Leptin administration exacerbates thioacetamide-induced liver fibrosis in mice

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AIM: To investigate the effects of leptin administration on liver fibrosis induced by thioacetamide (TAA).

METHODS: Twenty-four male C57Bl/6 mice were randomly allocated into four groups, which were intra-peritoneally given saline (2 mL/kg), leptin (1 mg/kg), TAA (200 mg/kg), and TAA (200 mg/kg) plus leptin (1 mg/kg) respectively, thrice a week. All mice were killed after 4 wk. The changes in biochemical markers, such as the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), malondialdehyde (MDA) in liver were determined. For histological analysis, liver tissues were fixed with 10% buffered formalin, embedded with paraffin. Hematoxylin-eosin (HE) staining and picric acid-Sirius red dyeing were performed. The level of α1(I) procollagen mRNA in liver tissues also increased (0.28±0.04 vs 1.91±0.14 nmol/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver increased (50.67±10.46 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01), and the level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01), and the level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01). The level of SOD in liver decreased (51.80±8.36 U/mg pro vs 50.67±10.46 U/mg pro, P<0.01).

RESULTS: Apparent liver fibrosis was found in TAA group and TAA plus leptin group. Compared to saline group, the levels of ALT and AST in serum and MDA in liver increased in TAA group (205.67±27.69 U/L vs 50.67±10.46 U/L, 177.50±23.65 U/L vs 76.33±12.27 U/L, 2.60±0.18 nmol/mg pro vs 1.91±0.14 nmol/mg pro, P<0.01) and in TAA plus leptin group (256.17±22.50 U/L vs 50.67±10.46 U/L, 234.17±27.37 U/L vs 76.33±12.27 U/L, 2.97±0.19 nmol/mg pro vs 1.91±0.14 nmol/mg pro, P<0.01). The level of SOD in livers decreased (51.80±8.36 U/mg pro vs 81.52±11.40 U/mg pro, 35.78±6.11 U/mg pro vs 81.52±11.40 U/mg pro, P<0.01) and in TAA group (205.67±27.69 U/L vs 50.67±10.46 U/L, P<0.05; 234.17±27.37 U/L vs 177.50±23.65 U/L, P<0.05; 2.97±0.19 nmol/mg pro vs 2.60±0.18 nmol/mg pro, P<0.05; 0.54±0.07 vs 0.28±0.04, P<0.01; 3.17 vs 2.00, P<0.05), and the level of SOD in liver decreased (35.78±6.11 U/mg pro vs 51.80±8.36 U/mg pro, P<0.05). There were similar changes in the degree of type I collagen deposition confirmed by picric acid-Sirius red dyeing.

CONCLUSION: Leptin can exacerbate the degree of TAA-induced liver fibrosis in mice. Leptin may be an important factor in the development of liver fibrosis.

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Key words: Liver fibrosis; Leptin; α1(I) procollagen

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INTRODUCTION
Leptin, an obese gene product, is a 16-kd peptide hormone expressed and secreted predominantly by adipose tissue[6], and plays an essential role in the regulation of body weight mainly by reducing food intake and increasing energy expenditure[3]. In addition to the effect of controlling body fat mass, leptin has a variety of other biological functions, such as wound healing, angiogenesis, immune response, etc.[3-6], thus exerting its effects on many tissues or organs, including the liver. Potter et al.[5], found that isolated hepatic stellate cells (HSCs) also produce leptin during the in vitro transactivation process in 1998. Since then researchers have paid more attention to the correlation between leptin and liver diseases. Recently, it was reported that the serum leptin levels are elevated in patients with chronic viral hepatitis, alcohol-induced cirrhosis, or non-alcoholic steatohepatitis (NASH)[8-11]. These observations suggest that leptin may be involved in the progression of liver fibrosis. Accordingly, in the present study we investigated the effect of leptin administration on liver fibrosis caused by thioacetamide (TAA).

MATERIALS AND METHODS

Animals and treatment
Twenty-four, 6-wk-old male C57Bl/6 mice, weighing 18.4-24.2 g, were obtained from Institute of Transplantation, Tongji Medical College, Huazhong University of Science and Technology. All mice were housed in a temperature-humidity-controlled environment in a 12 h light-dark cycle
with free access to food and water. The mice were randomly divided into four groups with six mice in each, which were given an intra-peritoneal injection of saline (2 mL/kg), recombinant murine leptin (1 mg/kg, R&D Systems Inc., USA), TAA (200 mg/kg), TAA (200 mg/kg) plus leptin (1 mg/kg) thrice a week. All mice were killed after 4 wk. Blood and livers were collected for further examination.

**Histological examination**

Liver tissues were fixed with 10% buffered formalin, embedded with paraaffin, and then hematoxylin-eosin (HE) staining and picric acid-Sirius red dyeing were performed. Liver fibrosis was evaluated by a semi-quantitative method to assess the degree of histological injury using the following criteria: grade 0: normal liver; grade 1: few collagen fibrils extended from the central vein to the portal tract; grade 2: apparent collagen fibril extension without encompassing the whole lobule; grade 3: collagen fibrils extended into and encompassed the whole lobule; grade 4: diffuse extension of collagen fibrils and formation of pseudo-lobule.

**Estimation of liver function**

Blood was obtained at the time of killing. The serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were measured by the Olympus AU-1000 biochemical autoanalyzer, as markers of hepatic damage.

**Level of malondialdehyde and superoxide dismutase**

Malondialdehyde (MDA) and superoxide dismutase (SOD) contents in liver tissue were assayed by assay kits (Jiancheng Biotech Ltd, Nanjing, China).

**RNA extraction and RT-PCR assay**

Expression of \( \alpha_1(I) \) procollagen mRNA was evaluated with RT-PCR. Total RNA was isolated from liver specimens with RNase reagent (Watson Biotechnologies, Inc., Shanghai, China) according to the manufacturer’s descriptions. Total RNA was quantified spectrometrically at 260 nm, and the quality of isolated RNA was analyzed on agarose gels under standard conditions. Two-step RT-PCR was performed as recommended by the suppliers. Primer sequences were \( \alpha_1(I) \) procollagen: forward 5'-CCT GGA CGC CAT CAA GGT CTA C-3' and reverse 5'-CCA AGT TCC GGT GTG ACT CG-3', fragment length 419 bp; \( \beta \)-actin: forward 5'-ACC ACA GCT GAG AGG GAA ATC G-3' and reverse 5'-AGA GGT CTT TAC GGA TGT CCA CG-3', fragment length 277 bp. Amplification conditions were as follows: pre-denaturation at 95 °C for 2 min, then in a thermal controller for 35 cycles (denaturation at 95 °C for 45 s, annealing at 56 °C for 45 s and extension at 72 °C for 1 min), and a final extension at 72 °C for 7 min after the last cycle. Ten milliliters of the PCR products was analyzed on 2% agarose gel containing ethidium bromide with TAE buffer at 80 V for 30 min and photographed under UV illumination. The band intensities were quantified by densitometry. \( \alpha_1(I) \) procollagen/\( \beta \)-actin quotient indicated the relative expression of \( \alpha_1(I) \) procollagen.

**Statistical analysis**

The results were expressed as mean±SD. One-way analysis of variance (ANOVA) with LSD post hoc comparison was used to test for differences in means of variables between groups. \( P<0.05 \) was considered statistically significant. All data were analyzed by SPSS 11.0 software.

**RESULTS**

**Animal model and liver histology**

Compared to saline group and leptin group, the mice in TAA group and TAA plus leptin group were thin and less haired. The surface of liver was rough and formation of small nodules was observed, indicating that liver fibrosis developed as expected. The degree of type I collagen deposition was confirmed by liver histology stained with picric acid-Sirius red. As anticipated, liver tissue specimens from both the saline group and leptin group showed no significant picric acid-Sirius red staining, indicating a lack of type I collagen deposition outside of central and portal blood vessels. Apparent hepatocyte degeneration, necrosis, infiltration of inflammatory cells, expanded portal tracts and collagen deposition were found in TAA group and TAA plus leptin group. The degree of liver fibrosis in these two groups significantly increased compared to that in saline group or leptin group (\( P<0.01 \)). But compared to TAA group, the deposition of type I collagen in TAA plus leptin group was more apparent and the degree of liver fibrosis also increased (\( P<0.05 \)). The details about the degree of liver fibrosis in each group are shown in Table 1. Type I collagen deposition in each group is shown in Figure 1.

**Serum ALT and AST level**

At the end of the study, there was no difference in serum ALT and AST levels between saline group and leptin group. But serum ALT and AST levels in TAA group and TAA plus leptin group significantly increased compared to those in saline group and leptin group (\( P<0.01 \)). Furthermore, treatment with TAA plus leptin increased the ALT and AST levels compared to treatment with TAA (\( P<0.05 \), Table 2).

**Table 1** Degree of liver fibrosis in each group of mice

| Group       | n | Grade of liver fibrosis | Average |
|-------------|---|------------------------|---------|
|             |   | 0 | I | II | III | IV |
| Saline      | 6 | 6 | 0 | 0 | 0 | 0 | 0 |
| Leptin      | 6 | 6 | 0 | 0 | 0 | 0 | 0 |
| TAA         | 6 | 6 | 0 | 0 | 0 | 0 | 0 |
| TAA+leptin  | 6 | 6 | 0 | 0 | 0 | 5 | 1 | 3.17<sup>a,b,d</sup> |

\(<P<0.05 \) vs TAA group; \(<P<0.01 \) vs saline group; \(<P<0.01 \) vs leptin group.
Changes of MDA and SOD in liver tissue

The amount of MDA and SOD did not obviously change in leptin group, compared to that in the saline group. The amount of MDA in TAA group and TAA plus leptin group was significantly higher than that in saline group ($P<0.01$), while SOD in the two groups was significantly lower than that in saline group ($P<0.01$). But MDA was higher in TAA plus leptin group than in TAA group ($P<0.05$), while SOD was lower in TAA plus leptin group than in TAA group ($P<0.01$, Table 3).

![Figure 1](image)

**Figure 1** Type I collagen deposition in saline group (A), leptin group (B), TAA (C), and TAA plus leptin group (D). 200× (red color indicates picric acid-Sirius red staining for collagen).

| Table 2 | Serum ALT and AST level (mean±SD) |
|---------|-----------------------------------|
| Group   | $n$   | ALT (U/L) | AST (U/L) |
| Saline  | 6     | 50.67±10.46 | 76.33±12.27 |
| Leptin  | 6     | 47.50±11.78 | 71.67±14.39 |
| TAA     | 6     | 205.67±27.69 | 177.50±23.65 |
| TAA+leptin | 6   | 256.17±22.50 | 234.17±27.37 |

* $P<0.05$ vs TAA group; $P<0.01$ vs saline group; $P<0.01$ vs leptin group.

Table 3 Changes of MDA and SOD in liver tissue (mean±SD)

| Group        | $n$ | SOD (U/mg pro) | MDA (nmol/mg pro) |
|--------------|-----|----------------|-------------------|
| Saline       | 6   | 81.52±11.40    | 1.91±0.14         |
| Leptin       | 6   | 83.63±10.68    | 1.93±0.13         |
| TAA          | 6   | 51.80±6.36     | 2.60±0.18         |
| TAA+leptin   | 6   | 35.78±6.11     | 2.97±0.19         |

* $P<0.05$ vs TAA group; $P<0.01$ vs saline group; $P<0.01$ vs leptin group.

**α1(I) procollagen mRNA expression**

To determine the expressions of collagen, mRNA transcripts for α1(I) procollagen were assessed. RNA extracts from livers were analyzed by reverse transcription PCR. Furthermore, the band intensity ratio of α1(I) procollagen to β-actin was evaluated and presented as percentage of β-actin. As shown in Table 4 and Figure 2, the expression of α1(I) procollagen in leptin group did not obviously change, compared to that in the saline group, but significantly increased in TAA group and TAA plus leptin group ($P<0.01$). The expression of α1(I) procollagen in TAA plus leptin group was much higher than that in TAA group ($P<0.01$, Table 4 and Figure 2).

**DISCUSSION**

In the present study, chronic administration of TAA or TAA plus leptin caused liver fibrosis as indicated by the changes of serum markers, histopathological changes, and molecular biological changes. But more overt liver fibrosis was observed, when leptin was used in combination with TAA. Our results are in line with other findings that leptin-deficient $ob/ob$ mice do not develop fibrosis during steatohepatitis or in response to chronic toxic liver injury,$^{12,13}$ indicating that leptin can exacerbate the degree of fibrosis in mouse liver induced by TAA, and may be an important factor in the development of liver fibrosis.
factor in the development of liver fibrosis.

The activities of serum fibrosis-associated enzymes, namely ALT and AST, significantly increased along with the increased expression of \( \alpha 1(1) \) procollagen mRNA in TAA group and TAA plus leptin group. But leptin could augment TAA-induced inflammatory response and increase the expression of \( \alpha 1(1) \) procollagen mRNA. The histological examination also found that leptin increased the TAA-induced deposition of type I collagen. Specific increase in procollagen mRNAs in liver fibrosis parallels increases in tissue collagen content. Since increased synthesis of procollagen contributes to the increase of collagen content in fibrotic liver, the expression of \( \alpha 1(1) \) procollagen mRNA can reflect the degree of hepatic fibrosis[14,15]. Activation of HSCs is the essential event in hepatic fibrogenesis, because HSCs transactivate to myofibroblast-like cells that produce a large, excess amount of matrix proteins (e.g., fibrillar collagen, fibronectin, laminin, and proteoglycans) in injured liver[20]. It was reported that HSCs express short forms of the leptin receptor (Ob-Ra), but not the functional long form of the leptin receptor Ob-Rb[17,18]. The activation of HSCs transmits the cytokine signal to Kupffer cells in the liver express the long form of the leptin receptor Ob-Rb[17,18], leptin may exert an indirect effect on the activation of HSCs mediated by TGF-\( \beta \). As Ob-Rb leptin can increase TGF-\( \beta \) expression in the sinusoidal endothelial cells and Kupffer cells, TGF-\( \beta \) activates type I collagen promoters, and upregulates the extracellular matrix such as collagen and fibronectin from HSCs[19]. Recently, it was reported that leptin also can activate HSCs directly[20]. However, the role of leptin in the activation of HSCs needs to be further analyzed.

TAA is a typical hepatotoxin and causes centrilobular necrosis by generation of ROS. ROS triggers lipid peroxidation chain reaction. MDA is the production of lipid peroxidation, the amount of MDA can express the degree of lipid peroxidation. SOD is a ubiquitous chain breaking anti-oxidant found in all aerobic organisms. It is a metalloprotein widely distributed in all cells and protects cells against ROS-induced oxidative damage. Oxidative stress contributes to the pathogenesis of hepatic fibrosis induced by alcohol and virus infection[21,22]. Furthermore, oxidative stress is involved in activation of HSCs and promotion of their proliferation, collagen synthesis, and migration[23]. The activation of quiescent HSCs plays an important role in the process of liver fibrosis[24]. In our experiment, MDA was higher in TAA plus leptin group than in TAA group (\( P<0.05 \)), while SOD was lower in TAA plus leptin group than in TAA group. The results suggest leptin can augment TAA-induced lipid peroxidation by lowering the level of enzymic antioxidant. But leptin administration alone has no apparent effect on lipid peroxidation. Whether leptin is a co-factor needs to be further studied. On the other hand, leptin can regulate macrophage function[24] and augment endotoxin-stimulated TNF production by macrophages[25]. Hematopoietic cells including macrophages and lymphocytes contain a functional leptin receptor[26], through which leptin regulates proliferation and maturation of immune cells[27,28] and elicits release of pro-inflammatory cytokines from macrophages[3]. These pro-inflammatory cytokines and immune responses play a critical role in the development of liver fibrosis. The above findings may explain, at least in part, why leptin increases the degree of liver fibrosis in mice induced by TAA.

It has been reported that hepatic cirrhosis is six fold more prevalent in obese individuals than in the general population[29,30] and obesity is an independent risk factor for the development of chronic liver diseases caused by alcohol, chronic hepatitis C, and NASH[31-34]. These observations indicate that there could be a pathogenic link between obesity and liver fibrosis. But the reason why obesity accelerates the development of hepatic fibrosis has not been elucidated. Circulating levels of leptin correlate well with body fat mass[35]. Obese people have remarkably high serum leptin levels. Increased serum leptin presumably enhances pro-inflammatory and pro-fibrogenic responses in the liver, and generate oxidative stress. Leptin may be a determinant of hepatic inflammation and fibrosis in obese individuals, especially in those with NASH. Our findings support the hypothesis that leptin is involved in the progression of liver fibrosis. However, additional studies are needed to elucidate the role of leptin in the progression of chronic liver fibrosis in obese patients.

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