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Mitigative effects of chloroquine treatment against hypoxia-induced intestinal injury: a histological and immunohistochemical study

Hipoksiye bağlı bağırsak hasarına karşı klorokin tedavisinin hafifletici etkileri: histolojik ve immünohistokimyasal bir çalışma

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ABSTRACT

Objective: Hypoxia has an important role in the disruption of intestinal mucosal integrity because of inflammation and apoptosis induced by inflammatory cytokines such as TNF-α (Tumor necrosis factor-alpha), IL-6 and IFN-y, and apoptotic regulatory proteins. Chloroquine (CLQ) is a drug used in the novel coronavirus disease (COVID-19) and is widely used for the treatment of many inflammatory diseases such as malaria and rheumatoid arthritis. In this study, we aimed to reduce the destructive effects of hypoxia-induced inflammation and apoptosis in the intestinal mucosa of rats with CLQ applications.

Methods: For this purpose, a total of 24 Wistar Albino rats were randomly divided into three groups; Group I: Control group (n=8), Group II: Hypoxia (n=8) and Group III: Hypoxia + CLQ (n=8). The control group was housed in plexiglass cages to keep the oxygen levels at 10% levels for 28 days, while the hypoxia and hypoxia+CLQ groups were housed in a normal atmospheric environment (21%

ÖZET

Amaç: Hipoksi, TNF-α (Tumor necrosis factor-alpha), IL-6 ve IFN-y gibi inflamatuvar sitokinler ve apoptotik düzenleyici proteinler tarafından indüklenen apoptoz neticesinde bağırsağın mukozal bütünlüğünün bozulmasında önemli bir role sahiptir. Klorokin (CLQ), yeni koronavirüs hastalığında (COVID-19) kullanılan bir ilaçtır ve sıtma ve romatoid artrit gibi birçok inflamatuvar hastalığın tedavisi için yaygın olarak kullanılmaktadır. Bu çalışmada, sıçanların bağırsak mukozasında hipoksi ile indüklenen inflamasyon ve apoptoza bağlı yıkıcı etkilerin CLQ uygulamaları ile azaltılması amaçlanmıştır.

Yöntem: Bu amaçla toplam 24 adet Wistar Albino rat rastgele üç gruba ayrılmıştır; Grup I: Kontrol grubu (n=8), Grup II: Hipoksi (n=8) ve Grup III: Hipoksi + CLQ (n=8). Kontrol grubu normal atmosferik ortamda (%21 O_2), hipoksi ve hipoksi+CLQ grupları oksijen seviyelerinin 28 gün boyunca %10 seviyelerinde tutulabilmesi için pleksiglas kafeslerde barındırılmış

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O_2), and the hypoxia+CLQ group was administered CLQ at a dose of 50 mg/kg every day for 28 days. At the end of the experiment, the intestinal tissues of the experimental animals, were extracted under the anesthesia and they were sacrificed.

Results: As a result of histopathological evaluations, it was determined that CLQ applications showed healing properties on the histopathological effects induced by hypoxia in the intestine. While an increase in TNF-α expression was observed in the hypoxia group, a statistically significant decrease was detected in the hypoxia+CLQ group. In addition, Bax expression was found to be statistically significantly lower in the hypoxia+CLQ group when compared to the hypoxia group. On the contrary, it was observed that Bcl-2 expression was statistically significantly increased in the hypoxia+CLQ group compared to the Hypoxia group.

Conclusion: We observed that hypoxia causes significant damage to the intestinal mucosa and triggers a severe inflammation that drives cells to apoptosis. Considering the curative effects of chloroquine on the intestinal mucosa, we suggest that this anti-inflammatory drug has a potential to use clinically to alleviate the deleterious effects of hypoxia in the intestine.

Key Words: Apoptosis, chloroquine, hypoxia, TNF-α, intestine

ve hipoksi+CLQ grubuna 28 gün boyunca her gün 50 mg/kg dozda CLQ uygulanmıştır. Deney sonunda anestezi altında bağırsak dokuları çıkarılan deney hayvanlarının yaşamlarına son verilmiştir.

Bulgular: Histopatolojik değerlendirmeler sonucunda CLQ uygulamalarının, hipoksinin bağırsakta indüklediği histopatolojik etkiler üzerinde iyileştirici özellikler gösterdiği belirlenmiştir. Hipoksi grubunda TNF-α ekspresyonunda artış gözlenirken, hipoksi+CLQ grubunda istatistiksel olarak anlamlı bir azalma tespit edilmiştir. Ayrıca hipoksi+CLQ grubunda Bax ekspresyonunun Hipoksi grubu ile karşılaştırıldığında istatistiksel olarak anlamlı derecede düşük olduğu belirlenmiştir. Bunların aksine, Bcl-2 ekspresyonunun hipoksi+CLQ grubunda hipoksi grubuna göre istatistiksel olarak anlamlı derecede artmış olduğu gözlenmiştir.

Sonuç: Hipoksinin barsak mukozasında önemli bir hasara neden olduğunu ve hücreleri apoptoza sürükleyen ciddi bir inflamasyonu tetiklediğini gözlemledik. Klorokinin bağırsak mukozası üzerindeki iyileştirici etkileri göz önüne alındığında, bu anti-enflamatuar ilacın, bağırsakta hipoksinin zararlı etkilerini hafifletmek için klinik olarak kullanım potansiyeline sahip olduğunu düşünmekteyiz.

Anahtar Kelimeler: Apoptozis, klorokuin, hipoksi, TNF-α, bağırsak

INTRODUCTION

Hypoxia is known as a restricted oxygen (O_2) in an organism or in a situation (1). In an organism, hypoxia means a state in which oxygen is not enough for the tissue oxygen necessity to sustain homeostasis. This may cause weak oxygen transportation into the tissues both owing to lower blood quantity and lower oxygen volume in blood. The level of hypoxia can be various, and it may be mild or hazardous to organisms. It can be applied as acute, chronic or combination of acute and chronic applications. Although some tissues overcome the destructive effects of hypoxia for a long time, other tissues are very sensitive even to relatively low oxygen levels (2-4).

In addition to digestion and absorption functions, studies have showed that the gastrointestinal tract have many other functions such as mucosal barrier,

endocrine and immunomodulatory functions. Intestinal mucosal barrier is one of the most substantial barrier systems of body and it includes biological barrier, chemical barrier and immune barrier. Thanks to collaboration of these different barriers which have different conformation, molecular structure and biological functions, a powerful defence mechanism is formed against pathogenic antigens or foreign microorganism via combined and complicated signalling pathways (5).

Hypoxic conditions can trigger a barrier dysfunction in gastrointestinal tract. As a consequence of this barrier dysfunction, a systemic inflammation can take place because of the bacterial and other pathogenic invasions; it is also the key factor which induces high-altitude multiple organ dysfunction syndrome (6). Moreover, it is considered that hypoxia is one of the important factors in the pathogenesis of bowel diseases such as Crohn's disease (7).

The restricted energy synthesis induced by hypoxia reduces cilia swinging, decelerates peristaltic movement and prevents the cleaning of the gastrointestinal tract. Furthermore, sufficient blood and oxygen demand of gastrointestinal mucosa for healthy function can vary under specific conditions. Therefore, hypoxia can damage to the intestinal villi (8). The anatomical and chemical structures of gastrointestinal microvilli are also very sensitive to hypoxic conditions (9, 10).

Many researches have shown that the immunomodulatory function of intestinal barrier is maintained by an organization of several cytokines such as ILs, IFNs and TNF-α (11). Hence, the imbalance between pro- and anti-inflammatory cytokines can be harmful to intestinal mucosa. It is shown that hypoxia can increase permeability of intestinal mucosa in vivo and it can induce the activation of inner immune cells. Activation of the immune cells leads the mucosal immune system response (12). TNF-α, IL-6, and IFN-γ are important inflammatory factors playing important roles in various inflammatory reactions and are highly correlated with the severity of inflammation (13).

Apoptosis plays an important role in the intestinal epithelium and it occurs in response to the stress of intestinal epithelial cells. Apoptosis can be induced in the epithelium of both crypt and villi (14). Bcl-2 family proteins are the first ones among discovered apoptosis regulators in mammalians and they can regulate apoptosis both negatively and positively(15, 16). Bcl-2 and Bcl-XL proteins are anti-apoptotic regulators of programmed cell death, and they inhibit apoptosis induced by several factors in most cell types while Bax and Bid induce or enhance apoptosis (17, 18).

CLQ is administered against malaria (19). CLQ is recommended and used in the treatment of COVID-19 pneumonia.(20) CLQ is also used in the treatment of rheumatoid arthritis (21). The effect of TNF-alpha is prevented by the lysosomal protease inhibitor chloroquine (22). CLQ pre-treatment could improve mortality in mice by inhibiting inflammatory pathways and lethal death (23).

In the present study, we investigated the impact of CQ treatment on normobaric hypoxia-induced intestine injury in rats. The aim of this study is to investigate the protective effects of CLQ in the gastrointestinal tract of rats exposed to normobaric hypoxia. For this purpose, we evaluated the histopathological changes in duodenum, and changes in the expression of TNF-α as a tissue inflammatory mediator. In addition, we evaluated changes in the expression of Bax and Bcl-2, which are the pro- and anti-apoptotic Bcl-2 family proteins.

MATERIAL and METHOD

Experimental Design

Mature 24 male Wistar inbred albino rats were obtained from the Experimental and Clinical Research Centre of Erciyes University. All procedures were carried out in accordance with the Universal Declaration of Animal Rights, with the approval of the Ethical Committee (Date: 14.02.2018, Decision no: 18/027) of Erciyes University Experimental Animals.

Rats were fed with pellet food and they had free access to drinking water. The room temperature was held constant at 23±1°C and 12 h night-day cycle was applied. Animals were treated in accordance with the Guidelines for Animal Experimentation of Jichi Medical University (Shimotsuke, Japan), based on the National Institutes of Health Guidelines for the Use and Care of Laboratory Animals.

The rats were randomly divided into three groups as follows: Control group (n=8), Hypoxia group (HX, n=8), Hypoxia plus Chloroquine (Sigma Aldrich, C6628) group (Hypoxia+CLQ, treated with 50 mg/kg/day chloroquine). Rats in Control group were kept in room air. Rat in Hypoxia and Hypoxia+CLQ groups were put in Plexiglas chambers which could allow maintaining oxygen levels at 10% and their oxygen levels were monitored in the chambers.

During the 28 days, all groups were given equal volumes of intraperitoneal (i.p.) injections. The CLQ preparation was diluted with distilled water. The control group was given the same volume of distilled water. The hypoxia group was given 10% oxygen and i.p. distilled water. Hypoxia+CLQ group were given 10% oxygen and 50 mg/kg/day CLQ intraperitoneally. The study was terminated on day 28. The rats were sacrificed by decapitation under i.p. ketamine (50 mg/kg) and xylazine (10 mg/ kg) anaesthesia. After decapitation, intestine tissues were rapidly excised and left in 10% neutral formalin fixation solution for histological and immunohistochemical examination.

Histological Evaluation

Intestine samples were fixed for 72 hr in 10% neutral formalin solution. After washing with water, samples were dehydrated following through a graded alcohol series. Xylol were used for clearing the samples and the tissues were embedded in paraffin blocks. The 5 µm thick sections were taken from the paraffin blocks and they were stained with haematoxylin-eosin for evaluating histological changes. The histological changes were scored from 0 to 3 according to the histological findings. Scoring was performed with these steps: 0 represents no pathological findings, and 1, 2 and 3 represent pathological findings and 1; mild, 2; moderate and 3 was severe. Moreover, presence of leukocyte infiltration and mucosal degeneration at intestinal mucosa were examined on the sections. Changes in the intestinal morphology was evaluated by measuring the length and width of the intestinal villi and height of intestinal mucosa. Images were taken with light microscope (Olympus BX51; Olympus, Tokyo, Japan) and photos were taken with camera attachment of this microscope. The histological examinations were performed by histologists of the study group.

Immunohistochemistry

TNF-α, Bax and BCL_2 expression were detected immunohistochemically using a polyclonal antibody and the streptavidin-biotin-peroxidase technique (24). The sections were deparaffinized in xylene, rehydrated through graded alcohols and washed in deionized water. Antigen retrieval was performed by microwave treatment in 0.01 M sodium citrate buffer, pH 6.0, at 95°C for 5 min. The slides were cooled and held at room temperature for 10 min. Sections were washed with PBS. Endogenous peroxide activity was inhibited by immersion in 3% (w/v) H_2O_2 for 12 min. Lab Vision™ UltraVision™ Large Volume Detection System (TP-125-HL; Thermo Fisher Scientific, Waltham, MA) was used. All sections were washed with distillated water, then Ultra V block was applied for 10 min at room temperature to block background staining. Sections then were incubated overnight at 4°C with an TNF-α (52B83) mouse monoclonal antibody diluted 1:50 (sc-52746; Santa Cruz Biotechnology), Bax rabbit polyclonal antibody diluted 1:200 (bs-0127R; Bioss), Bcl-2 rabbit plyclonal antibody diluted 1:200 (bs-4563R; Bioss) in antibody diluent buffer (TA-125-ADQ; Thermo Fisher Scientific). After washing with PBS, sections were incubated with biotinylated goat anti-polyvalent secondary antibodies (TP-125-BN) (TP-125-HL; Thermo Fisher Scientific). The immunoreaction was amplified using the streptavidin-

avidin-peroxidase complex and visualized using 3,3' p-diaminobenzidine tetrahydrochloride (TA-060-HDX; Thermo Fisher Scientific). After counterstaining with Gill's haematoxylin, sections were washed three times with deionized water. Then, the sections were dehydrated through rising alcohols, cleared in xylene and mounted with Entellan. Images were taken using a light microscope (Olympus BX51; Olympus). At least five randomly chosen fields in each slide were counted at the original ×20 magnification. The immunoreactivity was scored from 0 to 3 according to the histological findings and 0 represents no staining, and 1, 2 and 3 represent in 1; less staining, 2; moderate staining and 3; strong staining.

Statistical Analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 24.0) and graphs were drawn by using Graphpad Prism 8.0 software. The Kolmogorov-Smirnov test was used to identify normal distribution of the data. In case of normal distribution, quantitative variables were compared using One-way analysis of variance (ANOVA) and Tukey's post-hoc test. The data was presented as the mean of normalized data±standard deviation of the mean (SEM). p-value<0.05 was considered as statistically significant.

RESULTS

Histological findings

Intestinal mucosa showed a healthy histological structure in control group and there were no detected defects in the intestinal mucosa and villi. In the hypoxia group, it showed increased mucosal degeneration and inflammatory cells. The intestinal villi height in the hypoxia group were decreased and villi width was thinner than control groups. The intestinal mucosa of hypoxia group was shorter than the control group (Figure 1). Damage of intestinal mucosa ($p< 0.001$), the height of the intestinal villi ($p< 0.022$), the villi width ($p< 0.001$), thickness of the mucosa ($p<0.005$) was significantly decreased in the hypoxia group as compared to the control group. Treatment with CLQ significantly reduced the damage on the histological structure of the intestine in rats exposed to hypoxia (Table 1, Figure 1 and Figure 2).

Table 1. Changes in the histological damage score and measurements (μ) of the villi height, villi width and mucosa thickness among groups

Groups	Control	Hypoxia	Hypoxia+CLQ	*p*
Score of Damage	0.06±0.25	1.46±1.19[a]	0.64±1.28[b]	**0.001**
Villi Height	672.43±127.47	593.09±148.37[a]	645.81±84.44	**0.022**
Villi Width	177.17±54.39	140.36±36.98[a]	151.71±38.03[a]	**0.001**
Mucosa Thickness	915.13±158.60	822.38±162.11[a]	931.68±152.18	**0.005**

Data are expressed as mean±SD. Significant result was considered when $p < 0.05$. (Tukey's post-hoc test).

[a] Significantly different from control group.

[b] Significantly different from both control and hypoxia group.

CLQ: Chloroquine.

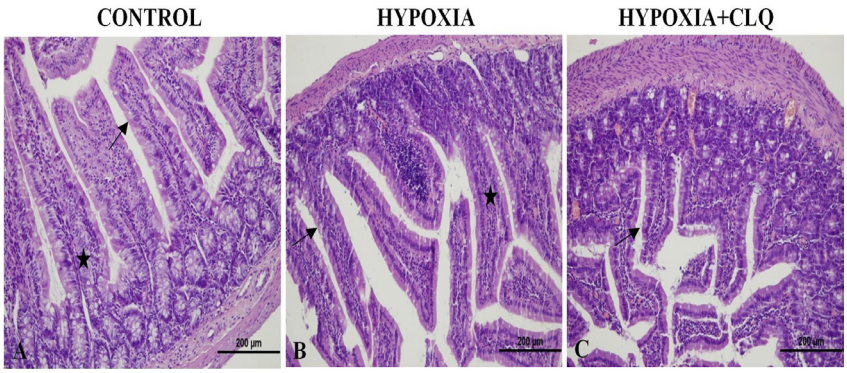

Figure 1. Haematoxylin-eozin (H&E) staining to evaluate the effects of hypoxia on the intestinal epithelium, intestinal villi height, villi width and thickness of mucosa.
(A) Control group given distilled water by i.p. injection.
(B) Hypoxia group exposed to reduced oxygen level (10%) and given same volume distilled water by i.p. injection for 28 days.
(C) Hypoxia+CLQ group exposed to hypoxic oxygen level (10%) and given chloroquine (50 mg/kg/day) by i.p. injection for 28 days.
Arrows show the intestinal epithelium and stars show the intestinal villi.
Stars show the villi width.
CLQ: Chloroquine

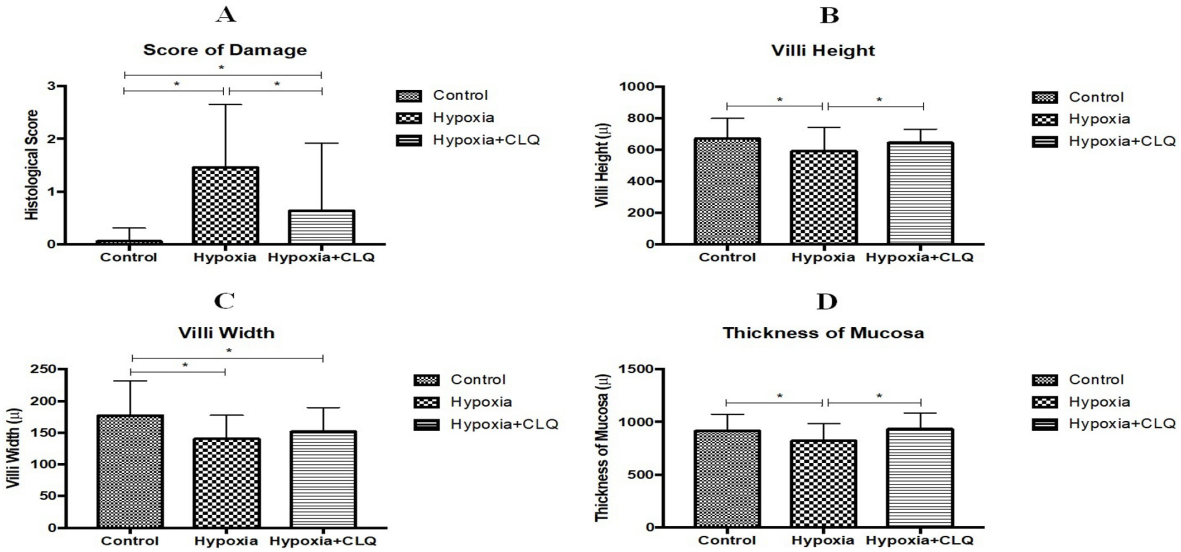

Figure 2. (A) Graphics of histopathological score of intestinal damage, (B) changes in measurements (µ) of the intestinal villi height (B) and villi width (C) and thickness of intestinal mucosa (D).
Measurements were statistically analysed with SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 24.0). Statistical significance among groups were determined by Tukey's post hoc test and results were transformed to graphs by using Graphpad Prism 8.0 software.
* shows the significant difference among groups.
CLQ: Chloroquine

Immunohistochemical Findings

TNF-α, Bax and Bcl-2 expressions were observed in the intestinal epithelium. In the hypoxia group, TNF-α and Bax expressions statistically increased when compared with control group while Bcl-2 expression significantly reduced compared to control group. In the hypoxia+CLQ group, TNF-α and Bax expressions statistically reduced compared with that in group hypoxia. Additionally, In the hypoxia+CLQ group, Bcl-2 expression statistically increased compared with that in group hypoxia (Table 2, Figure 3 and Figure 4).

Table 2. Immunreactivity score of TNF-α, Bax and Bcl-2 among groups

Groups	Control	Hypoxia	Hypoxia+CLQ	*p*
TNF-α	1.30±0.83	2.96±0.18[a]	1.32±0.74	0.001
Bax	0.43±0.56	2.83±0.37[a]	1.25±1.12[b]	0.001
Bcl-2	2.10±0.92	0.93±0.90[a]	1.96±1.11	0.001

Data are expressed as mean±SD. Significancy among groups were considered when $p < 0.05$.

[a] Significantly different from control group.

[b] Significantly different from both control and hypoxia group.

CLQ: Chloroquine; TNF-α :Tumor necrosis factor-alpha.

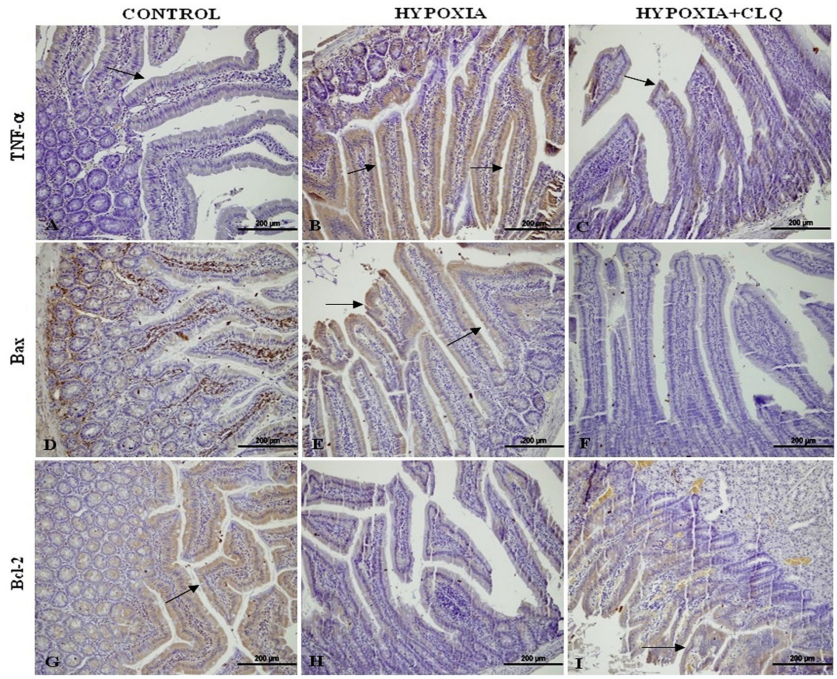

Figure 3. Immunohistochemical stainings of all groups by using TNF-α, Bax and Bcl-2 polyclonal antibodies and streptavidin-biotin-peroxidase technique.

Control group (A) and Hypoxia+CLQ group (C) of TNF-α staining show less immunoreactivity while Hypoxia group (B) of TNF-α shows strong immunoreactivity. Similarly, Hypoxia group (E) of Bax shows strong immunoreactivity when compared with Control group (D) and Hypoxia+CLQ group (F) of Bax. On the other hand, Hypoxia group (H) of Bcl-2 immunostaining shows less immunoreactivity while Control group (G) and Hypoxia+CLQ (I) group show strong immunoreactivity.

Arrows show the immunoreactive cells at the intestinal epithelium.

CLQ, Chloroquine; TNF-α, Tumor necrosis factor-alpha

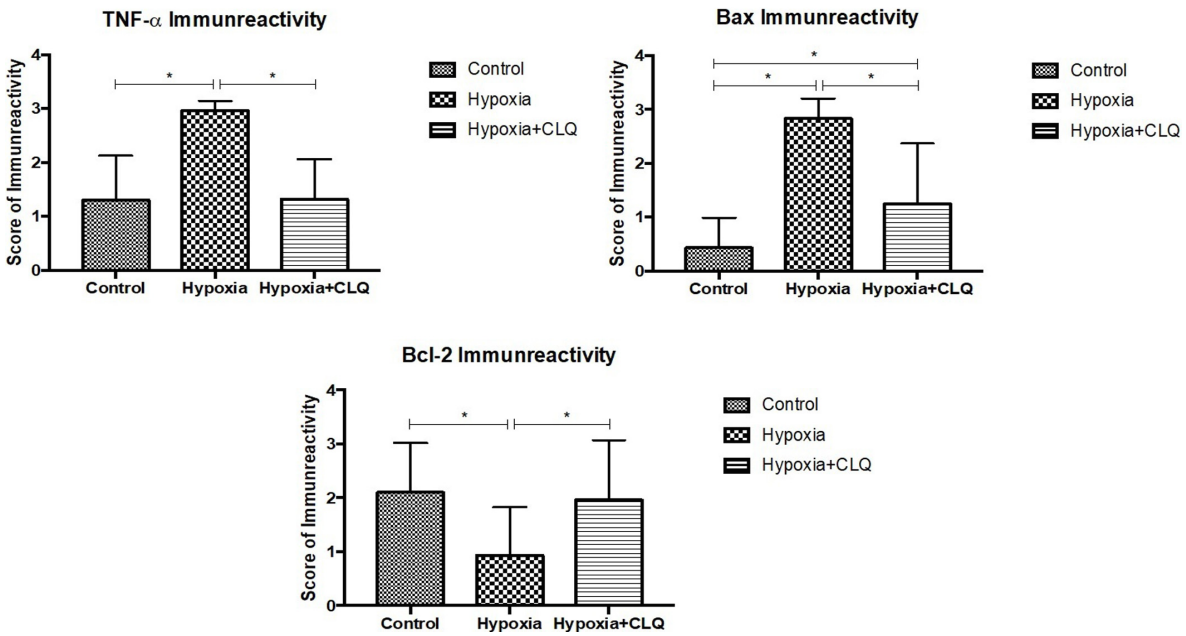

Figure 4. Graphics obtained from the analysis of TNF-α, Bax and Bcl-2 immunoreactivity scores.
Data scored by histologist of the study group were analysed with SPSS statistical software (SPSS for Windows, SPSS Inc, Chicago, IL, version 24.0).
Statistical significance among groups were determined by Tukey's post hoc test and results were transformed to graphs by using Graphpad Prism 8.0 software.
* shows the significant difference among groups.
CLQ, Chloroquine; TNF-α, Tumor necrosis factor-alpha

DISCUSSION and CONCLUSION

Instabilities between oxygen levels supplied to tissues and oxygen consumption result in tissue hypoxia. Hypoxia causes many pathophysiologic conditions in some process and situations such as ion homeostasis, proliferation, differentiation, erythropoiesis, angiogenesis (25).

Entireness of intestinal mucosa is a major element for normal physiologic function and disease prevention. Wu et al. (26) have reported that the intermittent hypoxia disrupts the intestinal structure. In this study, we show that the chronic hypoxia causes a significant damage in intestinal mucosa. Intestinal damage score is high numbered in hypoxia group when considered control group. Moreover, in hypoxia group, there is a significant decrease in intestinal villi height, villi width and mucosa thickness (Table 1).

Several studies have showed that hypoxia plays a role in cell death by apoptosis, oxidative stress and inflammation. As a major mechanism regulating the cell death, apoptosis has an important role in hypoxia-induced cell damage. Many results have suggested that there is a close link between hypoxia and apoptosis. Hypoxia may cause apoptosis by upregulation of hypoxia-inducible factor (HIF), increased free oxygen radicals, overloading calcium ions and mitochondrial damage (27). Recent studies have indicated hypoxia increases the production of mitochondrial reactive oxygen levels (ROS) and reduces the antioxidant defense (28). ROS can interact with biomolecules and can damage several cell component such as DNA, RNA, proteins and lipids (29, 30). In the light of several studies, we believe that the intestinal structure disruption such as reducing intestinal villi height, villi width and mucosal thickness is because

of apoptosis triggered by HIF and increased ROS at intestinal mucosal region.

Kalpana et al. (31) have suggested that hypoxia induces excessive production of inflammatory cytokines such as nuclear factor kappa-B (NF-κB), interleukin-1 alpha (IL-1α) and TNF-α causing systemic inflammation and cell death in their study. They used male Sprague-Dawley rats as a model organism, and they exposed them hypobaric hypoxia. As a result, they observed a significant increase in TNF-α level in brain tissue. In the present study, we have found that hypoxia induces excessive production of TNF-α in intestinal mucosa. According to our immunohistochemical stainings, TNF-α levels in intestinal epithelial cells increased and this may cause a significant systemic inflammation (Table 2).

The mitochondrial apoptotic pathway is regulated by Bax and Bcl-2, which are the pro- and anti-apoptotic Bcl-2 family proteins (32). Therefore, Bcl-2 and Bax ratios determining whether a cell undergo programmed cell death are very important for many cell types in mammalians. In their study, Aliparasti et al. (33) investigated the effects of chronic hypoxia on Bcl-2/Bax ratio in rat heart tissue. For this purpose, they expose rats to O_2 11% for two weeks and investigate the changes in expression of Bcl-2 and Bax proteins by Real Time Polymerase Chain Reaction (RT-PCR). As a one of their results, they suggested that chronic hypoxia negatively affected the mRNA expression of Bax protein but has no effect on the mRNA expression of Bcl-2 protein. In our study, we found that the Bax immunoreactivity significantly increased in intestinal epithelial cells in the hypoxia group while Bcl-2 immunoreactivity decreased according to our immunohistochemical staining (Table 2).

CLQ is widely used in treatment of *Plasmodium vivax malaria* as an anti-inflammatory agent. However, it is administered for treating some autoimmune defects such as rheumatoid arthritis and systemic lupus erythematosus (34). It is promising against the new SARS-CoV-2 coronavirus causing COVID-19 (35). In several studies with experimental animals, CLQ has been shown to have ameliorative effects against mucosal damage induced by various chemicals in the intestine (36). In addition, several studies have shown that CLQ inhibits oxidative stress and apoptosis in the intestinal tract after hepatic ischemia-reperfusion (37). Moreover, in many studies CLQ has been shown to suppress inflammation via the TLR pathway in inflammatory bowel disease (38). It is thought that this ameliorative effect of CLQ in the intestinal tract is due to its inhibitory effect on oxidative stress and apoptosis pathways due to its anti-inflammatory effect. In our study, we used CLQ to reduce negative effects of hypoxia on intestinal mucosa in rats. According to our histopathologic and immunohistochemical examinations, we found that CLQ significantly reduced the negative effects of hypoxia such as decreasing in height and width of intestinal villi and mucosal thickness in hypoxia+CLQ group when compared hypoxia group. Moreover, in hypoxia+CLQ group, a significant amelioration in terms of inflammation were determined by histopathological and immunohistochemical examinations; the number of inflammatory regions in intestinal mucosa disembarked and TNF-α immunoreactivity decreased significantly. CLQ inhibited apoptosis triggered by inflammatory cytokines such as TNF-α because it has an effect on Bcl-2/Bax ratio. In hypoxia group, Bax immunoreactivity significantly increased in intestinal epithelial cells while in hypoxia+CLQ group its immunoreactivity significantly decreased when compared to hypoxia group.

As a consequence, we suggested that the administration of CLQ after exposure to hypoxic conditions can reduce the negative effects of hypoxia on intestinal tract. By means of its anti-inflammatory feature, the devastating effects of overexpression of inflammatory cytokines overloading cell to apoptosis in the intestinal mucosa may be inhibited when it is used regularly and properly dosed administrations.

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ETHICS COMITTEE APPROVAL

* The study was approved by the Erciyes University's Experimental Animal and Local Ethics' Committee (Date: 14.02.2018 and Number: 18/027).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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