Original Article

Investigation of the Effects of Acute and Chronic PTZ Model Epilepsy in Rats Exposed to Neonatal Hyperoxia on *Bdnf, Ngf, Cyt c, Bax,* and *Bcl-2* Gene Expression Levels in the Brain

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Abstract

Objective: The aim of this study was to investigate the relationship between acute and chronic epilepsy that may occur in adulthood, gene expression levels, and the possible mechanism of neuronal loss in rats exposed to hyperoxia in the postnatal period.

Methods: The study was started with 12 female rats (mother rat). Two main groups were formed: six control and six hyperoxia groups. At the end of the experiment, brain tissue samples were collected and *Bdnf, Ngf, Cyt c, Bax*, and *Bcl-2* gene expressions were studied by quantitative polymerase chain reaction. *Bax* (Bcl-2 associated X-protein) and *Cytochrome (Cyt) c* gene expression levels were found to be significantly higher in the hyperoxia-epilepsy groups, especially in the male group, than in the other groups ($p<0.05$).

Results: While the *Ngf* gene expression level increases significantly in females due to epilepsy, it is independent of hyperoxy ($p<0.05$). *Bdnf* gene expression levels were found to be affected by hyperoxia in both males and females ($p<0.05$). In our study, a significant increase in *Bax* and *Cyt c* gene expression levels was observed in the neonatal hyperoxia and epilepsy group.

Conclusion: It is thought that this increase in gene expression levels molecularly supports neuronal loss, but the related pathways will be better clarified with further studies.

Keywords: Hyperoxia, neonatal hyperoxia, epilepsy, gene expression

INTRODUCTION

Preterm infants are relatively early to face hyperoxia because of their early delivery from the intrauterine environment and become more vulnerable to hyperoxic stress because of their insufficient antioxidant defense mechanisms.[1] Because they are susceptible to deterioration caused by reactive oxygen species (ROS), their endogenous radical scavenging systems are not fully mature.[2] In the sensitive period of brain development, supraphysiological oxygen therapy affects the developmental processes because of hyperoxia. The toxic effects of hyperoxia on the brain have been demonstrated in both experimental and clinical studies.[2,3]

Although individuals exposed to hyperoxia in the neonatal period may be prone to epilepsy, its mechanism has not yet been clarified. There are studies showing that hyperoxic brain damage in developed models causes widespread apoptosis and a decrease in the number of neurons in various regions of the brain because of increased oxidative stress and decreased activation of neurotrophin pathways. In the clinical setting, up to 50% of surviving premature infants exhibit cognitive deficits or behavioral problems in the later stages of development.[4-6]

Development of the mammalian brain is a dynamic process that includes structural and functional maturation processes. The evolution of the brain is characterized by neuronal cell development and proliferation, migration, glial cell proliferation, axonal and dendritic growth, synaptogenesis, and myelination of axons.[7] Although neuronal migration processes are usually completed in extremely preterm infants born at the limit of viability (about the 24th week of gestation), glial cell maturation, growth, and connection formation are still ongoing processes.[4,8] Neuronal electrical activity is strongly dependent on metabolic factors such as mitochondrial development, cerebral vascular density and blood flow, maturation of glucose utilization systems, and cytochrome oxidase activity.[9,10]

With the rapid development of neonatology in recent years, oxygen therapy has become the most important measure in the rescue and treatment of newborns and preterm infants. Studies have shown that prolonged exposure to hyperoxia in neonates can cause hyperoxic lung damage, hyperoxic retinopathy, hypoxic ischemic encephalopathy, and ultimately lead to worsening of the child's condition, lowering survival and quality of life, easily causing other diseases that can affect adulthood. Hyperoxic brain injury is common and more severe, particularly in very low birth weight and preterm infants. Studies have shown that high oxygen concentrations can affect the brain and reduce weight.[11]

Brain damage is more common in preterm newborns than in term newborns for various reasons, such as developmental and genetic weaknesses and different exposure to adverse perinatal environments.[12,13] However, the mechanisms of neonatal brain injury have not been fully understood so far.[14] Studies have proven that glutamate excitotoxicity is one of the main mechanisms of preterm-related brain injury.[15,16] To prevent the accumulation of extracellular glutamate, the brain relies on rapid uptake by sodium-dependent glutamate transporters such as excitatory amino acid transporters (EAATs) and vesicular glutamate transporters (VGLUTs).[17,18] In addition, γ-aminobutyric acid (GABA) is another important component of the balance between excitation and inhibition and plays an important role in different processes associated with brain development.[19] Zhao et al.[14] found hyperoxia-induced glutamate accumulation in the immature cerebrum and cerebellum of newborn rats. In the same study, increased oxidative stress and decreased expression of glutamate transporters, including EAATs and VGLUTs, within 2 weeks of hyperoxia threat contributed to impaired glutamate homeostasis in the rat brain. Under physiological conditions, astrocytes are necessary for the uptake of synaptic glutamate, thereby preventing neuronal hypersynchronization and glutamatergic excitotoxicity. However, reactive glia can promote neuronal apoptosis. Experimental models have shown the upregulation of the Bcl-2 protein in the sclerotic hippocampus in both neuronal and glial cells.[20-22] In addition, Bcl-xL has shown a positive correlation with seizure frequency in an amygdala-triggered seizure model, indicating an antiapoptotic response after recurrent seizures as a possible attempt to prevent neuronal loss.[23] One of the main biological functions of anti-apoptotic Bcl-2 proteins is to prevent the disruption of mitochondrial integrity. Members of the pro-and anti-apoptotic Bcl-2 family of proteins are expressed throughout the central nervous system during embryonic and adult life.[24] Regarding pro-apoptotic Bax-like proteins, Bax is widely expressed in the brain,[25] and Bax was the first Bcl-2 homologous gene that was found to act as an apoptosis executor. The Bax protein is expressed in various tissues as multiple alternative splicing variants that are normally localized in the cytosol or loosely attached to the mitochondria. Indeed, Bax expression in HeLa cells resulted in increased ER Ca^{2+} loading followed by Ca^{2+} release from ER, an increase in mitochondrial Ca^{2+} loading, and strengthening of mitochondrial Ca^{2+} responses, consequently triggering apoptosis.[26] These results agree with previous studies that reported that Bax/Bak overexpression facilitates the transfer of Ca^{2+} from the ER to mitochondria, makes mitochondria sensitive to absorb more Ca^{2+}, and thus induces cell death.[27,28] Bax also regulates dynamic Ca^{2+} signaling between the ER and cytosol in cortical neurons, regardless of its classical function in the mechanism of apoptotic cell death or its proposed involvement in the decoupling of mitochondrial PTP.[29] Emerging studies show that pro-and anti-apoptotic members of the Bcl-2 protein family not only modulate the mitochondrial pathway of apoptosis but also have important 'day-time' activities. These functions include the regulation of neuronal Ca^{2+} homeostasis and mitochondrial energy.[30] This studyaimed to investigate the susceptibility of rats exposed to postnatal high oxygen to convulsions and epilepsy in adulthood and the molecular mechanisms of this susceptibility. For this purpose, an acute and chronic epilepsy model was created in adulthood of rats exposed to hyperoxia during the neonatal period. In the case of epilepsy, which has any convulsive effects of exposure to hyperoxia in the newborn period, the expression levels of some proteins known to be responsible for neuronal loss in the brain were determined to obtain information about degeneration and regeneration in the brain, and some genes that are effective in degeneration were studied. At the same time, the differences between males and females, in this case, were considered.

MAIN POINTS

- Premature babies face hyperoxia relatively early due to inadequate antioxidant defense mechanisms and are more vulnerable to hyperoxic stress, exhibit cognitive deficits or behavioral problems in later stages of development, and become prone to epilepsy.
- Determination of different gene expression levels by applying an experimental epilepsy model in later stages of development in rats exposed to hyperoxia in the postnatal period may provide an understanding of the possible mechanisms of neuron loss.
- *Bdnf, Ngf, Cyt c, Bax,* and *Bcl-2* gene expression levels molecularly support neuron loss, but the relevant pathways will be better elucidated in future studies.

METHODS

Experimental Animals

The experimental animals used in the study were obtained from the Bolu Abant İzzet Baysal University Experimental Animals Application and Research Center. Until the start of the study and during the study, the animals will be kept in the Experimental Animals Application and Research Center for 12 h in a light/dark environment with a relative humidity of 60-70% and will be fed ad libitum. All experimental animals have been treated based on the guiding principles approved by the Animal Ethical Committee of Bolu Abant İzzet Baysal University, and all treatments comply with the recommendations provided in the Declaration of Helsinki (registration number: 2020/23, date: 26.08.2020). All experimental animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals. This study was started with 12 mother rats. Six of them were in the control group and others were in the hyperoxy group. Wistar albino female rats (n=12) was deposited by taking vaginal smears and determining the estrus period. The joining day was considered as day 0 of pregnancy. In the control groups, mother rats (n=6) and offspring (n=24). In the groups with hyperoxia, mother rats (n=6) and offspring (n=24) were subjected to controlled constant oxygen exposure with a concentration of 80% and a current rate of 4 L/h until the postnatal 5th day. When the rats were two months old, an acute and chronic pentylenetetrazole (PTZ) epilepsy model was created. Experimental Groups: It was composed of 2 main groups: control and hyperoxy. Control female (CF), control acute epilepsy female (CAEF), control chronic epilepsy female (CCEF), control male (CM), control acute epilepsy male (CAEM), control chronic epilepsy male (CCEM), and

hyperoxy female (HF), hyperoxy acute epilepsy female (HAEF), hyperoxy chronic epilepsy female (HCEF), hyperoxy male (HM), hyperoxy acute epilepsy male (HAEM), and hyperoxy chronic epilepsy male (HCEM) in a total of 12 subgroups. Four animals were used in each group. After the experiment, brain tissues of all animals were taken and *Cytochrome c, Bax, Bcl-2, Bdnf*, and *Ngf* gene expressions were determined by real-time polymerase chain reaction (RT-PCR). One-way analysis of variance was used for the statistical analysis of the results, and it was determined whether there was a difference between the groups and from which group this difference originated with the post-hoc LSD test. A value of $p<0.05$ was considered significant.

An acute and chronic PTZ model (kindling model) was created in 2-month-old female and male offspring. The animals were then decapitated, and their brains were removed. The cerebellum was separated, and the left hemisphere was stored in RNAase DNAase free tubes at -80 until gene expression was studied (Figure 1).

Creation of a Chronic PTZ Model of Epilepsy

To establish a chronic experimental epilepsy model, PTZ [35 mg/kg/intraperitoneally (i.p.)] was injected i.p. three times a week (Monday, Wednesday, and Friday) until the seizure behavior of the animals was observed. Severity of seizures was observed as stage 3 and stage 4 according to the Racine scale. For the Kindling model, 13 doses of 35 mg/kg were applied on Mondays, Wednesdays, and Fridays, 3 days a week. Stages 4 and 5 were observed according to the racing scale in the last three applications consecutively, and it was accepted that the ignition pattern was formed. It was evaluated according to the racing scale in both acute and chronic models (Figure 1).

Creation of an Acute PTZ Model of Epilepsy

A single dose of PTZ (50 mg/kg/i.p.) was administered to establish an acute experimental epilepsy model. It was evaluated according to the racing scale in both acute and chronic models (Figure 1).

Severity of seizures was scored as follows:

Stage 0: No response

Stage 1: Ear and facial twitching

Stage 2: Myoclonic body jerks without an upright position

Stage 3: Myoclonic tremors in theupright position with clonic forefoot convulsions

Stage 4: Tonic-clonic seizures

Stage 5: General tonic-clonic seizures and loss of postural control.

Q-PCR Method

To detect changes in gene expression levels, total mRNA was isolated, cDNA synthesis was performed, and quantitative RT-PCR (qRT-PCR) experiments were performe.

RNA isolation: For RNA isolation from tissue samples, 1 mL of Trizole solution was added to a 50-mg tissue sample and homogenized. The tubes were incubated at room temperature (Tm) for 5 minutes, then 200 µL chloroform was added, and the mixture was manually shaken for 15 s. The tubes were maintained at room Tm for 3 minutes, centrifuged at 12,000 g, and at 4 °C for 15 min. The transparent-colored upper phase was taken into a new tube and 500 µL of 100% isopropanol was added. After incubation at room Tm for 10 min, the tubes were centrifuged for 10 min at 12,000 g and 4 °C. At this stage, the RNA in the sample formed a white precipitate at the bottom of the tube. The liquid in the tube was removed, taking care not to touch this precipitate, and the RNA precipitate was washed with 1 mL of 75% ethanol and centrifuged at 7500 g and 4 °C for 5 min. The resulting RNA was dissolved with 20-50 µL of DEPC-ddH_2O, and its concentration was measured.

cDNA synthesis: For each sample, 1 µg of RNA, 2 µL of oligo dT, and DEPC-ddH_2O were mixed with a final volume of 8 µL and incubated for 5 min at 70 °C. After 10 µL of 2X reaction buffer and 2 µL of reverse transcriptase enzyme were added, the samples were incubated for 1 h at 42 °C and 5 min at 80 °C. The cDNA samples were stored at -20 °C.

qRT-PCR: Primers that bind with high specificity to the target gene regions to be tested for RT-PCR experiments were designed. The oligo design was performed using the Amplify program, and its properties such as melting Tm and primary-dimer formation were studied using the same program. To ensure that the selected primers did not bind to other unwanted regions (unspecific) in the genome, the primers were selected from the exon-intron junction regions. However, the specificity of the primers was confirmed using in silico PCR using the UC Genome Browser. To investigate the level of mRNA expression, 1 µL of cDNA, 1 µL of primer mixture (10 µM, forward+reverse), 10 µL of 2X SYBR Green, and 8 µL of ddH_2O were added to each qRT-PCR reaction. The following program was used for the reaction:

95 °C for 5 min, [95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 30 sec] x 40, 72 °C for 5 min

Analysis of the qRT-PCR results: Normalization with a housekeeping gene such as GAPDH was performed to prevent differences between samples and possible pipetting errors during the detection of mRNA expression levels. The analysis was performed using the ddCt[31] method using the following equation:

ddCt=Ct (target gene) Ct (housekeeping gene)

Target gene expression = 2^(- ddCt)

Statistical Analysis

Statistical analysis of the results determined whether there was a difference between the groups with One-way analysis of variance, and it was determined from which group this difference originated with the post hoc test. The LSD test was used as a post hoc test, and differences with a p value of 0.05 were considered significant (Table 1).

RESULTS

Nerve growth factor (Ngf) gene expression levels were evaluated, and no significant difference was found between female control and hyperoxy (control a CF) and HF, CAEF and HAEF, CCEF and HCEF groups (Figure 2a). In addition, *Ngf* gene expression levels

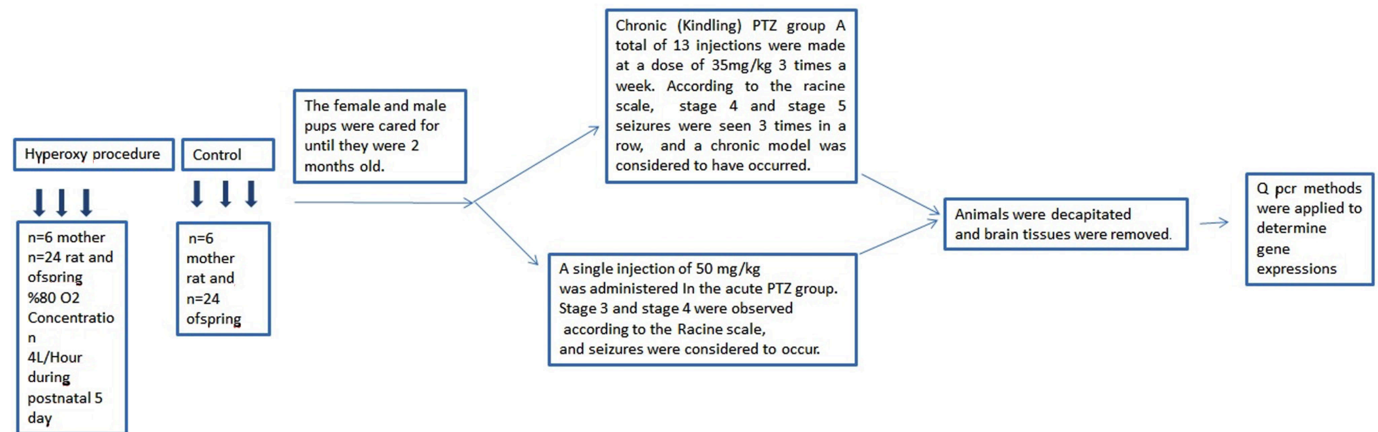

Figure 1. Experimental procedure
PTZ: Pentylenetetrazole

Table 1. Experimental groups

	Control (n=24)	Hyperoxy (n=24)
Female	**CF;** Healthy female rat (n=4)	**HF;** Female rat exposed to hyperoxy (n=4)
Acute female	**CAEF;** Healthy female acute PTZ created (n=4)	**HAEF;** Hyperoxy exposed female rat acute PTZ was established (n=4)
Chronic female	**CCEF;** Healthy female chronic PTZ created (n=4)	**HCEF;** Hyperoxy exposed female rat chronic PTZ was established (n= 4)
Male	**CM;** Healthy male rat (n=4)	**HM;** Hyperoxy exposed male rat acute PTZ was established (n=4)
Acute male	**CAEM;** Healthy male acute PTZ created (n=4)	**HAEM;** Hyperoxy-exposed female rat acute PTZ was induced (n=4)
Chronic male	**CCEM;** Healthy male chronic PTZ established (n=4)	**HCEM;** Hyperoxy exposed male rat chronic PTZ was established (n=4)

CF: Control female, CAEF: Control acute epilepsy female, CCEF: Control chronic epilepsy female, CM: Control male, CAEM: Control acute epilepsy male, CCEM: Control chronic epilepsy male, HF: Hyperoxy female, HAEF: Hyperoxy acute epilepsy female, HCEF: Hyperoxy chronic epilepsy female, HM: Hyperoxy male, HAEM: Hypeoxy acute epilepsy male, HCEM: Hyperoxy chronic epilepsy male, PTZ: Pentylenetetrazole

in the control group were evaluated among themselves, and *Ngf* gene expression levels were found to be significantly higher in the CAEF and CCEF groups than in the CF group ($p<0.05$) (Figure 2a). Then, *Ngf* gene expression levels in the hyperoxy group were evaluated among themselves. *Ngf* gene expression levels were found to be significantly higher in the HAEF and HCEF groups than in the HF group ($p<0.05$) (Figure 2a).

When the *Ngf* gene expression levels in males were evaluated, there was no significant difference between the CM and HM groups ($p<0.05$). There was no difference in Nfg levels in males when no convulsant substance was administered (Figure 2b).

The *Ngf* gene expression level was significantly lower in the CAEM and CCEM groups than in the CM group ($p<0.05$) (Figure 2b). *Ngf* gene expression levels were significantly higher in the HCEM group than in the HM group.

No significant difference was found between the CF group without epilepsy and the HF group in terms of *Ngf* gene expression levels. There was no significant difference between the CM and CF groups in the male groups without epilepsy. When the CF and CM groups were compared, the *Ngf* gene expression level was found to be higher in the CM group ($p<0.05$) (Figure 2a, 2b).

Brain-derived neurotrophic factor (Bdnf) gene expression levels were compared between the CF and HF groups, and there was no statistically significant difference. When the control group was evaluated within itself, the *Bdnf* gene expression level in the CAEF group was significantly higher than that in the CF and CCEF groups $p<0.05$ (Figure 3a).

The male groups were compared. The CM and HM groups were compared, and the Bdnf level was found to be significantly higher in the HM group. The control group is evaluated among themselves, there is no significant difference. *Bdnf* gene expression level was found to be significantly higher in HAEM and HCEM groups compared to HM group. The male and female groups were compared in terms of Bdnf, and there was no significant difference ($p<0.05$) (Figure 3b).

The *Cyt c* gene expression level in the CF group was significantly lower than that in the HF group ($p<0.05$) (Figure 4a).

There was no statistically significant difference in terms of *Cyt c* gene expression levels between the CAEF and HAEF groups ($p<0.05$). The *Cyt c* gene expression level in the HCEF group was significantly higher than that in the HF and HAEF groups ($p<0.05$) (Figure 4a).

In the male groups, the *Cyt c* gene expression level was significantly higher in the acute and chronic epilepsy groups in hyperoxia-treated rats ($p<0.05$) (Figure 4b).

When female and male groups were compared, *Cyt c* gene expression levels were found to be significantly higher in male acute and chronic epilepsy groups than in female acute and chronic epilepsy groups ($p<0.05$) (Figure 4a, 4b).

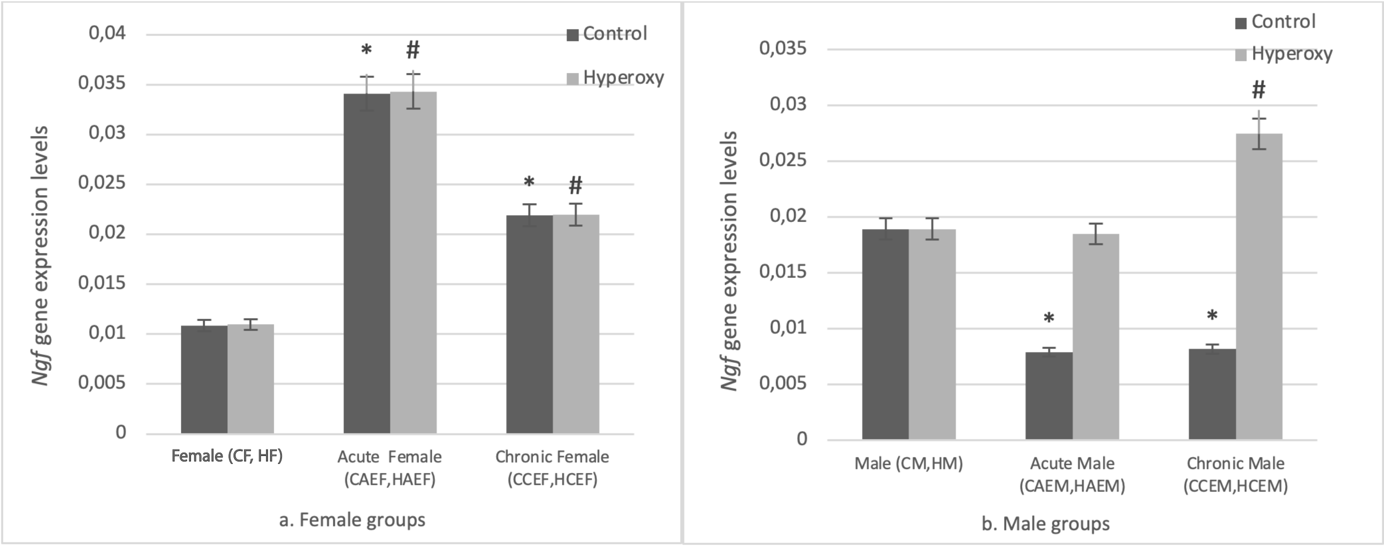

Figure 2. a) *Ngf* gene levels in brain in female groups *p<0.05 compare with control female groups, #p<0.05 compared with control hyperoxia female groups. **b)** *Ngf* gene expression levels in brain in male groups *p<0.05 compare with control male #p<0.05 compare with other with all hyperoxia groups

CF: Control female, CAEF: Control acute epilepsy female, CCEF: Control chronic epilepsy female, CM: Control male, CAEM: Control acute epilepsy male, CCEM: Control chronic epilepsy male, HF: Hyperoxy female, HAEF: Hyperoxy acute epilepsy female, HCEF: Hyperoxy chronic epilepsy female, HM: Hyperoxy male, HAEM: Hypeoxy acute epilepsy male, HCEM: Hyperoxy chronic epilepsy male

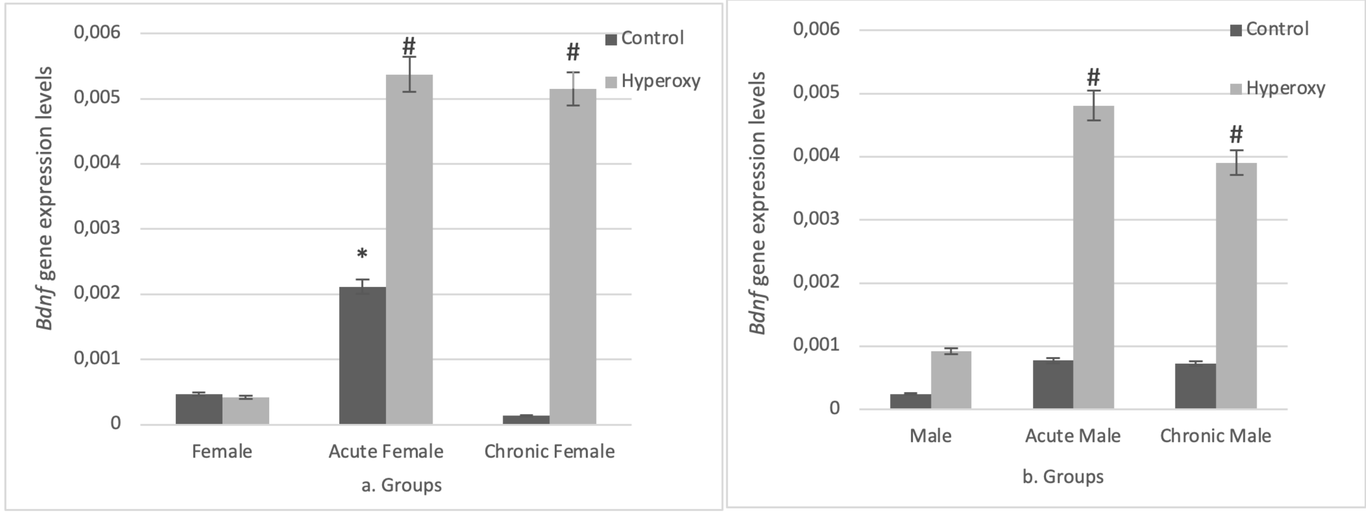

Figure 3. a) *Bdnf* gene expression levels in the brain in females *p<0.05 compared with control female groups, #p<0.05 compared with control hyperoxia female. **b)** *Bdnf* gene expression levels in the brain in males p<0.05 compared with other with all hyperoxia groups

There was no significant difference in *Bax* gene expression levels between the CF and HF groups.

When the control groups were evaluated among themselves, there was no significant difference between the groups. In the hyperoxy group, *Bax* gene expression levels were significantly higher in both acute and chronic epilepsy than in the HF group.

Both acute and chronic epilepsy *Bax* gene expression levels were significantly increased in the teeth exposed to hyperoxy compared with the control groups. According to this result, it was found that epilepsy developed in females exposed to hyperoxia, and the level of *Bax* gene expression in their brains increased. Together with hyperoxia, epilepsy had the effect of increasing the level of *Bax* gene expression (p<0.05) (Figure 5a).

Bax gene expression levels were significantly higher in the HM group than in the CM group (p<0.05).

When the HM group was evaluated among themselves, no significant difference was observed between the groups. When the CM group was compared among themselves, there was no significant difference between the groups. When the hyperoxia and control groups were compared, it was found that the number of male rats exposed to hyperoxia was significantly higher than that of the control group in all groups (p<0.05) (Figure 5b).

The *B-cell lymphoma 2 (Bcl-2)* gene expression level in the CAEF and CCEF groups was significantly higher than that in the CF group (p<0.05) (Figure 6a).

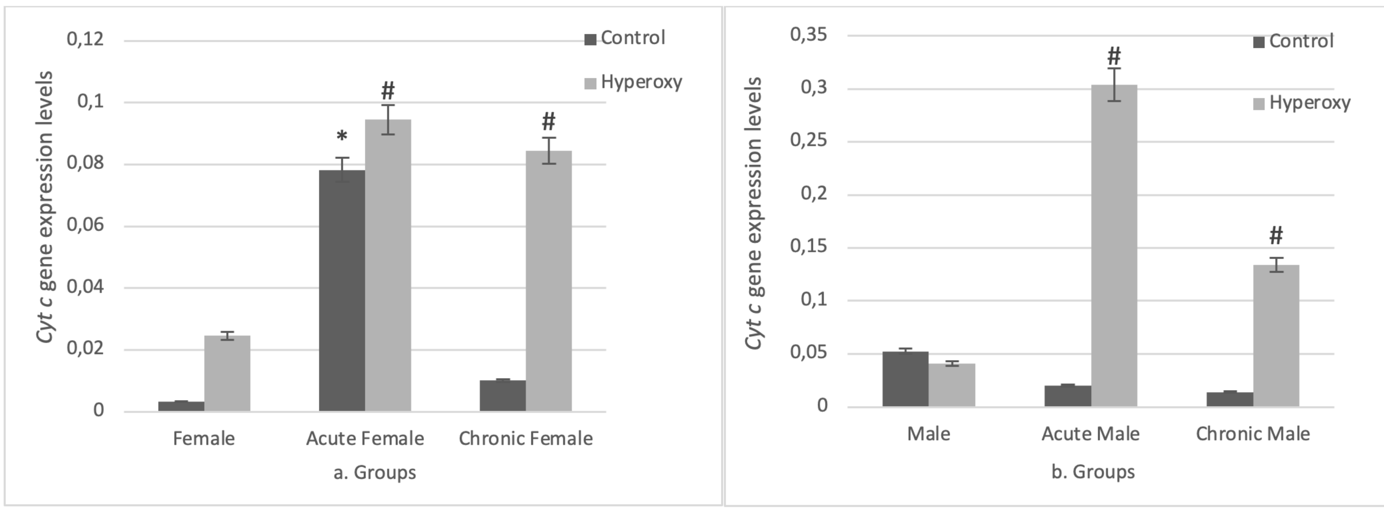

Figure 4. a) *Cyt c* gene expression levels in brain in female *p<0.05 compared with control female groups, #p<0.05 compared with control hyperoxia female. **b)** *Cyt c* gene expression levels in the brain in males p<0.05 compared with other with all hyperoxia groups

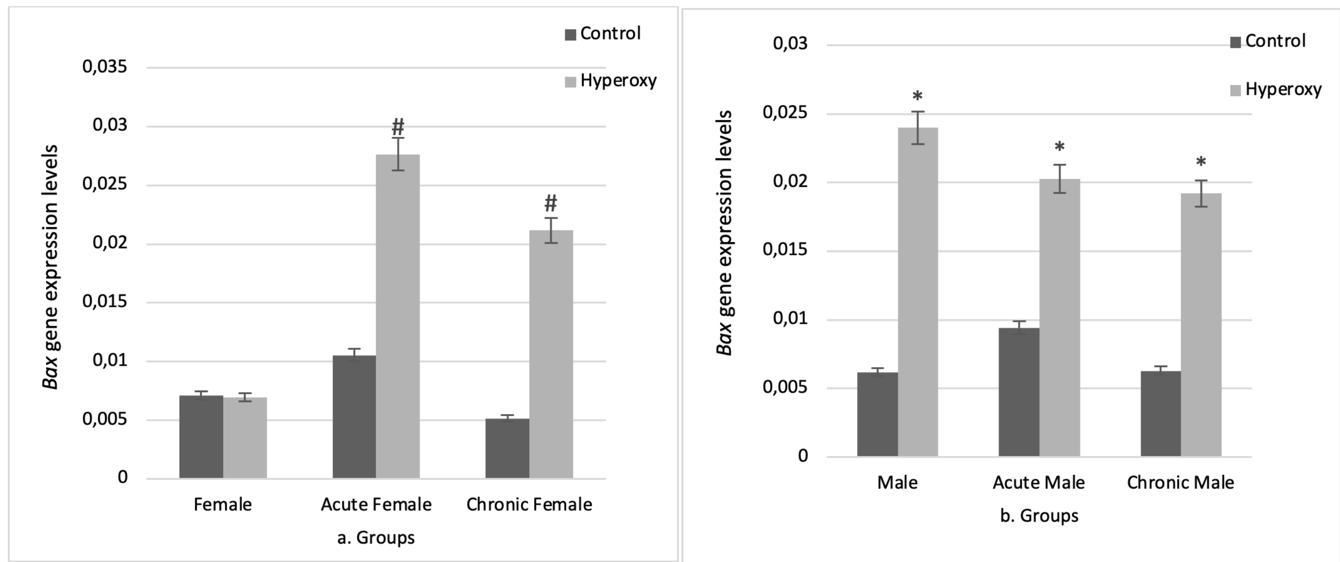

Figure 5. a) *Bax* gene expression levels in the brain in females, #compare with control hyperoxia female. **b)** *Bax* gene expression levels in the brain in males *compared with control groups.

The *Bcl-2* gene expression level was evaluated, and there was no significant difference between HF and CF. The *Bcl-2* gene expression level in the CAEF group was significantly higher than that in the CCEF and CF group (p<0.05) (Figure 6a). The *Bcl-2* gene expression level was found to be significantly higher in the HAEF group than in the CAEF group (p<0.05) (Figure 6a). The *Bcl-2* gene expression level was also found to be significantly higher in the HCEF group than in the CCEF group (p<0.05) (Figure 6a). It was found that *Bcl-2* gene expression levels increased in the acute and chronic epilepsy groups in females exposed to hyperoxia.

The HM group was evaluated among themselves, and no significant difference was observed between the groups. When the CM group was compared among themselves, there was no significant difference between the groups. When the hyperoxia and control groups were compared, it was found that the number of male rats exposed to hyperoxia was significantly higher than that of the control group in all groups (p<0.05) (Figure 6b).

DISCUSSION

Thanks to the developing technologies in the medical field, the birth and survival rates of preterm infants are constantly increasing. It is estimated that approximately 15 million preterm infants are born every year in the world,[32] of which have led to an increase in problems with the brain due to premature birth. Until now, many factors involved in hyperoxia have been associated with brain damage.[33] Oxygen has become an important treatment approach for these patients because atmospheric oxygen therapy concentrations significantly improve the neonatal hypoxic state. However, high amounts of oxygen[34] can stimulate the production of several active oxygen substances. In infants, especially preterm infants, the immune and antioxidant defense system is not adequately developed; therefore, preterm infants are more vulnerable to these substances.[35]

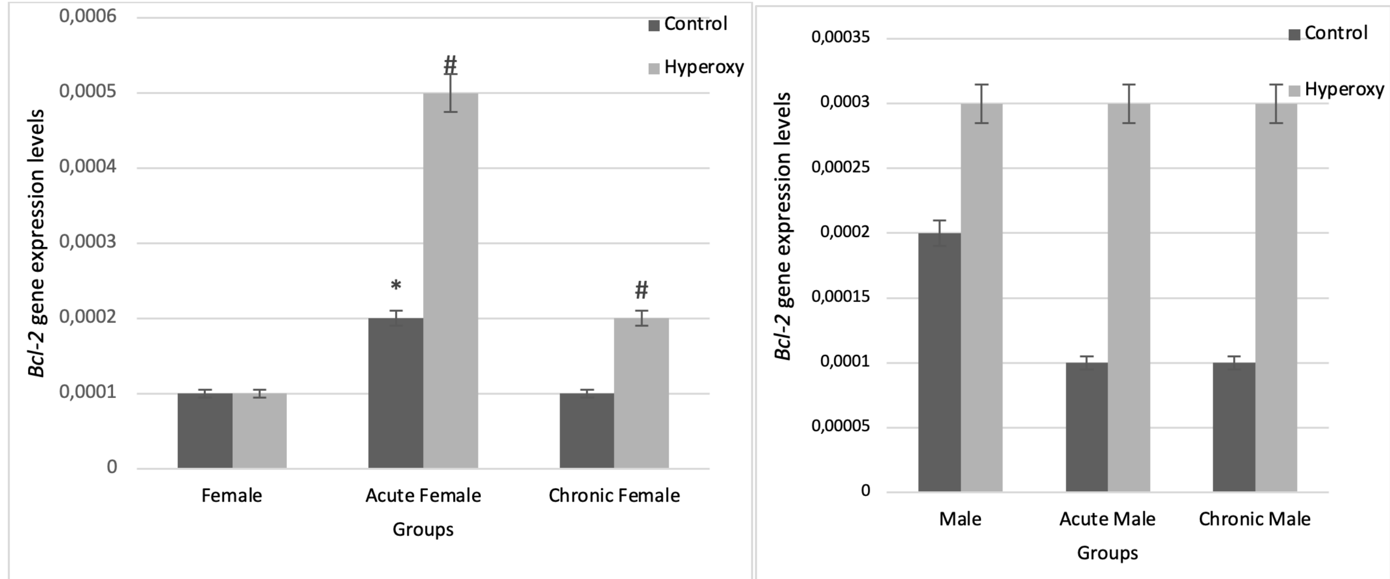

Figure 6. a) *Bcl-2* gene expression levels in brain in female groups *$p<0.05$ compare with control female groups, #$p<0.05$ compared with control hyperoxia female groups. **b)** *Bcl-2* gene expression levels in the brain in male

Oxidative stress indicates an imbalance between the formation of free radicals and resistance against oxidative substances, which leads to tissue damage.[36] Considering that preterm newborns exhibit higher levels of oxidative stress because of their inability to face the oxygen-rich environment when they are born, many studies have shown that oxidative stress has an important role in the formation and development of brain damage. During this condition, the body is unable to effectively eliminate the oxidative stress reaction, which produces ROS and leads to apoptosis.[34,37]

Epilepsy is defined as excessive and synchronous excitation of neurons. Its general mechanism is defined as a decrease in inhibitory neurotransmitters (GABA) and an increase in the number of excitatory neurotransmitters. Energy and mitochondria are essential in this mechanism. This is because ATP is required for neurons to be stimulated. During a seizure, the amount of ATP decreases, which causes intracellular calcium to pass to the mitochondria. In mitochondria, the amounts of anti- and pro-apoptotic protein increase. Mitochondrial permeability increases; the apoptosome, consisting of the triad of Cytochrome c, procaspase-9, and apaf 1 forms.[38] These trigger the formation of caspase-9. Caspase-9 leads to the formation of caspase-3, and cell death occurs. Caspase-6 is also found in the brain, which causes neuronal cell death and is more effective than caspase-3. Calcium enters this pathway directly through the endoplasmic reticulum (ER, Ca), and caspase-12 is formed if this pathway is activated. If the inflammation pathway is activated, caspase-8 is also formed Caspase-12 and caspase-8 can stimulate caspase-3, -6, and -7, or altogether (Caspase-8, Caspase-9, Caspase-12) can cause a stimulus, and irreversible cell death occurs.[20]

Ngf and Bdnf are important for the survival, maintenance, and regeneration of certain neuronal populations in the brain. Depletion of these neurotrophic factors is associated with disease pathology and symptoms and is considered a potential therapeutic approach for neurodegenerative diseases.[39] Bdnf was found to be high in the serum of epilepsy patients in direct proportion to the severity of the disease.[40] Studies have shown that Bdnf injection into the temporal and hippocampal brain regions of mice causes seizures, and when Bdnf transcription is blocked, seizures are almost completely eliminated.[41] Similarly, in our study, it was observed that *Bdnf* and *Ngf* gene expression increased and increased significantly, especially with hyperoxia, in the epilepsy group.

According to this result, the increase in the *Ngf* gene expression level in females is independent of hyperoxia. In this study, it was found that the increase in the *Ngf* gene expression level in females was related to epilepsy. Unlike in females, *Ngf* gene expression level increased with the combined effect of hyperoxia and epilepsy in males.

Oxidative stress indicates an imbalance between the formation of free radicals and resistance against oxidative substances, which leads to tissue damage.[36] Considering that preterm newborns exhibit higher levels of oxidative stress because of their inability to face the oxygen-rich environment when they are born, many studies have shown that oxidative stress has an important role in the formation and development of brain damage. During this condition, the body is unable to effectively eliminate the oxidative stress reaction, which produces ROS and leads to apoptosis.[34,37]

Bax and Bcl-2 are Bcl-2 family proteins that are key factors in the regulation of intrinsic apoptosis. Specifically, Bax is a pro-apoptotic protein, whereas Bcl-2 is an anti-apoptotic protein. Bcl-2 proteins are key regulators of the intrinsic apoptotic pathway. Each member of this family contains one or more Bcl-2 homology (BH) domains, BH1-BH4.[42] Bcl-2 family proteins are critical regulators of apoptosis for inhibiting or promoting cell death via the intrinsic pathway of apoptosis.[43] Bax is an important pro-apoptotic protein of the intrinsic pathway. Bax moves into mitochondria by inducing the release of Cyt c into the cytoplasm. Finally, Cyt c activate caspase 9, which destroys caspase 3. Bcl-2, an anti-apoptotic protein, can bind to Bax by inhibiting the release of Cyt c.[44] Studies involving Bax expression in temporal lobe epilepsy are controversial. Several studies have shown an increase in Bax, whereas others have shown similar immunostaining compared with control samples. Bcl-2 and

active caspases are overexpressed in both the neuronal and glial cytoplasm of the sclerotic hippocampus.[20,21,44]

When looking at the *Cyt c* gene expression level in general, it can be seen that the Cyt c level in males is significantly higher than that in females. According to this result, it has been shown that the *Cyt c* gene expression level is higher in cases of acute or chronic epilepsy in male individuals exposed to hyperoxia.

According to our study, while hyperoxia did not cause any effect in acute epilepsy, it was an important factor in females, and in chronic epilepsy, it was observed that the Cyt c level increased significantly. While there was no difference with the control group in acute epilepsy in hyperoxia-exposed female rats, the *Cyt c* gene expression level in chronic epilepsy was significantly increased compared with the chronic CF group. Cyt c gene expression levels increased significantly in both acute and chronic epilepsy in male rats exposed to hyperoxia compared with the control.

Study Limitations

The aim of this study was to investigate the relationship between acute and chronic epilepsy that may occur in adulthood, gene expression levels, and the possible mechanism of neuronal loss in rats exposed to hyperoxia in the postnatal period. However, due to lack of budget, the limitations of this study are the inability to verify protein and the inability to stain Bdnf, Ngf, Cyt c, Bax, and Bcl-2 in the tissue by immunohistochemistry, and the inability to perform TUNEL staining.

CONCLUSION

As a result, if any form of epilepsy develops in adulthood in males and females exposed to hyperoxia, high *Bax* gene expression levels in females with chronic epilepsy and high *Cyt c* gene expression levels in male individuals can be accepted as evidence of neuronal loss. In addition, neuronal loss may occur by different mechanisms in males and females. Ngf is higher in females exposed to hyperoxia, and neuronal loss in females may be less than that in males.

Ethics

Ethics Committee Approval: The study was approved by the Bolu Abant İzzet Baysal University of Animal Ethical Committee (registration number: 2020/23, date: 26.08.2020).

Informed Consent: Animal experiment.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: C.Ö., Ü.K., H.S., Concept: C.Ö., Design: C.Ö., Ü.K., Data Collection or Processing: C.Ö., Ü.K., H.S., Analysis or Interpretation: C.Ö., Ü.K., H.S., Literature Search: C.Ö., Ü.K., H.S., Writing: C.Ö., Ü.K., H.S.

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