

Original Research Article

JNK pathway promotes hepatocyte apoptosis by inhibiting Bcl-2 and upregulating expressions of Bim, caspase-3 and caspase-9 after cardiopulmonary bypass

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Sent for review: 18 December 2019

Revised accepted: 27 February 2020

Abstract

Purpose: To study the effect of Jun N-terminal kinase (JNK) signaling pathway on hepatocyte apoptosis *in vivo* and *in vitro*, and to elucidate the mechanism of action.

Methods: TdT-mediated dUTP Nick-End Labeling (TUNEL) method was used to determine apoptosis in control and cardiopulmonary bypass (CPB) groups at 0, 3 and 6 hours after rat surgery. The expressions of JNK and p-c-Jun in liver tissues at 0, 3 and 6 h after surgery, and the levels of p-c-Jun, Bcl-2 and Bim following overexpression of JNK, were determined using Western blot assay. Human liver cell line HL-7702 was cultured and transfected with over-expressed JNK plasmid and empty plasmid. Proliferation of HL-7702 cells after JNK over-expression was assessed by Cell Counting Kit-8 (CCK-8), while quantitative real-time polymerase chain reaction (RT-qPCR) was employed to evaluate mRNA expression levels of caspase-3 and caspase-9 mRNA after JNK over-expression. Apoptosis of the cells was determined by flow cytometry (FC) after JNK over-expression.

Results: FC results showed that the number of apoptotic hepatocytes increased after JNK over-expression in hepatocytes while TUNEL assay results demonstrated that hepatocyte apoptosis increased in CPB group, when compared to control group; furthermore, the number of apoptotic cells gradually increased within 6 h after surgery. The expressions of JNK and p-c-Jun were higher in CPB group than in control group, and increased gradually in both groups within 6 h after surgery. Overexpression of JNK decreased the proliferation of hepatocytes, and also lowered protein expression levels of p-c-Jun and Bim; on the other hand, the protein expression levels of Bcl-2 fell, while mRNA expression levels of caspase-3 and caspase-9 mRNA increased.

Conclusion: JNK pathway promotes hepatocyte apoptosis after cardiopulmonary bypass by inhibiting Bcl-2 pathway and promoting the expressions of Bim caspase-3 and caspase-9.

Keywords: Cardiopulmonary bypass, Apoptosis, JNK pathway, Bim, caspase-3 and caspase-9

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INTRODUCTION

During cardiac surgery, a series of changes take place in visceral organs due to cardiopulmonary bypass (CPB) and hemodilution [1]. Studies have found that this effect still occurs after CPB, especially in the liver [2]. Many studies have reported liver damage after CPB, which manifests as postoperative high bilirubin, transient changes in liver enzymes, and increased lactate levels due to insufficient tissue perfusion [3,4].

The c-Jun N-terminal kinase (JNK) is a member of the MAPK family, and it is activated by various stimuli such as cytokines, physical factors and ischemia-reperfusion [5]. Activated JNK signaling pathway phosphorylates different substrates, including c-Jun. Researchers have found that increased levels of activated c-Jun cause cell apoptosis or differentiation, and lead to cell proliferation [6].

Studies have demonstrated that embryonic fibroblasts derived from JNK1-/-and JNK2-/-mice are resistant to UV-induced apoptosis in JNK1 and JNK2 knockout mice [7]. The role of JNK3 in cell apoptosis has been demonstrated in JNK3-/-mice [8]. Other studies have found that JNK inhibitors reduced damage to liver cells, endothelial cells, and myocardial cells during ischemia-reperfusion [9,10]. Indeed, signals arising from JNK activation via phosphorylation are transmitted to the apoptosis signal pathway, resulting in regulation of the apoptotic process.

It has been hypothesized that the JNK pathway may be involved in hepatocyte apoptosis induced by extracorporeal circulation. The present study was carried out to verify this hypothesis. Rat experiments were used to study the effect of JNK pathway on mouse hepatocyte apoptosis *in vivo*. In order to further verify the specific effect of JNK pathway, the mechanism of JNK pathway involvement in hepatocyte apoptosis was studied *in vitro* so as to provide new molecular therapeutic targets for the treatment of diseases caused by apoptosis.

EXPERIMENTAL

Cells and animals

Human liver cell line HL-7702 was purchased from Zhengzhou Yizeng Biotechnology (Zhengzhou, China).

Thirty 30 adult male SPF SD rats weighing between 450 and 500g were provided by the Experimental Animal Center of Zhengzhou

University. All protocols used in the animal experiments were approved by the Ethics Committee on Animal Research of Zhengzhou University (approval no. ZZUCEARL-20180521). The study was performed in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals in China [11].

Animal model

All animal operations were performed in accordance with the guidelines for laboratory animal use approved by Zhengzhou University. The rats were randomly divided into CPB group and sham group, with 15 rats in each group. The experimental rats were fasted for 24 hours and denied access to water for 6 hours before surgery. The neck and thighs were depilated with 8 % Na₂S, and disinfected with iodophor. Cardiopulmonary bypass (CPB) rat model was established as previously described [12].

Hepatocyte apoptosis detection

Liver tissue was made into routine paraffin sections which were dewaxed twice with xylene, each for 10 minutes, and rehydrated with gradient ethanol (100, 95, 90, 80 and 70 %). The sections were then sealed with 3 % H₂O₂-methanol for 20 minutes and washed thrice with 3 PBS buffer (5 minutes each time). Then, 50 mL of TUNEL reaction buffer solution was added to each sample. The sections were sealed by sealing the membrane, followed by incubation in wet box at 37 ° C in dark for 1 hour. Thereafter, enzyme-labeled antibody was added to the sections, followed by incubation at 37 ° C for 30 min, and washing thrice with PBS. Thereafter, the sections were treated with DAB color development solution prepared in line with the instructions in the DAB color development kit (Beyotime Biotechnology, China). The reaction was performed at room temperature for 10 min, and the PBS solution was washed 3 times (5 min each time). The slides were sealed and observed under a microscope.

Western blot and real-time- quantitative polymerase chain reaction (RT-qPCR)

Proteins were extracted from liver tissue/cells using total-tissue protein extraction kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Bicinchoninic acid (BCA) Protein Assay Kit and ECL substrate color development kit were purchased from Beyotime Biotechnology (Shanghai, China). Mouse SP (SP-9002) detection kit was purchased from Zhengzhou Mengzhen Biotechnology (Zhengzhou, China). The bands were observed

using an ECL western blotting analysis system (GE Healthcare, USA) and exposed to Kodak x-ray film.

Quantitative real time polymerase chain reaction (RT-qPCR) assay was performed as previously described [13]. Reverse transcription kit was purchased from Roche company (Roche, Switzerland). The specificity of the amplification was determined using melting curve analysis. Data were calculated as threshold cycle (Ct) function. The relative mRNA expressions of caspase-3 and caspase-9 genes were determined with $2^{-\Delta\Delta C_t}$ method, with GAPDH as endogenous control. The primers used in the study are shown in Table 1.

Table 1: Primers used in the study

Primer	5' to 3' Sequence
GAPDH:	TGTTTCGTCATGGGTGTGAAC
Forward	
Reverse	ATGGCATGGACTGTGGTCAT
Caspase-3:	ACTGGACTGTGGCATTGAG
Forward	
Reverse	GAGCCATCCTTTGAATTCGC
Caspase-9:	CCAACCCTAGAAAACCTTACCC
Forward	
Reverse	TCTGCATTTCCCCTCAAATC

Cell proliferation and transfection

Cell proliferation was determined using CKK-8 kit (Beyotime Biotechnology, China) according to the manufacturer's instructions. Over-expressed JNK plasmid and empty plasmid were designed and synthesized by Suzhou Jima Gene Company (Suchow, China). Lipofectamine 2000 (Thermo Fisher, USA) was used to conduct cell transfection according to the manufacturer's instructions.

Flow cytometry

Cells were plated in 6-well plates. When the cell growth density was 50 - 60 %, over-expressed JNK plasmid and empty plasmid were transfected into the cells using Lipofectamine 2000 in line with the manufacturer's protocol. After 24 - 48 h of culture, the cells were subjected to apoptosis assay using TUNEL apoptosis assay kit (Beyotime Biotechnology, China).

Statistical analysis

Data collation and statistical analysis were done using SPSS 19.0 software, with GraphPad Prism 6 as the mapping software. Paired sample comparison was done with *t*-test, while multiple

group comparison was done using one-way-ANOVA (SNK-q test for pairwise comparison).

RESULTS

CPB-induced apoptosis of liver cells

Apoptosis in liver tissue was determined using TUNEL method at 0, 3, and 6 h after the establishment of the closed chest CPB model. It was found that apoptosis of liver cells in the CPB group increased gradually after 6 hours of surgery. There was no significant change in hepatocyte apoptosis in the sham group (sham group).

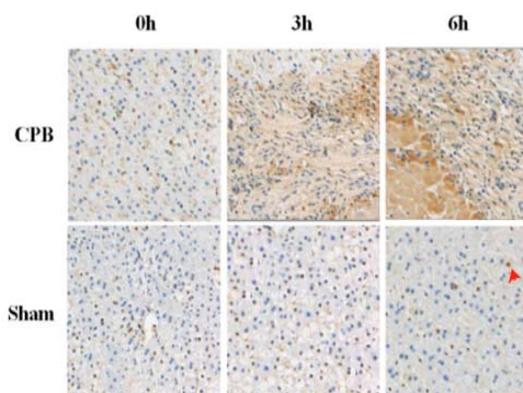


Figure 1: CPB-induced hepatocyte apoptosis

CPB activated JNK pathway

Western blot was used to assay the expressions of JNK and p-c-Jun in the liver tissues of mice in the CPB group and sham group within 6 hours after surgery. It was found that the expressions of JNK and p-c-Jun in the liver tissue of mice in the sham group were unchanged, while those in the liver tissue of the CPB group were gradually increased within 6 h after surgery. This indicates that CPB activated the JNK pathway.

Over-expression of JNK promoted c-Jun phosphorylation and suppressed Bcl-2 pathway

Human liver cells HL-7702 were cultured *in vitro*, and JNK over-expression plasmid was transfected into the cells. Western blotting was used to evaluate the effect of the over-expression on the expressions of anti-apoptotic Bcl-2 and pro-apoptotic proteins. It was found that after over-expression of JNK in liver cells, the expressions of JNK, p-c-Jun and Bim were increased, while the expression of Bcl-2 was decreased, as shown in Figure 3.

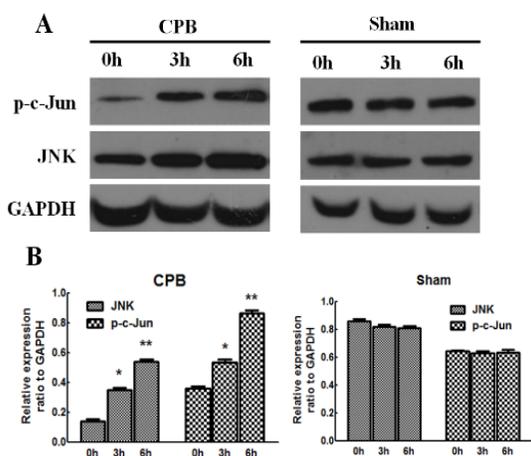


Figure 2: Effect of CPB on the JNK pathway. A: Results of Western blotting showed that the expressions of JNK and p-c-Jun in liver tissue of CPB group mice gradually increased within 6 hours after operation. B: Grayscale scanning values of protein bands; * $p < 0.05$, ** $p < 0.01$, compared with 0 h after surgery

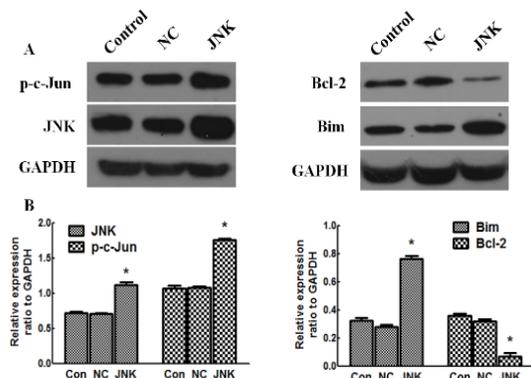


Figure 3: Effect of JNK overexpression on the expressions of Bcl-2 and p-c-Jun. A: Results of Western blotting demonstrated increased expressions of p-c-Jun, Bim and p-c-Jun, and decreased expression of Bcl-2 after overexpression of JNK. B: Grayscale scanning values of protein bands; * $p < 0.05$, compared with the control group

JNK over-expression reduced cell proliferation activity

After over-expression of JNK in liver cells, the mRNA expressions of caspase-3 and caspase-9 were determined using RT-qPCR. It was found that the levels of caspase-3 and caspase-9 mRNA in hepatocytes increased after JNK over-expression. At the same time, cell proliferation was determined using the CCK-8 method. It was found that after JNK over-expression in liver cells, the cell proliferation decreased, as shown in Figure 4.

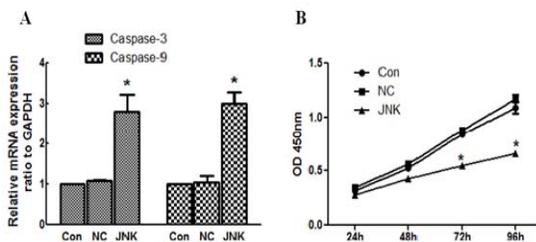


Figure 4: JNK overexpression increased mRNA levels of caspase-3 and caspase-9, and decreased cell viability of hepatocyte. A: Results of RT-qPCR demonstrated that caspase-3 and caspase-9 mRNA levels increased after the overexpression of JNK in hepatocytes. B: Results of CCK-8 showed that cell proliferation decreased after the overexpression of JNK in hepatocytes; * $p < 0.05$, compared with the control group

Over-expression of JNK promoted hepatocyte apoptosis

After JNK over-expression in hepatocytes, the apoptosis of the cells was determined with flow cytometry in combination with apoptosis detection kit. It was found that over-expression of JNK promoted hepatocyte apoptosis. These results are shown in Figure 5.

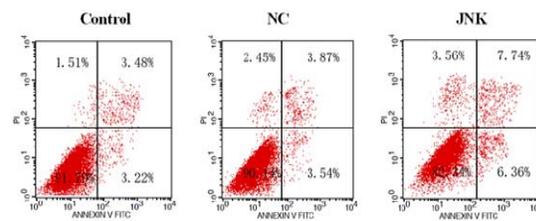


Figure 5: JNK overexpression promoted the apoptosis of hepatocyte

DISCUSSION

The JNK signaling pathway is involved in many cellular processes such as cell proliferation, cell differentiation, and apoptosis. Previous studies have reported that the JNK pathway promoted apoptosis during ischemia-reperfusion, leading to heart, kidney, brain, liver damage [14,15]. The present study found that hepatocyte apoptosis increased after CPB surgery, and the expressions of JNK and p-c-Jun in liver tissues increased, indicating that the JNK pathway was activated by CPB stimulation.

There are many regulatory mechanisms involved in the promotion of apoptosis by JNK. For example, the JNK pathway in the nucleus has been shown to promote the expressions of several pro-apoptotic proteins, e.g. TNF- α and Bak. Studies have found that in the nervous

system, apoptosis of neuronal cells requires the participation of JNK, a process which is inhibited by the expression of inhibitors of JNK in the nucleus [16]. This study found that the level of c-Jun phosphorylation increased after activation of JNK, indicating that the JNK-c-Jun pathway is involved in hepatocyte apoptosis. However, further studies are needed to elucidate the exact mechanism involved in JNK-induced hepatocyte apoptosis.

In addition to the regulatory mechanism in nucleus, JNK is also involved in the regulation of mitochondrial apoptosis. Studies have shown that the JNK peptide inhibitor D-JNKI-1 (XG-102) reverses some pathological changes in the mitochondria, almost completely eliminating cytochrome c release and PARP cleavage [17]. Other studies have found that during TNF- α -induced apoptosis of HeLa cells, JNK induced the decomposition of caspase-8-dependent Bid to form jBid with a molecular weight of 21 KDa, which was transferred to mitochondria where it promoted the expressions and release of pro-apoptotic proteins Smad and DIABLO [18]. In this study, after over-expressing JNK in hepatocytes, Bcl-2 expression was decreased, while expression of Bim was increased, indicating that JNK is likely to regulate the expressions of these factors through the mitochondrial pathway, thereby promoting apoptosis of hepatocytes.

Studies have found that taurine inhibited arsenic-induced liver damage [19]. Under the effect of taurine, the abnormal expressions of Bcl-2 and Bax were reversed, the activation of caspase-3 was inhibited, and the phosphorylation of JNK was also suppressed. These show that arsenic-induced liver damage was achieved by activating the JNK pathway, inhibiting Bcl-2 expression, and promoting the expressions of Bax and caspase-3, resulting in liver cell apoptosis. The role of the JNK pathway in promoting apoptosis is consistent with the results of this study. In a study by Xie, acute liver damage was induced in mice with carbon tetrachloride, and the mice were treated with dihydromyricetin. It was found that dihydromyricetin significantly reduced serum levels of ASL, AST and some pro-inflammatory factors [20].

The levels of caspase-3, caspase 6, caspase 8 and caspase 9, and the expression level of JNK were decreased. Results from TUNEL experiments showed that hepatocyte apoptosis was also decreased, indicating that dihydromyricetin resisted liver damage through a mechanism dependent on the JNK pathway [20]. In the present study, over-expression of JNK in liver cells led to increases in the transcription

levels of caspase-3 and caspase-9. This is consistent with the results obtained in [20], indicating that activation of the JNK pathway promoted the expressions of caspase-3 and caspase-9, thereby further promoting hepatocyte apoptosis. These studies have shown that the JNK pathway is an important anti-apoptotic target.

In this study, it was found that over-expression of JNK in liver cells altered the expressions of caspase-3 and caspase-9, Bcl-2 and Bim, and also increased the level of phosphorylation of c-Jun. The c-Jun N-terminal kinase (JNK) regulates the expression of related genes and promotes apoptosis of hepatocytes, but the upstream regulatory mechanism of JNK needs to be further investigated.

CONCLUSION

The results obtained in this study suggest that under stimulation by CPB, JNK pathway is activated and hepatocyte apoptosis is induced *in vivo*. Over-expression of JNK decreases the expression of anti-apoptotic protein Bcl-2, while the expression levels of pro-apoptotic proteins Bim, caspase-3 and caspase-9, and hepatocyte apoptosis increased *in vitro*. Therefore, JNK pathway may promote hepatocyte apoptosis by inhibiting the expression of anti-apoptotic protein Bcl-2, and promoting the expressions of Bim, caspase-3 and caspase-9.

DECLARATIONS

Acknowledgement

This work was supported by General Project 2014 of Health Commission of Henan (grant no. 201403087) and 5451 Overseas Training Project 2017 of Health Commission of Henan Province (grant no. 2017038).

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication. Haibin Yu, Haojie Zhang and Yan Cheng conceived and designed the study. Fangtao Zhu, Yachao Lin and Weihua Huang

collected and analyzed the data, while Haibin Yu wrote the manuscript.

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REFERENCES

- Davidson JA, Urban TT, Baird C, Tong S, Woodruff A, Twite M, Jagers J, Simões EAF, Wischmeyer P. Alkaline Phosphatase in Infant Cardiopulmonary Bypass: Kinetics and Relationship to Organ Injury and Major Cardiovascular Events. *J Pediatr* 2017; 190: 49-55.e2.
- Huffmyer JL, Groves DS. Pulmonary complications of cardiopulmonary bypass. *Best Pract Res Clin Anaesthesiol* 2015; 29(2): 163-175.
- Di Tomasso N, Monaco F, Landoni G. Hepatic and renal effects of cardiopulmonary bypass. *Best Pract Res Clin Anaesthesiol* 2015; 29(2): 151-161.
- Soliman R, Fouad E, Belghith M, Abdelmageed T. Conventional hemofiltration during cardiopulmonary bypass increases the serum lactate level in adult cardiac surgery. *Ann Card Anaesth* 2016; 19(1): 45-51.
- Kwon IS, Kim J, Rhee DK, Kim BO, Pyo S. Pneumolysin induces cellular senescence by increasing ROS production and activation of MAPK/NF- κ B signal pathway in glial cells. *Toxicol* 2017; 129: 100-112.
- SCHRECK I, AL-RAWI M, MINGOT J M, Scholl C, Diefenbacher ME, O'Donnell P, Bohmann D, Weiss C. c-Jun localizes to the nucleus independent of its phosphorylation by and interaction with JNK and vice versa promotes nuclear accumulation of JNK. *Biochem Biophys Res Commun* 2011; 407(4): 735-740.
- Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ. Requirement of JNK for stress-induced activation of the cytochrome c-mediated death pathway. *Science* 2000; 288(5467): 870-874.
- Yang DD, Kuan CY, Whitmarsh AJ, Rincón M, Zheng TS, Davis RJ, Rakic P, Flavell RA. Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the *Jnk3* gene. *Nature* 1997; 389(6653): 865-870.
- Uehara T, Bennett B, Sakata ST, Satoh Y, Bilter GK, Westwick JK, Brenner DA. JNK mediates hepatic ischemia reperfusion injury. *J Hepatol* 2005; 42(6): 850-859.
- Ferrandi C, Ballerio R, Gaillard P, Giachetti C, Carboni S, Vitte PA, Gotteland JP, Cirillo R. Inhibition of c-Jun N-terminal kinase decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats. *Br J Pharmacol* 2004; 142(6): 953-960.
- National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals. *Guide for the Care and Use of Laboratory Animals*. 8th edition. Washington (DC): National Academies Press (US); 2011.
- Madrahimov N, Boyle EC, Gueler F, Goecke T, Knöfel AK, Irkha V, Maegel L, Höffler K, Natanov R, Ismail I, Maus U, Kühn C, Warnecke G, Shrestha ML, Cebotari S, Haverich A. Novel mouse model of cardiopulmonary bypass. *Eur J Cardiothorac Surg* 2018; 53(1):186-193.
- Gu HF, Tang CK, Yang YZ. Psychological stress, immune response, and atherosclerosis. *Atherosclerosis* 2012; 223(1): 69-77.
- Shvedova M, Anfinogenova Y, Atochina-Vasserman EN, Schepetkin IA, Atochin DN. c-Jun N-Terminal Kinases (JNKs) in Myocardial and Cerebral Ischemia/Reperfusion Injury. *Front Pharmacol* 2018; 9: 715.
- Liang R, Nickkholgh A, Hoffmann K, Kern M, Schneider H, Sobirey M, Zorn M, Büchler MW, Schemmer P. Melatonin protects from hepatic reperfusion injury through inhibition of IKK and JNK pathways and modification of cell proliferation. *J Pineal Res* 2009; 46(1): 8-14.
- Coffey ET. Nuclear and cytosolic JNK signalling in neurons. *Nat Rev Neurosci* 2014; 15(5): 285-299.
- Zhao Y, Spigolon G, Bonny C, Culman J, Vercelli A, Herdegen T. The JNK inhibitor D-JNKI-1 blocks apoptotic JNK signaling in brain mitochondria. *Mol Cell Neurosci* 2012; 49(3): 300-310.
- Madesh M, Antonsson B, Srinivasula SM, Alnemri ES, Hajnóczky G. Rapid kinetics of tBid-induced cytochrome c and Smac/DIABLO release and mitochondrial depolarization. *J Biol Chem* 2002; 277(7): 5651-5659.
- Li S, Yang L, Dong G, Wang X. Taurine Protects Mouse Liver Against Arsenic-Induced Apoptosis Through JNK Pathway. *Adv Exp Med Biol* 2017; 975: 855-862.
- Xie J, Liu J, Chen TM, Lan Q, Zhang QY, Liu B, Dai D, Zhang WD, Hu LP, Zhu RZ. Dihydropyridinone alleviates carbon tetrachloride-induced acute liver injury via JNK-dependent mechanism in mice. *World J Gastroenterol* 2015; 21(18): 5473-5481.