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ADMINISTRATION OF LOW DOSES OF TUMOR NECROSIS FACTOR-ALPHA PROTECTS RAT LIVER FROM ISCHAEMIC DAMAGE AND REPERFUSION INJURY

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Liver ischaemia and reperfusion (IR) injury is a significant clinical problem. The aim of our study was to investigate the protective effect of tumor necrosis factor-alpha (TNF-α) on rat liver ischaemia-reperfusion injury. A TNF-α dose of 3 μg/kg body weight was injected into rats that had undergone partial (70%) ischaemia and reperfusion. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), total blood antioxidant level (using the FRAP test), and the concentrations of TNF-α, myeloperoxidase (MPO) and malondialdehyde (MDA) in liver homogenates after 1, 6, and 72 hours of reperfusion were measured. It was demonstrated that, rats subjected to IR, the administration of small doses of TNF-α significantly reduced ALT and AST activities after 60- minute liver ischaemia and 1 or 6 hour of reperfusion. The strongest reductions in ALT and AST activities were seen after 1 hour of reperfusion (30% and 35%, respectively). Exogenous TNF-α reduced the release of this cytokine in all observed periods, with the greatest reduction observed after 1 hour of reperfusion. Decreases in MPO concentration (by 40-45% in all periods of observation), as a marker of hepatic neutrophil infiltration, and in MDA concentration, the end-product of lipid peroxidation (by 55-60% at all time points), accompanied the reduction of TNF-α release. The administration of TNF-α to the rats after IR did not alter total plasma antioxidant potential, as assayed by the FRAP test, after 1 hour of reperfusion; however, at the later times a marked increase (~ 40-50%) occurred. We demonstrated that intraperitoneal injections of small doses of TNF-α protect rat livers from IR injury. The mechanism of this protection is related to reductions in the release of TNF-α during IR after injection of this cytokine, resulting in reductions in oxidative stress and inflammation during the later phase of reperfusion.

Key words: *liver, ischaemia/reperfusion injury, tumor necrosis factor-α, malondialdehyde, alanine aminotransferase, myeloperoxidase*

INTRODUCTION

Liver ischaemia and reperfusion (IR) injury is a serious clinical problem that occurs during resection surgery, organ transplantation and haemorrhagic shock (1-4). An early phase of liver injury (up to 2 hours after reperfusion) is characterised by the activation of Browicz-Kupffer cells (KC). Activated KC produce reactive oxygen species (ROS), which damage the liver; numerous inflammatory cytokines, such as TNF-α, interleukin 1 (IL-1) and IL-8; and platelet activating factor (PAF) (5-7). Apoptosis of hepatocytes and liver sinusoidal endothelial cells also takes place (8). During the late phase of liver injury (6 hours after reperfusion), an inflammatory process occurs, which results from neutrophil recruitment and accumulation in the liver. Activated neutrophils cause endothelial and hepatocellular damage through the release of ROS and proteases, and they exacerbate the structural damage and functional impairment of the liver (6, 9-11).

TNF-α is one of the key inflammatory mediators in the liver, as in others organs (12, 13). TNF-α is a pleiotropic cytokine whose biological effects range from causing cell death to inducing tissue regeneration (14). The release of TNF-α starts at the beginning of reperfusion and increases during the early phase of IR (15, 16). The inhibition of TNF-α production or its neutralisation with anti-TNF-α antibodies decreases the number of neutrophils infiltrating the liver and reduces liver IR injury (17, 18).

Studies on IR of the liver have partially focused on methods of preventing liver injury. Apart from surgical procedures, such as ischaemic preconditioning (IP) and intermittent portal triad clamping, numerous attempts at pharmacological prevention of IR-induced liver damage have been undertaken (19). TNF-α has been administered to limit reperfusion injury and to determine the mechanisms of ischaemic preconditioning in mice, demonstrating that injecting low doses of TNF-α (1 or 5 μg/kg body weight) prior to ischaemia and reperfusion reduces subsequent TNF-α release and liver injury and induces hepatocyte proliferation (20). It has also been found that applying IP briefly stimulates TNF-α production. Injections of low doses of this cytokine bring about a similar effect and protect the liver from IR injury (21). Further studies on TNF-α knock-out mice have revealed that IP is ineffective without TNF-

α, and supplementation with low doses of this cytokine restores the protective effect of IP (21).

The aim of our study was to investigate the protective effect of low doses of TNF-α in a rat model of partial ischaemia-reperfusion and to determine the mechanism of the protective effects of TNF-α. This study may also enable us to better understand the protective mechanism of IP. Evaluation of hepatic injury was performed by determining the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The damage resulting from ROS was evaluated by measuring the concentration of malondialdehyde (MDA) in liver homogenates and by measuring the plasma total antioxidant capacity by the FRAP test (ferric reducing ability of plasma). The liver- homogenate concentrations of TNF-α and myeloperoxidase (MPO), a marker of hepatic neutrophil accumulation and activation, were also measured.

MATERIAL AND METHODS

Experiments were conducted under the consent of the the local Ethics Committee for Animals Research (LECAR protocol No 15/05) of the Medical University of Silesia. The study was performed with male Wistar rats weighing between 200 and 250 g. During the experiment, all rats were kept in standard breeding conditions (temperature of 21- 23°C, relative humidity of 65-70%, day/night cycle of 12/12h).

The animals were divided into 10 experimental groups containing six rats each:

1. Controls (rats without medical intervention) (K group).
2-4. Controls subjected to anaesthesia, laparotomy and sham operation and examined after 1, 6 or 72 hours (SO1, SO6, and SO72 groups, respectively).
5-7. Rats subjected to 60- min partial hepatic ischaemia and 1-, 6-, or 72- hours reperfusion (IR1, IR6, and IR72 groups, respectively).
8-10. Rats treated with TNF-α (3 μg/kg body weight, intraperitoneally) 10 min before 60- min hepatic ischaemia and 1-, 6-, or 72- h reperfusion (TNF1, TNF6, and TNF72 groups, respectively).

All surgical procedures were conducted under general anaesthesia after intraperitoneal injection of ketamine (10 mg/kg body weight) and droperidol (0.25 mg/kg body weight). In the laparotomy control group, the rats were anaesthetised, and a midline incision was performed (sham operation). The rats in IR groups were subjected to partial hepatic ischaemia (70%) followed by reperfusion, as described by Asakawa *et al*. (22). Briefly speaking, under anaesthesia, after laparotomy, ischaemia was induced by occluding hepatic arteries, hepatic veins and bile ducts to the left and median lobes with an atraumatic vascular clamp (Aesculap). Reperfusion was caused by removing the vascular clamps after 60 min of warm ischaemia. The left and median lobes of the liver (after IR), as well as heart blood samples, were collected.

After centrifugation, plasma and serum samples obtained from whole blood were portioned and frozen at –85°C until the assay. Liver samples were homogenised in a volume of phosphorous buffer (50 mmol/L, pH 6), five times greater than the tissue volume, and they were aliquoted and frozen at –85°C.

Serum alanine aminotransferase (ALT) activity was determined by the method, of Wroblewski-LaDue modified by Henry and Bergmeyer using the LDH-NADH complex (23-25). Serum aspartate aminotransferase (AST) activity was determined by standard clinical automated analysis (Technicon RA-XT). Concentration of TNF-α and MPO in the liver homogenates were measured by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (R&D Systems, MN, USA; and HBT, Uden, Netherlands, respectively) according to the manufacturer's instructions. The MDA concentration in the liver homogenates was detected by the Ohkawa method, using the reaction with tiobarbiturate acid (26). Plasma total antioxidant capacity was determined by the FRAP test, which utilises Fe^{3+} ion reduction by antioxidants present in a sample.

All values are expressed as mean ±SD. Differences between the groups were analysed with the Mann-Whitney non-parametric U test for unpaired variables. The following symbols and significance levels were used: ns, $p \geq 0.05$; *, $p < 0.05$; **, $p < 0.01$.

RESULTS

Serum ALT activity of the controls without medical intervention (K group) was 140±27 U/L. In the sham-operated rats, after 1 hour of reperfusion (SO1), the activity of ALT was similar to the value obtained for the K group (*Table 1*). In the IR1 group, there was a marked increase in ALT activity (~1700%), to

Table 1. Serum activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in control and experimental rats subjected to 60 min. of ischaemia and 1, 6, or 72 hours of reperfusion (n=6).

Experimental groups	**ALT (U/L)**	**AST (U/L)**
K	140±27	184±42
SO1	146±33	214±48
SO6	145±29	205±41
SO72	145±25	203±22
IR1	2513±233	3390±400
IR6	2179±206	3031±313
IR72	1309±239	2246±140
TNF1	1596±226	2290±286
TNF6	1887±222	2435±260
TNF72	1160±191	2105±296
Differences between the two groups tested with the Mann-Whitney U test		
IR1 vs TNF1	p<0,01	p<0,01
IR6 vs TNF6	p<0,05	p<0,05
IR72 vs TNF72	ns	ns

2513±233 U/L, ($p<0.01$ *vs*. K). In the TNF1 group, the ALT activity decreased to 1596±226 U/L, 35% lower than IR1 ($p<0.01$).

After 6 hours of reperfusion, the ALT activity in SO6 was as low as in the SO1 group (*Table 1*). In the IR6 and TNF6 groups, ALT activities were 2179±206 U/L and 1887±222 U/L, respectively, which were lower than the IR1 and TNF1 values. The administration of TNF-α (TNF6 group) caused a slight decrease in ALT activity compared to IR6 (~13%), but this difference was statistically significant ($p<0.05$).

After 72 hours of reperfusion, ALT activities in the IR and TNF groups were the lowest of the three time points, at 1309±239 and 1160±191 U/L, respectively (*Table 1*). The difference between IR72 and TNF72 was not statistically significant.

Serum AST activity of the controls without medical intervention (K) was 184±42 U/L (*Table 1*). The AST activity in sham-operated rats (SO1) was slightly higher, 214±48 U/L. In the IR1 group, AST activity was 3390±400 U/L, an ~ 1700% and 1500% increase over K and SO1, respectively ($p<0.01$ for both). In the TNF1 group, AST activity decreased to 2290±286 U/L, a 32% reduction compared to IR1 ($p<0.01$).

After 6 hours of reperfusion, the AST activity in SO6 (205±41) was as low as in K and SO1 groups (*Table 1*). IR6 rats had an AST activity lower than the IR1 rats (3031±313 U/L), whereas the TNF6 AST activity was higher than in TNF1 (2435±260 U/L*).* The administration of TNF-α (TNF6) caused an ~20% decrease in AST activity in comparison with IR6 ($p<0.05$).

Serum AST activities in the IR72 and TNF72 groups were the lowest of the three periods of observation (2246±140 and 2105±296 U/L, respectively, *Table 1*). The difference between IR72 and TNF72 groups was not statistically significant.

TNF-α concentration in the liver homogenates in the controls (K) was 175±26 pg/mg. The concentration of TNF-α was slightly higher in sham-operated rats (SO1), at 195±41 pg/mg tissue (*Fig. 1*). In the IR1 group, the TNF-α concentration of 317±41 pg/mg was ~80% higher in the controls, and this difference was statistically significant ($p<0.01$). Administration of TNF-α (TNF1) decreased the TNF-α tissue concentration (250±37 pg/mg) by about 20% ($p<0.05$).

After 6 hours of reperfusion, liver concentrations of TNF-α in IR6 and TNF6 were lower than in IR1 and TNF1, at 266±41 pg/mg and 238±53 pg/mg tissue, respectively (*Fig. 1*). Liver TNF-α was slightly but not significantly lower in the TNF6 compared to the IR6 group.

TNF-α concentrations in IRI72 and TNF72 groups were the lowest of the three periods of observation (217±39 and 202±40 pg/mg tissue, respectively, *Fig. 1*). TNF-α concentration in the TNF72 group was similar (~7% lower) to IR72.

The MPO concentrations in the liver homogenates in the K and SO1 groups were similar (4.53±1.02 and 3.59±0.75 μg/g tissue, respectively, *Fig. 2*). In IR1 liver homogenates MPO was 15.62±1.52 μg/g, a 250% increase over the K group ($p<0.01$). As a result of TNF-α injection (TNF1), MPO concentration decreased by about 40% (9.26±1.5 μg/g tissue) when compared to IR1 ($p<0.01$).

After 6 hours of reperfusion, MPO concentration in the IR6 and TNF6 groups was lower than in IR1 and TNF1 (12.39±1.86 and 7.14±0.66 μg/g tissue, respectively, *Fig. 2*). In TNF6 group, MPO concentration decreased markedly (by over 40%) compared to IR6, and this difference was statistically significant ($p<0.01$).

The respective MPO concentrations of 9.43±1.44 and 6.19±0.93 μg/g in the IR72 and TNF72 groups were lower than at other observation times (*Fig. 2*). Nevertheless, both values were significantly higher than in the control groups K and SO72.

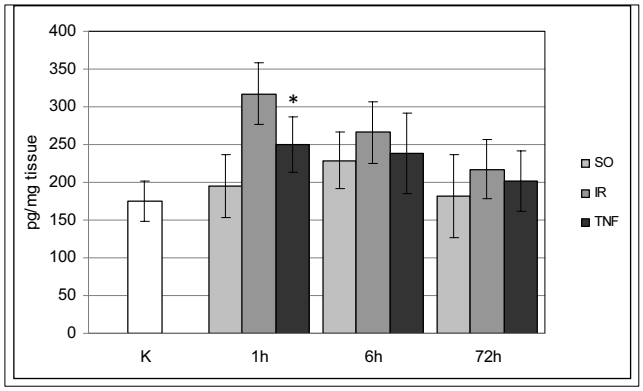

Fig. 1. TNF-α concentrations in the liver homogenates of control and experimental rats subjected to 60 min. of ischaemia and 1, 6, or 72 hour of reperfusion. Each bar represents the means±SD (n=6). * -$p<0.05$ compared to the IR groups.

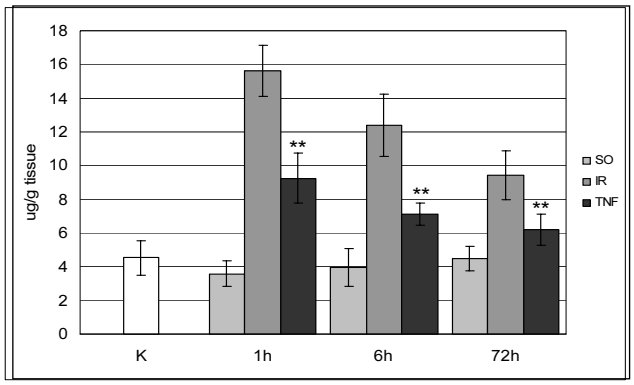

Fig. 2. Myeloperoxidase (MPO) concentrations in the liver homogenates of control and experimental rats subjected to 60 min of ischaemia and 1, 6, or 72 hours of reperfusion. Each bar represents the means±SD (n=6). **$p<0.01$ compared to the IR groups.

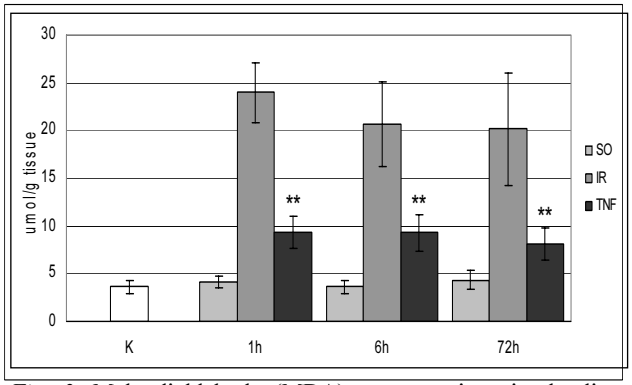

Fig. 3. Malondialdehyde (MDA) concentrations in the liver homogenates of control and experimental rats subjected to 60 min. of ischaemia and 1, 6, or 72 hours of reperfusion. Each bar represents the means±SD (n=6). **$p<0.01$ compared to the IR groups.

The injection of TNF-α (TNF72) caused an ~45% decrease in MPO concentration compared to IR72 ($p<0.01$).

MDA concentrations in the liver homogenates of the controls (K) and sham-operated (SO1) rats were similar, at 3.62±0.65 and 4.13±0.59 μmol/g tissue, respectively (*Fig. 3*). In IR1 rats, MDA was 23.99±3.14 μmol/g tissue an increase of 560% over the K group ($p<0.01$). After TNF-α administration (TNF1), MDA decreased to 9.35±1.63 μmol/g tissue, which was

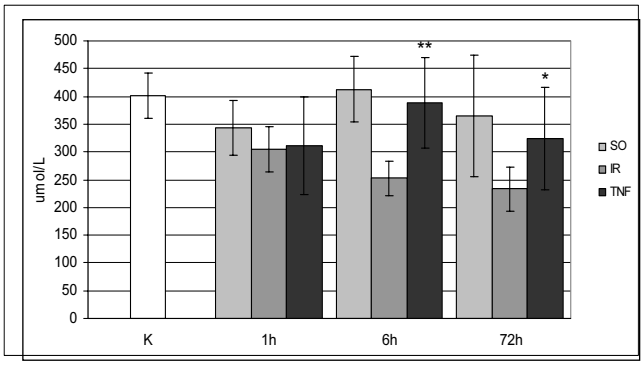

Fig. 4. Plasma total antioxidant capacities measured by FRAP in control and experimental rats subjected to 60 min. of ischaemia and 1, 6, or 72 hours of reperfusion. Each bar represents the means±SD (n=6). * -p<0.05, **p<0.01 compared to the IR groups.

69% lower than in the IR1 group (p<0.01) but 160% higher than in the K group.

After 6 hours of reperfusion, the MDA concentration in IR6 rats was lower (20.62±4,41 μmol/g tissue) than in IR1, whereas the MDA concentration in the TNF6 group (9.29±1.94 μmol/g tissue) was similar to the concentration in the TNF1 group (*Fig. 3*). In TNF6 rats compared to IR6 rats, the MDA concentration decreased markedly (by about 55%), a statistically significant difference (p<0.01). However, the concentration of MDA in this group was approximately160% higher than in the K group.

After 72 hours of reperfusion, MDA concentrations in IR72 and TNF72 groups were lower than at the other time points (20.13±5.85 and 8.09±1.64 μmol/g tissue, respectively) (*Fig. 3*). TNF-α injection (TNF72 group) caused an ~60% decrease in MDA concentration compared to IR72 (p<0.01). In the TNF72 group, the concentration of MDA was still ~125%higher than in the K group.

The plasma total antioxidant capacity, as measured with the FRAP test, in the controls without medical intervention (K group) was 401±41 μmol/L (*Fig. 4*). In sham-operated rats (SO1 group) this value was lower (344±49 μmol/L), but the difference was not statistically significant. When compared to the controls, the concentration of antioxidants in IR1 rats decreased by about 25% to 305±40 μmol/L (p<0.01). In comparison to IR1, administration of TNF-α (TNF1) caused an increase in antioxidant concentration to 311±88 μmol/L, but this increase was not significant.

After 6 hours of reperfusion, plasma total antioxidant capacity in SO6 rats was similar to the K group (*Fig. 4*). The antioxidant concentration in the IR6 group (253±31 μmol/L) was lower than in IR1 , while the TNF6 antioxidant capacity was higher than in TNF1, at 388±82 μmol/L. When compared to IR6, the injection of TNF-α resulted in a marked (~50%) increase in antioxidant concentration (p<0.01).

After 72 hours of reperfusion, the plasma total antioxidant capacities in the IR72 and TNF72 groups were 233±40 and 324±93 μmol/L, respectively; both lower than control values (*Fig. 4*). The injection of TNF-α (TNF72), increased plasma total antioxidant capacity (by about 40%) in comparison to IR72 group, and this difference was statistically significant (p<0.05).

DISCUSSION

In the liver, as in other internal organs, TNF-α is the key inflammatory reaction mediator during ischaemia an reperfusion (12, 13). TNF-α is secreted by activated macrophages, endothelial cells and B lymphocytes. It causes apoptosis, increased blood coagulability, increased expression of adhesion molecules on leukocytes and endothelial cells, as well as the release of numerous cytokines, chemokines, leukotrienes and ROS. In an assessment of TNF-α activity, its positive activities in IR of the liver, such as the activation of hepatocyte proliferation and production of IL-6, which induces an anti-inflammatory response, must be stressed (6). It is believed that TNF-α is not produced during ischaemia, but rather is produced just after circulation is restored, when the level of previously released IL-12 is the highest (15). However, studies conducted on mice have shown that TNF-α starts to be released at the beginning of ischaemia and increases with ischaemic time (20). Similar results have been obtained in partial hepatic ischaemia without reperfusion, where, after 2 hours, a 4-fold increase in TNF-α level has been observed in the lobes after ischaemia, and a 2-fold increase is observed with normal blood flow (27). In our study, after using warm reperfusion, the production of TNF-α in the ischaemic lobes increased. However, the increase was not great, since TNF-α concentration in homogenate after 1 hour of reperfusion exceeded the control value by about 80%. Over the course of the reperfusion, the concentration of TNF-α decreased, but after 72 hours of reperfusion it was still about 25% higher than the control values, which could indicate continuous inflammation of the liver. Intraperitoneal injection of TNF-α decreased the concentration of this cytokine in the liver homogenate after 1 hour of reperfusion, compared to the group of rats in which only IR was used. This confirms the results of Teoh *et al*., although the decrease in TNF-α concentration that we observed was not very as great (28). After the 6 and 72 hours of reperfusion, injecting TNF-α caused a much smaller (and not statistically significant) decrease in TNF-α concentration in the liver homogenates when compared to the values obtained after IR. The study of Peralty *et al*. shows that in mice that had undergone IR for 6 or 24 hours, TNF-α concentration following IP was decreased by a greater percentage than we recorded in our study (29). Our data indicate that injecting TNF-α prior to IR did not decrease TNF-α production in the liver to as great degree as in other studies. This may suggest that at an early stage of reperfusion, a short suppression of KC activation takes place. However, in subsequent periods of reperfusion, not only KC, but also activated neutrophils may contribute to the increase of TNF-α level. Ischaemic preconditioning may have a similar influence on the production and release of TNF-α. Therefore, it can be assumed that decreasing the production of TNF-α may contribute to the protective effect of IP.

It is well known that TNF-α promotes the accumulation, adhesion and activation of neutropfils (9, 10). Activated neutropfils damage hepatocytes and endothelial cells. Neutrophil infiltration into the liver has been observed within the first hour of reperfusion, and their accumulation in the liver reaches a maximum 24 h after circulation has been restored (30). In our study, we measured MPO concentration in the hepatic tissue as an indicator of neutrophil accumulation and activation in the liver. The injection of low doses of TNF-α in all periods of observation caused marked reductions in neutrophil infiltration into the liver after IR. Similar results have been recorded using IP in rats after IR (29, 31). A decrease in MPO after the 4th and 6th hour of reperfusion, accompanied by a decrease in CXC chemokine expression, was also observed in post-IP livers intended for transplantation (32). In contrast, the study by Glanemann *et al*. found that a decrease in MPO concentration due to IP occurred only directly after ischaemia and was not observed over longer periods of reperfusion (33). Moreover, Chouker *et al*., using IP in patients who underwent partial resection of the liver, recorded an increase in MPO concentration in comparison to the patients in whom IP was not used or in whom the Pringle manoeuvre was used (34). They suggested that IP inhibits cytotoxic functions of

neutrophils, which reduces liver damage, rather than affecting their accumulation in the organ (34). Not all results concerning MPO after the employment of IP correspond to our findings, which may suggest that the protective mechanism of IP is quite complex and does not depend only on the variations in the production and release of TNF-α. Administering low doses of TNF-α might activate only some mechanisms responsible for the protective effect of IP.

Liver IR injury is largely related to the development of oxidative stress. The main sources of ROS in the liver, after IR, are KCs, endothelial cells, and neutrophils (through the action of NADPH oxidase) in the later phases of reperfusion (5, 9, 35). MDA is the end-product of lipid peroxidation, and an increase in its level in tissue reflects an increased production of ROS and indicates oxidative damage of the organ (36). An increase in MDA in hepatic homogenate indicates organ damage and, along with increased ALT and AST activities, is a criterion of ischaemia-reperfusion damage. The marked increases that we observed in MDA concentration in IR rats over all periods of observation confirms the significance of oxidative stress in liver IR damage. Corresponding results have been obtained in many previous studies (37-42). In our study, the administration of TNF-α significantly decreased MDA concentration at all time points. This suggests that limiting liver injury may be related to inhibiting the release of ROS by activated KC in the first period of reperfusion, and their decreased production by, for example, neutrophils in the late phase of reperfusion.

TNF-α, which is produced and released in the liver shortly after reperfusion, has a strong influence not only on the liver, but also on other organs, especially the lungs (18, 43, 44). Liver IR induces an inflammatory reaction in the lungs, which is related to neutrophil activity. This leads to increased production of ROS and an intensification of systemic oxidative stress. Plasma total antioxidant capacity (the FRAP test) also allows us to evaluate oxidative damage outside the liver. In our study, after 1 hour of reperfusion, in spite of administering TNF-α, the total concentration of antioxidants was comparable to the concentration in IR rats. These findings suggest that after 1 hour of reperfusion, oxidative shock might be caused by decreases in antioxidant concentrations due to operative shock in the sham-operated rats and by ROS production in the liver and other damaged organs of IR rats. In contrast, after 6 and 72 hours of reperfusion, administration of TNF-α increased total antioxidant potential of the plasma. This seemed to result not only from limited production of ROS in the liver (which was indicated by MDA concentration in the liver) but also from limited operation-induced oxidative shock and from decreased ROS production in other remote organs, such as the lungs. An increase in plasma complete antioxidant potential has also been observed while administrating ascorbic acid and quercetin, which reduce the oxidative stress occurring in ischaemia and reperfusion of the rat liver (45).

The determination of serum activity of ALT and AST is one of the most commonly used indicators of liver damage. ALT is present only in the cytosol of hepatocytes, and its activity in blood serum indicates the damage of cytoplasmic membranes of liver cells, which can result in their death. AST is localised predominantly in mitochondria (about 80%), as well as in cytosol, and its activity increases after damage not only to the plasma membrane, but also to organelles. In our experiment, based on ALT and AST activity, the protective effect of TNF-α was strongest after 1 hour of reperfusion (TNF1 group). The smaller difference between IR6 and TNF6, and even smaller difference between IR72 and TNF72, indicates a lesser influence of TNF-α administration after 6 and 72 hours of reperfusion on mechanisms leading to hepatocyte damage.

The results of this study indicate that intraperitoneal injection of low doses of TNF-α protects the liver from IR injury in both the early and later phases of reperfusion. We conclude that a decrease in TNF-α release observed primarily after 1 hour of reperfusion, due to the earlier administration of TNF-α, is the key factor in inducing such protection. A decreased level of TNF-α at the beginning of reperfusion probably programs reductions in the activation of neutrophils and the expression of cellular adhesion molecules, and it reduces the accumulation of inflammatory cells in the liver, protecting the liver from injury. The protective effect of TNF-α is also related to reduced oxidative stress, which occurred throughout the period of reperfusion. Further studies will be required to explain the exact mechanism of the protective effect of TNF-α in IR-injured livers.

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