

Investigation of the protective effect of *Tarantula cubensis* extract on the liver and brain of rats exposed to gentamicin

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Abstract

This study aimed to investigate the possible protective effects of *Tarantula cubensis* extract (TCE) on the liver and brain of rats exposed to gentamicin (GM). A total of 40 male Sprague Dawley rats were randomly divided into four equal groups: control, TCE, GM, and GM+TCE. Some biochemical indices, apoptotic markers (B-cell lymphoma 2 [Bcl-2] and Bcl-2 associated X protein [Bax]) and histopathological changes were evaluated. In the GM group, serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), total bilirubin, brain and liver tissue malondialdehyde (MDA) concentrations increased; albumin, total protein, brain and liver tissue superoxide dismutase (SOD) concentrations and the total antioxidant status (TAS) decreased. Apoptosis induced down-regulation of Bcl-2 and up-regulation of Bax in liver and brain tissues in the GM group. GM-treated animals demonstrated several histopathological changes. TCE administration restored some histopathological changes. Lipid peroxidation and apoptosis decreased, antioxidant defense increased, concentrations of some serum biochemical indices (AST, ALT, ALP, GGT, and total bilirubin) decreased, and albumin and total protein levels increased in the TCE+GM group compared to the GM group. In conclusion, high doses of GM induce adverse effects on liver and brain tissue of rats. It was concluded that TCE administration can improve these adverse effects by reducing lipid peroxidation and apoptosis, improving the antioxidant defense system. TCE can be used to protect against the toxic effect of GM and other chemical agents in the liver and brain in veterinary medicine. However, additional studies are needed to confirm this assumption.

Antioxidant, apoptosis, immunohistochemistry, oxidative stress

Gentamicin (GM) is an aminoglycoside antibiotic used for the treatment of several pathogenic Gram-negative bacterial infections. It is widely used in veterinary medicine because of its efficacy (Laorodphun et al. 2022). It has been reported in previous studies that gentamicin causes nephrotoxicity, hepatotoxicity, and ototoxicity (Kaplan et al. 2017). These adverse effects may be induced by reactive oxygen species (ROS) and generation of free radicals in tissues via deoxyribonucleic acid (DNA) damage and membrane lipid peroxidation (Moreira et al. 2014). Enzyme systems, such as superoxide dismutase (SOD), protect cells against ROS damage. Some studies have reported that several antioxidants, including green tea extract, ascorbic acid, vitamin E, etc., reduced the side effects of gentamicin (Kadkhodae et al. 2005; Khan et al. 2009; Hakyemez et al. 2022).

The mechanism of gentamicin-induced toxicity is multifactorial and unclear. Oxidative stress is a major cause of toxicity. ROS destroys the cell components. An impaired antioxidant defense system and increased ROS levels cause oxidative stress, resulting in liver damage and toxicity (Arjinajarn et al. 2017). The brain is especially sensitive to oxidative damage because of the excess of polyunsaturated fatty acids (Almalki et al. 2022).

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The release of various alkenes and aldehydes during oxidative stress causes peroxidation of membrane lipids. Malondialdehyde (MDA) is an indicator widely used in the evaluation of oxidative stress (Karadogan et al. 2022). ROS production could lead to cell apoptosis through the modulation of B-cell lymphoma 2 (Bcl-2) family proteins. Apoptosis is a programmed cell death that mediates the controlled removal of unwanted damaged cells from the organism. Extrinsic and intrinsic factors, Bcl-2 associated X protein (Bax), caspases, and the Bcl-2 family contribute to apoptotic mechanisms. Bax plays a proapoptotic role, whereas Bcl-2 has an antiapoptotic function in liver and brain cells (Kalkan et al. 2012).

Previous studies have shown that an acute kidney injury can affect some organs, for example the brain and the liver, which is called a remote organ injury (Doi and Rabb 2016; Gardner et al. 2016). Renal ischaemia may lead to an increase in hepatic enzymes, cellular necrosis, and congestion in the liver (Najafi et al. 2017). The most dramatic and sensitive indicator of hepatocyte injury is the release of intracellular enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the circulation. During hepatic damage, serum concentrations of these enzymes increase (El-Houseiny et al. 2022).

Homeopathy is one of treatment options used in veterinary medicine. One of these options is *Tarantula cubensis* D6 extract (TCE) (Theranekron®) obtained from the *Tarantula cubensis* spider. *Tarantula cubensis* extract is used in veterinary medicine to treat traumatic necrotic disorders and some types of cancer, relieve inflammation and oedema, ensure rapid epithelialization, regeneration and resorption as well as produce antiphlogistic and demarcation effects (Corum et al. 2016).

Although some synthetic and natural antioxidant drugs have been used to prevent liver, kidney, and brain damage due to GM toxicity, there is no specific treatment agent against GM toxicity. There was also no scientific data on TCE's antioxidant and anti-apoptotic effects on liver and brain of rats exposed to GM toxicity. Therefore, this study was designed to investigate the protective effects of TCE on the brain and liver tissues of rats exposed to GM toxicity.

Materials and Methods

Animals

A total of 40 male Sprague Dawley rats weighing between 280 and 420 g were used. Rats were obtained from Balikesir University Experimental Animals Production, Care, Application and Research Center, Balikesir, Turkey. Standard commercial pellet food and water were provided *ad libitum*. All tests and procedures were performed according to the European Economic Community Guidelines for the care and use of laboratory animals and were approved by the Local Ethics Committee of Balikesir University (approval number: BAU-HADYEK 2020/3-4).

Experimental procedure

Rats were randomly divided into four equal groups: control (0.5 ml isotonic saline, intraperitoneally (i.p.) for 8 days), GM (100 mg/kg i.p. for 8 days), TCE (200 µl/kg/day, subcutaneously (s.c.) for 14 days), GM (100 mg/kg i.p. for 8 days) + TCE (200 µl/kg/day s.c. for 14 days). GM (Goldbio, St. Louis, MO, USA) dose was chosen based on previous studies (Kalkan et al. 2012; Khaksari et al. 2021). The TCE (Richter Pharma, Welss, Australia) dose was chosen based on the study by Karabacak et al. (2015). Twenty-four hours after the last application, blood samples were taken, and serum was separated and stored at -70 °C. The liver and brain were immediately dissected after rats were sacrificed under isoflurane anaesthesia (inhaling 3 Vol% isoflurane in a chamber for 3–5 min with maintained anaesthesia at 1–1.5 Vol% isoflurane with 0.8 l/min air as the carrier via a nose cone), rinsed from blood in isotonic saline and then half of them was stored at -70 °C until analysis; the other half of the tissues were fixed in 10% phosphate-buffered formalin for immunohistochemical and histopathological examinations.

Serum analysis

Serum ALP, GGT, ALT, AST, total protein, total bilirubin, and albumin concentrations were measured using the commercially available diagnostic kits (Archem, Istanbul, Türkiye) as instructed by the manufacturer in a biochemical autoanalyser (Sinnova D280, Nanjing, China).

Measurement of biochemical indices

The liver and brain tissues were weighed and homogenized in ice-cold 1.15% potassium chloride to prepare a 10% (w/v) tissue homogenate at 1,300 rpm for 3 min with homogenizator (Stuart SHM 1, UK). The half

of the homogenate was used for the determination of MDA described by Yoshiko et al. (1979). The other half of the homogenate was centrifuged at 5,000 g for 60 min at 4 °C and the supernatant was separated for the determination of SOD and the total antioxidant status (TAS) analysis. Total protein levels of the tissue homogenates and supernatants were analyzed using the Lowry method (Lowry et al. 1951). The levels of SOD in tissue supernatants were assessed following the methods of Sun et al. (1988). TAS levels of supernatants were measured using a commercially available kit (Rel Assay Diagnostics, Gaziantep, Turkey) according to the manufacturer's instructions.

Histopathological examination

Liver and brain tissue specimens in formalin fixation for 72 h were blocked by passage through alcohol and xylol, and were embedded in a paraffin block. Sections of 3 µm were taken in the microtome (Leica 2245, Nussloch, Germany) and then stained with haematoxylin and eosin (H&E). The preparations were closed with entellan. All slides of each group were examined under a light microscope. Histopathological evaluation scored in four categories based on the intensity of alterations: -, absent; +, mild; ++, moderate; +++, severe.

Immunohistochemical examination

After the follow up and blocking procedures, 3-µm sections were taken in polylysine-coated slides. Immunohistochemistry was performed on sections using avidin-biotin-peroxidase complex (ABC, Daco Cytomation, Denmark) method according to the manufacturer's instructions.

The sections were deparaffinized, rehydrated, and blocked with 3% hydrogen peroxide. And then incubated with Bax (dilution: 1:100, Santa Cruz, CA, USA) or Bcl-2 (dilution: 1:100, Santa Cruz, CA, USA) antibodies. Sections were incubated with biotinylated goat anti-rabbit secondary antibody (Dako Corporation, Carpinteria, USA) and streptavidin peroxidase complex (ABC; Dako Corporation, Carpinteria, USA). The slides were treated with 3,3-diaminobenzidine (DAB) in phosphate-buffered saline (PBS) (0.5 mg DAB/ml) containing hydrogen peroxide 30% v/v. The Bax and Bcl-2 positive cells were examined semi-quantitatively under a light microscope and then photographed.

Statistical analysis

The results were expressed as means in each group. The statistical analysis of differences between groups was analysed using ANOVA and the significance of differences was determined using Duncan's test. SPSS (Version 17.0, Chicago, IL, USA) program was used for all statistical analyses. The differences between the groups in terms of the indices examined were considered significant at the $P < 0.05$ level. All the values are presented as mean \pm SE.

Results

Biochemical results

Concentrations of ALT, AST, GGT, ALP, and total bilirubin increased, and albumin and total protein levels decreased in the GM group compared to the control group. AST, ALT, ALP, GGT, and total bilirubin levels decreased and albumin and total protein levels increased in the GM+TCE group compared to the GM group (Table 1). In addition, in liver and brain tissues, MDA levels increased whereas SOD and TAS levels decreased in the GM group compared to the control group. MDA levels decreased whereas SOD and TAS levels increased in the GM+TCE group compared to the GM group (Table 2). The levels of biochemical indices in serum and in the liver and brain tissues of all groups are presented in Tables 1 and 2.

Histopathological findings and immunohistochemical evaluation

Bcl-2 and Bax were assessed immunohistochemically and histopathological alterations in liver and brain tissues were examined. Immunohistochemical examination showed that apoptosis was induced in liver and brain tissue in the GM group. The Bcl-2 activity was increased, and the Bax positive intensity of liver and brain tissue was decreased in the GM+TCE group compared to the GM group. The immunohistochemical findings showed slight apoptotic activity in the control and TCE groups compared to GM and GM+TCE (Plates XV, XVI, Figs 1 and 2).

Microscopical examination of the brain and liver tissues of GM groups showed some histopathological findings (e.g., brain: gliosis, hyperaemia, and spongy changes; liver: portal inflammation, hepatic congestion, and necrosis in hepatocytes). TCE administration restored these histopathological changes (Table 3, Fig. 3, Plate XVII).

Table 1. Serum biochemical indices in study groups (mean \pm SE).

Indicator	Control (n = 10)	<i>Tarantula cubensis</i> extract (n = 10)	Gentamicin (n = 10)	Gentamicin + <i>Tarantula cubensis</i> extract (n = 10)
AST (IU/l)	65.1 \pm 1.02 ^d	79.9 \pm 1.51 ^c	135.1 \pm 1.55 ^a	100.7 \pm 1.16 ^b
ALT (IU/l)	70.3 \pm 1.03 ^d	88.3 \pm 2.44 ^c	159.7 \pm 1.47 ^a	111.5 \pm 1.52 ^b
ALP (IU/l)	145.7 \pm 1.43 ^d	162.5 \pm 2.5 ^c	248.5 \pm 2.50 ^a	202.9 \pm 2.45 ^b
Total bilirubin (mg/dl)	0.42 \pm 0.01 ^d	0.50 \pm 0.007 ^c	1.49 \pm 0.01 ^a	1.05 \pm 0.02 ^b
Total protein (g/dl)	6.77 \pm 0.02 ^a	6.39 \pm 0.04 ^b	4.54 \pm 0.04 ^d	6.01 \pm 0.02 ^c
Albumin (g/dl)	4.58 \pm 0.02 ^a	4.42 \pm 0.02 ^b	3.39 \pm 0.02 ^d	4.16 \pm 0.02 ^c
GGT (IU/l)	6.19 \pm 0.03 ^d	7.12 \pm 0.03 ^c	14.50 \pm 0.49 ^a	9.00 \pm 0.53 ^b

^{a, b, c, d} Significant difference between groups with different superscripts in the same row ($P < 0.05$).

AST - aspartate aminotransferase; ALT - alanine aminotransferase; ALP - alkaline phosphatase; GGT - gamma glutamyl transferase.

Table 2. Liver and brain tissue indices in study groups (mean \pm SE).

Tissue	Indicator	Control (n = 10)	<i>Tarantula cubensis</i> extract (n = 10)	Gentamicin (n = 10)	Gentamicin + <i>Tarantula cubensis</i> extract (n = 10)
Liver	MDA (μ mol/mg protein)	8.08 \pm 0.17 ^d	10.62 \pm 0.38 ^c	17.11 \pm 0.22 ^a	13.52 \pm 0.15 ^b
	SOD (U/mg protein)	47.11 \pm 0.32 ^a	44.30 \pm 0.49 ^b	30.68 \pm 0.23 ^d	39.27 \pm 0.33 ^c
	TAS (mmol trolox equiv./mg protein)	2.23 \pm 0.02 ^a	2.11 \pm 0.02 ^b	1.21 \pm 0.01 ^d	1.82 \pm 0.01 ^c
Brain	MDA (μ mol/mg protein)	13.26 \pm 0.14 ^d	13.99 \pm 0.06 ^c	25.87 \pm 0.09 ^a	19.07 \pm 0.20 ^b
	SOD (U/mg protein)	39.65 \pm 0.21 ^a	28.10 \pm 0.12 ^b	28.11 \pm 0.05 ^d	33.94 \pm 0.16 ^c
	TAS (mmol trolox equiv./mg protein)	1.10 \pm 0.02 ^a	0.98 \pm 0.02 ^b	0.62 \pm 0.01 ^d	0.79 \pm 0.01 ^c

^{a, b, c, d} Significant difference between groups with different superscripts in the same row ($P < 0.05$)

MDA - malondialdehyde; SOD - superoxide dismutase; TAS - total antioxidant status

Table 3. Histopathological alteration in liver and brain tissues of study groups (H&E staining).

Tissue	Indicator	Control (n = 10)	<i>Tarantula cubensis</i> extract (n = 10)	Gentamicin (n = 10)	Gentamicin + <i>Tarantula cubensis</i> extract (n = 10)
Liver	Portal inflammation	-	-	++	+
	Hepatic congestion	-	+	+++	++
	Necrosis in hepatocytes	-	+	+++	++
Brain	gliosis (cortex)	-	+	++	+
	Hyperaemia (cortex)	-	-	++	+
	Spongy changes (cortex)	-	-	++	+
	Necrosis (cortex)	-	-	-	-
	Degeneration/atrophy Purkinje cells (cerebellum)	-	-	-	-
	Basket and stellate cell, cellular vacuolation (cerebellum)	-	-	-	-
	Molecular layer vacuolation (cerebellum)	-	-	-	-

- Absent; + mild; ++ moderate; +++ severe

Discussion

Many drugs induce toxicity which limits their therapeutic benefits. Antibiotics such as aminoglycosides, sulphonamides, beta-lactams, tetracycline etc. can induce acute renal failure in animals. Since aminoglycosides cause severe toxicity, their clinical use is limited. As an aminoglycoside antibiotic, GM is one of the most widely used drugs and its toxic effect has been reported in previous studies. It is used in the management of Gram-negative bacterial infections (Kalkan et al. 2012).

Some researchers reported that GM causes reactive oxygen metabolites, particularly those of O₂⁻ and hydrogen peroxide (H₂O₂) (Martinez-Salgado et al. 2002). Increased ROS production leads to lipid peroxidation, protein denaturation, and oxidative damage. Under normal conditions, the antioxidant-oxidant system is in balance, while there is an increase in MDA and a decrease in SOD and TAS during oxidative stress. Intracellular SOD enzyme is a major antioxidant defence component (Yaman and Balıkcı 2010). ROS play a vital role as inducers of apoptosis. Apoptosis is a form of programmed cell death resulting in interconnected intracellular caspase proteins. Apoptosis plays an essential role in eliminating unnecessary cells (Tanyeli et al. 2019).

The toxicity induced by the administration of high doses of GM activates the intrinsic apoptosis pathway by acting on mitochondria and then increasing Bax expression, which induces apoptosis (Lopez-Novoa et al. 2011). GM toxicity decreased Bcl-2 expression and increased Bax expression. Bax is known as the proapoptotic protein and inhibits the antiapoptotic effects of Bcl-2. This effect of Bax protein can cause increased apoptosis in the liver and brain tissues of rats exposed to GM toxicity. TCE-treated rats experienced a reduction in apoptosis that occurs with GM toxicity. In this study, it has been shown that GM causes apoptosis as a result of toxicity, which is consistent with the literature (Khaksari et al. 2021). Both histopathological and immunohistochemical findings also confirm that TCE is a slightly effective drug for GM-induced toxicity. Histopathological changes in tissues with GM-induced toxicity were also in line with the results of previous studies (Akyüz et al. 2021). Histopathologically, a decrease in tissue damage was observed when the GM+TCE group was compared to the GM group. For example, portal inflammation, hepatic congestion, necrosis in hepatocytes, gliosis, hyperaemia, and spongy changes mildly decreased in the GM+TCE group compared to the GM group. Alterations of each group are presented in Table 3.

TCE has been reported to have demarcating and anti-inflammatory actions in cases of some injuries (Stampa 1986). It had a protective effect against toxicity, and decreased MDA levels in tissue and levels of serum hepatic markers (ALT, AST, ALP, and GGT). In this study MDA levels decreased, and SOD and TAS levels increased in the GM+TCE group compared to the GM group. It has been shown that TCE can reduce damage by activating antioxidant systems in the liver and brain tissues of rats exposed to GM toxicity. The results of the current study are consistent with previous studies (Karabacak et al. 2015; Makav et al. 2020). We reason this result is due to the curative effect on necrotic tissue, and the regenerative and anti-inflammatory properties of TCE.

This study demonstrated that gentamicin induced hepatotoxicity as evidenced by the elevation of serum AST, ALT, ALP, GGT, and total bilirubin and a decrease in serum albumin and total protein levels. Gentamicin-induced brain damage and hepatotoxicity were associated with increased oxidative stress and decreased antioxidant defence system. TCE treatment showed improvement in liver function. This study is the first to show that TCE treatment might provide slight protection against brain and liver injury by gentamicin-induced oxidative stress. This study showed an increase in brain and liver MDA level, reduction in antioxidant reserves, SOD and TAS together with some histopathological changes in the brain and liver tissue in GM-treated groups compared to the control group which is also in line with the previous study by El-Maddawy (2014).

A significant increase in hepatocyte damage indicators and a decrease in serum albumin and total protein concentrations were observed in the gentamicin group compared to the control group. In this respect, this study is in line with previous studies (Sivachandran and Hariharan 2012; Arjinajarn et al. 2017).

This study showed that GM toxicity significantly increased the product of lipid peroxidation (MDA) in liver and brain tissue, and decreased the antioxidant status (SOD and TAS). On the other hand, it has been shown in previous studies that acute kidney injury can also affect other organs and cause some tissue injuries (Yarjani et al. 2019).

There are no known reports describing the protective role of TCE against GM-induced liver and brain oxidative damage. In this study, it was observed that TCE has a protective effect on brain tissue as well as a hepatoprotective effect. This protective action of TCE is thought to be by destroying and scavenging ROS. Our results showed that increased oxidative stress led to low SOD, TAS and high MDA levels in the GM group, which is also consistent with the literature (Cenesiz 2020).

This study also showed that TCE inhibited GM-induced apoptosis in liver and brain tissue. In this study, the protective effect of TCE on lipid peroxidation in GM-induced toxicity was reported. Liver and brain SOD and TAS levels were significantly elevated in the GM+TCE group compared to the GM group, so TCE can protect by directly increasing the tissue antioxidant capacity, as well as providing regenerative, curative, and anti-inflammatory effects.

In conclusion, based on the results of this study, we determined that TCE treatment provided a mild protective effect on the liver and brain tissues of rats exposed to GM toxicity. The results of this study showed that TCE improved the deteriorated biochemical indices and had a protective effect against GM-induced liver and brain damage. This protective effect of TCE is thought to be due to its antiapoptotic, antioxidant, and regenerative properties, and it protects against liver and brain damage. The mechanism of these effects is through increased total tissue antioxidant capacity, reduction of oxidative stress, or other unknown pathways (the effect of TCE at the molecular level) that require further investigation. We propose that TCE can be used to protect against the toxic effect of GM and other chemical agents in the liver and brain. However, additional studies are needed to confirm this assumption.

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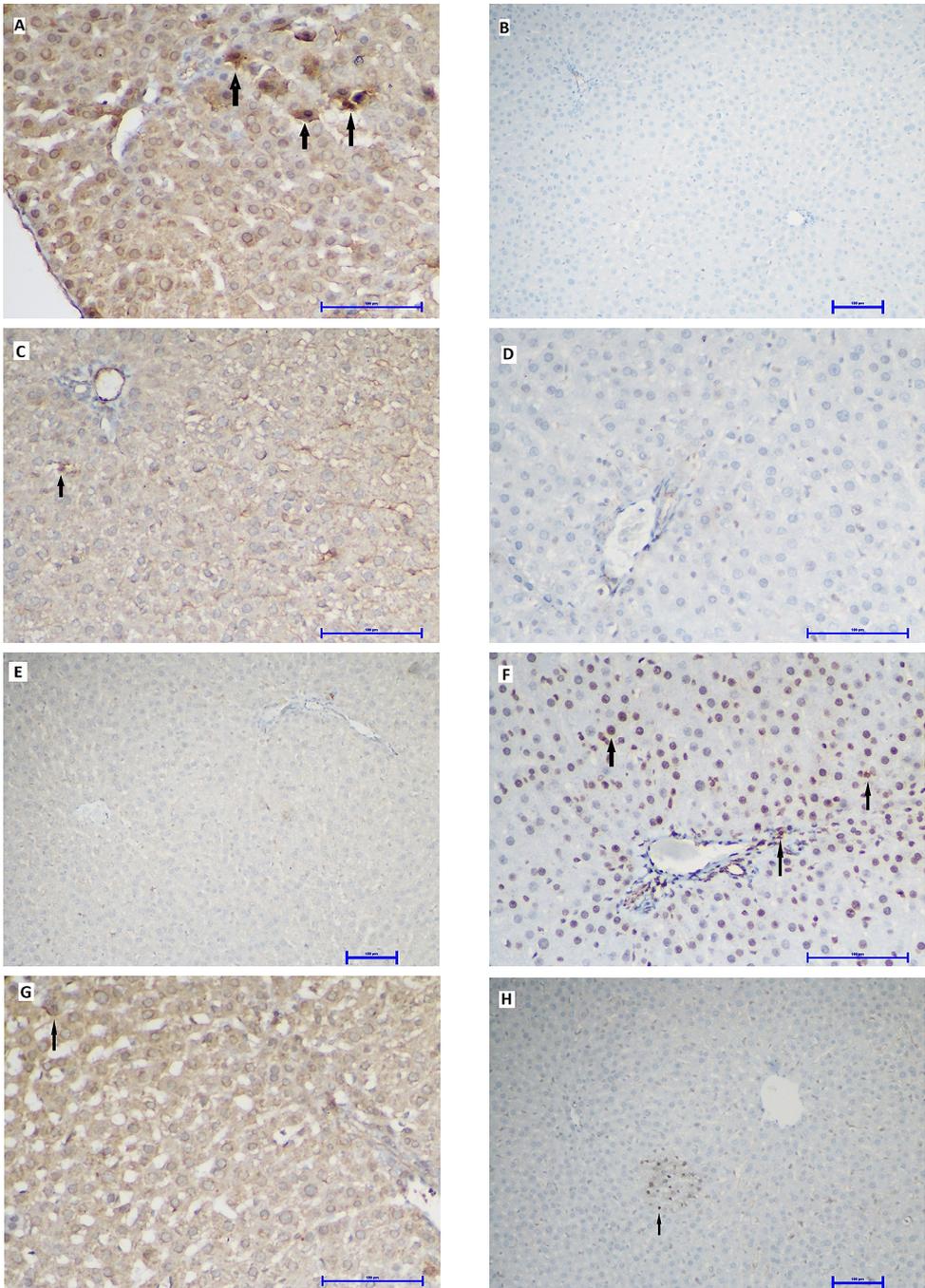


Fig. 1. Liver B-cell lymphoma 2 (Bcl-2) (A), Bcl-2 associated X protein (Bax) (B) in control group, Bcl-2 (C), Bax (D) in TCE group, Bcl-2 (E), Bax (F) in GM group and Bcl-2 (G), Bax (H) in GM+TCE group. Arrows indicate Bcl-2 or Bax positive cells.

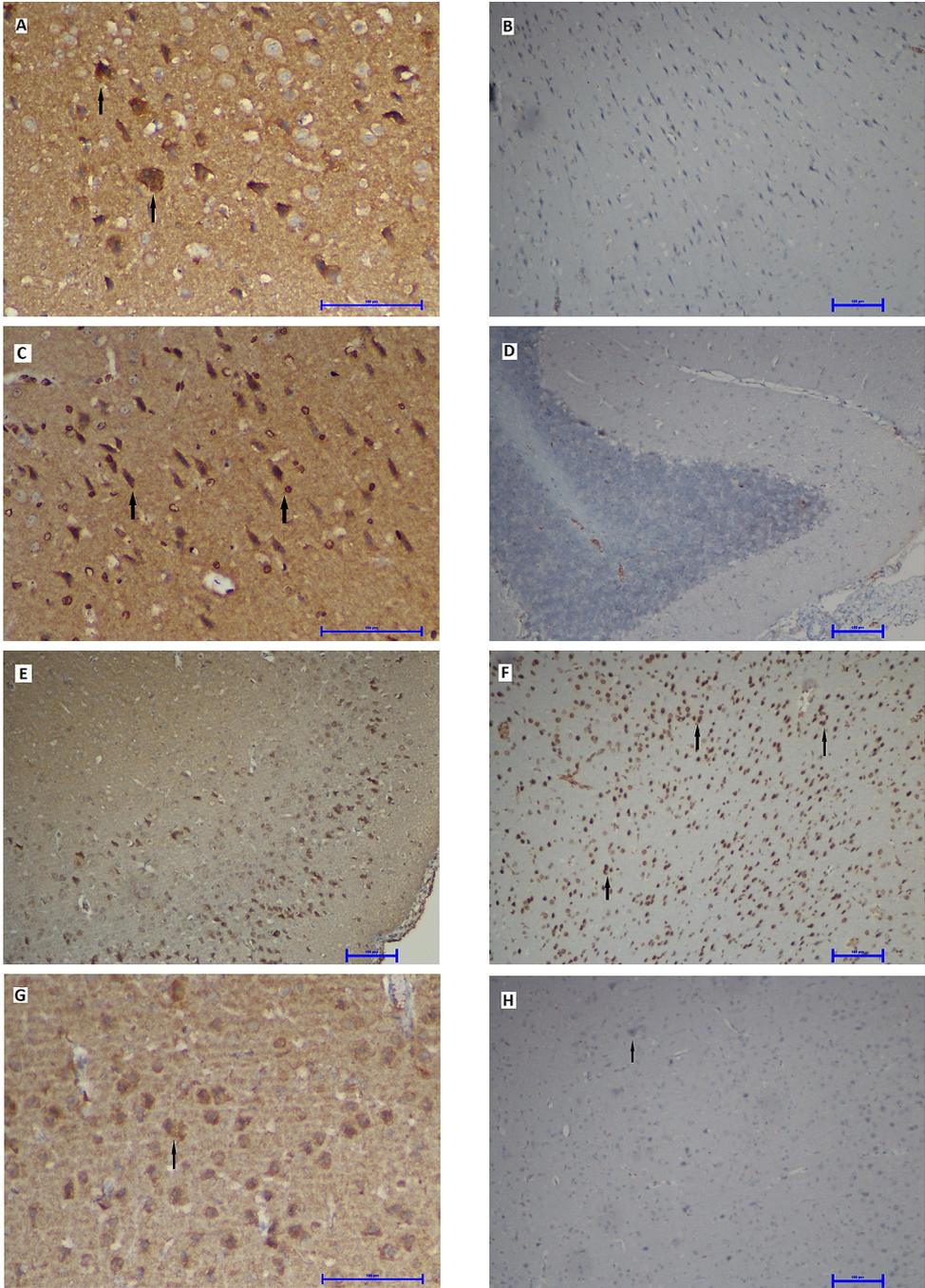


Fig. 2. Brain B-cell lymphoma 2 (Bcl-2) (A), Bcl-2 associated X protein (Bax) (B) in control group, Bcl-2 (C), Bax (D) in TCE group, Bcl-2 (E), Bax (F) in GM group and Bcl-2 (G), Bax (H) in GM+TCE group. Arrows indicate Bcl-2 or Bax positive cells.

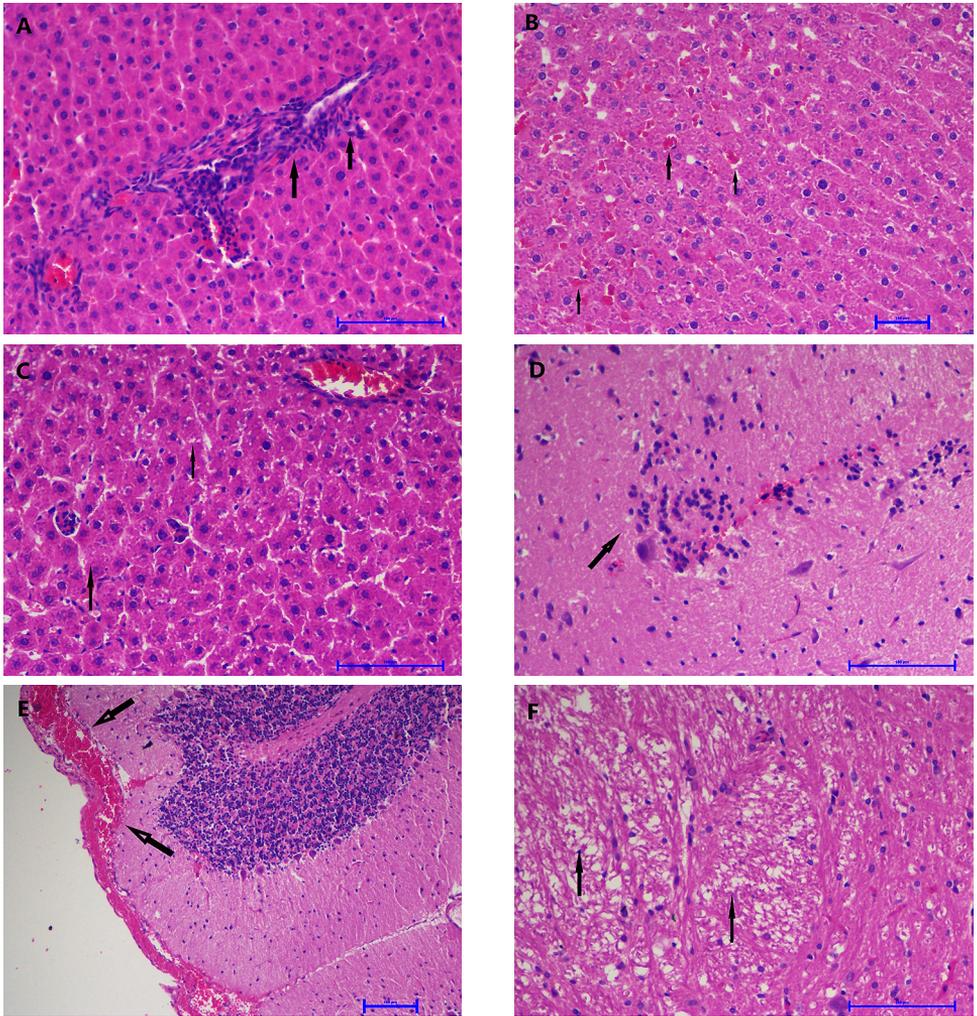


Fig. 3. Liver histopathology; portal inflammation (A) (arrows), hepatic congestion (B) (arrows), necrosis in hepatocytes (C) (arrows). Brain histopathology; gliosis (D) (arrows), hyperaemia (E) (arrows) and spongy changes (F) (arrows) (H&E staining).