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An antibody of TNF-alpha did not prevent thioacetamide-induced hepatotoxicity in rats

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Abstract

Tumor necrosis factor (TNF)-α antibodies have been shown to reduce liver damage in different models. We investigated the effects of infliximab (a TNF-α antibody) on liver damage in thioacetamide (TAA)-induced hepatotoxicity in rats. Group 1 ($n = 8$) was the control group. In group 2 ($n = 8$), the TAA group, the rats received 300 mg/kg intraperitoneal (ip) TAA daily for 2 days. In group 3 ($n = 8$), the TAA + Infliximab (INF) group, infliximab (5 mg/kg ip daily) was administered 48 hours before the first dose of TAA daily for 2 days and was maintained for 4 consecutive days. In group 4 ($n = 8$), the INF group, the rats received only ip infliximab (5 mg/kg) daily. Livers were excised for histopathological and biochemical tests (thiobarbituric-acid-reactive substances [TBARS], and myeloperoxidase [MPO]). Serum ammonia, aspartate transaminase (AST), alanine transaminase (ALT), TNF-α, liver TBARS and MPO levels, and liver necrosis and inflammation scores in the TAA group were significantly higher than in the control and INF groups (all $p < 0.01$). All parameters except AST were not significantly different between TAA and TAA + INF. In conclusion, our results suggest that oxidative stress plays an important role in TAA-induced hepatotoxicity, and infliximab does not improve oxidative liver damage.

Keywords

infliximab, tumor necrosis factor-α, thioacetamide, oxidative stress, liver

Introduction

The liver plays a central role in the transformation and degradation of endogenous and exogenous chemicals. Therefore, the liver is susceptible to toxicities caused by the products generated during these natural occurrences. In response to liver damage, specific intracellular processes are initiated to maintain liver integrity. Tumor necrosis factor (TNF)-α is a key mediator of these processes and activates different cellular responses such as proliferation, survival, and death.[1] In addition, accumulation of reactive oxygen species (ROS) has been shown to result in the progression of injury of hepatocytes as well as activation of stellate cells and consequently fibrosis. This accumulation causes lipid peroxidation of cell membranes or mitochondrial membranes that result in cell necrosis or apoptosis. Lipid peroxidation subsequently

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activates further inflammatory and immune responses and contributes to the progression of hepatic injury. This process has been best studied in alcoholic and nonalcoholic liver disease, ischemia reperfusion injury, and drug-induced hepatic injury.[2-6]

Infliximab is a TNF-α antibody and is widely used in the treatment of rheumatoid arthritis and inflammatory intestinal diseases. TNF-α antibodies have been shown to reduce liver damage in models such as carbon tetrachloride-induced hepatotoxicity[7] and experimental obstructive jaundice.[8]

Thioacetamide (TAA) has been extensively used to induce hepatotoxicity in rats. TAA inflicts tissue injury by producing ROS, thereby directly promoting the peroxidation of membrane lipids.[9,10] In this study, we aimed to investigate the effects of infliximab on liver damage in TAA-induced hepatotoxicity in rats.

Materials and methods

Animals

Healthy male Wistar rats weighing 250–300 g were used in this study. Animals were housed on a 12-hour light/dark cycle (lights on from 08:00 hours) at a constant ambient temperature (24 $\pm$ 1°C), with normal rat chow and water available ad libitum. The study protocol was in accordance with the guidelines for animal research and was approved by the Ethics Committee of our hospital.

Induction of hepatotoxicity

Hepatotoxicity was induced by intraperitoneal (ip) injection of 300 mg/kg TAA (Merck, Germany) daily for 2 days.[11] Supportive therapy by subcutaneous administration of 5% dextrose (25 mL/kg) and NaCl 0.9% with potassium (20 mEq/L) was given every 6 hours to avoid weight loss, hypoglycemia, and renal failure, as previously described.[12]

Experimental design

Thirty-two male Wistar rats were divided into four groups. Group 1 ($n = 8$), the control group, received 0.1 mL of NaCl (0.9%) ip for 4 consecutive days. In group 2 ($n = 8$), the TAA group, the rats received 300 mg/kg ip TAA daily for 2 days at intervals of 24 hours. In group 3 ($n = 8$), the TAA + Infliximab (INF) group, infliximab (at 5 mg/kg ip daily) was administered 48 hours before the first dose of 300 mg/kg ip TAA daily for 2 days at intervals of 24 hours like in group 2 and was maintained for 4 consecutive days. In group 4 ($n = 8$), the INF group, the rats received only ip infliximab at 5 mg/kg daily for 4 consecutive days.

Tissue samples

On day 5 of the study, a laparotomy was performed under strictly sterile conditions after the rats had been anesthetized with 50 mg/kg ketamine (Ketolar; Parke-Davis, Spain) and 10 mg/kg xylazine HCl (Alfazyne 2%; Alfasan, The Netherlands). After the skin had been sterilized with iodine and shaved, the abdomen was opened widely. Blood was taken from the right ventricle under sterile conditions for biochemical analysis (aspartate transaminase [AST], alanine transaminase [ALT], ammonia, and TNF-α levels. The liver was excised for histopathological and biochemical tests (Thiobarbituric-acid-reactive substances [TBARS] and myeloperoxidase [MPO]).

Liver histology

For liver histopathological analysis, the right lobes of the livers were processed for light microscopy. This processing consisted of fixing the specimen in a 5% neutral formol solution, embedding it in paraffin, slicing sections of 5-mm thickness, and staining the sections with hematoxylin and eosin and Masson trichrome. The tissue slices were scanned and scored by two expert pathologists who were unaware of the sample assignment to an experimental group. The degree of inflammation and necrosis was expressed as the mean of 10 different fields within each slide and classified on a scale of 0–3 (normal, 0; mild, 1; moderate, 2; severe, 3).[13]

Biochemical analyses

Preparation of serum and tissue homogenates. Blood samples of approximately 4 mL were taken from each rat. Each blood sample was used for the separation of serum after transfer into marked centrifuge tubes. These samples were kept at room temperature for 30 min and centrifuged at 3000*g* for 10 min. The serum samples obtained in this way were aliquoted for biochemical analyses and were stored at –20°C until analysis. Liver tissue was removed from the rats and washed three times with cold physiological saline. They were then weighed, and the wet weight was recorded. The tissues were sliced into approximately 0.5–1 g portions using a lancet, placed in covered plastic cups wrapped with aluminum foil, and stored

at –20 °C until analysis. Tissue samples taken from the freezer on the day of analysis were weighed after they had been thawed at an ambient temperature and then homogenized in a glass–teflon homogenizer (Tempest Virtishear, model 278069; Virtis, Gardiner, New York, USA) at 5000 rev/min for 2 min after the addition of 10 volumes of cold KCL (150 mM). The homogenates were used for TBARS analysis on the same day.

Determination of lipid peroxidation. The TBARS and other products of lipid peroxidation considered to be indicators of oxidative stress were analyzed using the method described by Buege and Aust.[14] Briefly, 250 μL of tissue or serum homogenate, 500 μL of thiobarbituric acid (TBA) reactant (3.7 g/L TBA dissolved in 0.25 mol/L HCL), and 1.5 mL of 15% trichloroacetic acid were added to screw-topped Pyrex centrifuge tubes ($\sim$10 mL) and mixed. The tubes were placed in a hot water bath at 95°C for 30 min and then immediately cooled under tap water. *n*-Butanol (3 mL) was added to each tube and mixed so that the pink chromogen separated into the butanol phase. The absorbance of the colored organic phase was read against a blank at 535 nm. 1,1,3,3-Tetramethoxypropane was used to prepare a calibration curve from which the serum and tissue levels of TBARS were calculated. Tissue levels were expressed as nmol/g wet tissue.

Serum AST and ALT levels. Serum levels of AST and ALT were measured using a kinetic UV method defined by the International Federation of Clinical Chemistry, with pyridoxal phosphate and NADH as the cofactors. An Olympus autoanalyzer and commercial kits of the same brand were used for the two analyses in the routine biochemistry laboratories of Turgut Ozal Medical Center (Malatya, Turkey). AST and ALT levels were expressed as U/L.

Determination of MPO. The tissue samples from the liver, previously prepared and stored at –20°C until analysis, were thawed, weighed, suspended (10% w/v) in 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and homogenized. The homogenate was sonicated three times for 10 sec (Bandelin Electronic, Sonopuls GM 750, Berlin, Germany). After sonication, the specimens were freeze–thawed three times and microcentrifuged at 14,000*g* for 15 min at 4°C. The supernatants were used for MPO analysis.

The MPO assay was commenced by mixing 0.1 mL of tissue material with 2.9 mL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.0005% hydrogen peroxide.[15] The reaction was carried out at room temperature for about 5 min. The absorbance change at 460 nm was recorded kinetically with an LKB-UV spectrophotometer (LKB Biochrom Ultraspec Plus, Cambridge, England) at 1 min intervals. One unit of MPO activity was defined as that required to degrade 1 μmol of peroxide per minute at 25°C. Protein concentrations in the supernatants were measured using Bradford's method.[16] MPO activity was expressed as units/g protein.

Blood ammonia analysis. Blood levels of ammonia were analyzed using the colorimetric Berthelot (indophenol) reaction.[17] In this reaction, free ammonia in previously deproteinized samples generates a blue-colored indophenol molecule by reacting with sodium nitroprusside in an alkaline environment containing phenol and hypochlorite ions. The color intensity of the chromogen, spectrophotometrically measured against blank samples at 625 nm, increases in direct proportion to the concentration of ammonia. $(NH_4)_2SO_4$ was used as a standard. Results are expressed in mg ammonia nitrogen/dl.

Serum TNF-α levels. Serum TNF-α levels were measured using appropriate commercial kits (Biosource International, Camarillo, California, USA) by the ELISA method. The results were expressed as pg/mL.

Survival rates. Mortality counts in the groups were recorded daily following the first dose of TAA. The survival rates were determined based on the numbers of live rats on the day of sacrifice.

Statistical analyses

Results are expressed as means $\pm$ SD. Comparisons of quantitative variables among the groups were made with one-way analysis of variance or the corresponding nonparametric test (Kruskal–Wallis), as required. Post hoc comparisons were made with the LSD test. Proportion comparisons were carried out using the χ^2 or Fisher test. For all comparisons, a statistically significant difference was defined as $p < 0.05$.

Table 1. Mean ± SD values of serum ammonia, AST, ALT, and TNF-α levels of the groups

Groups	Ammonia[a]	AST (U/L)	ALT (U/L)	TNF-α (pg/mL)
Control (n= 8)	557.12 ± 109.68	122.38 ± 31.61	46.38 ± 25.91	71.13 ± 40.50
TAA (n = 7)	910.71 ± 263.01*	6007.29 ± 3864.24b	2578.14 ± 1155.69b	547.29 ± 348.47*
TAA+INF (n = 7)	1156.40 ± 185.04	10247.80 ± 2513.12c	3210.60 ± 1795.85	463.20 ± 150.79
INF (n = 8)	514.50 ± 19.46	144.00 ± 48.36	47.25 ± 18.45	35.88 ± 18.51

AA, thioacetamide, TAA + INF, thioacetamide + Infliximab, INF, infliximab.
[a] Ammonia was expressed in mg ammonia nitrogen/dL.
[b] $p < 0.01$ compared with the control and INF groups.
[c] $p < 0.05$ compared with the TAA group.

Table 2. Mean ± SD values of liver TBARS and MPO levels in the groups

Groups	TBARS[a]	MPO[b]
Control (n = 8)	1.56 ± 0.19	191.13 ± 41.67
TAA (n = 7)	2.07 ± 0.32[c]	330.00 ± 54.88[c]
TAA+INF (n = 7)	2.14 ± 0.24	379.40 ± 52.57
INF (n = 8)	1.43 ± 0.07	143.50 ± 25.29

TAA, thioacetamide, TAA + INF, thioacetamide + Infliximab, INF, Infliximab.
[a] Liver thiobarbituric-acid-reactive substances (TBARS) levels were expressed as nmol/g wet tissue.
[b] Liver myeloperoxidase (MPO) activities were expressed as units/g protein.
* $p < 0.01$ compared with the control and INF groups.

Table 3. Mean ± SD values of the hepatic necrosis and inflammation scores of the groups

Groups	Necrosis	Inflammation
Control (n = 8)	0.00 ± 0.0.00	0.00 ± 0.0.00
TAA (n = 7)	2.14 ± 0.37[a]	2.00 ± 0.58[a]
TAA + INF (n = 7)	2.20 ± 0.45	1.80 ± 0.44
INF (n = 8)	0.00 ± 0.0.00	0.00 ± 0.0.00

TAA, thioacetamide, TAA + INF, thioacetamide + Infliximab, INF, Infliximab.
[a] $p < 0.01$ compared wcith the control and INF groups.

Results

Mortality and survival rates

The survival rates in the TAA (87.5%) and TAA + INF groups (87.5%) were lower than in the control and INF groups on the day of sacrifice. There was no mortality in the control and INF groups. There was no statistically significant difference between groups.

Serum ammonia, AST, ALT, and TNF-α levels

Table 1 shows the mean ± SD values for serum ammonia, AST, ALT, and TNF-α levels. Serum ammonia, AST, ALT, and TNF-α levels in the TAA group were significantly higher than in the control and INF groups (all $p < 0.01$). Serum AST levels in the TAA + INF group were only significantly higher than in the TAA group ($p < 0.05$). Serum ammonia, ALT, and TNF-α levels were not significantly different between TAA and TAA + INF.

Oxidative stress results

Liver TBARS and MPO levels in the TAA group were significantly higher than in the control and INF groups (both $p < 0.01$, Table 2). These parameters were not significantly different between TAA and TAA + INF.

Results of liver histopathology

In the TAA group, liver necrosis and inflammation scores were significantly higher than in the control and INF groups (both $p < 0.01$, Table 3). These parameters were not significantly different between TAA and TAA + INF. Figures 1 and 2 show liver histopathology of control and INF groups, respectively. As can be seen in Figures 3 and 4, necrosis and inflammation in the liver significantly increased in the TAA and TAA + INF groups, respectively.

Discussion

In the present study, we detected that infliximab (antibody of TNF-α) did not prevent thioacetamide-induced liver damage in rats. Administration of thioacetamide increased serum TNF-α levels and led to oxidative liver damage. However, infliximab treatment could not restore these parameters.

Many studies in rats and cultured cells have shown the involvement of oxidative stress in the aetiology of TAA-induced liver damage.[6,18] Oxidative stress occurs because of excessive free radical production

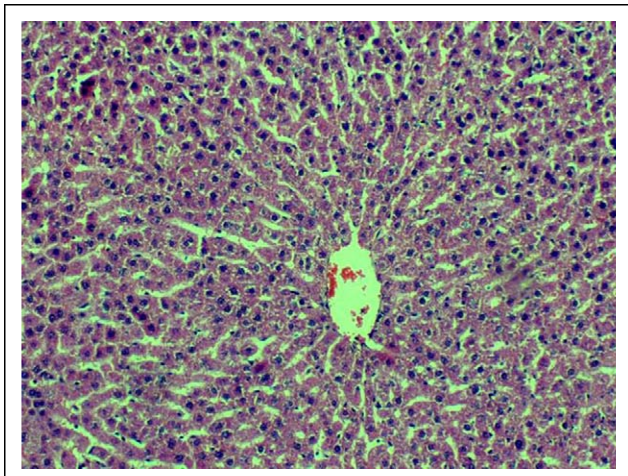

Figure 1. Normal liver histology in the control group (hematoxylin and eosin ×20).

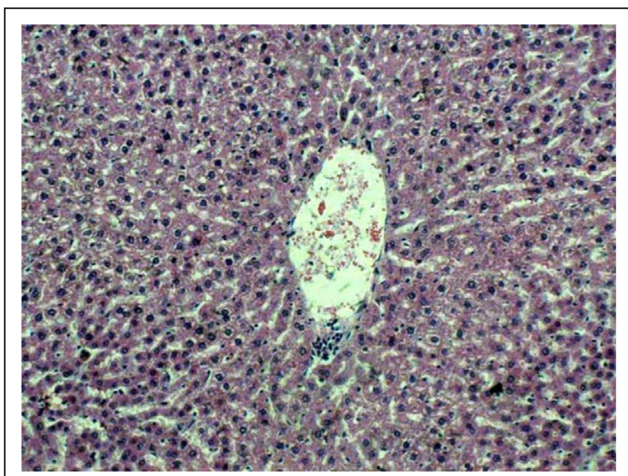

Figure 2. Normal liver histology in the INF group (hematoxylin and eosin ×20).

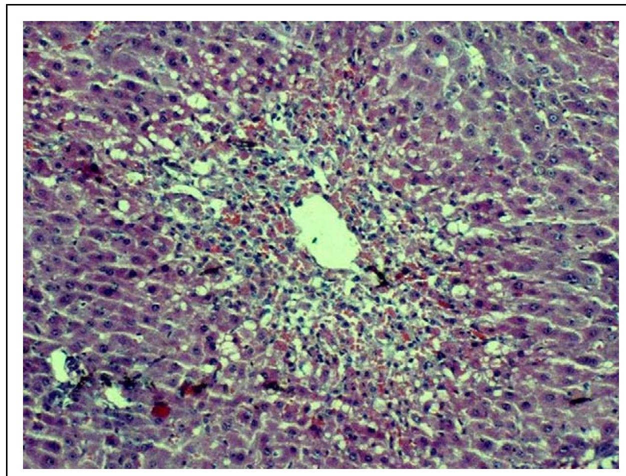

Figure 3. Severe liver necroinflammation in the thioacetamide (TAA) group (hematoxylin and eosin ×20).

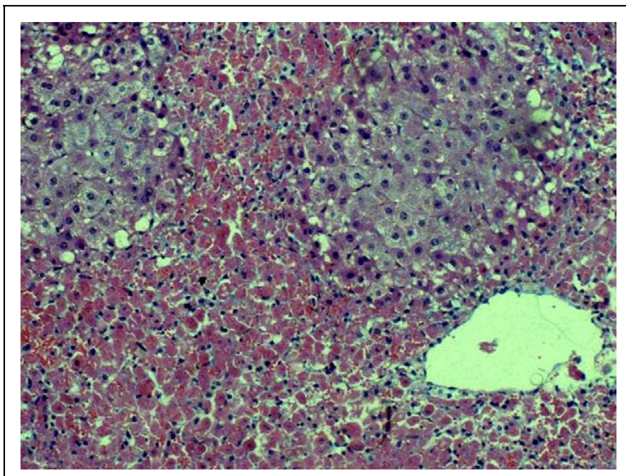

Figure 4. Severe liver necroinflammation in the thioacetamide (TAA) + Infliximab (INF) group (hematoxylin and eosin ×20).

and/or low antioxidant defence.[19-21] When hepatocytes are exposed to excess ROS, oxidative stress occurs and affects many cellular functions. An increase in free radicals causes overproduction of TBARS, which is, thus, commonly used as a marker of oxidative stress. It is well known that proinflammatory cytokines, oxidants, and reactive nitrogen moieties play important roles in liver damage. These mediators are released into the milieu secondary to inflammation, by inflammatory cells. MPO activity is used as an index of neutrophil infiltration. In our study, we found that TAA-induced liver damage was associated with increased liver TBARS and MPO activities. Additionally, high serum TNF-α levels accompanied increased oxidative stress parameters in the TAA group. It has been reported that TAA-induced hepatotoxicity increases serum TNF-α levels in rats.[22,23] TNF-α is a pleiotropic cytokine that induces cellular responses that include proliferation, production of inflammatory mediators, and cell death. In the liver, TNF-α is involved in the pathophysiology of viral hepatitis, alcoholic liver disease, and ischemia reperfusion injury. TNF-α may activate TNF receptors on hepatocytes to induce ROS production.[24] TNF-α antibodies have been shown to reduce oxidative liver damage in many studies.[8,25] However, there is no study investigating the effects of an antibody of TNF-α on thioacetamide-induced hepatotoxicity. Simpson et al. reported that inhibition of TNF-α did not prevent

experimental paracetamol-induced hepatic necrosis.[26] He et al. has reported that inhibition of TNF-α signaling by anti-TNF-α antibodies is unable to prevent fumonisin hepatotoxicity in mice.[27] Also, Sneed et al. reported that pentoxifylline (TNF-α synthesis inhibitor) protected rats from lipopolysaccharide-induced enhancement of allyl alcohol hepatotoxicity, while anti-TNF-α serum had no effect. They suggested that TNF-α is still produced by Kupffer cells after treatment with anti-TNF-α serum and can act locally before neutralization by the anti-serum.[28] Thus, they suggested that autocrine and paracrine hepatic effects of TNF-α may still ocur. Contrary to pentoxifylline, we used TNF-α antibody which cannot modulate TNF-α release like pentoxifylline. Infliximab only blocks released TNF-α in milieu. In our study, TNF-α antibody treatment did not significantly decrease TNF-α levels. This result suggested that used TNF-α antibody dose was not enough or there was a lack of cross reactivity with rat TNF-α because we used chimeric human TNF-antibody in this study.

It has been reported that Kupffer cells generate ROS, which in turn enhance the secretion of various chemokines and cytokines including TNF-α after ischemia/reperfusion. Increased TNF causes ROS overproduction in hepatocytes and leads to neutrophil infiltration.[24] In our study, thioacetamide increased liver TBARS, MPO levels, and necroinflammation scores, but TNF-alpha antibody treatment did not improve these parameters. The ineffectiveness of infliximab on increased oxidative stress may be considered a second factor for failure of infliximab on thioacetamide-induced liver damage.

In the present study, TNF-alpha antibody treatment did not alter serum TNF-alpha levels. Bahcecioglu et al. reported that an antibody of TNF-α had no significant effects on the serum TNF-α level in carbon tetrachloride-induced hepatotoxicity.[7] Iimuro et al. and Tilg et al. reported that an antibody of TNF-α did not change TNF-α levels in alcohol-induced liver damage.[29,30] In these models, it has been reported that an antibody of TNF-α treatment attenuates oxidative liver damage in contrast to our results. Interpretations for the relationship between serum TNF-α levels and liver oxidative injury in a thioacetamide model is difficult because there is no study regarding the effects of TNF-α antibodies in this model.

In our study, hepatotoxicity was induced by TAA. Thioacetamide causes hepatocellular necrosis after biotransformation to an active metabolite via the flavine adenine dinucleotidemono-oxygenase pathway, resulting in the formation of TAA-S-oxide. Also, it has been reported that hydroxyl radicals play a direct role in the induction of liver damage in TAA model.[13] These TNF-α-independent liver damage pathways may be contributed to ineffectiveness of TNF-α antibody treatment in this model.

In conclusion, our results suggest that oxidative stress plays an important role in TAA-induced hepatotoxicity, and an antibody of TNF-α does not improve oxidative liver damage in this model.

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