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Original article

Protective effects of anise in prevention of cerebral ischemia reperfusion injury in rats

E. Yildizhan[1], H. Ozesmer[2], M.M. Inan[2], F. Tatli[2], M. Rencber[3], A. Akbas[2], M. Kamar[2], E. Gündüz[4], N. Baksi[5], B.V. Ulger[2], M. Akkuş[1] , A. Kaydu[6]

[1] Department of Histology and Embryology, Faculty of Medicine, Dicle University, 21280, Diyarbakır, Turkey
[2] Department of General Surgery, Faculty of Medicine, Dicle University, 21280, Diyarbakır, Turkey
[3] Department of General Surgery, Viransehir State Hospital, 63700, Viransehir, Şanlıurfa, Turkey
[4] Department of Emergency Medicine, Faculty of Medicine, Dicle University, 21280, Diyarbakır, Turkey
[5] Department of Laboratuary Animals, Faculty of Veterinary, Dicle University, 21280, Diyarbakır, Turkey
[6] Department of Anesthesia, Faculty of Medicine, Dicle University, 21280, Diyarbakır, Turkey

Abstract

The aim of this study is to determine the protective efficacy of anise in cerebral ischemia and reperfusion injury in rats.

In this study, 28 Wistar Albino rats, weighing 250-300 grams (g), were used. Four groups were formed with 7 rats in each group. Group 1 (n=7): Control group, Group 2 (n=7): Anise group, 5 mL/kg/day of anise aqueous extract prepared according to Gamberini's protocol was given orally by gavage for 30 days. Group 3 (n=7): Cerebral ischemia reperfusion (CIR) group, at the beginning of the experiment, 30 minutes of cerebral ischemia and 1 hour of reperfusion were induced and the animals were sacrificed by exanguination. Group 4 (n=7): Anise+ CIR group, After administering 30 days of anise's aqueous extract, CIR was induced and the study was terminated.

TOS values of the Anise+ CIR group was significantly lower than that of the CIR group ($p<0.05$). Il-6 and TNF-α values of the CIR group were significantly higher than the Anise+ CIR group ($p<0,05$).

Our study revealed that anise ameliorates oxidative damage and inflammation due to cerebral ischemia/reperfusion, by reducing the levels of inflammatory cytokines (TNF-α, Il-6).

Keywords: anise, cerebral ischemia reperfusion, oxidative stress, inflammation

Correspondence to: E. Yildizhan, e-mail: blgc_eda@hotmail.com, tel.: +90 412 2488001- 4032, fax: +90 412 2488523

Introduction

Ischemia is characterized by lack of necessary oxygen and glucose to the tissues due to reduced blood flow (Kalogeris et al. 2016). Reperfusion is the restoration of decreased blood flow to tissues or organs (Kalogeris et al. 2012). Increased reactive oxygen species (ROS) production after Cerebral Ischemia Reperfusion (CIR) exceeds the capacity of the antioxidant system in the cerebrum (Sun et al. 2014). With the restoration of blood flow, oxygen reaches the cells again, and this increases the severity of ischemic damage by damaging DNA and plasma membrane, and finally apoptosis occurs (Wu et al. 2015).

Extracts of plants are being used for prevention and treatment of diseases for a long time (Shojaii et al. 2012). Anise is one of the widely used plants and is grown in warm regions around the world (Kucukkurt et al. 2009). It has therapeutic properties in neurological and respiratory diseases. Studies have shown that anise also has various pharmacological properties such as antioxidant, antifungal, analgesic, antimicrobial, antiviral (Özcan et al. 2006).

In this study, we examined the protective efficacy of anise in cerebral ischemia and reperfusion injury.

Materials and Methods

This study was approved by the Dicle University Animal Experiments Local Ethics Committee (DUHADEK) (2021/31).

Supply of animals and housing conditions

In our study, 28 Wistar Albino female rats weighing 250-300 grams (g) were used. Four groups were formed with 7 rats in each. The room and laboratory conditions in which the rats were housed were as follows: 24±2°C room temperature, 50±10% relative humidity, 12:12-hour day-night cycle (automatic lighting 07.00-19.00 day), red light to support night vision, daytime light intensity up to 200 lux, ventilation 10 changes/hour, sound level up to 75 dB. Physical contact with the room was not allowed except for a researcher and a keeper. Information about the macro-environmental conditions of the room (temperature, humidity, sound, light intensity) was checked and recorded on a daily basis. In order to provide equal conditions for all groups, the in-cage feeding and care criteria were as follows: Feeding: Standard irradiated feed with 12 mm diameter was in pellet form (Korkuteli, Antalya) and animals were provided with ad-libitum access to food and water throughout the study. Litter: Sawdust, 3-4 mm in size, not containing more than 2% moisture, moisture absorbing, mold-bad odor-free, was used. The cage and litter were changed once a week by the same person. Health checks: Visually done by the same person once a day. The welfare and living standards of animals were according to the "Regulation on the Welfare and Protection of Animals Used for Experimental and Other Scientific Purposes" of the TR Ministry of Agriculture and Forestry.

Preparation of anise solution

100 grams (g) of anise seeds were brewed in 1 liter (L) of distilled water at 70°C for 30 minutes. Afterwards, it was filtered through Whatman 1 filter paper and given to the rats orally by gavage as 5 mL/kg daily (Gamberini et al. 2015).

Surgery protocol

Before the surgical procedures, 90 mg/kg Ketamine Hydrochloride+10mg/kg Xylazine Hydrochloride was administered intraperitoneally (i.p.) to all experimental animals and the animals were put under general anesthesia. The rats were then fixed on an operating table in the supine position. External carotid artery (ECA) and internal carotid artery (ICA) were exposed by blunt dissection. ICA was temporarily occluded with an artery clamp. After 30 minutesof ischemia, the clamps were loosened and the rat brains were reperfused for 1 hour. During ischemia and reperfusion, body temperatures of the rats were measured with a rectal thermometer and maintained.

Formation of experimental groups

This study was approved by the local ethics committee of Dicle University (2021/31).

Group 1 (n=7): Control group, Group 2 (n=7): Anise group; anise aqueous extract prepared according to Gamberini's protocol was given orally 5 mL/kg/day by gavage for 30 days. Group 3 (n=7): CIR group; 30 minutes of cerebral ischemia and 1 hour of reperfusion were applied and the rats were sacrificed by exanguination on the first day. Group 4 (n=7): Anise+ CIR group; After administering 30 days of anise's aqueous extract, CIR was applied and the study was terminated.

The blood samples were centrifuged at 3000 rpm for 8 minutes, and then the serum portions were sent to the biochemistry laboratory. The excised brain tissues were placed into 10% buffered formaldehyde prepared in phosphate buffer (PBS) and sent to the Histology/Embryology laboratory. After routine histological follow-ups, they were embedded in paraffin blocks. Sections of 3-4 μm thickness were taken from each

Table 1. Statistical results of the rat brain tissue and serum total antioxidant status (TAS) and total oxidant status (TOS) values, mean ± standard deviation values of serum Il-6 and TNF-α values.

Parameters	Control	Anise	CIR	Anise+ CIR
TAS (nmol trolox equiv/mg protein) – Serum	$2.29\pm0.25^{c,d}$	$2.67\pm0.21^{c,d}$	$1.31\pm0.19^{a,b}$	$1.70\pm0.15^{a,b}$
TOS (nmol H_2O_2 equiv/mg protein) – Serum	$23.60\pm2.07^{c,d}$	$22.96\pm0.91^{c,d}$	$49.06\pm3.11^{a,b,d}$	$33.33\pm2.57^{a,b,c}$
TAS- Tisuue	0.28 ± 0.12^{c}	$0.32\pm0.02^{c,d}$	$0.22\pm0.01^{a,b}$	0.26 ± 0.01^{b}
TOS - Tissue	$1.75\pm0.05^{c,d}$	$1.85\pm0.24^{c,d}$	$5.52\pm0.61^{a,b,d}$	$4.20\pm0.52^{a,b,c}$
Il-6 (pg/mL)- Serum	$28.93\pm1.61^{c,d}$	$31.45\pm1.95^{c,d}$	$49.70\pm3.82^{a,b,d}$	$37.47\pm2.10^{a,b,c}$
TNF-α (pg/mL)- Serum	$155.66\pm1.34^{b,c,d}$	$132.53\pm6.21^{a,c,d}$	$232.09\pm23.32^{a,b,d}$	$179.77\pm12.97^{a,b,c}$

a – different from the control group, b – different from the anise group, c – different from CI/R group, d – different from anise+ CI/R group.

block and stained with hematoxylin and eosin (HE). The brain tissue was evaluated for histopathological changes such as inflammation, bleeding, edema, and cytoplasmic vacuolization.

Evaluation of total antioxidant (TAS) and total oxidant (TOS) capacities

The brain tissues of the rats were frozen at -80°C and preserved. TAS and TOS capacities in both tissue and serum were examined in the biochemistry laboratory together with the separated serum fractions. The kits were obtained from Rel Assay Diagnostics (Gaziantep, Turkey) and the automatic measurement method developed by Erel was used for measurement (Erel 2015, 2014).

Serum inflammation markers

Interleukin-6 (Il-6) and Tumor necrosis factor-alpha (TNF-α) levels were measured to examine inflammatory changes. For this, rat compatible ELISA kits were used.

Histopathological evaluation

After the excised brain tissues were fixed in 10% formaldehyde for 3 days, they were cut into 3 parts, in which the hippocampus was left in the middle part. The 3-4 μm thickness sections were cut and stained with HE. Sections containing the hippocampus were evaluated for inflammation, bleeding, edema, cytoplasmic vacuolization and degenerative changes.

Statistical evaluation

We used SPSS for Windows version 22 (Chicago, IL, USA) for statistical analyzes. One-Way Analysis of Variance (ANOVA) was applied to normally distributed measurements, while Kruskal Wallis-H test was applied to non-normally distributed measurements. A value $p<0.05$ was detemined as important.

Results

Analysis of TAS and TOS values

TOS values were found to be significantly lower in the Anise+ CIR group than in the CIR group ($p<0.05$), (Table 1).

Analysis of Il-6 and TNF-α values

Serum Il-6 and TNF-α values did not show a normal distribution. In the Mann Whitney-U test, both Il-6 and TNF-α values were found to be significantly higher in the CIR group than that in the Anise+ CIR group ($p<0.05$) (Table 1).

Histological evaluation

Hematoxylin and eosin staining of the brain tissues of the CIR group revealed that, inflammation, edema, bleeding, cytoplasmic vacuolization and degenerative changes in the hippocampus region were quite intense, but in the Anise+CIR group, these histopathological changes were milder compared to the CIR group. (Fig. 1).

Discussion

Cerebral ischemia is a serious condition that can cause permanent brain damage and develops after stroke, head trauma or resuscitation (Jeon et al. 2013). Ischemia and reperfusion injury is the creation of free radicals and inflammatory response as a result of oxygen deficiency in tissues (Neblina et al. 2005). Most of the tissue damage due to ischemia/reperfusion occurs during the reperfusion phase (Crack et al. 2005).

In general, bilateral arteria carotid ligation and middle cerebral artery ligation are preferred in order to create a CIR model (Rabuffetti et al. 2000). However, in our study, ischemia and reperfusion were performed with unilateral carotid artery ligation. TAS and TOS

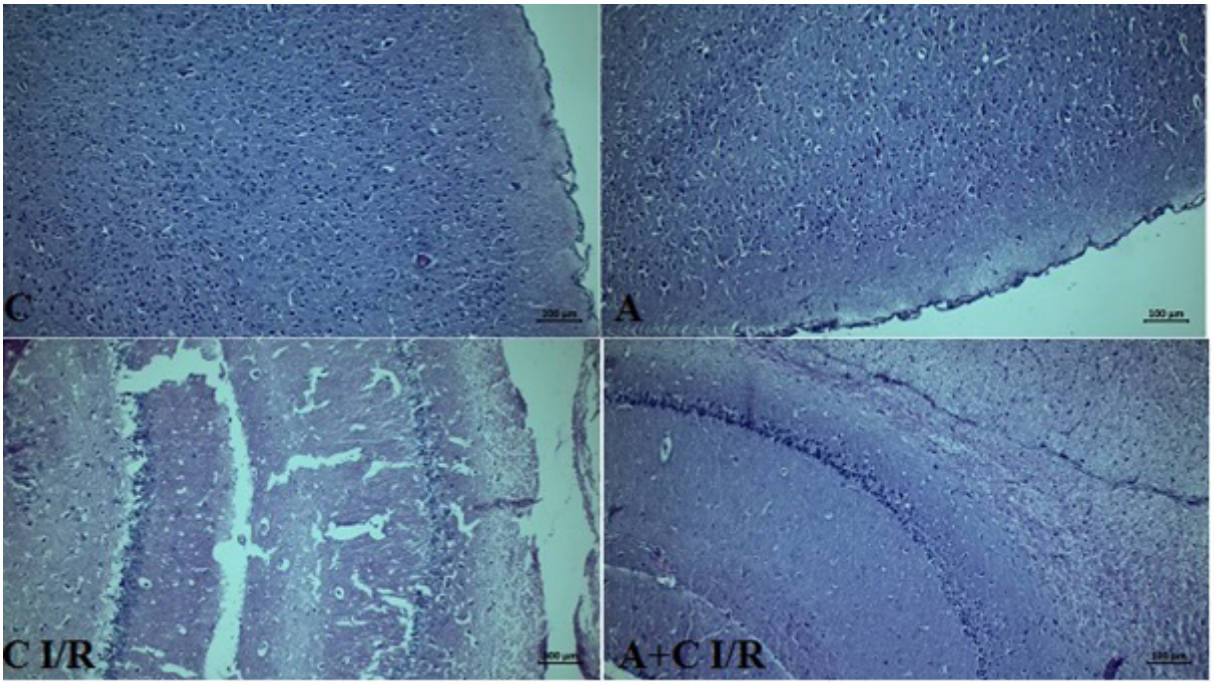

Fig. 1. C: Rat control group, A: Rat anise group, CIR: Rat cerebral ischemia/reperfusion group, A+CIR: Rat anise + cerebral ischemia/reperfusion group (hematoxylin and eosin; bar = 100 µm)

measurements were done in brain tissue and blood serum samples. For evaluation of the inflammation, serum TNF-α and Il-6 levels were measured. In addition, the hippocampus region was examined beneath a light microscope in order to examine the histopathological changes.

The basic molecules that make up TOS are hydrogen peroxide and lipid hydroperoxide. In their experimental studies, Sugawara et al showed that hydrogen peroxide and lipid hydroperoxide increase in ischemia/reperfusion condition (Sugawara et al. 2004). Similarly, we observed an increase in both tissue and serum TOS levels in the cerebral I/R condition. TOS levels of the serum and brain tissue of anise+CIR group were significantly lower than the CIR group. We observed that anise reduced the severity of oxidative damage.

In previous cerebral I/R studies, ischemia duration was kept between 5 and 30 minutes, and reperfusion time up to 72 hours (Selakovic et al. 2011). In our study, we determined the ischemia time to be 30 minutes and the reperfusion time to be 1 hour.

In studies on cerebral ischemia/reperfusion, cerebral cortex, hippocampus and corpus striadum are evaluated, while these areas are heavily affected by ischemia (Lopalco et al. 2020). In this study, we evaluated the histopathological changes in the hippocapus region. The hippocampus is the part where learning, cognitive functions and memory are controlled, and it has been reported that the hippocampus is the most affected part in previous CIR studies on rodents (Earnest et al. 1980). For evaluating degenerative changes due to CIR, H&E staining is widely used (Cao et al. 2011). The most common histopathological changes that occur in CIR are swelling of neurons, cytoplasmic vacuolization, apoptosis and necrosis. In addition, other studies have shown that edema, congestion and inflammation also develop (Johansson 2003). We observed that diffuse degenerative changes occurred in the hippocampus region and inflammation was intense in the CIR group, whereas inflammation and congestion were milder in the A+CIR group.

Conclusion

Ous study revealed that anise reduces the levels of inflammatory cytokines (TNF-α, Il-6) and thus reduces oxidative damage and inflammation resulting from cerebral ischemia/reperfusion. More studies are needed to reveal the celular mechanisms of anise in preventing cerebral ischemia-related damage.

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