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Investigation of Neurotoxicity Oxidative Stress and Oxidative DNA Damage in Cadmium-Induced Brain Injury in Rats

Sıçanlarda Kadmiyuma Bağlı Beyin Hasarında Nörotoksisite Oksidatif Stres ve Oksidatif DNA Hasarının Araştırılması

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ABSTRACT

Cadmium, which is one of the toxic metals widely used in many areas of the world, is taken into the body in different ways. In this study, the damage caused by cadmium in the brain and how this damage affects the levels of glial fibrial acidic protein, 8-Oxo-2'-deoxyguanosine (8-OHdG), neuronal nitric oxide synthase, Süperoksit dismutaz (SOD), Glutatyon (GSH), glutatyon peroksidaz (GPX), inducible nitric oxide synthase, and malondialdehid (MDA) expression in the brain tissue were investigated. A total of 16 male Wistar albino rats 200-220 g were used in the experimental study. Rats were divided into 2 groups. A single cadmium i.p. dose of 0.025 mmol/kg was given to rats in the cadmium group. Rat brain tissue samples were analyzed using biochemical analyses as well as histopathological and immunohistochemical methods. In the histopathological examination of the brain tissues, normal histological structure was observed in the brain samples belonging to the control group, while necrosis and degeneration of neurons in the brain, as well as hyperemia in the parenchyma and meningeal vessels were observed in the cadmium group. In immunohistochemical examinations, while glial fibrial acidic protein, 8-OHdG, and neuronal nitric oxide synthase expression was not observed in the brain samples of control group, severe expression of glial fibrial acidic protein, 8-OHdG, and neuronal nitric oxide synthase was observed in the brain tissue of the group receiving cadmium. In the biochemical analyses performed, it was observed that SOD, GSH, and GPx enzyme levels increased in cadmium groups, inducible nitric oxide synthase and MDA enzyme levels were decreased. As a result of this study, it is thought that markers' expression levels are important in understanding the oxidative stress in pathogenesis of cadmium toxicity and will provide a guide and important contributions to future studies.

Keywords: Biochemical, cadmium, histopathology, immunohistochemistry

ÖZ

Dünyada birçok alanda yaygın olarak kullanılan toksik metallerden biri olan kadmiyum, vücuda farklı şekillerde alınmaktadır. Bu çalışmada kadmiyumun beyinde oluşturduğu hasar ve bu hasarın beyin dokusunda GFAP, 8-OHdG, nNOS, SOD, GSH, GPx, iNOS ve MDA ekspresyon düzeylerini nasıl etkilediği araştırıldı. Bu deneysel çalışmada 200-220 gr ağırlığında 16 adet erkek Wistar albino sıçan kullanıldı. Sıçanlar iki gruba ayrıldı. Kadmiyum grubuna 0.025 mmol/kg'lık tek bir intraperitoneal Cd uygulandı. Rat beyin dokuları biyokimyasal, histopatolojik ve immünohistokimyasal boyama yöntemi ile incelendi. Beyin dokularının histopatolojik incelemesinde kontrol grubuna ait beyin örneklerinde normal histolojik yapı gözlenirken, kadmiyum grubunda beyinde nöronlarda nekroz ve dejenerasyon ile parankim ve meningeal damarlarda hiperemi gözlendi. İmmünohistokimyasal incelemelerde kontrol grubunun beyin örneklerinde GFAP, 8-OHdG ve nNOS ekspresyonu izlenmezken, Cd uygulanan grubun beyin dokusunda şiddetli GFAP, 8-OHdG ve nNOS ekspresyonu gözlendi. Yapılan biyokimyasal analizlerde Cd gruplarında SOD, GSH ve GPx enzim seviyeleri artarken, iNOS ve MDA enzim seviyelerinin düştüğü gözlendi. Bu çalışma sonucunda markerlerin ekspresyon düzeylerinin Cd toksisitesinin patogenezinde oksidatif stresin anlaşılmasında önemli olduğu ve gelecekte yol gösterici ve önemli katkılar sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Biyokimya, histopatoloji, immünohistokimya, kadmiyum

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INTRODUCTION

In the developing world, many conveniences have been provided in our lives with the innovations in various industries. However, this development has also caused many health problems. Among the causes of these health problems, heavy toxic metals such as iron, lead, arsenic, copper, and cadmium, which are industrial wastes, have an important place.[1-3] Cadmium (Cd) is a heavy metal with mutogenic, carcinogenic, and toxic effects on living organisms. Cadmium is taken into body through digestion, respiration, and skin. Although it varies according to the animal species, 0.5%-12% of Cd was taken through digestion. Almost all of the Cd vapor is absorbed from the lungs. This makes this element in the cigarette important to us. Moreover, Cd salts can be absorbed up to 4% through the skin.[1,4,5] Cd taken into the body is transported by binding to proteins and blood cells in the blood, and most of it accumulates in the liver and kidney, and a small part in the spleen.[6] Apart from these organs, Cd has also been reported to accumulate in the brain due to its ability to cross the blood brain barrier (BBB) and cause serious neurological damage.[7] Recent studies on cadmium have reported that it is also a risk factor for occurrence of Parkinson's[8] and Alzheimer's disease.[9-11]

Glial cells, one of the central nervous system cells, are of great importance in supporting neurons. Yet another cell structure, astrocytes act as a bridge between the BBB and neurons, providing great support to neurons and preventing foreign substances from entering the brain.[12-15] In addition, it has been reported that some toxic metals inhibit the cytotoxic effects they can create in brain tissue.[16] Glial fibrial acidic protein (GFAP) is a protein that is an intermediate filament in mature astrocytes. It is also an important protein that plays a role in structure of astrocytes and many functions during the development of astrocytes.[17-20] In some toxication studies conducted in brain tissue, it has been reported that determining the level of GFAP expression is important in determining the severity of toxicity.[21] It has been reported that GFAP expression increases in brain tissue in diseases such as Scrapi, Creutzfeldt-Jakob and Alzheimer's.[21] In some studies, in animals exposed to heavy metals such as Cd, Zn, Pb, and Tl, it was found that heavy metals accumulate in glial cells and cause morphological changes in these cells as a result of their toxic effects.[22,23]

Oxidative stress of cells in many tissues and organs causes permanent damage to the membranes, protein structures, and lipids of DNA.[24] Toxic substances are known to cause serious oxidative damage in the body. In particular, cadmium has been reported to cause serious damage to many tissues especially brain tissue, by increasing oxidative stress in the body.[25] It has been reported that cadmium causes significant decreases in SOD, GPx, and GSH enzyme levels, which are oxidative stress parameters in brain tissue.[26,27] 8-OHdG and 8-oxodG in nuclear and mitochondrial DNA determine the damage caused by oxidative stress on cell DNA in the body and are known to be the most common of the free radical-derived forms.[24] 8-OHdG marker was used to determine the oxidative damage caused by cadmium in the brain.[25,28]

Nitric oxide (NO) is synthesized by a reaction catalyzed by the nitric oxide synthase (NOS) enzyme in many cells, and L-arginine and O_2 molecules are converted into NO and citrulline molecules. The NOS enzyme has 3 isoforms, the first 2 being structural (cNOS) and the third being inducible (iNOS). While iNOS can be stimulated by cytokines and other compounds, cNOS is divided into 2 groups as vasodilation endothelial NOS (eNOS) in endothelial cells and neuronal NOS (nNOS) in the nervous system.[29] Neuronal NOS is reported as a highly sensitive marker in detecting the damage caused by oxidative stress in brain tissue.[30-32] Although the cellular and biochemical effects of cadmium in the brain have been revealed in many studies in the literature reviews, the pathogenesis of the disorders it causes has not been fully elucidated.[33] In this study, it was aimed to investigate the effects of GFAP, 8-OHdG, nNOS, SOD, GSH, GPx, iNOS, and MDA expression levels in brain damage caused by Cd toxicity by biochemical and immunohistochemical analyses in order to determine the pathogenesis of Cd intoxication in brain tissue. This study aimed to reveal the pathogenesis of oxidative stress caused by cadmium in the body and subsequent oxidative DNA damage caused in brain tissue.

MATERIALS AND METHODS

As the research material, 16 male Wistar-albino rats with a weight of 200-220 g were used. Rats were provided by Atatürk University, Medical Experimental Application and Research Center (ATADEM) unit. The rats were kept for a week in order to adapt to their environment, and ad libitum nutrition was applied with pellet feed and water. In the study using $CdCl_2 \cdot 5H_2O$ (cadmium chloride pentahydrate, CAS NO: 7790-78-5, Sigma, US) as a cadmium source, rats were divided into 2 groups, with n = 8 in each group.

Group I: Control group: The animals in this group were given standard pellet feed, drinking water, and saline (SF) intraperitoneally (i.p.).

Group II: Cadmium applied group: A single dose of i.p. 0.025 mmol/kg Cd was given to each animal.[34]

It was prepared by dissolving Cd in saline (SF). Group II was administered SF for 5 days, and on the 5th day, Cd i.p. was administered 1 hour after SF application. Animals were euthanized 24 hours after the last administration to collect brain tissue samples. In 6 days, rats were necropsied and brain tissue samples were taken (Ethics Number: 2014/12-b).

Biochemical Analysis

Homogenization was performed for brain tissues using Magna Lyser (Roche, Switzerland) homogenizer device. The obtained homogenates were centrifuged at 15 000 rpm. Then the supernatant was used for biochemical analysis. MDA level, GSH, CAT, iNOS, and GPx activity in the brain tissues were measured using the corresponding anti-rat ELISA kits (YL Biotech, Shanghai, China) according to the manufacturer's instructions.[34]

Histopathological Examination

Brain tissue samples were detected in 10% buffered formalin solution. Paraffin blocks were prepared after routine tissue follow-up procedures. Sections of 4 µm thickness were taken from the blocks. The preparations for histopathological examination were stained with hematoxylin-eosin and examined with a microscope (OLYMPUS BX51, Tokyo, Japan). Examined brain cortex areas were evaluated as none (–), mild (+), moderate (++), and severe (+++) according to their pathological findings.

Immunohistochemical Examination

Tissue sections were deparaffinized and dehydrated. The sections were kept in 3% H_2O_2 for 10 minutes and then incubated for 5 minutes with protein block. Primary antibody (GFAP Cat No: ab68428, dilution ratio (DR): 1/100, UK; 8-OHdG Cat No: sc-66036, DR: 1/100, US; nNOS Cat No: ab5586, DR: 1/100, UK) was dropped onto the sections and incubated for 1 hour. 3-3'

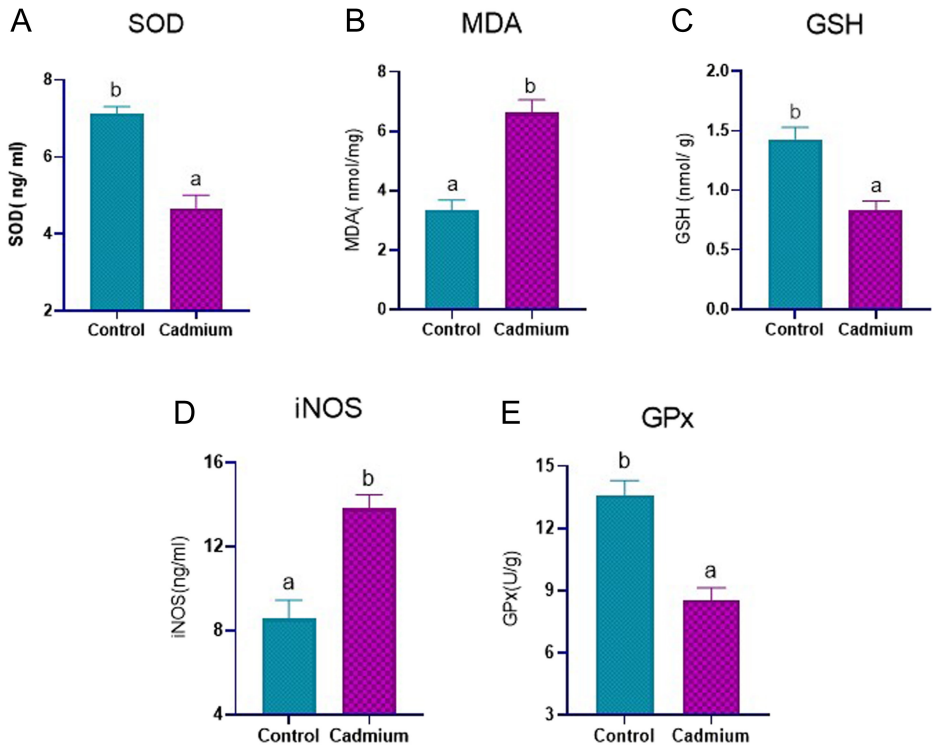

Figure 1. SOD, GSH, MDA, GPx, and nNOS levels in brain tissues. (A) SOD enzyme level, (B) MDA level, (C) GSH level, (D) iNOS level, and (E) GPx level; statistically, the differences between the groups were shown with letters ($P < .05$). The obtained values were expressed as mean ± SEM. nNOS, neuronal nitric oxide synthase; SEM, standard error of mean.

Diaminobenzidine (DAB) was used as a chromogen. The slides were kept in Mayer's hematoxylin and then washed. Then the prepared sections were examined with microscope (ZEISS AXIO, Jena, Germany).[34]

Statistical Analysis

For histopathological examination, Mann Whitney *U* test was used to compare binary groups using the Statistical Package for the Social Sciences version 20.0 (IBM SPSS Corp.; Armonk, NY, USA). The statistical comparison of the values obtained as a result of the biochemical analyses performed in the study was made with the GraphPad Prism 8.0.1 data program. One-way ANOVA and Tukey test were used to compare the mean values of more than 2 independent groups.

To determine the intensity of positive staining obtained as a result of immunohistochemical staining, 5 areas were selected from images and evaluated in the ZEISS Zen Imaging software. One-way ANOVA and Tukey test were used. As a result of the test, *P* value of <.05 was considered significant.

RESULTS

Biochemical Analyses Findings

As a result of the biochemical analyses performed on the brain tissues, it was observed that SOD, GSH, and GPx enzyme levels in the cadmium group decreased significantly when compared to the control group. The levels of nNOS and MDA showed a significant increase in the cadmium groups compared to the control group ($P < .05$) (Figure 1).

Histopathological Findings

In the histopathological examination, normal histological structure was observed (Figure 2) in group I (control group). In group II (cadmium applied group), in the brain tissues, severe degeneration and necrosis in the neurons and hyperemia in parenchyma and meningeal vessels were detected (Figure 2) When compared with the control group, a statistically significant difference ($P < .05$) was found. Histopathological results were shown in Table 1.

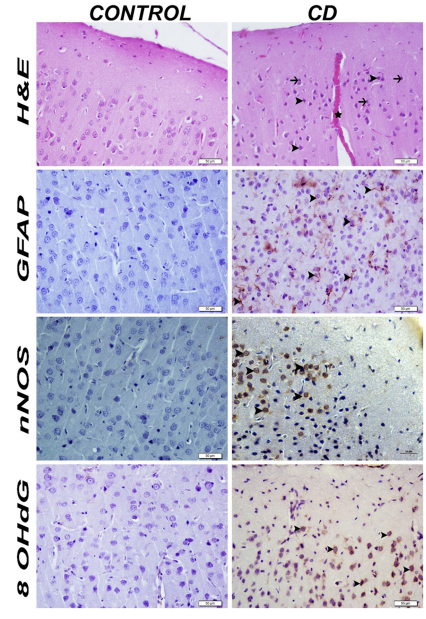

Figure 2. Brain tissue, degeneration in neurons (arrowheads), necrosis (arrows), hyperemia in vessels (stars), hematoxylin-eosin, severe level of GFAP, nNOS and 8 OHdG expression (arrowheads), IHC-P, Bar: 50 µm. CD, cadmium applied group.

Table 1. Scoring of Histopathological Findings in Brain Tissue

Parameters	Control group	Cadmium Applied Group
Degeneration in neurons	–	+++
Necrosis in neurons	–	++
Hyperemia of meningeal and parenchymal vessels	–	+++

Table 2. Scoring of Immunohistochemical Findings in Brain Tissue

Parameters	Control Group	Cadmium Applied Group
Glial fibrial acidic protein	21.52 ± 4.11^a	62.85 ± 5.46^b
Neuronal nitric oxide synthase	19.42 ± 4.96^a	58.55 ± 6.62^b
8-OHdG	20.39 ± 6.62^a	60.74 ± 7.45^b

[a,b]Differences between means with different letters in the same column are significant ($P < .05$).

Immunohistochemical Findings

In the control group, there was no GFAP, nNOS, and 8 OHdG expression in the brain tissues (Figure 2). In the Cadmium applied group, there was severe GFAP, nNOS, and 8 OHdG expression in the brain tissues (Figure 2). When compared with the control group, a statistically significant difference ($P < .05$) was found. Immunohistochemical results are shown in Table 2.

DISCUSSION

Cadmium, which is widely used in industry, accumulates in the environment and creates serious problems for human health. Volcanic mountains are reported to be the most important pollutants as a source of Cd. Cadmium, either intentionally or unintentionally, is taken into the body with contaminated food, water, soil, dust, or air,[35] digestion, respiration, and skin.[1] After entering the body, it is transported by binding to proteins and blood cells in the blood and most of it accumulates in the liver and kidney.[6] Apart from these organs, Cd has also been reported to accumulate in the brain due to its ability to cross the BBB and cause serious neurological damage.[7]

Cadmium causes necrotic hepatitis, hyperemia in the vessels, and mononuclear cell infiltration in the liver.[34] It has been reported to cause fatty degeneration, hydropic degeneration, and fibrosis[36] in animals treated with a daily dose of 1 mg/kg $CdCl_2$. It has been revealed that it causes severe intertubular hemorrhages in the medulla and cortex in the kidneys and degeneration and necrosis in the tubular epithelium.[34] It has been reported that neuronal atrophy, degeneration, and necrosis were observed in rats administered Cd at 5 mg/kg to brain tissues.[36] Podarcis siculus was shown to cause widespread edema in the brain tissue administered 2 mg/kg Cd daily for 7 days.[33] Histopathologically, in rat brain tissues where Cd toxicity was induced, it has been reported that neuronophagia, perineuronal vacuolization, vascular hyperemia, and satellitosis were observed especially in the cerebral cortex.[2] In this study, in accordance with the literature, degeneration and necrosis in neurons and hyperemia in parenchymal and meningeal vessels were detected in Cd toxicity in brain tissues.

Glial fibrial acidic protein (GFAP) is a protein that is an intermediate filament in mature astrocytes. It is also an important protein that plays a role in the formation of the skeleton of astrocytes and many functions during the development of astrocytes.[17,18] Astrocytes become reactive and react as a result of traumas that cause astrogliosis, various diseases, genetic disorders, or toxic effects of chemical substances. It has been reported that GFAP expression started to increase rapidly with the formation of astrogliosis.[37] In some studies, they have defined that the increase in GFAP levels in trauma or disease situations that cause astrogliosis is the most serious finding of this process.[38] In rats with severe traumatic brain injury, an increase in GFAP expression level was found in astrocytes in the cortex where brain tissue damage occurred 3 days after the injury.[39] It was also reported that GFAP expression increased in gliosis models created experimentally in rat brain tissues.[37] In this study, it was determined that GFAP expressions in brain tissues increased significantly in Cd toxicity.

It was reported in studies that oxidative stress occurring in the body causes neurotoxicity in brain tissue as well as in many tissues and organs. Studies have shown that many substances, including Cd, increase oxidative stress in the body and cause oxidative damage to the brain tissue. In some studies performed by creating neurotoxicity with Cd, while it has been reported that Cd causes a decrease in SOD, GSH, and GPx enzyme levels in brain tissues, it has also been reported to cause serious increases in iNOS and MDA enzyme levels.[26,27] The results of the present study and the literature were found to be compatible. With this result, the negative effects of Cd on some oxidative stress parameters in brain tissues were revealed.

8-OHdG is widely used in toxication studies to show DNA damage caused by oxidative stress.[24,40,41] There are many studies in which 8-OHdG shows DNA damage in cadmium toxicity.[25,28,42] In a study, it was shown that the expression level of 8-OHdG increased significantly as a result of oxidative stress in the brain tissues of rats administered 5 mg/kg cadmium chloride.[25] In another study, it was determined that cadmium administration at 5 mg/kg increased the expression level of 8-OHdG in rat brain tissues.[28] In this study, it was determined that 8-OHdG expressions increased in brain tissues with CD application.

Neuronal NOS is a very sensitive marker in detecting neurological damage in the brain due to oxidative stress.[43] In many toxicity studies, it has been reported that the level of nNOS expression in neurons in brain tissue increases depending on the severity of Cd toxicity.[44-46] In a study conducted in rats treated with Cd at concentrations 0, 5, 10, and 20 µmol/L, it was detected that the expression level of nNOS increased significantly in rats treated with 5 and 10 µmol/L Cd.[47] In this study, in accordance with the literature, it was determined that the level of nNOS expression in neurons increased due to the damage caused by Cd in rat brain tissues in which acute Cd intoxication was created.

As a result, it was observed in the study that a single dose of 0.025 mmol/kg Cd caused severe damage to the brain tissues and this damage increased GFAP, 8-OHdG, nNOS, iNOS, and MDA expression and decreased SOD, GSH, and GPx expression. The GFAP, 8-OHdG, nNOS, iNOS, MDA, SOD, GSH, and GPx expression levels has been an important study in understanding the pathogenesis of Cd toxicity, and it is considered and evaluated to provide a guiding and important contribution to future studies.

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