



Research article

Celecoxib-mediated attenuation of non-alcoholic steatohepatitis is potentially relevant to redistributing the expression of adiponectin receptors in rats

Guoying Zhu^a, Li Chen^{b,c,d}, Su Liu^e, Ling She^{c,d}, Yongnian Ding^{c,d}, Changqing Yang^b, Fengshang Zhu^{c,d,f,*}^a Department of Clinical Nutrition, Putuo People's Hospital, School of Medicine, Tongji University, Shanghai, 200060, China^b Department of Gastroenterology, Tongji Hospital, School of Medicine, Tongji University, Shanghai, 200065, China^c State Key Laboratory of Pathogenesis, Prevention and Treatment of High Incidence Diseases in Central Asia & Xinjiang Key Laboratory of Neurological Disorder Research, Xinjiang Medical University, Xinjiang Uygur Autonomous Region, Urumqi, 830000, China^d Department of Gastroenterology, Second Affiliated Hospital of Xinjiang Medical University, Urumqi, 830063, China^e Department of Gastroenterology, Zhabei Central Hospital of Jingan District, Shanghai, 200070, China^f Department of Gastroenterology, Tongji Hospital, School of Medicine, Tongji University, 389 Xincun Road, Shanghai, 200065, China

HIGHLIGHTS

- NASH is a watershed in the progression of NAFLD.
- Finding a therapy for NASH is in urgent need.
- Pharmacological inhibition of COX-2 activity ameliorated the severity of NASH.
- Low-dose celecoxib, a COX-2 inhibitor, can improve NASH by redistributing the expression of adiponectin receptors.

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ABSTRACT

Pharmacological inhibition of cyclooxygenase-2 (COX-2) activity ameliorated the severity of non-alcoholic steatohepatitis (NASH) rats. It is not completely understood that the role of COX-2 inhibitor celecoxib on adiponectin receptors (Adipo-R1/R2) expression in different tissues in NASH rats. Sprague-Dawley male NASH rats induced by a high-fat diet (HFD) were administrated with or without celecoxib for 8 weeks. Biochemical parameters of liver function, glucose, and lipid metabolism, and the levels of adiponectin, tumor necrosis factor- α (TNF- α), prostaglandin E₂ (PGE₂) in the serum or liver were collected according to the standard protocols. The mRNA and protein levels of Adipo-R1, Adipo-R2, and COX-2 in the liver, muscle, and visceral fat were performed by quantitative real-time polymerase chain reaction (q-PCR) and Western blot analysis, respectively. The results showed that celecoxib ameliorated the various clinical indicators and pathological characteristics in the NASH rats, including body weight, liver function, liver index, and redox activities in serum and hepatic samples. The serum concentrations of adiponectin and TNF- α and PGE₂ were negatively correlated. As expected, these ameliorative effects of celecoxib were associated with the gene and protein levels up-regulation of Adipo-R1, Adipo-R2 in the liver and visceral fat tissues, and seeming to be compensatory down-regulation expression in muscle tissues ($P < 0.05$). Additionally, COX-2 protein expression was negatively correlated with serum adiponectin levels, protein expression of adiponectin receptors from the liver and visceral fat, conversely, positively correlated with those from the muscle. Our current study demonstrate that celecoxib might effectively alleviate NASH rats in a unique manner closely relevant to redistributing the expression of adiponectin receptors in the liver, visceral fat, and muscle. However, the precise molecular mechanism needs further study.

* Corresponding author.

E-mail address: zhufengshang@126.com (F. Zhu).<https://doi.org/10.1016/j.heliyon.2022.e09872>

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1. Introduction

Non-alcoholic fatty liver disease (NAFLD), recently being renamed as metabolic-associated fatty liver disease (MAFLD), is the most common cause of chronic elevation of transaminases worldwide [1, 2]. The disease spectrum of NAFLD ranges from simple fatty liver to non-alcoholic steatohepatitis (NASH), and the latter is generally believed as a precursor for further severe liver diseases including hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [3]. Insulin resistance (IR) has been proved to play a crucial role in the occurrence and deterioration of NAFLD and NASH. On the contrary, clinical characteristics associated with NASH encompass a wide spectrum of metabolic components abnormalities, such as central obesity, hypertension, diabetes, hypertriglyceridemia, and all the spectrum of diseases mentioned tend to be IR and metabolic syndrome [3].

Celecoxib, a specific cyclooxygenase-2 (COX-2) inhibitor, has previously served as an analgesic and antipyretic, anti-inflammatory, and chemoprevention agent in colorectal and other cancer [4, 5]. Over the past decade, it has been shown that the expression of COX-2 in acute and chronic liver diseases was elevated [6, 7, 8, 9]. Abnormally elevated COX-2 expression in NAFLD and NASH suggested that that COX-2, as an important inducible enzyme, plays a pivotal role in controlling the production of downstream cytokines including interleukin-6 (IL-6), IL-1, tumor necrosis factor- α (TNF- α) [9, 10, 11]. Extensive studies have implied that pharmacological inhibition of COX-2 activity ameliorates the severity of high-fat diet (HFD)-induced NASH through suppressing the non-canonical Wnt signaling pathway, inhibiting nuclear factor- κ B (NF- κ B) activation and endoplasmic reticulum stress, and promoting hepatocellular apoptosis [3, 7, 9]. However, additional molecular details by which inhibition of COX-2 ameliorates NASH are not fully elucidated.

Adiponectin is a protein secreted by adipose cells that couples regulation of insulin sensitivity in energy metabolism and serves as a possible link between obesity and IR [12, 13]. Importantly, both obesity and IR are ineluctably associated with NASH [14, 15]. Generally, serum adiponectin exerts a potent insulin-sensitizing function by engaging two adiponectin receptors (Adipo-R1, Adipo-R2), and then the combination further acts through the activation of downstream signaling pathways associated with AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPAR α) [16, 17]. Our previous study has demonstrated that the genes and protein expression of Adipo-R1 and Adipo-R2 were reduced in the liver and visceral fat, and elevated in muscle in a rat NASH model [18]. Therefore, either up-regulating hepatic Adipo-R1 and Adipo-R2 expression or activating Adipo-R1 and Adipo-R2 activity with drugs, or a combination of both offer novel targets and approaches for treating IR-associated diseases such as NASH and type 2 diabetes [18, 19].

In line with our hypothesis, our previous study showed that PPAR γ agonists rosiglitazone and pioglitazone improved NASH by directly modulating the expression of Adipo-R1 and Adipo-R2 in different tissues [2, 18]. Additionally, specific COX-2 inhibitors have been considered to partially function as PPAR γ agonists [20, 21]. However, the effect of celecoxib on adiponectin receptors expression in NASH rats has not been unraveled. The present study aims to evaluate whether celecoxib's improvement effect in NASH rats is associated with redistributing the expression of adiponectin receptors in different tissues.

2. Materials and methods

2.1. Animals

Thirty Sprague-Dawley male rats (6–8 weeks of age), weighing 145–175g, were obtained from Sina-British SIPPR/BK Lab Animal Ltd., Co (Shanghai, China). The rats were reared in the Animal Experiment Center of Shanghai Putuo People's Hospital affiliated to Tongji

University. All rats were exposed to 12-hour cycles of light and darkness and were free to eat and drink throughout the experiments. The animal treatment protocols were approved by the Ethics Committee of Shanghai Putuo People's Hospital (PTY-2019001). The experiments were performed under the guidelines for animal experiments of Tongji University School of Medicine and conducted according to established animal welfare guidelines as the international practice.

2.2. Experimental protocol

This experimental research protocols also has been approved by the Ethics Committee of Shanghai Putuo People's Hospital affiliated to Tongji University (PTY-2019001). Rats were acclimatized for 5 days and randomly divided into three groups (the control group, NASH group, and celecoxib group, $n = 10$ in each group). The control group was fed with a normal laboratory diet and regular drinking water. Rats in the NASH group received an 88% normal diet plus 2% cholesterol and 10% lard for 12 weeks to induce completely the same animal model of NASH as we previously described [2, 18]. The celecoxib group was fed with the same diet as the NASH group supplemented with the solution of celecoxib (20 mg/kg of body weight, Pfizer Inc, USA) from the 12th week for 8 weeks, during which rats continued receiving the HFD [3, 9]. Rats in the control group and NASH group simply received normal rat chow and HFD respectively, until the endpoint of the experiment at 20 weeks. At the 20th week of the experiment, all rats were sacrificed after 12 h of fasting and water deprivation. Furthermore, cardiac puncture blood samples, liver, muscle, and visceral fat tissue were collected, weighed, immediately frozen in liquid nitrogen, and stored at -80°C . The sections from the right lobe liver were washed in cold saline and fixed in 10% buffered formalin phosphate solution for histopathological analysis according to our previous study [18].

2.3. Biochemical measurements in serum

2.3.1. Liver function contents

The assays were performed using corresponding kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu province, China) including alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT) by the Department of Biochemistry, Tongji Hospital, Tongji University (Shanghai, China).

2.3.2. Serum glucolipid metabolism parameters

Serum triglycerides, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), free fatty acids (FFA), fasting blood glucose (FBG), and fasting insulin (FINS) were measured using the corresponding assay kits (MyBioSource, San Diego, CA, USA), following manufacturer's instructions. HOMA-insulin resistance score was calculated and compared using the relevant formula ($\text{HOMA-IR} = \text{FPG} \times \text{FINS} / 22.5$) [18].

2.3.3. Adipocytokine and inflammatory mediators

The levels of serum adiponectin, TNF- α and prostaglandin E_2 (PGE $_2$) were implemented using enzyme-linked immunosorbent assay (ELISA) kits (R&D system INC., Minneapolis, MN, USA) with positive and negative controls that were previously described [22].

2.4. Antioxidant and oxidative stress markers in serum and liver

Serum and hepatic levels of antioxidant enzyme activities, including total anti-oxidation competence (T-AOC), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-PX), and malondialdehyde (MDA) were determined by antioxidant enzyme activities kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to our previous studies [2, 18].

2.5. Histopathological studies

Liver tissues fixed with 10% formalin solution were used for the preparation of pathological sections. Sections from each sample were paraffin imbedded, sliced into 4 μ m thickness, stained with hematoxylin-eosin (HE) and Masson's trichrome, and examined under a light microscope for assessment of steatosis, inflammation of lesions, and scoring fibrosis according to the previous reported [23, 24]. The score based on the degree of liver steatosis is as follows: 0 score (<5%), 1 score (5%–33%), 2 scores (34%–66%), and 3 scores (>66%). Intralobular inflammatory reaction was scored as follows: 0 (None), 1 (<2 lesion at 100-fold magnification), 2 (2–4 lesions at 100-fold magnification), 3 (2–4 lesions at 200-fold magnification), 4 (\geq 5 lesions at 200-fold magnification). Ballooning of hepatocytes was scored as follows: 0 (None), 1 (Rare), 2 (Common). The above three elements constituted the NAFLD activity score (NAS) [23]. Fibrosis was scored as follows: 0 (None), 1 (Limited to around central veins), 2 (Also found around Glisson's capsules), 3 (Associated with bridging fibrosis), and 4 (Nodular transformation).

2.6. Quantitative real-time polymerase chain reaction (q-PCR)

Approximately 100mg of the liver, muscle, and visceral fat tissues were excised and homogenized in 1ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA), respectively. Total RNA from different tissues was extracted and subsequently transcribed into cDNA using the Prime-ScriptTM RT reagent Kit with gDNA Eraser (Takara, Japan). The mRNA levels of Adipo-R1, Adipo-R2, and COX-2 were measured as our previous descriptions [2, 18]. Primers sequences for rat Adipo-R1 were as follows: forward 5'-GCT GGC CTT TAT GCT GCT CG-3' and reverse 5'-TCT AGG CCG TAA CGG AAT TC-3'; rat Adipo-R2, forward 5'-CCA CAA CCT TGC TTC ATC TA-3' and reverse 5'-GAT ACT GAG GGG TGG CAA AC-3'; COX-2, forward 5'-AGA TCC ACA ACG GAT ACA TT-3' and reverse 5'-TCC CTC AAG ATT GTC AGC AA-3'; Primers sequences for house-keeping gene GAPDH were: forward 5'-AGA TCC ACA ACG GAT ACA TT-3 and reverse 5'-TCC CTC AAG ATT GTC AGC AA-3' [18]. Data were normalized to GAPDH and PCR amplifications were simultaneously performed in triplicate to avoid systematic errors.

2.7. Western blot

The method was adopted as we previously reported with minor modifications [18]. Briefly, total protein extracts were obtained by homogenization of liver, muscle, and visceral fat tissues using lysis buffer and protease inhibitors (Roche, NJ, USA) at 4°C with sonication, respectively. Protein concentrations were measured by Coomassie brilliant blue G-250 (Amresco, OH, USA) staining. Protein samples were heated at 100 °C for 10 min, and protein samples (60 μ g per well) were separated on 12 percent SDS-polyacrylamide gels and electrophoretically transferred onto PVDF transfer membrane 0.45 μ m (Millipore, MA, USA). The membranes were stained with 0.5% Ponceau S to assure equal protein loading, blocked for 1h with 5% powdered nonfat dry milk in TBS-T (25 mmol/L Tris-HCl, pH8.0, 144mmol/L NaCl, 0.1% Tween 20), and incubated overnight at 1:1000 with the anti-COX-2 (Sigma-Aldrich Corp., St. Louis, MO, USA), anti-Adipo-R1 and anti-Adipo-R2 (Beijing Biosynthesis Biotechnology Co., Ltd, China). After incubation with the secondary antibodies, the membranes were briefly washed twice and then 3 times for 10 min each with TBS-T. The reactions were visualized with the enhanced chemiluminescence (ECL) according to the manufacturer's instructions and the blots were analyzed using the ChemiDoc MP imaging system (Bio-Rad, Santa Rosa, CA, USA). The results were approved by repeating the reactions three times.

2.8. Statistical evaluation

Statistical analysis was undertaken with SPSS version 19.0 software (Chicago, IL, USA). One-way ANOVA followed by Tukey's post hoc test

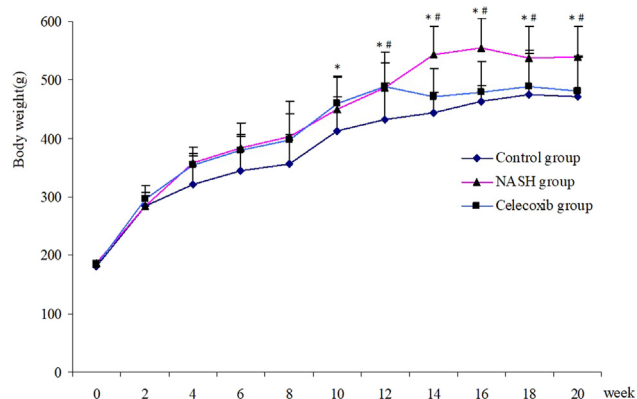


Figure 1. Celecoxib-mediated attenuation of body weight in NASH rats. Data are expressed as mean \pm SE ($n = 10$ in each group). One-way ANOVA was performed followed by Tukey's multiple comparison test. NASH = non-alcoholic steatohepatitis. * $P < 0.05$ in NASH group compared with the corresponding control group; # $P < 0.05$ in celecoxib group compared with the corresponding NASH group.

was used to analyze the differences between the three groups in body weight, liver index, biochemical parameters, antioxidant enzyme activities, and gene and protein expressions. Comparisons between groups of NAS and fibrosis scores were analyzed for statistical significance by the Wilcoxon signed-rank test. The potential correlation between TNF- α , PGE₂, and adiponectin was examined using linear regression. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of celecoxib on body weight and liver index in NASH rats

NASH group animals exhibited a significant increase of body weight from 12 to 20 weeks, and elevated liver index (liver weight/body weight) at 20 weeks when compared with the control group (6.03 ± 0.4 , 3.66 ± 0.36 , respectively) (Figure 1). Meanwhile, celecoxib treatment significantly decreased body weight as well as the liver index (6.03 ± 0.4 in the NASH group and 4.81 ± 0.52 in the celecoxib group, $P < 0.05$).

Table 1. Effects of celecoxib on biochemical parameters in rats.

	Control group	NASH group	Celecoxib group
ALT (U/L)	53.50 \pm 3.89	108.33 \pm 10.11 [#]	90.90 \pm 30.3 [§]
AST (U/L)	45.90 \pm 7.96	164.33 \pm 33.54 [#]	126.70 \pm 25.90 [§]
GGT (U/L)	42.30 \pm 17.83	91.11 \pm 19.63 [*]	84.80 \pm 15.63 [§]
Cholesterol (mmol/L)	1.53 \pm 0.34	3.57 \pm 0.21 [*]	3.48 \pm 0.29 [§]
Triglyceride (mmol/L)	0.59 \pm 0.19	1.04 \pm 0.18 [*]	0.78 \pm 0.06 [§]
HDL (mmol/L)	1.32 \pm 0.12	1.11 \pm 0.06 [*]	1.29 \pm 0.11 [§]
LDL (mmol/L)	0.19 \pm 0.01	0.29 \pm 0.07 [*]	0.21 \pm 0.09 [§]
FFA (mmol/L)	0.91 \pm 0.06	1.22 \pm 0.22 [*]	0.89 \pm 0.09 [§]
FBG (mmol/L)	6.69 \pm 0.31	11.43 \pm 0.18 [#]	9.71 \pm 0.85 [§]
FINS(μ U/ml)	18.59 \pm 0.83	28.37 \pm 0.91 [#]	22.83 \pm 3.29 [§]
HOMA-IR	1.71 \pm 0.09	2.67 \pm 0.05 [#]	2.27 \pm 0.20 [§]

Results are shown as mean \pm SE ($n = 10$ in each group). One-way ANOVA was performed followed by Tukey's multiple comparison test. ALT = alanine aminotransferase, AST = aspartate aminotransferase, GGT = gamma-glutamyl transferase, HDL = high-density lipoprotein, LDL = low-density lipoprotein, FFA = free fatty acids, FBG = fasting blood glucose, FINS = fasting insulin, HOMA-IR = HOMA-insulin resistance index, NASH = non-alcoholic steatohepatitis. * $P < 0.05$, # $P < 0.01$ compared with the corresponding control group; § $P < 0.05$, § $P < 0.01$ compared with the corresponding NASH group.

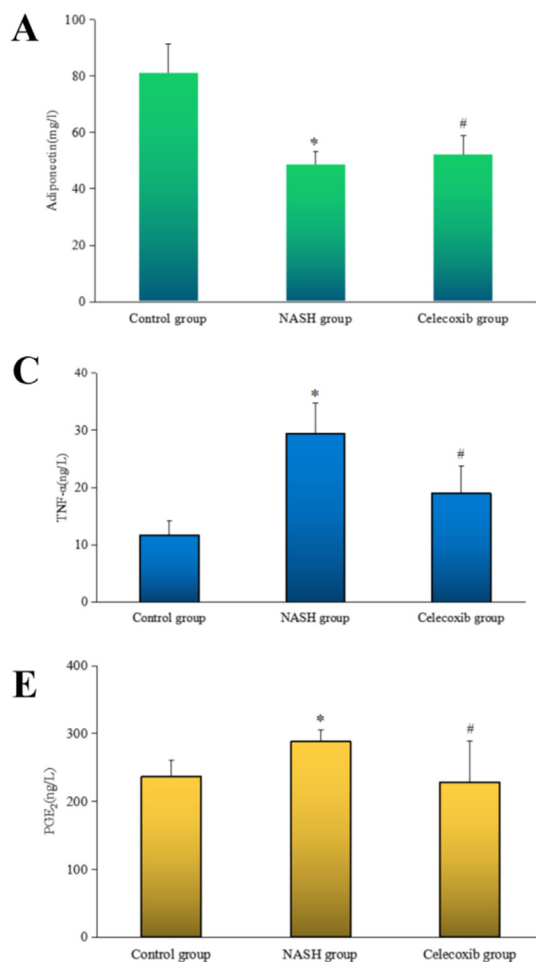
Table 2. Effects of celecoxib on serum and hepatic antioxidant enzyme activities.

		T-AOC	T-SOD	CAT	MDA	GSH-PX
serum	Control group	8.62 ± 0.88	250.02 ± 19.65	18.32 ± 0.70	15.67 ± 1.13	126.58 ± 7.65
	NASH group	7.58 ± 0.71*	278.27 ± 30.21*	15.92 ± 1.92*	19.62 ± 2.13*	97.76 ± 14.62 [#]
	Celecoxib group	8.37 ± 0.58 [§]	305.22 ± 17.70 [§]	18.39 ± 1.28 [§]	17.31 ± 1.60 [§]	112.92 ± 25.22 [§]
Liver	Control group	1.74 ± 0.40	228.42 ± 12.46	28.86 ± 5.94	23.20 ± 5.59	223.72 ± 27.23
	NASH group	1.08 ± 0.53 [#]	163.84 ± 12.96 [#]	14.01 ± 2.06 [#]	32.28 ± 8.51 [#]	277.86 ± 57.99*
	Celecoxib group	1.51 ± 0.34 [§]	198.00 ± 28.15 [§]	24.67 ± 3.52 [§]	24.40 ± 4.47 [§]	304.14 ± 37.00 [§]

Results are shown as mean ± SE ($n = 10$ in each group). One-way ANOVA was performed followed by Tukey's multiple comparison test. T-AOC = total anti-oxidation competence, T-SOD = total superoxide dismutase, CAT = catalase, MDA = malondialdehyde, GSH-PX = glutathione peroxidase, NASH = non-alcoholic steatohepatitis. Unit for T-AOC, T-SOD, and CAT is U/ml (serum) and U/mg-protein (liver), and for MDA is nmol/L (serum) and mol/mg-protein (liver), for GSH-PX is Unit of activity (serum and liver). * $P < 0.05$, [#] $P < 0.01$ compared with the corresponding control group; [§] $P < 0.05$, [§] $P < 0.01$ compared with the corresponding NASH group.

3.2. Biochemical and metabolic parameters changes

Serum levels of liver function parameters (ALT, AST, and GGT) were elevated in the NASH group compared with the control group and significantly declined in the celecoxib group (Table 1). In addition, serum lipids (total cholesterol, triglycerides, HDL, LDL, FFA) and glycometabolism indicators (FBG, FINS, HOMA-IR) exhibited significant elevation in the NASH group. As expected, serum lipids and glycometabolism indicators suggested noticeable improvement after celecoxib treatment (Table 1).



3.3. Antioxidant enzyme activities changes

Twelve weeks of HFD in the NASH group showed a statistically significant increase in serum and hepatic MDA levels, and a prominent decrease in T-AOC, CAT (serum and hepatic), and GSH-PX (serum). Interestingly, serum T-SOD and hepatic GSH-PX activities showed remarkable increases in comparison to the control group despite hepatic T-SOD activities decreasing in the NASH group. Celecoxib treatment resulted in not only a distinct decrease in serum and hepatic MDA levels

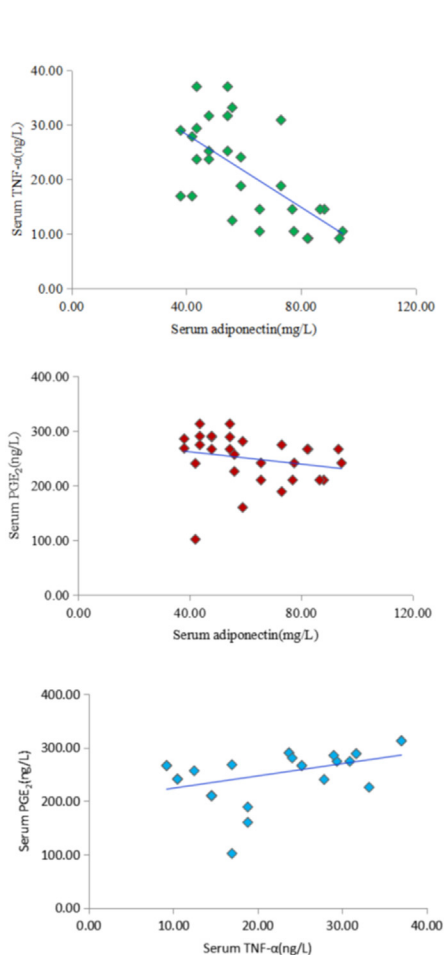


Figure 2. Effects of celecoxib on serum levels of adiponectin (A), TNF-α (C), and PGE₂ (E) and their correlation degrees (B, D, F). Results are shown as mean ± SE ($n = 10$ in each group). One-way ANOVA followed by Tukey multiple comparison tests and linear regression. There was a negative correlation in serum concentration between adiponectin and TNF-α, PGE₂ ($r = -0.460$, $P = 0.001$; $r = -0.292$, $P = 0.033$, respectively. B,D), and a positive correlation between TNF-α and PGE₂ ($r = 0.311$, $P = 0.023$). F). TNF = tumor necrosis factor, PGE₂ = prostaglandin E₂, NASH = non-alcoholic steatohepatitis. * $P < 0.01$ compared with the control group; [#] $P < 0.05$ compared with the NASH group.

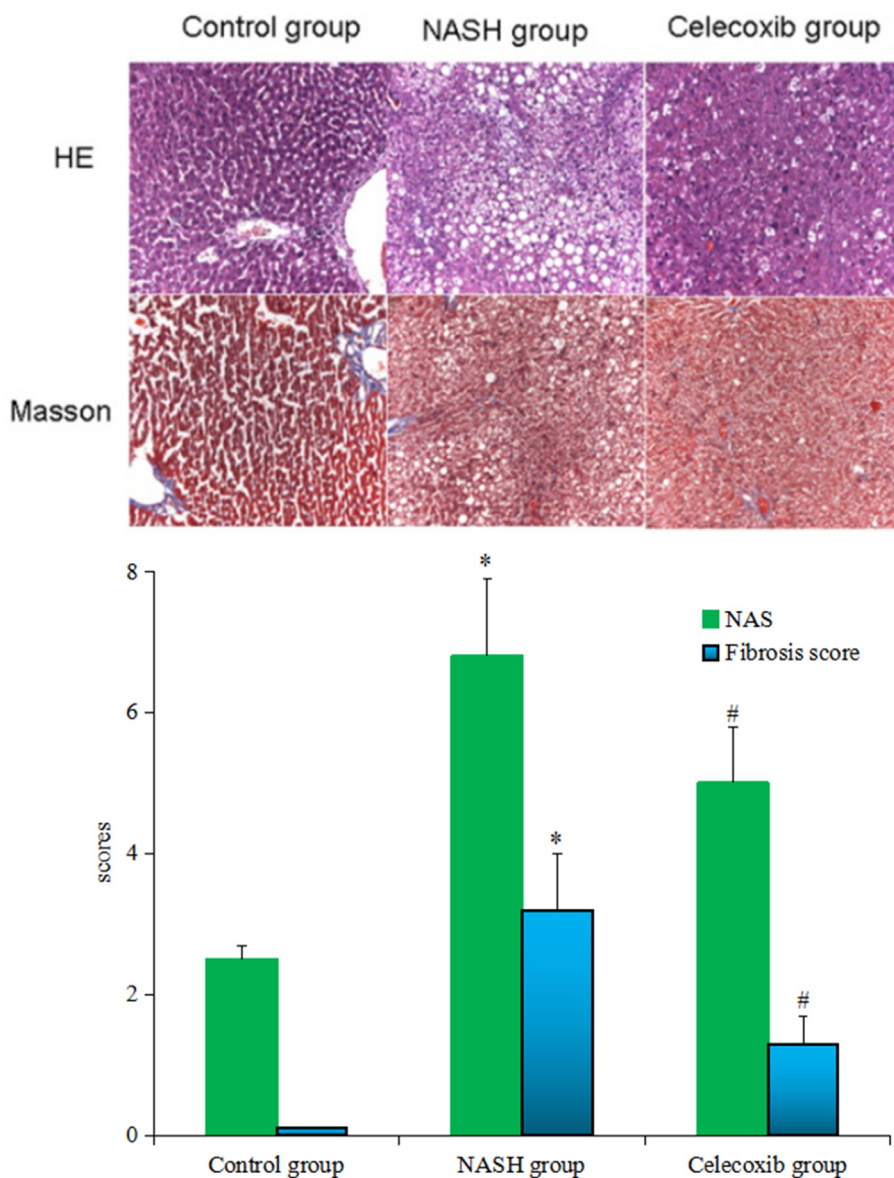


Figure 3. Histological improvement of celecoxib on the NAS and fibrosis score in NASH rats. Results are shown as mean \pm SE ($n = 10$ in each group). Original magnification $\times 200$. NASH = non-alcoholic steatohepatitis, HE = hematoxylin-eosin, NAS = NAFLD activity score, NASH = non-alcoholic steatohepatitis. * $P < 0.01$ in NASH group compared with the corresponding control group; # $P < 0.01$ in celecoxib group compared with the corresponding NASH group.

but also a significant increase in serum and hepatic T-AOC, T-SOD, CAT, and GSH-PX levels (Table 2).

3.4. Serum levels of adiponectin, TNF- α , and PGE₂ and their correlation degrees

Serum adiponectin level (Figure 2A) displayed a significant reduction in the NASH group. However, TNF- α (Figure 2C) and PGE₂ (Figure 2E) levels were significantly increased compared with the control group ($P < 0.01$). After 8-week treatment with celecoxib, serum adiponectin level was elevated. Meanwhile, the levels of TNF- α and PGE₂ in the celecoxib group were significantly decreased ($P < 0.05$) (Figure 2A, B, C). There was a negative correlation in serum concentration between adiponectin and TNF- α , PGE₂ ($r = -0.460$, $P = 0.001$; $r = -0.292$, $P = 0.033$, respectively. Figure 2B, D), and a positive correlation between TNF- α and PGE₂ ($r = 0.311$, $P = 0.023$. Figure 2F).

3.5. Histological improvement in liver

Mallory bodies, ballooning degeneration, inflammatory cells (macrophages and lymphocytes) infiltration, and bridging fibrosis were aggravated after HFD consumption (Figure 3). Of 10 rats in the NASH group, 6 showed severe steatosis, 7 showed moderate inflammations, and 6 showed moderate fibrosis. Celecoxib administration resulted in significant histological improvements in NAS ($P < 0.01$) and fibrosis score ($P < 0.01$) compared with the NASH group.

3.6. Effects of celecoxib on Adipo-R and COX-2 mRNA relative expression

A significant reduction of mRNA relative expression in Adipo-R1 and Adipo-R2 was observed in the liver and visceral fat in the NASH group, but their expression was notably increased in muscle tissues. After 8-week administration of celecoxib, the mRNA levels of the two re-

Table 3. Effects of celecoxib on Adipo-R mRNA relative expression.

Tissues	Group	Adipo-R1	Adipo-R2
Liver	Control group	2.13 ± 0.13	1.75 ± 0.19
	NASH group	0.98 ± 0.04*	0.34 ± 0.10*
	Celecoxib group	2.08 ± 0.22 [§]	0.75 ± 0.08 [#]
Muscle	Control group	2.47 ± 0.66	0.46 ± 0.15
	NASH group	6.98 ± 0.56*	4.52 ± 0.97*
	Celecoxib group	4.31 ± 0.36 [§]	3.19 ± 1.28 [#]
Visceral fat	Control group	1.39 ± 0.14	1.58 ± 0.18
	NASH group	0.19 ± 0.11*	0.51 ± 0.13*
	Celecoxib group	0.45 ± 0.11 [#]	1.46 ± 0.21 [§]

Results are shown as mean ± SE ($n = 10$ in each group). Results are shown as mean ± SE ($n = 10$ in each group). One-way ANOVA followed by Tukey multiple comparison tests. Adipo-R1 = adiponectin receptor 1, Adipo-R2 = adiponectin receptor 2, NASH = non-alcoholic steatohepatitis. * $P < 0.05$ compared with the control group; [#] $P < 0.05$, [§] $P < 0.01$ compared with the NASH group.

ceptors were noticeably up-regulated in the liver and visceral fat and down-regulated in muscle ($P < 0.05$) (Table 3). In addition, COX-2 relative expression in the NASH group was increased over 5-fold compared with the control group (2.83 ± 0.24 and 0.57 ± 0.08 , respectively), and this increase was inhibited by celecoxib treatment (2.83 ± 0.24 and 0.76 ± 0.10 , respectively, $P < 0.01$).

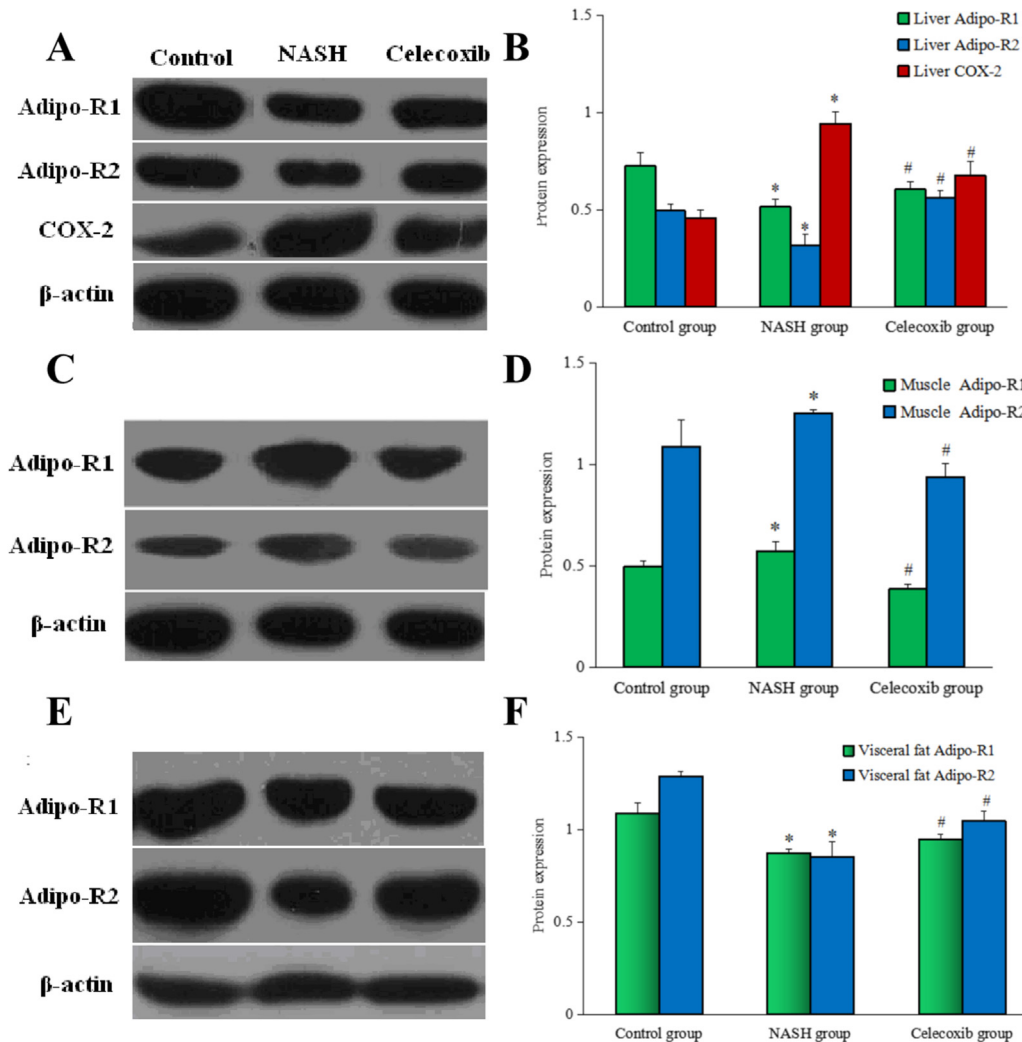


Figure 4. Effects of celecoxib on adiponectin receptors and COX-2 protein expression in the liver, muscle, and visceral fat. COX-2 and adiponectin receptors levels in liver (A), muscle (C), and visceral fat (E) were examined by Western blot analysis. The relative Adipo-R1, Adipo-R2, and COX-2 levels were determined by densitometric analysis (B, D, and F). Data are shown as mean ± SE ($n = 10$ in each group). Adipo-R1 = adiponectin receptor 1, Adipo-R2 = adiponectin receptor 2, NASH = non-alcoholic steatohepatitis. * $P < 0.01$ compared with the control group; [#] $P < 0.01$ compared with the NASH group.

3.7. Effects of celecoxib on Adipo-R and COX-2 protein levels

Compared with the control group, NASH rats showed a decrease of Adipo-R1 and Adipo-R2 proteins in the liver (Figure 4A, B) and visceral fat (Figure 4E, F), but an increase in muscle (Figure 4C, D), which was expectedly consistent with our q-PCR results. Importantly, celecoxib treatment significantly increased Adipo-R1 and Adipo-R2 protein levels in the liver (Figure 4A, B) and visceral fat (Figure 4E, F), and decreased their levels in muscle (Figure 4C, D). Meanwhile, COX-2 protein expression in the NASH group was increased over 2-fold compared with the control group. Celecoxib administration predictably results in a significant reduction of COX-2 protein (Figure 4A, B).

3.8. Correlation between COX-2 protein expression level and other parameters

The relative expression of COX-2 protein in the liver was negatively correlated with the Adipo-R1 (Figure 5A, C), Adipo-R2 (Figure 5B, D) in the liver and visceral fat, and a positive correlation with the above indexes in muscle (Figure 5E, F) $P < 0.05$. Moreover, there was a negative correlation between the protein expression of COX-2 and serum adiponectin (Figure 6A), and a positive correlation with serum TNF- α (Figure 6C) and PGE₂ (Figure 6E). In addition, serum adiponectin was negatively correlated with serum TNF- α (Figure 6B) and PGE₂ (Figure 6D), while the latter two indexes were positively correlated (Figure 6F).

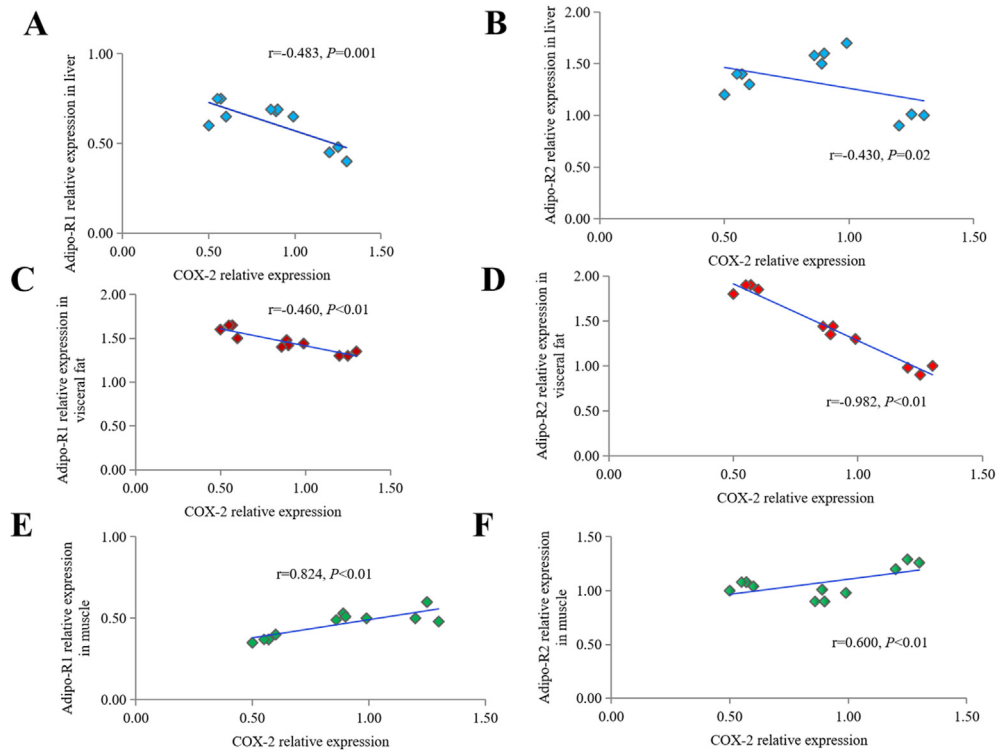


Figure 5. Correlation between COX-2 protein expression and Adipo-R in different tissues. A linear regression model was performed. There was a negative correlation between the protein expression of COX-2 and Adipo-R1 (A, C), Adipo-R2 (B, D) in the liver and visceral fat, and a positive correlation with the above indexes in muscle (E, F). Adipo-R1 = adiponectin receptor 1, Adipo-R2 = adiponectin receptor 2, COX-2 = cyclooxygenase-2.

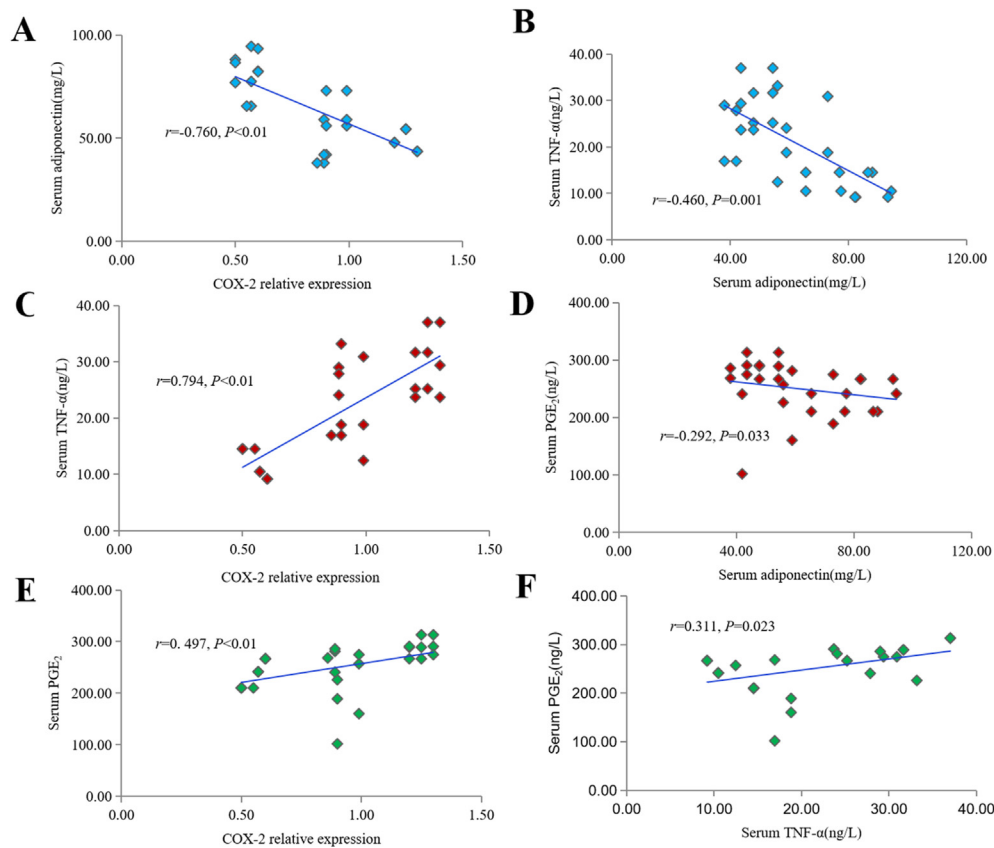


Figure 6. Correlation between COX-2 protein expression level and serum adiponectin, TNF- α , and PGE₂. A linear regression model was performed. There was a negative correlation between the protein expression of COX-2 and serum adiponectin (A), and a positive correlation with serum TNF- α (C) and PGE₂ (E). Furthermore, serum adiponectin was negatively correlated with serum TNF- α (B) and PGE₂ (D), while the latter two were positively correlated (F). COX-2 = cyclooxygenase-2, PGE₂ = prostaglandin E₂, TNF- α = tumor necrosis factor alpha.

4. Discussion

NASH is histologically characterized by hepatic fatty infiltration and inflammation [25, 26]. Early studies have suggested that COX-2 protein is expressed in hepatic cells in acute or chronic liver diseases [6, 7, 8, 9] and is involved in the pathogenesis of NAFLD and NASH [27]. A growing number of basic studies in recent years have confirmed that low-dose celecoxib effectively reduces steatogenesis, inflammation, and partial fibrosis in experimental NASH [3, 7, 9]. In the current study, according to our results, we found that oral treatment with celecoxib for 8 weeks did substantially ameliorate clinical parameters including body weight, NAS, and fibrosis score in the experimental NASH, and our findings on these indicators were consistent with previous studies. In contrast, a similar study from alcohol-induced fatty liver disease demonstrated that celecoxib further increased the steatosis, liver weights, and plasma ALT and TNF- α levels [25], which suggests that both the complexity of effects of COX-2 inhibitors on fatty liver disease and the need for further research. This discrepancy in hepatic histological changes may be ascribed to the different celecoxib doses, animal species, and experimental models used in different studies.

IR is the core initiating link of the NAFLD disease spectrum. It has been reported that COX-2 activation is crucial for the development of IR and steatohepatitis in a rat model of high fat-induced obesity [7, 28]. Our and other previous studies have demonstrated that liver functional parameters (ALT, AST, GGT), serum lipids (serum triglycerides, total cholesterol, LDL, HDL, FFA), and glycometabolism indicators (FBG, FINS, HOMA-IR) were altered in the NASH rats [18, 29]. The results presented in this study showed that the liver function parameters, serum lipids, and glycometabolism indicators were significantly improved after celecoxib treatment, indicating that celecoxib may play a vital role in alleviating IR in addition to its anti-inflammatory effect.

Oxidative stress and lipid peroxidation are considered as one of the crucial mechanisms in NASH occurrence and development [30, 31]. Our findings presented in this study also confirmed the anti-oxidation effect of celecoxib in NASH rats. The details of our results showed levels of liver "protective" parameters (T-AOC, T-SOD, CAT, and GSH-PX) were generally decreased, but the level of liver "damaging" parameter (MDA) was increased. After celecoxib intervention, the corresponding indicators displayed a significant improvement in the celecoxib group. However, interestingly, we also found that the levels of serum T-SOD and hepatic GSH-PX showed remarkable increases in comparison to the control group. Considering the two indicators were even higher in the celecoxib group, we deemed the results are compensatory.

Previous studies have reported that hepatic Kuffer cells are activated when exposed to inflammatory cytokines, endotoxin, and oxygen-free radicals [32, 33, 34]. Accompanied by the changes of Kuffer cells in morphology, size, and numbers, a series cascade inflammatory cytokines, such as TNF- α , IL-1, and IL-6, were continually released. Sequentially, inflammatory cytokines induced I κ B degradation and p65 translocation to the nucleus, which resulted in the further activation of the NF- κ B pathway [32] and consequences of COX-2 up-regulation. Furthermore, up-regulated COX-2 stimulated the secretion of inflammation mediators such as PGE₂ and thromboxane B₂ (TXB₂), and then stimulated the expression of TNF- α , IL-1, IL-6, and other cytokines that regulated by the inflammatory mediators mentioned above. Consequentially, a vicious positive feedback loop was formed and eventually led to severe liver injury [18, 35]. Our present results showed that a significant decrease in serum levels of TNF- α and PGE₂ after celecoxib administration, which seems to imply that the COX-2-mediated positive feedback vicious cycle potentially benefits from the COX-2 inhibitor.

As the only known adipocytokine that has a grand protective effect on IR and metabolic syndrome [36, 37], further studies have confirmed that adiponectin could augment insulin sensitivity by reinforcing the inhibitory effect of gluconeogenesis and enhancing fatty acid oxidation in a

PPAR γ -dependent manner [38]. The discovery of two 7-transmembrane proteins, Adipo-R1 and Adipo-R2, facilitates our understanding of the molecular mechanisms underlying the insulin-sensitizing effect of adiponectin [39, 40]. Either a decrease in serum adiponectin level or an underexpression of adiponectin receptors in the liver could deteriorate the transition from NAFLD to NASH [41]. Physical exercise, weight loss, and PPAR γ agonist analogues have been demonstrated to improve NASH [18] and IR-related cardiovascular events [26, 41]. In addition, previous studies have recognized that COX-2 inhibitors partially possess the ability to act as a PPAR γ agonist [42]. Meanwhile, the expression and function of adiponectin receptors vary in different tissues. Consequently, we observed the effects of celecoxib on adiponectin receptors from different tissues in NASH rats. However, in reality, Adipo-R1 and Adipo-R2 expression in different tissues in NASH are paradoxical in the reported studies [43, 44]. The majority of authors discovered Adipo-R2 was lowered in the liver. It was found in the present study that mRNA and protein expression of Adipo-R1 and Adipo-R2 was decreased in the liver and visceral fat tissues, and compensatively elevated in muscle tissue of the NASH rats. Celecoxib intervention lasting 8 weeks significantly increased adiponectin receptor expression in liver and visceral fat, and conversely decreased adiponectin receptor expression in muscle. In addition, as expected, COX-2 mRNA and protein expression were significantly decreased after celecoxib administration despite a significant increase in the NASH group. Our linear correlation analysis also demonstrated that COX-2 expression was negatively correlated with Adipo-R1 and Adipo-R2 in the liver and visceral adipose tissue, but positively correlated with the above markers in muscle. Combined with the relationship between COX-2 expression and serum adiponectin, TNF- α , and PGE₂ levels, we speculated that COX-2 inhibitors might regulate the redistribution of Adipo-R in different tissues through another intermediate link, thereby further improving IR and alleviating the NASH process.

In addition to the natural absence of in vitro studies, the limitations of the present studies include more observational data with no insights on the underlying molecular mechanisms, especially on the upstream and downstream relationship between COX-2 and Adipo-R. This suggests that the precise regulatory mechanism by which intermediate means or ways to connect COX-2 and Adipo-R will require further elucidation.

5. Conclusions

Taken together, our studies suggested that pharmacological inhibition of COX-2 activity elevates serum adiponectin levels, increases Adipo-R1 and Adipo-R2 expression in the liver and visceral fat tissue, and decreases Adipo-R1 and Adipo-R2 expression in muscle. Celecoxib-mediated attenuation of non-alcoholic steatohepatitis is associated with redistributing the expression of adiponectin receptors from the liver, visceral fat, and muscle in rats. This study further indicates that COX-2 inhibitors may represent a novel therapy for NASH. However, the clearer understanding of the effect of COX-2 inhibitors on adiponectin receptors in different tissues needs to be further explored.

Declarations

Author contribution statement

Fengshang Zhu: Conceived and designed the experiments; Wrote the paper.

Guoying Zhu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Li Chen, Su Liu, Ling She: Performed the experiments; Analyzed and interpreted the data.

Yongnian Ding: Analyzed and interpreted the data; Wrote the paper.

Changqing Yang: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data availability statement

Data included in article/supp. material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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