The effect of ozone (O3) therapy in addition to high dose methylprednisolone on hypoxia inducible factor-1 alpha (HIF-1α) expression in rabbit cornea

Tavşan korneasında yüksek doz metilprednizolonun yanı sıra ozon (O3) tedavisinin hipoksi ile indüklenebilir faktör-1 alfa (HIF-la) ekspresyonu üzerine etkisi

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ABSTRACT

Introduction: To investigate the effect of ozone (O3) therapy on cornea subjected to systemically used high dose methylprednisolone (MP) in a rabbit model.

Methods: Twenty-four New Zealand White adult male rabbits were randomly divided into three equal groups as containing eight animals. The first group (n = 8) was used as the control group and nothing was applied to them, whereas the other 2 groups named as steroid groups were subjected to IM methylprednisolone injection at a dose of 20 mg/kg/day for three days. After three days of MP administration, only the third group was treated with 50-µg/mL O3 (20 mL O3) through the rectal insufflation for 14 sessions. The histopathological examination of corneas of three groups were made, and they were also assessed regarding the expression of hypoxia-inducible factor-1 α (HIF-1α).

Results: It was determined that systemically administered high dose MP caused erosion and necrosis in corneal epithelium and stromal disintegrations in corneal stroma in steroid groups (Group 2 and Group 3). In the MP + O3 group (Group 3), the histopathological findings were mild. The expression of HIF1-α in the cornea of Group 1 (control group), Group 2 (MP), and Group 3 (MP+O3) was measured as, 17.9±9.6%, 3.1±1.0%, and 6.4±1.9% respectively.

Conclusions: MP and O3 therapy decreased HIF-1α expression in rabbit cornea in both intervention groups. Between these two groups, HIF-1α expression remained relatively high in the MP-O3 group than in the MP group alone.

Keywords: hypoxia inducible factor 1 alpha subunit, rabbits, ozone, methylprednisolone, cornea

ÖZ
Giriş: Bir tavşan modelinde sistem olarak uygulanan yüksek doz metilprednizolon (MP) uygulanmış kornea üzerinde ozon (O3) tedavisinin etkisini araştırırmak.

Yöntem: Yirmi dört Yeni Zelanda Beyaz yetişkin erkek tavşanı rastgele oda ve kornea epitelinde erozyon ve nekroza ve korneal stromada stromal parçalanmaya neden olduğu belirlendi (Grup 2 ve Grup 3). MP + O3 grubunda (Grup 3) histopatolojik bulgular hafifti. HIF-1-α'nın Grup 1 (kontrol grubu), Grup 2 (MP) ve Grup 3 (MP+O3) korneasında ekspresyonu sırasıyla %17.9±9.6, %3.1±1.0 ve %6.4±1.9 olarak ölçüldü.

Sonuç: MP ve O3 tedavisi, her iki müdahale grubunda da tavşan korneasında HIF-la ekspresyonu azalttı. Bu iki grup arasında, HIF-la ekspresyonu MP+O3 grubunda daha MP grubuna göre nispeten yüksek kaldı.

Anahtar kelimeler: hipoksi ile indüklenebilir faktör-1 alfa subüni, tavşanlar, ozon, metilprednizolon, kornea

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**Introduction**

The use of systemic steroids has been involved in almost all of the practice of medicine. Apart from administration of oral steroids, higher intravenous doses (1 mg/kg for three-days) of these drugs can also be applied in some specific forms of optic nerve diseases such as demyelinating optic neuritis due to multiple sclerosis, or arteritic anterior ischemic optic neuropathy [1]. The transcription factor Hoxia-inducible factor 1 alpha (HIF-1) is a key mediator of cellular homeostasis in response to hypoxia. HIF-1 transactivates genes that facilitate metabolic adaptation by shifting from oxidative phosphorylation to anaerobic glycolysis, and enhances oxygen delivery by inducing vasodilatation, increasing vascular permeability, enhancing erythropoiesis, and angiogenesis [2]. HIF-1 activates the transcription of many genes that code for proteins that are involved in angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis [2]. HIF-1, widely distributed in mammalian tissues and cells, is a transcriptional regulator for hypoxic response [3]. Moreover, there was evidence to support that the cardio-protection strategies from ischemia, like ischemic preconditioning and ischemic postconditioning were performed through up-regulation of HIF-1α [4]. HIF-1α would enter the nucleus to form a heterodimer with β subunit, which can bind to hypoxia response element to regulate the transcription and activation of over 200 downstream target genes, and thereby promoting the cell survival [5]. Moreover, the highly-expressed HIF-1α in myocardial I/R injury could reduce the oxidative stress, as revealed by Ong et al. and inhibit the opening of mPTP, thus achieving protection against I/R-induced injury [6].

In this respect, we aimed to investigate the effect of systemically used high dose MP on cornea, and the impact of ozone treatment on this cornea by assessing the HIF-1α expression in a rabbit model.

**Methods**

Twenty-four New Zealand White (NZW) adult male rabbits (weight: 2.5–3 kg) were obtained from the Experimental Animal Center of the Canakkale Onsekiz Mart University. The rabbits were housed in plastic cages, 21 ± 2 °C, 12 h light / 12 h daily dark photoperiod and standard rabbit diet (Bil-Yem Ltd. Co., Ankara, Turkey) throughout the experiment.

The rabbits were randomly divided into three equal groups (Control, MP, MP-O3) of eight animals. The first group (n = 8) was used as the control group, and nothing was applied to them. The other 2 groups named as steroid groups (Group 2 and Group 3) were subjected im methylprednisolone (MP) injection at a dose of 20 mg/kg/day for three days. Among the steroid groups, only the MP-O3 group was treated with 50-µg/mL ozone (20 mL O3) through the rectal insufflation every other day for 14 sessions. All animals were sacrificed at days 30.

**Surgical procedure**

During all applications, the administration of intramuscular 10 mg/kg ketamine (Ketalar, Eczacıbaşı, Istanbul, Turkey) was used for premedication. Then a 24 G artery catheter was placed, followed by a 26 G vein catheter. When sedation was not sufficient due to the length of the procedure, anesthesia induction was maintained with the use of 2 mg/kg ketamine, 1µg/kg fentanyl, and 0.5 mg/kg rocuronium bromide. The anesthesia was applied and maintenance of anesthesia was achieved with 1% isoflurane and 50%/50% oxygen/air mixture. 1 mg / kg rocuronium IV, 10 mg / kg ketamine IV and 2µg/kg fentanyl IV were used to sacrifice the rabbits. The right eyes of the rabbits were enucleated for histopathological evaluation of the cornea.

**Histopathological Examination**

The corneas were excised and fixed in 10% formaldehyde solution at room temperature for 48 hours. The cornea samples were dehydrated and embedded in paraffin according to standard histological procedures. Serial sections of 5 μm were prepared using microtome (SLEE cut-5062) from each eyes, and the sections were stained with hematoxylin-eosin (H&E) for histopathological assessment. Light microscopy Olympus CX41 (Olympus, Japan) and Kameram image analysis software (Kameram Gen III; Argenit, Istanbul, Turkey) was used in all histological examination.

**Immunohistochemical Examination**

The tissue sections were deparaffinized in xylene and rehydrated in descending concentrations of ethanol followed by antigen retrieval in sodium citrate buffer for 10 min. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide for 10 min at room temperature. The nonspecific binding of antibodies was blocked by incubation with a blocking serum (Ultra V Block, LabVision). Streptavidin peroxidase (Ultra Vision Detection System-HRP kit, Thermo Scientific/Lab Vision). Streptavidin peroxidase (Ultra Vision Detection System-HRP kit, Thermo Scientific/Lab Vision) was then added at room temperature for 10 min. The chromogen 3-amino-9-ethyl-carbazole (AEC Substrate System, Thermo Scientific/Lab Vision) was used and the sections were counterstained with Mayer’s hematoxylin. Immunohistochemical staining density of HIF1-α was measured as percentage (%) using image analyze software.

**Ethical Approval**

This study was approved by the Canakkale Onsekiz Mart University Local Ethics Committee for Animal Experiments (Date: 26.06.2015; no 2015/05-14).

**Statistical Analysis**

Data were analyzed using SPSS 16.0 statistical software package for Windows (SPSS Inc, Chicago, IL, USA). The results were reported as the means ± standard deviation. Differences among groups were analyzed using Student’s paired t-test or one-way analysis of variance, as appropriate. p<0.05 was considered statistically significant.
RESULTS

Histopathological Results
Substantial histopathological changes in terms of epithelial degenerations, squamous cell loss and disorganization in the corneal stroma were founded in MP group (figure 1-b). However, ozone treatment was found to provide a notable improvement in the corneal structure (Figure 1-c).

![Figure 1. H&E staining of the cornea. (a) Group 1 (control), (b) Group 2 (MP), (c) Group 3 (MP-O3). Arrow and arrowhead represents corneal epithelial degenerations and corneal stromal disintegrations respectively (Magnification; 100X).](image)

Immunohistochemical Results
HIF1-α immunoreactivity of slides were measured with image analysis software as a percentage (%). In the corneas of Group 1, Group 2 and Group 3, the expression of HIF 1-α was measured as 17.9±9.6, 3.1±1.0 and 6.4±1.9 respectively. When examined with anti-HIF1-α antibody immunohistochemical staining of cornea tissues, a significant decreased immunoreactivity observed in Group 2 (high dose MP group) compared to Group 1 (control group) (p<0.05). Increased corneal HIF1-α immunoreactivity was detected in the Group 3 (MP-O3) when compared to Group 2 (p<0.05, Figure 2).

![Figure 2. The effect of high dose MP-O3 treatment on HIF1-α expression in the cornea. (a) Group 1 (control), (b) Group 2 (MP), (c) Group 3 (MP-O3). HIF1-α expression was showed in corneal epithelium and stromal fibroblast. Arrow; anti-HIF1-α stained cells. *; p<0.05 compared to control group; ¥; p<0.05 compared to steroid group. Magnification; 400X.](image)

Discussion
The steroids are widely used drugs in almost all parts of the medicine, and among their various effects, they are particularly employed for the treatment of the inflammation and edema. However, besides the beneficial aspects of the steroids, sometimes they may also lead to undesired conditions arising from their suppression of collagen metabolism. Regarding the collagen metabolism, steroids have been suggested to cause decrease in the rate of mRNA coding procollagen chains, and additionally reduction in posttranslational modification involving the proline and lysine hydroxylation processes [7]. The previous studies investigating this influence of steroids on corneal layers have demonstrated that topically applied steroids can result in decreased proliferation of corneal epithelial cells due to triggering the apoptotic pathways in corneal epithelium [8]. In addition to in vivo studies, the induction of apoptosis in corneal epithelial cells owing to the use of dexamethasone has been revealed in cultured human corneal epithelial cells [9]. Moreover, this relation was also assessed by using either low, or high dose of dexamethasone, and it was found that dexamethasone might inhibit the corneal epithelial apoptosis in a dose-dependent manner, namely the effect of inhibition increases proportionally with the increment of dexamethasone dose [9]. Bourcier et al. have demonstrated the glucocorticoid receptor (GR) expression in human cultured keratocytes and they have reported that dexamethasone can display its apoptotic effect on keratocytes via this receptor [10].
In the current study, we found that corneal epithelial degeneration and corneal stromal disintigration occurred in corneas subjected to systemically high dose methylprednisolone. These results may indicate the different action of mechanism for the explanation of relation between steroids and corneal layers, in comparison with the other studies investigating the effect of topically administrated steroids on corneal layers. The previous studies attributed the harmful effects of steroids on corneal epithelium and corneal stroma to the higher dose of steroid reached to these layers. However, systemically applied steroids can not concentrated as much as the topically administrated route. Hence, it might lead to these pathological changes as a result of influencing the corneal limbal vasculature as the mechanism of steroid associated osteonecrosis of femoral head (ONFH). Because, the disturbance in the microcirculation of femoral head have been suggested to be one of the cause for the development of ONFH, it may be reasonable to think that corneal limbal cells, involving in the corneal epithelial healing and keratocyte nutrition may be adversely affected in the case of systemically used of high dose steroids. Another important finding of this study may be the determination of decreased HIF-1α expression in the corneas exposed to systemical high dose methylprednisolone. The expression of HIF-1α has been reported to provide tissue protection via suppressing the apoptosis [11]. Vidal et al. have demonstrated the increased expression of HIF-1α in adeno-cortico-tropine hormone (ACTH) secreting pituitary tumours [12]. Furthermore, Zhang et al. have suggested the relationship between GR and HIF-1α as increase in HIF-1α can lead to GR upregulation [13] Taken together the findings of these previous studies, in the present study, the decreased expression of HIF-1α in steroid groups might have been arisen from the suppression of ACTH by the negative feed back mechanism acts on hypothalamus-pituitary axes. Namely, the suppression in secretion of ACTH might have caused either reduction in HIF-1α expression, or increase in GR expression. The benefit of ozone therapy on the increased expression of HIF-1α in the corneas of Group 3 is not clear, but it may be hypothesized that the application of ozone therapy might have an impact on preventing the degradation of HIF-1α.

Limitations
In fact, major shortcomings of the current study may be the absence of examination of ACTH levels in the blood circulation, and the expression of GR and vascular endothelial growth factor (VEGF) in the corneal layers. Additionally, it would be better if the histopathological assessment of the corneas were made by using TUNEL dye, and caspase evaluation. However, if the current study is supported with the further ones eliminating these shortcomings, clear conclusions can be obtained about the effect of systemic steroids on cornea.

Conclusion
In conclusion, steroids may also lead to pathological alterations on corneal layers even when they are used in systemically route. Besides that, ozone therapy may be beneficial for the all palliation of these complications.

Conflict of interest: None

Financial disclosure: None

References