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Original article

Propolis ameliorates cerebral injury in focal cerebral ischemia/reperfusion (I/R) rat model via upregulation of TGF-β1

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ABSTRACT

Neuroprotective impact of transforming growth factor β1 (TGF-β1) is increasingly recognized in different brain injuries. Propolis exhibits a broad spectrum of biological and pharmacological properties including neuroprotective action. The objective of the investigation was to explore the involvement of TGF-β1 signaling in the neuroprotective mechanism of propolis in I/R rats. In this study, focal cerebral ischemia model was built by middle cerebral artery occlusion (MCAO) for 2 h followed by reperfusion. The investigation was carried out on 48 rats that were arranged into four groups (n = 12): the sham group, I/R control group, I/R + propolis (50 mg/kg) group and I/R + propolis (100 mg/kg) group. The results revealed that propolis preserved rats against neuronal injury induced by cerebral I/R. It significantly reduced neurological deficit scores and improved motor coordination and locomotor activity in I/R rats. Propolis antagonized the damage induced by cerebral I/R through suppression of malondialdehyde (MDA) and elevation of reduced glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), brain-derived neurotropic factor (BDNF) and dopamine levels in the brain homogenates of I/R rats. Other ameliorations were also observed based on reduction of neurodegeneration and histological alterations in the brain tissues. These results also proposed that the neuroprotective effect of propolis might be related to upregulation of TGF-β1 and suppressed matrix metallopeptidase-9 (MMP9) mRNA expression.

1. Introduction

Stroke is a major public health problem worldwide and is the third leading cause of death after heart disease and cancer (Feher et al., 2011). Ischemic strokes caused by an interruption of the blood supply to the brain and represent about 87% of stroke patients. Upon cerebral I/R, a cascade of pathophysiological events initiates in the infarct area including infiltration of peripheral inflammatory cells, activation of microglia cells, excessive production of inflammatory mediators, and matrix metalloproteases (Han et al., 2014). Thereby, several mechanisms play a role in the pathophysiology of ischemic stroke, such as, oxidative stress, inflammation and apoptosis. Likewise, oxidative stress has emerged as the most deleterious pathological characteristic in the pathogenesis of ischemic brain injury, therefore, specific neuroprotective therapies targeting oxidative stress are found to valuable for the treatment of cerebral I/R (Chen et al., 2011).

Propolis is a natural resinous mixture produced by honeybees. The main constituents of propolis are polyphenolics including flavonoids, esters such as caffeic acid phenethyl ester, amino acids, polysaccharides and hydroxybenzene (Ramadan et al., 2012). Propolis has marked biological activities such as antimicrobial,

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antioxidant anti-inflammatory and immune-stimulating activities (Ramadan et al., 2015; Mostafa et al., 2016). In addition, propolis was reported to speed up the tissue regeneration and repair of injured cells (Fuliang et al., 2005). Further, some of the chemical components of propolis such as galangin and caffeic acid phenethyl ester have been reported to have potential neuroprotective activity (Nanaware et al., 2017). Despite these promising findings, the mechanism of propolis upon acute brain injuries has not been thoroughly investigated.

Most of the present therapeutic strategies of ischemic stroke are directed to alleviate symptoms; however, damaged nerve cells cannot be restored. Accordingly, exploring a mechanism that is able to trigger the regeneration of nerve cells and repair ischemic cerebral apoplexy will have a considerable effect on clinical practice. The family of TGF-β is a small group of multiple functional cytokines represented by three isoforms: TGF-β1, TGF-β2 and TGF-β3. These isoforms are produced in the brain by the glial and neuronal cells. Among these isoforms, TGF-β1 has been found to be considerably expressed in brain of neurologic disorder patients (Pratt and McPherson, 1997). Recent reports provided evidences that increased TGF-β1 expression plays a protective function in ischemic stroke (Zhu et al., 2017). Increasing TGF-β1 expressions in ischemic brain may become a therapeutic strategy for ischemic cerebrovascular disorders (Yu et al., 2018).

The role of TGF-β1 in the protective effect of propolis against stroke has not been previously reported. The present study was conducted to assess the involvement of TGF-β1 signaling in the neuroprotective mechanism of propolis in a rat model of MCAO. To the authors' knowledge, this is the first investigation to explore the role of TGF-β1 signaling in the protective mechanism of propolis in cerebral I/R injury.

2. Material and methods

2.1. Animals

Animal procedures were performed according to the protocol approved by the National Research Centre (NRC) Ethics Committee, Egypt (MERC-18–086). Forty-eight adult male Wistar rats, five months of age (150–180 g) were obtained from the Animal House Colony at the NRC, Egypt. Animals were maintained for few days under standard environmental conditions (12 h dark/12 h light cycle; temperature 20–22 °C; relative humidity 40-60%) Table 1. The study was conducted according to regulations of the ethics committee of the NCR which gave its consent in accordance with the National Regulations on Animal Welfare and Institutional Animal Ethical Committee.

2.2. Drugs

Propolis (Biopropolis, 400 mg soft gelatin capsules; Sigma Pharmaceutical Industries SAE Company, Egypt) were used after dissolution in paraffin oil.

2.3. Induction of focal cerebral I/R injury

Focal cerebral I/R injury in rats was induced by the MCAO for 2 h followed by reperfusion as described by Bai et al. (2016) with slight modifications. Briefly, rats were anesthetized with an intraperitoneal injection of 10% chloral hydrate (350 mg/kg) and fixed on the surgery board in the supine position. The left carotid region was exposed through a midline cervical incision; the external carotid artery and the common carotid artery were exposed. A non-traumatic microvascular clamp was introduced from the carotid bifurcation into the internal carotid artery, thereby occluding the origin of the middle cerebral artery. After a 2-hours occlusion, reperfusion was initiated by withdrawal of the clamp. Finally, the incision was sutured and the animal was allowed to recover from anesthesia. During the operation, the rectal temperature of rats was maintained at 37 °C using a heating lamp. Rats in sham operated group received the same procedure but without MCA occlusion. The animals were allowed to recover from anesthesia prior to being returned to their original housing.

2.4. Experimental protocol

Rats were randomly assigned into four groups and each group consisted of 12 rats.

1. Sham group: Normal rats underwent identical surgical procedure, except for MCAO and treated with the vehicle (paraffin oil).
2. I/R control group: I/R rats treated with the vehicle.
3. I/R + propolis-50 group: I/R rats treated with propolis at 50 mg/kg.
4. I/R + propolis-100 group: I/R rats treated with propolis at 100 mg/kg.

The vehicle and propolis were orally administered by intragastric gavage for 7 consecutive days before MCAO, at the onset of reperfusion and extended for 3 days after MCAO.

Neurological deficit scores were evaluated in all rats, 24 h after MCAO surgery. Then, animals of each group (n = 12) were randomized into two subgroups (n = 6). The first subgroup of animals was used for rotarod test and biochemical estimation and the second subgroup for activity cage test, histopathological examination, neuronal cell count and gene expression analysis (Fig. 1).

2.5. Assessment of neurological deficit

After 24 h of MCAO surgery, neurological deficit scores were evaluated in rats of the first and second subgroups by an observer blinded to the treatment of experimental animals using a 5-point scale as described by Longa et al. (1989). The scoring pattern was as follows: grade 0, no neurological deficit; grade 1, failure to entirely extend the contralateral forelimb; grade 2, circling if pulled by tail but normal posture at rest; grade 3, circling spontaneously; grade 4, no spontaneous activity and a sluggish level of consciousness; grade 5, death. Rats scoring of 0 (except for sham operated group) or 5 were excluded from the study.

Table 1
Effect of propolis on oxidative stress biomarkers in focal cerebral ischemia-reperfusion model.

Groups	MDA (nmol/g)	GSH (μmol/g)	SOD (U/g)	GPx (U/g)	CAT (U/g)
Sham	16.7 ± 0.82[b]	4.3 ± 0.22[b]	73.9 ± 1.05[b]	235.3 ± 8.19[b]	2.14 ± 0.06[b]
I/R control	38.3 ± 1.30[a]	1.7 ± 0.10[a]	46.8 ± 1.23[a]	133.7 ± 3.19[a]	0.42 ± 0.01[a]
I/R + propolis-50	29.8 ± 1.42[a,b]	2.6 ± 0.10[a,b]	55.8 ± 1.22[a,b]	182.3 ± 5.17[a,b]	1.52 ± 0.04[a,b]
I/R + propolis-100	20.4 ± 1.20[b]	3.8 ± 0.24[b]	70.2 ± 1.12[b]	212.8 ± 7.73[b]	2.04 ± 0.06[b]

Data are represented as mean ± S.E.M. of 6 rats per group.
[a,b] are significant difference from sham and I/R control groups, respectively at $p \leq 0.05$.

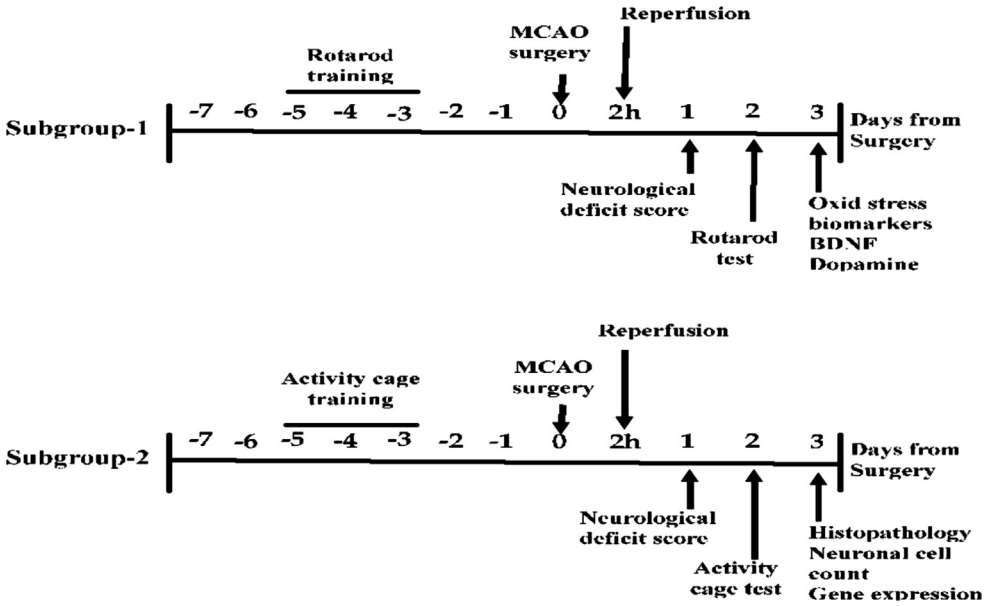

Fig. 1. Experimental design.

2.6. Behavioral assessment

2.6.1. Assessment of motor coordination and balance using rotarod

Motor coordination and balance of the rats was measured by using a rotarod apparatus (Model No. 7750; Ugo Basile) following the procedure described by Vijitruth et al. (2006) {Vijitruth, 2006 #47; Vijitruth, 2006 #47}. Before MCAO, each rat was trained on an accelerating rotating rod using an accelerating speed level (4–40 rpm). Three separate trials were given to each rat at 5 min interval and cut off time (300 s) to obtain stable baseline values (in seconds). On the test day, each rat was placed on the rod rotating from 4 to 40 rpm over 5 min by an observer who was blinded to the experimental groups. Each rat was tested 3 times with a 10 min intertrial interval. At each trial, the latency to fall was recorded with a maximum time of 300 s. The mean of three trials was calculated for each rat. Passive rotation, accompanying the rod without walking, was considered as a fall.

2.6.2. Assessment of locomotor activity using activity cage

Spontaneous coordinate activity of the rats was measured by using a grid floor activity cage (Model No. 7430, Ugo-Basile, Comerio, Italy) to detect the rat's movements. Movements by the rat that interrupted infrared beams were automatically detected, and the beam-interruption information was processed by the activity cage software to provide counts of horizontal movements. Before MCAO surgery, rats were acclimated to the test room for 1 h. Then, each rat was placed individually into the activity cage for a 5 min session and the basal activity counts were recorded. At the end of the session, each rat was gently removed from the activity cage, and then returned to its home cage. The grid floor was wiped out with a 70% (*v*/*v*) alcohol solution in distilled water between sessions to prevent olfactory cues. On the test day, each rat was re-exposed to the activity cage for a 5 min session and the final activity counts were recorded (El-Marasy et al., 2018).

2.7. Euthanasia and brain tissue samplings

Two hours after the last dose, all animals were sacrificed by decapitation using sharp scissors, which is rapid and painless. The brains of all rats were excised.

2.8. Biochemical analyses in brain homogenates

In the biochemical analysis, the fresh brain tissue was collected and washed with refrigerated saline. A 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 rpm at 4 °C for 15 min using a cooling centrifuge. The obtained supernatants were separated, frozen immediately in liquid nitrogen and then stored at −80 °C until further processing.

2.8.1. Measurement of lipid peroxidation in brain homogenates

The quantitative measurement of lipid peroxidation (LPO) in brain homogenates of rats was performed according to the method of Ruiz-Larrea et al. (1994). In brief, 0.5 mL of the homogenate supernatant were mixed with 4.5 mL of TCA-TBA reagent (20% TCA, and 0.8% TBA, 3:1) and heated for 20 min in a boiling water bath. After cooling, the mixture was then centrifuged at 3000 rpm for 10 min. The supernatant was collected, and the absorbance was read against blank (distilled water instead of sample), at 535 nm. The amount of MDA produced is calculated, using a molar absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and expressed as nmol/g tissue (Abdel-Salam et al., 2013).

2.8.2. Measurement of brain GSH content

Reduced glutathione content in brain homogenate was determined according to the method of Ellman (1959), modified by Bulaj et al. (1998). Briefly, 0.5 mL of the homogenate supernatant was added to 0.5 mL of trichloro-acetic acid 10%. The mixture was vortex-mixed and centrifuged at 4000 rpm/5 min at 4 °C. In a clean test tube, 1.8 mL of phosphate buffer pH 8.0 was added to 0.1 mL of the supernatant and 0.1 mL of Ellman reagent. After exactly 5 min, the absorbance was read at wave length 412 nm against blank (distilled water instead of sample). Reduced glutathione levels were calculated using the extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$. The results were expressed in μmol GSH/g tissue (Abdel-Salam et al., 2013).

2.8.3. Determination of SOD activity in brain homogenates

SOD activity was measured in brain homogenates as previously described by Mansour et al. (2015). In brief, 100 μl of brain super-

natant were added to Tris-HCl buffer (0.1 M, pH 8) containing 24 mmol/L pyrogallol and 30 μmol/L catalase in a total volume of 3 mL. The absorbance was measured at 420 nm.

2.8.4. Determination of GPx, CAT, BDNF and dopamine in brain homogenates

GPx, CAT, BDNF and dopamine levels were measured in brain homogenates according to the manufacturer instructions using commercial ELISA kit, Elabscience Biotechnology Co., Ltd-USA.

2.9. Histopathological examination

Samples of right-brain lobe including cerebral cortex and hippocampus were fixed in 10% neutral buffered formalin for 48 h then the samples were processed to get 4 μm paraffin embedded sections. Then, the sections were stained with hematoxylin and eosin (H&E). The infarct areas were determined, photographed and calculated in each histopathological section by using Lieca Qwin 500 Image Analyzer (Leica, Cambridge, England). The percentage of the ischemic lesion areas represented as ratio of the infarct area to the whole slide area was calculated according to El-Marasy et al., (2018).

2.10. Neuronal cell count

Other sections from the right-brain lobe of different experimental groups were stained by toluidine blue stain according to the method described by Kirino et al., (1984). The brain cortex and hippocampus were examined in 5 random fields/section from each group under high magnification power (X400). The number of intact neuronal cells and the total number of the neuronal cells were counted using Lieca Qwin 500 Image Analyzer, and then the percentage of intact neuronal cells was calculated.

2.11. Gene expression analysis

Left-brain lobe was dissected from a fresh brain and quickly kept at −80 °C before total RNA was isolated by Trizol Reagent (Invitrogen) as described by the manufacturer. Total RNA (2 μg/μl) was treated with DNase I (Invitrogen) and used for cDNA synthesis using Superscript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. The resulting cDNA was used as template in quantitative PCR performed under the following thermocycling procedure: 95 °C for 3 min, followed by 35 cycles of 95 °C for 0.5 min, 60 °C for 0.5 min, and 72 °C for 1 min. Primers were used for TGFβ1 (forward: GGACTCTCCACCTGCAAGAC and reverse: CTCTGCAGGCGCAGCTCTG), MMP9 (forward: CACTGTAACTGGGGGCAACT and reverse: CACTTCTTGTCAGCGTCGAA), and B-actin (forward: ATGGTGGGTATGGGTCAG and reverse: CAATGCCGTGTTCAATGG). The calculated lengths of the PCR products are 392 base pairs (bp) for TGF-β RI (GenBank accession No. NM_021578.2), 150 bp for MMP9 (GenBank accession No. NM_031055.1) and 97 bp for B-actin (GenBank accession No. NM_031144.3). Data were normalized to β-actin used as a reference gene. The relative mRNA expression of the target genes was calculated according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

2.12. Statistical analysis

Data are presented as the mean ± SEM for 8 rats per group in the behavioral tests and 6 rats per group in the biochemical tests. Comparisons between groups regarding the in biochemical analyses and behavioral tests (locomotor activity and rotarod test) were carried out using two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Data were analyzed with Graph Pad Prism v. 5.0 (Graph Pad Software, Inc., CA, USA). Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Assessment of neurological deficit

Neurological deficits were assessed 24 h post MCAO surgery (Fig. 2). Sham-operated rats exhibited no neurological deficits, whereas I/R control rats had the highest neurological deficit score suggesting that the deficits seen in I/R animals are due to ischemia and are not caused by the surgical procedure. Neurological deficit scores for animals treated with propolis (50 and 100 mg/kg) were significantly lower (2.7 ± 0.09 and 1.8 ± 0.05, respectively) than that of the I/R control rats (4.0 ± 0.15).

3.2. Behavioral assessment

3.2.1. Evaluation of motor coordination using rotarod

Neurobehavioral assessment was evaluated two days after MCAO surgery in rats (Fig. 3-a). I/R injury induced significant decrease in the falling latency time by 65.6% in comparison with sham-operated rats. The results indicated that propolis at 50 and 100 mg/kg could improve the motor coordination ability of MCAO rats compared with I/R control rats. Both doses of propolis significantly prolonged the final falling latency time to be 68.9% and 119.8% of the I/R control rats, respectively.

3.2.2. Evaluation of locomotor activity using activity cage

Likewise, the I/R control group manifested a marked reduction in their locomotor activity approximately by 4.3 folds as compared to sham-operated rats (Fig. 3-b). Propolis in doses of 50 and 100 mg/kg resulted in significant elevation in the locomotor activity of I/R rats by 150.6% and 246.6%, respectively as compared to I/R control group.

3.3. Effect of propolis on oxidative stress biomarkers

The activities of the antioxidant enzymes such as SOD, GPx and CAT along with GSH and LPO contents in the brains of sham and I/R rats are listed in Table 1. Activities of SOD, GPx and CAT were significantly ($p \leq 0.05$) reduced in the brain homogenates of I/R control rats as compared to sham group. I/R control rats manifested significantly ($p \leq 0.05$) decreased levels of GSH, an endogenous antioxidant, in their brain tissue as compared to sham group. Both doses of propolis resulted in significantly increased levels of SOD, GPx, CAT and GSH compared to I/R control rats. Interestingly, propolis at 100 mg/kg was able to successfully normalize the antioxidant markers in the brain tissues of I/R rats.

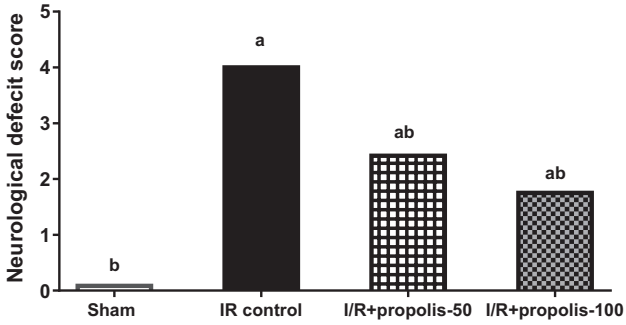

Fig. 2. Neurological deficit scores, 24 h after MCAO surgery. [a]$p \leq 0.05$ vs. the sham group. [b]$p \leq 0.05$ vs. the I/R control group.

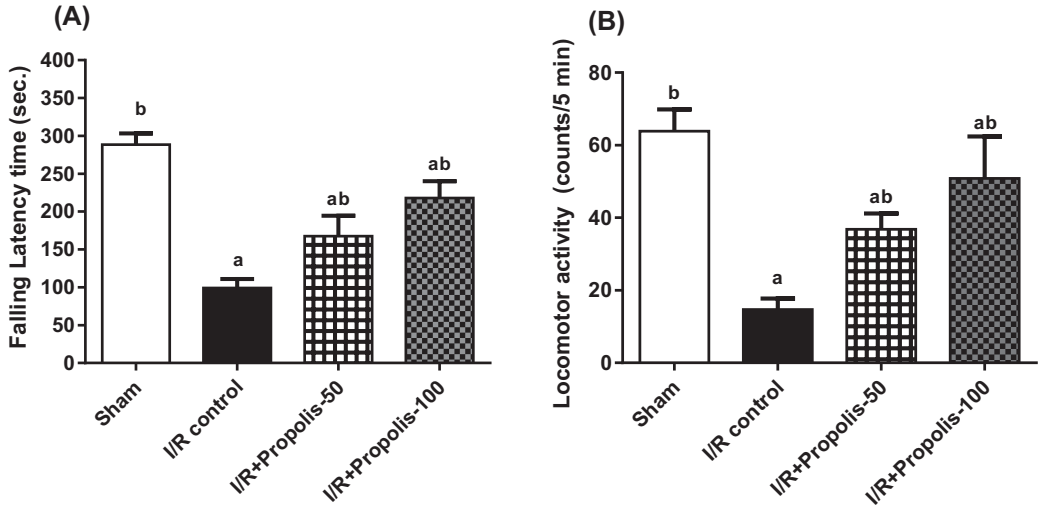

Fig. 3. Effect of propolis on (A) motor coordination (rotarod) and (B) locomotor activity (activity cage) in I/R rats.

The data in Table 1 indicated that cerebral I/R resulted in a severe LPO in brain tissues of I/R control rats as confirmed by significant elevation in the brain content of MDA compared with sham group. Medication of I/R rats with both doses of propolis resulted in a significant decrease in the brain contents of MDA compared to I/R control rats ($p \leq 0.05$). The high level of MDA in the brain tissue of I/R rats being restored back to normal level only in the group receiving the higher dose level.

3.4. Effect of propolis on brain BDNF and dopamine levels

Fig. 4 showed that brain contents of BDNF and dopamine were decreased in the I/R control group to be 58.6% and 53.4% of sham rats, respectively. These values were significantly corrected by propolis intervention at dose levels of 50 and 100 mg/kg in varying degrees. The higher dose of propolis showed a better improvement in brain tissue BDNF (66.2%) and dopamine (71.1%) compared with the I/R control rats.

3.5. Histopathology of the brain

The sham group showed normal histological picture of cerebral cortex (Fig. 5-A), and hippocampus (Fig. 5-E). The grey matter of the cerebral cortex of I/R control group revealed areas of coagulative necrosis with inflammatory cell infiltration, massive number of degenerated and necrosed neurons with neuronophagia, multiple focal areas of gliosis, astrogliosis and microgliosis (Fig. 5-B). Whilst the cerebral cortex grey matter showed axonal fragmentation and demyelination of nerve fibers. The hippocampus of the same group showed massive number of degenerated pyramidal cells (Fig. 5-F). All previous described lesions were minimal in I/R + proposal-50 and I/R + proposal-100 groups as shown in (Fig. 5-C, D, G, H).

Fig. 6 summarized the percentage of infarct area in the different groups. The groups treated with propolis (50 and 100 mg/kg) revealed significant reduction in the percentage of the infarct area comparing with I/R control animals.

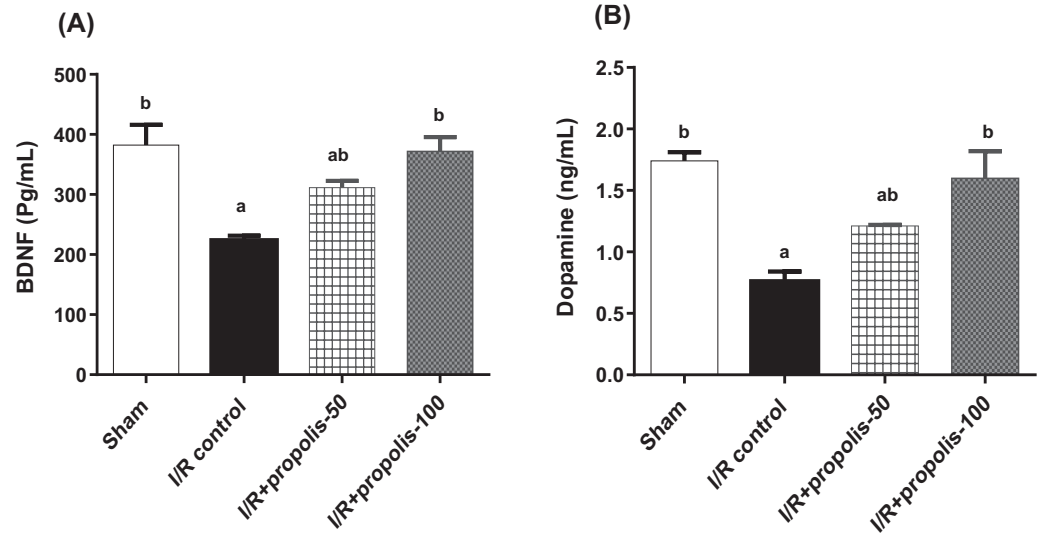

Fig. 4. Effect of propolis on brain contents of BDNF (A) and dopamine (B) in I/R rats.

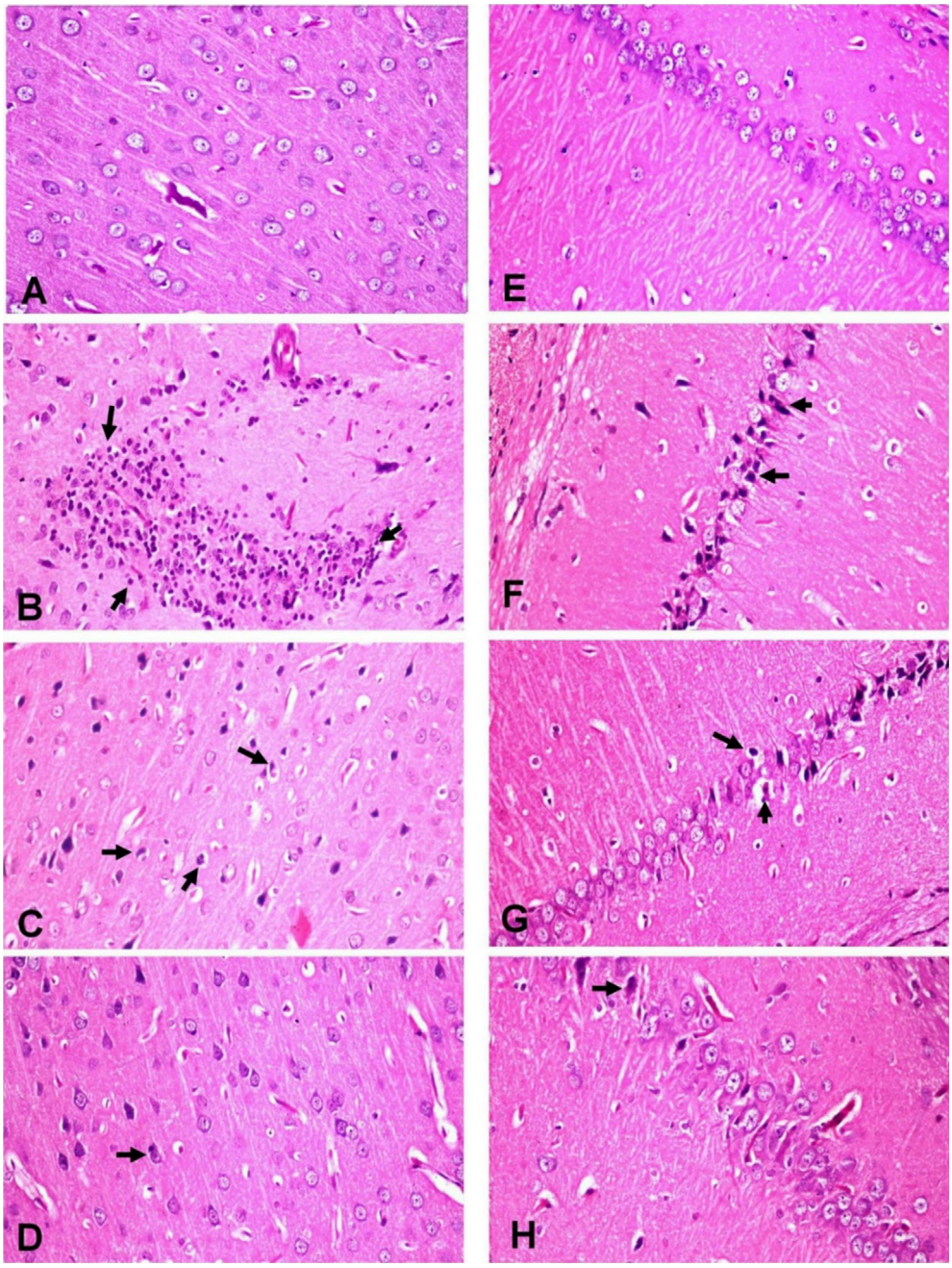

Fig. 5. The histopathological picture of brain cortex (A–D) and hippocampus (E–H) CA1 region in different groups. A: Sham group showing normal histologic picture of brain cortex (H&E X400). B: I/R control group showing large area of gliosis (arrow) with marked neuronal degeneration (H&E X400). C: I/R + propolis-50 group showing moderate number of degenerated and necrosed neurons (arrow) (H&E X400). D: I/R + propolis-100 group showing slight number of degenerated and necrosed neurons (arrow) (H&E X400).

E: Sham group showing normal histologic findings of brain hippocampus (H&E X400). F: I/R control group showing massive number of pyramidal cell degeneration and necrosis (arrow) (H&E X400). G: I/R + propolis-50 group showing moderate number of degenerated and necrosed pyramidal cells (arrow) (H&E X400). H: I/R + propolis-100 group showing few numbers of degenerated and necrosed pyramidal cells (arrow) (H&E X400).

3.6. The neuronal cell count

The cerebral cortex and hippocampus of the sham rats showed intact neurons with vesicular nucleus (Figs. 7-A & 8-A, respectively). While the I/R control rats revealed massive number of darkly stainedand shrunken cells in both cerebral cortex and hippocampus regions (Figs. 7-B & 8-B, respectively). The I/R + propolis-50 (Figs. 7-C & 8-C, respectively) and I/R + propolis-100 (Figs. 7-D & 8-D, respectively) revealed significant elevation in the percentage of intact neuronal cells comparing to I/R control rats.

3.7. Effect on TGF-β1 gene expression

Brain tissue TGF-β1 levels were significantly elevated in I/R control rats (1.7 folds) in comparison with the sham animals (Fig. 9-A). This result indicates that activation of TGF-β1 signaling were associated with cerebral I/R injury. Propolis at both dose levels could

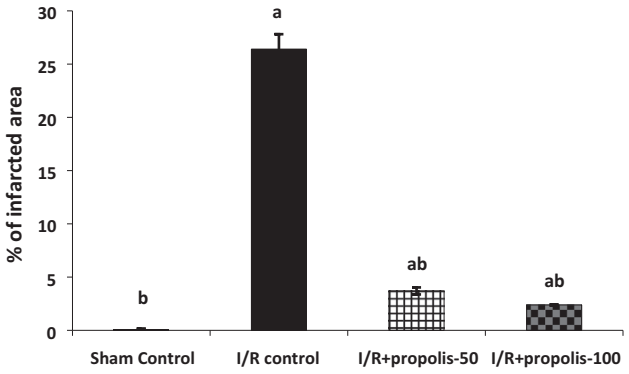

Fig. 6. The percentage of the infarct area represented as ratio of the infarct area to the whole slide area. Values are expressed as mean ± SEM; $^{a}P \leq 0.05$ versus the sham group, $^{b}P \leq 0.05$ versus I/R control group.

accelerate the TGF-β1 expression in brain tissue of I/R rats in comparison with I/R control animals. Again, higher dose level of propolis showed better improvement (5 folds of that in the sham-operation group).

3.8. MMP9 gene expression

MMP9 mRNA expression showed a significant increase in I/R control rats as compared with the sham animals ($p \leq 0.05$; Fig. 9-B). MMP-9 mRNA expression levels in the I/R + propolis groups were reduced as compared to I/R control animals ($p \leq 0.05$; Fig. 9-B).

4. Discussion

The brain is highly sensitive to ischemic insults. Cerebral ischemia is a result of scanty blood flow to an area of the brain. In spite of the variety of the available therapeutic options, high prevalence rates of stroke are still recorded due to lower efficacy of neuroprotective drugs. Thereby new alternative therapeutic strategies are urgently required to reduce the incidence and to treat the aftermath of stroke as well. Recently, TGF-β1 has been reported as a neuroprotective target that is mostly pointed toward the modulation of the endogenous pathways involved in ischemic processes (Zhu et al., 2017; Yu et al., 2018). This study aim to investigate the possible role of TGF-β1 in the mechanism of propolis neuroprotection in rats with cerebral I/R injury.

In the current study, MCAO as a cerebral I/R model in rats resulted in neurological deficit, decreased locomotor activity in the activity cage test and motor incoordination in rotarod test. Consistent with previous reports, the current study demonstrated that neurological deficit score in control animals were significantly elevated compared to sham group (Ishrat et al., 2009). Treatment of I/R rats with propolis improved the neurologic performance and motor coordination and increased the final locomotor activity as compared to I/R control group. These results confirmed the neuroprotective effects of propolis in the rat model of cerebral I/R injury.

It is well known that oxidative stress induced as a result of activation of reactive oxygen species (ROS) is a crucial pathological constituent of cerebral ischemic damage (Moro et al., 2005). Additionally, the resultant oxidative stress and overproduction of ROS during I/R event has a major role for the degree of brain damage (Irmak et al., 2003). Various enzymatic antioxidant molecules such as SOD, GPx and CAT, nonenzymatic antioxidants such as GSH and LPO products such as MDA are usually being used as possible oxidative stress biomarkers. In oxidative stress, GSH is converted to glutathione disulfide and depleted leading to LPO. Therefore, the role of GSH as a sensible marker for the evaluation of oxidative stress is important (Eldesoky et al., 2018).

In case of cerebral ischemia, estimation of oxidative stress biomarkers is very important for a better understanding of the mechanisms of neuroprotective agents (Xiong et al., 2016). Our results supported the existence of oxidative stress and the subsequent reduction in SOD, GPx, CAT and GSH levels and increase in LPO in ischemic rats' brain tissues following I/R, indicating an over induction of oxidative damage to brain. In this investigation, we confirmed the anti-oxidant activity of propolis in cerebral I/R injured rats. The results revealed that propolis antagonized the cerebral I/R induced decrease in brain SOD, GPx, CAT and GSH contents and increase in brain MDA levels. Various investigations have proved that alcoholic and aqueous extracts of propolis have antioxidant effects and protect the neurons against oxidative damage by reducing the generation of free radicals and LPO (Ramadan et al., 2015). In addition, the results of Bazmandegan et al., (2017) indicated that Iranian propolis confers neuroprotection on the stroke-induced neuronal damage via an antioxidant mechanism. A previous report by Liu et al. (2008) indicated that one of the propolis constituents; pinocembrin has the potential to reduce ischemic injury in MCAO rats. They attributed the reductions in injury to the antioxidative and antiapoptotic effects of pinocembrin.

Brain-derived neurotrophic factor (BDNF) is one of the neurotrophins that exerts a major role in neuronal maturation and endurance, and synaptic functions through extracellular signal-regulated kinase (ERK), intracellular signaling pathways including phosphoinositide 3-kinase/Akt (PI3K/ Akt), and phospholipase C-γ (PLC-γ) pathways (Adachi et al., 2018). BDNF has received much attention as a target of many brain disorders. Decrement of BDNF levels is associated with various neurological disorders (Varendi et al., 2015). In this study, a significant reduction of BDNF level after MCAO was noticed in the brain of the I/R control rats. Treatment of I/R rats with propolis significantly increased BDNF levels in their brains in comparison with I/R control rats. Some *in vitro* data proposed that BDNF plays an essential role in recovery from ischemia (Heurteaux et al., 2010). In addition, previous reports indicated that BDNF could protect the cells against injury via its antioxidant properties (Heaton et al., 2011). Moreover, various reports indicated that the antioxidant property of BDNF might be due to its capability to scavenge free radicals' ions or modulate antioxidant enzymes (Wu et al., 2016).

Dopaminergic system disturbance has also been involved in many types of neurological disorders (Adachi et al., 2018). Dopamine plays fundamental roles in the nervous system, controlling motor and cognitive functions through activating some types of a G protein-coupled receptor family (Tritsch and Sabatini, 2012). Our findings showed that propolis significantly elevated dopamine levels in brain tissue compared to I/R control rats. These results are in agreement with those of Safari et al. (2015), who reported that re-intake of propolis markedly improved dopamine and dopaminergic neurons in the striatum of a rat model of Parkinson's disease. Propolis also restored the changes in locomotors and rotary activity, suggesting that neuroprotective effect of propolis may be due to an increase in dopamine metabolism in the striata.

It is well established that MCAO as a cerebral I/R model in rats resulted in formation of cerebral infarct area (El-Marasy et al., 2018) as shown in ischemic model. The percentage of cerebral infarcted area was dramatically reduced in the I/R rats treated with propolis (50 and 100 mg/kg) which confirmed the neuroprotective effect of propolis. In our study, MCAO initiated severe neuronal cell loss and histopathological lesions in both cerebral cortex and hippocampus regions in I/R control group. These findings come in harmony with previous studies (El-Marasy et al., 2018). Toluidine blue

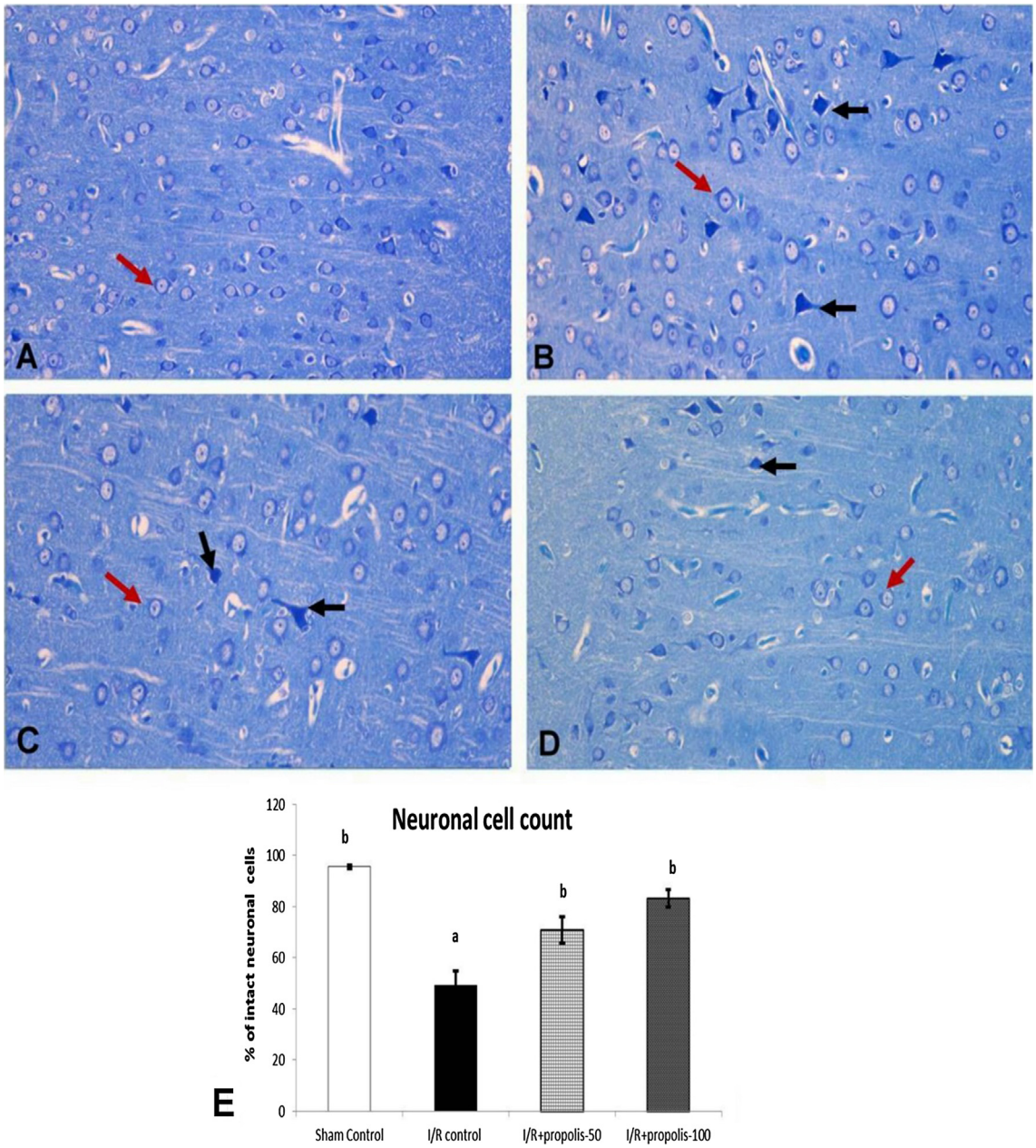

Fig. 7. Histological sections of the cerebral cortex in the different groups (Toluidine blue stain, X 400). Intact cell (red arrow), degenerated cell (black arrow). A: Sham group. B: I/R control group. C: I/R + propolis-50 group. D: I/R + propolis-100 group. E: The bar chart represents the percentage of intact neuronal cells in cerebral cortex. All values are expressed as mean ± SE (n = 6); $^{a}P \leq 0.05$ versus the sham group, $^{b}P \leq 0.05$ versus I/R control group.

stain is a well identified histopathological technique for detection of neuronal cell damage in brain (Kirino et al., 1984). Pretreatment with propolis (50 and 100 mg/kg) caused marked reduction in the histopathological changes and the neuronal cell loss in brain cortex and hippocampus suggesting that propolis can reduce the brain damage caused by stroke.

TGF-βs superfamily is a large family of pleiotropic cytokines that can control cell growth, proliferation and apoptosis. TGF-βs were shown to be neuroprotective in the MCAO model of stroke (Pál et al., 2014). Among the TGF-β family members, TGF-β1 is a cytokine that is generated in the brain in response to ischemia. TGF-β1 is a master regulator of glial activation and scar formation after brain ischemia (Luo et al., 2019). In addition, TGF-β1 also has been shown to protect hippocampal neurons from injuries, including neurotoxins, as well as excitotoxic and hypoxia/ischemia injury (Cho et al., 2012). In this investigation, we found that the expression of TGF-β1 was up-regulated in ischemic cerebral tissues of I/R control rats than that in the sham group. Similar observations have been made in MCAO rats, an animal model of stroke (Popp et al., 2009). Abundant evidence also demonstrates elevated expression of TGF-β1 in animal models of cerebral ischemia (Vincze et al., 2010). Increased level of TGF-β1 in brain tissue was also found in patients with ischemic stroke (Krupinski et al., 1996). The increased TGF-β1 expression in cerebral I/R damage may be resulted from the hypoxia-induced stress of the CNS (Wang et al., 2016). Our findings revealed that administration of propolis at doses of 50 and 100 mg/kg to I/R rats elevated the expressions of TGF-β1 compared with I/R control group. This indicates that propolis treatment could further upregulates the expression of TGF-β1 in brain tissue, playing a protective role in ischemic

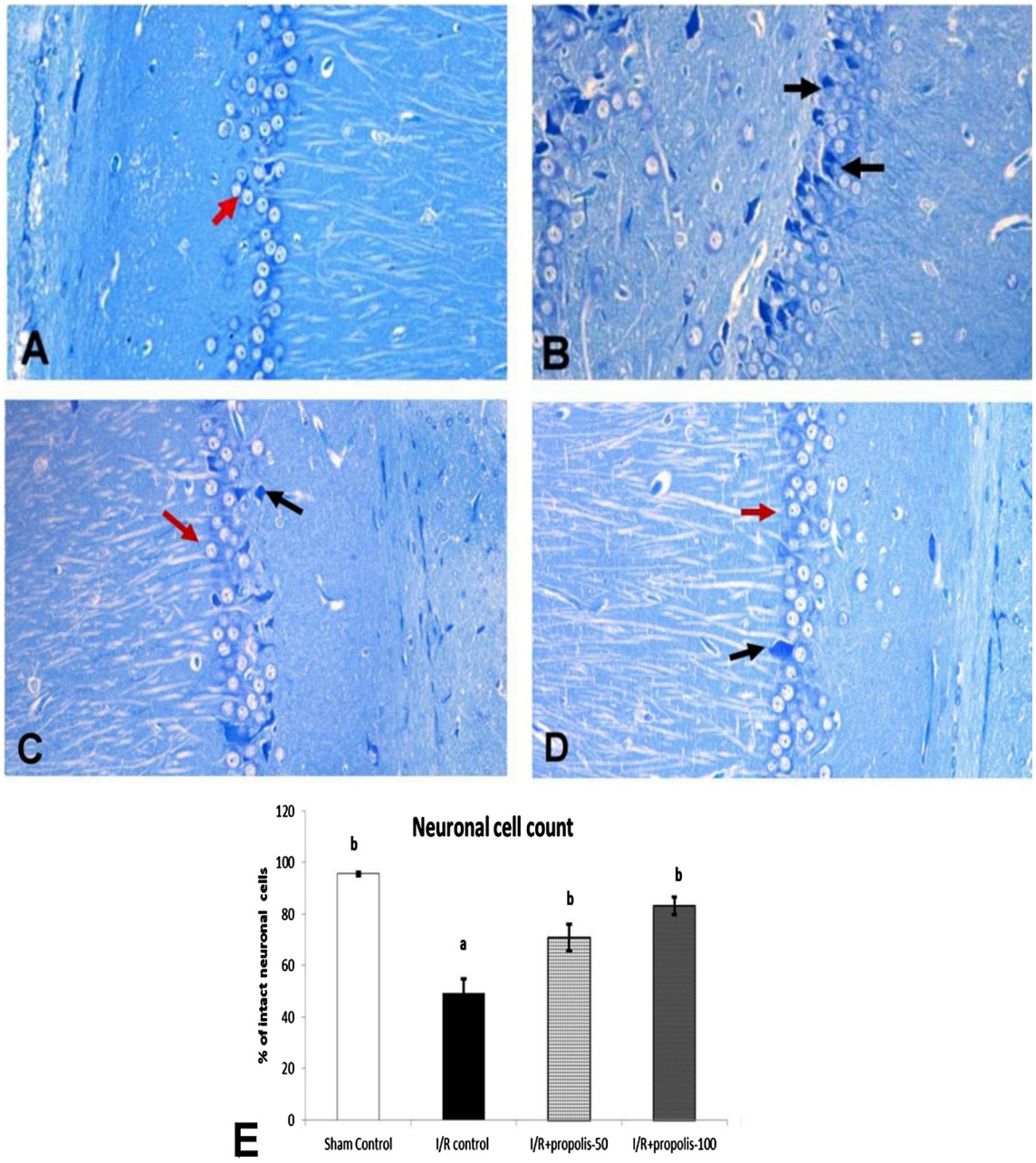

Fig. 8. Histological sections of the hippocampus in the different groups (toluidine blue stain, X 400). Intact cell (red arrow), degenerated cell (black arrow). A: Sham group. B: I/R control group. C: I/R + propolis-50 group. D: I/R + propolis-100 group. E: The bar chart represents the percentage of intact neuronal cells in the hippocampus. All values are expressed as mean ± SE (n = 6); $^{a}P \leq 0.05$ versus the sham group, $^{b}P \leq 0.05$ versus I/R control group.

stroke. These results are in agreement with those reported by Dobolyi et al., (2012) who mentioned that TGF-β1 had a marked role in protecting hippocampal neurons against ischemia. Overall, the present results elucidate that propolis shows neuroprotective effect in case of ischemic brain injuries. According to the present results, the elevated expression of TGF-β1 in cerebral tissues following cerebral ischemia is among the various possibilities of the neurotrophic effect of propolis.

MMP9 (gelatinase B) is a member of MMPs which is a family of calcium- and zinc-dependent proteolytic enzymes responsible for extracellular matrix remodeling (Murphy and Nagase, 2008). Several studies reported that MMP-9 is up-regulated at both gene and protein levels after cerebral I/R injuries (Xu et al., 2015). Excessive MMP9 expression has been shown to play a deleterious role in brain injuries and positively associated with blood brain barrier disruption after ischemic injuries (Brouns et al., 2011). In line with previous studies (Xu et al., 2015), a significantly increased level of MMP9 mRNA was noted in I/R control animals compared to sham-operation group. With one week of propolis pre-treatment and 3 days post-treatment, the elevated expression of MMP9 was markedly downregulated, indicating that the protective effect of propolis by promoting tissue repair and extracellular matrix reconstruction. These results come in accordance with da Silveira et al. (2016) who reported that some components of propolis such as caffeic acid phenyl ester possess a significant inhibition of MMP9 gene expression and activity. Suppression of MMP9 expression

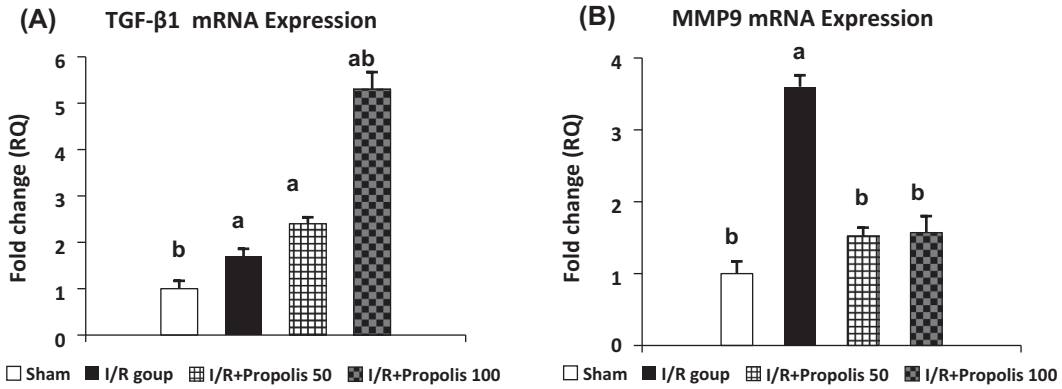

Fig. 9. mRNA expression levels of TGF-β1 (A) and MMP-9 (B) in the I/R rat brain of the different experimental groups determined by real time RT-PCR with b-actin used as an internal control gene. All values are expressed as mean ± SE (n = 06); $^{a}P \leq 0.05$ versus the sham group, $^{b}P \leq 0.05$ versus I/R control group.

could be attributed to its inhibition by TGF-β1 indicating a cross-talk between TGF-β1 and MMP9 in the pathogenesis of I/R brain lesions (Ronaldson et al., 2009).

5. Conclusion

In conclusion, this study indicates that propolis exhibits neuroprotective effect against cerebral I/R injury in rats, as shown by the increase in the brain levels of BDNF and dopamine and the improvement in histopathological parameters. The neuroprotective effect of propolis is likely to be mediated through its antioxidant effect and its capability to upregulate TGF-β1. The results proposed that propolis may represent a new prototype agent of potential neuroprotective agents in the treatment of cerebral I/R damages.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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