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EFFECTS OF LEPTIN ON HEPATIC ISCHEMIA/REPERFUSION DAMAGE IN RATS

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ABSTRACT

Objectives: The liver is inevitably exposed to ischemia during transplantation, trauma, surgical interventions, and vascular pathologies. Recent studies have shown that leptin has protective effects against ischemia/reperfusion (I/R) injury in the pancreas, stomach, and kidneys. In our study, we investigated the effects of leptin on liver I/R injury. **Methods:** For this purpose, 32 Sprague-Dawley rats weighing between 250-300 grams were randomized into four groups. Group I (Control Group, n=8), Group II (I/R Group) underwent 45 minutes of liver ischemia followed by 45 minutes of reperfusion (n=8), Group III (Leptin Group, n=8), and Group IV (Leptin + I/R Group, n=8). Intracardiac blood samples were taken from all subjects to measure serum levels of Gamma Glutamyl Transferase (GGT), Alkaline Phosphatase (ALP), Alanine Transaminase (ALT), malondialdehyde (MDA), nitrite, and leptin. Following blood collection, the rats were decapitated. The liver tissues from each group were divided into two equal parts. One part was stored at -70°C for the measurement of MDA, Reduced Glutathione (GSH), leptin, and nitrite, while the other part was preserved in a 10% formaldehyde solution for histopathological examination. **Results:** The tests indicated that leptin has protective effects against liver ischemia-reperfusion injury, reduces lipid peroxidation, and diminishes histopathological damage in the liver. It is suggested that leptin may exert these protective effects by increasing nitric oxide (NO) synthesis. **Conclusion:** Administering leptin in liver ischemia-reperfusion injury appears to prevent or reduce tissue damage by decreasing Reactive Oxygen Species (ROS) and/or increasing NO synthesis. Therefore, leptin seems to be a promising agent for future applications in liver-related pathologies.

KEYWORDS: Leptin, Hepatic I/R, GSH.

INTRODUCTION

Liver exposure to ischemia is inevitable during various conditions such as transplantation, trauma, and vascular pathologies. Additionally, during liver surgery, methods inducing ischemia are employed to achieve a clean operative field and minimize blood loss.

The consequences of ischemia-reperfusion (I/R) injury encompass local and distant tissue damage.[1] Recent studies on I/R injury can be categorized into two groups: those aimed at preventing and those aimed at reducing I/R damage.[2] Although several methods have been effective in reducing damage, none have completely eliminated it.

I/R injury in the liver can lead to significant hepatocellular damage and organ dysfunction. Following reperfusion, the initial reaction involves the activation of Kupffer cells, which release reactive oxygen radicals and proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-2, IL-6, IL-10, and IL-12.[3, 4]

Leptin, identified through positional cloning in rats, is a peptide protein product of the obesity gene.[5,6] It exhibits both central and peripheral effects, including increasing the proliferative activity of cytokines and inhibiting apoptosis.[7] Studies have also reported its angiogenic activity.[8] Furthermore, recent research has shown that leptin increases nitric oxide (NO) release from endothelial cells, leading to vasodilation.[9,10]

Leptin has been found to have protective effects on pancreatic, gastric, and renal I/R injuries in previous studies.[11, 12, 13] However, its effects on liver I/R injury have not been extensively studied. Therefore, the aim of our study was to investigate the effects of leptin on liver I/R injury and explore the underlying mechanisms of action.

MATERIALS AND METHODS

The study was conducted at the Medical and Surgical Research Center (TİCAM) Laboratory of the Osmangazi University Faculty of Medicine with the approval of the Ethics Committee. In this study, 32 Sprague-Dawley rats weighing between 250-300 grams were used. The experimental animals were fed standard rat chow and tap

water under standard laboratory conditions. After 12 hours of fasting, all rats were intraperitoneally administered 50 mg/kg of Sodium Pentothal anesthesia. Following anesthesia, the rats were placed in the supine position, and after cleansing the area with 10% povidone iodine, median laparotomy was performed.

The rats were randomly divided into 4 groups.
1. Control group (Group C) (n=8)
2. Ischemia/Reperfusion group (Group I/R) (n=8)
3. Leptin group (Group L) (n=8)
4. Leptin + I/R group (Group L+I/R) (n=8)

In Groups I and III, only liver pedicle dissection was performed, while in Group III, 0.1 ml of leptin (Recombinant Leptin (OB), Sigma L-4146, St. Louis, MO, USA) at a dose of 10µg/kg was intraperitoneally administered 60 minutes before pedicle dissection. Leptin was dissolved in 15 mMol 0.5 ml sterile HCl solution and prepared by adding 7.5 mMol 0.3 ml sterile NaOH solution to achieve a pH of approximately above 5.2. Leptin was stored at 2-4°C until injection. In Groups II and IV, liver ischemia was induced by placing small vascular bulldog clamps around the portal vein, common bile duct, and hepatic artery for 45 minutes. In Group IV, 0,1 ml of leptin at a dose of 10µg/kg was intraperitoneally administered 60 minutes before ischemia. Subsequently, reperfusion was performed for 45 minutes in Groups II and IV.

After obtaining intracardiac blood samples from all subjects to assess serum Gamma Glutamyl Transpeptidase (GGT), Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT), Malondialdehyde (MDA), nitrite, and leptin levels, the rats were decapitated. Liver tissues obtained from each group were divided into two equal parts. One part was stored at -70°C for MDA, Reduced Glutathione (GSH), leptin, and nitrite measurements, while the other part was preserved in 10% formaldehyde solution for histopathological examination.

BIOCHEMICAL ANALYSIS

The sera of the rats were centrifuged at 5000 rpm for 3 minutes. Tissue samples were homogenized in 0.1M phosphate buffer (pH 7.4) using an Ultra Turrax homogenizer (IKA T18 basic, Wilmington NC, USA). Homogenates were centrifuged at 5000 rpm for 10 minutes at +4°C, and the supernatants were separated for analysis.

Serum ALT, GGT, and ALP levels were determined using a Hitachi 917 automatic analyzer (Boehringer Mannheim, Germany) (R&D systems kit, Minneapolis, MN, USA). Leptin levels in blood and tissue were measured using the ELISA method (murine ELISA kit, DSL, USA).

Malondialdehyde (MDA) Measurement The MDA level in blood and tissue was measured by a modified version of the method described by Ohkawa et al., based on the measurement of the absorbance of the color produced by MDA with thiobarbituric acid at 532 nm in acidic medium. The absorbance of the upper layer was measured at 532 nm. The MDA quantity in the sample was calculated from a calibration curve drawn using 1,1,3,3-tetraethoxypropane as a standard and expressed as umol/L in serum and nmol/mg in tissue.[14]

Nitrite measurement serum and tissue nitrite levels were measured using the Griess reagent method described by Green et al. The concentrations were calculated from a calibration curve prepared with sodium nitrite.

Reduced Glutathione (GSH) Measurement The GSH level in tissue was measured by modifying the method of Beutler et al.[15] The optical density of the color produced was read at 412 nm.

HISTOPATHOLOGICAL EVALUATION

Histopathological examinations were performed at the Department of Pathology, Osmangazi University Faculty of Medicine. Preparations stained with hematoxylin-eosin were evaluated by a single pathologist blinded to the groups. Parameters commonly used to evaluate changes in liver I/R damage were recorded. These parameters included congestion, hemorrhage, vacuolization, sinusoidal dilatation, necrosis, and neutrophil infiltration. Histopathological scoring was performed using the Suzuki scale.[16]

STATISTICAL ANALYSIS

Statistical analysis was performed using the SPSS 15.0 for Windows package program (SPSS Inc., Chicago, IL, USA). The Kruskal-Wallis test was used to compare the biochemical and histopathological parameters of the groups. The Bonferroni-corrected Mann-Whitney U test was used for the pairwise comparison of groups. A p-value of less than 0.05 was considered statistically significant. Data were expressed as median (minimum-maximum) for continuous variables and as a percentage for categorical variables.

RESULTS

BIOCHEMICAL FINDINGS

When the groups were compared in terms of the mean ALT levels, a statistically significant difference was found between the ischemia group and the other groups ($p<0.001$).

When the groups were compared in terms of the mean GGT levels, the mean GGT values in the Leptin+I/R group were lower than those in the ischemia group ($p<0.001$). However, they were significantly higher than those in the control and leptin groups ($p<0.01$).

When the groups were compared in terms of the mean ALP levels, the mean ALP values in the Leptin+I/R group were lower than those in the ischemia group

(p<0.001). However, they were significantly higher than those in the control and leptin groups (p<0.01).

When the groups were compared in terms of the mean serum MDA levels, the mean MDA values in the Leptin+I/R group were lower than those in the ischemia group (p<0.001). However, they were significantly higher than those in the control and leptin groups (p<0.01).

When the groups were compared in terms of the mean serum leptin levels, a statistically significant difference was found between Group III and IV and Group I and II (p<0.001).

When the groups were compared in terms of the mean Nitrite levels, a statistically significant difference was found between the Leptin+I/R group and the other groups (p<0.001).

When the groups were compared in terms of the mean tissue GSH levels, the mean GSH values in the Leptin+I/R group were higher than those in the ischemia group (p<0.05). However, they were significantly lower than those in the control group (p<0.01). A statistically significant difference was found between the I/R group and all other groups (p<0.001).

When the groups were compared in terms of the mean tissue MDA levels, the mean MDA values in the Leptin+I/R group were lower than those in the ischemia group (p<0.001). However, they were significantly higher than those in the control and leptin groups (p<0.05).

When the groups were compared in terms of the mean tissue leptin levels, a statistically significant difference was found between Group III and IV and Group I and II (p<0.001).

When the groups were compared in terms of the mean tissue nitrite levels, the mean nitrite values in the I/R group were lower than those in the Leptin+I/R group (p<0.001). However, they were significantly higher than those in the control group (p<0.05).

HISTOPATHOLOGICAL EXAMINATION

Liver samples taken from the groups were evaluated for changes in liver I/R damage, focusing on congestion, hepatocellular changes, necrosis, and portal inflammation, which are the most commonly used parameters.[17] Scores were given by adapting the semi-quantitative scale used by Hauet et al.[18]

When the groups were compared in terms of congestion, the total congestion scores in the ischemia group were significantly higher than those in the other groups (p<0.05). In both the control group and the L+I/R group, only 1+ congestion was detected in 3 samples each.

When the groups were compared in terms of hepatocellular changes, the total hepatocellular change scores in the ischemia group were significantly higher than those in the other groups (p<0.05).

When the groups were compared in terms of necrosis, the mean necrosis scores in the ischemia group were significantly higher than those in the other groups (p<0.05). No necrosis was observed in any samples in the control group. However, only 1+ necrosis was detected in 1 sample in the L+I/R group.

When the groups were compared in terms of portal inflammation, the total portal inflammation scores in the ischemia group were significantly higher than those in the other groups (p<0.05). In both the control group and the L+I/R group, only 1+ portal inflammation was detected in 1 sample each. We reported these comparisons in Table 1.

Table 1: Histopathological scoring of all groups.

Rat Number	Congestion	Hepatocellular changes	Necrosis	Portal İnflammation
C-1	1	0	0	0
C-2	1	1	0	0
C-3	0	0	0	1
C-4	1	1	0	0
C-5	0	1	0	0
C-6	0	0	0	0
C-7	0	0	0	0
C-8	0	1	0	0
IR-1	3	2	2	2
IR-2	2	3	2	1
IR-3	2	3	2	1
IR-4	3	3	3	1
IR-5	3	3	2	2
IR-6	2	3	2	3
IR-7	2	3	3	2
IR-8	3	3	3	1
L-1	1	0	0	0

L-2	0	0	0	1
L-3	0	0	0	0
L-4	0	1	0	0
L-5	1	1	0	0
L-6	0	0	0	0
L-7	0	1	0	0
L-8	0	0	0	0
LIR-1	0	1	0	0
LIR-2	1	2	0	0
LIR-3	0	0	0	0
LIR-4	0	0	0	1
LIR-5	0	1	0	0
LIR-6	1	0	1	0
LIR-7	0	0	0	0
LIR-8	1	1	0	0

DISCUSSION

In recent years, as the number of liver transplantations has increased, exposure to ischemia during traumas and vascular pathologies has become inevitable. Regardless of its cause and duration, the liver undergoes reperfusion following the ischemic period, and the primary damage to the liver occurs during this reperfusion.[19] Especially with the increasing number of transplantations, the importance of ischemia-reperfusion (I/R) injury in the liver is growing. We investigated the effects of leptin, a gene product of obesity with known benefits in renal, pancreatic, and gastric I/R injuries, on liver I/R injury. In doing so, we hypothesized that leptin's angiogenetic effect and its ability to increase NO synthesis in various tissues, including vascular endothelium, could potentially mitigate or reduce I/R injury.[7]

The liver's response to ischemia varies depending on the types and experimental methods.[20] To investigate the effect of leptin on liver I/R injury, we created an experimental liver I/R model. For this model, we chose a 45-minute total portal pedicle clamping method.

In some I/R studies, especially those with prolonged ischemic durations, rats were prophylactically administered 500 U/kg heparin.[21] Since we assumed that the short duration of ischemia in our study would not lead to the development of the no-reflow phenomenon, we did not apply any prophylactic medication.

ALT is an enzyme that catalyzes the reversible transport of α-ketoacids to amino acids. It is a liver-specific enzyme produced only in the cytosol of hepatocytes. It increases in hepatocellular necrosis and is indicative of damage causing leakage of hepatocyte plasma membrane.[22] The increase in ALT has been suggested to be due to lipid peroxidation caused by cytolysis and secondary to the lipoperoxidation caused by SOR in the reperfusion phase.[23]

We observed that liver function values reached high levels at the 45th minute of reperfusion after applying ischemia to the liver for 45 minutes. When compared with the control group, statistically significant differences were found between the I/R group and the control group in terms of ALT values.

GGT is a peptidase that catalyzes the transfer of amino acids from one peptide to another and is a heterodimeric protein. GGT is the only enzyme present in the cytoplasm, containing glutathione (GSH) and GSH residues. GGT plays an important role in leukotriene metabolism. Following GSH conjugation, GGT, together with LTA4, forms the potent pro-inflammatory and vasoconstrictor LTC4.[24] GGT is primarily located in the periphery of lobules in the liver. It shows activity in the membranes of hepatocytes, especially in the canalicular and sinusoidal parts, and in the epithelial membranes of large bile ducts. Therefore, it increases in hepatocyte membrane damage and all cholestatic processes.[25]

We observed that liver function values reached high levels at the 45th minute of reperfusion after applying ischemia to the liver for 45 minutes. When compared with the control group, statistically significant differences were found between the I/R group and the control group in terms of GGT values.

ALP is an enzyme produced by hepatocytes. With increased mRNA synthesis in hepatobiliary diseases, ALP synthesis in the cell membrane also increases.[26]

We observed that liver function values reached high levels at the 45th minute of reperfusion after applying ischemia to the liver for 45 minutes. When compared with the control group, statistically significant differences were found between the I/R group and the control group in terms of ALP values.

The reason for the increase in these enzymes may be increased permeability due to cytolysis and/or cell necrosis resulting from I/R injury. Energy stores of the cell and ATP are consumed during ischemia.[27]

Perhaps the increase in these enzymes is secondary to lipid peroxidation caused by SOR during reperfusion.

One of the most harmful effects of free oxygen radicals is lipid peroxidation, which results in structural and functional cell damage, leading to cell death. Phospholipids and polyunsaturated fatty acids are present in the structure of cell membranes. Lipid peroxidation is a complex process that begins with the removal of an H atom from a methylene group between two unsaturated bonds in a lipid molecule. As a result of this process, carbon-centered lipid radicals are formed in the presence of oxygen, which then produce lipid peroxides or hydroperoxides. Malondialdehyde (MDA), a relatively stable end product, is formed later. MDA can be used as an indicator of lipid peroxidation.[28]

We observed that when groups were compared in terms of serum and tissue MDA levels, MDA levels in the I/R group were found to be significantly higher compared to the control group.

The high MDA levels in our study indicated increased lipid peroxidation due to oxidative stress. Perhaps this is done through increased SOR following the peroxidation of phospholipids in the membrane structure disrupted after I/R injury.

In some studies, GSH levels were found to be significantly lower in the I/R group after 30 minutes of complete ischemia followed by 30 minutes of reperfusion. In one study, when a group subjected to 30 minutes of ischemia followed by 60 minutes of reperfusion was compared with a group subjected to 60 minutes of ischemia followed by 60 minutes of reperfusion, it was observed that GSH levels were significantly lower in both groups, but in the second group, these levels were even lower.[29, 30]

In our study, when comparing the groups in terms of GSH levels, GSH levels in the IR group were significantly lower compared to the control group.

NO is produced in endothelial cells with the help of iNOS (inducible nitric oxide synthase).[31] NO then stimulates soluble guanylate cyclase and increases intracellular guanosine 3',5'-cyclic monophosphate (cGMP) production in vascular smooth muscle and platelets, causing vasodilation.[32] NO modulates leukocyte, platelet, and endothelial cell adhesive interactions, thereby modulating tissue blood flow, arterial pressure, and neurotransmission.[33]

Endothelial NO protects against vasoconstriction caused by hormones circulating in the vessel walls or activated platelets under physiological conditions. In endothelial damage such as I/R, NO release is impaired. During adaptation to hypoxia in ischemic tissues, NO production increases.[34] It competes with O2 in cytochrome oxidase during adaptation to hypoxia in ischemic tissues, reversibly inhibiting mitochondrial respiration. This metabolic suppression mediates O2 consumption, glucose and protein synthesis, and inhibition of ion transport.[35] All these cellular processes regulate NO production during ischemia.

During the reperfusion phase, NO levels decrease due to increased average blood flow and decreased acidotic period.[36] Another mechanism is the reaction of superoxide radicals with NO during reperfusion.[37]

In our study, when comparing the mean serum nitrite levels between groups, no significant difference was found between the IR group and the control group. When comparing the mean tissue nitrite levels, the nitrite level in the IR group was significantly higher than in the control group.

In light microscopy-based histopathological evaluation, after 45 minutes of ischemia followed by 45 minutes of reperfusion, liver tissue samples taken at the 45th minute of reperfusion showed congestion, portal inflammation, hepatocellular changes, and necrosis, unlike the control group. When both groups were compared in terms of total score, significant IR damage in the liver was detected after 45 minutes of reperfusion.

We did not come across a study investigating the effects of leptin on liver I/R injury in the literature. Therefore, to investigate the effects of leptin on other organs in I/R injury, we administered intraperitoneal leptin at a dose commonly used in studies, 10µg/kg, 60 minutes before ischemia. When comparing the mean ALT, GGT, and ALP levels between the groups, ALT, GGT, and ALP values in the L+IR group were lower than those in the ischemia group but higher than those in the control and leptin groups.

Leptin administration reduced oxidative damage. The reason for this may be the replacement of NO, which causes lipid peroxidation by interacting with superoxide anions during I/R damage, by increasing NO release, or another possible mechanism may be the inhibition of apoptosis by leptin.[7, 38]

When comparing the groups in terms of mean tissue GSH levels, the mean GSH levels in the Leptin+IR group were higher than those in the ischemia group but significantly lower than those in the control group. This can be considered as an indicator of the reducing effect of leptin on SORs.

In a study, it was found that 60 minutes after exogenous leptin administration, NO concentration increased and lipolysis increased in adipose tissue. In the same study, it was observed that the lipolytic effect of leptin decreased with NOS inhibition and did not change with pharmacological ganglion blockade.[39]

When comparing the mean serum and tissue nitrite levels between groups, a statistically significant difference was found between the Leptin+IR group and the other groups. This may indicate that leptin reduces tissue

oxidative damage not only by reducing SOR levels but also by increasing NO synthesis.

In histopathological evaluation, liver tissue samples from the L+IR group, when compared with samples from the IR group, showed significantly lower congestion, portal inflammation, hepatocellular changes, and necrosis. When both groups were compared in terms of total score, it was found that the total score was significantly lower in the L+IR group after 45 minutes of reperfusion. These data are consistent with studies showing that leptin reduces damage in gastric I/R injury, although it does not fully correct it. Leptin administration appears to reduce I/R-induced liver tissue damage by reducing SORs and/or increasing NO synthesis. Leptin is a promising agent for reducing I/R tissue damage.

CONCLUSION

Liver exposure to ischemia is inevitable during various conditions such as transplantation, trauma, surgical interventions, and vascular pathologies. Protective effects of leptin on ischemia-reperfusion (I/R) injury have been demonstrated in recent studies, particularly in pancreatic, gastric, and renal I/R injuries. In our study, we investigated the effects of leptin on liver I/R injury.

Examinations revealed that leptin has protective effects on liver ischemia-reperfusion injury, reducing lipid peroxidation and histopathologically reducing damage in the liver. It was speculated that leptin may exert these protective effects by increasing nitric oxide synthesis.

In conclusion, leptin administration in liver ischemia-reperfusion injury appears to reduce tissue damage by decreasing superoxide radicals and/or increasing NO synthesis. Therefore, leptin represents a promising agent that could be used in the future for liver-related pathologies.

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