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Article

Effects of *Urtica dioica* Seeds on Oxidative/ Nitrosative Stress Levels and Myeloperoxidase Activity in Muscle Ischemia/Reperfusion Injury

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The purpose of this study is to evaluate the preventive effects of *Urtica dioica* (UD) on muscle ischemia/reperfusion (I/R) injury. A total of 27 male Wistar rats were divided into three groups as the control group (1), I/R + saline group (2), and I/R+UD group (3). Group 1 did not receive any treatment. Group 2 was administered a total of 2mL/kg saline (1mL/kg before ischemia and 1 mL/kg after reperfusion), and group 3 was given a total of 2mL of UD (1mL/kg before ischemia and 1mL/kg after reperfusion) as treatment. Saline and UD were administered via intraesophageal canula once a day for five days. At the end of five days, all the rats were exposed to muscle ischemia for 60 min followed by 60 min of reperfusion of the bilateral hindlimbs induced using a tourniquet. Muscle tissue histopathologies were evaluated by light microscopy. Furthermore, oxidative/nitrosative stress biomarkers such as catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), nitrotyrosine (3-NT), nitric oxide (NO), and myeloperoxidase (MPO) as an inflammatory marker in tissue samples were measured. UD treatment significantly decreased oxidative/nitrosative stress biomarker levels and MPO ($p<0.05$). We established that UD treatment could alleviate muscle injury induced by muscle I/R in rats by inhibiting the inflammation and oxidative/nitrosative stress.

Keywords: Muscle Ischemia/Reperfusion Injury. Myeloperoxidase. Oxidative/Nitrosative Stress. *Urtica dioica*.

INTRODUCTION

Ischemia-reperfusion (I/R) injury is cellular damage that causes the release of toxic metabolites and several inflammatory substances into the systemic circulation (Patterson, Klenerman, 1979; Akcil, Tug, Doseyen, 2000). Upon the reperfusion of ischemic tissue, there may be more severe damage than the ischemic injury itself. An acute inflammatory response is the basis of the physiopathology of I/R injury. Complex mechanisms such as emerging free oxygen radicals and leukocyte aggregation cause cellular death, organ dysfunction, and finally, organ failure (Seyama, 1993; Erdogan *et al*., 1999). A tourniquet is commonly used in operations, injury, and organ transplantation, which poses some risks inherent to its use as most complications are directly related to the duration of ischemia. The use of a tourniquet exposes muscles to significant risk due to being more vulnerable to ischemia. Some studies have demonstrated that the period of ischemia could have the probability to produce pathologic changes in skeletal muscle. Changes similar to **i**nflammation have also been observed in ischemic tissue (Cetinus *et al*., 2005).

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) may be generated during the reperfusion of ischemic tissue, which presents considerable evidence that injury to ischemic tissue occurs almost exclusively during the reperfusion phase. The damage is caused by

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the large flux of superoxide radicals generated when oxygen is reintroduced to the ischaemic tissue (Akcil, Tug, Doseyen, 2000; Dhaliwal *et al.*, 1991; Kirshenbaum, Singal, 1993; Kurutas, 2015; Salvemini, Cuzzocrea, 2002). Studies have shown that foods containing phenolic compounds have important antioxidant activities, such as *Urtica dioica (UD)* (Karakaya, El, Taş, 2001; Pieroni *et al.*, 2002; Rechner *et al.*, 2002).

Numerous studies of plants and drugs illustrate their protective antioxidant and anti-inflammatory effects on I/R injury. One such plant is UD – a typical green plant that grows worldwide. From the distant past to the present day, it has been used in many different applications such as alternative medicine, food, paint, fibre, fertiliser, and cosmetics (Karakaya, El, Taş, 2001). In addition, studies have shown that UD has anti-microbial, anti-inflammatory, analgesic, and antioxidant effects (Cetinus *et al.*, 2005). As far as we know, no studies were reported in the literature that investigated UDs antioxidant and inflammatory effect on muscle I/R status. Therefore, we aimed to evaluate the levels of oxidative/nitrosative stress biomarkers such as catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), nitrotyrosine (3-NT), nitric oxide (NO), and myeloperoxidase (MPO) as inflammatory markers in muscle tissues.

MATERIAL AND METHODS

UD Ethereal Oil Isolation

UD seeds were collected by a botanist, converted to granules in an electrical grinder, and added to diethyl ether. The mixture was filtered following extraction for half an hour. The filtrate was separated from the ether with a rotary evaporator under a vacuum. The resulting etheric oil extract was then collected. The extract was centrifuged at 4.000rpm for 15 minutes. Then the grounds at the bottom of the tube were removed, and the remaining clear fluid was used in this study.

Animals

This study was approved by the local ethics board of Experimental Animals Laboratory of the Faculty of Medicine of Kahramanmaras Sutcu Imam University in 2019. For this study, twenty-seven Wistar male rats with an average mean weight of 228.9±31.5 (ranging between 180-276g) were utilised. The rats were cared for per the Guide for the Care and Use of Laboratory Animals. Rats were given standard rat food and pipe water with a 12h light/12h dark rhythm in a room at a temperature of 22±2°C. Rats were randomly divided into three groups containing nine rats each, namely a control group (group 1), I/R+Saline group (group 2), and Treatment (I/R+UD) group (group 3). In group 2, a total of 2mL/kg 1.15 % KCI solution (1mL/kg before ischemia and 1mL/kg after reperfusion), and in group 3, a total of 2.0mL of UD (1mL/kg before ischemia and 1 mL/kg after reperfusion) was administered as treatment. Saline and UD were given via intraesophageal canula once a day for five days. However, group 1 were not administered anything. Tourniquet ischemia was applied to all rats after five days. An elastic rubber band (1x3x90 mm) was used as a tourniquet applied onto the right thigh as proximal as possible. Before the tourniquet ischemia, rats were anaesthetised intraperitoneally with 50mg/kg/weight of ketamine (Ketalar) and 5mg/kg/ /weight of xylazine (Rompun). These doses were repeated as necessary. Tourniquet was kept in place for 60 min while the rats were under anaesthesia. After the ischemic period, the tourniquet was released by cutting the rubber band, and both groups were reperfused for about 60min. The rats were then sacrificed with an overdose of ketamine-xylazine. The tibialis anterior muscles were excised from the legs of the rats. All specimens were washed with saline to remove the haematoma and then dried. The tibialis anterior muscles weighed 259.4±47.5mg (X±SD) (min-max: 198-358mg) and were individually stored in plastic bottles at -20˚C until biochemical analyses were carried out.

Homogenate Preparation

Muscles were then weighed, perfused with 1.15% ice-cold KCl, minced, and homogenised in 5 volumes (w/v) of the same solution using a Heidolph 50110 R2R0 homogeniser. Oxidative/nitrosative stress biomarkers and MPO assays were performed on the supernatant

preparation in a Sorvall RC-2B centrifugation of the homogenate at 14.000 rpm for 30min at +4 °C.

Oxidative/Nitrosative Stress Biomarkers Measurements

MDA levels that reflect lipid peroxidation rate in tissue samples were measured according to the procedure by Ohkawa, Ohishi, Tagi (1979). The reaction mixture contained a 0.1ml tissue sample, 0.2ml of 8.1% sodium dodecyl sulphate, 1.5ml of 20% acetic acid, and 1.5ml of 0.8% aqueous solution of TBA. The mixture pH was adjusted to 3.5, and the volume was finally made up to 4.0ml with distilled water, and then 5.0ml of the mixture of n-butanol and pyridine (15:1 v/v) were added. The mixture was shaken vigorously. After centrifugation at 4000rpm for 10min, the absorbance of the organic layer was measured at 532nm. The protein concentration of the tissue samples was measured with Spectronic-UV 120 spectrophotometer by Lowry's method (Lowry *et al.*, 1951). MDA levels were expressed as nmol/mg protein in tissue samples.

SOD activity was measured according to the method described by Fridovich in 1947. This method employs xanthine and xanthine oxidase to generate superoxide radicals that react with p-iodonitrotetrazolium violet (INT) to form a red formazan dye measured at 505nm. Assay medium consisted of the 0.01M phosphate buffer, CAPS (3-cyclohexilamino-1-propanesulfonic acid) buffer solution (50mM CAPS, 0.94mM EDTA, saturated NaOH) with pH 10.2, solution of substrate (0.05mM xanthine, 0.025mM INT) and 80UL xanthine oxidase. SOD activity was expressed as U/mg protein in tissue samples. CAT activities were determined by measuring the decrease in hydrogen peroxide concentration at 230nm by t Beutler's method (Beutler, 1975). Assay medium consisted of 1M Tris HCI, 5mM Na_2EDTA buffer solution (pH 8.0), 1M phosphate buffer solution (pH 7.0) and 10mM H_2O_2. CAT activity was expressed as U/mg protein in tissue samples.

NO level in tissues samples was measured using the Griess reaction (Ridnour *et al.*, 2000). NO levels were expressed as μmol/mg protein. 3-NT levels in tissues samples were determined with an ELISA kit (Bioxytech Nitrotyrosine-EIA, OxisResearch, Portland, OR, USA) according to the manufacturer's protocol. 3-NT levels were given as nmol/mg protein. MPO activity in tissue samples was measured at 460nm was by the O-dianisidine method (Krawisz, Sharon, Stenson, 1984). One unit of MPO was defined as giving an increase in absorbance of 0.001 per min, and specific activity was shown as U/mg protein.

Histological Methods

The muscle tissues were fixed in 10% formaldehyde and processed routinely. They were embedded in paraffin. Five-micrometre sections were obtained, stained with Harris hematoxylin-eosin and examined under a light microscope (Fischer *et al.*, 2008).

Statistics

Complete measured information was analysed using SPSS 25.0 (Statistical Package for Social Science). Data were given as mean ± standard deviation. One way analysis of variance (post hoc Bonferroni test) was used in multiple group analysis, and Mann-Whitney-U tests were used to compare two independent groups. $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

Biochemical Findings

We found in our study that I/R significantly increased MDA, NO, 3-NT and MPO ($p<0.05$). However, the activities of CAT and SOD enzymes decreased ($p<0.05$). MDA, MPO, NO, and 3-NT were increased while the contents of antioxidant-related factors (SOD and CAT) were markedly dropped in the I/R+saline group compared to the control group ($p<0.05$) (Figure 1, Table I). Table I also exhibited that I/R+UD treatment significantly reduced MDA, NO, and 3-NT contents and enhanced SOD and CAT contents compared with the I/R+saline group ($p<0.05$). The MPO level of group I/R+UD dropped compared to group I/R+saline, but there was no statistically significant difference ($p>0.05$) (Figure 1).

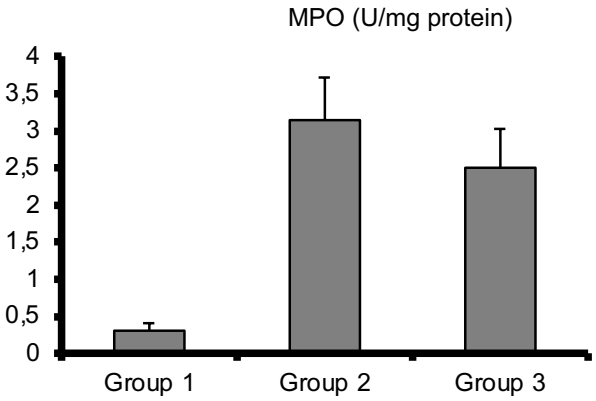

FIGURE 1 - The level of MPO in groups. [a]: p<0.05 compared to group 1 (control group).

TABLE I - The levels of oxidative/nitrosative stress biomarkers in groups

	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD
CAT (U/mg protein)	0.812 ± 0.128	0.271 ± 0.104 [a]	0.473 ± 0.100 [ab]
SOD (U/mg protein)	2.882 ± 0.705	1.216 ± 0.236 [a]	2.028 ± 0.280 [ab]
MDA (nmol/mg protein)	1.841 ± 0.428	5.202 ± 0.419 [a]	3.490 ± 0.335 [ab]
NO (µmol/mg protein)	14.070 ± 3.952	47.251 ± 9.090 [a]	32.820 ± 8.796 [ab]
3-NT (nmol/mg protein)	40.111 ± 8.131	167.889 ± 22.779 [a]	120.778 ± 24.914 [ab]

[a]: p<0.05 compared to group 1 (control group). [b]: p<0.05 compared to group 2 (I/R+saline group).
*SD: standard deviation

Histopathological Findings

Histopathological findings of the study are shown in Figures 2 (A, B, and C). These results indicated that pathological changes did not occur in muscles tissues of group 1. There was necrosis, congestion, or inflammation findings (Figure 2A). Muscle necrosis and congestion of the sinusoids and central vein were evident in group 2 (Figure 2B). In group 3, Tissue with UD appeared similar to group 1 (Figure 2C). In addition, tissue damage in group 3 was lower than in group 2.

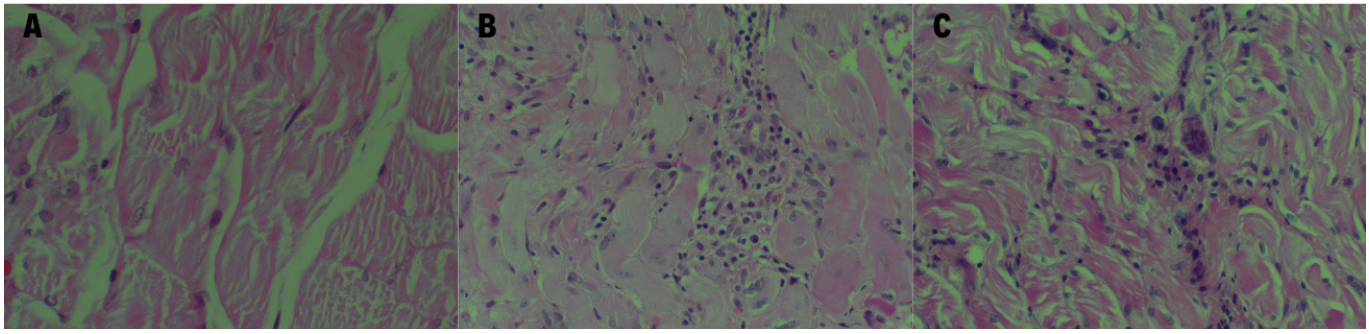

FIGURE 2 - Light microscopy of muscle tissues of experimental groups for H&E staining. (A) In control rats, normal muscle structure was seen. (B) After I/R, severe muscle damage was detected. (C) Therapy of UD-ameliorated muscle tissue injury. (H&E, scale bar, 50 µm).

Ischemia was used for a different purpose. It was aimed to diminish bleeding during the operation and injury. Biochemical changes occurred in the ischemic tissue either during the ischemia or reperfusion phase. Some studies demonstrated that hypoxia causes the inhibition of mitochondria respiratory chain enzyme and increases xanthine oxidase enzymes (Seyama, 1993; Opie, 1990; Repine, 1991). This enzyme stimulates the production of ROS in ischemic tissue. Cell damage commonly occurs during the reperfusion phase (Seyama, 1993; Erdogan *et al*., 1999; Kirshenbaum, Singal, 1993; Kurutas, 2015). Some studies reported the relationship between myocardial injury and lipid peroxidation. It was shown that oxygen-free radicals have not changed or decreased during the ischemic period but increased significantly during the reperfusion period (Kirshenbaum, Singal, 1993; Dhaliwal *et al*., 1991). This study underlines three points: firstly, the emergence of ROS/RNS that arise after muscle I/R and cause injury can be prevented by UD; (Akcil, Tug, Doseyen, 2000); secondly, the increase in MPO that is an indication of tissue damage in muscle I/R injury is lower after the UD treatment; (Seyama, 1993); and thirdly, in the early stages of muscle I/R injury, histopathological examination of muscle tissue shows considerably less muscle injury with the UD treatment.

ROS/RNS have a significant mediator role in I/R injuries of several organs (Lindsay, Romaschin, Walker, 1989*;* Molyneux, Glyn, Ward, 2002; Ortadeveci, Öz, 2017). Some research suggests that antioxidant molecules may provide protection from I/R injury (Grieve, 1981; Tahri *et al*., 2000; Sökeland, 2000). UD is known to be a potent antioxidant, breaking up free radicals. For this reason, it is expected to be protective in muscle I/R injury of rats (Kirshenbaum, Singal, 1993; Kurutas, 2015; Opie, 1990). Studies of UD have used different doses and methods of application. So far, they have not agreed upon a standard dose. UD is given internally in most studies (Repine, 1991, Guilpain *et al*., 2008). We used a total dose of 2mL/kg administered via gavage (Kirshenbaum, Singal, 1993; Repine, 1991; Salvemini, Cuzzocrea, 2002). In order to decrease I/R injury, some studies administered the drug only before ischemia or after reperfusion, but others have given it for both ischemia and reperfusion (Kirshenbaum, Singal, 1993; Lindsay, Romaschin, Walker, 1989; Sökeland, 2000). We preferred in our study to use 2 ml/kg UD (1 mL/kg before ischemia and 1 mL/kg after reperfusion). We aimed to benefit from a prophylactic effect by attempting to reach a definite tissue level of UD before we created ischemia and to detect the protective effect on immediate damage that will occur after reperfusion.

Oxidative/nitrosative stress plays a crucial role in the tissue damage of I/R, and the reduction in this negative effect by antioxidant therapies were shown in experimental studies published recently (Beutler, 1975). Lipid peroxidation is known to play an essential role in damage to the cell membrane through reactive oxygen radicals. I/R toxicity was shown to increase MDA levels, a necessary index of lipid peroxidation, in experimental studies and this increase was suppressed by antioxidant therapies (Ohkawa, Ohishi, Tagi, 1979; Armstrong, Browne, 1994). Besides their direct, damaging effects on tissues, free radicals seem to trigger the accumulation of leukocytes in the tissues involved and thus aggravate tissue injury indirectly through activated neutrophils. The activated neutrophils located in the inflammatory foci and secreting MPO into the extracellular space were demonstrated to convert hydroperoxides into free radicals, initiating lipid peroxidation (Sayhan *et al*., 2012). MPO is an enzyme found predominantly in neutrophils and used as an effective quantitative index of inflammation. In our study, elevated MPO activity, MDA, NO, and 3-NT levels in group 2 tissue may indicate that neutrophil accumulation and lipid peroxidation contributes to I/R-induced oxidative/nitrosative muscle injury. Hence, the results also suggest that UD has a preventive effect by suppressing neutrophil infiltration and lipid peroxidation. Only one study on the protective effects of UD on the muscle was reported after I/R injury occurred. Cetinus *et al.* (2005) measured only MDA levels to determine whether or not protective UD on muscle I/R.

In our study, a histopathological examination detected no pathological changes in group 1. Particularly severe necrosis, moderate inflammation, vacuolar degeneration, and vascular congestion were seen in group 2. Analysis of group 3 showed that the pathological changes present in group 2 almost completely disappeared. These results indicate that UD has a protective effect on muscle damage created with I/R. Sayhan *et al.* (2012) reported that UD

treatment had a protective effect against renal damage induced by renal I/R. This protective effect is possibly due to its ability to inhibit I/R induced renal damage, apoptosis and cell proliferation (Sayhan *et al*., 2012). Shackebaei *et al.* (2010) reported that the aqueous extract of UD significantly increased the tolerance of isolated rat hearts against damage caused by I/R. Moreover, regarding the cardioprotective effect of UD extract, the consideration of its clinical usage is suggested, and a complementary study of its cellular effect is warranted (Shackebaei *et al*., 2010).

Treating diseases using plants dates back to ancient times. Various antioxidants were given in experimental rats or humans on I/R status. Some studies indicated positive effects of antioxidants such as allopurinol, SOD, carnitine, nicotinic acid vitamins C and E on I/R status (Karakaya, El, Taş, 2001; Pieroni *et al*., 2002; Molyneux, Glyn, Ward, 2002). UD was experimentally used on humans and animals in various illnesses. UD has good diuretic, natriuretic, hypotensive, antioxidant, anti-inflammatory, antirheumatic and antiprostatic effects (Cetinus *et al*., 2005; Sökeland, 2000; Sayhan *et al*., 2012; Shackebaei *et al*., 2010). We believe that antioxidants like UD (exogenous) may cause a decrease the cell damage by oxidative/nitrosative stress.

CONCLUSION

This study had some limitations. Due to the study protocol, muscle I/R-injured rats were sacrificed just after reperfusion to observe the early effects of UD. If we could have kept the rats alive, we could also have observed the long-term effects of UD in muscle I/R injury. We expect that further studies on the long-term effects of UD will enhance the value of our positive findings. In addition, it is crucial to examine the possible ability of UD to reverse I/R-induced damage in muscle tissue and its effects on levels of antioxidant enzymes.

In conclusion, it was found that UD increased the antioxidant ability and decreased oxygen free radicals in the early period of muscle I/R injury in rats. Also, evaluation of histopathological findings of muscle tissue indicated that UD had beneficial effects on muscles. Hence, UD can be considered a preventive treatment agent in muscle I/R injury. As this study does not contain information about the long-term results of UD treatment of muscle I/R injury, further experimental and clinical studies are needed.

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