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RESEARCH ARTICLE

THE EFFECT of OMEGA 3, 6,9 and STEARIC ACID on TRACE ELEMENTS in ISCHEMIA/REPERFUSION-INDUCED HEART TISSUE in a RAT HIND LIMB MODEL

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ABSTRACT

In this study, the effect of omega fatty acids (3, 6, 9) and stearic acid on some trace elements (Cu, Mn and Zn) in heart tissue, which is a distant organ, by creating ischemia/reperfusion in the hind legs of rats was investigated. Animals were divided into ten groups: Control (C), I/R, I/R+Omega 3, I/R+Omega 6, I/R+Omega 9, I/R+stearic acid, omega 3, omega 6, omega 9 and stearic acid groups. I/R was applied to the right hind legs of I/R, I/R+omega 3, I/R+omega 6, I/R+omega 9 and I/R+stearic acid groups under anesthesia. In this study, the levels of Cu, Mn and Zn elements were studied in samples obtained from heart tissue in animal models divided into ten groups using an inductively coupled plasma optical emission spectroscopy (ICP-OES) device. Compared to the control group, heart tissue's Cu and Zn levels were low, Mn levels were high but not important in the ischemia group ($p>0.05$). When ischemia and omega 3, 6, 9 and stearic acid applied groups were compared with the ischemia group, heart tissue's Cu level was found to be low and Mn level was higher, but this result was not important ($p>0.05$). Compared to the ischemia group, heart tissue's Zn levels were found to be notably higher in omega 6+IR, omega 9+IR and stearic acid groups ($p<0.05$). As a result, I/R application changes trace element levels in heart tissue, which is a distant organ. In the case of I/R, omega fatty acids and stearic acid treatment may provide a protective effect by improving trace element levels.

Keywords: *Omega fatty acids, Stearic acid, Heart tissue, Rat, Trace elements*

1. INTRODUCTION

The term ischemia/reperfusion (I/R) is described as the restoration of blood circulation to certain organs after blood supply has been impaired for a certain period of time. IR injury is a common and important clinical problem affecting many organs, especially the brain (stroke and head injury), heart (myocardial infarction) and skeletal muscle. Skeletal muscle has high metabolic activity and is for this reason sensitive to reperfusion damage after ischemia. I/R damage to skeletal muscle can cause severe injury to the extremities, including severe necrosis major to amputation and severe life-threatening necrosis [1]. Inflammatory variables seen in patients under chronic ischemia and surgical treatment may change the concentrations of macro and trace elements in the blood [2]. While copper (Cu) is necessary to maintain the structure and function of some proteins and antioxidants, it also affects the development of atherosclerosis. Marginal copper intake or deficiency has been suggested as a risk factor for cardiovascular illnesses, and serum copper concentration has been found to increase in

atherosclerosis obliterans [3]. Zinc (Zn) is one of the most abundant trace elements in our organism. In addition to growth, improvement, differentiation, immune response and receptor activity, Zn functions as a component of more than 300 enzymes [4]. Disruption of Zn homeostasis, which is a necessary trace element required for normal cellular structure and functions, is associated with various health problems including cardiovascular diseases. Both exogenously and endogenously released zinc may be effective in cardiac protection after ischemia/reperfusion injury [5]. Trace elements are necessary for the proper function of living organisms. Some elements may have a preservative impact on target organs during ischemia and reperfusion [6-11] and have a favorable role in stabilizing organ protection solutions [12-14]. Manganese (Mn) has important roles in protein, polysaccharide and cholesterol metabolism, fetal development and lactation, as well as hydrolases, transferases and kinases [15]. It is an integral compound of Mn-SOD, an antioxidant enzyme that converts the superoxide radical to hydrogen peroxide (H_2O_2). Therefore, manganese superoxide dismutase has protective properties especially against oxidative stress. Manganese deficiency causes respiratory system diseases, nervous system diseases and infertility in humans (16). In a study, it was revealed that the increase in Mn-SOD activation mediated by free radical production during hyperthermia is important in providing early phase and late phase cardio preservation against ischemia/reperfusion damage in rats [17].

Polyunsaturated fatty acids (PUFAs) are necessary for many biochemical cases. Although omega-3 and omega-6 fatty acids come from the same origin, they have opposite physiological effects. A-linolenic acid (ALA), docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) are essential omega-3 fatty acids. Dietary DHA deficiency results in elevated levels of zinc in the hippocampus and overexpression of the putative zinc transporter ZnT3 in the rat brain [18].

Ischemia causes devastating health and economic burdens brought about by disorders characterized by reduced organ-specific blood flow. Mechanisms and treatments underlying tissue damage caused by ischemia have been studied for many years to reduce these. Therefore, in our work, we wanted to determine the effects of omega 3, omega 6, omega 9 and stearic acid (SA) fatty acids on some trace element levels in heart tissue after experimental limb ischemia reperfusion. Our aim in the study is to examine the possible usability of some elements in rats with hind limb ischemia reperfusion model.

2. MATERIALS and METHODS

2.1. Animal Material

67 Wistar albino female rats (200-250 gr and 6-7 months old) were obtained from Experimental Animals Production Center. It was housed in standard conditions with a 12-hour light-dark cycle and free access to water and food was provided. All experimental studies were started in line with the animal ethics committee guidelines and after the approval of the Van Yüzüncü Yıl University Experimental Animals Local Ethics Committee (YÜHADEK) dated 28/04/2022 and numbered 2022/04-05.

2.2. Experimental Procedures

Rats were separated into ten different groups in accordance with the procedures to be performed. Fatty acids (omega 3, 6, 9) and stearic acid were given to rats orally daily for 14 days in the amount of 300 mg/kg [19]. Two hours of ischemia-2 hours of reperfusion was performed over the leg (quadriceps muscle) under anesthesia in rats that underwent ischemia-reperfusion procedure [20].

JSR A
Journal of Scientific Reports

Rats divided into 10 different groups were arranged as follows: 1. Group: Control group: The rats in this group were fed normally (standard rat pellet food and water) for 14 days without any treatment. 2.Group: Ischemia-reperfusion group: At the end of the 14-days ad libitum feeding with standard rat pellet food and water, 2 hours of reperfusion under anesthesia was applied to the leg (quadriceps muscle) with a tourniquet after 2 hours of ischemia. 3. Group: Omega-3 group: Daily 300 mg/kg Omega 3 (O-3) was given by gastric gavage for 14 days. 4.Group: Omega-6 group: Daily 300 mg/kg Omega 6 (O-6) was given by gastric gavage for 14 days. 5. Group: Omega-9 group: 300 mg/kg Omega -9 (O-9) was given daily for 14 days by gastric gavage. 6. Group: Stearic acid group: 300 mg/kg stearic acid (SA) was given daily by gavage for 14 days. 7. Group: Omega-3+ischemia-reperfusion group: 300 mg/kg Omega-3 (O-3) was given daily for 14 days by gavage. At the end of the 14th day, 2-hour ischemia-2 hours reperfusion under anesthesia was administered to the rats on the leg (quadriceps muscle). 8.Group: Omega-6+ischemia-reperfusion group: 300 mg/kg O6 per day was given by gavage and ischemia reperfusion was applied on the 14th day. 9. Group: Omega-9+ischemia-reperfusion group: 300 mg/kg Omega-9 (O-9) was given daily for 14 days by gavage. At the end of the 14th day, 2 hours ischemia-2 hours reperfusion under anesthesia was applied to the rats on the leg (quadriceps muscle). 10. Group: Stearic acid+ischemia-reperfusion group: 300 mg/kg stearic acid (SA) was given daily by gavage for 14 days. At the end of the 14th day, 2-hour ischemia-2 hours reperfusion under anesthesia was administered to the rats on the leg (quadriceps muscle).

2.3. Preparation of Tissues

After the procedures applied for 14 days, the rats were euthanized and their heart tissues were surgically separated and placed in ziplock bags and stored in the freezer at -80^{o}C until experimental studies were conducted. Tissue samples taken from the hearts of rats were dried at 105^{o}C for 1 hour and weighed correctly and placed in 25 mL experimental tubes. Then, nitric acid and perchloric acid were added in a 1:1 ratio to dissolve the solid form. The solution was digested thoroughly using a microwave digestion oven. After digestion, the samples were cooled at room temperature. 1 ml of the cooled samples was taken and diluted by adding 9 ml of distilled water. Analysis of Cu, Mn and Zn elements in digested samples was carried out. The analyzes were measured using the ICP-OES (ICP-OES, Thermo ICAP 6300 DUO Scientific) device at Van Yüzüncü Yıl University Science Application Center. The device was calibrated using standard solutions.

2.4. Statistical Analysis

All data were given as mean±standard error and statistical analysis was performed with SPSS 20.00 (SPSS Inc, Chicago, IL) package program. Groups were compared by using one-way analysis of variance followed by post hoc Tukey Test.

3. RESULTS

In the study, the levels of Cu, Mn and Zn elements were determined in the heart tissues of rats fed with different fatty acids for 14 days.

Table 1. Heart tissue Cu, Mn and Zn levels of all groups.

	Cu (ppm)	Mn (ppm)	Zn (ppm)
Control	0,122±0,082 $^{a, b}$	0,020±0,007 ^{a,b}	0,687±0,107 ^{c}
Ischemia	0,141±0,086 ^{a}	0,014±0,004 ^{b,c}	0,713±0,063 ^{c}
Omega3+IR	0,053±0,020 ^{c}	0,016±0,004 ^{a,b,c}	0,691±0,042 ^{c}
Omega6+IR	0,080±0,034 $^{a, b,c}$	0,016±0,002 ^{a,b,c}	1,176±0,484 ^{a}

Omega9+IR	0,101±0,021 [a, b,c]	0,016±0,004 [a,b,c]	1,010±0,255 [a, b]
SA +IR	0,069±0,012 [a, b,c]	0,021±0,004 [a]	1,194±0,457 [a]
Omega3	0,079±0,035 [a, b,c]	0,012±0,003 [c]	0,774±0,150 [b, c]
Omega6	0,047±0,015 [c]	0,015±0,004 [a,b,c]	0,749±0,074 [b, c]
Omega9	0,113±0,060 [a, b,c]	0,016±0,004 [a,b,c]	0,734±0,045 [c]
SA	0,069±0,012 [b,c]	0,014±0,001 [c]	0,822±0,149 [b, c]

*, different letters in the same line denote statistical significance
Note: Different characters in the same column indicate statistical significance.

The heart tissue Cu level in the ischemia group was higher than all groups, but it was found to be significant only when compared with the omega 6, Omega 3+IR and Stearic acid groups ($p<0.05$). The heart tissue Cu level of the control group was found to be remarkably higher than that of the omega 6 group ($p<0.05$).

Heart tissue Mn levels in the control group and stearic acid+IR group were found to be significantly higher than omega 3 and stearic acid groups ($p<0.05$). The heart tissue Mn level in the stearic acid+IR group was found to be significantly higher than the ischemia group ($p<0.05$). While the heart tissue Mn level in the ischemia group was lower than the control group, this decrease was not significant ($p>0.05$)

Although the heart tissue Zn level in the ischemia group was higher than the control group, this was not significant ($p>0.05$). The heart tissue Zn level in the ischemia group was found to be significantly lower than the Omega 6+IR, Omega 9+IR and stearic acid+IR groups ($p<0.05$). While the heart tissue Zn level was higher in the omega 3, omega 6, omega 9 and stearic acid groups than in the control and ischemia groups, this elevation was not meaningful ($p>0.05$).

4. DISCUSSION

Ischemia is described as insufficient systemic blood providing to a local tissue because of occlusion of the vascular system in that tissue; these cases are among the most widespread reasons of heart attack and stroke in humans [22–24]. Reperfusion is described as restoring blood flow to a previously absent tissue; restoration of nutritional reinforcement to previously starved areas underlies many important pathogenic manifestations [23]. Thanks to ischemia-reperfusion studies in experimental animals, it has become easier to understand the molecular and physiological changes accompanying stroke and heart attack [26, 21–25]. Although IR can occur in every tissue, its harmful effect on skeletal muscle is much more severe compared to other body tissues due to devastating systemic complications [27].

Polyunsaturated fatty acids (DHA), are essential for human metabolism but cannot be produced *de novo*. Therefore, people have to take these fatty acids from the diet [28]. The levels and composition of these substances can be determined directly by diet or by dietary intake of fatty acid precursors. Fatty acid precursors are converted endogenously into physiologically active long chain polyunsaturated fatty acids by extension and desaturation by fatty acid desaturase enzymes. Reports have shown that dietary DHA supplementation has a protective effect on retinal ischemia damage and oxidative damage after post-ischemic oxidative stress in fetal rat brain [21, 22]. Evidence of interaction between trace elements and essential fatty acid (EFA) metabolism has been revealed in studies [29]. For example, it has been shown that zinc may be needed as a cofactor in desutarase 6 (Δ6) enzyme activity in rats [30]. According to the data obtained in a study, especially plasma EFA

composition shows that plasma zinc, calcium and magnesium levels are related [31]. EFAs are significant in the absorption of zinc, calcium or magnesium. Therefore, trace elements appear to make an important contribution to fatty acid metabolism [32, 33].

Zinc is very plentiful trace element in the body system after iron and involved in many physiological processes such as neuronal death, immunity and cancer [34]. In animal studies, it has been shown that Zn deficiency causes atherosclerosis by causing the release of proatherogenic factors in mice [35]. It has been shown that ischemic stroke increases the serum Zn level [36]. Some studies suggest that although zinc is not the initiator of damage, changes in its levels can lead to the damage process [37]. Other studies have suggested that it may cause excessive zinc release from neurons in stroke, as in irregularities occurring in neurotoxicity [38, 39]. De Paula et al., [40] stated that massive and transient zinc accumulation during cerebral ischemia is importantly related in brain injury by promoting neuronal apoptotic death, and therefore they suggested that zinc removal may be a way to reduce ischemic brain injury. Akçıl et al., [41] showed that the plasma zinc level of wistar albino rats increased significantly who underwent ischemia with 30 minutes clamping of the superior mesenteric artery followed by 20 minutes of reperfusion when compared to the control group. It has been demonstrated that the increase in plasma Zn concentration can be attributed to severe tissue damage and peroxidant and antioxidant properties of Zn, considering the size of the affected area after ischemia-reperfusion. In another study, it was shown that serum Zn level decreased in the I/R group when compared to the control group [42]. Zn is also very significant in cell viability as it has antioxidant, antiapoptotic and anti-inflammatory effects. The increase in plasma zinc level after ischemia may be the result of its anti-atherogenic properties preventing vascular endothelial derangements and its role in signaling pathways involved in apoptosis. The role of Zn in ischemia as a cytoprotective by reducing the formation of oxygen radicals has also been suggested [43]. In the present study, although the heart tissue Zn level in the ischemia group was higher than the control group, this highness was not significant ($p>0.05$). The heart tissue Zn levels of the groups in which ischemia was induced and omega 6, omega 9 and steraic acid were applied were found to be higher than the ischemia group, but only omega 6+IR and stareic acid+IR groups were found to be significantly higher ($p<0.05$). In our study, a significant increase was observed in rat heart tissue Zn levels with omega 6, omega 9 and stearic acid treatment and subsequent ischemia application to rats. It can be said that this treatment protects the tissues by increasing the antioxidant, antiapoptotic and anti-inflammatory properties of Zn.

Copper is an essential element for biological systems. The redox cycle between Cu^{+1} and Cu^{+2} catalyzes the production of highly toxic hydroxyl radicals [42]. Cu is also a metal cofactor of various enzymes. When rats with superior mesenteric artery I/R were compared with the control group, it was indicated that the plasma copper level increased significantly. Increased plasma copper level may be the cause of excessive tissue damage and a prooxidant characteristic [34]. In experimental animal studies, it has been shown that copper levels increase due to oxidative stress in I/R models [44,42]. Increased Cu concentration in serum in lower extremity ischemia may result from the acute phase response [43]. In the presented study, the heart tissue Cu level in the ischemia group was seen to be higher than in all other groups, but it was found to be significantly higher only in the omega 6 and omega 3+IR groups ($p<0.05$). Ischemia and the application of omega 3, omega 6, omega 9 and stearic acid treatment caused a decrease in the heart tissue Cu level. The increase in copper level in the ischemia group may have increased the acute phase reaction and the treatment may have prevented the acute phase response. According to the data obtained, it can be concluded that omega 3, omega 6, omega 9 and staeric acid applications may be beneficial in ischemic conditions.

Manganese superoxide dismutase (Mn-SOD) is an enzyme that protects mitochondria, an important organelle for cellular respiration, from reperfusion damage and limits mitochondria-associated apoptosis. It has been shown that plasma Mn levels increase after I/R injury, but this increase is not significant [41]. In one study, they observed that rised activity of mitochondrial Mn-SOD during I/R damage is important in mitochondrial protection and reduction of apoptosis [45]. In another study, Mn-SOD activity was seen to be significantly reduced by I/R administration to the kidney of rats that underwent 45 minutes of ischemia followed by 3 hours of reperfusion [46]. It has been described that changes in the calcium, potassium, magnesium, sodium, and phosphorus content of the liver in rats undergoing hepatic ischemia-reperfusion are associated with injury to cell membranes, which can be confirmed by the quantity of diene conjugates produced [47]. In rats undergoing a liver ischemia/reperfusion model, pretreatment of all-trans retinoic acid (atRA) has been demonstrated to reduce liver I/R injury by inhibiting malondialdehyde (MDA) release and increasing the activity of manganese superoxide dismutase (Mn-SOD) [48]. In the present study, the heart tissue Mn level of the ischemia group was found to be significantly lower than the control group ($p<0.05$). Performing ischemia and applying omega 3, omega 6, omega 9 and stearic acid treatment caused an increase in the heart tissue Mn level, and the heart tissue Mn level in the group that was treated only with stearic acid was seen to be significantly higher than the ischemia group ($p<0.05$). The treatments applied may have caused an increase in the antioxidant level due to the increase in the heart tissue Mn level.

These results show that ischemia/reperfusion injury causes significant changes in trace element concentrations in heart tissue, which is a distant organ. In line with the data we have obtained, it can be concluded that trace element levels can be corrected with omega fatty acids and stearic acid applications in case of ischemia.

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ATTACHMENTS

VAN YÜHADYEK
VAN YÜZÜNCÜ YIL ÜNİVERSİTESİ
Hayvan Deneyleri Yerel Etik Kurulu

ÇALIŞMA ONAY BELGESİ

VAN YUZUNCU YIL UNIVERSITY (TURKEY)
ANIMAL RESEARCHES LOCAL ETHIC COMMITTEE
APPROVAL CERTIFICATE

Araştırmanın Adı: ***Research Title:***	Şıçan arka bacak iskemi/reperfüzyona bağlı indüklenen kalp dokusunda eser elementler üzerine omega 3, 6,9 ve stearik asitlerin etkisi Effect of omega 3, 6,9 and stearic acids on trace elements in rat hind limb ischemia/reperfusion-induced heart tissue.	
Araştırıcı(lar): ***Investigator(s)***	**Yürütücü / *Chief investigator:***	Dr. Öğr. Üyesi Tuğba Gür
	Yardımcı Araştırıcı(lar) / *Co-investigator(s):*	
Araştırmada kullanılacak hayvanlar / *Animals to be used in the research:*		
Tür / species: *Rat*	**Sayı / *Numbers:* 67**	
Yaş /*Age: 8-12 hafta/weeks*	**Cinsiyet / *Sex:* Dişi/Female**	
Araştırmanın Öngörülen Başlama Tarihi / *Proposed Research Starting Date:01.05.2021*		
Araştırmanın Öngörülen Bitiş Tarihi / *Proposed Research Completion Date:02.06.2022*		

Karar:
Yukarıda bilgileri verilen planlanan araştırma projesi için Hayvan Deneyleri Etik Kurul Onayı gerekmemektedir.
Tarih: 28/04/2022 Karar No: 2022/04-05
Decision:
The proposed research project detailed above does not need Animal Researches Ethic Committee Approval. Date:.
28/04/2022 *Decision number:* 2022/04-05

	BAŞKAN/CHAIR Prof. Dr. Semiha DEDE	
ÜYE/Member Prof. Dr. N. Tuğba BİNGÖL	**ÜYE/Member** Prof. Dr. Sıddık KESKİN	**ÜYE/Member** Prof. Dr. Nalan ÖZDAL
ÜYE Prof. Dr. Atilla DURMUŞ	**ÜYE/Member** Doç. Dr. Ferda KARAKUŞ	**ÜYE/Member** Doç. Dr. Yıldıray BAŞBUĞAN
ÜYE/Member Doç. Dr. Canser Yılmaz DEMİR	**ÜYE/Member** Doç. Dr. Abdulahad DOĞAN	**ÜYE/Member** Dr. Öğr. Üyesi Dicle ALTINDAL
ÜYE/Member Dr. Öğr. Üyesi Şükrü ÖNALAN	**ÜYE/Member** Vet. Hek. İsmail Hakkı BEHÇET	**ÜYE/Member** Zir. Müh. Kenan YILDIRIMOĞLU