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Therapeutic effects of a recombinant mutant of the human ciliary neurotrophic factor in a mouse model of metabolic syndrome

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Metabolic syndrome (MS) is highly prevalent in developed countries and becoming a serious worldwide public health issue. In this study, we established a MS model by feeding male C57BL/6J mice with a high-fat diet (10%) for 18.5 weeks, studied the therapeutic effects of a recombinant mutant of the human ciliary neurotrophic factor (rhmCNTF) 0.1 (C-0.1) or 0.3 (C-0.3) mg·kg^{-1} per day subcutaneously or pair feeding (PF, which mice were restricted to the same amount of food as eaten by C-0.3 treated mice) in MS mice. After 10 days treatment, rhmCNTF reduced obesity related indices, ameliorated glucose and lipid metabolism abnormality, and enhanced insulin sensitivity. In addition, liver function and antioxidant ability of MS mice were improved by rhmCNTF. Pair feeding revealed the same effects as C-0.3 on obesity related indices and insulin sensitivity, but aggravated hepatic steatosis and hepatic function. The results suggest that rhmCNTF could serve as an effective therapeutic agent for MS and related diseases.

1. Introduction

Metabolic syndrome (MS) is defined as a cluster of abnormalities including abdominal obesity, insulin resistance, type 2 diabetes mellitus, dyslipidaemia, hypertension, non-alcoholic fatty liver diseases, and/or cardio-cerebrovascular diseases, *etc* (Unger 2002; Dandona et al. 2005). It is highly prevalent in developed countries (Meigs 2002) and becoming a serious worldwide public health issue (Haffner and Taegtmeyer 2003). Among these disorders, abdominal obesity and ectopic fat deposition are the central feature and main reason for MS (Lebovitz 2003; Turkoglu et al. 2003; Grundy 2004).

The ciliary neurotrophic factor (CNTF) was first used as a trophic factor for amyotrophic lateral sclerosis to attenuate disease progression (ALS CNTF Treatment Study Group 1996). But patients treated with CNTF underwent marked weight loss and nausea. Since then, CNTF was studied as an anti-obesity agent at a lower dose. Previous reports indicated that CNTF or CNTF analogs decreased body weight and ameliorated the metabolic abnormalities induced by obesity in *ob/ob* mice (Gloaguen et al. 1997; Lambert et al. 2001), *db/db* mice (Gloaguen et al. 1997; Sleeman et al. 2003), diet-induced AKR/J obese mice (Gloaguen et al. 1997; Lambert et al. 2001) and obese diabetic KK/Ay mice (Liu et al. 2007a,b). But in a phase III trial, the efficacy of recombinant human CNTF (Axokine, Regeneron Pharmaceuticals, Tarrytown, NY) for obesity treatment was limited by the development of antibodies in two thirds of the subjects (Korner and Aronne 2004).

To enhance biological activity and decrease immunogenicity of CNTF, a novel mutant of recombinant human CNTF (rhmCNTF) was obtained, which is a truncated form of CNTF with the last 14 C-terminal amino acids removed and glycine at position 185 and isoleucine at position 186 are replaced by lysine and methionine, respectively. In this study, we established MS models by feeding C57BL/6J mice with high-fat diet (HFD) for 18.5 weeks and studied the therapeutic effects of rhmCNTF.

2. Investigations and results

2.1. Characteristics of MS model

After 18.5-week feeding with HFD and 10 days injection with saline plus feeding with HFD, MS mice were abdominal obesity, hypercholesterolemia, hyperglycemia, hyperinsulinemia, and insulin resistance (Table). In addition, mice had significantly increased liver weight and hepatic steatosis (Figs. 1 and 2). These symptoms were similar to those in humans. No animal died prior to the time of examination from any groups.

2.2. Effects of rhmCNTF on obesity related indices

The treatment of rhmCNTF decreased body weight in dose-dependent manner in MS mice (Fig. 3). After 10 days treatment, rhmCNTF 0.3 mg kg^{-1} per day (C-0.3) produced greater weight loss than pair-feeding (PF, which restricted food intake to same amount as eaten by the C-0.3 treated mice) (Table). The wet weight of visceral fat and fat coefficient of C-0.3 and pair feeding mice were both reduced significantly (Table).

2.3. Effects of rhmCNTF on serum lipid levels

The cholesterol in HFD led to decrease in serum triglyceride (TG) level. This result is consistent with that reported by Savransky et al. (2007). The serum level of TG was significantly decreased in C-0.1, C-0.3 and PF group by 38.5%,

Table: Effects of rhmCNTF or pair-feeding on obesity related indices, serum levels, and insulin sensitivity in metabolic syndrome (MS) mice

	Normal $n = 10$	Vehicle $n = 14$	PF $n = 14$	C-0.1 $n = 14$	C-0.3 $n = 14$
Body weight (g)	27.5 ± 1.9	28.6 ± 1.6	$25.6 \pm 2.1^{**}$	$26.3 \pm 1.2^{**}$	$23.5 \pm 1.8^{**‡}$
Wet weight of visceral fat (g)	$0.78 \pm 0.07^{*}$	1.04 ± 0.32	$0.71 \pm 0.32^{*}$	0.98 ± 0.34	$0.68 \pm 0.19^{**}$
Visceral fat coefficient (%)	$2.84 \pm 0.33^{*}$	3.77 ± 1.00	$2.71 \pm 1.04^{*}$	3.70 ± 1.21	$2.87 \pm 0.69^{**}$
Triglyceride ($mmol \cdot L^{-1}$)	$1.06 \pm 0.13^{*}$	0.83 ± 0.19	$0.61 \pm 0.12^{*}$	$0.51 \pm 0.16^{**}$	$0.53 \pm 0.15^{**}$
Total cholesterol ($mmol \cdot L^{-1}$)	$2.91 \pm 0.32^{**}$	3.81 ± 0.29	3.54 ± 0.12	3.58 ± 0.24	$3.44 \pm 0.19^{*}$
FBG ($mmol \cdot L^{-1}$)	$4.49 \pm 0.69^{**}$	6.03 ± 1.33	$4.07 \pm 1.18^{**}$	5.31 ± 1.10	$4.88 \pm 1.09^{*}$
FINS ($\mu g \cdot L^{-1}$)	$0.24 \pm 0.05^{**}$	0.42 ± 0.12	$0.29 \pm 0.09^{**}$	0.38 ± 0.10	$0.32 \pm 0.08^{*}$
FIRI	$1.21 \pm 0.39^{**}$	2.65 ± 1.29	$1.21 \pm 0.61^{**}$	2.02 ± 0.74	$1.62 \pm 0.61^{*}$

MS mice were treated for 10 days with saline (vehicle) or rhmCNTF 0.1, 0.3 mg kg^{-1} per day subcutaneously, or were pair-fed to the food intake of C-0.3 group (PF). FBG: fasting blood glucose, FINS: fasting serum insulin level; FIRI: fasting insulin resistance index, FIRI = FBG ($mmol \cdot L^{-1}$) × FINS ($mIU \cdot L^{-1}$)/25. All data are mean ± S.D.

* $P < 0.05$

** $P < 0.01$ versus vehicle group

‡ $P < 0.01$ when C-0.3 group versus PF group

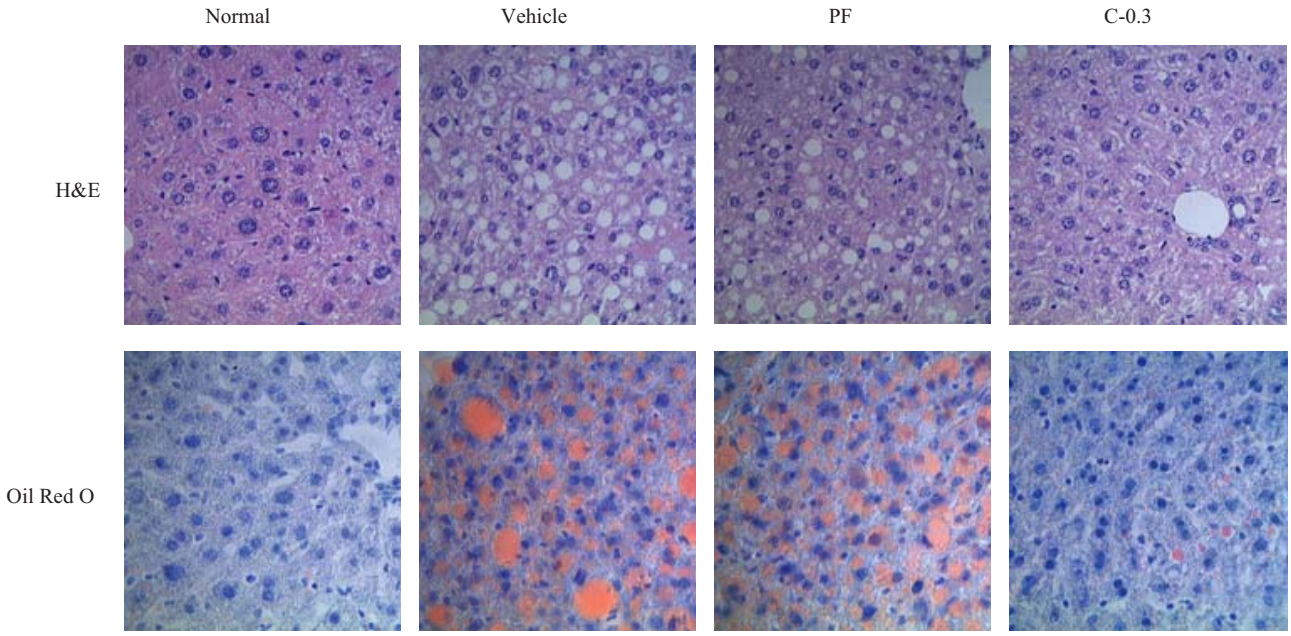

Fig. 1: Effect of rhmCNTF or pair-feeding on hepatic steatosis in metabolic syndrome (MS) mice. MS mice were treated for 10 days with saline (vehicle), or rhmCNTF 0.3 mg kg^{-1} per day subcutaneously (C-0.3), or were pair-fed to the food intake of C-0.3 mice (PF). Liver sections were H&E or oil red O stained (magnification ×400)

36.1%, and 26.5%, respectively (Table). The serum level of total cholesterol (TC) was significantly decreased in the C-0.3 group by 9.7% as compared with the MS model group ($P < 0.05$). TC level decreased slightly in C-0.1 and PF groups but had no statistically significant difference as compared with MS model group (Table).

2.4. Effects of rhmCNTF on insulin sensitivity

The hyperglycemia, hyperinsulinemia, and insulin resistance in MS mice were corrected effectively in C-0.3 and PF groups, but not in the C-0.1 group (Table). Insulin sensitivity in the PF group was improved closely to that in normal group and seems superior to that of the rhmCNTF 0.3 mg kg^{-1} treatment group (Table).

2.5. Effects of rhmCNTF on hepatic steatosis and hepatic function

The MS mice had hepatic steatosis and significantly increased liver weight (both $P < 0.05$) in conjunction with slightly elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels ($P > 0.05$). The treatment of rhmCNTF showed a trend of improvement on hepatic steatosis ($P > 0.05$, Figs. 1 and 2*A*) associated with a significant reduction of serum levels of ALT in both C-0.1 and C-0.3 groups (Fig. 2*C*) and AST in C-0.1 group (Fig. 2*D*). On the contrary, pair feeding had a aggravated tendency on hepatic steatosis ($P > 0.05$, Figs. 1 and 2*A*) associated with the significant elevation of liver free fatty acid (FFA) concentration (Fig. 2*B*) and serum ALT level (Fig. 2*C*). The difference of the hepatic steatosis degree, liver FFA content, serum levels of ALT and AST had statistical significance between C-0.3 group and PF group (Fig. 2*A*, *B*, *C*, *D*). The liver weight loss of C-0.3 group was equivalent to that in PF group (Fig. 2*E*).

2.6. Effects of rhmCNTF on antioxidant ability in MS mice

The MS mice had a significantly elevated liver total antioxidant capacity (T-AOC), but not a significantly changed liver malondialdehyde (MDA) level. The 10 days injection of rhmCNTF increased liver T-AOC and decreased liver MDA level significantly (Fig. 4). The effects of pair-feeding on liver T-AOC and MDA were similar to that of rhmCNTF (Fig. 4).

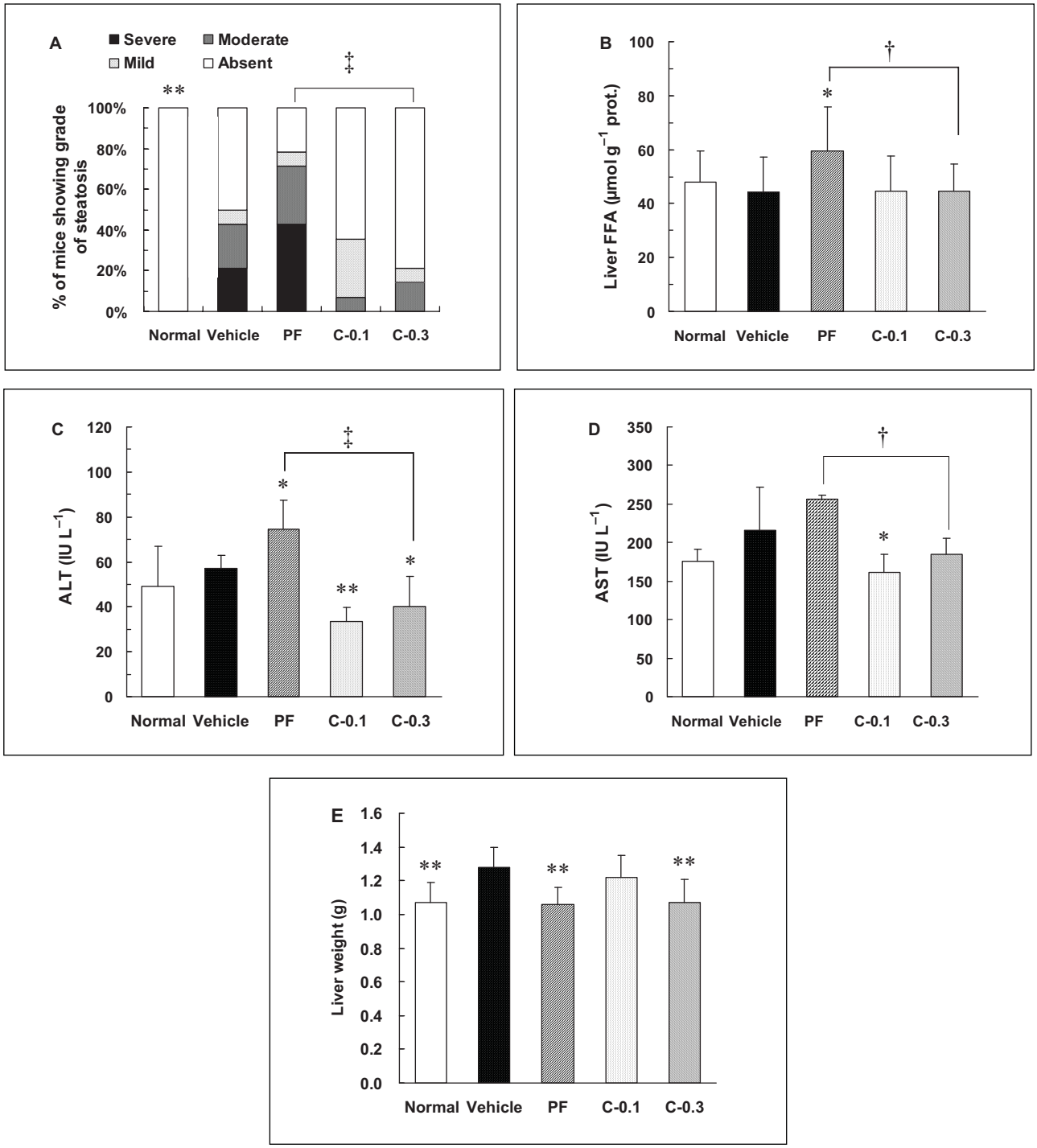

Fig. 2: Effect of rhmCNTF or pair-feeding on hepatic steatosis and hepatic function in metabolic syndrome (MS) mice. MS mice were treated for 10 days with saline (vehicle), or rhmCNTF 0.1, 0.3 mg kg^{-1} per day subcutaneously (C-0.1, C-0.3), or were pair-fed to the food intake of C-0.3 mice (PF). (*A*) The degree of steatosis was classified as absent, or present in < 30% hepatocytes (mild), 30–60% hepatocytes (moderate), or > 60% hepatocytes (severe). Animals were 10 in normal group and 14 in other groups. Chi square test was used for the ranked data statistical analysis. (*B*) Liver free fatty acid (FFA) content, $n = 10–14$. (*C*) The level of serum alanine aminotransferase (ALT) and (*D*) aspartate aminotransferase (AST), $n = 5–6$. (*E*) Liver weight, $n = 10–14$. Data are mean ± S.D. in figure *B*, *C*, *D*, and *E*. $^{*}P < 0.05$, $^{**}P < 0.01$ versus vehicle group; $^{\dagger}P < 0.05$, $^{\ddagger}P < 0.01$ when C-0.3 group versus $PF_{C\text{-}0.3}$ group

3. Discussion

Metabolic syndrome (MS) is characterized by abdominal obesity complicated by insulin resistance, type 2 diabetes mellitus, and dyslipidaemia, *etc*. In this study, MS mice were abdominal obesity, hypercholesterolemia, hyperglycemia, hyperinsulinemia, insulin resistance and hepatic steatosis. These symptoms are similar to those in humans. In addition, the results showed that model mice had hepatic triglyceride accumulation, but had no obviously increased aminotransferase level, hepatic FFA content and oxidative stress. The level of hepatic T-AOC even compensatory increased. These results indicated that mice simply showed hepatic steatosis alone. The triglyceride synthesis actually helps to protect hepatocytes from lipotoxicity by buffering the accumulation of FFA (Yamaguchi et al. 2007).

The treatment with rhmCNTF reduced obesity related indices, ameliorated glucose and lipid metabolism abnormality, enhanced insulin sensitivity and hepatic antioxidant ability, and slightly improved hepatic steatosis and function. Sleeman et al. (2003) also reported that $CNTF_{Ax15}$ lowers the degree of hepatic steatosis in conjunction with improved liver function,

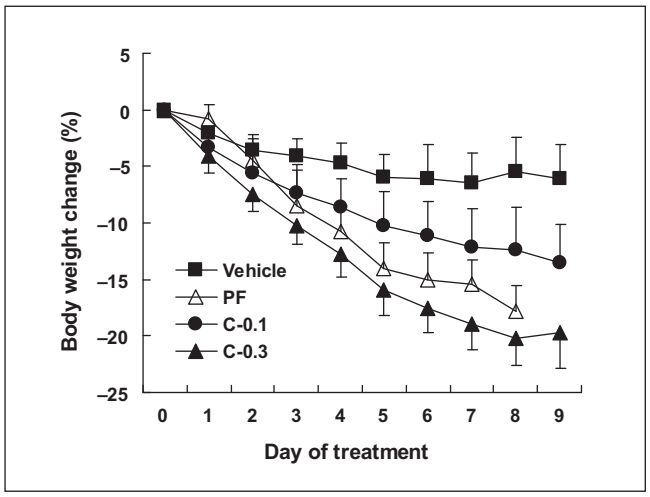

Fig. 3: Effect of rhmCNTF or pair-feeding on body weight in metabolic syndrome (MS) mice. MS mice were treated for 10 days with saline (vehicle) or rhmCNTF 0.1, 0.3 mg kg^{-1} per day subcutaneously (C-0.1, C-0.3), or were pair-fed to the food intake of C-0.3 mice (PF). Body weight is shown as percentage difference from that of day 0. All data are mean ± S.D., $n = 10$–14

liver insulin signaling, and metabolic rate in *db/db* mice. One reason for enhanced insulin sensitivity after CNTF treatment is increased glucose uptake and insulin signaling in skeletal muscle (Watt et al. 2006). RhmCNTF enhanced hepatic antioxidant ability shown in this study may be a mechanism for improvement of hepatic function.

Pair feeding, during which mice were restricted to the same amount of food as eaten by C-0.3 treated mice revealed the same effects as C-0.3 on weight loss, liver weight and visceral fat weight reduction, and improvement of insulin sensitivity, but aggravated accumulation of FFA, hepatic steatosis and liver damage. The results showed that caloric restriction reduced visceral fat mass and thus enhanced insulin sensitivity. But short-term severe caloric restriction exaggerated fat mobilization from peripheral adipocytes, which in turn increased the content of FFA in liver and led to liver function damage. This indicates that rhmCNTF has other important mechanisms not limited in food intake reduction. Administration of $CNTF_{A\times15}$ produced a marked improved insulin and lipid homeostasis in DIO mice without elevating corticosterone levels but food restriction increased circulating adrenal corticosteroids (Lambert et al. 2001).

4. Experimental

4.1. Protein

The genetically engineered recombinant human mutant of ciliary neurotrophic factor (rhmCNTF) was manufactured by the Lanzhou Institute of Biological Products (Lanzhou, China). The purity of protein is higher than 95%.

4.2. High-fat diet

The high-fat diet (HFD) containing 10% fat (w/w), 2% cholesterol (w/w), and 0.4% sodium cholate in basic diet was obtained from Suzhou Shuangshi Laboratory Animal Feed Science Co. Ltd. (Suzhou, China).

4.3. Animals

Nine-week-old male C57BL/6J mice weighing 18–22 g were obtained from the Shanghai Laboratory Animal Center (Shanghai, China) housed under standard laboratory condition with a 12 h/12 h light-dark cycle (7:00–19:00), at a temperature of 20–22 °C, and a humidity of 40-60%. All animal procedures were conducted in compliance with the guidelines for animal care and use of Gansu Province Key Laboratory of Preclinical Study for New Drug, China.

4.4. Experimental protocol

After 7 days adaptation, mice were fed with HFD *ad libitum* for 18.5 weeks to produce the MS model, except for the mice which were fed with basic diet as normal control group. MS mice were subcutaneously injected with saline/vehicle (in model control group) or rhmCNTF 0.1 (C-0.1), 0.3 (C-0.3) mg·kg^{-1} per day, or pair-fed (PF, where mice were restricted to the same amount of food as eaten one day before by C-0.3 mice) for 10 days. Body weight and food intake were measured daily. To facilitate measurement of food intake, mice were transferred from group housing to single housing. On day 11, 16 h after the last administration, all mice were killed for serum and tissues analysis.

4.5. Serum samples analysis

Serum samples were taken to determine fasting blood glucose (FBG) by colorimetry, fasting serum insulin (FINS) by rat/mouse insulin ELISA kit (the product of Linco Research, Inc., St. Charles, Missouri, USA), triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) with an automatic biochemistry analyzer. Fasting insulin resistance index (FIRI) were calculated as in equation: FIRI = FBG (mmol·L^{-1}) × FINS (mIU·L^{-1})/25.

4.6. Tissues analysis

The liver and visceral fat (perirenal fat and epididymal fat pads) wet weight was measured. The liver tissue of every animal was divided into three parts. One part was fixed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin (H&E). One part was for frozen section and stained with oil red O. The degree of hepatic steatosis was evaluated

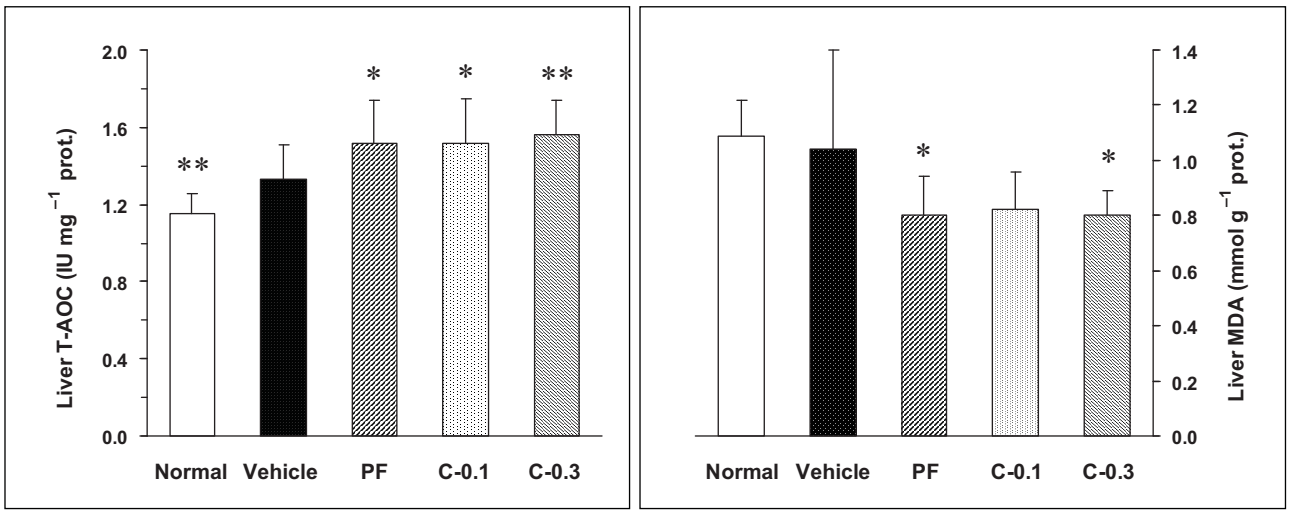

Fig. 4: Effect of 10 days subcutaneous injection of rhmCNTF or pair-feeding on Liver total antioxidant capacity (T-AOC) and content of malondialdehyde (MDA) in metabolic syndrome (MS) mice. All data are mean ± S.D., $n = 10$–14. *$P < 0.05$, ** $P < 0.01$ versus vehicle group

by light microscopy and graded as follows: absent (no hepatocyte is steatosis), mild (present in <30% hepatocytes), moderate (30–60% hepatocytes), or severe (>60% hepatocytes). The last part tissue was minced and homogenized in cold physiological saline on ice to determine free fatty acid (FFA) and total antioxidant capacity (T-AOC) by a colorimetric method, malondialdehyde (MDA) by thiobarbituric acid reactive substance assay. Kits were products of Jiancheng Bioengineering Institute, Nanjing, China),

4.7. Statistical analysis

The data were expressed as mean ± S.D. Statistical comparisons between groups were carried out using ANOVA. Chi square test was used for the ranked data statistical analysis. $P < 0.05$ was considered significant.

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