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ORIGINAL RESEARCH

The Effect of Hepcidin on Cardiac Ischemia-Reperfusion Injury

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ABSTRACT

Background/aim: Hepcidin is the main hormone in the regulation of iron metabolism which is also released from the heart. The aim of our study was to investigate the effects of hepcidin on the cardiac ischemia-reperfusion injury.

Materials and methods: In this study, 12 Wistar albino rats were divided into two groups ($n = 6$ each): 1) The ischemia-reperfusion group (Group 1); 2) Hepcidin-treated group (Group 2). Rat hearts were perfused on Langendorff system with KH (Krebs-Henseleit) and subjected to 30 min stabilization, 30 min global ischemia, and 30 min reperfusion. Hepcidin (- M) was applied to group 2 at the onset of ischemia. Malondialdehyde (MDA), glutathione (GSH), and nitric oxide (NOx) levels were measured in heart tissue for NOx levels, viscosity, and ion content of perfusate were collected before ischemia and the 1st, 5th, 10th, 20th, and 30th minutes of reperfusion were determined. Apoptosis in heart was evaluated.

Results: NOx and MDA levels significantly decreased in heart tissue in Hepcidin-treated group. NOx and viscosity of perfusate were not significantly different between the groups. Perfusate iron, calcium, magnesium, potassium, and sodium levels in group 2 were more homogeneous. Histologic structures of heart tissue were regularly in group 2. Apoptosis were increased in control group compared to hepcidin treated group.

Conclusion: These results suggest that hepcidin may have a protective effect on the heart for the ischemia-reperfusion injury.

Keywords: hepcidin; ischemia; reperfusion; heart; caspase-9; apoptosis

INTRODUCTION

Ischemic disorders are the leading causes of death in Western countries. Ischemic heart disease or myocardial ischemia is characterized by insufficient blood flow, with respect to the metabolic requirements of the myocardium. In other words, the reduction in coronary blood flow is the result of reduced oxygen and nutrient support in the heart [1–3].

Reperfusion is the restoration of blood flow to an ischemic organ. Timely reperfusion decreases cardiac morbidity and mortality. However, reperfusion may result in cardiomyocyte dysfunction, also known as "ischemia-reperfusion (*I/R*) injury" [4–6].

Hepcidin is a small peptide hormone and plays a central role in iron metabolism and internalization and destruction of ferroportin (FPN) [7].

Hepcidin secretion, induced by the increase of plasma transferrin bound iron levels, decreases iron release from macrophages and enterocytes. These homeostatic circuits maintain a constant range of plasma iron with preventing iron absorption from the intestine and iron overload in tissues [8]. Hepcidin inhibits ferroportin channels of enterocytes which are located on basolateral part [9, 10].

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Myocardial ischemia results in increased ferritin associated with the degree of ischemia [11, 12].

Iron deposition in heart myocytes causes oxidative stress and changes in myocardial function due to DNA damage [13].

Hepcidin is an intrinsic cardiac hormone that can be released from the heart, and its release is regulated by hypoxia and inflammation [11, 14].

Increased hepcidin levels in the heart with hypoxia; may create a protective mechanism against free radicals by controlling intracellular ferritin concentration of free cytosolic iron (Fe^{+2}) concentration [15].

There is limited literature on the effect of hepcidin on cardiac ischemia-reperfusion. In this study, we aimed to investigate the effects of hepcidin on the cardiac ischemia-reperfusion injury with regard the tissue MDA, NOx, perfusate ion levels and viscosity and caspase 9 activity and histological evaluations.

MATERIALS AND METHODS

Animals

The recommendations of the Declaration of Helsinki (1964) for animal care were taken into consideration. In this study, we used 12 mature Wistar albino rats weighing 250–300 g each. Animals were obtained from the Animal Breeding and Experimental Research Laboratory of Gazi University. The experimental protocol was approved by the Ethical Committee (GU ET-10.021) of Gazi University. The animals were housed under (12 h light/dark cycle, 20–25 °C) designed laboratory conditions and were fed with a standard rat pellet and allowed to drink water ad libitum.

Experimental Protocol

In the experiment, 12 rats were randomly divided into two groups:

1. The Ischemia-Reperfusion control group (Group 1; $n = 6$)
2. Hepcidin-treated group (Group 2; $n = 6$).

NOx levels as nitrite (NO_2^-), plus nitrate (NO_3^-) in tissue and perfusate, tissue MDA as an indicator of lipid peroxidation, and GSH level as an indicator of antioxidant capacity were measured.

The Isolated Perfused Heart

Animals were heparinized and anesthetized with Ketamine HCI (60 mg/kg, intraperitoneal). The hearts were excised and put in to ice cold Krebs-Henseleit (KH) and immediately cannulated from aortae and perfused with the non-circulating modified Krebs-Henseleit solution containing (in mM) 118 NaCl, 4.7 KCl, 1.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25.2 $NaHCO_3$, and 11.1 glucose in reverse. The perfusion pressure was kept constant at 90 cmH_2O. The KH was bubbled with 95% O_2-5% CO_2 mixture and kept at 37 °C during experiment.

Perfusate and Tissue Preparation

The fluid passing from the heart (perfusate) was taken to the eppendorf tubes during reperfusion period at the 1st, 5th, 10th, 20th, and 30th min and stored in deep freeze at –20 °C for biochemical analysis. At the end of the reperfusion, the hearts were unloaded from langendorff, were immediately frozen in liquid nitrogen and were stored in the deep freeze for biochemical analyzes at –80 °C. NOx levels were measured in the perfusate. In addition, MDA, GSH, and NOx levels were determined in the heart tissue.

Measurement of NOx Content (Nitrate and Nitrite) in Tissue and Perfusates

NOx concentrations were measured by the Griess reaction. The tissue was diluted with PBS and were homogenized. After homogenization, it was centrifuged at 2,000 relative centrifugal force (RCF) at 4 °C for 5 minutes. Then, 500 μL of supernatant was removed and 250 μL 0.3 N NaOH was added, then incubated for 5 min at room temperature. After incubation, 250 μl of 10% ZnSO4 was added and vortexed. After vortexing, the supernatant was centrifuged at 3,000 RCF at 4 °C for 5 minutes. It was then centrifuged at 14,000 RPM for 5 minutes at 4 °C. After centrifugation, vanadium III chloride ($VaCl_3$), sulfanilamide (SULFA), and N-(1-naphtyl) ethylenediamine dihydrochloride (NEDD) were added to the supernatant and the mixture was kept at 37 °C for 30 minutes. The samples were read by a spectrophotometer at 540 nm. The same method was applied to the perfusate samples and was studied by spectrophotometer at 540 nm [16].

Measurement of Tissue MDA Levels

Tissues were diluted with 10% trichloroacetic acid (TCA) and homogenized. After homogenization, the samples were centrifuged at 3,000 RCF at 20 °C for 15 minutes. The supernatant was removed and again centrifuged at 3,000 RCF at 20 °C for 8 minutes.

Then, 750 μl of samples were taken to the glass tube and 10 μl of 1% butylated hydroxytoluene (BHT) was added on the samples. TBA (750 μl) was added to the mixture and vortexed for 20 seconds. After vortexing, the mouths of the tubes were covered with glass marbles and boiled at 120 °C for 15 minutes. The mixture was centrifuged at 3,000 RCF for 8 minutes. After centrifugation, the samples were analyzed by a spectrophotometer (ELISA) (BioTek ELx405 Washer-Synergy HT Reader-Gen5 Data Analysis Software) at 535 nm [17].

Measurement of Tissue GSH Levels

Tissues were diluted with 10% TCA and homogenized. After homogenization, the samples were centrifuged at 3,000 RCF at 20 °C for 15 minutes. The supernatant was removed and again centrifuged at 3,000 RCF at 20 °C for 8 minutes. After centrifuged, Na_2HPO_4 (0.3M) and 5′,5′-dithiobis 2-nitrobenzoic acid (DTNB) were added and the samples were vortexed. The samples were kept at room temperature for 10 minutes. After the whole procedure, the samples were analyzed by a spectrophotometer (ELISA) (BioTek ELx405 Washer-Synergy HT Reader-Gen5 Data Analysis Software) at 412 nm [18].

Histologic and Immunohistochemical Procedures

The heart tissues of group 1 and group 2 were rapidly fixed in 10% formalin for 72 h at room temperature and embedded in paraffin blocks. Then, routine histological procedures were applied. Tissue sections (4 μm in thickness) were stained with Hematoxylin and Eosin (H&E) and Masson's Trichrome.

For immunohistochemical analyses, sections at 4 μm thickness were used for the primary antibody; Caspase-9 (LAP6 Ab-4, Abcam) was diluted at 1:100 and incubated overnight at 4 °C. Then, the tissue sections were incubated with secondary antibody (LabVision, Thermo Scientific, Fremont) for 10 min each. The reaction product was revealed by streptavidin peroxidase complex (LabVision, Thermo Scientific, Fremont) with 3,3'-diaminobenzidine (DAB). Finally, Mayer's hematoxylin was used as a counter staining.

For each animal, caspase-9 was examined in six randomly selected areas with 40× magnification. The scores were derived semiquantitatively by using light microscopy on the preparations of each animal and were reported as follows: none: 0, mild: 1, moderate: 2, severe: 3, and very strong: 4.

All the sections were examined and photographed with a light microscope (Leica DM 4000B, Germany). The images were evaluated with the Leica Q Win 3 Software (Cambridge, UK).

Perfusate Viscosity

The viscosities of the perfusates obtained from both groups were determined before ischemia and during reperfusion at the 1st, 5th, 10th, 20th, and 30th minutes with a viscometer (BioProfiller Vilastic).

Determination of Elements

Iron, calcium, magnesium, potassium, and sodium ions were analyzed in the perfusates collected from both groups, before ischemia and during reperfusion at the 1st, 5th, 10th, 20th, and 30th minutes by using Inductively Coupled Plasma-Optical Emission Spectrometer.

Statistical Analysis

Experimental data analysis was performed by using SPSS version 15.0 for Windows. Whether the distribution is normal or not was tested by using Kolmogorov-Smirnov test. Analysis of variance (ANOVA) was used in order to analyze the differences for each element within group. Student's *t*-test was used to compare the *I/R* and hepcidin groups for each data. $p < 0.05$ was considered to indicate statistical significance. Data were presented as means ± SEM and median (min – max).

RESULTS

Tissue NOx, MDA, and GSH Levels

The differences in tissue NOx levels between *I/R* and hepcidin group rats were found to be statistically significant ($p < 0.05$) (Table 1). Tissue NOx levels were significantly lower in the Hepcidin-treated group compared to the control (*I/R*) group ($p < 0.05$) (Table 1).

Tissue MDA levels were significantly lower in Hepcidin group compared to the control (*I/R*) group

TABLE 1. Comparison of tissue NOx, MDA, and GSH levels between study groups

Parameters	Group 1	Group 2
NOx (μM/g tissue)	33.76 ± 2.45	30.14 ± 1.24*
MDA (nM/g tissue)	19.59 ± 0.82	17.87 ± 1.26*
GSH (nM/g tissue)	1.01 ± 0.07	0.93 ± 0.11

*$p < 0.05$. All data were expressed as mean ± SD.

TABLE 2. Perfusate NOx levels and viscosity values of experimental groups

Parameters	Preischemia	1th min	5th min	10th min	20th min	30th min
Perfusate NOx levels (nM/ml)						
Group 1	18.32 ± 2.47	17.92 ± 1.63	17.98 ± 1.5	17.44 ± 1.44	17.7 ± 0.86	17.4 ± 1.11
Group 2	17.06 ± 1.44	17.06 ± 0.79	17.3 ± 0.76	17.6 ± 0.31	17.92 ± 1.46	17.3 ± 0.74
Perfusate viscosity (cp)						
Group 1	0.61 ± 0.18	0.76 ± 0.10	0.75 ± 0.08	0.71 ± 0.01	0.76 ± 0.06	0.72 ± 0.02
Group 2	0.72 ± 0.02	0.71 ± 0.01	0.72 ± 0.01	0.71 ± 0.01	0.72 ± 0.01	0.71 ± 0.02

All data were expressed as mean ± SD; cp: centipoise.

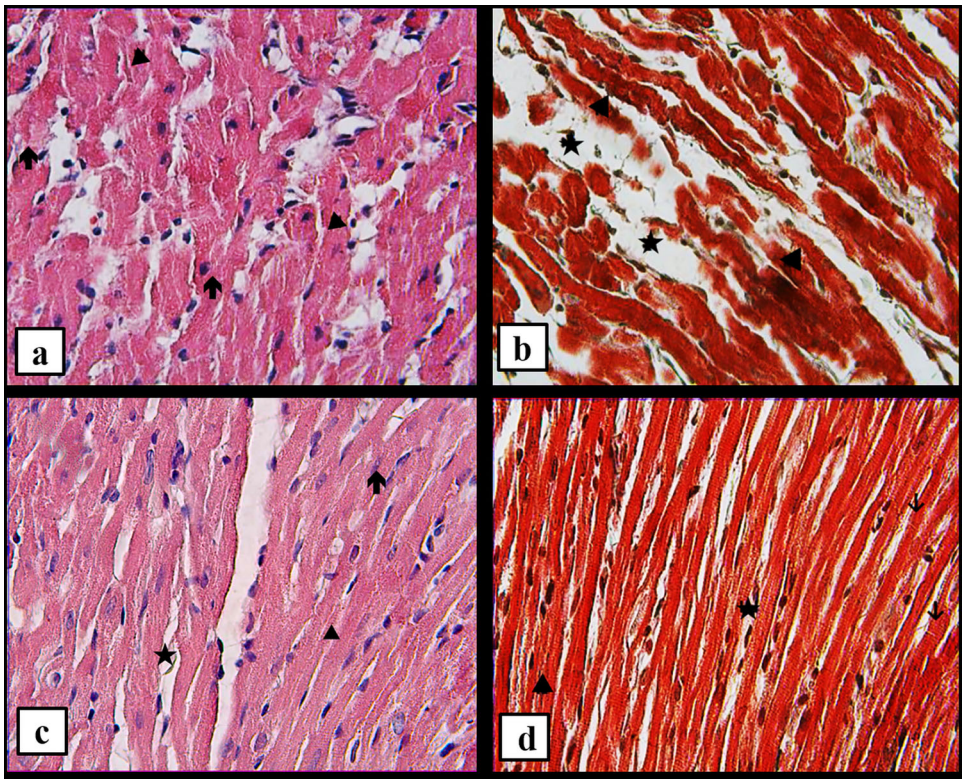

FIGURE 1. H&E and Masson's Trichrome staining of the experimental groups. (a,b) *IR* group showing disorganization of cardiac muscle fibers (arrowhead), morphology deformation of nuclei (arrow), edematous areas in the connective tissue (star); (c, d) hepcidin treated group showing recovery of cardiac muscle fibers (arrowhead), regularity of the organization of the fiber nuclei (arrow), cross striations (thin arrow) (magnification 40×).

($p < 0.05$) (Table 1). Tissue GSH levels were lower in the hepcidin-treated group in comparison to the control (*I/R*) group. However, the difference was not statistically significant ($p > 0.05$) (Table 1).

Perfusate NOx Levels, Viscosity, and Element Levels

There was no difference between intergroups and intragroups in perfusate NOx levels and viscosity values (Table 2). Iron, calcium, magnesium, potassium, sodium, and thallium levels were observed to be more uniform in the hepcidin group compared to the control (*I/R*) group. A significant decrease in Ca levels ($p < 0.05$) was observed in group 2 at 30 minutes compared to group 1.

Histochemical Results

In Hematoxylin-Eosin and Masson's Trichrome staining, the control (*I/R*) group showed the disorganization of cardiac muscle fibers, loss of cross striations, and intercalated disc (Figure 1a,b). Moreover, the nuclei of cardiomyocytes from *I/R* group showed eccentric location. HE staining disclosed pyknotic and heterochromotic nucleus structure (Figure 1a). The presence of edematous areas in the connective tissue between the bundles of cardiac muscle fibers and degenerative alterations were

TABLE 3. Perfusate element levels

Element (ppm)	Preischemia	1st min	5th min	10th min	20th min	30th min
Fe						
Group 1	0.055 ± 0.004	0.054 ± 0.003	0.056 ± 0.002	0.057 ± 0.001	0.050 ± 0.01	0.052 ± 0.01
Group 2	0.055 ± 0.002	0.052 ± 0.007	0.056 ± 0.001	0.056 ± 0.003	0.056 ± 0.001	0.056 ± 0.001
Ca						
Group 1	35.84 ± 0.47	36.13 ± 0.51	36.02 ± 0.41	36.25 ± 0.45	36.35 ± 0.65	35.84 ± 0.34
Group 2	36.45 ± 0.43	36.42 ± 0.32	36.12 ± 0.40	36.44 ± 0.58	36.6 ± 0.49	36.47 ± 0.48*
Mg						
Group 1	26.01 ± 3.69	27.97 ± 1.68	27.81 ± 1.31	24.96 ± 5.41	27.08 ± 2.54	28.02 ± 1.90
Group 2	26.21 ± 0.98	26.20 ± 0.78	26.42 ± 2.55	25.78 ± 2.00	25.79 ± 1.66	26.60 ± 1.49
K						
Group 1	1513.19 ± 292.01	1606.93 ± 136.03	1665.41 ± 138.53	1359.125 ± 665.75	1611.45 ± 181.62	1682.05 ± 243.9
Group 2	1551.86 ± 117.1	1522.38 ± 115.19	1605.22 ± 283.09	1551.55 ± 129.85	1489.26 ± 148.59	1565.24 ± 140.13
Na						
Group 1	15.83 ± 1.24	15.25 ± 0.31	15.17 ± 0.22	16.79 ± 3.66	15.35 ± 0.38	15.19 ± 0.43
Group 2	15.4 ± 0.37	15.34 ± 0.22	15.53 ± 0.81	15.44 ± 0.35	15.57 ± 0.54	15.36 ± 0.24

*$p < 0.05$. All data were expressed as mean ± SD.

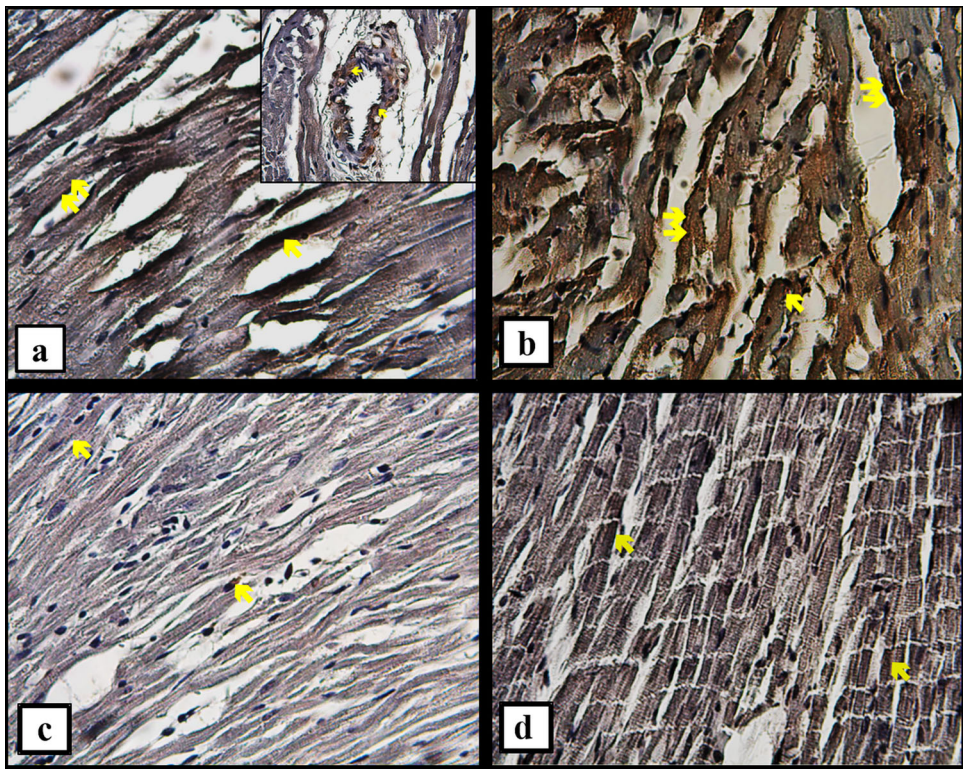

FIGURE 2. The immunoreactivity of caspase-9 in rat heart from each group. (a, b) The immunoexpression (yellow arrow) was detected in cardiac muscle fiber, sarcolemma, and endothelial cells in group 1; (c,d) cytoplasmic and membranous caspase-9 immunoreactivity was observed (yellow arrow) in the muscle fibers in group 2 (magnification 40×).

observed (Figure 1b). In the hepcidin-treated group, although some certain areas were similar like *I/R* group because of the ischemia-reperfusion injury, rest of the tissue was histologically regular and was similar to the normal cardiac muscle structure (Figure 1c,d).

In areas which appear to be regular, the recovery of cardiac muscle fibers, regularity of the organization of the myocardial fibers nuclei, regular cross striations, and myocardial intercalated disc appearance were noticed. The connective tissue between the cardiac muscle fibers was regular and there was no edematous area (Figure 1b,d). The myocardial tissue in the hepcdin group showed fewer pathological changes, as compared with the *I/R* group (Table 3).

TABLE 4. Mean staining intensity of caspase-9 in heart tissue

Parameter	Group 1	Group 2
Caspase-9 immunoreactivity	2 (1–3)	1 (0–2)*

*$p \leq 0.000$. All data were expressed as median (min – max).

Immunohistochemical Evaluation

Immunoreactivity of caspase-9 in *I/R* group, was detected in cardiac muscle fiber and sarcolemma (Figure 2a,b). Also, immunoreactivity was observed on endothelial cells in the connective tissue between the bundles of cardiac muscle fibers (Figure 2a, inset).

Immunohistochemical staining of caspase-9 in hepcidin group did not reveal increased cytoplasmic and membranous immunoreactivity in the muscle fibers (Figure 2c,d). Caspase-9 activity was significantly decreased in group 2 compared to group 1. The results of staining with caspase-9 are given in Table 4.

As a result, physiological apoptotic findings were increased in control group compared to hepcidin treated group.

DISCUSSION

Hepcidin is a novel peptide hormone and studies have largely focused on hepcidin in a broader perspective in recent years [19]. The aim of this study was to examine the effect of hepcidin on the cardiac ischemia-reperfusion injury. In our experimental study, we did not create a basal (control) group, because The use of any untreated heart as a control group in cardiac ischemia reperfusion studies by using the Langendorff mechanism will not provide any information for our purpose. Since it is more informative to compare the effect of the given substance with the ischemia reperfusion group, untreated ischemia heart tissue has not been used. Since the control group had *I/R* damage, no additional basal group was needed [20–23].

The literature indicates that iron metabolism contributes to the ischemic damage after myocardial infarction. After myocardial infarction, the hepcidin mRNA expression is temporarily upregulated in the ischemic and in the nonischemic myocardial cells. The increase was specifically for hepcidin, no change was observed in other iron-related genes (hemoguvelin, IREG-1). The alteration of hepcidin protein expression in the ischemic area of infarct rats is associated with serum hepcidin level [23]. These data show that hepcidin is significantly increased in ischemic and nonischemic myocardial cells, after myocardial infarction. This finding indicates that hepcidin upregulation reduces the infarct area expansion by decreasing the iron toxicity of the infarcted heart.

Moreover, hepcidin is expressed in cardiomyocytes in experimental autoimmune myocarditis (EAM) and acute myocardial infarction (AMI) rat models. In the hearts of EAM and AMI, the ratio of hepcidin expression is significantly increased with interleukin-6/gamma-actin expressions. The same significantly higher rate is histologically found in human myocarditis hearts compared to those without myocarditis. Hepcidin expression is induced under myocarditis and myocardial infarction in cardiomyocytes. Increased inflammatory cytokine levels play an important role in iron homeostasis and free radical formation [24].

We also examined the generation of NOx, MDA, and GSH, which are the indicators for oxidant and antioxidant system.

Ischemia increases inflammatory cell infiltration and inducible nitric oxide synthase (iNOS) activity in vascular endothelium. Thus, while NO level increases during ischemia, large amounts of molecular oxygen transport to the tissues by the resumption of blood flow. A large amount of free oxygen radical and superoxide O_2.–, which are thought to be responsible for reperfusion injury, occurs [25–27].

The excess amount of NO which is formed during ischemia may react with increased O_2 radical during reperfusion resulting in peroxynitrite ($ONOO^-$) formation or turns into end products as nitrite and nitrate. Peroxynitrite is also responsible for some tissue damage. During reperfusion, both superoxide (O_2.–) and NO are produced and these two molecules form **peroxynitrite.** Thus, while the physiological effect of NO is inhibited, oxidative stress increases [25–28]. A study conducted by Wang et al. showed that NO's toxic effect in the heart; superoxide and a reaction with peroxynitrite. The activity of peroxynitrite such as NO is very low, however, it is a strong oxidant and causes lipid peroxidation and protein oxidation reactions in biological systems and membranes [29].

It has been observed that NO and peroxynitrite produced in the late phase of reperfusion are much higher than the early phase of reperfusion. This delayed increase in NO levels leads to an even greater increase in tissue damage [30].

In many studies, it has been suggested that NO and peroxynitrite formation, which occur in high amounts due to ischemia-reperfusion, can be reduced and reperfusion injury can be prevented [31].

In our study, NOx levels of tissues were significantly lower in the hepcidin-treated group than in

the control group; therefore, it was suggested that hepcidin may have a protective effect during *I/R*.

Also, the peptide hormone hepcidin is expressed in the heart and regulated by hypoxia and inflammation [15]. Hepcidin production regulated by tissue hypoxia may have a protective role in the heart with increased ferritin and reduced free O_2 radical production [11]. When tissue MDA levels, which are the indicators of oxidative stress, were analyzed, hepcidin has been shown to decrease oxidant stress in ischemia-reperfusion. Although the GSH levels decreased, it was not significant [15]. Our data showed that hepcidin has a positive effect during *I/R* and prevents oxidative damage. These findings were correlated with the study of Merle et al. (2007). A similar effect was observed in varies of ions in the perfusate. More uniform ion outputs that were similar to the preischemia levels in the hepcidin group was observed. This finding can be interpreted as less damage to the cells.

Cardiac ischemia results in an impaired balance in Ca levels in myocardial cells. As a result of this damage, increased Ca levels arising from extracellular and intracellular stores may occur at toxic levels during the reperfusion period. Excessive levels of cytoplasmic Ca may lead to contracture development by reducing the susceptibility of myofibrils [5, 32, 33]. In our study, significantly higher perfusate calcium levels were found in hepcidin group than in the control (*I/R*) group at the 30th minute. This finding can be explained as the use of Ca in a lesser damaged cells. Ischemia disrupts oxidative phosphorylation in the cell, leading to a decrease in intracellular adenosine triphosphate (ATP) and phosphocreatine synthesis. This condition leads to the ATP-dependent ionic pump dysfunction of the cell membrane and presence of more calcium, sodium, and water in the cell [34].

Na-K-ATPase is an important membrane enzyme that plays a key role by providing the sodium and potassium gradient on the cell membrane. Benkoel et al. showed that hepatic ischemia-reperfusion induces a decrease in Na^+, K ATPase expression [35].

In our study, increased Na and decreased K levels were observed in the hepcidin treated group. This demonstrates that Na-K ATPase activity is close to regular in hepcidin-treated group.

With the deterioration of membrane permeability, the concentrations of potassium and magnesium ions, which are very important for protein synthesis, are changed and accordingly protein synthesis is inhibited [36]. Our findings about ion concentrations are in agreement with a previous study [37].

Lowered magnesium levels in the perfusate at 30th minute from the hepcidin group were observed, but it was not significant compared to controls indicating the possible protective role of hepcidin.

In the hepcidin-treated group, histological findings also indicated less cellular damage because of less immunoreactivity for caspase 9 which is an indicator of apoptosis. Ma and Xu (2010) showed that in osteoblast, in comparison with the control, apoptosis rate of cells treated with hepcidin were lower [38]. A similar effect was also detected in our immunohistochemical findings. In our study, immunoreactivity score of cardiomyocytes for caspase 9 was lower in hepcidin group indicating the preventive effect of hepcidin.

In our model of cardiac ischemia reperfusion, externally applied hepcidin at 10^{-5} M caused antioxidative, antiapoptotic, and protective effect on heart. These results were conflicting with the findings of Sasai *et al*. In this study, authors presented adverse effect of hepcidin^{-25} on plaque rupture and endothelial cell death at the onset of acute myocardial infarctus [39]. In addition to, hepcidin has not only a protective effect on heart but also on kidney. Hepcidin treatment reduces inflammation with protection in renal ischemia–reperfusion [40].

In conclusion, ischemia–reperfusion causes local and systemic inflammatory response associated with oxidant production, increased microvascular permeability, complement activation, leukocyte–endothelial cell adhesion, and decreased endothelium-dependent relaxation [41, 42]. Moreover, *I/R* may result in multiple organ dysfunction syndromes (MODS) and death. Therefore, new clinical research and new therapeutic strategies are needed. According to our biochemical, histological and immunohistochemical results we found that hepcidin, which is administered at the beginning of ischemia at 10^{-5} M concentration, may have a cardioprotective effect on *I/R* damage. In the future, in vivo studies will be a guide for the protective impact of hepcidin. New therapeutic strategies may help to prevent or limit ischemia–reperfusion injury in human.

DISCLOSURE STATEMENT

The authors declare that they have no conflict of interest.

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