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Araştırma Makalesi

Immunoexpression of CD34, CD68, and CD3 in Cadmium-Induced Liver Damage and Protective Effectiveness of Bee Bread (Perga)

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ABSTRACT

Cadmium (Cd) is one of the potent environmental toxicants that causes oxidative stress in many organs of the body, including the liver. Perga (bee bread) is used for apitherapeutic purposes due to its medicinal properties. This study was conducted to investigate the effectiveness of perga on endothelial damage and inflammatory cell activation in the liver as a result of exposure to Cd. For this purpose, 32 male Wistar rats (8 rats/group) were randomly divided into 4 groups, as the control, perga (0.5 g/kg of perga), Cd (5 mg/kg of CdCl2), and Cd + perga (0.5 g/kg of perga + 5 mg/kg of CdCl2) groups. Daily intragastric Cd and/or perga was administered for 4 weeks. At the end of the study, the rats were euthanized and liver tissue sections were taken and stained with hematoxylin-eosin and Masson's trichrome. Immunohistochemically, the reactivity of the liver sinusoidal endothelium was determined using CD34 antibody, the reactivity of the Kupffer cells was determined using CD68 antibody, and the levels of T-lymphocytes were determined using CD3 antibody. Exposure to Cd caused significant histological changes in the liver. Immunohistochemically, exposure to Cd caused an increase in the expressions of CD34, CD68, and CD3. On the other hand, the co-treatment of Cd and perga caused partial improvement in some histopathological changes. Compared to the Cd group, there was a decrease in CD34 and CD68 positivity in the Cd + perga group. The results revealed that the histopathological changes and inflammation in the rat liver could partially improve with perga supplementation.

Key words: cadmium, immunohistochemistry, liver, perga, rat

Kadmiyumun Neden Olduğu Karaciğer Hasarında CD34, CD68 ve CD3 İmmunekspresyonları ve Arı Ekmeğinin (Perga) Koruyucu Etkinliği

ÖZ

Kadmiyum (Cd), karaciğer dahil olmak üzere vücudun birçok organında oksidatif strese neden olan güçlü çevresel toksik maddelerden biridir. Perga (Arı ekmeği), tıbbi özellikleri nedeniyle apiterapötik amaçlarla kullanılmaktadır. Bu çalışma, Cd maruziyeti sonucu karaciğerde meydana gelen endotelyal hasar ve yangısal hücre aktivasyonu üzerine perganın etkinliğini araştırmak amacıyla yapıldı. Bu amaçla 32 adet erkek wistar sıçan rastgele kontrol, perga (0.5 g/kg perga), Cd (5 mg/kg CdCl2) ve perga + Cd (0.5 g/kg perga + 5 mg/kg CdCl2) olmak üzere 4 gruba (8 sıçan/grup) ayrıldı. Dört hafta boyunca günlük intragastrik Cd ve/veya perga uygulandı. Çalışma sonunda ratlar sakrifiye edilerek karaciğer doku kesitleri hematoksilen-eozin ve Masson's Trichrom ile boyandı. İmmunohistokimyasal olarak karaciğer sinüzoidal endotelindeki reaktivite CD34, Kupffer hücrelerindeki reaktivite CD68 ve T-lenfosit seviyeleri CD3 antikorları kullanılarak belirlendi. Cd'ye maruz kalma, karaciğer önemli histolojik değişikliklere neden oldu. İmmunohistokimyasal olarak Cd maruziyeti CD34, CD68 ve CD3 ekspresyonlarında artışa neden oldu. Öte yandan, Cd ve perga'nin birlikte uygulanması bazı histopatolojik

değişiklikleride kısmi iyileşmeye neden oldu. Cd grubu ile kıyaslandığında, Cd + perga grubunda CD34 ve CD68 pozitifliğinde azalma meydana geldi. Sonuçlar, rat karaciğerinde meydana gelen histopatolojik değişikliklerin ve yangının perga takviyesi ile kısmen iyileşme gösterebileceğini ortaya koydu.

Anahtar kelimeler: immunohistokimya, kadmiyum, karaciğer, perga, rat.

INTRODUCTION

Cadmium (Cd) is one of the most toxic heavy metals released into the environment (Stohs et al., 2001). Exposure to Cd, which is widely used in industrial production (Sanjeev et al., 2019), occurs through air, water, and food and enters the body through the skin, gastrointestinal tract, and respiratory system (Rahimzadeh et al., 2017). As a result, it may cause damage to many organs, including the liver, kidneys, testicles, and brain (Rinaldi et al., 2017; Wang et al., 2018). Cd has been classified as a potent carcinogen due to its ubiquity in the environment and its cumulative toxic effect in humans (Waisberg et al., 2003).

Hepatotoxicity resulting from exposure to Cd occurs in two ways. The binding of Cd to the sulfhydryl group on critical molecules in the mitochondria causes primary damage to the mitochondria. Secondary damage results from inflammatory processes initiated by the activation of Kupffer cells (KCs) (Rikans and Yamano, 2000). KCs are activated as a result of many different types of liver damage and are involved in inflammation (Gehring et al., 2006). Activated KCs produce a number of inflammatory mediators, such as proinflammatory cytokines, which activate liver sinusoidal endothelial cells (LSECs), and hepatocytes, promote liver fibrogenesis, and attract circulating inflammatory cells. Thus, it initiates a chain of cellular and humoral responses that lead to inflammation and secondary damage in the liver (Friedman, 2000; Rikans and Yamano, 2000). Therefore, it is important to detect KC activation, endothelial damage, and inflammation in Cd-induced damage. Since CD68 is generally expressed on the surface and cytoplasm of macrophages, anti-CD68 antibodies have been used as a marker for KCs (Omar and Mohammed, 2017). CD34 is widely recognised as a diagnostic endothelial cell marker and is a transmembrane glycoprotein found on lymphohematopoietic stem and progenitor cells, leukemic cells, endothelial cells, and fibroblasts (Wood et al., 1997). CD3 is a surface marker expressed by all T lymphocytes.

There is increasing interest in the use of naturally occurring phytochemicals with hepatoprotective and antioxidant activity in the treatment of Cd intoxication (Flora et al., 2007). Perga is a fermented bee product produced from plant pollen, honey, and bee saliva. Worker bees use bee bread as a protein source for larvae and young bees (Urcan et al., 2018). Perga is produced in Türkiye (Sobral et al., 2017; Othman et al. 2019) as well as different countries of the world and is used as a functional food product or for medicinal purposes. Many effects of perga have been reported, such as antibacterial, antioxidant, and antitumoral effects, the modulation of depression, and its protective effect against cardiovascular diseases (Sobral et al. 2017, Urcan et al. 2018).

In our previous study, it was revealed that perga may have beneficial effects on kidney damage caused by Cd (Yaman et al., 2024a). As far as could be determined, there are no studies examining the effect of perga on Cd-induced hepatotoxicity. Thus, the aim of this study was to determine the endothelial damage, and KC and T cell activations in the liver resulting from Cd toxicity via the immunohistochemical method using CD34, CD68, and CD3 antibodies and investigate the effectiveness of perga in this regard.

MATERIAL and METHODS

Animals

Male Wistar albino rats were obtained from Van Yüzüncü Yıl University Experimental Animal Research Center. Humane care according to the criteria expressed in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institute of Health, were followed throughout the experiment. The ethics regulations followed were in accordance with national and institutional guidelines for the protection of animal welfare during the experiment. The local ethics committee of Van Yüzüncü Yıl University Animal Experiments approved this study (27/06/2024, 2024/06-11).

Experimental design

The 32 male rats were randomly divided into 4 groups (with 8 in each) and the experiment was conducted for 28 days. Cd was administered via orogastric gavage at a dose of 5 mg/kg/body weight (bw) daily throughout the experiment (Tripathi and Srivastav, 2011; Famurewa et al., 2021). Freshly obtained perga was dried at 35 °C for 4 h, ground into fine powder, and stored at –20 °C (Suleiman et al., 2021). During the trial period, the perga was administered daily via oral gavage at a dose of 0.5 g/kg/bw (Othman et al., 2019; Zakaria et al., 2021). The rats were divided into groups and the experiment was applied as shown in Table1. At the end of the experiment, the rats were anesthetized using ketamine (50 mg/kg) and xylazine (10 mg/kg), and liver tissue samples were taken for histopathological and immunohistochemical examination.

Table 1. Experimental animal groups.

Group	Treatment	Route of Administration
Control group	Distilated water	Orogastric gavage
Perga group	0.5 g/kg of perga	Orogastric gavage
Cd group	5 mg/kg of Cd	Orogastric gavage
Cd + Perga	0.5 g/kg of perga + 5 mg/kg of Cd	Orogastric gavage

A standard pellet diet was provided ad libitum during the experiment.

Histopathological examination

Histological examination of the liver tissue samples was performed using hematoxylin-eosin (H&E) stain to evaluate the degree of liver tissue architecture, vacuolar and granular degeneration, necrosis, hemorrhage, hyperemia, and inflammatory reactions, and Masson's trichrome staining was used to evaluate the degree of hepatic fibrosis (Deniz et al., 2021). Liver tissue samples obtained from the rats were fixed in 10% neutral buffered formalin. Then, the fixed samples were dehydrated in alcohol, embedded in paraffin, and 5-µm sections were cut. Histopathological findings were evaluated subjectively as negative (0), mild (1), moderate (2), and intense (3) (Yaman et al., 2024b).

Immunohistochemical staining

Expressions of the CD34, CD68, and CD3 markers were evaluated in the prepared sections according to the avidin-biotin-peroxidase complex (ABC) method. For this purpose, after the sections were placed onto adhesive slides, they were passed through the xylene and alcohol series. The sections were kept in 3% hydrogen peroxide (H_2O_2) for 20 min to block endogenous peroxidase inactivity, followed by washing 3 times in phosphate-buffered saline (PBS) for 5 min. Antigen retrieval was performed with citrate buffer (pH 6.0) for 30 min at 95 °C in a water bath, and then cooling for 20 min. Subsequently, the slides were incubated with blocking serum (Histostain Plus Bulk Kit; Zymed Laboratories Inc., Oxnard, CA, USA) for 15 min to block nonspecific binding sites. The sections were incubated with CD34, CD68 and CD3 primary antibodies at 4 °C overnight (Table-2). The next day, the slides were washed 4 times in PBS, incubated with biotinylated secondary antibody (Histostain Plus Bulk Kit; Zymed Laboratories Inc.) for 20 min at room temperature, and then washed 4 times in PBS for 5 min. Next, the sections were incubated with streptavidin-peroxidase conjugate (Histostain Plus Bulk Kit; Zymed Laboratories Inc.) at room temperature for 20 min. Finally, the slides were incubated for 5–15 min with diaminobenzidine, rinsed in distilled water (3 times for 5 min), and counterstained with Gill's hematoxylin for 3 min. Then, the sections were passed through alcohol and xylene and mounted directly with Entellan mounting medium. Immunohistochemical findings were evaluated subjectively according to the intensity of the staining in the tissue as negative (0), mild (1), moderate (2), and intense (3) (Yaman et al., 2024b).

Statistical analyses

Statistical evaluations were performed using IBM SPSS statistics for Windows (version 22.0; iBM corp., armonk, NY,Usa) and GraphPad Prism for Windows (version 6.0; Boston,Ma, Usa). Data are presented as the mean ± standard deviation, and statistical significance was set at $p < 0.05$. The histopathological and

immunohistochemical findings were analyzed using the Kruskal–Wallis test, followed by the Mann–Whitney U test to define the diversity among the groups.

Table 2. Antibody specificity, host, dilution rates, and incubation times.

Antibody	Host	Dilution	Incubation	Source
CD34	Mouse/Monoclonal	1:100 dilution	Overnight	Cell Marque
CD68	Mouse/Monoclonal	1:100 dilution	Overnight	Novus Biologicals
CD3	Mouse/Monoclonal	1:100 dilution	Overnight	Novus Biologicals

Heat-induced antigen retrieval was performed with citrate buffer.

RESULT

Effects of Cd and perga on liver histopathology

The histopathological lesions and number of affected rats are summarized in Table 3. Normal histopathological structure was observed in the liver sections of the control (Figure 1a) and perga (Figure 1b) groups. Severe histopathological lesions were found in the liver sections of the Cd group. There was severe deterioration in the structure of the liver tissue. The Remark cord structure was disrupted, and the sinusoids were not evident. Granular and vacuolar degeneration was commonly present in the hepatocytes. The hepatocyte cytoplasm was light colored. Additionally, necrosis was observed in some hepatocytes. Pyknosis, karyorrhexis, and karyolysis were observed in the nuclei of the necrotic hepatocytes. Inflammatory cells were observed focally in some places and sometimes spread throughout the liver parenchyma. A slight increase in fibrous tissue was detected in some portal areas. Additionally, hemorrhagic foci were detected in some areas (Figure 1c). When the liver tissues of the Cd + perga group were compared with the Cd group, partial improvement was observed in some findings. In particular, the liver structure appeared to be preserved. Although there was degeneration in the hepatocytes, it was significantly reduced (Figure 1d).

Table 3**.** Incidence and severity of the lesions in the liver of the control, perga, Cd, and Cd + perga groups.

Changes/lesions in the liver	Control	Perga	Cd	Cd + Perga
Deterioration of liver tissue architecture	0/8	0/8	8/8	8/8
Mild	0	0	0	1
Moderate	0	0	3	4
Intense	0	0	5	3
Mean	0.00±00 ^{b}	0.00±00 ^{b}	2.63±0.51 ^{a}	2.25±0.70^{a}
Vacuolar/granuler degeneration	0/8	0/8	8/8	8/8
Mild	0	0	0	2
Moderate	0	0	4	5
Intense	0	0	4	1
Means	0.00±0.00 ^{c}	0.00±00 ^{c}	2.50±0.53 ^{a}	1.87±0.64 ^{b}
Necrosis	0/8	0/8	5/8	5/8
Mild	0	0	3	4
Moderate	0	0	1	1
Intense	0	0	1	0
Means	0.00±0.00 ^{b}	0.00±0.00 ^{b}	1.60±0.89 ^{a}	1.20±0.45 ^{a}
Inflammatory cell infiltration	0/8	0/8	8/8	6/8
Mild	0	0	3	3
Moderate	0	0	4	3
Intense	0	0	1	0
Means	0.00±0.00 ^{b}	0.00±0.00 ^{b}	1.75±0.70 ^{a}	1.50±0.55 ^{a}
Hyperemia/hemorrhagia	0/8	0/8	6/8	6/8
Mild	0	0	4	4
Moderate	0	0	2	2
Intense	0	0	0	0
Means	0.00±0.00 ^{b}	0.00±0.00 ^{b}	1.33±0.52 ^{a}	1.33±0.52 ^{a}
The values present the number of rats showing change/number of rats examined in each treatment group.				
^{a,b,c}: Values with different letters in same row are significantly different at p<0.005				

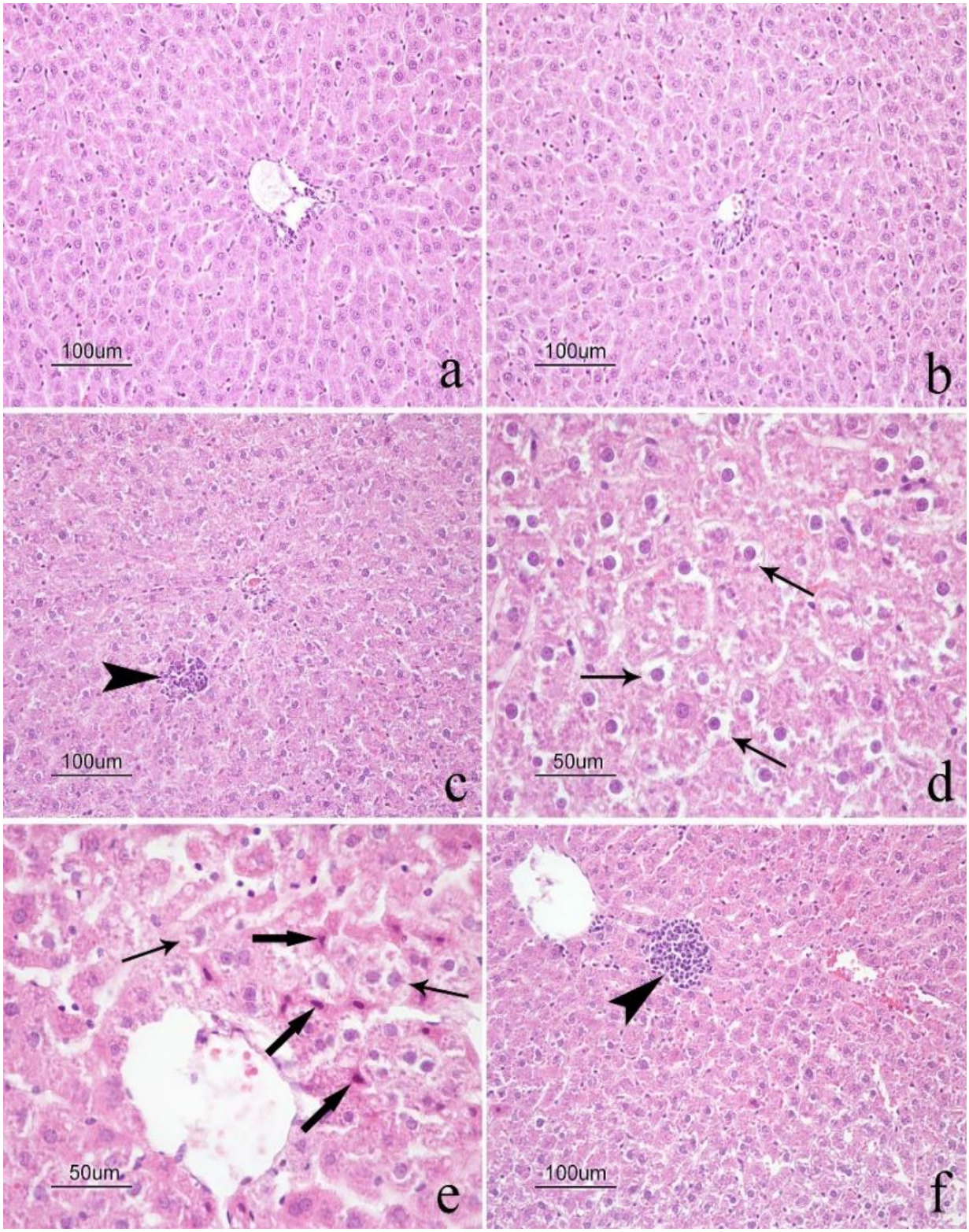

Figure 1. Effects of Cd and perga on the structure of the liver stained with H&E. (a) Control and (b) perga groups: normal histological appearance of the liver tissue. (c-e) Cd group: the presence of severe histopathological lesions is observed. (f) Cd + perga group: relatively fewer histopathological lesions. Description: inflammatory cell foci (arrowheads), vacuolated hepatocytes with light-colored cytoplasm (thin arrows), and necrotic hepatocytes (thick arrows).

In the examination performed with Masson's trichrome, normal staining was detected in the tissue sections of the control (Figure 2a) and perga (Figure 2b) groups. There was an increase in fibrous tissue in some portal areas in the sections of the Cd group (Figure 2c). Similarly, in the Cd + perga group, an increase in fibrous tissue was detected in some portal areas. There was no significant difference when compared to the Cd group (Figure 2d).

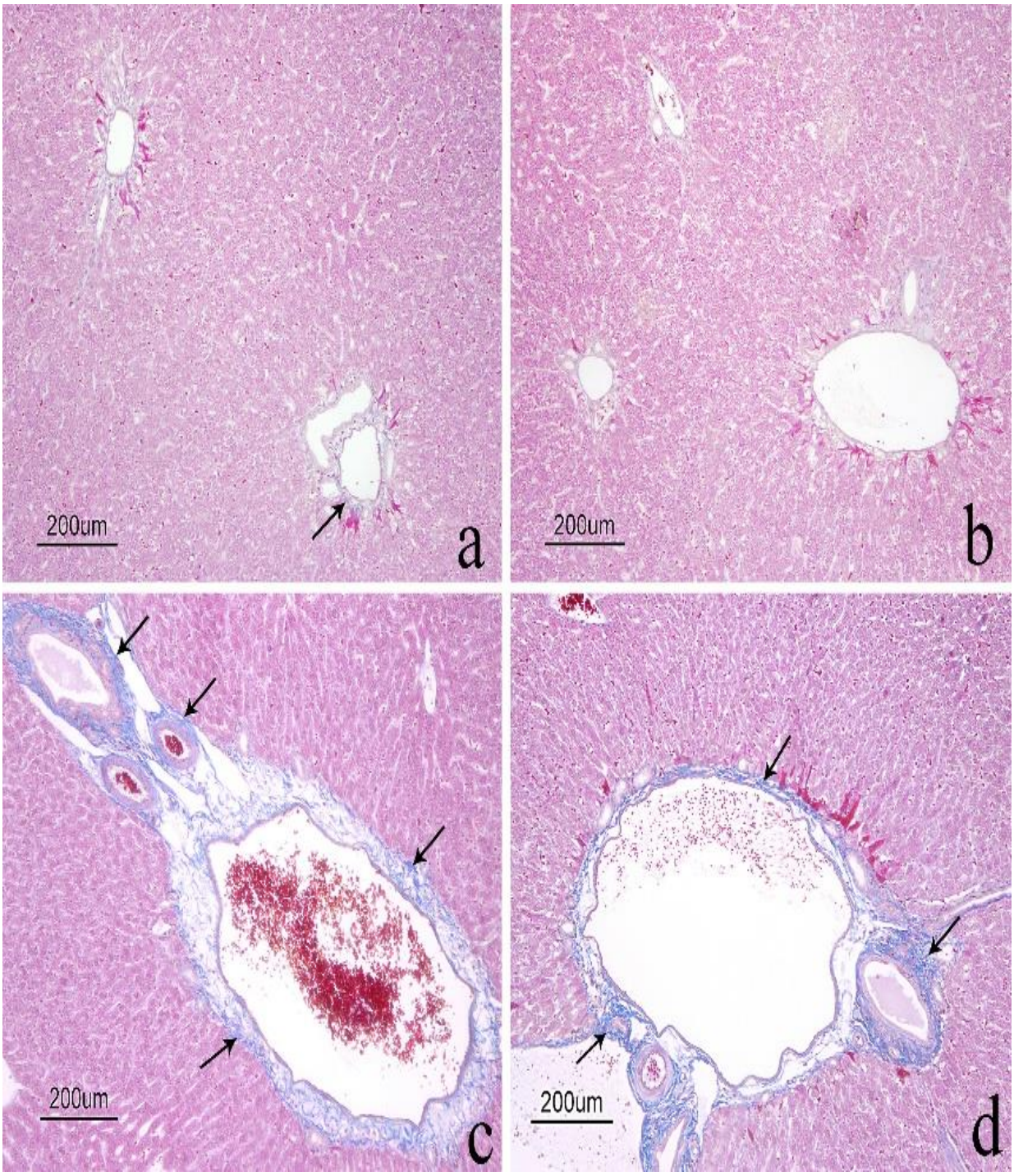

Figure 2. Masson's trichrome staining of the rat liver tissues. (a) Control and (b) perga groups: normal distribution of green-stained minimal fibrous tissue in portal areas (arrow). (c) Cd and (d) Cd + perga groups: a marked increase in fibrosis is observed in the portal area (arrows).

Effects of Cd and perga on the CD34, CD68, and CD3 immunoexpressions in the liver

CD68 immunoreactivity was not observed in the tissue sections of the control (Figure 3a) and perga (Figure 3b) groups. Compared with the control group, there was a significant increase in CD68 reactivity in the Cd group (Figure 3c). In the Cd + perga group, a significant decrease in CD68 reactivity was detected (Figure 3d). Minimal CD34 immunoreactivity was detected in the tissue sections of the control (Figure 4a) and perga (Figure 4b) groups. Compared with the control group, there was a significant increase in CD34 reactivity in the Cd group (Figure 4c). In the Cd + perga group, there was a significant decrease in CD34 reactivity (Figure 4d). When the tissue sections of the control (Figure 5a) and perga (Figure 5b) groups were examined, a few CD3-positive cells were seen scattered in the liver parenchyma. CD3-positive cells were abundantly found in the Cd group. These cells were densely present in the inflammatory cell foci and in the perivascular inflammation areas around the central veins. In addition, these cells were also commonly spread throughout the liver parenchyma (Figure 5c). Similar scenes were observed in the Cd + perga group (Figure 5d). The intensity of antibody immunoreactivity in the groups is presented in Table-4.

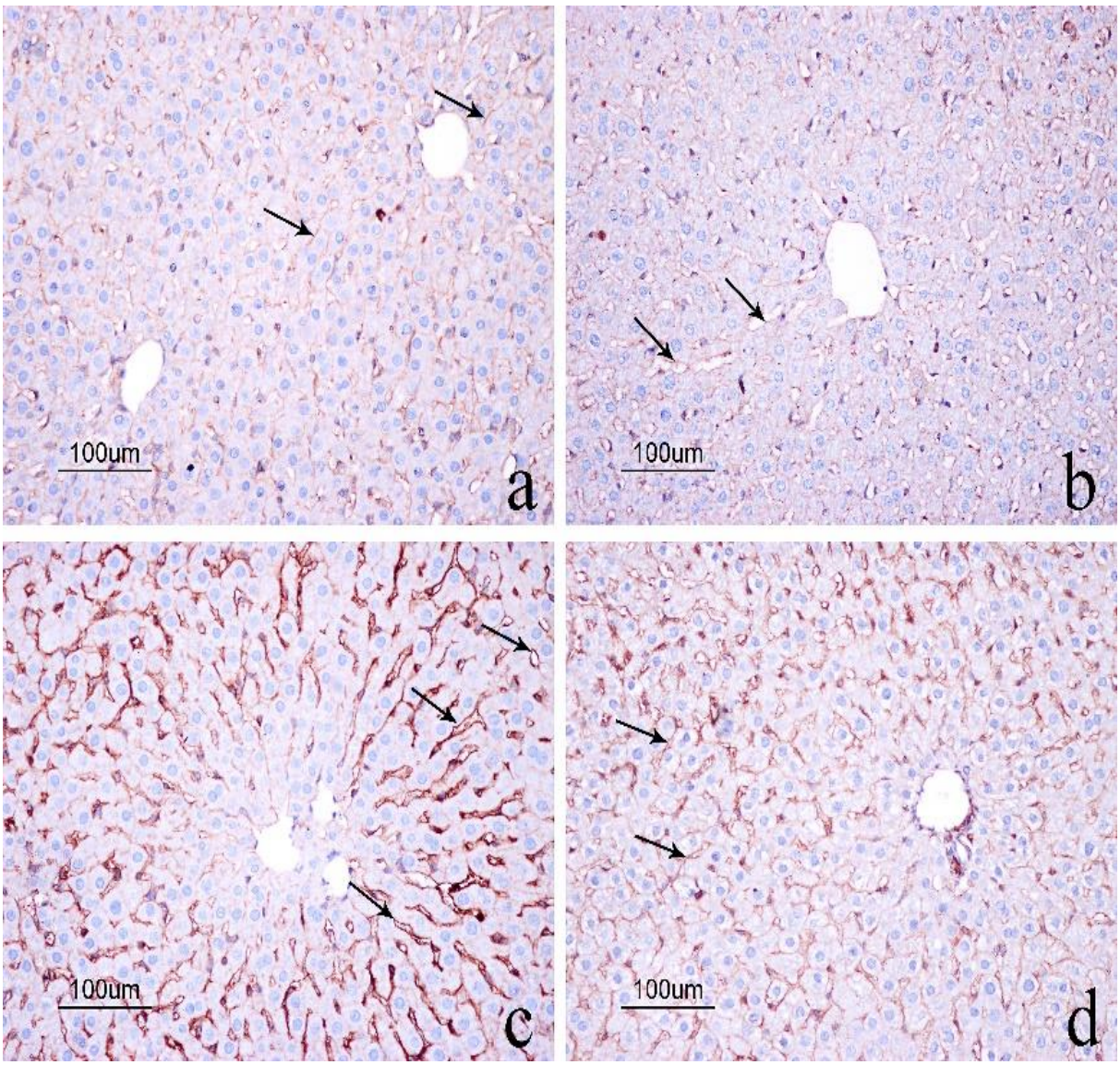

Figure 3. Effects of Cd and perga on the immune expression of CD34 in the rat liver tissues: (a) control group, (b) perga group, (c) Cd group, and (d) Cd + perga group. ABC method, counterstained with hematoxylin. Description: CD34 positivity (arrows).

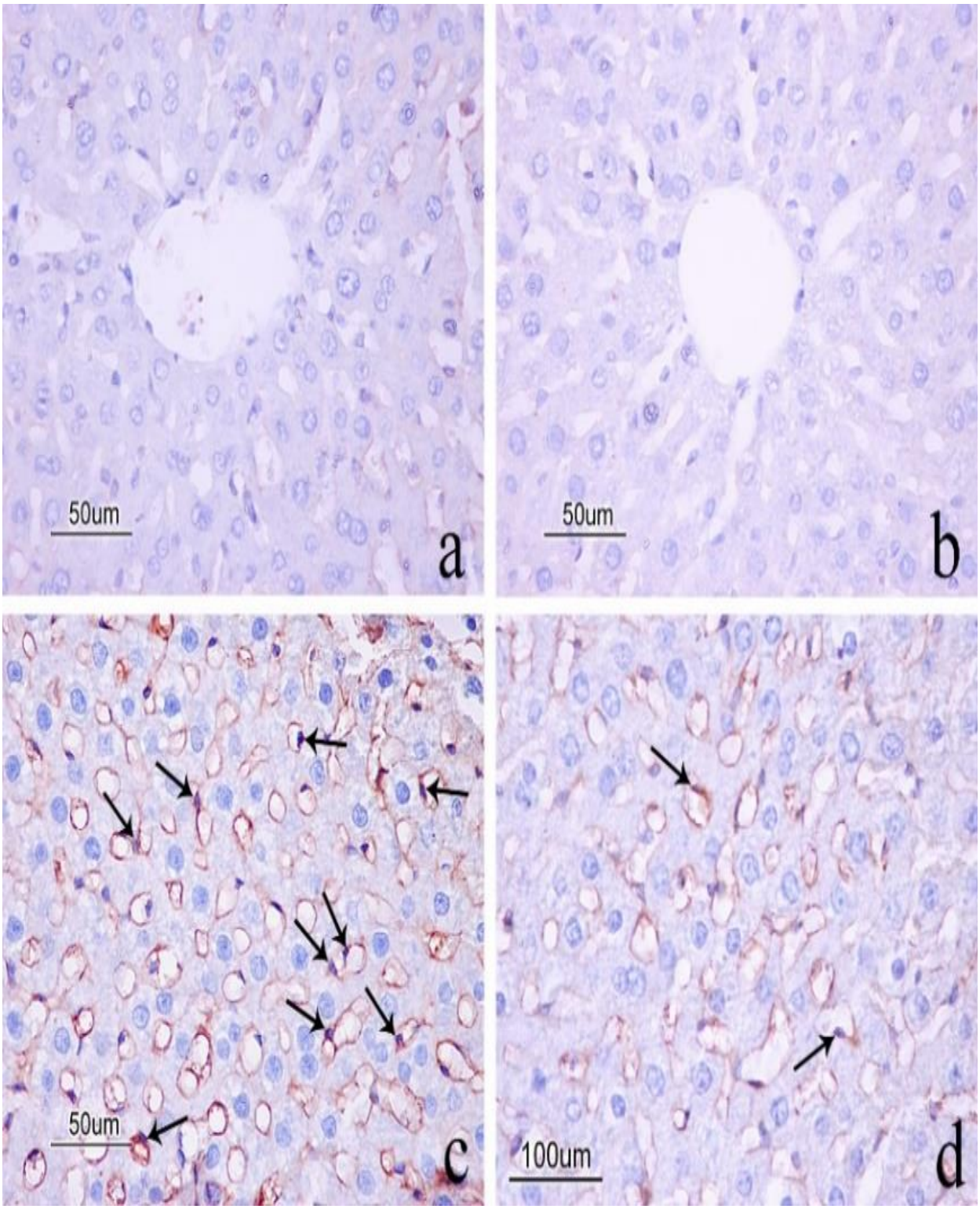

Figure 4. Effects of Cd and perga on the immune expression of CD68 in the rat liver tissues: (a) control group, (b) perga group, (c) Cd group, and (d) Cd + perga group. ABC method, counterstained with hematoxylin. Description: CD68-positive cells (arrows).

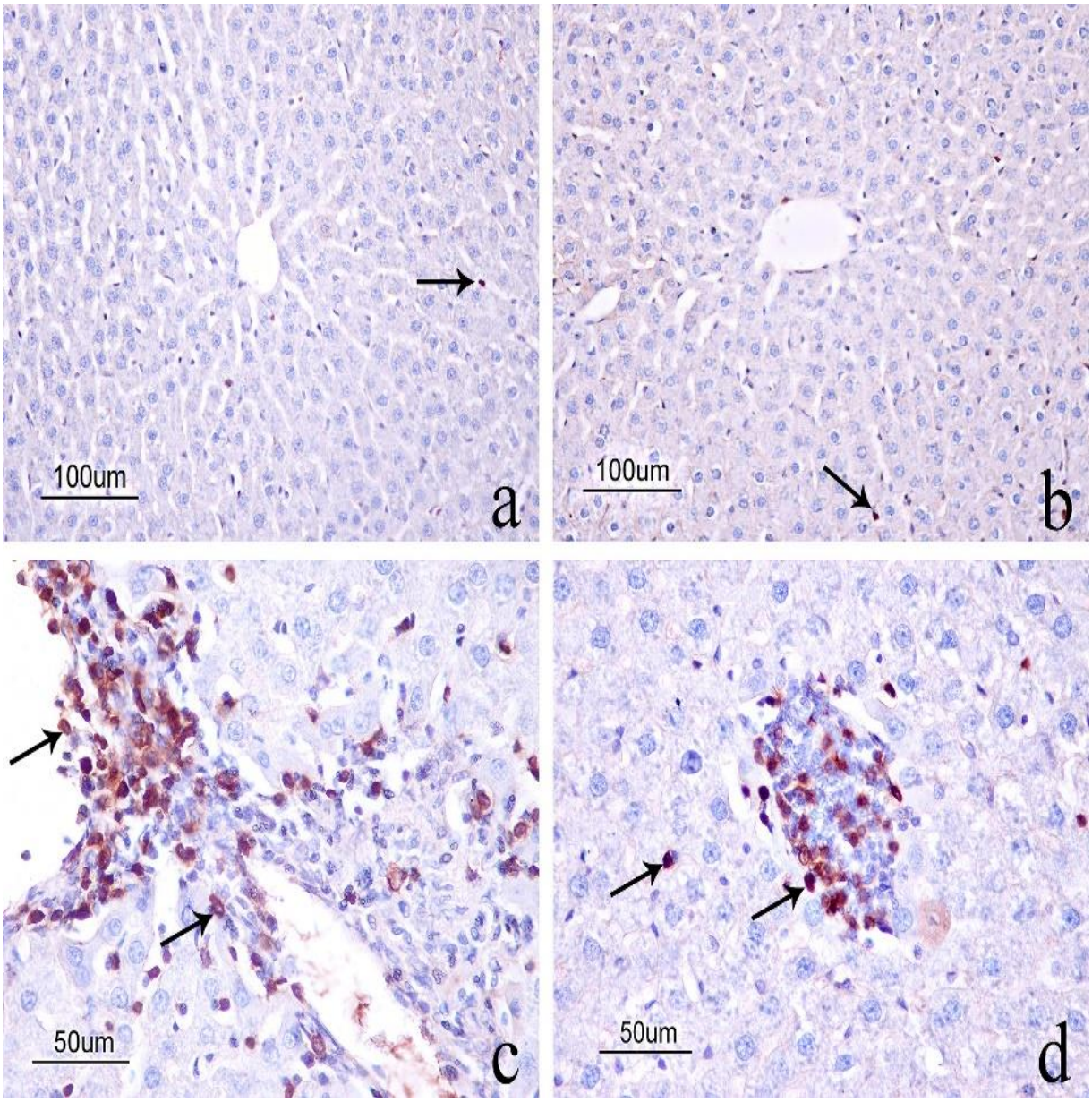

Figure 5. Effects of Cd and perga on the immune expression of CD3 in the rat liver tissues: (a) control group, (b) perga group, (c) Cd group, and (d) Cd + perga group. ABC method, counterstained with hematoxylin. Description: CD3-positive cells (arrows).

Table 4. Intensity of the CD34, CD68, and CD3 immunoreactivity in the rat liver tissues.

Antibodies	Control	Perga	Cd	Cd + perga
CD34	0.62±0.52 ^{c}	0.75±0.45 ^{c}	2.87±0.35 ^{a}	1.75±0.46 ^{b}
CD68	0.25±0.46 ^{c}	0.12±0.35 ^{c}	2.50±0.53 ^{a}	1.50±0.53 ^{b}
CD3	0.12±0.35 ^{b}	0.25±0.46 ^{b}	2.50±0.53 ^{a}	2.62±0.51 ^{a}

^{a,b,c}: Values with different letters in same row are significantly different at $p<0.005$.

DISCUSSION

Cd plays an important role in triggering hepatocellular toxicity, as the liver contributes to the rapid clearance of Cd from the blood. Hepatotoxicity has been suggested to include the direct toxic effect of metal,

ischemia due to endothelial cell damage, KC activation, and inflammatory damage (Yamano et al., 1998). Cd is also a potent immunotoxic agent and causes the production of reactive oxygen species and oxidative damage in the hepatocytes (Miltonprabu and Manoharan, 2016). Since oxidative stress is one of the basic mechanisms of Cd-induced damage, it has been stated that protection against Cd toxicity can be provided via the addition of some antioxidants (Karbownik et al., 2001). The present study identified evidence for the protective efficacy of perga as an antioxidant rich in flavonoids, against endothelial damage and inflammation in Cd-induced hepatotoxicity.

Hepatocytes are assumed to be the primary cellular targets for Cd toxicity (Kayama et al., 1995). Loss of the normal structure of parenchymatous tissue, focal necrosis and vacuolar degeneration in hepatocytes, inflammatory cell infiltration, and collagen fiber formation have been reported in rats exposed to Cd at a dose of 5 mg/kg/bw (El-Sokkary et al., 2010; Baskaran et al., 2018). In the current study, similar findings were seen in the Cd group. On the other hand, some findings were found to be significantly reduced in the Cd + perga group. This may have been related to the antioxidant properties of perga, because oxidative stress caused by Cd may contribute to hepatocellular necrosis and apoptosis (Ye et al. 2007). In this regard, different natural substances with antioxidant properties, especially those rich in phytochemicals, may have possible protective effects on the tissue damage caused by Cd. It has been reported that hepatic oxidative stress can be ameliorated by vitamin E (Fang et al., 2021). The protective functions of catechin (Choi et al., 2003), and quercetin (Vicente-Sanchez et al., 2008) from green teas against Cd intoxication have also been reported.

Although Cd can directly damage hepatocytes, it has been suggested that Cd-induced hepatocellular damage occurs as a result of ischemia caused by damage to LSECs (Rikans and Yamano, 2000). Cd acute toxicity is caused by the initial interaction of the metal with the vascular endothelium (Nolan and Shaikh, 1986). Accordingly, Cd induces degeneration of the hepatic endothelium, eroding the endothelial lining and resulting in the extrusion of damaged cells into the capillary lumen (McKim et al., 1992). Immunohistochemical CD34 expression, which is negative in most LSECs in the normal liver, increases in chronic inflammatory diseases, cirrhosis, hepatocellular carcinomas, and other pathological conditions (Kawanami et al., 2016; Arakelian et al., 2023). Consistent with previous studies, a significant increase in CD34 expression was detected in the Cd group. However, with perga treatment, CD34 reactivity in the Cd + perga group decreased significantly compared to the Cd group. This shows that perga is effective in reducing the damage to the liver endothelium.

KCs have been reported to be involved in Cd hepatotoxicity (El-Mansy et al., 2016). Increased cytoplasmic vacuolization, a morphological feature of activated mononuclear phagocytes, has been observed in KCs after Cd administration to rats (Sauer et al., 1997). Normally, the main functions of KCs are phagocytosis and antigen presentation (Vrba and Modrianský 2002). However, activated macrophages play an important role in liver damage and necrosis by secreting hundreds of products, some of which are mediators of the inflammatory response and some of which are cytotoxic (Hassoun and Stohs, 1996; Roberts et al. 2007). Immunohistochemical analysis of CD68 showed that the number of activated KCs increased in Cd-treated mice (He et al., 2020). In the present study, it was observed that CD68 activation increased in the Cd group compared to the control group. On the other hand, perga treatment resulted in a significant decrease in CD68 expression. Resveratrol, an antioxidant, has been reported to reduce the number of CD68 (+) KCs (Chan et al., 2011). Therefore, the decrease in CD68 activation may have been due to the antioxidant properties of perga.

Inflammatory processes play an important role in secondary damage caused by Cd (Kayama et al., 1995). Cd causes leukocyte influx into the liver tissue, which causes an inflammatory response that is the hallmark of Cd toxicity (Kataranovski et al. 2009). Apart from leukocytes, Cd can also affect lymphocyte development, subgroup distribution, and activity and representation in lymphoid tissues and blood (Djokic et al., 2015; Zhang et al., 2016). In the present study, an increase in the number of CD3 positive cells was detected in the liver sections of the Cd and Cd + perga groups. It has been reported that endothelial cells with high CD34 positivity activate a stronger proliferation of regulatory T lymphocytes compared to CD34 low cells (Arakelian et al., 2023). Therefore, the increase in the number of CD3-positive cells in the current study may have been related to the increase in the CD34 expression.

CONCLUSION

Taken together, the findings revealed that the perga was effective in partially reducing histopathological changes and partially preventing endothelial damage and KC activation in Cd toxicity. No definitive conclusion could be reached regarding the T lymphocyte activation. Further studies are needed to fully understand the hepatoprotective effects of perga.

Disclosure statement

The authors declare that they have no conflicts of interest

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