Dose-dependent ultrastructural changes in rat cornea after oral methylphenidate administration

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ABSTRACT

الأهداف: التحقق من تغيرات البنية الفوقية للجرعة–المستقلة في قرنية الجرذ بعد تلقي عقار ميثيلفينيديت عبر الفم.

الطريقة: أجريت هذه الدراسة بقسم التشريح بكلية الطب بجامعة غازي في الفترة ما بين مارس إلى مايو 2005م على إجمالي عدد 27 أنثى الجرذ من نوع ويستار ألبينو. قسمت الجرذان إلى ثلاث مجموعات مختلفة حسب الجرعة (20mg/kg، 10mg/kg، 5mg/kg) ومجموعات التحكم لديهم. تمت معالجتهم بعقار ميثيلفينيديت عن طريق الفم وتمت إزالة نسيج العين لإجراء الدراسة بالمجهر الإلكتروني.

النتائج: لاحظنا جميع الخلايا ولكن بشكل بارز أظهرت ظهارة الخلايا القاعدية وجود متغيرات تنكسية للجرعة–المستقلة مثل الأجسام البيبتيكية وتكاثف الكروماتين. في السداة، كان أكثر دليل وجدناه هو زيادة ليف الكولاجين، إلى جانب تغيرات الجرعة– المستقلة المتعلقة بالعملية البيبتيكية والتي هي عبارة عن تكاثف الكروماتين في نواتهم، كما تبين أيضاً كثافة تراكم مادة الإلكترون وخاصة الوذمة في السيتوبلازم. كما لوحظ أيضاً خطوط الخلية البطانية وتمزق مركبات الموصلي وتفجج في سيتوبلازم الخلية وكراتيلوزيس الميتوكندريا مع نشاط خشن لحوض شبكة هيولي.

خاتمة: يحرض عقار ريتالين التنكس الواضح خاصة في الخلايا الظهارية مع الجرعات الزائدة. لدى تنكس التأليف الجزيء لتركيب الخلية مع التليف السدوي تأثير سلبي على تجفاف القرنية. وعلى ضوء هذه النتائج، نعتقد أن العلاج بجرعات الريتالين يحتاج إلى إبقاءه على شكل محدود وذلك للحفاظ على صحة القرنية والبنية الفوقية والفسيولوجية ذات الصلة.

Objective: To investigate dose-dependent ultrastructural changes in rat cornea after oral methylphenidate (Ritalin©) administration.

Methods: This study was conducted in the Department of Anatomy, Gazi University Faculty of Medicine, Ankara, Turkey between March and May 2005, with a total of 27 female prepubertal Wistar albino rats, divided into 3 different dose groups (5mg/kg, 10 mg/kg, 20 mg/kg), and their control groups. They were treated orally with methylphenidate, and eye tissue was removed to process for electron microscopic studies.

Results: We observed that all cells, and prominently basal cells of the corneal epithelium show dose-dependent degenerative changes such as apoptotic bodies, chromatin condensation, and ondulation in their nuclei and crystolysis of the mitochondrion. In the stroma, the most evident finding was the increase of the collagen fiber. In addition to dose-dependent changes related to the apoptotic process, which is chromatin condensation in their nuclei, electron dense material accumulation, and pericellular edema in the cytoplasm were also seen. In the endothelial cell lines, disruption of the junctional complexes, vacuolization in the cell cytoplasms, and crystolysis of the mitochondrion's with rough endoplasmic reticulum cisternae activity were observed.

Conclusion: Ritalin© is inducing an evident degeneration, especially in epithelium cells with increasing doses. Ultrastructural cell organelle composition degeneration with stromal fibrosis has a negative effect on cornea dehydration. In light of these findings, we believe that the Ritalin© treatment doses need to be kept to a minimum to maintain healthy cornea ultrastructure and related physiology.

Saudi Med J 2008; Vol. 29 (4): 498-502

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Received 2nd October 2007. Accepted 9th March 2008.

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Disclosure: This work was supported by a grant from Gazi University Research Foundation, Ankara, Turkey (project code no. 01/2004-68). All the drugs mentioned in the manuscript are provided by the researchers, and are not provided by any drug company.

Attention-deficit hyperactivity disorder (ADHD) is the most prevalent adolescent psychiatric disorder affecting 3-7% of children, and characterized by a persistent pattern of alteration in one or more of the following behaviors: inattention, hyperactivity, and impulsivity.[1] Methylphenidate, commonly known by brand name as Ritalin©, and amphetamine are the most frequently used treatments for ADHD, and like cocaine, they are the most commonly abused stimulant drugs.[2-5] This psychostimulant binds and inhibits the dopamine transporter,[6,7] like cocaine,[8] and increases interstitial dopamine levels in rats. In neuronal and non-neuronal tissues, the membrane-bound receptors such as monoamine receptors, are mediating several responses to the endogenous catecholamines, which are epinephrine, norepinephrine, dopamine, and serotonin.[9] Cavalotti et al[10] showed D1-like and D2-like dopamine receptors in sections of the rabbit cornea suggesting their possible role in the control of corneal functions while other researchers investigated their role on the retina.[11]

The use of the Ritalin© for the treatment of attention deficit/hyperactivity disorder has increased dramatically in recent years, since its first use in the 1950's.[12] Since then, only few studies have been made on the potential for serious side effects, such as mutagenicity and carcinogenicity, in animals or in humans.[12] The great concern of the treatment of methylphenidate is that since in children the central nervous system (CNS) continues its maturation and growth well into the second decade of life, the risk therefore exists for adverse interactions between the developing CNS as well as cornea and long-term psychostimulant treatment.[13-15] Our aim is to investigate dose-dependent ultrastructural changes in the rat cornea, to demonstrate possible toxicity of the long-term and high dose use of the methylphenidate.

Methods. This study was conducted in Gazi University Faculty of Medicine, Department of Anatomy, Ankara, Turkey between March and May 2005. The experimental protocol was approved by the local Ethical Committee for animal studies. In the experimental protocol, 27 female Wistar albino rats with a weight of 110 g (±20), divided into 3 different dose groups (5mg/kg, 10 mg/kg, and 20 mg/kg) and their control groups, were used. Prepubertal (35 day-old) rats, as indicated in the literature, were treated orally with methylphenidate hydrochloride (MPH) dissolved in saline solution for 5 days per week over 3 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 hours to 20:00 hours) 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 anesthetized 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 eye tissue was removed.

Electron microscopic study. All tissues 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 (OsO_4), 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 (benzyl dimethyl amine), and dibutylphitalate. Semi-thin sections were cut and stained with toluidine 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).

Results. *Ultrastructural findings.* In the control group, the endothelium cell volume and organelle composition appears in their normal structure. The ultrastructural evaluation of the corneal stroma shows the long spindle-shaped keratocytes with their oval nuclei and cytoplasm in their normal structure. Collagen fibers were forming bundles, which were layered circular and longitudinal over the corneal stroma. New synthesized collagen fibers were observed around the cell, and accepted as an indicator of active keratocyte existence. The corneal epithelium appears in its normal ultrastructural appearance with normal organelle composition and junctional complexes. In the low dose treated group (5mg/kg/day), the ultrastructural evaluation of the corneal endothelium cells show minimal hypertrophy with normal structure of the junctional complexes, and crystolysis of the mitochondrion was seen in these cells. The most remarkable changes observed in this group are the decrease of the keratocyte volume due to collagen fiber increase in the corneal stroma. An abundant amount of new synthesized collagen fibers was interpreted as an indicator of high activity of the cells. The nucleus shape was damaged with the degeneration of the cytoplasmic organelle structure. In addition, we observed crystolysis in some areas of the mitochondria and dilatation of the rough endoplasmic reticulum (rER) cisternae related to increased metabolic activity (**Figure 1**). The corneal epithelium shows

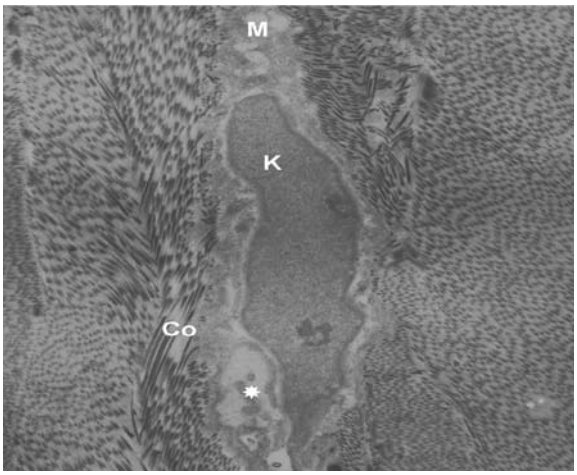

Figure 1 - Low dose treated group: M - crytolysis of the mitochondrion, K - keratocyte with decreased volume, CO - increased collagen fibers, * – dilatation of the endoplasmic reticulum cisternae, (Uranyl acetate-lead citrate X3000).

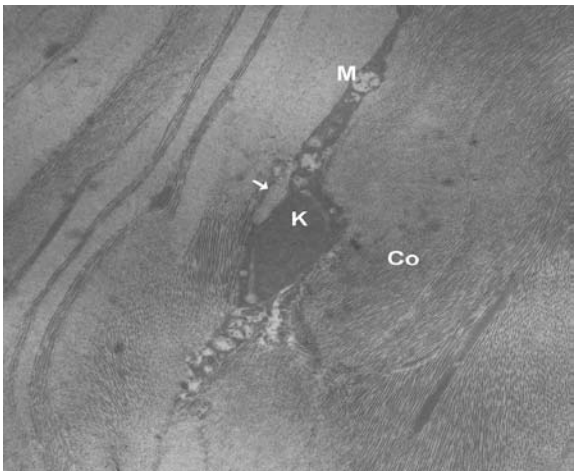

Figure 3 - High dose treated group: M - mitochondrion, CO – collagen, V - vacuoles, K - keratocyte in the apoptotic process, → – detachment between the cytomembranes of the nuclear membrane, (Uranyl acetate-lead citrate X3000)

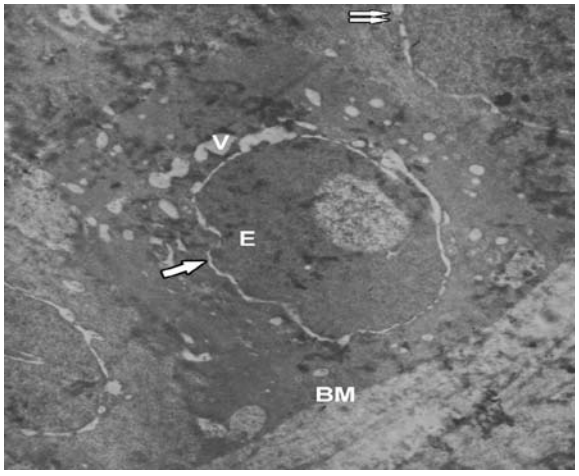

Figure 2 - Low dose treated group: E - epithelium, M - crystolysis of the mitochondrion, single arrow – detachment between the cytomembranes of the nuclear membrane, double arrow – disruption of the epithelial junctional complexes, V - vacuolization, BM - basement membrane, (Uranyl acetate - lead citrate X3000).

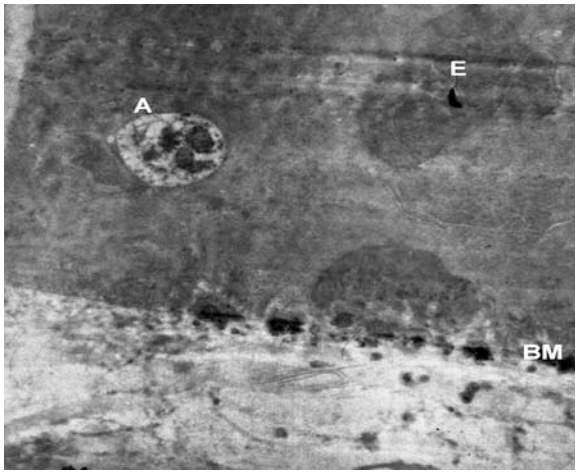

Figure 4 - High dose treated group: E - epithelium, BM - basement membrane, A - apoptotic body, (Uranyl acetate - lead citrate X3000).

evident degenerative changes such as vacuolization of the basal cells, disruption of the junctional complexes, and detachment between the cytomembranes of the nuclear membrane while surface cells and intermediary cells appear in their normal structure (**Figure 2**). In the curative dose treated group (10mg/kg/day), the corneal endothelium cells show a similar structure compared to the low dose treated group with highly active rER. Crystolysis of the mitochondrion was seen in some cells with hypertrophy of the cells. The junctional complexes show normal structure. The collagen fiber increase in the corneal stroma was observed similar to the low dose treated group. Some keratocytes show activity as in the low dose group, however, others show increased activity. We also observed the active cells rER cisternae highly dilatated. The cell activity was more increased along these observations and crystolysis of the mitochondria was also prominent in this group. The corneal epithelium degenerative changes were increased compared to the low dose treated group. Especially in some areas, basal cell junctional complexes disruption was observed and these sites show large vacuolizations in the cells that

were localized in the perinuclear site of the cytoplasm. Other degenerative changes, such as disruption of the junctional complexes, crystolysis of the mitochondria, and detachment between the cytomembranes of the nuclear membrane were also seen in this group similar to the low dose treated group, however, these changes were observed in all layers of the epithelium (data not shown). In the high dose treated group (20mg/kg/day), in the endothelial cell lines, we observed degenerated cells with disruption of their junctional complexes, and accepted this appearance as an indicator of the lysis and in some cells, crystolysis of the mitochondria were also seen with activity of the rER cisternae. The collagen fiber increase in the stroma was very prominent. A group of keratocytes was found to be swollen with detachment between the cytomembranes of the nuclear membrane and intracellular edema. Cellular organelle composition was not observed, and this finding observed in many cells was considered as a necrotic process. While a group of cells show changes related to apoptotic process, which is chromatin condensation in their nuclei, electron dense material accumulation, and crystolysis of the mitochondria in some area was also observed (**Figure 3**). The corneal epithelium, especially basal cell shows highly degenerative changes compared to other groups. A group of basal cell lines contained apoptotic bodies (**Figure 4**). We also observed in cells of all layers, chromatin condensation and ondulation in their nuclei. Crystolysis of the mitochondrion was also seen in this group.

Discussion. Methylphenidate is routinely used for the treatment of ADHD.[16] The pharmacodynamic of this psychomotor stimulant drug is primary activation of the noradrenergic and dopaminergic systems and has similar effects to cocaine and amphetamine.[16] Pilon and Scheiffle[17] reported a case of ulcerative keratitis associated with crack-cocaine abuse, and suggested that the practitioners should be aware of possible toxic effects of such agents on ocular tissues, such as corneal epithelial disruption, and stromal ulceration on cornea. Like cocaine in their case, Lu et al[18] reported another case of Ritalin-associated cataract and glaucoma.

The catecholamines dopamine, norepinephrine, and epinephrine are synthesized from the L-amino acid tyrosine, and are present in several nervous system areas, including the retina, uvea scleral tissue, and cornea.[9,19,20] D1-like and D2-like receptors subfamilies of the dopaminergic receptors are members of the G-protein-coupled receptor superfamily. While the D1-like receptors can stimulate adenylyl cyclase, the D2-like receptor subtypes inhibit adenylyl cyclase, and also stimulate mitogenesis and extracellular acidification.[10] Several researchers demonstrated the presence of the D1-like and D2-like receptors using autoradiographic techniques or freshly fixed human corneal tissue.[9,10] Cavalotti et al,[10] reported that the dopamine system is controlling the corneal function. Crosson et al,[20] suggested that chloride secretion in the rabbit corneal epithelium can be modulated by preterminal dopamine receptors located on the sympathetic nerve fibers, therefore, dopamine stimulation appears to be a serial process mediated by the release of norepinephrine from sympathetic nerve terminals in the epithelium.

In our study, we observed that all cells, prominently basal cells of the corneal epithelium show dose-dependent degenerative changes such as apoptotic bodies, chromatin condensation, and ondulation in their nuclei and crystolysis of the mitochondrion. In the stroma, the most evident finding was the increase of the collagen fiber. In addition to dose-dependent changes related to apoptotic process, which is chromatin condensation in their nuclei, electron dense material accumulation and pericellular edema in the cytoplasm were also seen. In the endothelial cell lines, disruption of the junctional complexes, vacuolization in the cell cytoplasm, and crystolysis of the mitochondria with rER cisternae activity were observed. As a result, Ritalin© is inducing an evident degeneration especially in epithelium cells, and this degeneration is occurring in the stroma and endothelial layer with increasing doses. Ultrastructural cell organelle composition degeneration with stromal fibrosis has a negative effect on cornea dehydration.

In the light of these findings, we believe that the Ritalin© treatment doses need to be kept at a minimum to maintain healthy cornea ultrastructure and related physiology.

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