Effect of zinc sulfate supplementation on metallothionein levels in rat heart tissue with induced ischemia-reperfusion injury

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ABSTRACT
Metallothioneins are remarkable proteins with regard to their role in the regulation of intracellular zinc balance and mediation of the physiological effects of zinc, as well as their antioxidant effects. The objective of the present study is to examine how zinc supplementation impacts metallothionein levels in ischemia-reperfusion injury in rat heart tissues obtained from 30 Wistar-Albino adult male rats already used in another project. Experimental animals were equally divided into three groups as follows: Group 1: Control (H-Cont); Group 2: Heart Ischemia/Reperfusion (H-I/R); and Group 3: Zinc supplemented Ischemia/Reperfusion (H/Zn-I/R). Cardiac ischemia-reperfusion procedures were carried out under general anesthesia. In the zinc-supplemented cardiac I/R group (Group 3), the animals were supplemented with 5 mg/kg/day intraperitoneal (i.p.) zinc sulfate per 21 days. At the end of the procedures, all animals were decapitated and heart tissues were collected. The tissues were subjected to immunohistochemical staining procedures using rat metallothionein antibody. The stained preparations were photographed and cells stained with metallothionein were counted at a light microscope to calculate their percentages. The analyzed heart tissue samples of the groups did not have any significant difference in terms of their metallothionein levels. Results obtained from the study indicate that 21-day zinc supplementation does not have a critical effect on metallothionein synthesis in cardiac ischemia-reperfusion. This result may be attributed to the dose of zinc, length of supplementation and/or duration of ischemia-reperfusion.

Keywords: Cardiac ischemia-reperfusion, zinc supplementation, metallothionein, rat

1. INTRODUCTION

Having a critical part in the antioxidant system, zinc is described as the versatile antioxidant. The centrality of zinc in the antioxidant system has been shown in both animal and cellular experiments through the increased oxidative stress in zinc deficiency. In both animal and cell models, zinc deficiency causes oxidative stress and leads to changes in the antioxidant enzyme levels [1,2]. Besides, copper-zinc superoxide dismutase (Cu-Zn SOD), as an antioxidant enzyme, is a major scavenger of reactive oxygen species (ROS). Although copper is important for the catalytic activity of this enzyme, zinc plays a structural role in it and is necessary for its functioning [3]. Cu-Zn SOD activity was reported to be lower in zinc deficiency. The critical roles that zinc plays in the antioxidant system can be seen in its stabilizing the protein that it is bound to against oxidation due to lack of redox activity, the impairment of GSH metabolism, and increased the vulnerability of cells to oxidative stress in zinc deficiency [4,5]. Hence, zinc is considered a component of the antioxidant system at many cellular levels. Zinc also serves an indirect function in the antioxidant defense system. By binding to metals through its pro-oxidant activity, zinc acts as an antioxidant that stimulates MT synthesis which can eliminate hydroxyl radicals and oxygen radical [6]. The protein family rich in cysteine groups and described as low-molecular-weight MTs plays critical roles in a number of physiological and pathological processes, including particularly oxidative stress [7]. MTs bind heavy metals and prevent the damage they can cause, catch free radicals like the hydroxyl radical, superoxide anion and peroxynitrite, and inhibit the DNA damage caused by hydroxyl radical and superoxide anion. This effect of MTs is 800 times greater than that of GSH [8]. Furthermore, binding zinc with high affinity, MTs function as a major intracellular zinc reserve. When intracellular free zinc is needed, MT releases zinc and thus mediates its unique physiological roles [9]. MT expression is induced by elevated zinc and thus, zinc homeostasis is maintained. Besides its strong radical catching character, its mediation of the effects of zinc point to the importance of MT in the prevention of oxidative stress.

In our study, it is aimed to examine how zinc supplementation to rat heart tissue with induced ischemia/reperfusion affects the metallothionein levels in the heart.

2. EXPERIMENTAL SECTION

2.1. Experimental Animals. The study included the heart tissues of rats which had been used in a previous project which had been approved by the ethics committee. For the tissues to be used, Ethics Committee Report for Research Involving Non-Living Animals was taken from the Ethics Committee of Selcuk University’s School of Veterinary Medicine.
2.2. Grouping of Animals. The rats to be used in the study were randomly grouped and were subjected to the following procedures:
Group 1: Heart Control Group (H-Cont, “n:10”): The rats in this group were not subjected to any procedure. General anesthesia was induced with 1.2-1.4 gr/kg intraperitoneal urethane. Heparin was injected and hearts were rapidly removed.
Group 2: Heart I/R Group (H-I/R, “n:10”): The rats in this group did not receive any supplementation.
Group 3: Heart Zinc I/R Group (H/Zn-I/R, “n:10”): The rats in this group were supplemented with 5 mg/kg zinc sulfate through an intraperitoneal injection daily for 21 days.

2.3. Feeding of Experimental Animals. The experimental animals were fed in special steel cages which were washed clean daily. Feed was given in special steel bowls and water (normal tap water) in glass feeding bottles. The animals were provided approximately 10 gr of feed per 100 gr of their body weight daily. They were kept in a medium with standard room temperature (21±1°C) and 12-hour light/dark cycle. At the end of experimental procedures, blood and heart tissue samples were collected from all animals to be used in analyses. The blood and heart tissue samples were stored at -80°C until the time of analysis.

2.4. Surgical Procedures. The rats in Groups 2 and 3 were placed under general anesthesia with the injection of sodium pentobarbital (50 mg/kg, i.p.). The induction of anesthesia was followed by trachea cannulation and connection of rats to a respiratory pump. After making an incision of about 1 to 1.5 cm in length on the right side of the breast, the heart was rapidly removed. Applying light pressure with fingers, the right thoracotomy was performed. After making an incision of about 1 to 1.5 cm in length on the left side of the breast, the heart was rapidly removed, and cutting the fourth left costa on the left side of the sternum, left thoracotomy was performed. Since the internal negative balance is lost as soon as the thorax is opened, positive pressure respiration was started by supplying the air in the room with a 1,5 ml/100 gr volume and at a rate of 60 pulses/minute through a ventilator (Harvard Animal Rodent Ventilator) in order to ensure that respiration continues, and pCO₂, pO₂ and pH values are protected. After the pericardium was pushed gently aside, the heart was freed. Applying light pressure on the right side of the breast, the heart was taken out. A 10 mm, round-tipped needle and 6/0 silk was used to rapidly suture the bottom of the coronary artery including a small part of the myocardial tissue. The heart was placed back into the chest, and 20 minutes were left to pass for stabilization. The edges of the silk suture were passed through a tiny plastic tube 1 mm in diameter and 1 cm in length. At the end of the 20-minute stabilization period, the silk placed at the bottom of the vessel was pulled using the plastic tube and a clamp to induce occlusion. After 30 minutes of ischemia, the clamp was removed, loosening the silk passed through the tube to restore blood supply, that is reperfusion. Reperfusion was continued for 120 minutes. Before ending the experiment, the rats were injected with heparin and their hearts were rapidly removed.

2.5. Evaluation of Hemodynamic Parameters. Systolic (SBP) and diastolic (DBP) blood pressure were measured directly using a transducer and recorder through a cannula placed on the carotid artery. Additionally, 40% of SBP and 60% of DBP were added to calculate the mean blood pressure (MBP). When evaluating the blood pressure of the rats, basal values in the period between the insertion of the cannula and drug administration, values after drug administration, values at the beginning and end of ischemia, as well as values before and after reperfusion were all used. Heart rate and ECG findings during surgical procedures were also recorded.

2.6. Zinc Sulfate Supplementation. Of the zinc sulfate (ZnSO₄·7H₂O), 0.89 gr was weighed and completed to 250 ml by adding serum physiologic. The animals in group 3 were administered 5 mg/kg intraperitoneal zinc sulfate per rat for 21 days.

2.7. Immunohistochemical Staining Procedure. Immunohistochemical staining procedures were carried out at the Pathology Department of Necmettin Erbakan University’s Meram School of Medicine. Following the surgical operation, cardiac tissue samples collected from the rats were placed in 10% formaldehyde buffer for fixation. After fixation, samples collected from the same sites as much as possible were put into cassettes and placed in the automatic tissue processor (Leica ASP300 S-Tissue Processing). Then the samples were buried into paraffin blocks. Cross-sections of 5 µm were obtained from the blocks using a microtome (Leica RM 2025 microtome) and placed on lysine-coated slides. One sample from each preparation was stained with hematoxylin& eosin for histological evaluation. Other preparations were left to wait in the drying oven at 60°C overnight. Then the preparations were subjected to standard immunohistochemical staining in an automated slide processing system (VENTANA BenchMark XT Automated Slide Processing System) using a DAB Detection Kit (Ref 760-500 LOT CD1520 ROCH 05269806001). For the primary antibody staining of metallothionein, Monoclonal Primary Metallothionein Antibody (clone E9; DAKO Copenhagen, Denmark) diluted at the ratio of 1:100 was incubated. After staining, the slides were washed in two alcohol series and xylene, mounted with balsam, and covered with glass.

2.8. Photographing of Preparations and Evaluation of Immunoreactivity. All stained preparations were examined under an Olympus DP 72 (U-TV0.63XC; OLYMPUS, Tokyo, Japan) light microscope.

Figure 1. Calculation of the area magnified 20 times in cardiac tissue samples using the Clemex Vision Lite 3.5 image analysis system and labelling and counting of MT-stained myocytes. The black arrow shows MT-stained cells.

While doing the examination, images of the preparations were obtained with the Olympus DP 72 (U-TV0.63XC;
3. RESULTS SECTION

The number of positively stained cells in the cardiac preparations was fairly low (total mean 0.06 ± 0.04) and there was not any color intensity difference in staining. Therefore, without considering the color intensity in the tissue, total MT positive cells in the identified area were counted and by calculating the ratio of the stained cells to the total number of cells, percentage values were obtained. After determining their homogeneity and distribution normality, the values were subjected to one-way variance analysis. Based on the analysis results, no statistical difference was established between the levels of MT-stained cells of the experiment groups (p<0.520) (Table 1).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Percentage of cells stained with MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-Cont (G1)</td>
<td>0.06 ± 0.03</td>
</tr>
<tr>
<td>H/I/R (G2)</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>H/Zn-I/R (G 3)</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>Total</td>
<td>0.06 ± 0.04</td>
</tr>
</tbody>
</table>

The values are presented as percentages of positively stained cells ± standard deviation. There is no statistical difference among groups.

The number of positively stained cells in the heart preparations was quite low (total mean 0.06± 0.04) and there was not any color intensity difference in staining. Therefore, total MT positive cells in the study groups were compared without giving consideration to color intensity in the tissue. No significant difference was established among the study groups in terms of their metallothionein levels. Zinc supplementation was reported to protect the heart in diabetic mice [10]. It is accepted that the two mechanisms underlying this cardiac protection result from the insulin-like action of zinc [11]. The essence of both events can be explained by the effect of zinc on the insulin signal [10,12]. In the first of these events, a zinc-dependent molecule, insulin-responsive aminopeptidase (IRAP), has been described and characterized in fatty and muscle tissues, which are the target tissues of insulin [12]. This zinc-dependent molecule (IRAP) is required to maintain the levels of glucose transporter 4 (GLUT 4) [12]. Ezaki [13] has shown that zinc stimulates the transport of GLUT to the cell surface and this stimulation results in increased glucose entry into the cell, which in turn leads to reduced blood glucose levels. A further target molecule for zinc in the insulin signaling pathway is GSK-3β (glycogen synthase kinase-3β) [10]. The level and activity of this molecule increases especially in type II diabetes patients [14]. Elevated GSK-3β, in turn, leads to impaired glycogen levels and insulin resistance in patients with type II diabetes [14]. Zinc, on the other hand, inhibits glycogen synthase kinase-3β (GSK-3β) and increases glucose intake into the cell, thereby reducing blood glucose [14]. Consequently, the protective effect of zinc on cardiac functions in diabetic humans and animals is associated with the glucose metabolism [15]. It was shown by Sun et al. [10] that, besides its cardiac protective effect in diabetic mice, zinc is more importantly involved in the stimulation of the metallothionein synthesis. Cong et al. [16] also reported that the metallothionein synthesis-stimulating effect of zinc might be a mechanism in its ameliorative effect on cardiac functions in diabetic rats. Similarly, Li and colleagues [17] noted that the stimulation of metallothionein synthesis and the increase in glucose metabolism were important factors in the protective effect of zinc on the cardiac tissue of diabetic rats. Besides, it was stated that the prevention of lipid peroxidation seen in the cardiac tissue by zinc through increased metallothionein synthesis was significant [17]. The studies on the topic seem to concentrate on the relationship between zinc and metallothionein in the inhibition of the impairment in cardiac functions in diabetes. There are only a few studies exploring the possible effects of zinc on the heart tissue in non-diabetic cases. It was suggested that zinc administration to isolated hypothermic heart preparations could affect lactate dehydrogenase enzyme, but still exercise a protective effect on cardiac functions [18]. Trace elements which are antioxidant enzyme co-factors, including zinc, were shown to be able to prevent tissue damage in cardiac ischemia-reperfusion injury [19]. Itoh and Kimura [20], for one, reported that zinc could be critical in preventing the tissue damage seen in myocardial ischemia-reperfusion [21] and the major role of zinc in this process could be increasing the cytokine-origin metallothionein synthesis. Highlighting the clinical importance of zinc supplementation in the prevention of cardiac ischemia-reperfusion, Karagulova et al. [22] reported that the prevention of lipid peroxidation in the cardiac tissue by zinc was mediated by the increase in metallothionein synthesis [23]. In our study,
metallothionein levels in the ischemic cardiac tissue were not affected by zinc supplementation. Our med-line research did not show any study which we could use to directly compare ours where we investigated the effect of zinc supplementation on metallothionein in cardiac ischemia-reperfusion. However, reports suggesting that tissue injury in the cardiac tissue can be inhibited by zinc supplementation [15,18,19,22] are not direct, and neither are they consistent with the results of our study.

4. CONCLUSIONS

The results of our study indicate that 21-day zinc supplementation does not have a substantial effect on metallothionein synthesis in cardiac ischemia-reperfusion. These results may be explained by the dose of zinc, length of supplementation and/or duration of heart ischemia-reperfusion used in the study.

5. REFERENCES