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PARP Inhibitor PJ34 Attenuated Hepatic Triglyceride Accumulation in Alcoholic Fatty Liver Disease in Mice

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a) Running title: PJ34 Attenuated Hepatic TG Accumulation in AFLD in mice

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d) Abbreviations:

AFLD, alcoholic fatty liver disease; ALD, alcoholic liver disease; DGAT1/2, diglyceride acyltransferase 1/2; NAD, nicotinamide adenine dinucleotide; NR, nicotinamide riboside; pADPr, polymeric adenosine diphosphate ribose; PARP, poly ADP ribose polymerase; PJ34, N-(5,6-Dihydro-6-oxo-2-phenanthridinyl)-2-acetamide hydrochloride; SREBP-1c, sterol regulatory element binding protein 1c.

Abstract

Poly ADP ribose polymerase (PARP) is a NAD-consuming enzyme and its specific role in the pathogenesis of alcoholic fatty liver disease (AFLD) is still elusive. In current study, we applied PJ34 to inhibit hepatic PARP activity to examine the corresponding pathological alteration in AFLD in mice and the underlying molecular mechanism. We found that PJ34 decreased the intracellular TG content in hepatocyte. Moreover, PJ34 suppressed the gene expression of DGAT1 and DGAT2 and elevated the intracellular NAD^+ level in hepatocyte. These mechanistic observations were validated in alcohol-fed mice injected with PJ34 intraperitoneally. Results indicate that the PJ34 injection attenuated hepatic TG accumulation in alcohol-fed mice. Furthermore, the gene expression of hepatic SERBP-1c, DGAT1 and DGAT2 were lowered by PJ34 injection, while the hepatic NAD^+ level was augmented by PJ34 injection in alcohol-fed mice. Finally, the nicotinamide riboside supplementation alleviated hepatic TG accumulation in alcohol-fed mice. These data indicate that applying PARP specific inhibitor PJ34 by intraperitoneal injection attenuated hepatic NAD^+ depletion and TG accumulation in alcohol-fed mice, which may be a potential candidate for AFLD therapy.

Introduction

Alcoholic fatty liver disease (AFLD) is one of the most prevalent forms of chronic liver disease worldwide and has a widespread incidence. It may progress to alcoholic liver disease (ALD), which is a major cause of illness and death in the United States(Purohit et al., 2004;Livero et al., 2016). The pathogenesis of AFLD is not well established, but is associated with excessive ethanol consumption which causes hepatic lipid accumulation, enhanced hepatic lipogenesis, and inhibits fatty acid oxidation (Rogers et al., 2008). AFLD can also progress to hepatitis, fibrosis, cirrhosis, which may then lead to hepatocellular carcinoma and liver-related death. Nevertheless, the mechanisms of AFLD are complicated and remain not completely understood.

Chronic alcohol consumption enhances lipogenesis and decreases fatty acid oxidation which destroys the balance of intracellular fatty metabolism. Alcohol exposure induces the generation of reactive oxygen species (ROS) which causes oxidative stress and lipid accumulation in the liver (Roskams et al., 2003). Excessive alcohol consumption reduces the hepatic nicotinamide adenine dinucleotide (NAD^+) level, which disrupts the balance of the NAD^+/NADH radio and the elevation of the lactate/pyruvate ratio (Gao et al., 2011). Ethanol metabolism mediates cellular redox status and disturbs several major hepatic lipid metabolism transcriptional regulators, such as the sterol regulatory element binding protein 1c (SREBP-1c), lipin-1, AMP-activated kinase (AMPK), Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), and Forkhead box protein O1 (FOXO1) (You et al., 2015; Harris et al., 2011; Everitt et al., 2013).

Poly ADP ribose polymerase (PARP) is an abundant nuclear protein that plays key roles in a variety of cellular processes, including transcriptional regulation and DNA repair, and programmed cell death. It detects and initiates an immediate cellular response to single-strand

DNA breaks (SSB) by signaling DNA-repairing enzymes to repair the SSB. It is composed of four domains: a DNA-binding domain, a tryptophan-glycine–arginine-rich (WGR) domain, an auto-modification domain, and a catalytic domain (Isabelle et al., 2010). PARP1 is the best-studied protein of the PARP family. Human PARPs (hPARPs)-1 consists of six functional domains: three Zinc finger DNA-binding domain (DBD: Zn1, Zn2, and Zn3), the automodification domain, the WGR domain and the catalytic domain (Schreiber V et al., 2006; Li X et al., 2014). As one of the NAD^+ consumers (Sirtuins, PARPs, and CD38), PARP catalyzes the synthesis of polymeric adenosine diphosphate ribose (pADPr) from NAD^+ to the target proteins, thereby regulating their activity (Kraus et al., 2013). Excessive hyperactivation of PARP causes the depletion of intercellular NAD^+ and ATP levels, which leads to cellular metabolistic disorder and death. Inhibition of PARP activity promotes atherosclerotic plaque regression, attenuates high-fat diet-induced dysfunction of lipid metabolism, and diabetes (Hans et al., 2009; Zakaria et al., 2017). Chemical or genetic inhibition of PARP1 attenuates carbon tetrachloride (CCl_4)-induced liver injury, inflammation and fibrosis (Mukhopadhyay et al., 2014). Meanwhile, the pharmacological inhibition of PARP-1 reduces hepatocellular carcinoma (HCC) growth (Quiles-Perez et al., 2010; Zhang et al., 2012). PARP1 knock out in mice protects against non-alcoholic steatohepatitis by restoring the hepatic NAD^+ content and sirtuin1 activity (Mukhopadhyay et al., 2017; Ray et al., 2017), but the specific role of PARP in AFLD progression is still yet to be thoroughly investigated.

This study aimed to investigate the effect of the PARP inhibitor N-(5,6-Dihydro-6-oxo-2-phenanthridinyl)-2-acetamide hydrochloride (PJ34, $C_{17}H_{17}ClN_2O_2$) on hepatic lipid accumulation induced by chronic alcohol feeding and its underlying mechanisms. We found that PJ34 attenuated hepatic NAD^+ depletion and triglyceride (TG) accumulation in both

hepatocyte and alcohol-fed mice. Our results suggest that the PARP inhibitor PJ34 could be applied as a potential therapeutic chemical for AFLD by modulating intracellular NAD^+ levels.

Materials and Methods

Animal Care and Treatment

Male C57BL/six mice (eight weeks) weighting 20 ± 0.5 (SD) g were obtained from the Harbin Medical University Experimental Animal Center. All studies were approved by the animal Ethical Committee of Harbin Medical University, Daqing, China. The mice were housed in conventional conditions, maintained on a 12 h light/dark cycle, at a temperature 23 ± 2 °C, humidity at 50 ± 5% and fed standard food and water ad libitum at the animal facility for one week before the experiment began. All mice were fed for four weeks with liquid diets. All diets were purchased from Research Diets Inc. (TROPHIC Animal Feed High-Tech, China). The pair-fed diet (TP 4030A, PF) contained 18% protein, 35% fat, and 47% carbohydrate; and the alcohol-fed diet (TP 4030C, AF) contained 18% protein, 35% fat, 11% carbohydrate, and 36% alcohol as per Lieber-DeCarli (Fernando et al., 2013). Forty-five male C57BL/6 mice were then randomly divided into six groups and were fed respective diets for model development. The groups included: (1) PF + physiologic saline (PF, n = 6); (2) PF + PJ34 (Selleck Chemicals, USA), (n = 6); (3) PF+ nicotinamide riboside (NR, Hangzhou LZ Chemical, China) (n = 6); (4) AF + physiologic saline (AF, n = 9); (5) AF + PJ34 (n = 9); and (6) AF + NR (n = 9). Treatment consisted of PJ34 as a PARP activity specific inhibitor (10 mg/kg/day) which was injected intraperitoneally three times a week throughout the experiment. The same volume of physiologic saline (0.1 mL/20 g/day i.p.) was injected intraperitoneally three times a week as vehicle-treated. NR as a natural NAD^+ precursor (10 mg/kg/day) was supplemented in the liquid diet throughout the experiment. Food consumption was recorded daily. Mice were weighed weekly on the same day. All animals were sacrificed after an overnight fast. At the end of the experiment, plasma, liver samples and

epididymal fat pad samples were harvested.

Histological Analysis

The liver samples were fixed in 4% paraformaldehyde for two days, transferred to 70% ethanol, and dehydrated through a serial alcohol gradient. Tissues were embedded in a paraffin wax block and cut into six μm thin sections. All sections were dewaxed in xylene, rehydrated through decreasing concentrations of ethanol, and washed in distilled water. Finally, tissue sections were stained with hematoxylin and eosin (H&E). The fields were randomly selected and were photographed under x400 magnification. The quantitative statistics of lipid droplets fold change were analysed by Image-Pro plus.

Circulating Biochemical Measurement

The levels of circulating TG and total cholesterol (TC) were performed with commercially available kits (BioSino, China) as per the manufacturer's instructions. We took a certain amount of plasma into the working liquid in proportion. The mixture was incubated in 37 °C water bath for 10 min. Next, a SpectraMAX 190 instrument (Molecular Devices, USA) was used to detect the absorbance of TG and TC at a wavelength of 505 nm. To measure the alanine transaminase (ALT) and aspartate transaminase (AST) concentration, we used the commercially available enzyme-linked immunosorbent assay (ELISA) kit (Shanghai jinma laboratory equipment, China). The plasma was diluted five times, and incubated with a relative working liquid for 30 min in a 37 °C incubator. The absorbance was detected by the SpectraMAX 190 instrument at a wavelength of 450 nm. The concentrations of TG, TC, AST and ALT were calculated by related equation.

Hepatic Triglyceride and Total Cholesterol measurement

The liver tissues were washed by PBS twice and 800 mg of liver tissue was homogenized in 1 mL 50 mM NaCl. Liver total lipids were extracted in a 7 mL heptanes:isopropanol (3:2) mixture at room temperature in a shaking stable for 2 h. They were centrifuged at 3000 x g for 5 min. The organic layer was collected in a new tube and dried under a stream of air in the hood. Liver TG and TC content were determined by the above-mentioned commercially available kit as described previously.

Cell Culture and Treatment

HepG2 cells, a human hepatoma cell line, were purchased from the Institute for Biological Sciences (Shanghai, China). HepG2 cells were cultured in DMEM high glucose culture fluid (Gibco Company, USA) containing 10% (v/v) FBS, supplemented with 2 mM glutamine, 100 IU/mL penicillin and 100 mg/mL streptomycin. They were incubated at 37 °C in a humidified incubator with 5% CO_2 and 95% air. For pharmacological studies, cells were washed with phosphate-buffered saline (PBS) three times and replaced with a serum-free medium for serum starvation. After 12 h culture, they were treated with the PARP inhibitor PJ34 (1 μM) in the absence or presence of 3.3 μM oleic acid (OA, Sigma, USA) for 24 h. The same volume of serum-free medium acted as the control group.

Intracellular TG Determination

After treatment with PJ34 in the presence OA for 24 h, cells were harvested at the end of the experiment. For intracellular TG measurement, HepG2 cells were dissolved in 0.3 mL NaOH (50 mM) solution per well (24-well plate). Intracellular total lipids were extracted in 1 mL heptanes:isopropanol (3:2) mixture at room temperature in a shaking stable for 2 h. They were centrifuged at 3000 x g for 5 min. The organic layer was placed in a new tube and dried under a

stream of air in the hood. Intracellular TG content was determined by the above-mentioned commercially available kit as described previously.

Hepatic and Intracellular NAD^+ Analysis

Liver tissue samples and intracellular NAD^+ levels were extracted by a commercially available kit (Suzhou Comin Biotechnology, China). Liver tissue samples and HepG2 cells were harvested after treatment. Five million cells were collected in the centrifugal tube and added to 1 mL acid extract liquor. For the liver tissues, 0.1 g tissue was homogenized in 1 mL acid extract liquor. They were used ultrasonic to broken for 1 min in an ice bath, then bathed in a boiling bath for 5 min, after cooling in ice bath and 10,000 x g centrifuge in 4 °C for 10 min. The supernatant was put in a new tube and the same volume of alkaline extract liquor was added before being centrifuged at 10,000 x g centrifuge in 4 °C for 10 min. The supernatant was used to test as per the manufacturer's instructions. Finally, the absorbance was detected by SpectraMAX 190 instrument at a wavelength of 570 nm. The NAD^+ concentration in each sample was calculated by related equation.

Western Blot Analysis

Cells were washed three times with PBS and lysed in RIPA buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, Beyotime Biotechnology, China), supplemented with phenylmethanesulfonyl fluoride (PMSF) (Beyotime Biotechnology, China) for 30 min. Liver tissues were ground with a glass homogenizer which was lysed in RIPA buffer. Whole cell extracts or tissue extracts were collected by centrifugation at 10,000 x g at 4 °C for 15 min. Protein concentration was determined using an Enhanced BCA Protein Assay Kit (Beyotime Biotechnology, China). Equal protein amounts (30 μg) were subjected to 10% or 8%

SDS–PAGE depending on the molecular weight of the desired proteins and transferred to a nitrocellulose transfer membrane (Pall Corporation, USA). The membranes were blocked with 5% powdered milk diluted in Tris-buffered saline (TBS) with 0.05% Tween 20 (TBST) for 1 h at room temperature and incubated with primary antibodies at 4 °C overnight. The primary antibodies used were mouse monoclonal IgG3 anti-pADPr (10H) (Santa Cruz Biotechnology, USA), rabbit monoclonal anti-PARP (46D11) (Cell Signaling Technology, USA), rabbit polyclonal anti-SREBP-1c (Bioss, China), mouse monoclonal anti-SREBP-1 (Santa Cruze Biotechnology, USA), rabbit polyclonal anti-DGAT1 (Bioss, China), rabbit polyclonal anti-DGAT2 antibody (Bioss, China), and mouse monoclonal anti-β-actin (ZSGB-BIO, China). The antigen-primary antibody complexes were washed and then incubated with HRP-conjugated secondary antibodies at room temperature for 1 h. Secondary antibodies used were goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated (ZSGB-BIO, China). Peroxidase was detected using enhanced chemiluminescence (ECL) Western blotting detection reagents (Haigene, China). Densitometric analysis was performed using the Quantity One software (Bio-rad, USA).

RNA Extraction and Real-Time PCR Assays

Total RNAs were extracted from the liver tissues or HepG2 cells. Total RNAs were isolated using TRlzol Reagent (HaiGene, China) as per the manual. For each sample, 1 μg of total RNAs were reverse transcribed using a Golden 1st cDNA Synthesis Kit (HaiGene, China) as per the manufacturer's instructions (30 °C for 15 min; 55 °C for 50 min; 95 °C for 10 min). Real-time PCR of SREBP-1, diglyceride acyltransferase 1 (DGAT1), DGAT2 and 18s rRNA mRNA were performed in an Applied Biosystems 7300 Real-time PCR system (Applied Biosystems, USA) by using the SYBR Green qPCR Mix (Roche, Switzerland) under the following conditions: 95 °C for

10 min, 45 cycles of 95 °C for 10 s, 58 °C for 20 s, and 72 °C for 30 s. To amplify the target genes, all primers were purchased from Genscript Biotechnology (Nanjing, China). Quantitative normalization of the cDNA in each sample was performed using the 18s rRNA gene as an internal control. Real-time PCR assays were performed in duplicate for each sample, and the mean value was used for the calculation of mRNA expression levels.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance and the Newman-Keuls test by OriginPro7.5 software (OriginLab company, Austin, USA) and Graph Pad Prism version 5 (Graph Pad Software Inc. San Diego, California). Data were presented as the mean ± standard error. Values of $P < 0.05$ were considered to indicate a statistically significant difference.

Results

Chronic Alcohol Consumption Induced Hepatic Fat Accumulation and Liver Injury in Mice

C57BL/6 mice were fed with a Lieber-DeCarli alcohol-containing diet for four weeks. Liver samples were harvested for histological examination and the hepatic TG content was detected. As shown in our results, chronic alcohol feeding significantly decreased body weight and epididymal fat pad weight/body weight rate (Figures 1A and 1C) in comparison to the PF group, where the liver weight/body weight rate was significantly increased (Figure 1B). Chronic alcohol consumption increased hepatic TG content and circulating TG levels (Figures 1F and 1G). Chronic alcohol exposure increased the levels of circulating ALT, AST and TC (Figures 1D 1E and 1I), and hepatic TC content (Figure 1H). The severity of AFLD was analyzed with H&E staining (Figure 1J and 1K, x 400). In the AF group, the lobular structures were destroyed and widely distributed, with more lipid droplets when compared with the PF group. The data showed that chronic alcohol consumption induced hepatic lipid accumulation and liver injury.

Chronic Alcohol Feeding Enhanced PARP Expression, pADPr Synthesis and NAD^+ Depletion in Mice

After feeding with an ethanol-containing liquid diet for four weeks, liver samples were harvested for further experiments. Total proteins were extracted from liver samples. Western blot was used to detect PARP expression and activation in alcohol-fed mice. Our results showed that chronic alcohol feeding elevated PARP protein expression (Figures 2A and 2B) and activity, which was shown by higher pADPr conjugated with proteins (Figures 2A and 2C), in comparison to the PF group. Furthermore, chronic alcohol consumption decreased hepatic NAD^+ levels (Figure 2D) and increased the gene expression and protein abundance in TG anabolism, such as SREBP-1c,

DGAT1 and DGAT2 (Figures 2E–2J) compared with the PF group. SREBP-1 precursor (125KDa) and mature SREBP-1 (68KDa) were significantly increased in alcohol-fed mice and PJ34 injection reduced SREBP-1 expression and activation in comparison to the AF group (Supplemental Figure S7). Thus, all results indicated that chronic alcohol consumption-induced hepatic TG accumulation was associated with hepatic PARP overexpression and activation, and the following hepatic NAD^+ depletion.

Inhibition of PARP Activity Increased NAD^+ Level and Lowered Intracellular TG Content in Hepatocyte

The HepG2 cells were used to investigate whether PARP inhibition modulated hepatic lipid accumulation and the underlying mechanisms.As shown in our results, PJ34 exposure (1 μM) for 24 h decreased pADPr synthesis (Figure 3C), but had no effect on the protein abundance of PARP (Figures 3A and 3B) in hepatocyte. Furthermore, we found that intracellular NAD^+ content was elevated by PJ34 (Figure 3D) while intracellular TG content was lowered by PJ34 treatment (Figure 3E). These results demonstrated that PARP inhibition augmented intracellular NAD^+ and decreased intracellular TG accumulation in HepG2 cells.

Inhibition of PARP Activity Suppressed the Expression of the Genes in TG Anabolism

To gain insight into the mechanism underlying PJ34 decreased intracellular TG accumulation, HepG2 cells were exposed to PJ34 (1 μM) in complete DMEM medium with OA addition for 24 h. The critical genes of TG anabolism were detected by RT-PCR. We found that the gene expression of DGAT1 and DGAT2 were significantly lowered by PJ34 treatment (Figures 4B and 4C), but the gene expression of SREBP-1c changed little (Figure 4A) in HepG2 cells. Consistent with the PJ34-triggered elevation of intracellular TG content (Figure 3E), our results demonstrated that

applying PJ34 to inhibit PARP activity decreased TG accumulation, and the underlying mechanism is involved in the PJ34-inhibited gene expression of DGAT1 and DGAT2 in TG anabolism in HepG2 cells.

PJ34 Injection Attenuated Hepatic TG Accumulation in Alcohol Fed Mice

To detect the specific role of PARP inhibition in the pathogenesis of AFLD, C57BL/6 mice were exposed to alcohol-containing liquid diet with/without PJ34 intraperitoneal injection for four weeks. As shown in our results, PJ34 injection increased body weight (Figure 5A) and decreased the liver weight/body weight rate (Figure 5B), but the epididymal fat pad weight/body weight rate had no significant changes (Figure 5C) when compared with the AF group. Moreover, PJ34 injection decreased hepatic TG content and the level of circulating TG in comparison to the AF group (Figures 5F and 5G). In our results, PJ34 injection had no role in hepatic TC content and the levels of circulating TC and ALT in comparison to the AF group (Figures 5D, 5H and 5I). However, PJ34 injection significantly decreased the level of circulating AST in comparison with the AF group (Figure 5E). Additionally, H&E staining showed that PJ34 injection alleviated hepatic lipid accumulation and the lobular structures were more intact in the AF + PJ34 group compared with the AF group (Figure 5J and 5K).

PJ34 Injection Elevated Hepatic NAD^+ Level and Lowered Gene Expression in TG Anabolism in Alcohol-Fed Mice

To detect the underlying mechanism for PJ34-alleviated TG accumulation in the pathogenesis of AFLD, C57BL/6 mice were fed with liquid diets containing alcohol with PJ34 injection for four weeks. We found that PJ34 injection suppressed hepatic PARP activation by decreasing the level of pADPr in comparison to the AF group (Figures 6A–6C). PJ34 injection alleviated hepatic

alcohol-induced NAD^+ depletion compared with the AF group (Figure 6D). Furthermore, the expression of critical genes including SREBP-1c, DGAT1 and DGAT2 were significantly lowered by PJ34 injection in comparison to the AF group (Figures 6E–6G). All data demonstrated that PJ34 injection alleviated hepatic TG accumulation via increasing NAD^+ content and inhibiting TG anabolism in the liver in alcohol-fed mice.

NR Supplementation Attenuated Hepatic TG Accumulation in Alcohol Fed Mice

To further demonstrate the specific role of NAD^+ in TG anabolism, C57BL/6 mice were fed with alcohol-containing liquid diet supplemented with NR for four weeks. We found that NR supplementation increased body weight (Figure 7A) and decreased liver weight/body weight rate (Figure 7B) in comparison to the AF group. No significant change was observed in the epididymal fat pad weight/body weight rate (Figure 7C) and hepatic TC content (Figure 7E) in alcohol-fed mice after NR supplementation. Importantly, NR supplementation elevated hepatic NAD^+ content (Figure 7F) and alleviated hepatic TG accumulation (Figure 7D) in alcohol-fed mice. All our data suggested that elevating hepatic NAD^+ content by NR supplementationattenuated chronic alcohol consumption-induced hepatic TG accumulation.

Discussion

In this study, we demonstrated that the PARP inhibitor PJ34 alleviated TG accumulation in both hepatocyte and the liver of alcohol-fed mice; and the underlying mechanism was involved in the PJ34-elevated NAD^+ level and PJ34-inhibited gene expression of SREBP-1c, DGAT1 and DGAT2 in the liver of alcohol-fed mice. All our data suggested that PJ34 had a therapeutic potential in AFLD by increasing the hepatic NAD^+ content and decreasing hypertriglyceridemia and hepatic TG accumulation.

Various publications have been shown that PARP has been gaining recognition as a central regular signaling molecule in numerous diseases including cancer, energetic metabolism, inflammation, shock, cardiovascular diseases, diabetes and fatty liver disease (Sistigu et al., 2015; Islam et al., 2016; Vida et al., 2017; Huang et al., 2017). High-fat high-sucrose (HFHS)-diets leds to insulin resistance, oxidative stress, steatosis and inflammation and lowers hepatic NAD^+ levels driving reductions in hepatic mitochondrial dysfunction and increases in hepatic lipid content in mice (Gariani et al., 2016; Verbeek J et al., 2015). HFHS-diets also increased PARPs activity in mice, shown by the ADP-ribose polymerization of proteins catalyzed by PARPs. NAD^+ was used to produce ADP-ribose for PARPs-catalyzed ADP-ribose polymerization, followed by a reduced intracellular NAD^+ level which disrupted the mitochondrial function and the resulting hepatic lipid accumulation (Gariani et al., 2017). Consistent with these investigations, we found that chronic alcohol fed mice caused PARP over activation and higher levels of ADP-ribose polymers of proteins compared to the PF group. This suggested that PARP activation was involved in the pathogenesis of AFLD. As a NAD^+ consumer, PARP activation promoted the accumulation of ADP-ribose polymers and NAD^+ depletion in many diseases (Rappou et al., 2016; Shetty et al.,

2014; Banasik et al., 2012; Martire et al., 2015). NAD^+ depletion impaired cellular redox capacity and metabolic transformation (Mouchiroud et al., 2013; Yu et al., 2016). In our investigation, chronic alcohol feeding for four weeks caused the decline of the hepatic NAD^+ level resulting from hepatic PARP activation. It was reported that NAD^+ overconsumption led to the metabolic disorder of fatty acids and the following hepatic lipid accumulation (Mukhopadhyay et al., 2017; Rappou et al., 2016). Increasing evidence showed that the alteration of PARP expression and activity was associated with the lipid metabolism (Pang et al., 2015; Kiss et al., 2015; Bai et al., 2015). Therefore, both the data and literature indicate that chronic alcohol consumption-induced activation of hepatic PARP and the decline of hepatic NAD^+ content have critical roles in hepatic TG accumulation. Thus, the pharmacological modulation of hepatic PARP activity might be a potential candidate for AFLD therapy.

Similar considerations have been seen in several recent reports. The PARP inhibitor Olaparib (4-[[3-[[4-(Cyclopropylcarbonyl)-1-piperazinyl]carbonyl]-4-fluorophenyl]Methyl]-1(2H)-phthalazinone, $C_{24}H_{23}FN_4O_3$) improved hepatic fatty acid oxidation and NAFLD induced by a HFHS-diet (Gariani et al., 2017). The pharmacological inhibition of PARP, or genetic deletion of PARP1 can restore the hepatic NAD^+ content and increase SIRT1 activation, which decreases hepatic TG accumulation in both alcoholic and non-alcoholic steatohepatitis (ASH/NASH) (Mukhopadhyay et al., 2017). In our study, we found that a PJ34 injection for four weeks lowered both circulating TG and hepatic TG level in alcohol-fed mice, but no significant changes were observed for circulating and hepatic TC levels in the same conditions. The mechanistic investigations in HepG2 cells also showed that PJ34 significantly decreased the intracellular TG level; however, the expression level of PARP had little change in PJ34-treated cells. Furthermore, PJ34 reduced the gene expression of

DGAT1 and DGAT2, both of which are key enzymes in TG anabolism. Nevertheless, there was no significant change in the gene expression of SREBP-1c, the lipogenic transcriptional factor in PJ34-treated hepatocyte, in comparison to the control group. Whether the transcriptional activity of SREBP-1c was lowered by PJ34 exposure needs to be confirmed in further studies. All the mechanistic observation in hepatocyte was validated in alcohol-fed mice. PJ34 injection for four weeks decreased hepatic gene expression of SREBP-1c, DGAT1 and DGAT2 in alcohol-fed mice. Overall, our results suggested that the PARP inhibitor PJ34 attenuated hepatic TG accumulation in AFLD progression and the underlying mechanism was involved in the PJ34-suppressed gene expression of DGAT1 and DGAT2 in TG anabolism.

PARP2 is another isoform of PARP. PARP1 and PARP2 act as a metabolic regulator by interacting with many transcription factors regulating lipid metabolism and oxidation (P. Bai et al., 2012). In liver, PARP2 as a negative regulator of SIRT1, PARP2 deletion or inhibition via enhanced SIRT1 expression and promoted mitochondrial biogenesis in mice (P. Bai et al., 2011). Hepatic cholesterol levels were higher in $PARP2^{-/-}$ mice. PARP2 deletion or inhibition increased hepatic cholesterol homeostasis and lower HDL levels in plasma via enhanced SREBP-1 expression (Magdolna Szántó et al., 2014). In our experiments, PJ34 injection could not change hepatic and plasma TC levels in alcohol-fed mice (Figure 5 H and 5I). All the data indicate that PJ34, as a chemical inhibitor for the activity of both PARP1 and PARP2, might exert differentiated roles between PARP1 and PARP2 in hepatic lipid metabolism, which needed to be confirmed in further investigation.

Since hepatic PARP-related NAD^+ alteration was associated with the dysfunction of TG accumulation in the progression of non-alcoholic steatohepatitis (Mukhopadhyay et al., 2017;

Gariani et al., 2017), whether it accounted for the progression of alcoholic steatohepatitis needed to be demonstrated. In our study, chronic alcohol feeding significantly decreased the hepatic NAD^+ level in comparison to the PF group. Additionally, PARP inhibitor PJ34 injections elevated hepatic NAD^+ level in alcohol-fed mice. The data from the HepG2 cell lines also showed that PJ34 elevated intracellular NAD^+ content. All changes of hepatic NAD^+ by PARP inhibition were associated with hepatic TG alteration. To further demonstrate PARP-regulated NAD^+ in alcoholic hepatic TG metabolism, we supplemented NR in the alcohol-diet for four weeks simultaneously. Recent studies have shown that NR as a NAD^+ precursor increased NAD^+ in both skeletal muscle and the liver, which enhanced oxidation metabolism and protected against high-fat diet-induced metabolic dysfunction via activated sirtuin1 and sirtuin3 (Canto et al., 2012). In our study, we found that NR supplementation increased the body weight and lowered the liver weight in alcohol-fed mice. NR supplementation elevated hepatic NAD^+ content and attenuated hepatic TG accumulation in alcohol-fed mice. All data suggested that a high level of NAD^+ supplementation alleviated alcoholic hepatic NAD^+ depletion and TG accumulation in the progression of AFLD. A low level of PARP activation-induced hepatic NAD^+ might be one reason for the pathogenesis of AFLD.

PARP inhibitors are currently being tested in clinical trials with approval from the Food and Drug Administration (FDA) and European Medicines Agency (EMA) (Parkes et al., 2016). A Phase III trial with the PARP1 and PARP2 inhibitor Niraparib (MK-4827) for ovarian cancer showed that Niraparib inhibited PARP-triggered DNA damage and cell death, and the ultimate oncogenic progression (Lin et al., 2017; Kanjanapan et al., 2017). Another oral PARP inhibitor Olaparib is undergoing Phase III trials for patients with ovarian cancer (Ledermann et al., 2016a)

and results demonstrated that Olaparib prolonged survival in some patients with serious ovarian cancer (Ledermann et al., 2016b). Aside from ovarian cancer, this inhibitor was also used for the treatment of breast and colorectal cancer and showed promising results (Robert et al., 2017). In *vivo*, C57BL/6 mice were fed with alcohol-containing liquid diets and injected intraperitoneally with/without Olaparib or Rucaparib (8-fluoro-5-(4-((methylamino)methyl)phenyl)-3,4-dihydro-2H-azepino[5,4,3-cd]indol-1(6H)-one phosphoric acid, $C_{19}H_{18}FN_3O_5P$) for four weeks. Olaparib and Rucaparib injection attenuated hepatic fat droplet accumulation in alcohol-fed mice (Supplemental Figure S1). Both of them attenuated hepatic TG accumulation and decreased the levels of circulating TG and TC and reduced liver injured by chronic alcoholic consumption in alcohol-fed mice (Supplemental Figure S2). Besides, we found that injected with Olaparib or Rucaparib elevated hepatic NAD^+ level in alcohol-fed mice and attenuated hepatic NAD^+ depletion by chronic alcoholic exposure (Supplemental Figure S3). The same result were found via treating with PJ34 in alcohol-fed mice. In *vitro*, H2O2 as a activator of PARP, HepG2 cells were exposed to complete DMEM medium with or without H_2O_2. H_2O_2 reduced intracellular NAD^+ level and enhanced intracellular TG content in hepatocyte. PARP activity by H_2O_2 enhanced the expression of critical genes in TG anabolism in HepG2 cells (Supplemental Figure S4). Moreover, PARP inhibition by PJ34 attenuated hepatic TG accumulation and inflammation (Supplemental Figure S5) in chronic alcohol-fed mice. Similarly, chronic alcohol-feeding increased the expression of pro-inflammatory cytokines IL-6, MCP-1 and TNF-α. Olaparib, Rucaparib or PJ34 injection markedly attenuated liver inflammation in liver tissues by immunohistochemical method (Supplemental Figure S6). These results suggest that PARP inhibition might be a promising therapeutic strategy for alcoholic

steatohepatitis. All our data indicated that PARP inhibitor, Olaparib, Rucaparib and PJ34 injection significantly elevated hepatic NAD^+ level and attenuated hepatic TG accumulation in alcohol-fed mice.

In general, our investigation provided primary evidence that the exogenous PARP pharmacological inhibitor PJ34 was effective against alcohol-induced hepatic TG accumulation by enhancing the hepatic NAD^+ content and suppressing the gene expression of DGAT1 and DGAT2 in the liver in alcohol-fed mice. Thus, the chemical inhibitor PJ34 has a therapeutic potential in AFLD progression by restoring hepatic NAD^+ depletion and lowering hepatic TG anabolism.

Authorship contributions

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Conducted experiments: Shishun Huang, Bing Zhang, Yingli Chen and Zhigang Wang.

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*Performed data analysis:*Yingli Chen, Huan Liu, Yang Liu, Xin Li and Zhiwei Bao.

*Wrote or contributed to the writing of the manuscript:*Shishun Huang, Zhenyuan Song and Zhigang Wang.

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Footnotes

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Competing financial interest

The authors declare no competing financial interest.

Legends for figures

Fig. 1. Chronic alcohol consumption induced hepatic fat accumulation and liver injury in C57BL/6 mice. Mice were fed with a Lieber-DeCarli alcohol-containing diet for four weeks. Chronic alcohol feeding lowered body weight (A) and increased liver/body weight rate (B) in comparison to the PF group. Epididymal fat pad/body weight rate (C) also decreased through chronic alcohol feeding. The levels of circulating ALT (D), AST (E), hepatic TG (F), hepatic TC (H), the level of circulating TG (G) and TC (I) were significantly increased by chronic alcohol consumption. Chronic alcohol feeding enhanced hepatic fat accumulation by staining of liver sections with H&E (x 400) (J). The quantitative statistics of lipid droplets fold change were showed (K). Values displayed are expressed as the mean ± SD (n = 6 per group). Asterisk (* $p < 0.05$) indicates significant differences compared to the pair-fed group. PF: pair-fed; AF: alcohol-fed; ALT: alanine transaminase; AST: aspartate transaminase;TC:cholesterol; TG: triglyceride.

Fig. 2. Chronic alcohol feeding enhanced PARP expression, pADPr synthesis and NAD^+ depletion in alcohol-fed mice. Total proteins were extracted from liver tissues and subjected to Western blot for expression of PARP, pADPr, SREBP-1c, DGAT1 and DGAT2 (A) with specific antibodies. Chronic alcohol exposure increased the expression of hepatic PARP protein (B) which catalyzed the pADPr formation (C) and lowered hepatic NAD^+ content in comparison to the PF group (D). The densitometric analysis showed that chronic alcohol feeding increased the expression of SREBP-1c (E), DGAT1 (F), and DGAT2 (G) proteins. Relative genic expression of SREBP-1c (H), DGAT1 (I), and DGAT2 (J) were significantly higher in chronic alcohol consumption when compared to the PF group. The data are expressed as the mean ± SD (n = 6 per group). Asterisk (*

$p < 0.05$) indicates significant differences compared with the PF group. PF: pair-fed; AF: alcohol-fed; DGAT1/2: diglyceride acyltransferase 1/2;pADPr:polymeric adenosine diphosphate ribose; PARP: poly ADP ribose polymerase; SREBP-1c: sterol regulatory element binding protein 1c.

Fig. 3. Inhibition of PARP activity augmented NAD^+ level and decreased intracellular TG content in hepatocyte. HepG2 cells were exposed to complete DMEM medium containing PJ34 (1 μM) for 24 h. The expression of intracellular PARP and pADPr were detected by Western blot (A). PJ34 treatment had no significant change on the expression of PARP (B) and decreased the synthesis of pADPr (C). PJ34 treatment elevated intracellular NAD^+ content (D). Moreover, PJ34 treatment also decreased intracellular TG content (E). All values were denoted as means ± SD from three or more independent batches of cells. * $p < 0.05$ compared to control group.pADPr:polymeric adenosine diphosphate ribose; PARP: poly ADP ribose polymerase;TG: triglyceride.

Fig. 4. Inhibition of PARP activity suppressed the expression of critical genes in TG anabolism. HepG2 cells were treated with PJ34 (1 μM) in present OA for 24 h. Total RNAs were extracted from cells. The mRNA of critical genes was detected by RT-PCR. PJ34 treatment changed little in intracellular SREBP-1c (A) and lowered intracellular gene expression of DGAT1 (B), and DGAT2 (C). All values were denoted as means ± SD from three or more independent batches of cells. * $p < 0.05$ compared to control group. DGAT1/2: diglyceride acyltransferase 1/2;SREBP-1c: sterol regulatory element binding protein 1c.

Fig. 5. PJ34 injection attenuated hepatic TG accumulation in alcohol-fed mice. C57BL/6 mice were fed with alcohol-containing liquid diet with/without PJ34 intraperitoneal injection for four

weeks. In comparison to the AF group, PJ34 injection augmented body weight (A) and lowered liver/body weight rate (B). However, PJ34 injection had no significant change on epididymis fat/body weight rate (C) and the levels of circulating ALT and AST (D and E). PJ34 intraperitoneal injection decreased hepatic TG content (F) and the level of circulating TG (G), but had no difference on hepatic TC content (H) and the level of circulating TC (I) in comparison to the AF group. Liver sections were stained with H&E (x 400) (J). The quantitative statistics of lipid droplets fold change were showed (K).The data are expressed as the mean ± SD (n = 6 per group); * $p < 0.05$ compared to the AF group. AF: alcohol-fed; ALT: alanine transaminase; AST: aspartate transaminase;TC:cholesterol; TG: triglyceride.

Fig. 6. PJ34 injection elevated hepatic NAD^+ level and lowered gene expression in TG anabolism in alcohol-fed mice. Male C57BL/6 mice were fed with alcohol-containing liquid diets and injected intraperitoneally with/without PJ34 for four weeks. Total proteins were extracted from liver tissues and used to detect the expression of PARP and the synthesis of pADPr (A) with specific antibodies. PJ34 injection had no effect on PARP expression (B) and decreased pADPr synthesis (C). PJ34 injection reduced hepatic NAD^+ depletion in comparison to AF group (D). PJ34 injection significantly alleviated hepatic critical genes expression of SREBP-1 (E), DGAT1 (F), and DGAT2 (G) in comparison to the AF group. The data are expressed as the mean ± SD (n = 6 per group); * $p < 0.05$ compared to the AF group. AF:alcohol-fed; DGAT1/2: diglyceride acyltransferase 1/2;pADPr:polymeric adenosine diphosphate ribose; PARP: poly ADP ribose polymerase; SREBP-1c: sterol regulatory element binding protein 1c.

Fig. 7.NR supplementation attenuated hepatic TG accumulation in alcohol feeding mice. Mice were fed alcohol-fed liquid diets with NR supplementation for four weeks. NR supplementation

augmented body weight (A) and decreased liver/body weight rate compared with the AF group (B), whereas NR had no change in the epididymal fat pad/body weight rate (C). NR supplementation decreased hepatic TG content (D) and had no change in hepatic TC content (E) in comparison to the AF group. Supplement with NR elevated hepatic NAD^+ content (F) in the AF + NR group. Asterisk (* $p < 0.05$) indicates significant differences compared to the AF group. NR: nicotinamide riboside. AF: alcohol-fed; NR:nicotinamide riboside;TC:cholesterol; TG: triglyceride.

Fig. 1

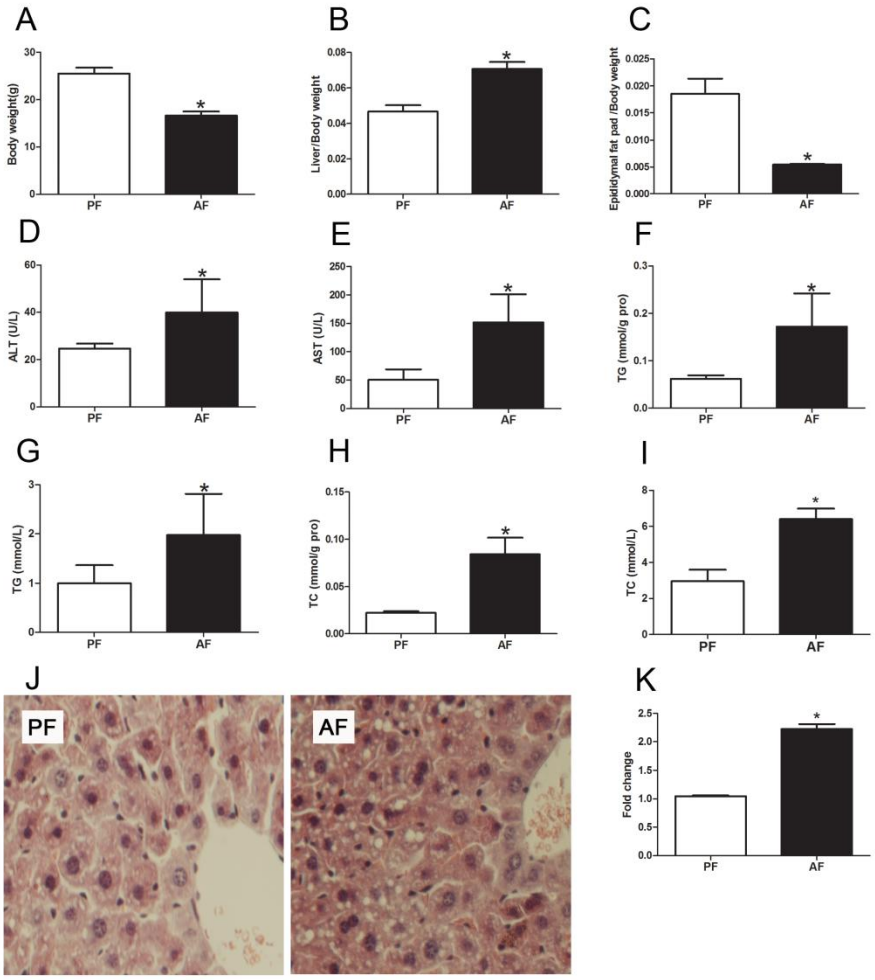

Fig. 2

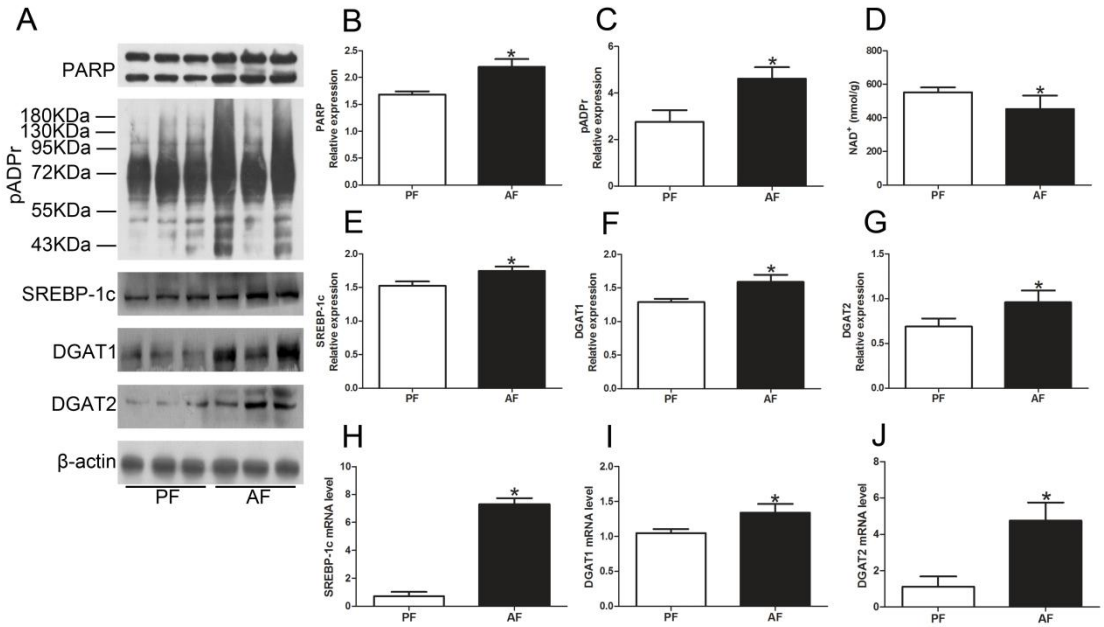

Fig. 3

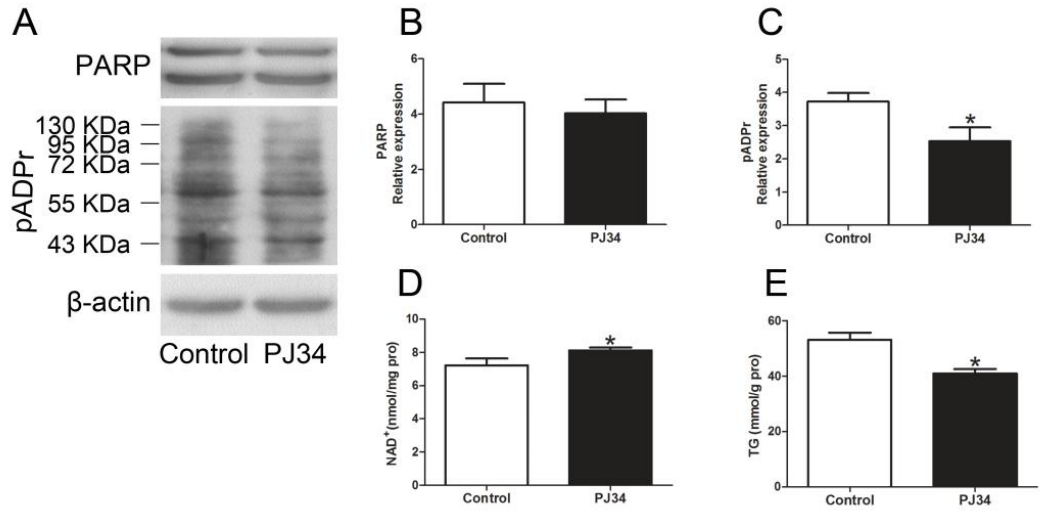

Fig. 4

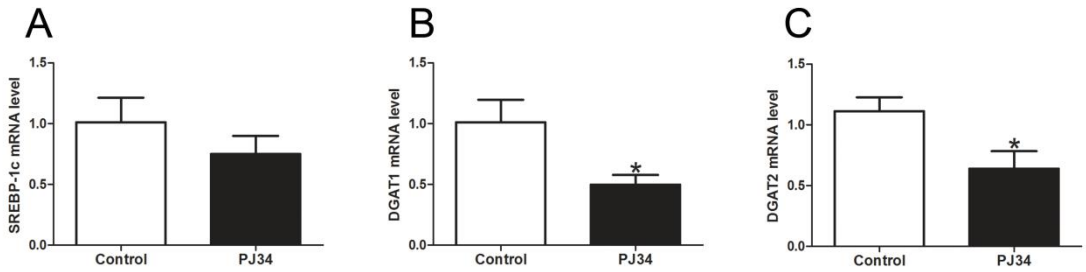

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Fig. 5

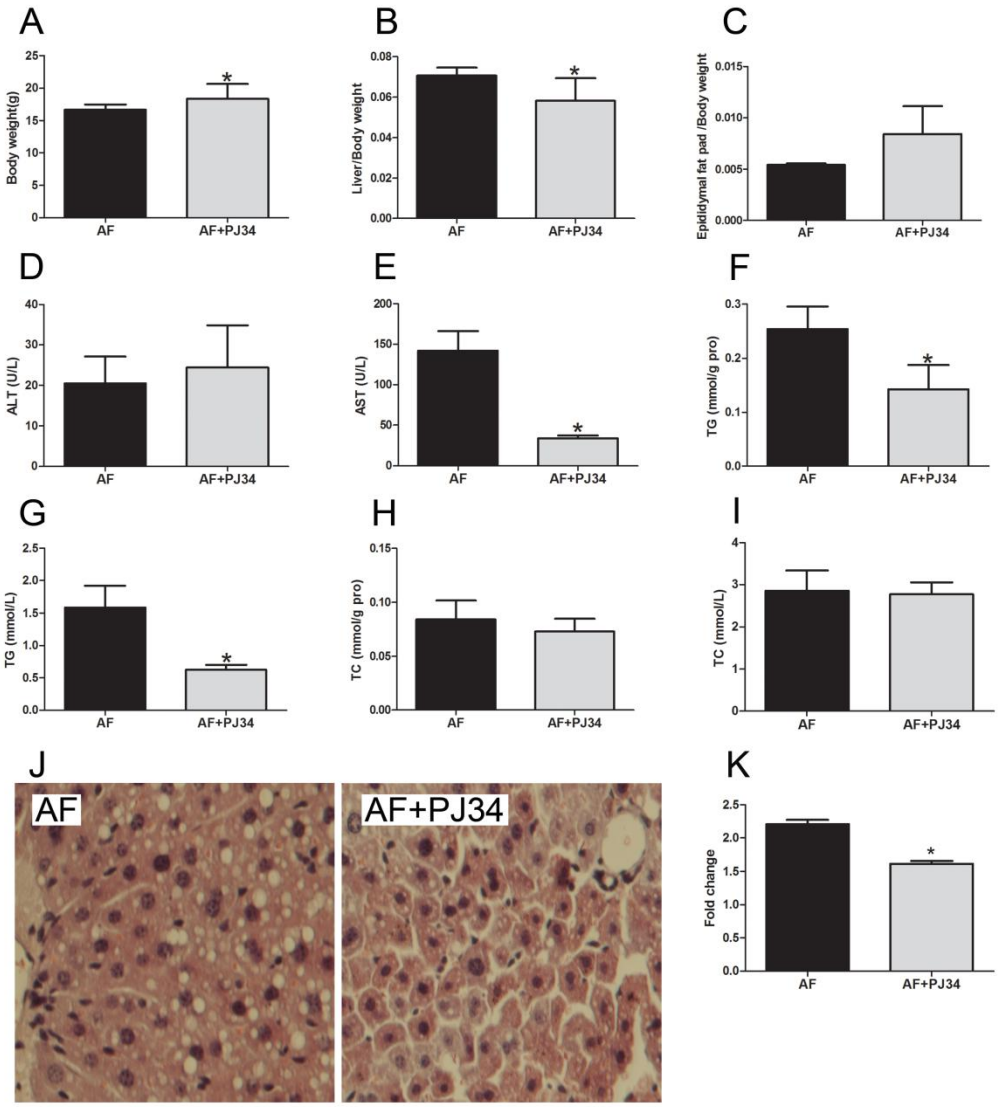

Fig. 6

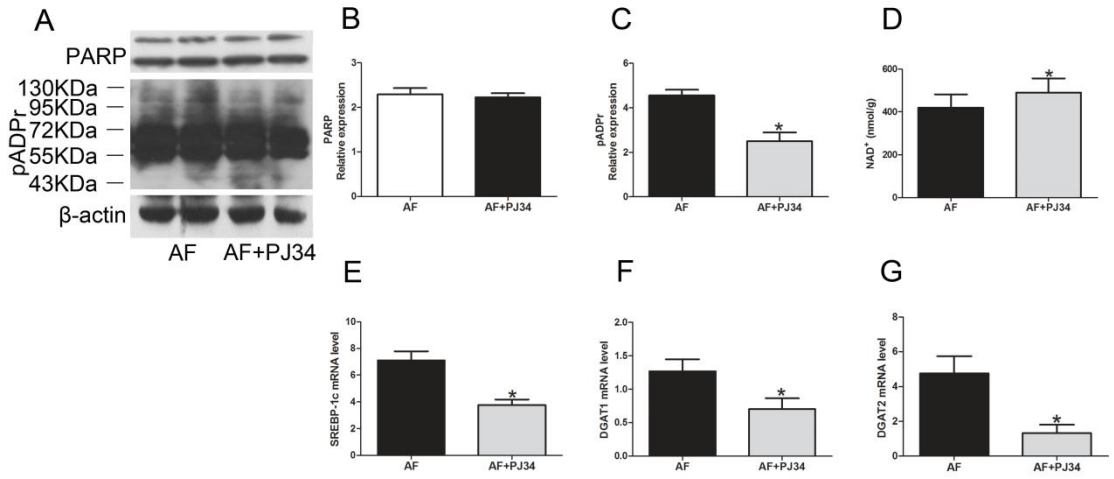

Fig. 7