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The role of hepcidin and its related genes (*BMP6*, *GDF-15*, and *HJV*) in rats exposed to ischemia and reperfusion

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Background/aim: To determine the roles of hepcidin and its related genes in a renal ischemia/reperfusion model.

Materials and methods: A total of 20 Wistar albino rats were equally divided into 2 groups: Group I was the control group and Group II was the ischemia and reperfusion (I/R) group (60 min of ischemia + 48 h of reperfusion). I/R was performed on the left kidneys of these rats and then the I/R-treated kidneys were removed. The levels of serum biochemical markers were evaluated after renal I/R. The expression levels of hepcidin-linked genes [growth differentiation factor 15 (*GDF-15*), bone morphogenetic protein 6 (*BMP6*), and hemojuvelin (*HJV*)] were also measured by RT-PCR technique. In addition, the tissues were evaluated histopathologically.

Results: No significant association was found between renal dysfunction and I/R when compared to biochemical parameters ($P > 0.05$). However, differences in platelet values were statistically significant ($P < 0.05$). Expression levels of *GDF-15*, *BMP6*, and *HJV* genes increased, but this increase was not statistically significant. In addition, histopathological evaluation was performed using hematoxylin and eosin stain. This showed a significant relationship between the control group and I/R group for ischemic and nonischemic kidney scoring.

Conclusion: Hepcidin and *BMP6*, *HJV*, and *GDF-15* should be taken into account when investigating the process of I/R.

Key words: Renal ischemia/reperfusion, hepcidin, *BMP6*, *GDF-15*, *HJV*

1. Introduction

Ischemia and reperfusion (I/R) injuries cause severe tissue damage, and many researchers have focused on their deteriorative effects. Previous studies have shown that reactive oxygen species (ROS) play a critical role in I/R injuries (1). ROS are produced within or outside of the cells as a result of some cellular reactions. Iron is one important factor that contributes to the production of ROS, due to its interaction with other compounds. Specifically, nonlocalized iron has an important role in the formation of ROS, which occurs in Fenton and Haber–Weiss reactions and may cause potentially deleterious effects in humans due to its redox ability (2). Therefore, ROS can damage various cellular macromolecules including lipids, DNA, carbohydrates, and amino acids. This means that the iron levels in cells and tissues must be regulated in order to prevent the ROS.

Iron is essential for the growth and development of all organisms and is of vital importance. Two-thirds of the iron is used in the production of hemoglobin. The remaining iron is used for myoglobin, respiratory enzymes, and hepatic ferritin. It also plays an important role in the immune system, energy production, RNA, DNA, and protein synthesis because of its electron exchange features. Scarcity or excess of iron in living organisms leads to many metabolic disorders. Therefore, regulation of iron homeostasis is very important. Previous studies have reported that hepcidin is responsible for regulating iron homeostasis. Hepcidin is an iron regulatory hormone that is produced by many organs including the heart, kidney, and pancreas (2–5). Serum iron levels and iron's tissue distribution are controlled by hepcidin. Moreover, it is known that hemojuvelin (HJV), bone morphogenetic proteins

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(BMPs), and growth differentiation factor 15 (GDF-15) are related to the expression of hepcidin (6,7). HJV, which has an essential role in iron homeostasis, is a member of a family of glycosylphosphatidylinositol-linked cell surface proteins. HJV is a co-receptor for BMPs. It binds to various forms of BMP to form a complex with BMP receptors. On the other hand, GDF-15 suppresses expression of the iron regulatory protein hepcidin (7).

The roles of diseases that cause I/R in the production and metabolism of hepcidin are not understood. Pathogenesis of renal I/R injury has been widely studied due to its clinical importance and incidence. As a result of I/R occurring in response to scarcity or excess of iron (iron deficiency anemia, inflammation anemia and thalassemia, hemochromatosis types), deterioration of blood flow, physiopathological changes such as endothelial/epithelial dysfunction in the kidneys, inflammation, and tubular obstruction may develop. Although there are many studies reporting the relationship between iron homeostasis and hepcidin in I/R models such as myocardial (8) and liver (6) models, there is no report on a renal I/R model. Therefore, in the present study, we examined renal dysfunction, injury, and serum biochemical markers (erythropoietin, reticulocyte, iron, transferrin, hepcidin, and IL-6) in the renal I/R model. The kidney tissues were evaluated histopathologically. Expression levels of *GDF-15*, *BMP6*, and *HJV/HFE2* genes were also examined.

2. Materials and methods

2.1. Study groups

In this study, 20 male Wistar rats weighing 200 to 250 g were used. The rats were put into a circadian rhythm with the following conditions before the experiment: 24–26 °C temperature and 50%–60% moisture rate. All experimental protocols were approved by the Experimental Animal Committee of Gaziantep University, Faculty of Medicine (voucher number: 26.12.2011/57).

In Group I, the control group, none of the rats were treated with any substance and they were not exposed to I/R.

In Group II, the I/R group, none of the rats were treated with any substance. Ischemia was performed to the right renal artery and vein with a clamp during the 60 min after laparotomy. After reperfusion of 48 h, all of the rats were operated on for relaparotomy. The kidneys were removed and blood samples were drawn. In the genetic analysis, the left kidneys represented the I/R model and the right kidneys represented the control group.

2.2. Laboratory measurements

2.2.1. Renal I/R model

All the rats were anesthetized with an intraperitoneal injection of xylazine (10 mg/kg) and ketamine (40 mg/kg). After anesthesia, the animal was fixed on the operating table and the abdominal skin was shaved and sterilized with 70% ethyl alcohol. A midline incision was then made, and the right renal artery was located and dissected free from its surrounding structures. The right renal artery was clamped for 60 min using a nontraumatic vascular clamp. The edges of the abdominal incision were then brought together and covered with a piece of gauze soaked with warm isotonic saline to prevent undue loss of body fluids. After removal of the vascular clamp on the right renal artery, the right kidney was removed, and then the abdomen was properly irrigated with isotonic saline. The abdominal incision was closed by continuous stitches using 3/0 Vicryl sutures. Forty-eight hours after ischemia, the animals were anesthetized with ketamine/xylazine intraperitoneally (40/10 mg/kg), and then kidney specimens were harvested.

2.2.2. Renal histopathological evaluation

After I/R, the kidneys were removed and placed in formalin. Tissues were then embedded in paraffin according to the standard protocol after alcohol and xylene treatments. Samples of 5-µm sections were cut and stained with hematoxylin and eosin and were visualized with a light microscope.

Tissue samples were observed histopathologically and scored as a result of semiquantitative evaluation. The scoring was as follows: 0 = normal tissue; 1 = swollen tubular epithelium cell areas, fewer than from 25% in cases of necrosis; 2 = between 25% and 50% in similar cases; 3 = between 50% and 75% in similar cases; 4 = between 75% and 100% in similar cases.

2.2.3. RNA isolation and cDNA preparation

Total RNA samples were isolated using a modified method by QIAGEN (Mainz, German). Total RNAs were reverse-transcribed with a Roche AMV Reverse Transcription Kit according to the procedures as provided by the supplier. Briefly, 10X RT buffer (2.5 µL), $MgCl_2$ (4 µL), dNTP (2.5 µL), random nonamer (5.25 µL), RNase inhibitor (0.5 µL), AMV reverse transcriptase (0.5 µL), mRNA, and RNase-free water were mixed to obtain cDNA. The reaction mixture was incubated at 45 °C for 45 min for reverse transcription and heated at 94 °C for 2 min to inactivate AMV reverse transcriptase. Obtained cDNAs were stored at −20 °C until tested. cDNAs were then denatured at 94 °C for 3 min; annealed at 59 °C for 45 s (*BMP6*), at 63 °C for 45 s (*GDF-15*), at 61 °C for 45 s (*HJV*), or at 59 °C for 45 s (*beta-actin*); and extended at 72 °C for 30 s (all of them). PCR mixtures (10 µL) were electrophoresed on 2% agarose gel, which was subsequently stained with 0.5 µL/mL ethidium bromide. Gels were scanned on an imaging analyzer and the corresponding band densities were relatively measured. At least 2 independent reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed. The primer sequences used are given in Table 1.

Table 1. Primer data of the targeted genes.

Genes	Primer	Sequence	Tm	bp
BMP6	Sense	5'-CGACACCACAAGGAGTTCAA-3'	58.0	407
	Antisense	5'-ACCTCGCTCACCTTGAAGAA-3'	58.9	
GDF-15	Sense	5'-CCCAGCTGTCCGGATACTC-3'	59.2	628
	Antisense	5'-ATCATAAGTCTGCAGTGACA-3'	54.0	
HJV/HFE2	Sense	5'-CATGGCAGTCCTCCAACTCTA-3'	61.4	1037
	Antisense	5'-AGACGCAGGATTGGAAGTAGGC-3'	62.3	

Tm: Melting temperature.

2.2.4. Quantitative RT-PCR

The mRNA expression levels of *BMP6*, *GDF-15*, and *HJV/HFE2* genes were analyzed by quantitative RT-PCR technique using an Applied Biosystems GeneAmp PCR System 9700 Thermal Cycler. The PCR products were run on 3% agarose gel electrophoresis, stained with ethidium bromide, and photographed after having been visualized with an ultraviolet transilluminator.

2.2.5. Investigation of biochemical parameters

At the end of study, 2–3 mL of blood were transferred to test tubes by intracardiac method. Samples were held at room temperature for 30 min and then centrifuged at 4000 rpm for 10 min. The obtained blood serum was stored at –80 °C until tested. The whole blood and reticulocyte and the iron (Fe) values were then determined with equipment from Beckman Coulter (LH780EPO) and Architech (c16000), respectively. The levels of erythropoietin (EPO), interleukin-6 (IL-6), and soluble transferrin receptor (sTfR) (R&D Systems Europe, Ltd., UK.) were determined by sandwich enzyme-linked immunosorbent assay (ELISA). Transferrin (Assaypro LLC, St Charles, MO, USA) and hepcidin (EIAab Science Co., Ltd., Wuhan, China) were determined by quantitative immunoassay method with an ELx 800 (BioTek Inc., Winooski, VT, USA).

2.3. Statistical analysis

All values are reported as mean ± standard deviation. Statistical analysis for gene expression and biochemical parameters was carried out by nonparametric Wilcoxon test using the program GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) and the Mann–Whitney U test, respectively. For comparison, the left kidney was used as an internal control. $P < 0.05$ was considered significant.

3. Results

In this study, a total of 20 rats were used for control and I/R groups. The expression levels of *BMP6*, *GDF-15*, and *HJV* genes were measured. Blood samples were also tested for hemoglobin (Hb), red blood cell (RBC), platelet, iron, reticulocyte percentage (Ret %), transferrin (TfR), sTfR, EPO, IL-6, and hepcidin levels. In addition to these results, kidney tissues were scored histopathologically.

3.1. Histopathological evaluation

The kidney tissues were observed by light microscope according to standard procedures. Histopathological evaluation of tissues was as follows: 0 = normal tissue; 1 = swollen tubular epithelium cell areas, fewer than 25% in cases of necrosis; 2 = between 25% and 50% in similar cases; 3 = between 50% and 75% in similar cases; 4 = between 75% and 100% in similar cases according to previous studies applied in a renal I/R model.

Light microscope pictures of the control, nonischemic right kidney and I/R kidneys are showed in Figures 1A–1D. I/R kidney tissues were more deteriorated than nonischemic and control tissues. Swollen tubular epithelium cell areas, vacuolar deterioration, intercellular cast formations (“), and necrosis in the I/R group were determined to be average in tissues with a score of 3. Moreover, swollen tubular epithelium cell areas, vacuolar deterioration, and necrosis were lower than 25% for nonischemic kidneys.

3.2. Gene expressions analysis

Expression levels of *BMP6*, *GDF-15*, and *HJV* genes were measured by using the RT-PCR method.

As shown in Table 2, the expression of *BMP6*, *GDF-15*, and *HJV* genes increased in the I/R group. The expression levels of these genes were statistically insignificant in the ischemic group compared to the nonischemic group ($P > 0.05$).

3.3. Biochemical parameters

As can be seen in Table 3, hepcidin, transferrin, IL-6, EPO, and thrombocyte levels were increased and Hb, sTfR, erythrocyte, and reticulocyte levels were decreased with I/R treatment when compared to the control group. Taken together, the relationship between these increases and decreases was not statistically significant ($P > 0.05$). There was only a statistically significant positive relationship in the increase of thrombocyte levels ($P < 0.05$).

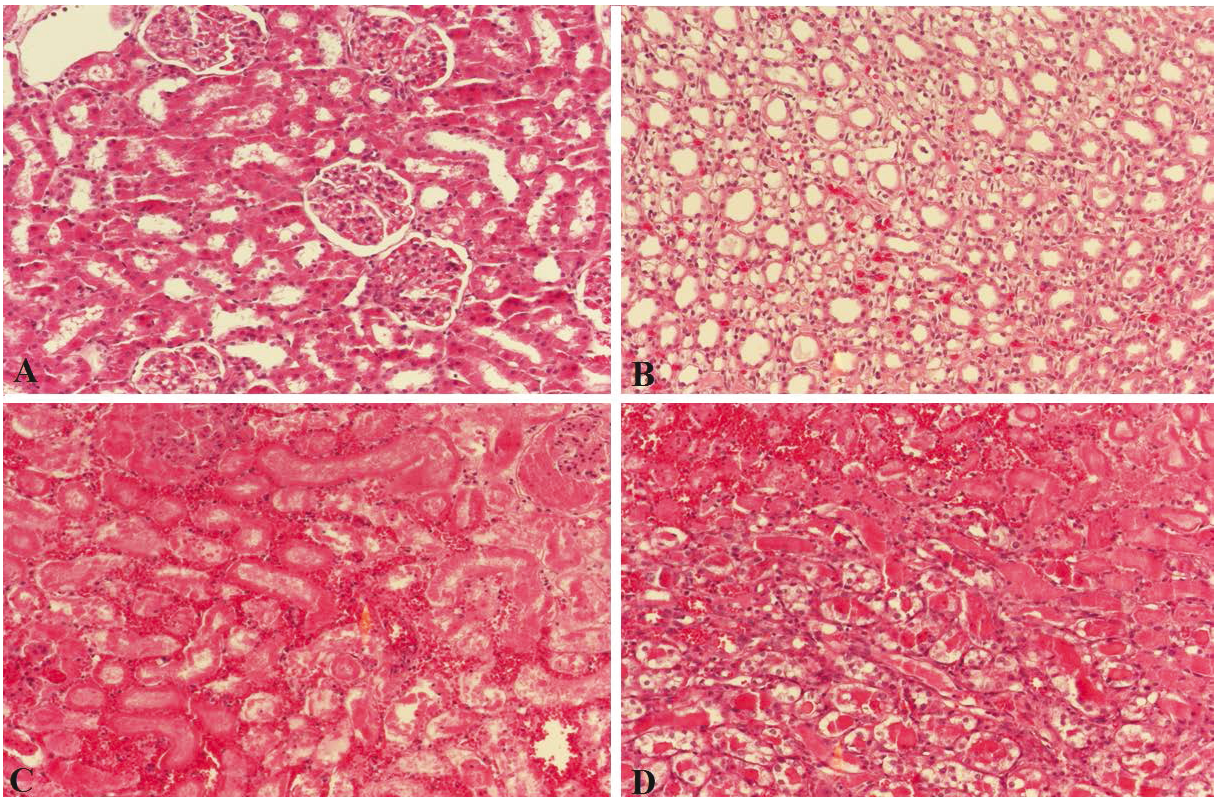

Figure 1. Histological analysis of renal tissue with hematoxylin and eosin staining. A and B: nonischemic right kidney; C and D: ischemic left kidney.

4. Discussion

I/R causes serious tissue disorders in many organs, including the heart, muscle, liver, lungs, and kidneys. In the renal system, a reduction in or stoppage of the blood flow is the main reason for cell damage.

Tubular injury, tubular cell necrosis, and tubular dysfunction in kidney tissues have been reported in various I/R models (1,9,10). In the present study, extravasation, tubular structures, loss of the nucleus in tubular epithelial cells, tubular dilation, interstitial lymphocyte accumulation, and tubular necrosis were determined by using the scoring method in ischemic and nonischemic groups. As shown in Table 4, the kidney tissue damage increased in the I/R group when compared

Table 2. Results of targeted gene expressions.

Genes	Groups	Expression	SD (±)	P
BMP6	Nonischemic	0.257	0.209	P > 0.05
	IR	0.37	0.317	
GDF-15	Nonischemic	0.42	0.379	P > 0.05
	IR	0.66	0.202	
HJV	Nonischemic	0.406	0.266	P > 0.05
	IR	0.454	0.227	

Table 3. Biochemical results of the rat blood.

Compounds	Control	IR	P
Hepcidin	139.5	167.2	P > 0.05
Transferrin	0.985	1.045	P > 0.05
IL-6	6.169	7.432	P > 0.05
Erythropoietin	102.4	111.5	P > 0.05
Platelets	549.1	749.2	P < 0.05
Hemoglobin	130.5	118.1	P > 0.05
sTfR	6.36	6.25	P > 0.05
Erythrocytes	6.92	6.87	P > 0.05
Reticulocytes	4.27	3.83	P > 0.05

Table 4. Histopathological scores of ischemic and nonischemic groups.

	Control	I/R nonischemic right kidney	I/R ischemic left kidney
1.	0	1	3
2.	0	2	4
3.	0	2	4
4.	0	2	4
5.	0	2	4
6.	0	2	4
7.	0	1	3
8.	0	1	3
9.	0	1	3
10.	0	1	3
P	0 ± 0.0	1.5 ± 0.1	3.5 ± 0.1

to the control. Chiang et al. (10) reported that MDA levels in renal tissue increased due to an increase of oxidative stress and H_2O_2 after I/R. The increase is an indicator of structural deterioration since oxidative stress and H_2O_2 were the causes of the damaged tissues in the I/R group. Deteriorated structures in nonischemic right kidney tissues were also observed. It has been reported that other organs are affected in cases of renal I/R injury. As a result, the present study shows that the right kidney, which was not exposed to I/R, was damaged when compared to the control. Therefore, I/R may affect other organs or tissues.

Our findings showed that the hepcidin level increased after approximately 48 h of reperfusion. Since the increase of hepcidin prevents uptake and release of iron, the level of iron decreased in the serum. It is known that iron is essential for erythropoietic activity and Hb formation (11), and, therefore, a deficiency of iron may reduce the levels of RBCs, Hb, and Ret %. The level of sTfR may also be reduced due to the decreasing erythroid proliferation in serum. When iron levels are reduced in the serum, the level of TfR increases for more uptake of iron (12,13). In the results of the present study, TfR level increased in serum. Moreover, levels of IL-6 and EPO may be increased due to hypoxia and inflammation in the process of I/R. EPO is a hormone produced in the liver and kidneys. In order to meet the organs' needs, EPO suppresses hepcidin expression when production of EPO increases in the liver and kidneys. However, the suppression mechanism has not been clarified yet. In addition, the platelet level increased, and this was found to be statistically significant. This increase is probably due to platelet-derived growth factor secreted by renal tubular cells to repair damage in the kidneys after reperfusion.

Hepcidin synthesis and expression of the *BM-6* gene increased in the I/R group. Wang et al. (14) reported that *BMP6* reduces ischemia-induced brain damage due to its prevention of H_2O_2 activity. It has been reported that *BMP6* protects renal proximal tubule cells (HK-2) against H_2O_2 in the kidney (15). Despite these reports, the effect of *BMP6* expression in the kidney has yet to be documented exactly. Moreover, expression of *HJV*, which is a co-receptor of *BMP6* (16,17), also increased. It is known that *HJV* regulates the hepcidin expression and blood iron homeostasis via BMP signaling (6). As a result, *BMP6* and *HJV* gene products have an important role in protecting tissue after I/R. In addition to *BMP* and *HJV*, expression of *GDF-15* also increased. The increased expression of *GDF-15* may be associated with hepcidin synthesis and Fe homeostasis; however, the mechanism still remains unclear.

In conclusion, there is severe tissue damage after I/R. The present study shows that the most severe effects were observed histopathologically in the I/R group. On the other hand, levels of hepcidin and expression of its related genes increased in the I/R group; however, the increase was insignificant statistically. While levels of TfR, IL-6, EPO, platelets, and Hb increased in the I/R group, those of sTfR, erythrocytes, and reticulocytes reduced. Moreover, only the increase in the level of platelets was significant statistically. Consequently, although they were insignificant statistically, it can be said that both hepcidin and the *BMP6*, *HJV*, and *GDF-15* genes may have important roles in I/R. To better understand these roles, it is suggested that the protein levels of these genes be determined by western blot method in further studies. Our study can be considered as the first detailed document on the level of hepcidin and expressions of *BMP6*, *HJV*, and *GDF-15* genes in a renal ischemia reperfusion model.

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