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Adiponectin alleviates liver injury in sepsis rats through AMPK/MTOR pathway

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Abstract. – OBJECTIVE: To investigate the influences of adiponectin (APN) on the liver injury in sepsis rats and to explore whether it exerts a therapeutic effect through the adenosine monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway.

MATERIALS AND METHODS: A rat model of sepsis was established through cecal ligation and puncture (CLP) (CLP group), and APN treatment group (APN group) and control group were also set. The changes in the liver function-related indicators, serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were determined by automatic biochemistry analyzer, and the levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1, and IL-6 were measured via enzyme-linked immunosorbent assay (ELISA). Hematoxylin-eosin (HE) staining was employed to detect liver tissue injury, and the hepatocyte apoptosis and necrosis after intervention with APN were evaluated using in situ fluorescence staining. Moreover, the mRNA expression of APN in liver tissues was detected via quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR), and the expression levels of phosphorylated AMPK and mTOR proteins in liver tissue samples were determined using Western blotting.

RESULTS: In terms of changes in liver function-related indicators, the concentrations of ALT and AST were substantially raised in the CLP group, and compared with those in the control group, the concentrations of the two indicators significantly declined in the APN group, showing statistically significant differences ($p<0.05$). CLP and APN group had evidently higher levels of inflammatory factors than the control group, but their levels in APN group were notably lower than those in the CLP group ($p<0.05$). It was found through the HE staining that the sepsis rats in CLP group had massive inflammatory cell infiltration, and that the inflammatory cells were remarkably decreased in the APN group after APN treatment. According to the in-situ fluorescence staining detection results, CLP group exhibited a notable increase in the cell apoptosis rate, and APN group had substantially reduced apoptotic cells ($p<0.05$). The determination results of APN expression revealed that CLP group had a lowered level of APN, and that the level of APN in APN group was markedly higher than that in the control group. Based on the results of Western blotting, the level of phosphorylated AMPK was remarkably elevated, and that of phosphorylated mTOR was lowered in the CLP group compared with those in the control group, while in comparison with CLP group, APN group showed a considerable elevation of phosphorylated AMPK level and a distinct decline in the phosphorylated mTOR level.

CONCLUSIONS: APN can activate the AMPK/mTOR pathway and reduce hepatocyte apoptosis to alleviate liver injury in sepsis rats.

Key Words:
Adiponectin, AMPK/mTOR pathway, Sepsis, Liver injury.

Introduction

Sepsis is an invasive infection-induced systemic inflammatory response characterized by inflammatory changes, progressive hypoxemia, and tissue injury, further progressing into single organ or multiple organ dysfunction syndrome[1-3]. In spite of the latest understanding of pathophysiology of sepsis, sepsis-associated complications are still difficult to be promptly treated, and the

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leading causes of morbidity and death are dysfunctions in the lung, liver, heart, kidney, and other vital organ due to the responses of the host to infectious injury[4]. The liver, as the target of inflammatory response disorder, tends to be the most severely affected organ. According to a literature report, adiponectin (APN) has a therapeutic effect on sepsis[5], but its role in sepsis-induced liver injury remains elusive.

APN, a hormone secreted by adipose tissues, has a strong ability to regulate various biological responses[6,7], and it is associated with obesity, coronary heart disease, atherosclerosis, and diabetes[8,9]. In addition to anti-atherosclerosis and anti-inflammatory effects, APN has an anti-apoptotic property[10,11]. APN not only functions well in adipose tissue metabolism and insulin resistance, but also plays a crucial role in the survival and proliferation of multiple cells[12], and its anti-apoptotic effect brings many benefits[13,14]. APN weakens the phagocytic activity of lipopolysaccharide-stimulated macrophages and inhibits the production of inflammatory cytokines[15,16]. APN deficiency in the APN-knockout mice can promote the activation of endothelial cells, raising the sepsis-related mortality rate[17]. APN can suppress the generation of tumor necrosis factor-α (TNF-α) and interleukin (IL)-6 to decrease the mortality rate in sepsis mice[18]. However, few studies have reported the influences of APN on the liver injury in sepsis. Yamauchi et al[19] reported that APN receptors activate intracellular signaling pathways to mediate cell functions. The downstream molecular pathways of APN receptors have not yet been elucidated, but the research on the metabolic responses of cells demonstrated that the activation of pleiotropic adenosine monophosphate-activated protein kinase (AMPK) is implicated in the downstream signaling cascades of APN receptors[20].

As a serine/threonine kinase activated by APN[21], AMPK plays a key role in regulating energy homeostasis and serves as a metabolic sensor adjusting adenosine triphosphate concentration[22]. Phosphorylated (p)-AMPK inhibits the target of the mammalian target of rapamycin (mTOR) signaling pathway, and mTOR is an enzyme in the downstream of AMPK. Active AMPK is an inhibitor of the mTOR pathway[23]. MTOR, a conservative protein kinase, is vital for the regulation of cell proliferation, growth, differentiation, migration, and survival[24]. APN can alleviate inflammatory responses and inhibit the growth of cancer cells through the AMPK/mTOR pathway[5,25]. Therefore, it was speculated in this study that the influences of APN on the liver injury in sepsis rats may be related to the AMPK/mTOR signaling pathway. The present study seeks to explore the influences of APN on the liver injury in sepsis rats through the AMPK/mTOR pathway.

Materials and Methods

Main Materials

Enzyme-linked immunosorbent assay (ELISA) kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), hematoxylin-eosin staining (HE) staining kit (Solarbio, Beijing, China), Annexin V-fluorescein isothiocyanate (FITC) *in situ* cell apoptosis assay kit (Partec AG, Arlesheim, Switzerland), ribonucleic acid (RNA) extraction reagent TRIzol (Shanghai Sangon Biotech, Shanghai, China), protease K (Nanjing Sunshine Biotechnology Co., Ltd., Nanjing, China), fluorescence quantitative Polymerase Chain Reaction (PCR; Beijing TransGen Biotech Co., Ltd. , Beijing, China), bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA), p-AMPK, AMPK, p-mTOR and mTOR antibodies (Abcam), and APN (BioVision, Inc., Milpitas, CA, USA).

AU 2700 automatic biochemistry analyzer (Olympus, Tokyo, Japan), microplate reader, and carbon dioxide incubator (Thermo Fisher Scientific, Waltham, MA, USA), RM2015 slicer (Leica, Wetzlar, Germany), electrophoresis apparatus (Bio-Rad, Hercules, CA, USA), fluorescence microscope (Olympus, Tokyo, Japan), tissue agitator (Wuxi Voshin Instruments Co, Ltd., Wuxi, China) and StepOne fluorescence quantitative PCR instrument (ABI, Applied Biosystems, Foster City, CA, USA).

Establishment of Animal Models and Sampling

After adaptively fed for 7 d, male Sprague-Dawley rats were used to establish the sepsis model by cecal ligation and puncture (CLP; CLP group, n=20). In control group (n=20), the cecum of rats was only separated, without ligation and puncture after the abdomen was opened, and those in APN treatment group (APN group, n=20) were intraperitoneally injected with APN at 3 mg/kg after modeling. Each group of rats was killed *via* arterial bleeding. Following centrifugation, the serum was harvested to detect the serum biochemistry parameters. After dissection, an appropriate number of liver tissues were taken and fixed in formalin for HE staining, and the remaining tissues

were stored in liquid nitrogen for the detection of messenger RNA (mRNA) expressions of proteins to be detected. This study was approved by the Animal Ethics Committee of Jinan City People's Hospital Animal Center.

Detection of Liver Function-Related Indicators

The serum samples collected earlier and cryopreserved at -80°C were taken out, and the changes in the concentrations of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using an automatic biochemistry analyzer.

Detection of Inflammatory Factors in Rats

The serum samples collected and cryopreserved at -80°C in advance were taken out, slowly thawed at 4°C, and then, centrifuged at low speed, and the supernatant was harvested. Subsequently, the levels of serum inflammatory factors TNF-α, IL-10, and IL-6 were determined using the ELISA kit, Finally, the absorbance in each group was measured with a microplate reader. The specific experimental steps were based on the particular conditions and instructions.

HE Staining of Liver Tissues

The fixed liver tissues were rinsed using running water overnight, dehydrated in gradient alcohol, embedded in paraffin, and routinely sectioned. Then, the sections were soaked in xylene and hydrated using a series of ethanol. Subsequently, the thin sections baked dry were stained using the HE staining kit according to the instructions. Finally, the obtained sections were mounted in neural resin, and the pathological changes in the structure of liver tissues under a light microscope.

Evaluation of Hepatocyte Apoptosis

The fresh liver tissues were cut into pieces using ophthalmic scissors on a plate, and washed with normal saline. Then, cell suspension was harvested for routine cell culture. The cells cultured in a 96-well plate were taken out, re-suspended, rinsed in phosphate buffered saline (PBS), and digested by a moderate amount of 0.25% trypsin. After pipetting and centrifugation, the cells were collected, and an appropriate number of cells were taken and centrifuged. After the supernatant was discarded, the morphologic characteristics and distribution of apoptotic hepatocytes in each group of rats were preliminarily detected using the Annexin V-FITC apoptosis assay kit, and the Annexin V-FITC was green fluorescence.

Detection of mRNA Expression of APN in Liver Tissues by qRT-PCR

A total of 100 mg of liver tissues were carefully and accurately weighed, ground in liquid nitrogen, and swashed by a homogenizer. Then, RNAs were extracted using TRIzol reagent, and reversely transcribed into complementary deoxyribonucleic acids (cDNAs). Subsequently, PCR amplification was performed in a system (20 μL) comprising 2 μL of cDNAs, 10 μL of mix, 2 μL of primers, and 6 μL of ddH_2O according to the procedures: pre-denaturation at 95°C (2 min), and reaction at 95°C (2 s), 60°C (20 s), and 72°C (30 s) for 40 cycles. Based on the sequences from GeneBank, the primers of the target gene and the internal reference β-actin were designed and synthesized by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) (Table I). Finally, the relative expression levels of genes were statistically analyzed using $2^{-\Delta\Delta Ct}$.

Determination of p-AMPK and p-mTOR Protein Levels in Liver Tissues by Western Blotting

The liver tissues were taken out and completely lysed with lysis buffer. The homogenate was transferred into a centrifuge tube and centrifuged at high speed and 4°C for 5 min, and the supernatant obtained was the protein to be detected. Then, the concentration of proteins was determined by the BCA method. After preparation of samples and gels, the samples were loaded for electrophoresis. Next, the protein gels containing the target proteins were cut,

Table I. Primer sequence.

Gene	Primer	Sequence 5′-3′
APN	Sense	GTCAGTGGATCTGACGACACCAA
	Anti-sense	ATGCCTGCCATCCAACCTG
β-actin	Sense	CCGCGAGTACAACCTTCTTG
	Anti-sense	TGACCCATACCCACCATCAC

Table II. Changes in serum inflammatory factors ($\bar{x} \pm s$).

Group	IL-6 (pg/mL)	IL-1 (pg/mL)	TNF-α (pg/mL)
Control	9.73±2.3	16.7±3.4	13.5±2.8
CLP	60.2±5.7*	81.3±4.1*	97.3±5.1*
APN	40.3±3.8#	33.9±5.7#	53.6±3.9#

Note: Compared with those in the control group, the levels of the inflammatory factors are significantly raised in both CLP group and APN group, and the inflammatory factor levels in the APN group are drastically lower than those in the CLP group ($p<0.05$). *$p<0.05$ *vs.* control group, #$p<0.05$ *vs.* CLP group.

transferred onto a membrane, and blocked at room temperature for 1 h. Subsequently, the primary antibodies were diluted and added into the membrane for incubation at 4°C overnight. The resulting proteins were added into the secondary antibodies and incubated at room temperature for 1 h. Finally, the color was developed using diaminobenzidine (DAB) substrate solution (Solarbio, Beijing, China) for 15 min, and the protein bands were scanned and detected by a gel imaging system, followed by analysis and quantification using Image Lab software to calculate the expression levels of the corresponding proteins in each group.

Statistical Analysis

All data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA), and presented as mean ± standard deviation. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). $p<0.05$ represented that the difference was statistically significant.

Results

Detection Results of Liver Function-Related Indicators

The concentrations of liver function-related indicators, serum ALT and AST, were determined using the automatic biochemistry analyzer. The results revealed that CLP group had substantially higher concentrations of ALT and AST than the control group, and the concentrations of liver function-related indicators in APN group were remarkably lower than those in the CLP group, showing statistically significant differences ($p<0.05$) (Figure 1).

Serum Inflammatory Factors

As shown in Table II, the levels of inflammatory factors in both CLP group and APN group were remarkably higher than those in the control group, while compared with those in the CLP group, the inflammatory factor levels in APN group were considerably lowered ($p<0.05$).

HE Staining Results of Liver Tissues

According to the HE staining results (Figure 2), the rats in the control group had orderly arranged hepatocytes and almost no inflammatory cell infiltration, while the sepsis rats in CLP group exhibited massive inflammatory cell infiltration in liver tissues, which was more evident in the portal area, with erythrocyte deposition. After treatment with APN, APN group showed remarkably alleviated inflammatory cell infiltration, without visible congestion.

Hepatocyte Apoptosis Detected

Based on the preliminary detection the results of apoptosis and necrosis *via in situ* fluorescence staining, there were almost no apoptotic hepatocytes in the control group, the apoptosis rate rose considerably in the CLP group, and the APN

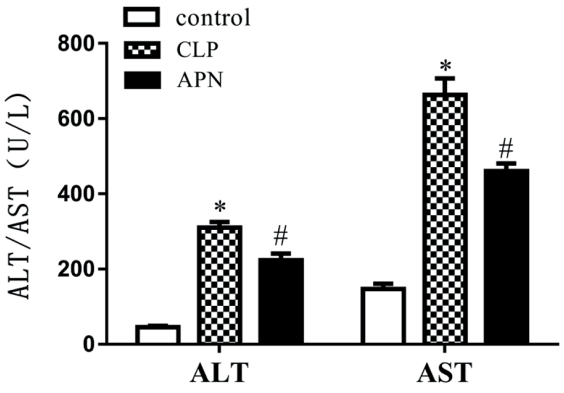

Figure 1. Detection results of liver function-related indicators: the concentrations of ALT and AST are notably elevated in CLP group, and they decline evidently in APN group compared with those in CLP group. *$p<0.05$ *vs.* control group, #$p<0.05$ *vs.* CLP group.

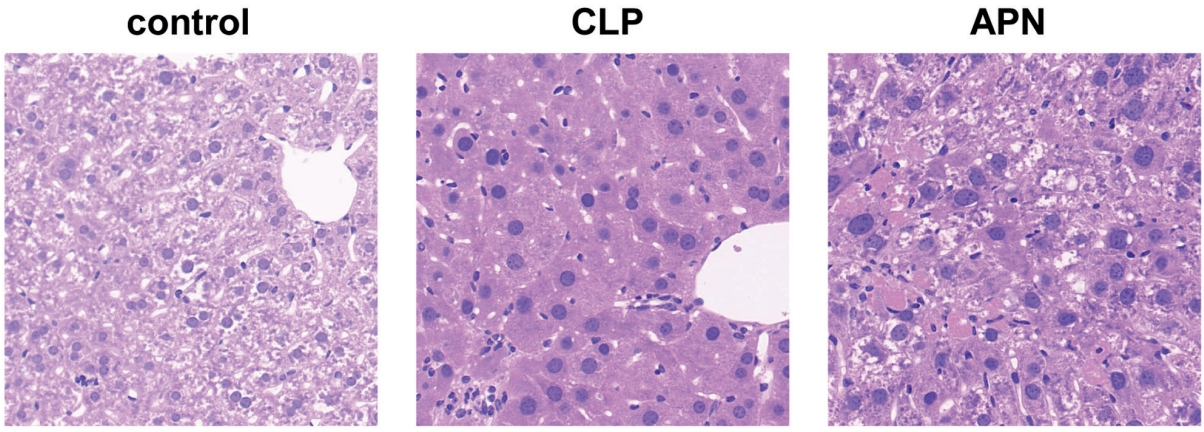

Figure 2. HE staining results of liver tissues (magnification: 200×): the sepsis rats in CLP group have massive inflammatory cell infiltration, whereas the inflammatory cells notably decline in APN group after APN treatment.

group showed a substantial decline in the apoptotic cells ($p<0.05$) (Figure 3).

MRNA Expression of APN in Liver Tissues

Figure 4 presents the mRNA expression of APN in liver tissues. It was discovered that compared with that in the control group, the level of APN was lowered in the CLP group, but significantly increased in the APN group, indicating that the expression of APN declined in the sepsis model.

P-AMPK and p-mTOR Levels in Liver Tissues Determined

Western blotting results revealed that CLP group had a remarkably higher level of p-AMPK and a lower level of p-mTOR than the control group, and compared with those in the CLP group, the level of p-AMPK was remarkably raised, and that of p-mTOR was evidently lowered in the APN group, displaying statistically significant differences ($p<0.05$) (Figure 5), implying that AMPK negatively regulates mTOR.

Discussion

As sepsis develops and progresses, invasive infection can cause damage to multiple organs. The liver is a common organ involved in multiple organ dysfunction syndrome, and liver dysfunction can lead to death[26]. In sepsis-induced inflammatory responses, hepatocyte injury is mainly attributed to leucocytes that release various harmful mediators. The inflammatory response in very severe sepsis may be more intense than the resistance of host, and such an overwhelming inflammatory

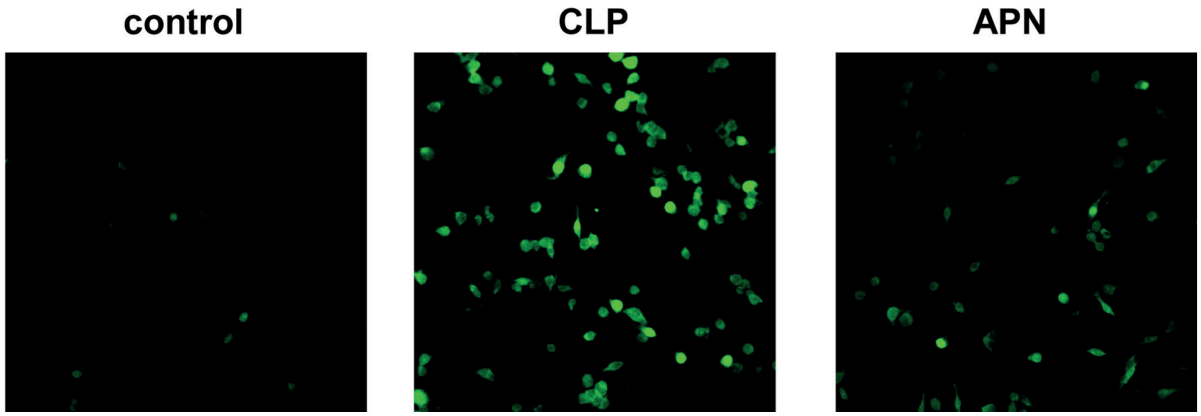

Figure 3. Detection results of hepatocyte apoptosis (magnification: 100×): the apoptosis rate is notably raised in CLP group, while the apoptotic cells decline substantially in APN group ($p<0.05$).

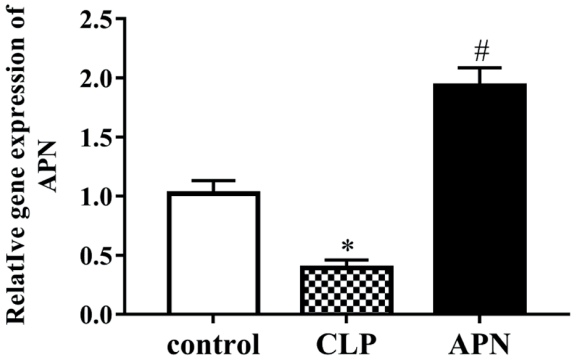

Figure 4. MRNA expression of APN: the expression level of APN declines in CLP group, but rises considerably in APN group ($p<0.05$). $^{*}p<0.05$ *vs.* control group, $^{\#}p<0.05$ *vs.* CLP group.

response is likely to cause organ injury. The generation of inflammatory mediators such as TNF-α is generally considered to play a vital role in the pathophysiological process of sepsis, and TNF-α is regarded as the major factor that may trigger the cytokine cascades and harmful physiological changes in organ structure and function in sepsis[27]. In this study, the levels of the inflammatory factors in the CLP group were significantly higher than those in the other two groups, and the inflammatory responses and liver tissue injury were alleviated in the APN group. The above results suggest that APN can inhibit the expression of pro-inflammatory cytokines to lower the mortality rate, exerting the anti-inflammatory effect in the rat model of sepsis, which are consistent with the previous reports[5].

APN, as an anti-inflammatory agent, has the anti-inflammatory activity *in vivo* and *in vitro*[28], thereby inhibiting the production of inflammatory cytokines, neutralizing lipopolysaccharide activity, stimulating numerous anti-inflammatory substances, and weakening the viability of macrophages and endothelial cells. It was observed in the present study that the expression of APN was considerably lowered in the rat sepsis model. The substantial decline in APN expression promotes the responses of inflammatory cytokines after inflammatory injury, thus adversely affecting organisms. The histological analysis of the HE staining of rat liver tissues showed that there were numerous infiltrating inflammatory cells, and that APN relieved the inflammation. Based on the histological evaluation, the liver injury score declined substantially after APN treatment. Besides, Ayala et al[29] showed that the necrosis and apoptosis in sepsis-induced hepatocyte injury have no evident boundary. Sugiyama et al[30] demonstrated that APN suppresses the growth of cancer cells by regulating AMPK. It was also found in the present study that APN could inhibit cell apoptosis and notably reduce sepsis-induced hepatocyte apoptosis.

The AMPK/mTOR pathway is essential for the changes in sepsis. According to research, this pathway plays a crucial role in the immune defense mechanism, and its activation is vital for reducing inflammation and organ dysfunction in sepsis[31]. MTOR is an enzyme in the downstream of AMPK. It is reported that APN is able to inhibit the mTOR pathway after the activation of AMPK, and that AMPK, as the inhibitor of the mTOR pathway, can suppress the progression of tumors[30]. Additionally, APN can activate autophagy to protect chondrocytes, which is associated with the process that AMPK inhibits mTOR to regulate the autophagy of chondrocytes. Since the mechanism by which APN exerts effects through the AMPK/mTOR pathway has not yet been fully clarified, based on the above reports, the in-

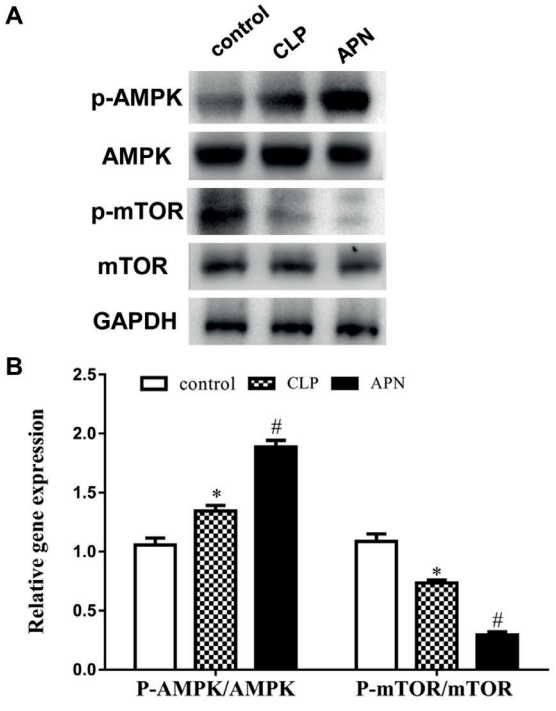

Figure 5. Phosphorylation levels of AMPK and mTOR pathway proteins in liver tissues: compared with those in control group, the level of p-AMPK is remarkably elevated, and that of p-mTOR declines. $^{*}p<0.05$ *vs.* control group, $^{\#}p<0.05$ *vs.* CLP group.

fluence of APN treatment on the AMPK/mTOR pathway was explored in this study. It was discovered that APN group exhibited a notable increase in the p-AMPK level and a substantial decrease in the p-mTOR level, suggesting that APN can activate AMPK and that p-AMPK suppresses the expression of the mTOR pathway. Further research is needed to evaluate each downstream molecular pathway of APN.

Conclusions

In summary, the present study reveals that APN can activate the AMPK/mTOR pathway and reduce hepatocyte apoptosis to attenuate the liver injury in sepsis rats, corroborates the important role of the AMPK/mTOR pathway in sepsis, and emphasizes the novel mechanism of APN in the pathological progression of sepsis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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