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Investigation of the protective effect of anzer propolis in cerebral ischemia-reperfusion injury

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Abstract. – OBJECTIVE: Globally, stroke is the leading cause of disability and death. With the use of thrombolytic therapy, reperfusion injury, and its consequences came to the fore. We aimed to find out how anzer propolis, which can only be obtained in Turkey's Eastern Black Sea region, affected ischemia-reperfusion injury using biochemical and histological techniques.

MATERIALS AND METHODS: **32 female Wistar albino rats were divided into 4 groups, including a control group. Three of the groups underwent 30 minutes of induced ischemia via clamping of the common carotid artery, followed by ischemia-reperfusion injury through the release of the clamp. One group received no treatment, another received oral administration of 100 mg/kg of anzer propolis one hour before surgery, and the third group received oral administration of 40 mg/kg of acetylsalicylic acid just before surgery. Histopathological examination assessed apoptosis and tissue necrosis, while serum and brain tissue were evaluated for levels of nerve growth factor (NGF), Interlokin 1β (IL-1β), Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), total antioxidant capacity (TAS), and total oxidant capacity (TOS).**

RESULTS: **Anzer propolis and acetylsalicylic acid significantly reduced hyperemia in vessels, vacuolization in neurons, glial cell infiltration, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positivity. The anzer propolis group had the highest NGF levels. The anzer propolis and acetylsalicylic acid groups had lower levels of TNF-a and IL-6 in the brain tissue than the ischemia-reperfusion group, while TAS levels were higher.**

CONCLUSIONS: **The findings obtained in this study suggest that anzer propolis has a neuroprotective effect against ischemia-reperfusion injury and will have beneficial effects on neurodegeneration. We believe our findings will contribute to the clinical treatment of ischemia-reperfusion injury.**

Key Words:
Stroke, Ischemia-reperfusion injury, Anzer propolis.

Introduction

The World Health Organization (WHO)[1] defines stroke as clinical manifestations of acute focal damage to the central nervous system (CNS), caused by a vascular event, which is a significant contributor to disability and mortality worldwide. According to the most recent assessments from the Global Burden of Disease (GBD)[2] of 2019, stroke is the second biggest cause of death and the third biggest cause of "disability-adjusted life years" losses.

It has been clinically proven[3] that vascular recanalization restores blood supply to tissue, and reperfusion can cause brain tissue damage. This process has been named ischemia-reperfusion (IR) injury. With the use of thrombolytic therapy in stroke patients, reperfusion injury and its consequences came to the fore. Despite the restoration of blood flow to ischemic tissue, the functional outcomes were not as significant as anticipated[4]. Patients receiving thrombolytic therapy or endovascular recanalization should exercise caution against ischemia-reperfusion injury. Reperfusion reoxygenation of tissue results in excessive formation of free radicals[5]. Antioxidants have not been sufficiently researched for their effects on the ischemia-reperfusion injury that could develop in brain tissue following revascularization.

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Propolis is a natural substance obtained from bees that has gained popularity recently. It has a high concentration of antioxidants[6]. In the treatment of cardiovascular disease, diabetes, and cancer, its anti-neurotoxic, antiviral, antibacterial, anticancer, and antioxidant activities have been demonstrated[7-9]. Anzer propolis has been shown[10] to reduce Interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and myeloperoxidase (MPO) levels, and to have antioxidant effects as measured by total antioxidant capacity (TAS) and total oxidant capacity (TOS) activities, and to inhibit cell apoptosis in a model of spinal ischemia-reperfusion injury. The purpose of this study was to investigate the effects of anzer propolis, which is native to the Eastern Black Sea region of Turkey, on ischemia-reperfusion injury in an ischemic stroke model.

Materials and Methods

Animal Group and Experimental Model

The study utilized 32 randomly selected adult female Wistar Albino rats weighing 250-300 grams. Prior to the experiment, rats were housed in separate polypropylene cages for seven days with *ad libitum* access to food and water with a natural day-night cycle.

Rats were randomly divided into 4 (n=8) groups; control (C) group, ischemia-reperfusion (IR) group, ischemia-reperfusion with anzer propolis (IR+AP) group, and ischemia-reperfusion with acetylsalicylic acid (IR+AA) group.

In order to generate an experimental model of an ischemic stroke in patients from the IR, IR+AP, and IR+AA groups, the common carotid artery was clamped, and 30 minutes of ischemia were induced. Then, the clamp was released, and ischemia-reperfusion injury was induced. The group assigned as the control group did not undergo any type of treatment or intervention. One hour prior to surgery, the IR+AP group received 100 mg/kg of anzer propolis dissolved in ethanol orally, and the IR+AA group received acetylsalicylic acid 40 mg/kg orally. For clamping, 50-g closing pressure Bulldog clamps were utilized. After the approximately two-hour period of anesthetic recovery following surgery, subjects were permitted to eat normally. At the 48th hour of the sacrification, blood samples were collected. Additionally, after the sacrification process, tissue samples were taken.

All animal manipulations were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals published by the United States National Institutes of Health. All animal experiments were performed and reported according to the Animals in Research: Reporting *In Vivo* Experiments (ARRIVE) guidelines.

Anesthesia and Monitoring

Standard anesthesia with intramuscular administration of ketamine HCl (87 mg/kg) and xylazine HCl (13 mg/kg) was performed on all rats. The doses of ketamine (25 mg/kg) and xylazine (5 mg/kg) were repeated in combination as necessary. During the anesthesia, the individuals' spontaneous breathing without the need for respiratory support was monitored, and vital values were recorded.

Surgical Technique

Prior to surgery, the rats' necks were shaved. After anesthesia, subjects were placed supine on the operating table under sterile conditions. Before and after the procedure, the surgical region was cleaned with 10% povidone-iodine (Batticon®; Adeka, Samsun, Turkey) and then wiped with sterile gauze. After staining, the surgical area was covered with a sterile, perforated green sheet. 3.2x-500 loupe (Carl Zeiss 3.2x-500 Loupe, GmbH, Oberkochen, Germany) was utilized in the surgical procedures. A midline incision was made in the neck. Deep dissection was used to reach the common carotid artery after superficial microdissection. The common carotid artery was dissected and clamped, and 30 minutes of ischemia were induced. After removing the clamp, an ischemia-reperfusion injury was induced. Following management of the hemorrhage, the neck incision was closed according to the method with 3/0 prolene sutures.

Sacrification

The sacrification procedure was carried out on all groups 48 hours after surgery. Blood was extracted from the left ventricles of the rats under general anesthesia (87 mg/kg ketamine hydrochloride and 13 mg/kg xylazine hydrochloride) prior to the sacrification procedure. The subjects were euthanized with a 150 mg/kg dose of intravenous thiopental.

Postoperative Neurological Evaluation

On a 5-point scale, the neurologic deficit assessment at 6, 24, and 48 hours after reperfusion was evaluated by a researcher who was unaware of the treatment administered[11]. 5-point scale: 0:

No neurologic deficit, 1: Failure to extend the contralateral forepaw fully; 2: Circling to the contralateral side, 3: Falling or leaning to the opposite side, 4: No spontaneous walking and a depressed level of consciousness.

Histopathological Examination

The brain tissue samples were collected and fixed in 10% formaldehyde and glutaraldehyde solutions. Histopathological examination was conducted to evaluate apoptosis and tissue necrosis. For the histopathological examination, the brain tissue samples were stained with hematoxylin-eosin (HE) and analyzed under a binocular light microscope (Nikon, Eclipse Ci, Tokyo, Honshu, Japan) in 10% buffered formalin solution. Microscopic images of the relevant samples were captured using a digital camera (Nikon DS F1, microscopic digital camera systems, Tokyo, Honshu, Japan). Hyperemia in vessels, vacuolization in neurons, areas of glial cell infiltration, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) positivity were evaluated histopathologically in all groups. The results were grouped as "no", "weak", "moderate" and "severe".

Biochemical Examination

Venous blood was collected at 48th hours postoperatively. Blood gas analysis and hemograms were made in the blood taken, and the samples from which the serum was removed were stored at -80°C. Serum Nerve growth factor (NGF) (Bioassay Technology Laboratory, catalog No.: E0539Ra, Shanghai, China), Interleukin 1β (IL-1β) (Andy Gene catalog number: AD3022Ra, Beijing, China), IL-6 (Andy Gene, catalog No.: AD3249Ra, Beijing, China), TNF-α (Sunred) Biological Technology, catalog no: 201-11-0765, Shanghai, China), TAS (Bioassay Technology Laboratory, catalog No.: E1710Ra, Shanghai, China), TOS (Bioassay Technology Laboratory, catalog No.: E1512Ra, Shanghai, China) measurements were performed by VGt Lambda Scan 200 (Bio-Tech Instrument, Winooski, VT, USA) ELISA device. In addition, after weighing the brain tissue samples, they were homogenized in 10 times phosphate buffer (pH 7.4; 1/10 g/ml), and crude extracts (homogenate) were obtained. Supernatants were obtained by centrifuging at 15,000 rpm for 15 minutes in a homogenate-cooled centrifuge. The obtained materials were stored in aluminum foils at -80 degrees. NGF, IL-1β, IL-6, TNF-α, TAS, TOS measurements in brain tissue were performed by ELISA method in homogenized samples.

Statistical Analysis

The obtained data were analyzed with Statistical Package for the Social Sciences (SPSS) version 25.0 (IBM Corp., Armonk, NY, USA). The normal distribution of numerical variables was examined using the Shapiro-Wilk Test. One-Way Analysis of Variance was used to compare numerical variables suitable for normal distribution, and Duncan's test was used for post-hoc analysis. The relationship between categorical variables was investigated by Chi-Square Analysis. Statistical significance was set at $p<0.05$.

Results

One rat each from the IR, IR-AP, and IR-AA groups died of anesthesia during the surgery; these rats were dropped from the study. Since one rat from all groups died, the control group was also taken as 7 rats. In the comparison of histopathological data and examinations, control groups were excluded from the analysis because they showed completely normal findings. The control group was included in the comparison of biochemical analyses.

Histopathological Findings

Hyperemia in vessels, vacuolization in neurons, areas of glial cell infiltration, and TUNEL positivity were evaluated histopathologically in all groups, and the results are summarized in Table I. Hyperemia in vessels was moderate in 1 (14.3%) rat, severe in 6 (85.7%) rats in the IR group. In the IR+AA group, it was weak in 2 (28.6%) rats, moderate in 4 (57.1%) rats, and severe in 1 (14.3) rat. In the IR+AP group, it was weak in 3 (42.9%) rats and moderate in 4 (57.1%) rats. There was a statistically significant difference between the groups ($p=0.008$). Vacuolization of neurons was moderate in 3 (42.9%) rats, and severe in 4 (57.1%) rats in the IR group. In the IR+AA group, it was weak in 2 (28.6%) rats, moderate in 4 (57.1%) rats, and severe in 1 (14.3) rat. In the IR+AP group, it was weak in 2 (28.6%) rats, moderate in 4 (57.1%) rats, and severe in 1 (14.3) rat. A statistically significant difference was observed between the groups ($p<0.001$). Glial cell infiltration was moderate in 3 (42.9%) rats, and severe in 4 (57.1%) rats in the IR group. In the IR+AA group, moderate severity was ob-

Table I. Comparison of histopathological parameters according to groups (n=7).

	IR	IR+AA	IR+AP	*p*-value*
Hyperemia in vessels				
Weak	0	2 (28.6%)	3 (42.9%)	
Moderate	1 (14.3%)	4 (57.1%)	4 (57.1%)	0.008
Severe	6 (85.7%)	1 (14.3)	0	
Vacuolization in neurons				
Weak	0	2 (28.6%)	2 (28.6%)	
Moderate	3 (42.9%)	4 (57.1%)	4 (57.1%)	<0.001
Severe	4 (57.1%)	1 (14.3%)	1 (14.3%)	
Glial cell infiltration				
None	0	5 (71.4%)	0	
Weak	0	1 (14.3%)	3 (42.9%)	<0.001
Moderate	3 (42.9%)	1 (14.3%)	3 (42.9%)	
Severe	4 (57.1%)	0	1 (14.3%)	
Tunel positive cells				
Weak	0	4 (57.1%)	2 (28.6%)	
Moderate	0	3 (42.9)	5 (71.4)	<0.001
Severe	7 (100%)	0	0	

*Chi-square test. IR: Ischemia reperfusion, IR+AA: Ischemia reperfusion+Acetylsalicylic acid, IR+AP: Ischemia-reperfusion+anzer propolis.

served in 1 (14.3%) rat and severe in 1 (14.3) rat. No glial cell infiltration was observed in 5 rats in the IR+AA group. Glial cell infiltration was weak in 3 (42.9%) rats, moderate in 3 (42.9%) rats, and severe in 1 (14.3) rat in the IR+AP group. A statistically significant difference was observed between the groups ($p<0.001$). TUNNEL positivity was detected in all 7 (100%) rats in the IR group. In the IR+AA group, 4 (57.1%) rats had weak, and 3 rats had moderate TUNEL positivity. In the IR+AP group, 2 (28.6%) rats had weak, and 5 (71.4) rats had moderate TUNEL positivity. A statistically significant difference was observed between the groups ($p<0.001$). The histopathological findings are shown in Figures 1-2.

Biochemical Findings

The levels of NGF, TNF-α, IL-6, IL-1, TAS, and TOS in rat serum and brain tissues are shown in Table II. Significant differences in NGF, TNF-α, and TAS levels were found between groups in serum analysis ($p=0.001$, $p=0.019$, and $p=0.018$, respectively). Other parameters revealed no difference between the groups' serum analyses. Significant differences in NGF, TNF-α, IL-6, and TAS levels were found in brain tissue analysis ($p=0.034$, $p=0.035$, $p=0.001$, and $p=0.031$, respectively). In other parameters, no difference was observed in the brain tissue analyses of the groups. The IR+AP group had significantly higher serum NGF levels than the other groups ($p=0.001$). Similarly, NGF levels in brain tissue were found to be significantly higher in the IR+AP group than in the other groups ($p=0.034$). Serum and tissue TNF-α levels were found to be similar in the IR+AA and IR+AP groups, higher than in the control group, and lower than in the IR group. This difference between the groups was found to be statistically significant ($p=0.019$, $p=0.035$, respectively). IL-6 levels in the brain tissue were 45.69±3.12 in the control group, 56.77±7.42 in the IR group, 31.49±1.72 in the IR+AP group, and 40.94±4.43 in the IR+AA group. The difference between the groups was statistically significant ($p<0.001$). TAS levels in brain tissue were found to be statistically significantly higher in the IR+AA and IR+AP groups than in the control and IR groups ($p=0.031$).

Neurological Evaluation Results

A statistically significant difference was found between the IR group, IR+AA, and IR+AP groups in the postoperative 6th-hour, 24th-hour, and 48th-hour neurological examination findings ($p=0.006$, $p=0.012$, $p=0.002$, respectively). One rat in the IR group, 2 rats in the IR-AA group, and 2 rats in the IR-AP group had seizures during postoperative follow-up. There was no statistically significant difference between the groups ($p=0.769$) (Table III).

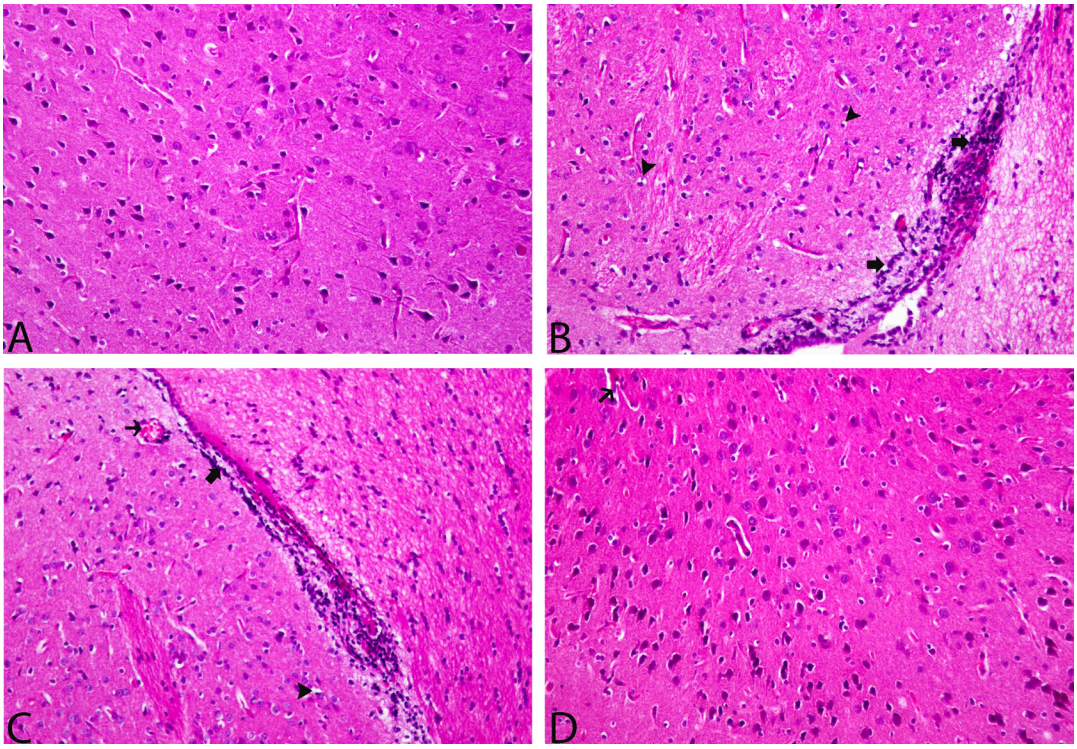

Figure 1. Histopathological findings. **A**, Control. **B**, IR thick arrow: areas of glial cell infiltration. Thin arrow: hyperemia in vessels. Arrowhead: vacuolization formation in neurons. **C**, IR+AP thick arrow: areas of glial cell infiltration. Thin arrow: hyperemia in vessels. Arrowhead: vacuolization formation in neurons. **D**, IR+AA thin arrow: in vessels hyperemia (20×-scale bar 100 μm).

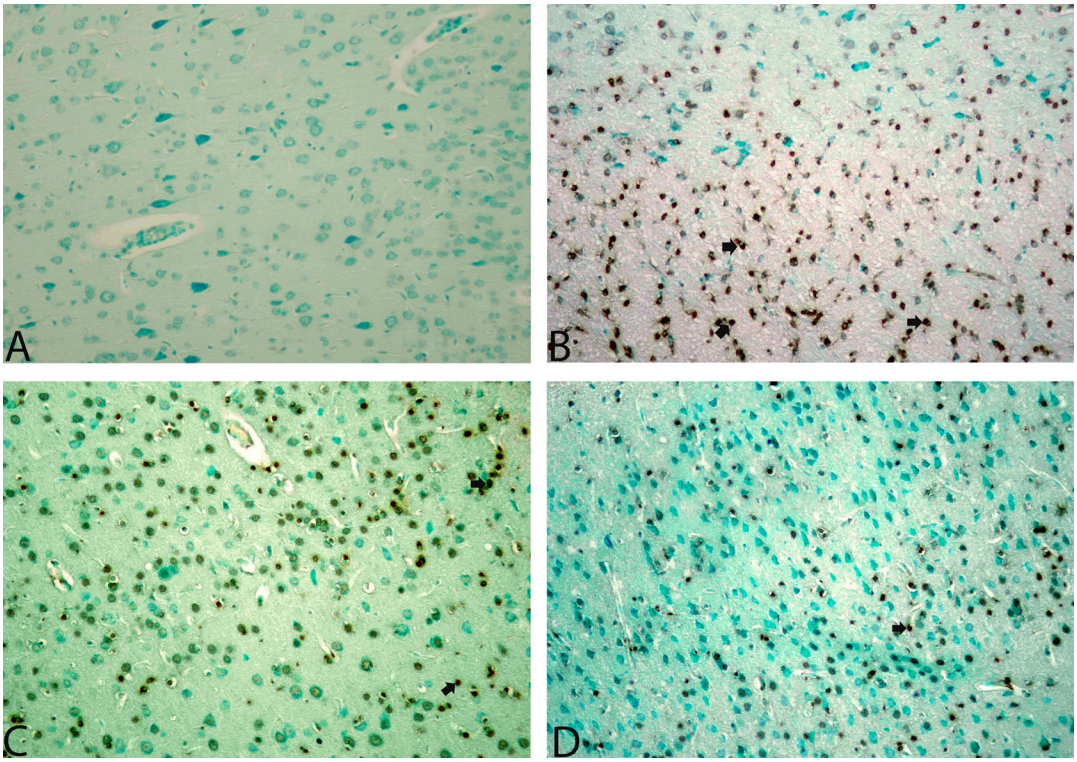

Figure 2. Histopathological findings (tunel). **A**, Control. **B**, IR thick OK: tunnel positive cells. **C**, IR+AP thick OK: tunnel positive cells. **D**, IR+AA thick OK: tunnel positive cells (20×-scale bar 100 μm).

Table II. Comparison of biochemical parameters according to groups (n=7).

	Control	IR	IR-AA	IR-AP	p*
NGF Tissue (ng/L)	730.12±30.22[b]	716.18±34.47[b]	757.38±61.30[b]	912.80±64.27[a]	**0.034**
NGF Serum (ng/L)	337.12±31.56[b]	377.20±41.82[b]	436.18±36.76[b]	575.42±42.83[a]	**<0.001**
TNF- α Tissue (ng/L)	87.64±3.47[a]	99.91±6.17[b]	91.85±1.37[a]	88.09±4.55[a]	**0.035**
TNF-α Serum (ng/L)	67.77±3.23[b]	83.78±2.03[a]	76.71±4.41a[b]	76.00±3.27[ab]	**0.019**
IL-6 Tissue (ng/L)	42.44±2.00[b]	57.34±4.38[a]	40.94±4.43[bc]	31.49±1.72[c]	**<0.001**
IL-6 Serum (ng/L)	45.9±3.12	56.77±7.42	40.95±1.84	49.86±1.99	0.083
TAS Tissue (U/ml)	6.40±0.58[a]	6.55±0.41[a]	7.06±0.67[ab]	7.94±0.47[b]	**0.031**
TAS Serum (U/ml)	6.04±0.41[b]	6.13±0.28[b]	5.85±0.23[b]	7.51±0.48[a]	**0.018**
TOS Tissue (U/ml)	8.445±0.44	7.95±0.54	8.76±0.24	7.73±0.56	0.426
TOS Serum (U/ml)	6.273±0.16	7.08±0.41	5.90±0.40	6.20±0.36	0.126
IL-1β Tissue (ng/L)	12.22±0.64	12.60±0.89	13.84±1.15	11.48±1.26	0.441
IL-1β Serum (ng/L)	15.03±1.06	11.07±0.73	13.64±2.37	13.27±0.95	0.273

*One-way analysis of variance, [a-c]: There is no difference between groups with the same letter for each line. Data are expressed as mean±sd. IL-1β=Interleukin 1β; IL-6=Interleukin-6; IR= Ischemia reperfusion; IR+AA: Iskemi reperfüzyon+Asetilsalisilik Asit; IR+AP= Ischemia reperfusion+anzer propolis; NGF: Nerve growth factor TAS=total antioxidant capacity; TNF-α=tumor necrosis factor-alpha; TOS=total oxidant capacity.

Table III. Neurological evaluation results according to the groups (n=7).

	6th-hour			24th-hour			48th-hour		
	IR	IR+AA	IR+AP	IR	IR+AA	IR+AP	IR	IR+AA	IR+AP
No neurologic deficit	0	0	0	0	0	0	0	3	3
Failure to extend the contralateral forepaw fully	0	4	3	0	5	4	1	4	4
Circling to the contralateral side	0	3	3	3	2	3	6	0	0
Falling or leaning to the opposite side	4	0	1	4	0	0	0	0	0
No spontaneous walking and a depressed level of consciousness.	3	0	0	0	0	0	0	0	0

IR: Ischemia reperfusion, IR+AA: Ischemia reperfusion+Acetylsalicylic acid, IR+AP: Ischemia reperfusion+Anzer propolis.

Discussion

Cerebral ischemia results from a complex interplay of various physiopathological mechanisms, such as excitotoxicity, calcium overload, free radical formation, and lipid peroxidation. Following ischemia, reperfusion can exacerbate the resulting damage. To prevent ischemia/reperfusion (IR) injury and mitigate existing damage, multiple strategies have been investigated[12-21], including the use of glutamate receptor antagonists, calcium channel blockers, membrane stabilizers, free radical scavengers, anti-edema therapy, and anti-inflammatory and antiaggregant agents, among others.

In ischemia-reperfusion studies[22,23] in rats, it has been shown that acetylsalicylic acid is a potent neuroprotective agent that reduces infarct size by approximately 50% when given at a relatively high dose of 40 mg/kg.

In a study[10] on spinal ischemia-reperfusion, propolis was found to reduce the levels of IL-6, TNF-α, and MPO in at least one animal model. According to this study, anzer propolis exhibited antioxidant effects and inhibited cell apoptosis in the spinal cord.

Our literature review yielded limited results, with only three publications[24-26] examining the effect of propolis on cerebral ischemia. Notably, the propolis used in those studies differed from anzer propolis.

Moreover, we did not encounter any studies investigating the impact of anzer propolis on cerebral ischemia/reperfusion injury during our literature review.

Following cerebral ischemia, inflammatory responses such as the release of IL-1β, IL-6, and TNF-α lead to the upregulation of adhesion molecules in both white blood cells and vascular endothelial cells, as observed in cerebral ischemia/reperfusion[27]. These events contribute to tissue damage and neurological deficits[28].

TNF-α levels increased in studies[29-34] where the IR model was used while agents that reduce ischemia decreased TNF-α levels. Similarly, in our study, TNF-α levels were found to be significantly higher in the IR group compared to the control group, while TNF-α levels were found to be significantly lower in the treatment groups (p=0.035).

IL-6 levels are elevated in blood samples from patients with ischemia. It has been shown[35] that IL-6-defective mice have a smaller infarction and improved neurological function after IR injury. In our study, we also observed an increase in serum and tissue IL-6 levels, and we showed that anzer propolis and acetylsalicylic acid significantly reduced IL-6 levels and cytokine levels in brain tissue (p<0.001).

Enzymatic agents such as the endogenous antioxidant systems glutathione peroxidase and superoxide dismutase reduce the damage arising from oxidative processes caused by free radicals during ischemia and reperfusion. They work by transforming free radicals into more harmless compounds or by inhibiting radical formation[36]. In our study, TAS (U/ml) capacity in brain tissue was statistically significantly lower in the IR group than in the IR+AP group (p=0.031). TOS (U/ml) in brain tissue was higher in the IR group than in the IR+AP group, but there was no statistical difference between the groups (p=0.426).

NGF and other members of the neurotrophin family of growth factors activate specific tyrosine kinase (Trk) receptors to stimulate cell survival[37,38]. Wang et al[37] showed that NGF protects peripheral sensory nerve cells. Some studies[39,40] have revealed that NGF has neuroprotective functions in the cerebral cortex and hippocampus, which are particularly vulnerable to cerebral ischemia and can improve neuronal degeneration. It is accepted[41] that NGF expression is up-regulated in the brains of rats after middle cerebral artery occlusion and plays a crucial role in the protection of ischemic damaged neuronal cells. In our study, NGF was found to be statistically significantly higher in both brain tissue and serum in the IR+AP group and IR+AA group compared to the IR group (respectively p=0.034, p<0.001). The highest NGF level was found in the IR+AP group. Oral administration of anzer propolis may possibly strengthen this protective effect by increasing the NGF level.

Apoptosis is a type of programmed cell death that triggers various metabolic and physiological processes to cause self-destruction. Over the years, it has been linked to certain pathological conditions. In the case of cerebral ischemia followed by reperfusion, apoptosis can affect neurons. However, rescuing apoptotic neurons can improve the pathological outcome. Our study found that brain tissue exhibited less apoptosis with the TUNEL method after ischemia/reperfusion in the IR+AP group compared to the IR group (p=0.001). Furthermore, significant differences were noted in hyperemia in vessels, vacuolization in neurons, and glial cell infiltration between the IR+AP and IR groups (p=0.008, p<0.001, p<0.001, respectively).

Conclusions

Our findings show that anzer propolis significantly reduces TNF-α and IL-6 levels in ischemic brain tissue while increasing NGF and TAS levels. The histological findings also support the therapeutic efficacy of this agent. Our results have the potential to be translated into clinical practice for managing IR injury.

Ethics Approval

The Afyon Kocatepe University Animal Experiments Local Ethics Committee approved the experiment procedures on October 4, 2022, with the approval number AKÜ HADYEK 49533702/89.

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Informed Consent

Not applicable.

Conflict of Interest

All authors have completed and submitted the International Committee of Medical Journal Editors form for disclosure of potential conflicts of interest. No potential conflict of interest was disclosed.

Authors' Contributions

Conceptualization: Cansu Köseoğlu Toksoy, Zülfükar Kadir Sarıtaş, Ülkü Türk Börü, Methodology: Zülfükar Kadir Sarıtaş, Fatma Görücü, Investigation: Cansu Köseoğlu Toksoy, Zülfükar Kadir Sarıtaş, Gökçe Zeytin Demiral, Fatma Görücü, Aziz Bülbül, Hasan Hüseyin Demirel, Yusuf Koç, Project administration: Cansu Köseoğlu Toksoy, Funding acqusition: Cansu Köseoğlu Toksoy, Writing-original draft: Cansu Köseoğlu Toksoy, Writing-review and editing: Zülfükar Kadir Sarıtaş, Ülkü Türk Börü, Supervision: Zülfükar Kadir Sarıtaş, Ülkü Türk Börü.

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