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Chloroquine attenuates chronic hypoxia-induced testicular damage via suppressing endoplasmic reticulum stress and apoptosis in experimental rat model

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Abstract

Chronic hypoxia negatively affects male fertility by causing pathological changes in male reproductive system. However, underlying mechanisms of this damage are unknown. Chloroquine (CLQ) is an anti-inflammatory agent that is widely used in the treatment of inflammation-related diseases such as malaria and rheumatoid arthritis. This study aimed to investigate the therapeutic effects of CLQ in the hypoxia-induced testicular damage via assessment of hypoxic response, endoplasmic reticulum stress and apoptosis. For this purpose, 32 Wistar albino rats were divided into 4 groups as control (given 20%-21% O_2, no treatment), CLQ (given 50 mg/kg and 20%-21% O_2 for 28 days), hypoxia (HX) (given 10% O_2 for 28 days) and HX + CLQ (given 50 mg/kg and 10% O_2 for 28 days). After the experiment, blood samples and testicular tissues were taken. Histopathological evaluation was performed on testicular tissues and hypoxia-inducible factor 1-α (HIF1-α), heat shock proteins (HSPs) HSP70, HSP90 and growth arrest and DNA damage-inducible gene 153 (GADD153) expression levels were detected via immunohistochemistry. Moreover, apoptotic cells were detected via terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining and serum testosterone levels were determined by enzyme-linked immunosorbent assay (ELISA) assay. Histopathological changes, apoptotic cell numbers and HIF1-α, HSP70, HSP90 and GADD153 expressions significantly increased in HX group ($P < .05$). Moreover, serum testosterone levels decreased in this group ($P > .05$). However, CLQ exerted a strong ameliorative effect on all parameters in HX + CLQ group. According to our results, we suggested that CLQ can be considered as an alternative protective agent for eliminating the negative effects of hypoxic conditions on male fertility.

KEYWORDS
apoptosis, chloroquine, heat-shock proteins, hypoxia, male infertility

1 | INTRODUCTION

People living at high altitudes are exposed to chronic hypobaric hypoxia, which is considered a condition where reduced pressure resulted in decreased oxygen availability causes low blood oxygen levels.[1,2] Several sportive activities such as mountain sports, tourism and jobs performed in areas over 1500 m above sea level cause exposure of intermittent hypobaric hypoxia.[3–5] Hypoxic conditions may

cause weak oxygen transportation into the tissues both owing to lower blood quantity and lower oxygen volume in blood. The level of hypoxia is important, and it can be mild or hazardous to organisms. Experimental hypoxia can be applied as acute, chronic or combination of acute and chronic applications. Although some tissues recover the detrimental effects of hypoxia for a long time, other tissues are very sensitive even to relatively low oxygen levels.[6–8] Glomus cells of carotid body detect the changes in oxygen pressure in arteries and this triggers hyperventilation and activates the sympathetic nervous system. Moreover, skeletal muscle can also respond to hypoxia by dilating vessel lumens to provide higher blood flow as a local defence.[9]

Several studies have reported that hypoxia-induced tissue damages may cause inflammation, oxidative stress, necrosis and apoptosis resulting in cellular damage. Because it is a major mechanism of programmed cell death, apoptosis has come to the forefront.[10]

The cells detect the reduced oxygen level with cellular pathways that begin with an enzyme class called prolyl hydroxylase domain (PHD) proteins.[11] These enzymes comprise of oxygen-sensing hydroxylases that hydroxylate specific proline domains on the α-subunit of the hypoxia-inducible factor (HIF). Hypoxia-inducible factor 1-α (HIF1-α) and HIF2-α are subunits of HIF heterodimer that dimerize with HIF1-β. Hypoxia-inducible factor 1-α subunit is expressed by many cell types, and it activates glycolytic genes and stimulates apoptosis and regulates pH with carbonic anhydrase.[12]

Folding of proteins and combining of multimeric structures is performed by molecular chaperones. Molecular chaperones are generated by heat shock proteins (HSPs). Heat shock proteins are classified according to their molecular weight. HSP70 and HSP90 are members of the HSPs family, make up major groups of molecular chaperons and occur in protein folding and during times of stress.[13] In addition, these two proteins have been shown to be expressed in diseases such as chronic heat stress,[14] toxicity,[15] cancer[16] and in models such as testicular torsion.[17] Moreover, disrupted protein synthesis leads endoplasmic reticulum stress (ERS) in the cells. Glucose regulated protein-78 chaperon arranges ERS.[18] If ERS cannot be improved, C/EBP homologous protein (CHOP, growth arrest and DNA damage-inducible gene 153 [GADD153]) induces apoptosis.[19]

Chloroquine (CLQ) is a drug used as an anti-inflammatory[20] and it is commonly use in treatment of many inflammation associated diseases such as malaria[21] and rheumatoid arthritis.[22] Moreover, it is recently used for the treatment of the pneumonia caused by coronavirus disease (COVID)-19.[23] The effect of tumour necrosis factor-α (TNF-α) inducing the upregulation of the other interleukins is prevented by the lysosomal protease inhibitor CLQ.[24] Chloroquine treatment could improve mortality in mice by inhibiting inflammatory pathways and lethal death.[25]

We know that HSPs are overexpressed when cell function does not maintain regularly and is disrupted by various reasons, and apoptosis is induced because of ERS. Therefore, it is inevitable that oxidative stress caused by hypoxia in the testis tissue will not activate HSPs and cause the overexpression of the GADD153 and HIF1-α. After a detailed literature review, it was concluded that studies of testicular injury induced by hypoxia are insufficient. Therefore, we aimed to show testicular damage because of hypoxia. We evaluated the testicular tissue damage histologically and showed the hypoxia-induced apoptosis in the testicular tissue. Moreover, we compared the expression levels of HIF1-α, HSP70, HSP90 and GADD153 and in the testis. We also measured serum testosterone levels to investigate the ameliorative effects of CLQ on the reduced testicular steroidogenesis by hypoxia. This study aimed to demonstrate the protective effects of CLQ against hypoxia-induced testicular damage.

2 | RESULTS

2.1 | Hypoxia-induced testicular damage attenuated by chloroquine

According to our histopathological examinations of the testicular sections, many histopathological changes were found in testicular tissue in the hypoxia (HX) group compared to the control group and CLQ group. In the HX group, narrowing of the seminiferous tubule diameters, a decrease in the spermatogenic cell line and the cells pouring into the lumen were observed. Moreover, haemorrhage is also observed in the interstitial region in the testicular tissues of HX group. Apart from these histopathological conditions, Johnsen's Testicular Biopsy Scoring (JTBS) performed by 2 independent histologists was found to be statistically significantly lower in the HX group compared to the control group and the CLQ group. In the HX + CLQ group, seminiferous tubules were found to be closer to tissue morphology in the control and CLQ groups despite haemorrhage in the interstitial area. In addition, it was determined that the seminiferous tubule diameters in the HX + CLQ group increased significantly compared to the HX group (Figure 1). Johnsen's Testicular Biopsy Score and mean seminiferous tubule diameter (MSTD) data of all groups are given in Table 2.

2.2 | Increased hypoxic response were reduced by CLQ via downregulation of HIF1-α

The immunohistochemical analysis of HIF-1α expression showed that HIF-1α was expressed in almost every seminiferous tubule in experimental groups (Figure 2). However, positively stained cells were hardly seen in control and CLQ groups (Figure 3). According to our immunoreactivity density measurements obtained with Image J program, the positive rate of HIF-1α in the seminiferous tubules was significantly higher in HX group than other experimental groups ($P < .05$). In addition, in the HX + CLQ group HIF-1α expressions were significantly weak in the seminiferous tubules when compared with the HX group ($P < .05$), suggesting that CLQ inhibits the hypoxic response in the testicular tissue (Figure 4A).

2.3 | Chloroquine decreased the increasing expressions of HSPs in hypoxic testicular damage

Results of immunohistochemical staining performed on testicular sections tissue via HSP70, and HSP90 antibodies show that the expressions of these factors were immunohistochemically detected in the seminiferous tubules of the experimental groups (Figure 3). According to immunohistochemical staining and the measurements of the immunoreactivity of these factors, it was observed that in the HX group, the expression levels of the HSP70 and HSP90 were statistically significantly increased in the spermatogenic cell line compared to the control and CLQ groups ($P < .05$). On the contrary, it was detected that the expression levels of these factors were significantly lower in the testicular sections of the HX + CLQ group when

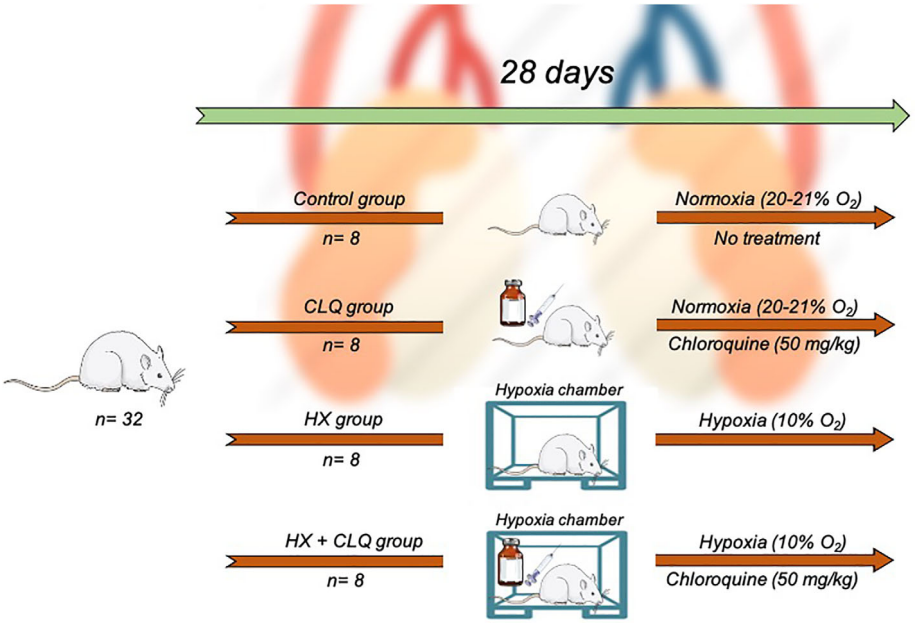

FIGURE 1 Schematic representation of the experimental design

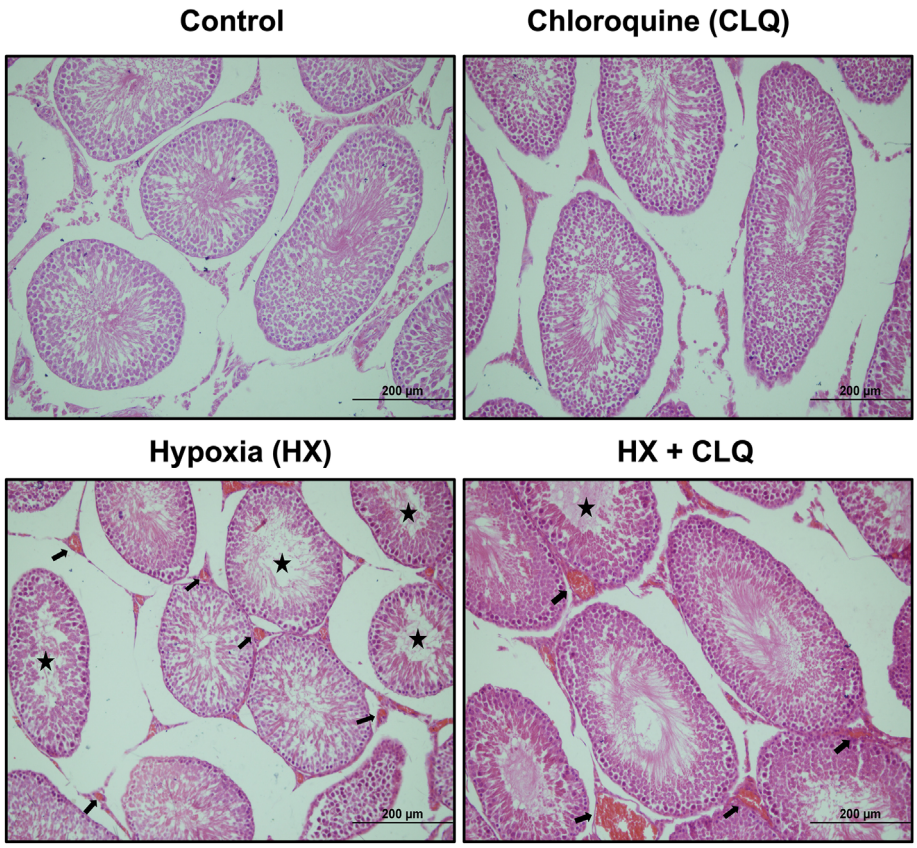

FIGURE 2 Light microscopy of testicular tissue stained with H&E staining. Control group and CLQ group showed normal histological tissue structure. In the HX group, damaged seminiferous tubules where spermatogenic cell line clearly reduced (stars) are seen. Moreover, this group showed excessive haemorrhage in the interstitial region (black arrows). Unlikely, in the HX + CLQ group, it is clearly seen the damage were substantially less when compared to HX group. Scale bar = 200 µm. CLQ, chloroquine; H&E, haematoxylin-eosin; HX, hypoxia

compared with the HX group ($P < .05$). Statistical analyses of the data of immunoreactivity measurements of spermatogenic cell line in the experimental groups were shown in the Figure 4B,C.

2.4 | Chloroquine decreased the ERS via reducing increased GADD153 expression

As a result of immunohistochemical staining, GADD153 expression was detected in the spermatogenic cell line in the seminiferous tubules of the testes of the experimental groups (Figure 3). When the immunoreactivity density measurements were compared among the groups, it was observed that GADD153 expressions increased significantly in HX group compared to control and CLQ groups ($P < .05$). Moreover, it was determined that the expression of GADD153 in the HX + CLQ group was significantly decreased compared to the HX group ($P < .05$), indicating that CLQ reduces the endoplasmic stress induced by hypoxia in testicular tissue. The graphs showing the statistical comparison of the immunoreactivity densities measured with the Image J program among the groups are shown in Figure 4D.

2.5 | Hypoxia-induced apoptosis reduced by CLQ administrations

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining was performed to determine apoptotic cells in testes tissue (Figure 5). The apoptotic cell number in the testicular tissues of control and CLQ group were found 0 (0-3) and 0 (0-3), respectively. Moreover, there was not statistically significance between these groups. The increase in the apoptotic cell number in HX group was found as 1 (0-7) and was statistically significant when compared to control group ($P < .05$). In HX + CLQ group, there was a decrease in TUNEL-positive cells and the apoptotic cell number as 0 (0-05). This decrease in the

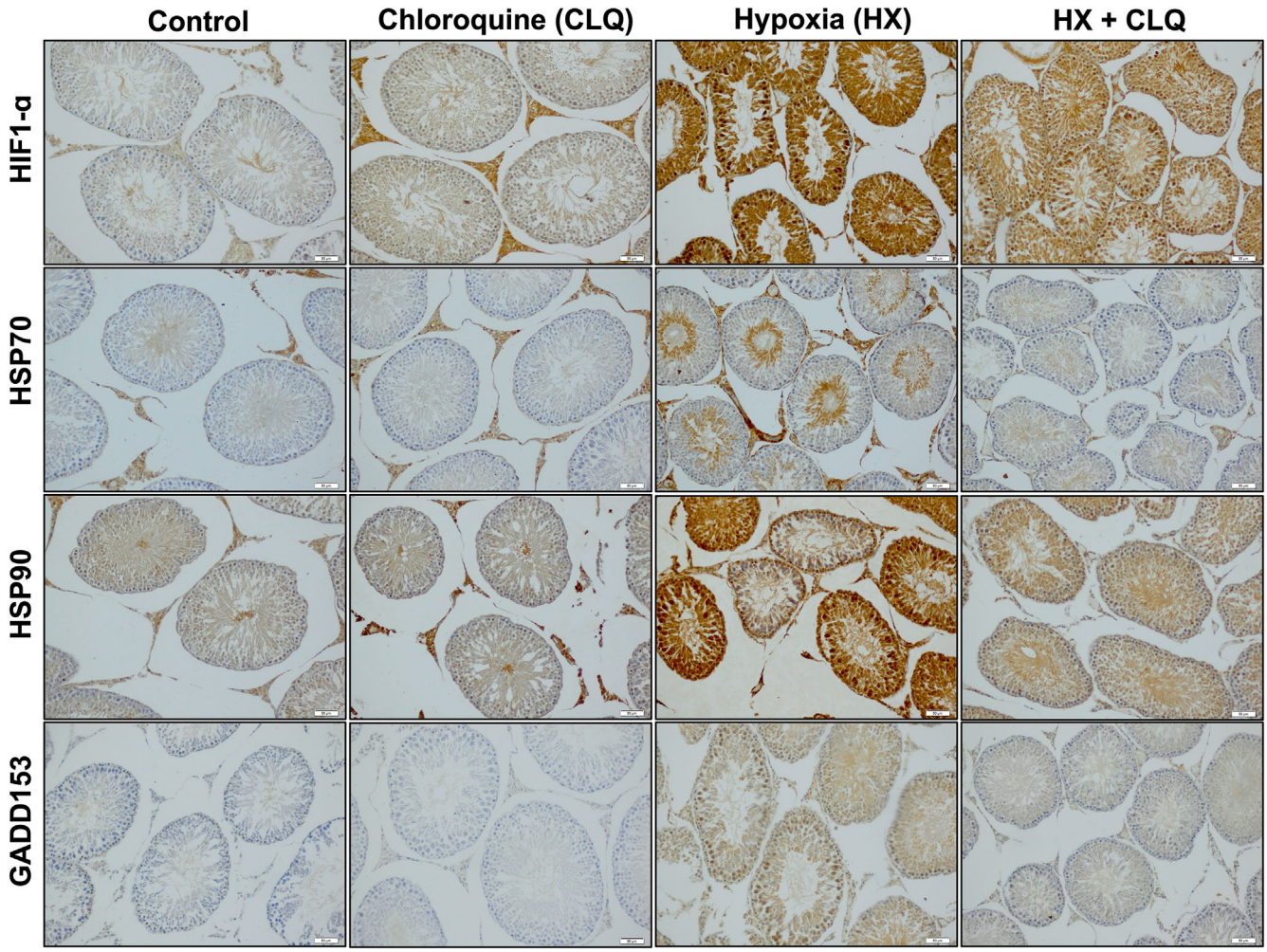

FIGURE 3 GADD153, HIF1-α, HSP70 and HSP90 immunostaining of testis tissues in experimental groups. Control (n = 8) and CLQ (n = 8) groups show weak GADD153, HIF1-α, HSP70 and HSP90 immunoreactivity in testis sections; HX ($n = 8$) group shows strong GADD153, HIF1-α, HSP70 and HSP90 immunoreactivity. Unlikely, in the HX + CLQ (n = 8) group, GADD153, HIF1-α, HSP70 and HSP90 immunoreactivity were substantially less compared to those in the HX group. Scale bar = 50 μm. Abbreviations: HX, hypoxia; CLQ, chloroquine; GADD153, growth arrest and DNA damage-inducible gene 153; HIF1-α, hypoxia-inducible factor 1-α; HSP70, heat-shock protein 70; HSP90, heat-shock protein 90

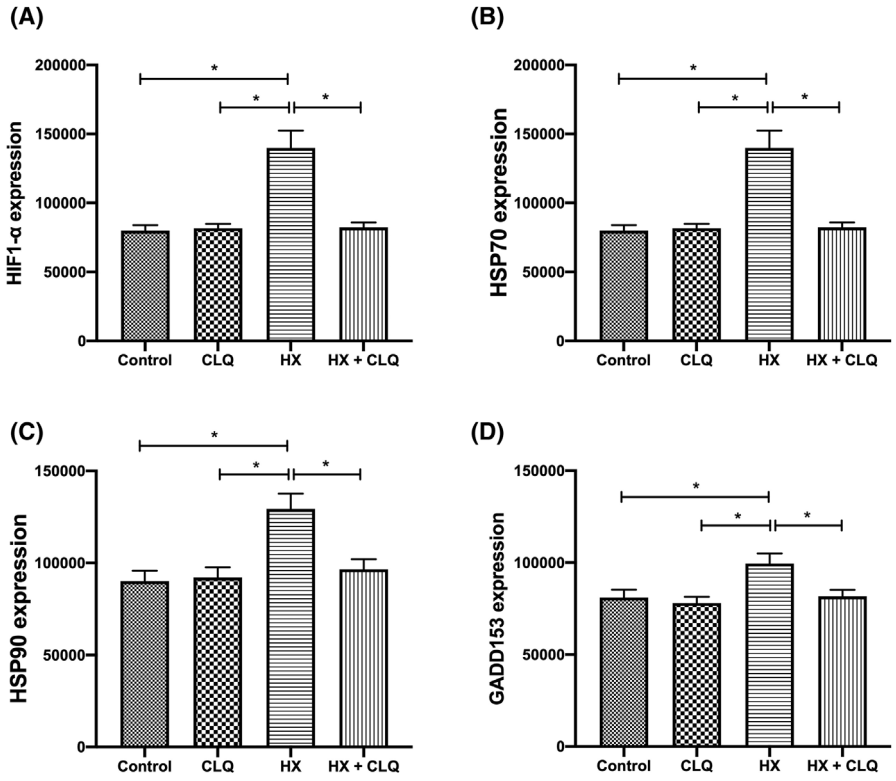

FIGURE 4 A-D, Statistical analysis of the immunoreactivity measurements of the GADD153, HIF1-α, HSP70 and HSP90 in the testis tissue sections of experimental groups. $P < .05$ was considered as significant. The expression levels of GADD153, HIF1-α, HSP70 and HSP90 were significantly higher in the HX (n = 8) group compared to control (n = 8) and CLQ (n = 8) groups ($P < .05$). Moreover, CLQ significantly reduced the increased expression levels of the GADD153, HIF1-α, HSP70 and HSP90 in the HX + CLQ group when compared with the HX group ($P < .05$). *Shows the statistically significance among experimental groups. Abbreviations: HX, hypoxia; CLQ, chloroquine; GADD153, growth arrest and DNA damage-inducible gene 153; HIF1-α, hypoxia-inducible factor 1-α; HSP70, heat-shock protein 70; HSP90, heat-shock protein 90

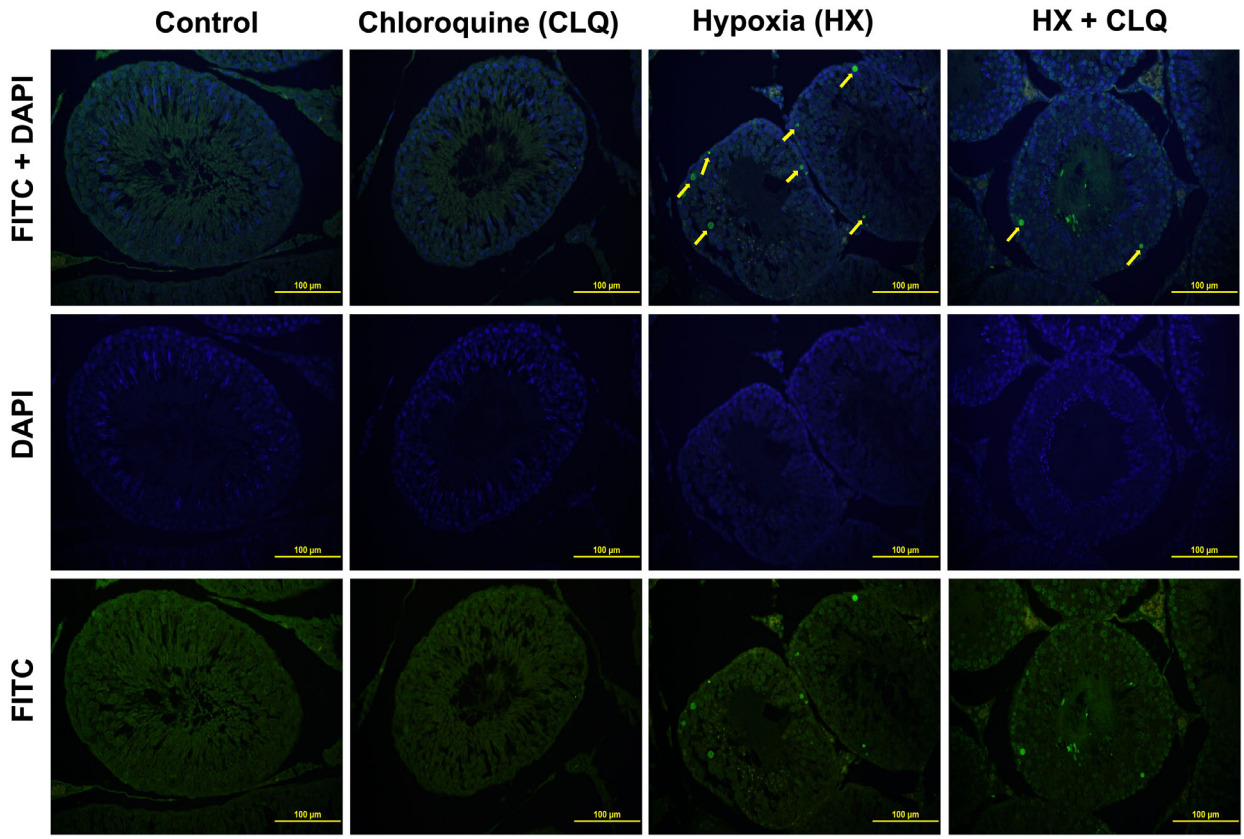

FIGURE 5 TUNEL staining of testicular tissue sections. In the control ($n = 8$) and CLQ groups, normal testis tissues and a few apoptotic cells were observed. However, in the HX (n = 8) group TUNEL-positive cells (yellow arrow) were mainly observed in testicular tissue sections. On the contrary, in the HX (n = 8) group TUNEL-positive cells significantly decreased compared to HX (n = 8) group. Scale bar = 100 µm. Abbreviations: CLQ, chloroquine; HX, hypoxia; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling

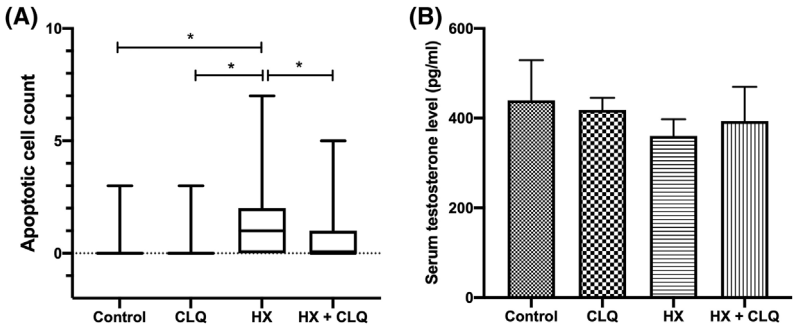

FIGURE 6 A, Statistical analysis of the apoptotic cell count shows that the apoptotic cell numbers significantly increased in the HX (n = 8) group compared to control (n = 8) and CLQ (n = 8) groups ($P < .05$). Moreover, it is clearly seen that CLQ caused a significant decrease in the apoptotic cell numbers in the HX + CLQ (n = 8) group when compared with those in the HX (n = 8) group ($P < .05$). (B) Statistical analysis of the serum testosterone levels of experimental groups. HX group showed a decrease in serum testosterone levels. However, it is observed that CLQ positively affected serum testosterone levels in the HX + CLQ group ($P > .05$). *Shows the statistically significance among experimental groups. $P < .05$ was considered as significant. Abbreviations: CLQ, chloroquine; HX, hypoxia

apoptotic cell number in the HX + CLQ group was significantly different when compared to HX group ($P < .05$). Statistical analysis of the apoptotic cell number in the seminiferous tubules of the testicular tissues among experimental groups were given in the Figure 6A.

2.6 | Decreased serum testosterone levels recovered by CLQ

Serum testosterone levels of the experimental groups and the statistical analyses of them were given in the Figure 6B. According to measurements obtained by enzyme-linked immunosorbent assay (ELISA) assay, it was observed that exposure of hypoxia decreased the plasma testosterone level of the experimental animals in the HX group compared to control and CLQ group, suggesting the inhibitory role of hypoxia on testicular androgenesis ($P > .05$). Although there was also no statistically significant difference between the HX and HX + CLQ groups, it was observed that serum testosterone levels were higher in the HX + CLQ group when compared to HX group ($P > .05$).

3 | DISCUSSION

In the present study, the ameliorative effect of CLQ has been investigated to eliminate the harmful effects of hypoxia on the testis. In previous studies, CLQ, which is widely used in the clinical treatment of inflammatory diseases such as malaria and rheumatoid arthritis, has been shown to play an attenuative role in various tissue damage models at low doses.[26] In this study, we tested the hypothesis that CLQ can protect testicular tissue from damage induced by hypoxia.

It is known that the hypoxic response triggered by low oxygen levels seriously damages many tissues and organs in the body. Many studies have shown that low ventilation levels caused by chronic hypoxia can be tolerated by increasing red blood cell production or increasing plasma erythropoietin levels.[27,28] Therefore, we think that the cause of haemorrhage in the abdominal region during the removal of testicular tissues of experimental animals is the increase in red blood cells because of chronic hypoxia. We also suggest that the presence of excessive haemorrhagic areas in the interstitial area in the HX group is also because of this reason as a response to hypoxic conditions.

The pathological low oxygen content or pressure in the testicular tissue of mammals is one of the causes of male infertility, especially in chronic hypoxia. Studies on the negative effects of hypoxia in testicular tissue have shown that chronic hypoxia has serious effects on spermatogenesis in testicular tissue by inducing oxidative stress and apoptozis.[29] In healthy men, it has been reported that reversible oligozoospermia caused by chronic hypoxia occurs with the increase in germ cell apoptosis induced by this hypoxic state.[30] According to our MSTD and JTBS data, we suggest that the decrease in the spermatogenic cell series that we determined in the HX group and the narrowing of the seminiferous tubules occurred because of the atrophy in the spermatogenic cell line based on inflammation, oxidative stress and apoptosis induced by chronic hypoxia in the testicular tissue. However, these parameters were significantly higher in the HX + CLQ group compared to the HX group. Therefore, we think that CLQ, which is used in the treatment of inflammatory diseases in the clinic, may cause inhibition of apoptotic pathways associated with inflammation and oxidative stress in the testicular tissue at dose of 50 mg/kg, protecting it from the harmful effects of hypoxia.

Under low oxygen (9%-10% O_2), HIF-1α binds to HIF-1β and the HIF-1α/HIF-1β heterodimer interacts with hypoxia response elements to control the expression of genes involved in glucose metabolism and transport, cell cycle and apoptosis.[31] In this study, it was determined that CLQ has an inhibitory effect on the hypoxic response caused by chronic hypoxia at a dose of 50 mg/kg. According to our immunohistochemical results, chronic hypoxia in the HX group significantly increased HIF-1α expression in the testicular tissue of the experimental animals, causing a strong hypoxic response. In the HX + CLQ group, HIF-1α expressions, which increased as a result of hypoxic response, decreased with CLQ administrations. In line with these results, we propose that CLQ protects the testicular tissue from the destructive effects of chronic hypoxia by reducing the HIF-1α expressions in

testicular tissue, therefore, playing a role in reducing the hypoxic response, which will eventually result in inflammation and apoptosis.

In many studies, it has been reported that HSP70 is upregulated in response to hypoxia and is related to the protection and survival of cells.[32,33] Inducible HSP70 is recognized as a useful marker of hypoxic condition in the cell. However, the role of HSP70 in a model of testicular injury induced by chronic hypoxia remains unclear. In this study, expression levels of HSP70 in testicular tissue under chronic hypoxia conditions were investigated. In line with our immunohistochemical data, we suggest that the significantly increased HSP70 expressions in testicular tissues of experimental animals exposed to chronic hypoxia indicate a serious response to chronic hypoxia in the testicular tissue. Therefore, we evaluated the increase of HSP70 expression in the testis under hypoxic conditions as activating the survival mechanisms of cells in response to hypoxia. In addition, CLQ reduced HSP70 levels increased by hypoxia in testicular tissue at a dose of 50 mg/kg. We think that this is caused by the decrease in hypoxic response in testicular tissue because of the healing properties of CLQ in testicular tissue. We propound that CLQ reduces the hypoxic response in testicular tissue and contributes to the maintenance of cells by preventing apoptosis, which will occur with the activation of a series of genes that play a role in apoptosis pathways.

Heat-shock protein 90 is known to be an abundant and ubiquitous molecular chaperone in the body that functions to ensure proper folding and conformational maturation of proteins. In addition to its chaperone property, HSP90 is also known to regulate cell signalling and function in eukaryotic cells.[34] Recent studies have reported increased HSP90 expressions in testicular tissue in various pathological conditions associated with inflammation, oxidative stress and apoptosis.[35] It has also been reported that HSP90 expressions are significantly increased in cardiomyocytes exposed to hypoxia.[36] In our study, we believe that the increase in the expressions of HSP90 in testicular tissue of experimental animals exposed to chronic hypoxia may have occurred because of protein misfolding because of oxidative stress induced by hypoxia. In addition, we suggest that CLQ, which we determined to have a healing effect on testicular tissue against the harmful effects of hypoxia, reduces HSP90 expressions, and this therapeutic agent has effects on the elimination of protein folding disorders caused by oxidative stress. These results indicate that CLQ has positive effects on the accuracy of translation in the testicular tissue under hypoxic conditions.

Hypoxia and nutrient deficiency are two conditions that can trigger ERS because of disturbance in endoplasmic reticulum (ER) homeostasis, which determines the accumulation of folded or misfolded proteins in the lumen of the ER. Like hypoxia, ERS can also transform into two different states: activation of pro-apoptotic pathways and cell death in the case of acute ERS or activation of survival responses in the case of chronic and mild ERS.[37] Growth arrest and DNA damage-inducible gene 153 is one of the modulatory components of the ERS-mediated apoptosis pathway. Growth arrest and DNA damage-inducible gene 153 upregulation has been shown to cause decreased sperm count and motility in adult male rats.[38] In addition, many studies have reported that GADD153 significantly increases in various cell types under hypoxic conditions.[39,40] In this study, it was determined that chronic hypoxia induces ERS by increasing the expression of GADD153 in rat testicular tissue. Moreover, it was determined that CLQ applied as a therapeutic agent reduces ERS through downregulation of GADD153 in seminiferous tubules in the HX + CLQ group. These results show that CLQ inhibits the activation of several genes that will eventually result in apoptosis by reducing ERS in testicular tissue.

In our study, it was determined that the number of apoptotic cells in the testicular tissues of the experimental animals exposed to chronic hypoxia in the HX group was significantly higher compared to the other groups. These results, which we obtained with apoptotic cell count with TUNEL staining method in testicular tissue, show that chronic hypoxia causes serious damage by inducing apoptosis in testicular tissue. In addition, CLQ, a powerful anti-inflammatory drug, significantly reduced the number of apoptotic cells in the HX + CLQ group at doses of 50 mg/kg. These results obtained from TUNEL staining, suggest that CLQ protects the testicular tissue from the destructive effects of chronic hypoxia by reducing the increasing number of apoptotic cells. We propose that this inhibitory effect of CLQ on apoptosis occurs because of inhibiting the activation of other genes in apoptotic pathways through inhibition of ERS.

Recent studies have indicated that chronic hypoxia reduces nuclear respiratory factor 1 (NRF1) expression in mice, resulting in downregulation of steroidogenic acute regulatory protein (StAR) levels playing an important role in the acute regulation of steroid hormone synthesis. This downregulation ultimately leads to a decrease in testosterone synthesis.[41] According to our biochemical data, chronic hypoxia reduced serum testosterone levels in the HX group compared to the control and CLQ groups. Therefore, we think that chronic hypoxia has detrimental effects on steroidogenesis and spermatogenesis by causing downregulation of some factors playing a key role in the testosterone synthesis in testicular tissue. In addition, we think that the decreased serum testosterone levels in experimental animals in the HX group are also because of Leydig cell loss in the interstitial region in the testicular tissue. The reason why the decrease in serum testosterone levels is not significant may be because of the number of animals used in the experimental groups. Using more animals in experimental groups could make the trend toward decrease in serum testosterone level statistically significant in the HX group. However, CLQ exerted a positive effect on serum testosterone levels, which decreases with hypoxia at a dose of 50 mg/kg. We think that the increase in serum testosterone levels in the HX + CLQ group compared to the HX group is because of the protective effects of CLQ on the Leydig cells so that steroidogenesis-related factors is not downregulated in the testicular tissue.

4 | CONCLUSION

In conclusion, chronic hypoxia caused histopathological changes in testicular tissues of experimental animals used in this study. Significant increase in HIF-1α and HSP70 expressions indicate cellular response to hypoxia in testicular tissue. We think that the increase in HSP90 expressions is because of the increase in the amount of misfolded protein because of hypoxia-induced oxidative stress. In addition, we think

that increased GADD153 expressions, together with the impairment of ER homeostasis because of misfolded protein accumulation, induce apoptosis in testicular tissue. Therefore, the significant increase in the number of TUNEL-positive cells in the groups treated with chronic hypoxia supports this idea. In this study, it has been proven that 50 mg/kg CLQ administration is a chemical agent that affects all parameters in testicular tissue. According to our immunohistochemical and biochemical results, CLQ reduced the hypoxic response and endoplasmic stress in the testicular tissue, causing inhibition of these processes that would result in apoptosis and protecting the testicular tissue from the damaging effects of chronic hypoxia. It also had a relatively curative effect on serum testosterone levels, which decreased with hypoxia. According to our results, it should not be ignored in future clinical studies that CLQ can be used as an ameliorative agent at low doses in the treatment of reproductive disorders because of low oxygen levels, especially in men living at high altitudes.

5 | MATERIALS AND METHODS

5.1 | Animals

This study was designed and performed in Histology-Embryology Department, Faculty of Medicine, Erciyes University, Turkey. The experimental protocol performed in the present study was accepted by the Erciyes University's Experimental Animal and Local Ethics' Committee with number 21/135/2021. A total of 32 male Wistar albino rats (8 weeks old, weighing 200-250 g) were obtained from Hakan Cetinsaya Experimental and Clinic Research Center, Erciyes University. The rats were kept in cages in the normal order of the day at 21°C and 12 hour of light/dark environment, and their water and nutrient requirements were fulfilled ad libitum.

5.2 | Chemicals

Chloroquine diphosphate (CLQ) salt was purchased from Sigma-Aldrich. The purity of powdered CLQ was ~98.5% according to the manufacturer's statement and it was dissolved in distilled water for the intraperitoneal injections to experimental animals.

5.3 | Experimental design

The rats were randomly divided into four groups, with eight rats per group. The groups were determined as follows:

1. Control group (n = 8): no medication was administrated to the rats and they were kept in normal room air (normoxia, 20%-21% O_2) for 28 days.
2. Chloroquine group (n = 8): 50 mg/kg CLQ was given to rats intraperitoneally per day for 28 days and they were kept in normal room air for 28 days.[42]
3. Hypoxia group (n = 8): rats were exposed to moderate–severe chronic hypoxia with 10% O_2 in the hypoxia chambers (BioSpherix) for 28 days.[43] No injection was administrated to the rats.
4. Hypoxia + CLQ group (n = 8): rats were exposed to moderate–severe chronic hypoxia with 10% O_2 in the hypoxia chamber and 50 mg/kg chloroquine was given intraperitoneally to rats for 28 days.

At the end of the experimental procedure (Figure 1), animals were anaesthetised with 30 mg/kg ketamine and 4 mg/kg xylazine combination and blood samples were collected for serum isolation before they were killed. Collected blood samples were centrifuged for 10 minutes at 3000 rpm. After killing, testis tissues were extracted from the animals for the histopathological and immunohistochemical examinations. Serum samples were kept at −80°C for further ELISA assays.

5.4 | Histological evaluation

Histological evaluation of the testis tissues was performed according to routine histological methods. Samples were fixed in 10% formaldehyde for 24-48 hours, dehydrated with alcohol series, cleared with xylene and embedded in paraffin blocks. Next, they were cut into 5-μm thick sections.

5.4.1 | Haematoxylin and eosin staining

Haematoxylin and eosin (H&E) staining was performed for determining the histopathological changes in the testicular tissue. Images were obtained with a light microscope (Olympus BX51; Olympus) and analysed. The structure of the testis tissue was evaluated by the study group.

5.4.2 | Histopathological score

Histopathological changes in the testicular tissues of experimental groups were scored by JTBS from 1 to 10. A total of 100 seminiferous tubules were examined and scored and analysed for each group by two independent histologists. In this scoring system, a score from 1 to 10 is given for each seminiferous tubule and the histopathological changes are shown in the spermatogenic cell line. The criteria used in the Johnsen's scoring of the seminiferous tubules were shown in the Table 1.[44]

5.4.3 | Measurement of the mean seminiferous tubule diameters

Mean seminiferous tubule diameters were measured in micrometres with Analysis LS Research Program. For the measurement of the

MSTD, at least 80 seminiferous tubules were measured and analysed to determine the differences among experimental groups.

5.5 | Immunohistochemistry

The immunohistochemistry method was applied according to previous studies[45,46] to investigate the changes in the immunoreactivities of HIF1-α, HSP70, HSP90 and GADD153 antibodies in seminiferous tubules and interstitial region of testis tissues. A total of 5 μm sections were cut from the paraffin blocks. The sections were treated with xylene for the deparaffinization, and they were hydrated along with alcohol series. Sections were taken into a sterile urine container with 0.01 M citrate buffer and heated in a microwave oven at 350 W for the antigen retrieval. For washing, the sections were exposed 3 times to phosphate-buffered saline (PBS) for 5 minutes. The sections were treated with 3% (w/v) H_2O_2 for 10 minutes to inhibit the endogenous peroxidase activity. After rewashing 3 times with PBS, Ultra V Block solution was applied to the sections, and they were stored in the incubation tank for 5 minutes. Next, HIF1-α (Anti HIF1-α antibody, Bioss), HSP70 (Anti HSP70 antibody, Bioss), HSP90 (Anti HSP90 antibody, Bioss), and GADD153 (Anti GADD153 antibody, Bioss) antibodies diluted in the ratio of 1:100 were administrated to the tissues and incubated overnight at 4°C. On the following morning, sections were rewashed 3 times with PBS, and the secondary antibody (TA-125-HDX, Thermo Fisher Scientific) was applied to sections for 10 minutes. After rewashing with PBS, the immunoreaction was amplified by using the streptavidin–avidin–peroxidase solution, and 3,3-*p*-diaminobenzidine tetrahydrochloride (TA-060-HDX, Thermo Fisher Scientific) was used for the visualization of the testis sections. Photographs were taken with a light microscope. To measure the density of immunoreactivities on the images, Image J (1.45 s, National Institutes of Health, RRID: SCR_003070) program was used to obtain quantitative data and obtained measurements were analysed by the study group in accordance with literature.

TABLE 1 Johnsen's score for the evaluation of spermatogenesis

Score	Histological findings
10	Complete spermatogenesis, numerous spermatozoa, germinal epithelium of regular height, tubular lumen of normal diameter
9	Numerous spermatozoa, germinal epithelium disorganized with sequestration of germinal cells, tubular lumen obturated
8	Less than 5 ± 10 spermatozoa per tubular cross-section
7	No spermatozoa, numerous spermatids, spermatocytes and spermatogonia
6	No spermatozoa, 5 ± 20 spermatids, numerous spermatocytes and spermatogonia per cross-section
5	No spermatozoa and spermatids, numerous spermatocytes and spermatogonia
4	No spermatozoa and spermatids, less than 5 spermatocytes, but numerous spermatogonia per cross-section
3	Only spermatogonia
2	No germinal cells, only Sertoli cells
1	No cells at all within the tubules

5.6 | Terminal deoxynucleotidyl transferase-mediated d-UTP nick end labelling assay

To detect the apoptosis in testicular tissue, TUNEL assay was performed according to the kit procedure (Roche, In Situ Cell Death Detection Kit). Sections were taken into sterile urine container with 0.01 M citrate buffer and heated in microwave oven at 350 W, consequently antigen retrieval was obtained. After washing with PBS, 450 μL of the purple-capped label solution and the blue-capped enzyme solution were mixed. We used 50 μL of solution remaining in the label solution for negatives. These solutions are added to the tissues in the dark and then incubated in a 37°C oven for 1 hour. After washing with PBS, it is covered with 4′,6-diamidino-2-phenylindole (DAPI) in the dark and TUNEL-positive cells were detected using fluorescent microscope. Moreover, apoptotic cells were count in a total of 150 microscopic areas for each group and statistical analyses were performed to compare the experimental groups.[47]

5.7 | Enzyme-linked immunosorbent assay

Rat Testosterone ELISA Kit (Cat. No: 201-11-5126, Sunredbio) was used to detect the serum testosterone level. Blood samples obtained from rats were centrifuged at 10 000 rpm at 4°C for 15 minutes. A total of 150 μL of the supernatant was taken into Eppendorf tubes

TABLE 2 JTBS and MSTD data of testicular tissue among experimental groups

Groups	Control	CLQ	HX	HX + CLQ	*P*
JTBS (1–10)	10 (8–10)	10 (8–10)	7 (5–9)[a]	9 (6–10)[b]	.001
MSTD (μ)	256.2 ± 36.78	259.6 ± 32.97	237.8 ± 31.95[a]	255.4 ± 30.44[b]	.001

Notes: JTBS data are expressed as median (min-max), and MSTD data are expressed as mean ± standard deviation. $P < .05$ was considered as significant.
[a]Shows the statistically significant difference when compared with control, CLQ and HX + CLQ groups.
[b]Shows statistically significant difference between HX and HX + CLQ groups.
Abbreviations: HX, hypoxia; CLQ, chloroquine; JTBS, Johnsen's Testicular Biopsy Scoring; MSTD, mean seminiferous tubule diameter.

separately for each group. In standard preparation, standard diluent was added to five tubes. A total of 120 μL of standard solution was added to the first tube and mixed. A total of 120 μL from the previous tube was added to the other four tubes, respectively. A total of 40 μL of supernatant was added to the samples section. Next, we added 10 μL of testosterone antibody to the samples and incubated at 37°C for 60 minutes. We added 50 μL of streptavidin HRP to both samples and standard section and incubated for 10 minutes at 37°C then, solution A, B and stop solution were added, respectively, and measured by ELISA reader.

5.8 | Statistical analysis

All statistical analyses were carried out by using GraphPad Prism v9.0 for MacOS (GraphPad Software). D'Agostino Pearson omnibus test was used to identify the normal distribution of the data. In the case of normal distribution, quantitative variables were compared using one-way analysis of variance (ANOVA) and Tukey's post hoc test. When data show abnormal distribution, Kruskal-Wallis test and Tukey's post hoc test were used for comparison of the experimental groups. The data with normal distribution were expressed as the mean of normalized data ± standard deviation of the mean, and the abnormal distributed data were expressed as median (minimum value-maximum value). $P < .05$ was considered as statistically significant.

AUTHORS CONTRIBUTIONS

A.T.A., E.K., S.O. and K.E.B. designed the study and A.T.A., E.K., and E.O. and T.C. performed experiment. A.T.A., E.K. and D.K. contributed to analyse the data. A.T.A. performed the histological analyses. A.T.A. wrote the manuscript. D.K. and B.Y. revised the final version of the manuscript.

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CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare that are relevant to the content of this article.

TRANSPARENCY PEER REVIEW STATEMENT

The corresponding author affirms that this manuscript is an honest, accurate, and transparent account; that no important aspects of the study have been omitted.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author on reasonable request.

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