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Protective effect of *Lumbricus rubellus* Hoffmeister extract in experimental renal ischemia/reperfusion injury in the nephrectomy rats

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Acute kidney injury (AKI), an immediate loss of renal function, leads to high mortality, and ischemia-reperfusion (I/R) injury is considered as one of the main causes of AKI. Inflammation and oxidative stress are known to play an important role in AKI. On the other hand, the earthworm extract, used in traditional medicine, is known to possess various biological and pharmacological activities viz. antiapoptotic, anticoagulative, fibrinolytic, anti-inflammatory, antioxidative stress, peripheral nerve regeneration, bone regeneration and wound healing. Hence, in this study, we investigated the protective effect of the earthworm *Lumbricus rubellus* Hoffmeister extract (LE) after nephrectomy, against oxidative stress occurring during renal ischemia/reperfusion (I/R) injury. A total of 10-12 weeks old *Sprague Dawley* male rats were divided into five groups (n=8). Group I (control), Group II (I/R), Group III (I/R + 10 mg/kg LE), Group IV (I/R + 20 mg/kg LE) and Group V (I/R + 40 mg/kg LE). All rats except in Gr. I were applied ischemia for 45 min and reperfusion for 24 h. At the end of the experiment, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) enzyme activities and malondialdehyde (MDA) levels were evaluated. In addition, kidney tissues were evaluated histologically. In results, the MDA and GPx level of the I/R group were found to be significantly higher than the control and LE groups. SOD activity of the control group did not differ when compared to LE groups and CAT levels were not significantly different between all groups. In addition, in Gr. III-V we observed nearly normal renal cortex and renal tubules. The present study, thus demonstrates that the extract of *L. rubellus* prevents renal I/R injury and induced biochemical and histological changes in the renal tissues in rats.

Keywords: Antioxidants, Earthworm extract, Kidney, Reactive oxygen species (ROS)

Acute kidney injury (AKI) is an immediate loss of renal function which associated with high mortality[1]. The ischemia-reperfusion (I/R) injury is one of the main causes of AKI, which usually occurs during kidney surgery and its pathophysiological process is quite complex[2]. An I/R injury may lead to production of reactive oxygen species (ROS), which can eventually bring about increased vascular permeability, cell death, cell damage interstitial edema tissue necrosis, impaired vasoregulation inflammatory cell infiltration and multi organ dysfunction[1,3-6]. It has been suggested that various cellular pathways, such as inflammation and oxidative stress, play an important role in AKI. In particular, excessive ROS formation leads to the production of cytotoxic metabolites that can induce irreversible disorders, such as DNA damage and lipid peroxidation[7,8]. Furthermore, ROS overproduction following I/R can be induced by the release of multiple enzymes capable of reducing molecular oxygen, forming superoxide and/or hydrogen peroxide[9]. By restoring blood flow to ischemic tissues, cells are able to heal in cases where the damage that resulted is not permanent. However, there may be differences in the number of cell death depending on the severity and duration of ischemia[10].

The antioxidant defence systems, the antioxidant scavenging enzymes [superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT)] and non-enzymatic free radical scavengers (vitamin

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C, vitamin E, bilirubin and uric acid), protect cells and tissues against oxidative injury. The first defence against ROS consists of enzymatic antioxidants such as CAT, SOD and GPx[11,12]. The earthworm *Lumbricus rubellus* Hoffmeister extract (LE) has been used for treating various diseases for many years in traditional medicine throughout the world, particularly in Asia, including China, Korea and India[13]. The coelomic fluid which is present in the earthworm's body cavity exhibits several biological activities, such as hemolytic, agglutinative, mitogenic activities, as well as antibacterial characteristics. Tissue homogenate obtained from earthworms contains several growth factors, including immunoglobulin-like growth factor, epidermal growth factor and insulin-like growth factor[14-16]. Earlier studies have shown the various beneficial pharmacological activities of earthworm extract, including fibrinolytic and anticoagulative activities, anti-inflammatory, antioxidative stress and anticancer effects[13,17-19]. In addition, different types of earthworm extract have peripheral nerve regeneration, bone regeneration and anti-inflammatory effects, including wound healing[20,21]. Lumbrokinase is an important protease containing a group of bioactive proteolytic enzymes derived from LE extracts[15,22]. In recent studies, it has been shown that the antiapoptotic effect of lumbrokinase is due to the activation of the JAK1/STAT1 pathway and has anti-ischemic activity[14,18,23,24]. However, to the best of our knowledge, no study is currently available that has investigated LE against oxidative stress induced during kidney I/R injury. In this study, it was thus aimed to investigate its protective effect in terms of biochemical and histology.

Materials and Methods

Animals

All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and ARRIVE guidelines; the experimental protocols were approved by the institutional animal ethics committee. Forty adult male Sprague-Dawley rats weighing between 250 and 280 g were obtained from the 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 darkness. Rats were fed laboratory pellet chows and given water ad libitum[25]. The animals were divided into five groups of eight animals each: Group I (Control), Group II (I/R), Group III (I/R + LE 10 mg/), Group IV (I/R + LE 20 mg/kg) and Group V (I/R + LE 40 mg/kg).

Experimental protocol

A right kidney nephrectomy was performed under anesthesia (100 mg/kg ketamine and 0.75 mg /kg chlorpromazine) to all rats in the experimental groups. After a 15-day recovery period, saline (I/R groups) or LE was administered intraperitoneally to the groups for seven days. The renal pedicle was occluded for 45 min to induce ischemia and then subjected to 24 h of reperfusion (I/R groups). LE was purchased from the Tong Ren Tang Drug Store in China and dissolved in saline at 2 cc/kg and administered to rats. All groups except Group I were administered 45 min of ischemia and 24 h of reperfusion. All rats were then decapitated under anaesthesia, and blood and kidney tissue samples were taken for biochemical and histological analysis.

Histopathological evaluation

Left nephrectomy specimens were fixed in 10% formalin solution and embedded in paraffin after routine tissue follow-up[26]. Tissue sections of 5 μm were taken from paraffin blocks and the sections were stained with hematoxylin-eosin. Histopathological examinations of all experimental groups were performed under a light microscope (NIKON®, Japan). At least ten fields per kidney slide were examined at minimum 50X magnification, to determine the level of morphological changes.

Biochemical analysis

Erythrocyte hemolysate was prepared from fresh blood drawn from the left cardiac ventricle of rats into 2 mL EDTA tubes. Erythrocyte and plasma were separated by centrifugation of blood. Hemolysate was then formed following three washings of erythrocyte with 0.9% NaCl. The hemolysate was separated out carefully and used for the SOD, CAT, GPx and MDA analyses.

Protocol of lipid peroxidation measurement

MDA production is an end product of lipid peroxidation which reacts with thiobarbituric acid to form a red coloured complex. The measurement of MDA levels by thiobarbituric acid reactivity is the most widely used method for assessing lipid peroxidation. MDA measurement was carried out according to the method developed by Uchiyama and

Mihara (1978) and the results were expressed as nmol MDA/mg Hb[27].

Determination of SOD/CAT/GPx activities

The SOD activity was determined with a ready-to-use reagent kit (Superoxide Dismutase Assay Kit, Calbiochem) according to the manufacturer's instructions. The results were expressed as U/mg of Hb.

The CAT activity measurement was carried out according to the method of Goth (1991)[28]. CAT activity (kU/L) was calculated as = $[(Abs_{blank1} - Abs_{blanksample}) / (Abs_{blank\ 2} - Abs_{blank\ 3})] \times 271$. Results were divided to sample hemoglobin amount ml/mg Hb.

GPx activity was spectrophotometrically assayed with commercial kits (GPx, Calbiochem kit, USA). Cellular glutathione peroxidase (c-GPx) is a member of a family of GPx enzymes whose function is to detoxify peroxides in the cell, thus playing a critical role in protecting the cell from free radical damage, chiefly lipid peroxidation. GPx enzymes catalyze the reduction of peroxides to the corresponding stable alcohols using glutathione (GSH) as a source of reducing equivalents. The oxidation of NADPH to NADP + is accompanied by a decrease in absorbance at 340 nm, thus providing a spectrophotometric means for monitoring GPx enzyme activity, expressed as U/mg of hemoglobin.

Statistical analysis

All statistical analysis was performed using the SPSS Version 21.0 (IBM Corporation, Armonk, New York, USA) and the GraphPad 7 Prism software (GraphPad Software, Inc., San Diego, CA, USA). All of the data was expressed as means ± SD. For normally distributed data, the difference between the experimental groups was demonstrated using a one-way ANOVA test. The significance was tested at $P > 0.05$, $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$.

Results

The SOD, CAT and GPx activity results and MDA levels of the experimental groups are provided comparatively in Fig. 1. SOD activity of the control group did not differ when compared to the LE groups ($P > 0.05$). SOD activity of the I/R group was significantly lower than the control group ($P < 0.01$). CAT activity levels did not differ between the control and the I/R groups ($P > 0.05$). When the 10 mg/kg LE group was compared with the control group, a significant increase was observed in CAT levels ($P < 0.01$). GPx activity levels of the I/R group was significantly higher than the control and LE groups ($P < 0.0001$). GPx levels decreased in the LE treated groups as compared to the I/R group, depending on the increasing dose. MDA level of the I/R group was significantly higher than the control and LE groups ($P < 0.0001$). MDA levels decreased significantly with LE application compared to the I/R group ($P < 0.0001$) (Fig. 1).

The control group did not show any morphological changes. Group II (I/R) showed severe tubular epithelial desquamation and tubular dilatation in the

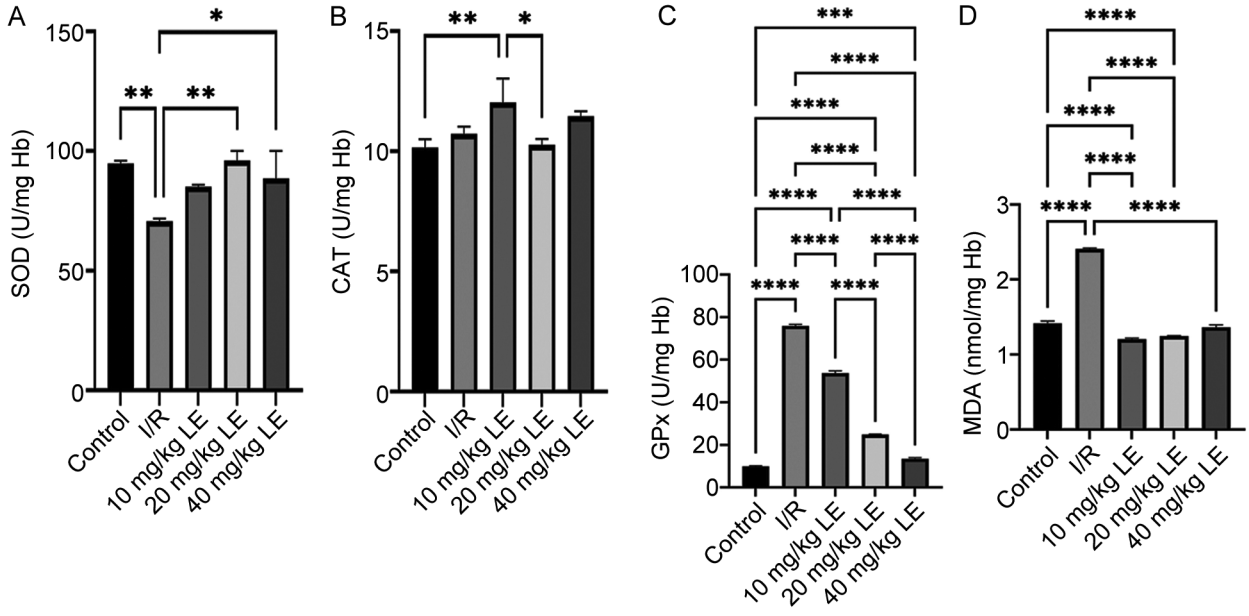

Fig. 1 — Comparison of the (A-C) SOD, CAT and GPx enzyme activities; and (D) MDA values of the experimental groups. Mean ± standard error (SE) plot (n=8).

renal cortex. In addition, in the Gr. III-IV and V, we observed near to normal renal cortex with Malpighi corpuscle and renal tubules in 10, 20, 40 mg/kg/day LE, respectively. As a result, the I/R group showed severe degeneration on light microscopic examination, when compared to control group. The protective effects of LE were observed, even with 10 mg/kg in Group III and with increased dose of LE in Gr. IV and V, to prevent the morphological changes in all rats; further dose increments did not alter the morphological renoprotective effects of LE (Fig. 2).

Discussion

Renal ischemia and reperfusion induce oxidative stress and free radical formation. Previous research has shown that excessive amounts of free radicals are generated immediately after the onset of reperfusion[29,30]. The protective effects of antioxidants on kidney tissue, including renal I/R injury, have yielded that it reduces lipid peroxidation and positive effects to the antioxidant system status[31]. 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[29-32]. 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 (LPO)[33,34]. In our study, LE treatment in renal ischemia-reperfusion decreased the MDA level. SOD activity was increased with low and high doses of LE treatment in kidney I/R injury. There was no difference between CAT levels in LE application in all groups compared to the I/R group and the GPx level was decreased by the LE treatment. On the other hand, MDA, SOD levels remained unaffected between different LE doses. This could indicate that different doses of LE may not be beneficial in decreasing oxidative injury with the exception of the GPx level. These results suggest that LE may be effective in preventing oxidative injury. When all groups were compared histopathologically in the kidney, different doses of LE administration did not show any significant difference in the renal structures. The protective effects of LE were observed in all LE administration group and further dose increment did not alter the morphological renoprotective effects of LE.

LE has been discovered to exist in the biology of a great number of compounds, such as the mitogenic effect of insulin-like proteins[13-16] fibrinolytic, protease and anticoagulative activities, the presence of immuno-globulin structures which reacted with anti-IgG, anti-IgA and anti-IgM17-19. Moreover, LE induces synthesis of EGF (epidermal growth factor) and FGF (fibroblast growth factor) during the wound healing on mice skin[35]. Previous studies on Lumbricus extract have shown its

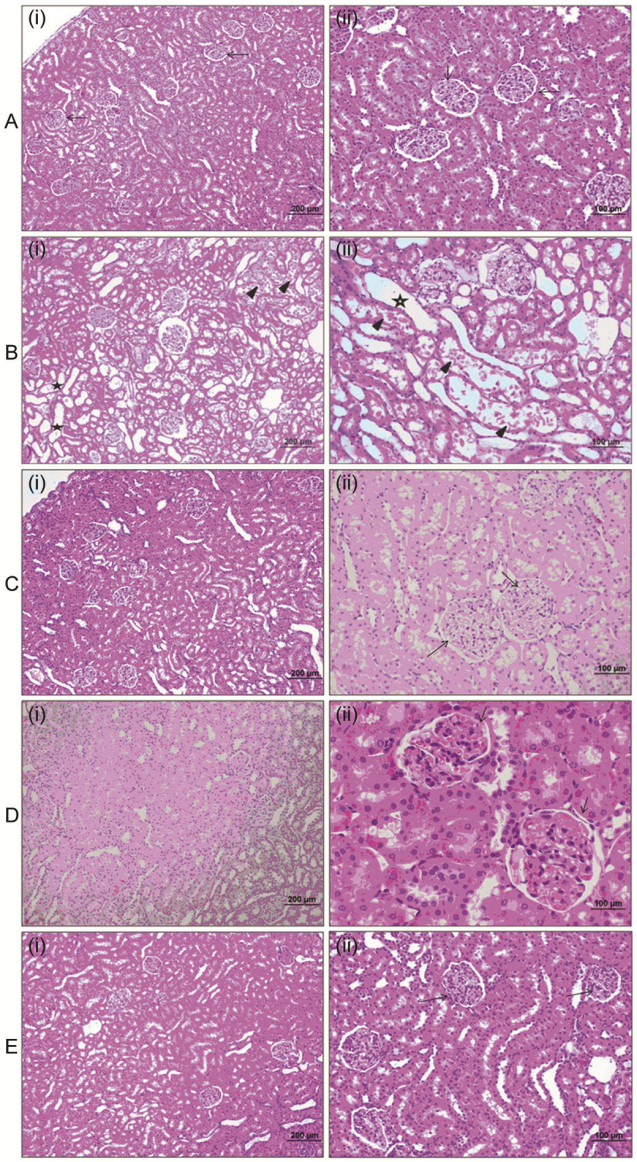

Fig. 2 — Histological images of the experimental groups. Light micrograph showing **(**A**)** Normal Malpighi corpuscle (arrow) and renal tubules in renal cortex of control group; . **(**B**)** Severe tubular epithelial desquamation (arrowhead), tubular dilatation (*) in renal cortex of I/R group. **(**C-E**)** Near to normal renal cortex with Malpighi corpuscle (arrow) and renal tubules in 10, 20 and 40 mg/kg, respectively [HE, bar: 200µm, bar: 100 µm].

antipyretic, antispasmodic, detoxic, diuretic, anti-hypertensive, antiallergic, antiasthmatic, spermatocidal, antioxidative, antimicrobial, anticancer, antiulceral and anti-inflammatory activities[13-16]. In addition, antioxidative effects has been observed, which possible that glycolipoprotein extract (G-90) or some of its components could have also antioxidative potential[36]. The Lumbricus extract has also been shown to be 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[13]. Lumbricus extract appears to enhance sciatic nerve regeneration and function recovery following injury[37].

Lumbrokinase (LK) is an enzyme derived from earthworms *Lumbricus rubellus* and it 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[38]. Recently, the 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[9]. Oral lumbrokinase has also been shown to ameliorate myocardial perfusion in patients with stable angina[40]. The mechanisms of lumbrokinase in the protection of cerebral ischemia have also been studied. One study 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[41].

Conclusion

The present study demonstrates that the earthworm *Lumbricus rubellus* (LE) extract prevents renal I/R injury, induced biochemical and histologic changes in the renal tissues of rats. LE in 10 mg/kg was observed to be enough to significantly prevent renal injury. Additional experimental studies are required to evaluate the effects of LE.

Conflict of interest

Authors declare no competing interests.

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