The Protective Effects of Lumbricus Extract in Experimental Unilateral Carotid Artery Comminus Ischemia/Reperfusion Model in the Rats

SIBEL CANBAZ KABAY¹ SEMIH OZ² HILMI OZDEN², DILEK BURUKOGLU³, GOKHAN KUS⁴, BENGI YEGIN² MEHMET CENGIZ USTUNER⁵ HAKAN SENTURK⁶ METE MISIRLIOGLU⁶ FATMAYILDIZ⁷ DILEK AYDEMIR²

¹Department of Neurology, Dumlupinar University Faculty of Medicine, Kutahya, Turkey.
²Department of Anatomy, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey.
³Department of Histology-Embryology Eskisehir Osmangazi University Faculty of Medicine, Turkey.
⁴Anadolu University Open Education faculty, Eskisehir, Turkey.
⁵Department of Medical Biology, Eskisehir Osmangazi University Faculty of Medicine, Eskisehir, Turkey.
⁶Eskisehir Osmangazi University Faculty of Science Biology Department, Eskisehir, Turkey.
⁷Toros University, Department of Medical Laboratory Tecniques, Mersin, Turkey.

Key words: brain, ischemia-reperfusion, antioxidant enzyme, lumbricus extract, lumbricus rubellus

Running Title: Protective effects of Lumbricus Extract on the brain
ABSTRACT

Background: This study aimed to investigate the protective effect of LE against oxidative stress induced during cerebral ischemia-reperfusion (CI/R) injury, by determining biochemical parameters and evaluating histological examinations.

Methods: The animals were divided into five groups (10 animals each); Group I was the control group which was applied only dissection of the left common carotid artery. Group II (I/R on left common carotid artery), Group III (I/R injury+ LE 20 mg per kg day); Group IV (I/R injury+ LE 40 mg per kg day) Group V (I/R injury+ LE 80 mg per kg day).

Results: The control group did not show any morphological changes. In Group II (I/R) severe damage and necrotic neurons were observed in the cortical area. The normal looking neurons and also together with very few necrotic neurons in the cortical area were observed in the 20 mg/kg LE treatment group. Normal-like neurons and glial cells as well as decreased damage in the cortical area were observed for the 40 and 80 mg/kg LE treatment group. MDA level of control group didn’t differ when compared to LE groups. MDA level of I/R group was significantly higher than control and LE groups. SOD activity and CAT levels of control group didn’t differ when compared with LE groups. SOD activity and CAT levels of I/R group were significantly lower than control groups.

Conclusions: The present study demonstrates that LE prevents CI/R injury induced biochemical and histologic changes brain tissues in the rat.

BACKGROUND

Cerebrovascular ischemia is a serious and common disease, [1] which leads to serious long-term disability in adults and it is one of the main cause of death around the worldwide [2]. Cerebral ischemia-reperfusion (CI/R) injury may lead to production of reactive oxygen species (ROS), which can eventually bring about cell damage, cell death, increased vascular
permeability, interstitial edema, tissue necrosis, impaired vasoregulation inflammatory cell infiltration, cell apoptosis, mitochondrial dysfunction [3,4]. The increased reactive oxygen species (ROS; e.g. superoxide anion, hydroxyl radicals, peroxynitrite, hydrogen peroxide) can alter the neuronal function because of neuronal death through protein oxidation, DNA damage, elevated nonenzymatic glycosylation, peroxidation of membrane lipids, play important roles in cerebral injury [5, 6]. Improvement of blood flow to ischemic tissues can result in recovery of cells if the injury is not permanent. However, depending on the intensity and the duration of the ischemia, variable number of cells may still die after the blood flow begins. CI/R leads to a complex cascade of events which also known as including the activation of nuclear factor kappa β (NF-κB), which is an important transcription factor which can regulate a variety of cytokines and protein gene transcription [7]. The antioxidant defence systems, none enzymatic free radical scavengers (vitamin E, vitamin C, uric acid, and bilirubin) and the antioxidant scavenging enzymes, (catalase [CAT], superoxide dismutase [SOD], and glutathione peroxidase [GPx]) protect cells and tissues against oxidative injury [8]. Lumbricus extract (LE) has been used for treating various diseases for many years in traditional medicine throughout the world more particularly in Asia, including India, Myanmar, China, Korea and Vietnam [9]. The coelomic fluid which is present in the earthworm’s body cavity has several biological activities like antibacterial, hemolytic, agglutinative, and mitogenic activities [10-12]. Tissue homogenate which obtained from earthworms contains several growth factors, including insulin-like growth factor, immunoglobulin-like growth factor, and epidermal growth factor [13]. Recent studies showed the various beneficial pharmacological activities of earthworm extract. These are fibrinolytic and anticoagulative activities, hepatoprotective activity, and antioxidative activity [9, 13-16]. In some previous studies it was reported that different types of earthworm extract have peripheral nerve regeneration, bone regeneration and anti-inflammatory effect including
wound healing [9, 14-22]. It was demonstrated that earthworm extract can induce the
differentiation of neurite-bearing cells and the proliferation and migration potency of RSC96
schwann cells [20, 23, 24]. In a study it was found that earthworm extract significantly
enhanced the nerve growth factor mediated neurite outgrowth from PC 12 cells [20]. RSC96
cells proliferation were induced by the earthworm extract treatment in a study [23].
Lumbrokinase is an important protease derived from earthworms [24]. In recent studies it was
shown that lumbrokinase has protective effects on hippocampus apoptosis, therapeutic
potential in diabetic nephropathy and mechanisms of lumbrokinase in protection of cerebral
ischemia have been studied. It was demonstrated that the anti-ischemic activity of
lumbrokinase was due to its antiplatelet activity by elevating cAMP level and attenuating the
calcium release from calcium stores, the antithrombosis action due to inhibiting of ICAM-1
expression, and the antiapoptotic effect due to the activation of JAK1/STAT1 pathway [25-27]. In the light of this literature knowledge we aimed to investigate the protective effect of
LE against oxidative stress induced during CI/R, by determining biochemical parameters and
evaluating histological examinations.

MATERIALS and METHODS

Animals

All animal protocols were approved by the institutional animal ethics committee of Eskisehir
Osmangazi University (Permit Number: 2015, 446-1). The experimental protocols were
approved by. Fifty adult male Sprague-Dawley rats weighting 250 to 280 g were obtained
from Medical and Surgical Experimental Research Center (Eskisehir-Turkey) and housed in
polycarbonate cages in a room with controlled temperature (22±2°C), humidity (50±5%), and
a 12 h cycle of light and dark (07:00 AM to 07:00 PM). Rats were fed laboratory pellet chows
and given water ad libitum. The animals were divided into five groups (10 animals each);
Group I was the control group which was applied only dissection of the left common carotid
artery. Group II (I/R on left common carotid artery), Group III (I/R injury+ LE 20 mg per kg day); Group IV (I/R injury+ LE 40 mg per kg day) Group V (I/R injury+ LE 80 mg per kg day).

**Experimental protocol**

Under anaesthesia (100 mg/kg ketamine and 0.75 mg/kg chlorpromazine), dissection of the left common carotid artery was performed and the left common carotid artery was occluded for 15 min to induce ischemia and then subjected for 24 h of reperfusion (I/R groups). Lumbricus rubellus was purchased from and identified by Tong Ren Tang Drug Store®, China. Lumbricus rubellus extract was prepared dried lumbricus with distilled water without heating MR Hei-Standard Magnetic Stirrer® (Germany) and the mixed solution was dissolved in saline as 2 cc/kg. LE or saline (I/R group) was administered gavages 45 min before ischemia and 24 h after reperfusion. The animals were decapitated after 24 h of reperfusion period. After induction of I/R injury, on brain histopathological examinations were performed.

**Histopathological evaluation**

Brain specimens were processed routinely in 10% formalin solution, and embedded in paraffin. Tissue sections of 5 µm were obtained, and stained with hematoxylin and eosin (H&E). All histopathological examinations were performed under a light microscope (NIKON®, Japan) by the histologist of the institute, who was blinded to all tissue specimens regarding their group. A minimum of 10 fields for each brain slides with minimum x50 magnification were examined to assign the severity of the morphological changes.

**Biochemical analysis**

The erythrocyte hemolysate was prepared from fresh blood from the left cardiac ventricle of the rats. 2 ml cardiac blood was taken into EDTA-contained tubes from each rat. Plasma and erythrocyte were separated with centrifugation. Then, as result of three times
washing of erythrocyte with 0.9 % NaCl, hemolysate was formed. The hemolysate was separated out carefully and used for malondialdehyde (MDA), SOD and CAT estimation.

**Protocols of lipid peroxidation and enzyme activities measurement**

MDA production is an end product of lipid peroxidation reacts with thiobarbituric acid to form a red colored complex. The measurement of MDA levels by thiobarbituric acid reactivity is the most widely used method for assessing lipid peroxidation. 0.1 ml of hemolysate, 3 ml of 1% phosphoric acid, and 0.5 ml of distilled water and 1.0 ml of 0.6 % 2-thiobarbituric acid were added. The mixture was boiled in water bath for 45 minutes. Afterward, the mixture was cooled in an ice, followed by an addition of 4.0 ml of n-butanol to extract the cold thiobarbituric acid reactants. The optical density of the n-butanol layer was determined at 532 nm after centrifugation at 1,000g for five minutes and expressed as nmol MDA/ mg Hb [28].

**Determination of SOD activity**

SOD activity was spectrophotometrically assayed with commercial kits. The Fluka SOD kit USA contains all reagents and solutions required for determining superoxide dismutase activity in an indirect assay method based on xanthine oxidase and a novel color reagent. The hemolysate SOD activity was determined by inhibition of Formosan dye (450 nm) employing the xanthin-xanthin oxidase enzymatic method to generate superoxide radicals and expressed as U/mg of hemoglobin.

**Determination of CAT activity**

One unit (1U) of CAT equals the enzyme activity that recognized 1 µmol of hydrogen peroxide in 60 sec at 37°C. CAT activity was measured with determination of absorbance of blank sample (1.0 ml substrate, 1.0 ml ammonium molybdate and 0.2 ml hemolysate), blank 1 (1.0 ml substrate, 0.2 ml hemolysate and ammonium molibdate added after 60 sec) and blank 2 (1.0 ml substrate, 1.0 ml molybdate and 0.2 ml buffer) against blank 3 (1.0 ml buffer, 1.0 ml
ammonium molybdate and 0.2 ml buffer) at 405 nm in spectrophotometer. CAT activity (kU/L) was calculated as $\left(\frac{Abs_{blank1} - Abs_{blanksample}}{Abs_{blank2} - Abs_{blank3}}\right) \times 271$. Results were divided to sample hemoglobin amount ml/mg Hb [29].

**Statistical analysis**

All statistical analysis was performed with the computer program “SPSS for Windows” (SPSS Inc; Release 11.5; Sep 6, 2002). All of the data were expressed as means ± SD. Differences between groups were evaluated by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison tests. The significance was tested at $p>0.05$, $p<0.05$, $p<0.01$ and $p<0.001$

**RESULTS**

The control group did not show any morphological changes that there were normal like neurons and glial cells in the cortical area. In Group II (I/R) severe damage and necrotic neurons were observed in the cortical area. However, the normal looking neurons and also together with very few necrotic neurons in the cortical area were observed in the 20 mg/kg LE treatment group. It was also observed that the damage in the cortical area continued. On the other hand, normal-like neurons and glial cells as well as decreased damage in the cortical area were observed for the 40mg/kg LE treatment group. In 80mg/kg LE treatment group it was seen that cortical damage were decreased and there were nearly normal neurons and glial cells (Figure 1). MDA level of control group didn’t differ when compared to LE groups ($p>0.05$). MDA level of I/R group was significantly higher than control and LE groups ($p<0.01$). But it didn’t change significantly MDA level when compared with group III, IV, V ($p>0.05$). SOD activity of control group didn’t differ when compared with LE groups ($p>0.05$). SOD activity of I/R group were significantly lower than control groups ($p<0.001$). CAT levels of I/R group were significantly lower than control groups ($p<0.01$). MDA levels, SOD and CAT activities of all groups were given in figure 2 and table 1.
DISCUSSION

CI/R injury which depends on vessel occlusion predominantly affects the brain and has been considered as the major pathogenic mechanism of various cerebrovascular diseases [30]. CI/R injury induces oxidative stress and free radical formation. Previous research shows that excessive amount of free radicals are generated immediately after the onset of reperfusion [3, 4]. Reperfusion after cerebral ischemia leads to cerebral edema, brain hemorrhage and neuronal death. [31]. Although successful clinical applications, there are very few effective treatments for CI/R injury, currently it must be the main aim to develop the new medical interventions [32]. Several strategies and drugs have been shown to decrease CI/R damage in animal models [33]. The protective effects of antioxidants on brain tissue including CI/R injury have yielded that it reduces lipid peroxidation and it has positive effects to antioxidant system status [34]. ROS produced in oxidative stress is scavenged by SOD, GPx and CAT. It has been demonstrated in numerous studies that ROS are directly involved in oxidative damage of cellular macromolecules such as lipids, proteins, and nucleic acids in tissues. MDA is the breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids and thus serves as a reliable marker of oxidative stress-mediated lipid peroxidation [35, 36]. In our study, LE treatment in brain ischemia-reperfusion decreased MDA level. SOD and CAT activity was increased with low and high doses of LE treatment in brain I/R injury. On the other hand, MDA, SOD levels didn’t show any difference between different LE doses. This may reflect that, different doses of LE may not be beneficial in decreasing oxidative injury. These results suggested that LE may be effective in preventing oxidative injury. When all groups were compared histopathologically in brain; different doses of LE administration didn’t show any significant difference about brain structures. The protective effects of LE were observed in all LE administration group and further dose increment did not alter the morphological neuroprotective effects of LE. LE has been discovered exist various biological
of a great number of compounds such as the mitogenic effect of insulin-like proteins [19, 37], fibrinolytic, protease and anticoagulative activities [15, 38], the presence of immunoglobulin structures which reacted with anti-IgG, anti-IgA and anti-IgM [39]. Moreover, LE induces synthesis of epidermal growth factor (EGF) and fibroblast growth factor (FGF) during the wound healing on mice skin [17]. Previous studies on Lumbricus extract have shown its antipyretic, antispasmodic, detoxic, diuretic, antihypertensive, antiallergic, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral and anti-inflammatory activities [21, 40-42]. In addition, antioxidative effects has been observed, which possible that glycolipoprotein extract (G-90) or some of its components could have also antioxidative potential [16]. Also, the Lumbricus extract has been shown to hepatoprotective as it enhances the activities of liver function such as the enhancement in the levels and activities of GSH, SOD, GPx and CAT, and decreases the levels and activities of serum ALP, AST, ALT, bilirubin and liver TBARS [9]. Lumbricus extract appears to enhance sciatic nerve regeneration and function recovery following injury [19]. Lumbrokinase (LK) is an enzyme derived from earthworms Lumbricus rubellus. Lumbrokinase consists of a group of proteolytic enzymes including plasminogen activator and plasmin extracted from a specific species of earthworm. Lumbrokinase is resistant to degradation by some cellular enzymes, and thus, it could be transferred intact and across the cell membrane by pinocytic vesicles or epithelial cells [43]. Recently, cardioprotective effect of lumbrokinase against myocardial ischemia has been researched on a rat model with acute myocardial infarction. The results show that it has protective actions on myocardial infarction in rats [44]. Also, oral lumbrokinase has been shown to ameliorate myocardial perfusion in patients with stable angina patients [45]. In conclusion, the present study demonstrates that LE prevents CI/R injury induced biochemical and histologic changes renal tissues in the rat. LE in 20 mg/kg
was observed to be enough to significantly prevent CI/R injury. According to these findings, it is necessary to make more experimental studies for the evaluation of LE effects.

Competing interests

The authors declare that there are no competing interests.

Authors’ contributions

SCK, SO and HO planned and carried out the experiments, analyzed the data and drafted the manuscript. DB, MCU and HS carried out the histopathological studies, participated in the sequence alignment and drafted the manuscript. GK participated in the sequence alignment and participated in the design of the study and performed the statistical analysis. BY and MM participated in the design of the study and helped in the interpretation of the results. FY and DA conceived of the study, and participated in its design and coordination and helped to draft the manuscript.

Acknowledgments

The authors declare that there are no acknowledgments.

LEGENDS

Figure 1

A; Group I (control group); Normal appearing neurons and (→) glial cells in cortical area are seen (►) (a, b). (HE, scale bar: 200μm, scale bar: 50.0μm)

B; Group II (I/R); Severe injury (→) and necrotic neurons (►) are seen in cortical area (a, b) (HE, scale bar: 100μm, scale bar: 50.0μm).

C; Group III (I/R injury+ LE 20 mg per kg day); Normal appearing neurons with a few number of necrotic neurons in cortical area are seen (►). Also ongoing injury can be seen in cortical area (a, b) (HE, scale bar: 100μm, scale bar: 50.0μm).
D; Group IV (I/R injury+ LE 40 mg per kg day); Decreased injury and nearly normal neurons and glial cells can be seen (a, b) (HE, scale bar: 100µm, scale bar: 50.0µm).

E; Group V (I/R injury+ LE 80 mg per kg day); Decreased injury and nearly normal neurons and glial cells can be seen (a, b) (HE, scale bar: 100µm, scale bar: 50.0µm)

Figure 2. Mean MDA, SOD, CAT activities of all groups.

Table 1. Multiple comparisons of MDA levels and antioxidant enzyme activities for all groups.

REFERENCES


Figure 2
Additional files provided with this submission:

Additional file 1: Table 1.doc, 37K
http://www.biomedcentral.com/imedia/3579676261667341/supp1.doc