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Türk Hijyen ve Deneysel Biyoloji Dergisi

Effect of copper sulphate in rat brain tissue: Kir channels

Sıçan beyin dokusunda bakır sülfat etkisi: Kir kanalları

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ABSTRACT

Objective: Copper plays an important role in the catalyzing of enzymes involved in the function of many enzymes in the cell, especially in mitochondria, where energy is produced in the cell, and is involved in mechanisms such as iron homeostasis, scavenging of free radicals, collagen, and elastin bond formation. The expression of internal potassium channels (Kir) is known in various tissues, especially in brain tissue. In this context, the aim of this study was to evaluate the morphological effect of Copper Sulphate ($CuSO_4$) in male and female rat brain tissue and the expression of Kir2.1 and Kir4.1 in the prefrontal cortex and hippocampus regions of the brain.

Methods: In this study, female and male Sprague dawley rats were divided into control group (n=10) and $CuSO_4$ group (n=10) and administered saline to the control group and $CuSO_4$ (100 mg/kg) to the $CuSO_4$group by oral gavage daily for 14 days. The animals were then euthanised and the brain tissues were examined histologically for morphological and tissue integrity and the expression of Kir channel proteins Kir2.1 and Kir4.1 was evaluated by immunohistochemical method.

ÖZET

Amaç: Bakır, hücredeki birçok enzimin işlevinde rol oynayan ve özellikle hücrede enerjinin üretildiği mitokondride etkili olup demir homeostazı, serbest radikallerin temizlenmesi, kolajen ve elastinin bağ oluşumu gibi mekanizmalarda yer alan enzimlerin katalizlenmesinde önemli bir rol oynar. İnternal potasyum kanallarının (Kir) ise çeşitli dokularda özellikle beyin dokusundaki ekspresyonu bilinmektedir. Bu bağlamda bu çalışmada, dişi ve erkek sıçan beyin dokusunda Bakır Sülfatın ($CuSO_4$) morfolojik etkisi, beyin prefrontal korteks ve hipokampüs bölgelerindeki Kir2.1 ve Kir4.1'in ekspresyonunu değerlendirmek amaçlanmıştır.

Yöntem: Bu çalışmada dişi ve erkek Sprague dawley ırkı sıçanlar, kontrol grubu (n=10) ve $CuSO_4$ grubu (n=10) olarak ayrıldı ve 14 gün boyunca günlük olarak ağızdan gavaj yoluyla kontrol grubuna serum fizyolojik, $CuSO_4$ grubuna ise $CuSO_4$ (100 mg/kg) uygulandı. Daha sonra hayvanlara ötenazi uygulandı ve ardından beyin dokuları morfolojik ve doku bütünlüğü açısından histolojik olarak incelenerek Kir kanalı proteinleri olan Kir2.1 ve Kir4.1'ün ekspresyonu ise immünohistokimyal yöntem ile değerlendirildi.

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Results: Histomorphological characteristics of $CuSO_4$ group brain tissues were observed to change and tissue integrity was disrupted. In both the control group and $CuSO_4$ group, Kir2.1 expression in the prefrontal cortex region was more intense than in the hippocampus region in both sexes. Especially in female rats, intense Kir2.1 expression was observed in neurons in the prefrontal cortex region ($p=0.001$). In $CuSO_4$ group, the main effect of region was found to be significant for Kir2.1 expression ($p=0.014$). Kir4.1 expression was observed to be intense in the hippocampus region of the control group, while there was a statistically significant difference between the regions in the control group ($p<0.001$).

Conclusion: We found that brain tissue exposed to excessive $CuSO_4$ could not fully preserve its morphology. In addition, we determined that Kir2.1 was intensely expressed especially in the prefrontal cortex region of the brain, but the expression of Kir4.1 was decreased in both prefrontal cortex and hippocampus regions of male rat brain tissues by $CuSO_4$ treatment. The relationship between the damage caused by intensive $CuSO_4$ application in brain tissue and the activation/inhibition of Kir channels needs to be investigated in more detail.

Key Words: Copper sulphate, Brain, Kir2.1, Kir4.1

Bulgular: $CuSO_4$ grubu beyin dokularının histomorfolojik özelliklerinin değiştiği ve doku bütünlüğünün bozulduğu gözlendi. Hem kontrol grubunda hem de $CuSO_4$ grubunda, prefrontal korteks bölgesindeki Kir2.1 ekspresyonu her iki cinsiyette de hipokampus bölgesine göre daha yoğun olduğu izlendi. Özellikle dişi sıçanlarda prefrontal korteks bölgesindeki nöronlarda yoğun Kir2.1 ekspresyonu gözlenmiştir ($p=0,001$). Kir2.1 ekspresyonu için $CuSO_4$ grubunda bölgenin ana etkisi anlamlı bulunmuştur ($p=0,014$). Kir4.1 ekspresyonunun kontrol grubu hipokampüs bölgesinde yoğun olduğu izlenirken, aynı şekilde kontrol grubunda bölgeler arasında istatistiksel olarak anlamlı bir farkın olduğu belirlenmiştir ($p<0,001$).

Sonuç: Aşırı $CuSO_4$'e maruz kalan beyin dokusunun morfolojisini tam olarak koruyamadığını tespit ettik. Ayrıca bulgularda Kir2.1'in özellikle beyin prefrontal korteks bölgesinde yoğun eksprese olduğu ama $CuSO_4$ uygulamasıyla özellikle erkek sıçan beyin dokularında gerek prefrontal korteks gerekse hipokampüs bölgesinde Kir4.1 ekspresyonunun azaldığını belirledik. Yoğun $CuSO_4$ uygulamasının beyin dokusunda yol açtığı hasar ile Kir kanallarının aktivasyon/inhibisyon arasındaki ilişkinin daha detaylı araştırılması gerekmektedir.

Anahtar Kelimeler: Bakır Sülfat, Beyin, Kir2.1, Kir4.1

INTRODUCTION

Copper (Cu) is an important trace element that plays a role in the function of many enzymes in the cell (1). It is especially effective in mitochondria, where energy is produced in the cell, and plays a role in catalyzing enzymes involved in mechanisms such as iron homeostasis, clearance of free radicals, and bond formation of collagen and elastin (2). Copper has many functions; it is one of the metals involved in biological processes such as cell proliferation, cellular oxidation and reduction events, angiogenesis, cell migration, and signal transduction (3). The copper required for the body has been suggested to be between 0.6 and 1.6 mg of copper per day in humans (4). Excess Cu in the body can come from food, drinking water and other environmental source (5). High intake of copper into the organism leads to serious poisoning and is reported to cause serious toxicity. Cu toxicity has been reported to induce oxidative stress and tissue damage as a result of Fenton reaction (6). Also, in the brains of Parkinson's

and Alzheimer's patients, copper levels decreased in brain regions such as substantia nigra, whereas copper accumulation occurred in Lewis bodies or senile plaques (7, 8). In another study, it was reported that chronic copper toxicity caused differential copper accumulation associated with cognitive dysfunction in the cerebral cortex, striatum and cerebellum of male Wistar rats (9). Furthermore, excess copper intake by oral administration has been shown to increase the amount of non-ceruloplasmin-bound copper in plasma, cortex, and hippocampus, which may cause oxidative damage through overproduction of Reactive Oxygen Species (ROS) (10).

The most important physiological feature of ion channels is electrical signals and they provide membrane potential formation in cells such as nerve and muscle where electrical stimulation is high (11). Internal potassium channels (Kir), which are ion channels that play an active role in excitable cells such as neurons and cardiomyocytes, actively conduct K+ ions into the cell toward the end of the action potential (12). Important functions of Kir channels; control cell proliferation, regulate hormone secretion in the central nerve system, modulate neurotransmitter release, and can act as hypoxia sensors (13). Studies to date have identified seven structurally distinct subfamilies of the Kir family, ranging from Kir1 to Kir7 (14). Kir2.1 channels are known to be highly expressed in the brain (15), and may regulate the excitability of granular cells in the hippocampus during development (16). Kir4.1 channels are reported to be centrally located in oligodendrocytes, where the myelin sheath is produced, and in astrocytes surrounding neurons, but also in the cortex, thalamus, hippocampus, and brain stem (17). Immunohistochemical evidence exists that oligodendrocytes express Kir4.1 (17) and a study on global Kir4.1 knock-out mice indicated that it is required for oligodendrocyte development and myelination (13, 18).

Therefore, in this study, we aimed to investigate the immunoreactivity changes of Kir channels; Kir2.1 and Kir4.1 with copper sulphate ($CuSO_4$) in the prefrontalcortex and hippocampus regions of male and female rat brain tissue. We think that the findings obtained in this study may be effective in changing the activity of Kir channels in brain tissue, especially with $CuSO_4$, and may help to elucidate the changes in the activity of Kir2.1 and Kir4.1 in both prefrontal cortex and hippocampus regions.

MATERIAL and METHOD

Animals

In all experimental procedures, 30-40 days old male and female Sprague dawley rats with body weights ranging from 50 to 70 g were used and all experimental animals were obtained from Erciyes University DEKAM. Rats were housed in cages at 24 °C ± 2 °C, 12 h light/dark cycles, with food and water in the cages. At the end of the experimental procedure, rats were sacrificed under ketamine-xylazine induced anaesthesia. Every effort and care was carefully taken to minimise animal suffering.

Experimental model induced by copper sulphate ($CuSO_4$)

In the study, male (n = 10) and female (n = 10) rats were randomly divided into 2 groups:

i) Control groups receiving saline by oral gavage (n = 10; 5 male- 5 female),

ii) $CuSO_4$ groups receiving $CuSO_4$ dissolved in saline at a dose of 100 mg/kg/day by oral gavage (n = 10; 5 male- 5 female).

The required dosage of $CuSO_4$ was 100 mg/kg for 14 days following the standard protocol of Liu et al. (19). The rats were then sacrificed and brain tissues were removed for histological and immunohistochemical examinations.

Histological analysis

The brain tissue was first preserved in 10% formaldehyde, and after that, it was flicked and cleaned with running water for an entire night. Tissues were immersed in a succession of progressively stronger alcohol solutions to extract water from

them (20). The tissues were dewatered, rinsed with xylol (amounting to the size of the tissue), and then embedded in paraffin blocks at the proper depth. From the paraffin-embedded blocks, sections with a thickness of 5 µm were cut using a microtome instrument. The tissue sections were then put on poly-L-lysine-coated slides for immunohistochemical analysis and on regular slides for hematoxylin and eosin staining. Following all staining procedures, an Olympus BX53 light microscope was used to assess tissue integrity, oedema and histological changes in the morphology of the prefrontal cortex and hippocampus regions.

Immunohistochemical analysis

The variations in Kir2.1 and Kir4.1 expressions in brain tissues were identified by labeling using immunohistochemical techniques using the avidin-biotin-peroxidase method (21) The 5 µm thick sections from the paraffin tissue blocks were stored at 60 °C for an entire night. They were then rehydrated using xylene and a series of graduated alcohols. After five minutes of distilled water washing, they were boiled in citrate buffer (pH 6.0; Thermo Fischer Scientific, UK, AP-9003-500) for five minutes in the microwave at 600 W, and they were allowed to cool for about fifteen minutes at room temperature. Tissue sections were kept in 3% hydrogen peroxide in methanol for 10 minutes to prevent endogenous peroxidase. Tissue sections were then treated with Ultra V Block solution (Thermo Fischer Scientific, UK, TA-125-UB) to prevent non-specific staining. Sections were then incubated with Kir2.1 (1:200 dilution ratio; Alomone Labs, APC-026) and Kir4.1 (1:200 dilution ratio; Alomone Labs, APC-035) overnight at 4 ◦C. After rinsing with PBS 3 times for 5 min each, biotinylated goat anti-polyvalent secondary antibody (Thermo Fischer Scientific, UK, TP-125-BN) was incubated in an oven at 37◦ C for 40 min. After rinsing in PBS 3 times for 5 min each, the sections were incubated with streptavidin peroxidase (Thermo Fischer Scientific, UK, TS-125-HR) at 37◦ C for 30 min in an oven. A solution of diaminobenzidine (DAB) chromogen (Thermo Fischer Scientific, UK, TA-125-HD) was applied to the sections in order to visualize the combined effect of the antibodies. The slides were counterstained using a Hematoxylin solution that was adjusted in accordance with Gill III (Merck, Germany, 1.05174.1000). After exposing each portion to progressively higher alcohol concentrations for ten minutes at a time, entellan was used to completely dehydrate it. With an Olympus BX53 light microscope, the sections were inspected. With Image J Version 1.46 (National Institutes of Health, Bethesda, Maryland), immunoreactivity levels in the hippocampal and prefrontal cortex of the brain were assessed. In each of the three experimental groups, a minimum of ten distinct locations were assessed and visualized for every rat.

Statistical analysis

Experimental data were analysed in SPSS 11.5 and presented as mean ± SD. Normality was tested using Shapiro-Wilks test and homogeneity of variances were tested using Levene test. Two-way analysis of variance was used to examine the effect of group, gender and region factor on Kir2.1, Kir 4.1 variables $p<0.05$ was considered statistically significant.

The study was approved by the Animal Ethics Committee of the Erciyes University (Date: 08.02.2024 and Number: 24/021).

RESULTS

Histological analysis

As shown in Figure 1, the tissue integrity was better preserved in the control group compared to the $CuSO_4$ group. In the experimental group, it was observed that the neuron body structure in the hippocampus region was not completely preserved. (Figure 1). Especially in the $CuSO_4$ group, we observed that vascular regions were opened in the prefrontal cortex region and also in the hippocampus region (Figure 1).

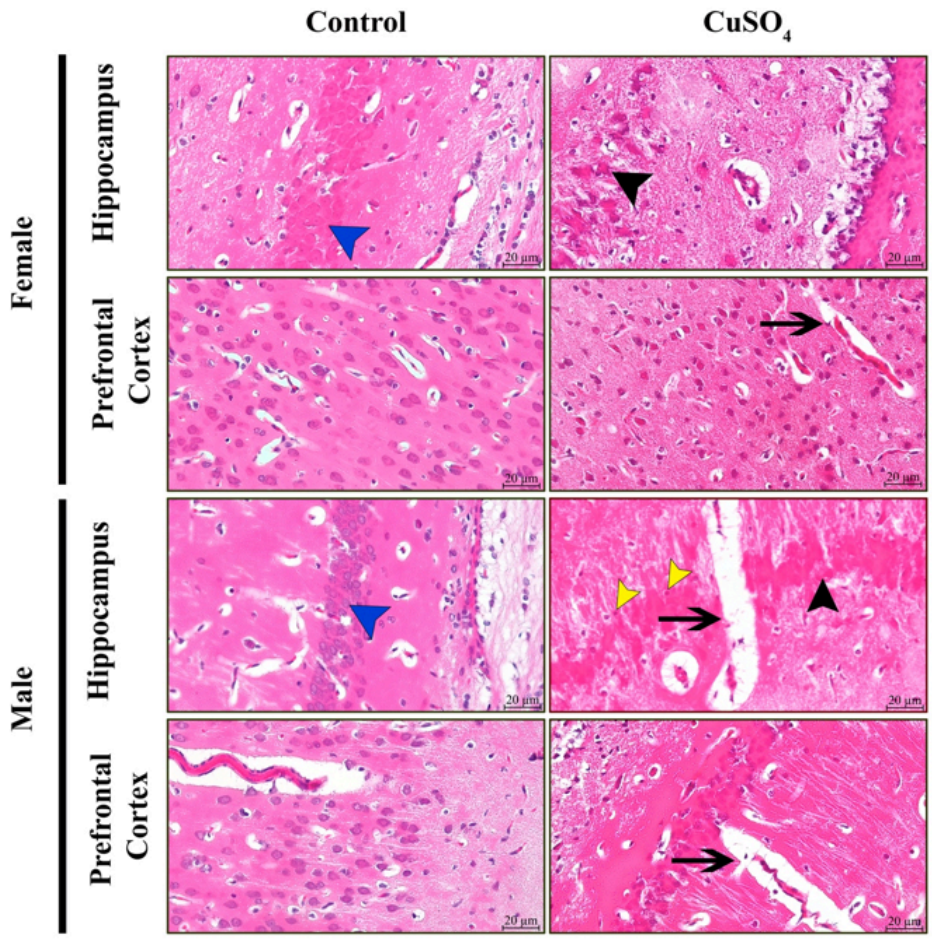

Figure 1. Hematoxylin and Eosin staining images of brain tissues including hippocampus and prefrontal cortex regions of control and CuSO4 groups. Arrow: Vascular dilatation. Black Arrowhead: Structurally disrupted neuron body. Blue arrowhead: Normal neuron body. Yellow arrowhead: Neuron degeneration. 20 µm: Measuring bar

Immunohistochemical analysis

Kir2.1 expression

Kir2.1 expression in the prefrontal cortex region was more intense than in the hippocampus region in both sexes, but statistically significant difference was observed only in female mice (p=0.001) (Figure 2 and Figure 4). However, in the male prefrontal cortex region of the experimental group, Kir2.1 immunopositivity was observed intensely in both cellular and intercellular areas (Figure 2). Moreover, Kir2.1 expression was also observed in neurons in the prefrontal cortex region of control and experimental group females (Figure 2). In the $CuSO_4$ group, Kir2.1 expression was more intense in the prefrontal cortex region than in the hippocampus region and the main effect of region (p=0.014) was significant (Figure 2 and Figure 4). As a result of the analysis; Region main effect (p<0.001), Group*Gender interaction (p=0.001), Group*Gender*Region interaction (p=0.001) were found significant. As a result, analyses were performed separately for each group. For Control group, Region main effect (p<0.001), Gender main effect (p=0.003), Region*Gender interaction (p<0.001) were found statistically significant.

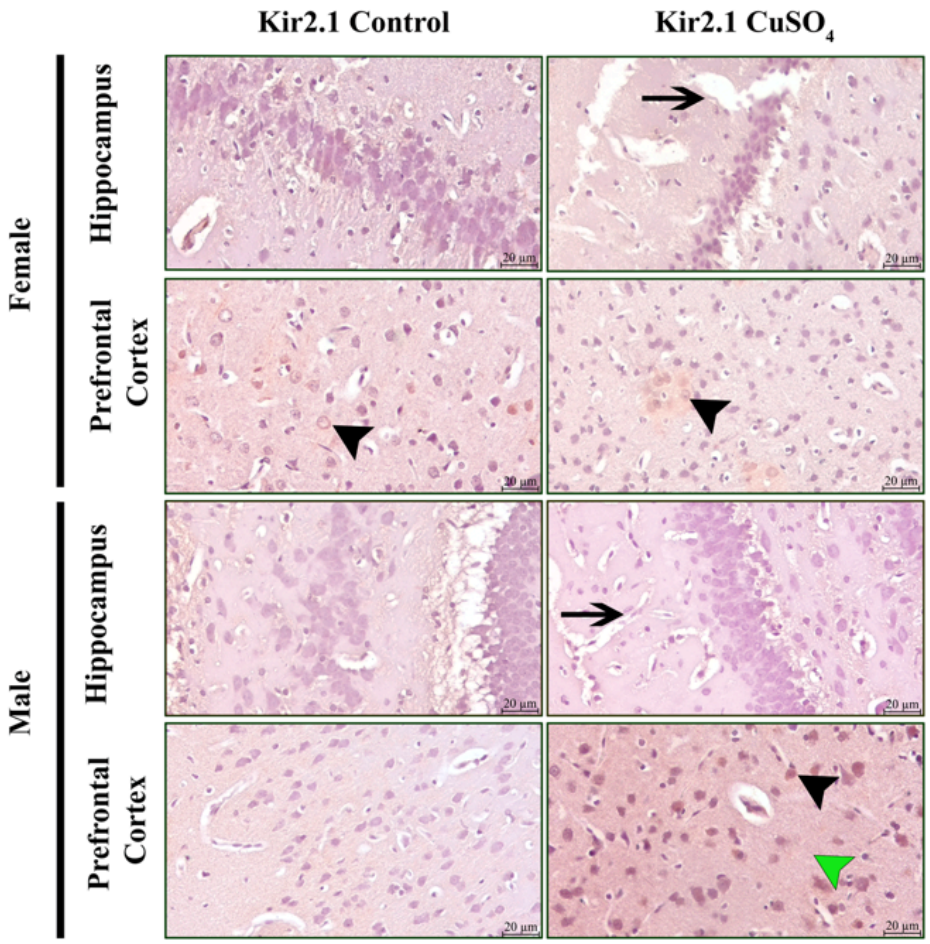

Figure 2. Immunohistochemical staining images showing Kir2.1 in the rat brain tissue. Arrow: Vascular dilatation. Black Arrowhead: Intense Kir2.1 expression in the prefrontal cortex and neuronal cell body. Green arrowhead: Intercellular region. 20 µm: Measuring bar.

Kir4.1 expression

In general, Kir4.1 expression in the intercellular region of the hippocampus was higher in male and female control group brains (Figure 3). In the experimental group, Kir4.1 expression was observed in the neuron body located in the female prefrontal cortex region (Figure 3). In the experimental group, the intensity of Kir4.1 expression was reduced in both cellular and intercellular regions of brain tissue in both hippocampus and prefrontal cortex regions in both males and females (Figure 3). As a result of the analysis; Region main effect ($p<0.001$) and Region*Group interaction ($p<0.001$) were found significant. As a result, analyses were performed separately for each group. While there were statistically significant difference between regions in control group ($p<0.001$), this difference could not be found statistically significant in the $CuSO_4$ group ($p=0.232$) (Figure 4).

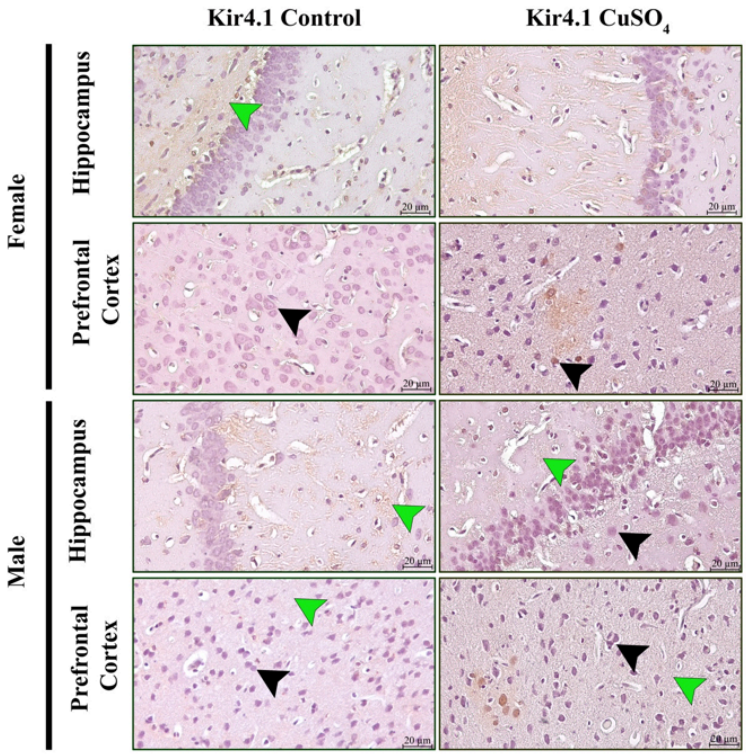

Figure 3. Immunohistochemical staining images showing Kir4.1 in the rat brain tissue. Black Arrowhead: Neuron cell body. Green arrowhead: Intercellular region. 20 µm: Measuring bars.

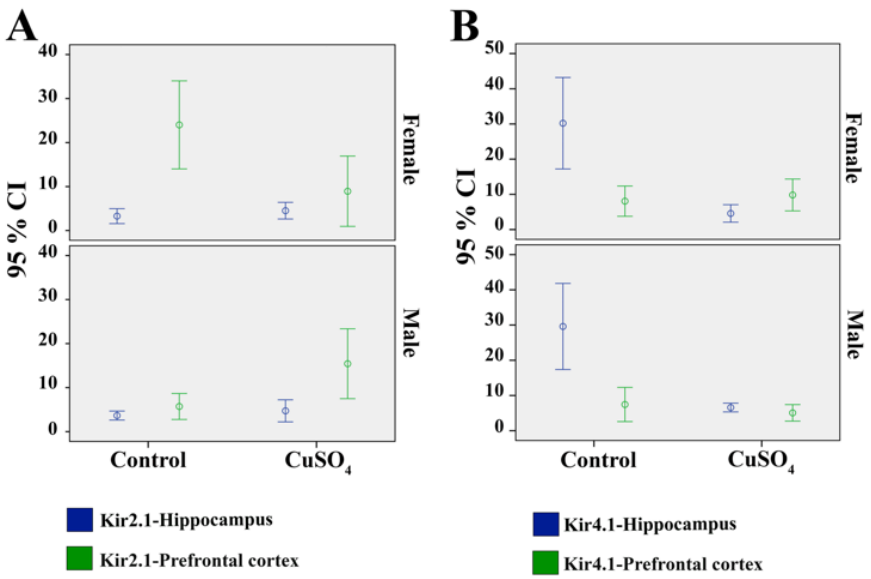

Figure 4. Statistical evaluation of $CuSO_4$ on Kir2.1 and Kir4.1 in rat brain tissue. CI: Confidence Interval. A: Confidence interval plot for Kir2.1. B: Confidence interval plot for Kir4.1

DISCUSSION

Enzymes related in the control of numerous physiological processes and biochemical activities have been reported to require copper as a cofactor (22). Cu is also crucial for the manufacture of hormones and for the growth and activity of the central nervous system (23). However, Cu excess has been linked to neuronal cell damage and the development of neuropsychiatric disorders. High Cu concentrations have also been reported in patients with Alzheimer's, Parkinson's and Wilson's diseases (24, 25). Additionally, a number of investigations have demonstrated that an excess of copper in Wilson's illness results in structural alterations in the thalamus, cerebral cortex, cerebellum, and caudate nucleus, among other brain regions (26, 27). A copper overdose combined with ethanol consumption has been linked to neuron death in the rat brain's lower regions of the hippocampus (28). Moreover, it has been documented that Cu toxicity can impair memory and learning by directly triggering apoptosis and astrocytosis in brain areas involving the frontal cortex and hippocampus, or indirectly through the glutamate and oxidative stress pathways (29).

According to research, Cu has been reported to cause hepatotoxicity such as oxidative stress, ER stress and apoptosis (30). $CuSO_4$ application may also be effective in reproductive organs and may increase autophagy level in testis and spermatogonia cell line (31). A study looking at the effect of $CuSO_4$ in rat brain tissues, brain histology showed degenerated neurons and damage to the cerebral cortex in rats treated with Cu alone (32). In a different study, the total volume of cerebellar structures in rats treated with $CuSO_4$ (1 and 8 mM) decreased significantly, and the total number of cells in the cerebellum cortex decreased in a dose-dependent manner (33). Based on this study, $CuSO_4$ dose of 100 mg/kg was administered to rats for 14 days in our study. In order to make the morphological evaluation of $CuSO_4$ in brain tissue, we performed histological evaluation with haematoxylin-eosin staining. According to the results we obtained, we observed that the tissue integrity of the brain tissues of the $CuSO_4$-treated group could not be fully preserved. We also observed that there were areas of blood supply in the brain tissues treated with $CuSO_4$. Especially in the $CuSO_4$ group, we determined that vascular regions were opened in the prefrontal cortex region.

A vital element for cellular physiology, copper plays numerous important functions in respiration, activity of antioxidants, tissue honesty, and synapse activity (22). With the growing awareness that copper is an essential signalling modulator, its role in intracellular trafficking is a major topic of research. However, there are still many areas to be explored as to how copper exerts such a diverse and widespread control over cell signaling. Thus, understanding the effect of excessive copper intake on Kir channels in brain tissue will be important. Composed of seven different subunits from Kir1 to Kir7 (12) and control cell proliferation, regulate hormone secretion in the central nervous system (CNS), modulate neurotransmitter release and can act as hypoxia sensors (12, 34). Kir channels are tetramers of four subunits and K+ ions cross the cell membrane by travelling across their electrochemical gradient downward (35). Kir channels are localised in specific regions of a cell, such as apical or basolateral membranes in epithelial cells and pre or postsynaptic regions in neurons; and consist of a structural form such as ion flow and channel pore opening kinetics (12). Kir2.x channels have been reported to be predominantly expressed in neurons (36). It has been documented that during hippocampal development, Kir2.1 channels influence the activity of granular cells (16). The Kir4.1 channel subunit is being distributed in glial cells of the CNS (37) and Kir4.1 plays an essential role in preserving K+ stability for proper neuronal function (38). In our study, we investigated the effect of $CuSO_4$ on Kir2.1 and Kir4.1 from Kir channels in rat brain tissues. Immunohistochemical analysis revealed that Kir2.1 expression in the

prefrontal cortex region was more intense than in the hippocampus region in both sexes in both the control and $CuSO_4$ groups. Regarding sex differences, we observed intense Kir2.1 expression in neurons in the prefrontal cortex region, especially in female rats.

In our study, Kir4.1 immunohistochemical analyses revealed that Kir4.1 expression in the area of hippocampus was higher in male and female control group brains. We also observed Kir4.1 expression in the vascular endothelium in the hippocampus region of control group males. When we looked at the $CuSO_4$ group, we found that the intensity of Kir4.1 expression was decreased in both hippocampus and prefrontal cortex regions in male and female brain tissue. Participation of Cu in the redox cycle and reactive oxygen species (ROS) production have been associated with macromolecular damage and altered sulfhydryl homeostasis (39). Damage caused by intense $CuSO_4$ intake may have activated especially Kir2.1 channels in neurons located in both hippocampus and prefrontal cortex in brain tissue. In addition, we suggest that the morphological structure of brain tissues exposed to intense $CuSO_4$ was not preserved and therefore Kir2.1 and Kir4.1 expression may have varied in different regions of the brain according to sex difference.

In conclusion; this study shows that intensive $CuSO_4$ treatment in rats causes a decrease in Kir4.1 channel protein expression and morphological damage in the brain. In addition, the importance of sex difference, which is one of the parameters examined in our study, is important here. We think that the relationship between the damage caused by intensive $CuSO_4$ application in brain tissue and the activation of Kir channels should be investigated in more detail. In addition, pathways affecting dirt channels, effects such as apoptosis or autophagy will need to be examined in future studies.

AUTHOR CONTRIBUTIONS

In order to recognise the participation of the authors, we highlight each individual contribution: O.Ö; Z.D, S.Y; Conceptualisation, Methodology, Writing - Review and Editing and designed the study, S.K; S.U; A.O.O: Conducted and supervised the experiments, S.K; (corresponding author); S.U; Z.D: Conceptualisation, Methodology, S.K; Z.D.: Contributed to formal analysis, experimentation, filtering and follow-up of histopathological evaluations. D.G; Performed statistical analyses. S.K; O.Ö; S.U; A.O.O; D.G; S.Y; Z.D: Writing - Original Draft, commented on figures, visualised and revised the manuscript. All these authors made significant contributions to the final manuscript and have approved this submission.

ETHICS COMMITTEE APPROVAL

* The study was approved by the Animal Ethics Committee of the Erciyes University (Date: 08.02.2024 and Number: 24/021).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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