخامت کپسول تیموس و عقده‌های لنفاوی، همچنین ضخیم شدگی قشر و بخش مرکزی غده فوق کلیه مشهود بود. بعلاوه کاهش ناحیه رتیکولاریس در قشر غدد فوق کلیه و بخش مرکزی تیموس و نیز کاهش لنفوسیت‌ها در خون محیطی در گروه های تجربی مشاهده گردیدند. همچنین تغییرات معنی‌داری در کورتیزول سرم نیز مشاهده گردید. **نتیجه گیری نهایی:** با توجه به اطلاعات بدست آمده که نشان دهنده برخی اثرات پاتولوژیک و نقش ممانعتی و تضعیف گر متیل فنیدیت بر روی دستگاه ایمنی و اندام‌های مورد مطالعه می‌باشد، پیشنهاد می‌گردد در تجویز این دارو احتیاط لازم صورت گیرد.

**واژه های کلیدی:** غدد فوق کلیوی، هیستوپاتولوژی، اندام‌های لنفاوی، متیل فنیدیت، مورفومتری

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