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should be addressed to
J.-P.G.
(gongjianping11@
126.com)

Impact of Recombinant Globular Adiponectin on Early Warm Ischemia-Reperfusion Injury in Rat Bile Duct after Liver Transplantation

Yang Xia & Jian-Ping Gong

Department of Hepatobiliary Surgery, The Second Affiliated Hospital of Chongqing Medical University, Chongqing, 400010, China.

Adiponectin (APN) is an adipocyte protein with anti-diabetic properties, which has been recently revealed to have anti-inflammatory activity in organ ischemia- reperfusion injury (IRI). However, little is known about its function in bile duct IRI after liver transplantation. Therefore, we investigated whether APN affects early warm IRI in rat bile duct using a liver autologous transplantation model. In our study, rats were randomly divided into three experimental groups: a sham group, a IRI group, and a APN group. The serum enzyme levels and BDISS scores of bile duct histology associated with bile duct injury, decreased after administration of APN. Subsequently, the expression of proinflammatory cytokines, such as tumor necrosis factor(TNF-α),.interleukin-6(IL-6) and myeloperoxidase (MPO) decreased. Furthermore, pretreatment with APN suppressed the activation of nuclear factor-kappa B (NF-κB) (p65), a transcription factor involved in inflammatory reactions, compared to other two groups. Administration of APN also downregulated the expression of Fas protein and attenuated caspase-3 activity to decrease bile duct apoptosis. Our results illustrate that APN protects the rat bile duct against early warm IRI by suppressing the inflammatory response and hepatocyte apoptosis, and NF-κB (p65) plays an important role in this process.

Advances in surgical techniques and immunosuppression have made liver transplantation a first-line treatment for many patients with end-stage liver disease. Of all liver transplantation, a mortality rate of 8%–15% is associated with biliary complications[1,2]. Biliary complications may be attributed to a variety of factors which includes hepatic artery thrombosis[3], prolonged cold injury time[4–6], warm ischemia- reperfusion injury[4,6,7], and immunological rejection[8,9] and have been variously associated with structural changes and functional lesions of the biliary tract following grafting. Among these pathogenic factors, warm IRI, especially, is regarded as the major concern of the early phase of biliary lesion development[4,10].

The process of biliary tract warm IRI is an inflammatory cascade involving multiple interconnected events[4–6,11]. NF-κB (p65), a critical transcription factor involved in inflammatory reactions, is observed predominantly located in the nucleus after ischemia-reperfusion. Many studies have indicated that activated NF-κB(p65) up-regulated the secretion of large amounts of inflammatory cytokines, such as TNF-α, and IL-6 during the initial phase of warm IRI[12–14]. The interaction of cytokines leads to the non-functioning epithelium by increasing neutrophil adherence, causing disturbance of the biliary tract microcirculation, and inducing bile duct cell apoptosis[11,15,16]. Consequently, activation of NF-κB(p65) has been considered to be a critical event in the initiation and perpetuation of bile duct warm IRI.

APN, derived from adipocytes, is a secreted hormone which was considered firstly as an important regulator of energy use and metabolism in endocrinology[17]. Recently, an increasing number of research papers have been involved in exploring the anti-inflammatory and anti-apoptotic properties of APN[18,19]. Although mounting evidence has confirmed the role of APN in other organ ischemia-reperfusion injury, it is still unclear whether APN is involved in liver IRI. In a study, a combined APN and FTY720 therapy in a small-for-size fatty liver transplant model significantly improved liver graft survival[20]. In this work, APN exerted a possible protective activity in fatty liver suffering IRI. However, direct effects of APN on bile duct ischemia-reperfusion injury after liver transplantation are rare.
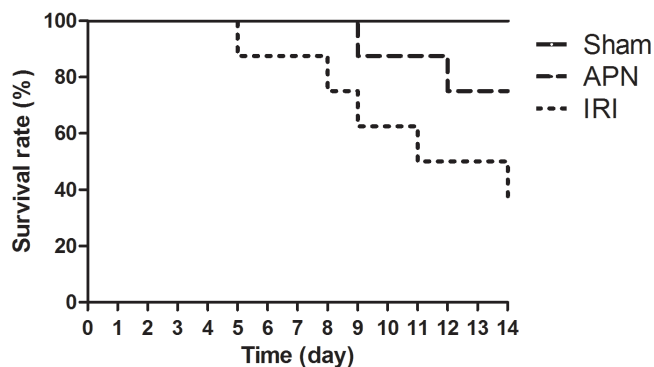
Figure 1 | The survival rates between rats following transplantation. Sham: The sham group (n=8) APN: Adiponectin group (n=8) IRI: Ischemia-reperfusion group (n=8).

Our laboratory has established a model of rat autologous liver transplantation that simulates the entire process of clinical liver transplantation and avoids the effects of infection and immune suppression while accurately controlling bile duct warm ischemia-reperfusion time. This model is therefore suitable for investigation of the effect of APN on rat bile duct during early warm IRI of transplantation.

Results

Survival and Pathological Examination. Survival curves are shown in figure 1. None of the sham group (n=8) died, while 2 of 8 in APN groups, 5 of 8 in IRI groups died after two weeks followed by liver transplantation (p= 0.0181). (Fig. 1) Statistical significance was found among groups. The bile duct showed a normal appearance in sham group, and more marked histological changes occurred in IRI group. From histological changes at 24 h after operation, our study results showed that bile duct injury in IRI group was more serious than sham group, such as edema, necrosis and ablate in epithelial cells of bile duct. Administration of APN decreased the injury(Fig. 2).

Serum Transaminases. Alanine transaminase (ALT) is an enzyme mainly synthesized in the liver. Significantly elevated levels of ALT often suggest the existence of other medical problems such as viral hepatitis, liver damage, bile duct problems. Alkaline phosphatase (ALP) is an enzyme which present in all tissues throughout the entire body, but is particularly concentrated in liver, bile duct, kidney, and bone. High ALP levels can occur if the bile ducts are damaged. Direct bilirubin (DBIL) is a conjugated version of bilirubin which conjugated with glucuronic acid by the enzyme glucuronyl-transferase. Biliary diseases such as ischemic injury and obstruction may result in in release of the substances into the circulation. Therefore the level of serum ALT, ALP and DBIL could be used as biochemical markers to evaluate the function of bile duct. Serum
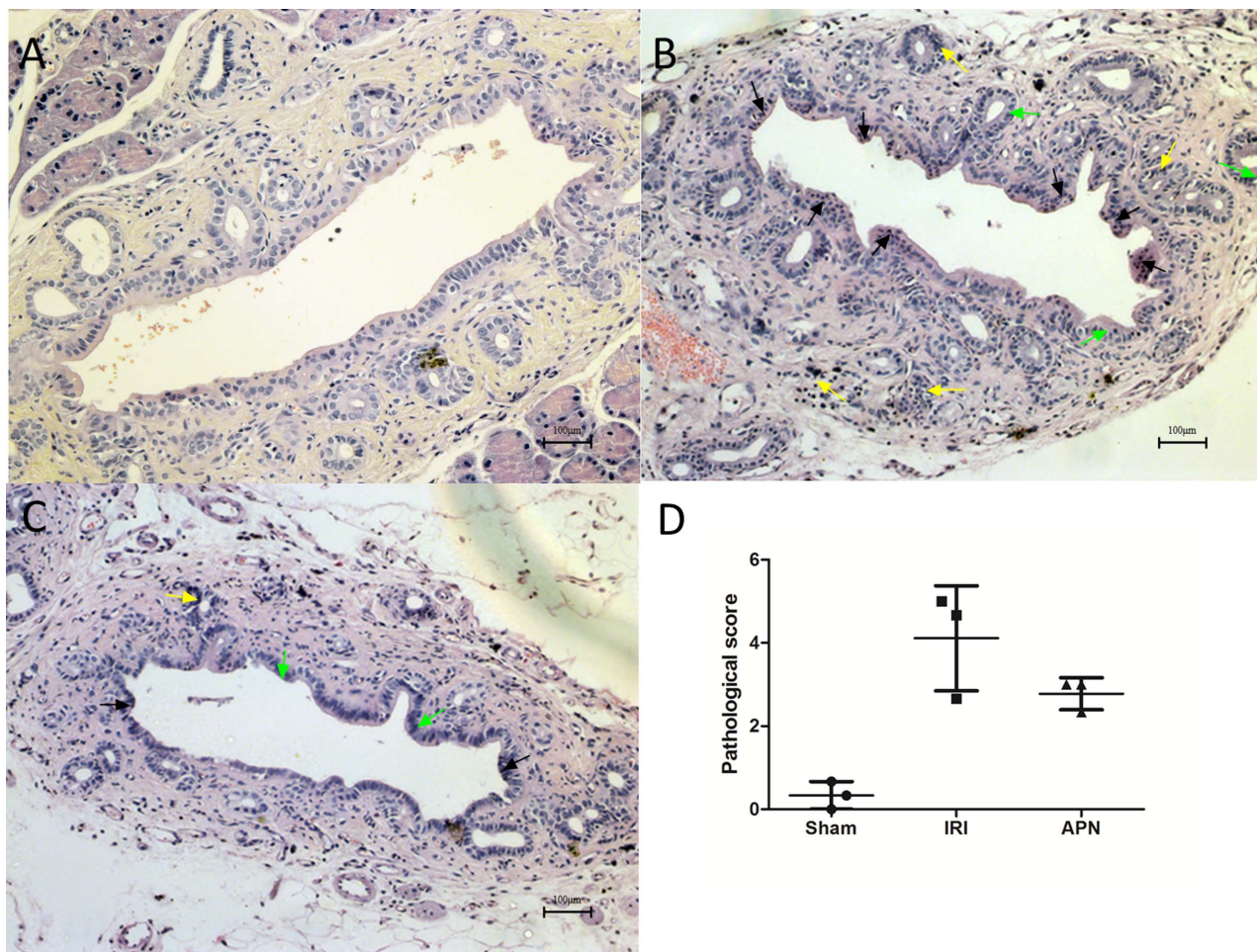
Figure 2 | Bile duct architecture at 24 h after ischemic-reperfusion (HE stain, ×200). (A) The sham group (n=4); (B) Ischemia-reperfusion group (n=6); (C) Adiponectin group (n=6). (D) Pathological score in all groups. More severe bile duct tissues damage with epithelial cells edema (green arrow), neutrophil infiltration (yellow arrow) and necrosis (black arrow) were seen in I/R groups (Fig 2B), whereas I/R with APN treatment showed a marked reduction in the severity of these features (Fig 2C).
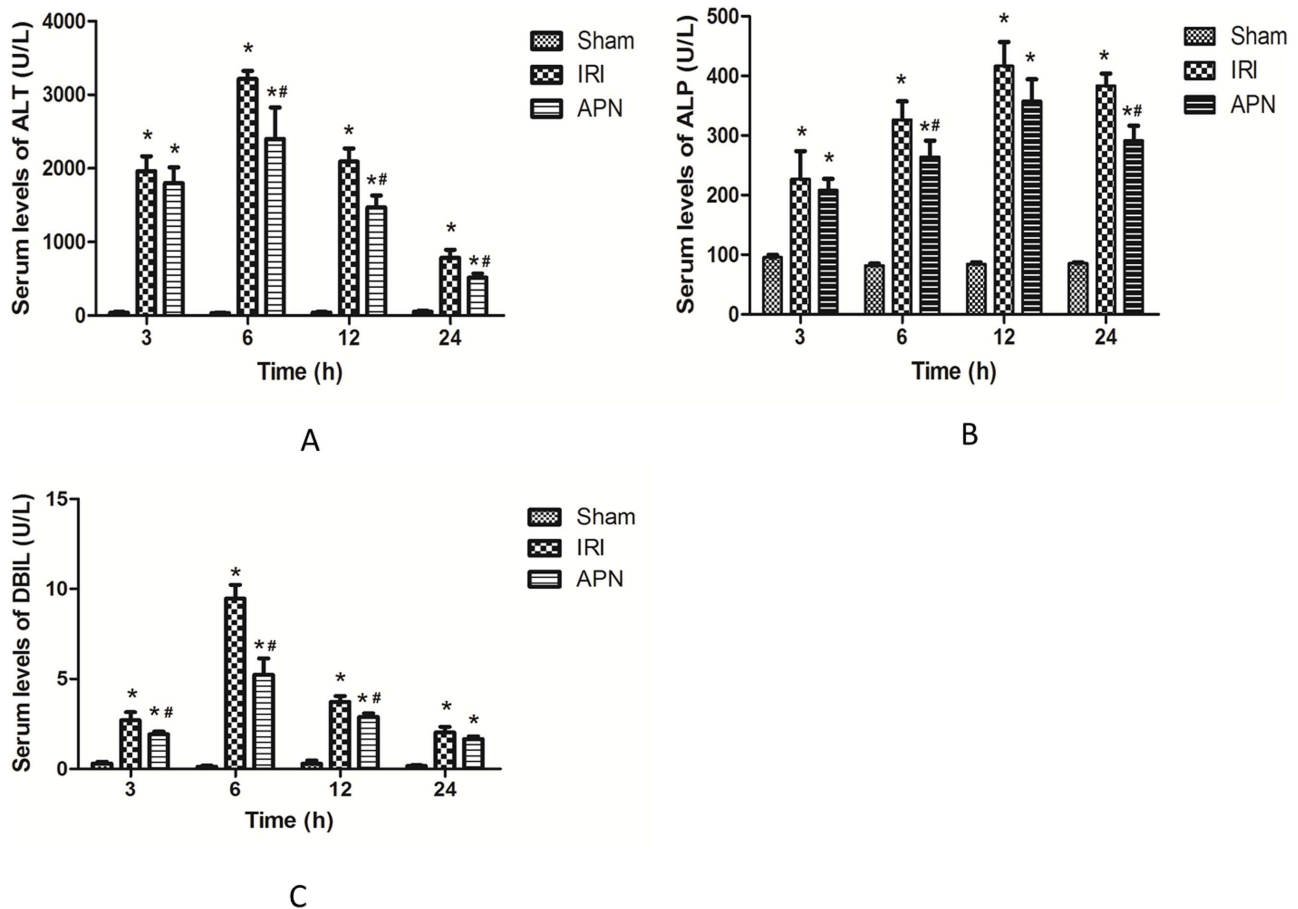
Figure 3 | Serum transaminases concentrations in different groups at different time points. Serum levels of ALT, ALP and DBIL were dramatically increased at the early stage of warm ischemia-reperfusion injury. Serum transaminases concentrations were decreased in APN groups. Sham: The sham group IRI: Ischemia-reperfusion group APN: Adiponectin group. There are three rats in each group at single time point. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, #p<0.05 vs. IRI group.

ALT, ALP and DBIL concentrations at different time points are shown in Fig. 3. Those for ALT, ALP and DBIL levels were significantly higher in the IRI group than in the sham group at all indicated time points (P<0.01); Bile duct function was evaluated with ATL, ALP and DBIL, respectively. In most case, the damage was most evident in IRI group. Pretreatment with APN significantly alleviated the damage at 3, 6, 12 and 24 h after operation (P<0.05).

TNF-α and IL-6 levels. As shown in Fig. 4, the serum level of TNF-α was markedly increased during warm ischemia-reperfusion, reaching a plateau 6 h after operation. Pretreatment with APN significantly decreased this level at 6, 12 and 24 h after operation (P<0.05). In addition, the IL-6 level was also increased in the animals induced by bile duct warm IRI. Preadministration of APN led to a significant decrease in IL-6 levels between 6 and 12 h after operation (P<0.05).

Myeloperoxidase (MPO) Activity. The myeloperoxidase activity in the ischemic tissue at 12 h after reperfusion was markedly increased by IRI, but this induction was significantly less in the adiponectin-treated group than in the IRI group. MPO levels were reduced in APN group compared with those in IRI group, suggesting that APN exerted an effect on inhibition of infiltration of neutrophils after operation. (P<0.05) (Fig. 5).

Effect of APN on the activation of NF-κB. Immunohistochemistry was used to observe NF-κB activation in bile duct tissue directly. The high expression of NF-κB p65 in bile duct tissue was observed in the IRI group. As shown in Fig. 6, the expression of NF-κB protein in IRI group was markedly elevated at 12 h after reperfusion, whereas APN remarkably inhibited the activation of NF-κB. The degradation of IκBα(a major inhibitor of NF-κB) in IRI group was significantly increased in western blot(P<0.05). APN administration was found to suppress the degradation of IκBα, further demonstrating that activation of NF-κB was inhibited (P<0.05). Furthermore, APN administration markedly inhibited the phosphorylation of NF-κB p65. It illustrated that activation of NF-κB was inhibited, and the result was the same with immunohistochemistry.

Apoptosis of Bile Duct Cell. The bile duct sections at 24 h after operation were stained by the terminal UDP nick end labeling (TUNEL) assay to evaluate cell apoptosis. The number of TUNEL-stained cells increased in the ischemia-reperfusion groups compared with sham group (P<0.05). Administration of APN prevented the increase in bile duct apoptosis after surgery (P<0.05)(Fig. 7).

Expression of Fas Protein. To elucidate the mechanisms of apoptosis, the concentration of apoptotic Fas protein was measured by immunohistochemistry at 24 h after surgery in all three groups (Fig. 8). IRI induced an increase in the number of Fas-positive cells compared with the sham group at 24 h after surgery (P<0.05). The expression of Fas in APN group was significantly lower than in IRI group after surgery (P<0.05).

Caspase-3 Activity. As shown in Fig. 9, the Caspase-3 activity in IRI and APN groups gradually increased and reached a peak at 12 hours
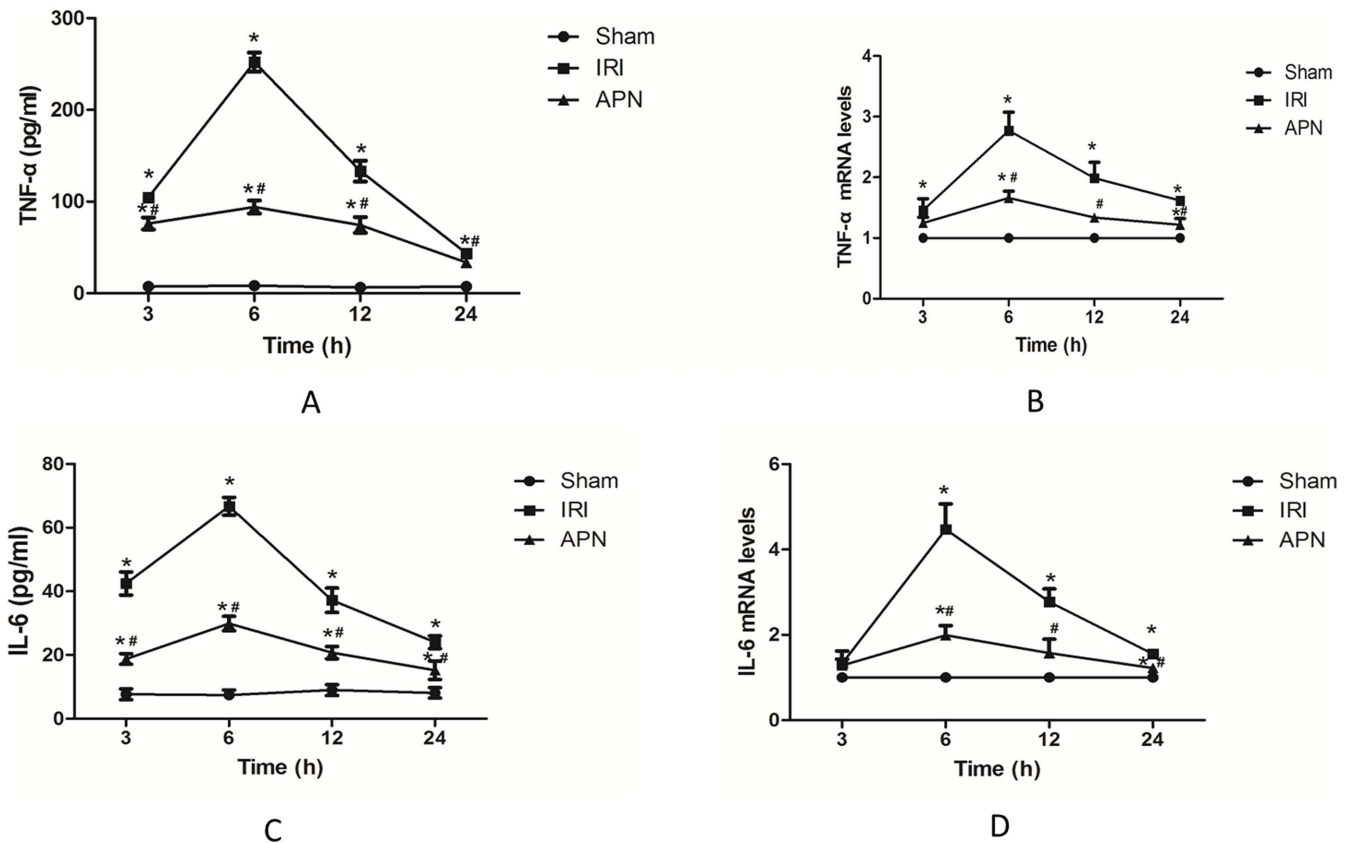
Figure 4 | Time course of changes in pro-inflammatory cytokines levels. (A) Serum TNF-alpha level. (B) Bile duct tissue TNF-alpha mRNA level. (C) Serum IL-6 level. (D) Bile duct tissue IL-6 mRNA level. Sham: The sham group. IRI: Ischemia-reperfusion group. APN: Adiponectin group. There are three rats in each group at single time point. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, #p<0.05 vs. IRI group.

after the operation. The Caspase-3 activity in APN group was lower than in IRI group at 6 h, 12 h and 24 h (P<0.05).
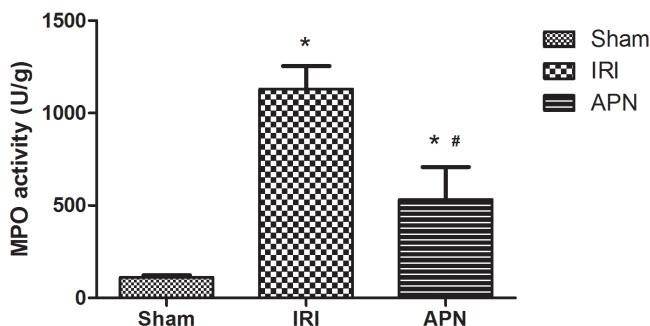
Discussion

Experimental studies have demonstrated the involvement of APN in ischemia-reperfusion injury, such as protecting kidney from acute kidney injury, reducing cerebral ischemia-reperfusion injury, and preventing fatty liver graft dysfunction after transplantation[20–22]. In our research, survival for rats treated with APN during ischemia and subsequent reperfusion in autologous liver transplantation significantly improved compared with rats that received only normal saline (Fig. 1). Therefore, alterations in the circulating APN levels may have important long-term transplantation implications.


Figure 5 | The MPO activity in the three experimental groups at 12 h after surgery. Sham: The sham group (n=4). IRI: Ischemia-reperfusion group (n=6). APN: Adiponectin group (n=6). There are three rats in each group at single time point. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, #p<0.05 vs. IRI group.

From pathomorphologic changes(Fig. 2), we found that the biliary tract injury in APN group was less serious than that in IRI group. Likewise, serum enzyme assays of clinical indicators, e.g. ALT, ALP and DBIL, were supplemented to determine bile duct cells injury after transplantation. In the present study, the levels of ALT, ALP and DBIL were significantly elevated and then slowly decreased in all of the three groups. And serum enzyme levels in APN group were constantly lower than those in IRI group(Fig. 3). Together, the analysis of serum enzymes and pathomorphologic changes revealed that the bile duct ischemia-reperfusion injury took place in the early stage, but recovery occurred in the middle and late stage after transplantation. Meanwhile, APN treatment leaded to a marked decrease in serum enzyme activity which indicated that bile duct damage was alleviated.

The mechanism involved in the protection of APN is multifactorial, including anti-inflammatory and anti-apoptosis properties. As illustrated by the decreased production of MPO and the inflammatory cytokines, e.g. TNF-α and IL-6. APN can markedly suppress the inflammation responses while it also prevents bile duct cells apoptosis. Therefore, upregulation of APN in the circulating might have important beneficial effects on biliary lesions.

Inflammatory reactions, as manifested by the infiltration of inflammatory cells play an important role in the progression of bile duct injury after IRI. As assessed both by ELISA and RT-PCR, the proinflammatory cytokines, such as TNF-α and IL-6, were obviously higher in the IRI group than in the sham group. However, treatment with APN significantly decreased those proinflammatory cytokines (Fig. 4). Something similar occurred with myeloperoxidase, which is one of the distinct indicators for the tissue infiltration of neutrophil granulocytes[23]. Our data also showed that APN could significantly ameliorate the inflammation responses as manifested by the decreased production of MPO(Fig. 5).
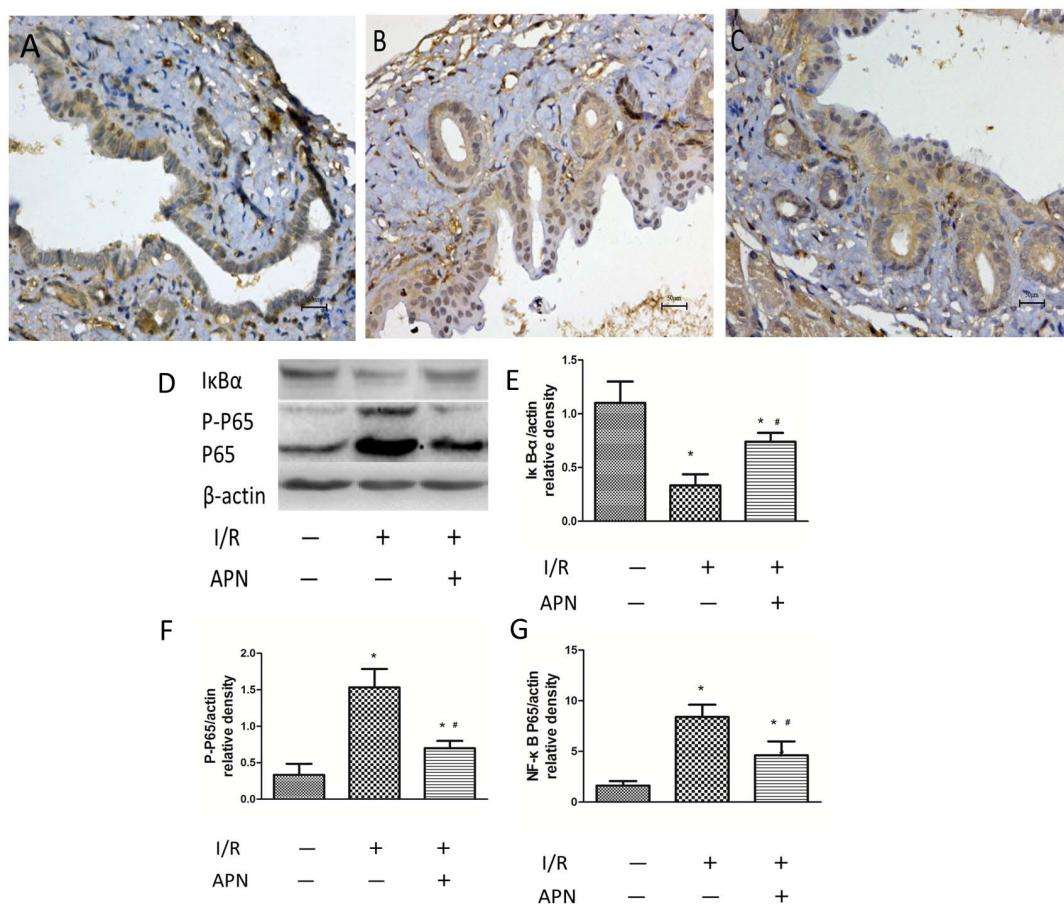
Figure 6 | Protein expression of NF-κB in bile duct tissues after surgery (magnification, ×400). (A) Sham group (n=8) (B) IRI group (n=12) (C) APN group (n=12). (D) IκBα, phospho-p65, p65 expression in western lot. (E) Expression of IκBα in bile duct tissues by Western blot analysis. (F) Expression of phospho-p65 in bile duct tissues by Western blot analysis. (G) Expression of p65 in bile duct tissues by Western blot analysis. Sham: The sham group. IRI: Ischemia-reperfusion group. APN: Adiponectin group. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, # p<0.05 vs. IRI group.

NF-κB is a key transcriptional factor regulating the transcription of inflammation associated genes. It has been reported that NF-κB (p65) signal activation is closely related to aberrant inflammatory responses in various biological processes[22,24,25]. With regard to an important role in many pathophysiologic responses, NF-κB (p65) caused inflammatory cascade after reperfusion[26]. This inflammatory cascade resulted in endothelial cells degeneration and blood coagulation in the biliary tract microcirculation[27], which induced bile duct injury. To further elucidate the anti-inflammatory mechanism of APN, we investigated the activation of NF-κB. Our results showed that IRI increased the activity of NF-κB and treatment with APN suppressed NF-κB activation, as assessed by p65 phosphorylation in Ser536 residue, by the levels of IκBα and by immunostaining for p65(Fig. 6). Furthermore, our experiment demonstrated that pre-administration of APN could enhance the degradation of IκBα proteins, translocation of the p65 subunit to the nucleus, and the binding activity of NF-κB.

Apoptosis also plays a major role in IRI of liver transplantation. The number of apoptotic bile duct cells were measured by the TUNEL assay. Our results demonstrated that the number of apoptotic bile duct cells increased substantially in the early stage of warm IRI and markedly decreased after APN treatment(Fig. 6). These findings suggested that APN exerted another important property in suppressing bile duct cell apoptosis in the early stage of warm IRI.

In order to evaluate the protective mechanism of APN against IRI-induced bile duct cell apoptosis, we also monitored the levels of Fas protein and the levels of caspase-3. It is well known that Fas-FasL

ligand is another pathway associated with apoptosis[28]. Fas/FasL interaction induce cell death through activation of the caspase signaling cascade, leading to cleavage of chromatin and loss of structural integrity[29]. So we hypothesized fas-mediated apoptosis was regarded as an important effector process in bile duct injury. In this study, Fas immune-reactivity was seen in both ischemia reperfusion groups and we observed a more significant enhancement in the IRI group than in the APN group. Morever, according to our experiment administration of APN significantly decreased the expression of Fas in rats after IRI. The caspase-3 results were conformed with the apoptosis from the TUNEL method. After the operation for 6, 12 and 24 hours, APN group had a lower Caspase-3 activity than the IRI group. In our study, pretreatment with APN might markedly decrease the caspase-3 activity after reperfusion of liver transplantation. So we conjectured that APN could inhibit bile duct apoptosis through depressing the expression of Fas protein and caspase-3 activity.

In the field of liver transplantation, NF-κB (p65) has been considered as a critical transcription factor regulating cell apoptosis, which is associated to the production of TNF-α[30,31]. It has been demonstrated that increased TNF-α levels strongly induced apoptosis and necrosis[15], through triggering leukocyte chemotaxis and activating many of the proteins involved in apoptosis, such as the proteases caspase-3 and caspase-8[32]. Activated caspase-3, along with mitochondria cytochrome-C released into the cytoplasm and cleaved cellular substrates tomorphological changes in cells and nuclei during apoptosis[33,34]. In accordance with the findings from the TUNEL assay, our data showed that TNF-α was overexpressed during the
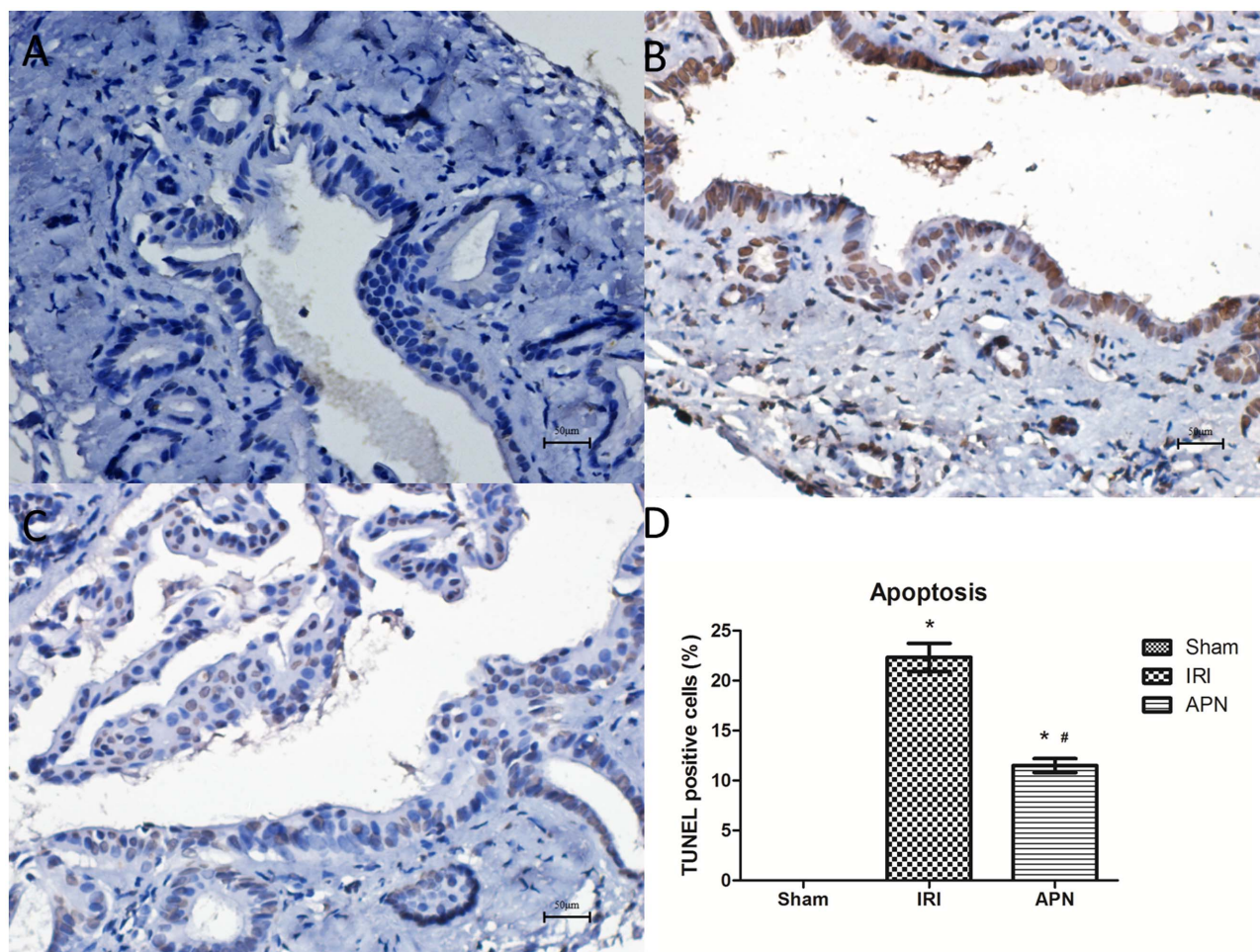
Figure 7 | Apoptosis of bile duct cells in the three experimental groups at 24 h after surgery. (magnification, ×400) (A) Sham group (n=4) (B) IRI group (n=6) (C) APN group (n=6). (D) Quantitative analysis of TUNEL-positive hepatocyte nuclei per total nuclei in each experimental group is presented. Sham: The sham group. IRI: Ischemia-reperfusion group. APN: Adiponectin group. Values are expressed as the mean ± SD. *$p < 0.05$ vs. sham group, # $p < 0.05$ vs. IRI group.

early phase of WIRI whose expression could be significantly down-regulated by pretreatment with APN. These data suggested that APN inhibited the activity of NF-κB (p65), consequently inhibiting the transcription of TNF-α[15,35].

In summary, our experiment demonstrated that APN exerted potential biliary protective functions against IRI, which were considerably attributed to the anti-inflammatory and anti-apoptosis properties of APN and that NF-κB (p65) acted as a critical transcriptional factor in this event. Our results further confirmed that APN protected the rat bile duct against early warm IRI during liver transplantation.

As our understanding of the mechanisms for ischemic cholangiopathy as well as for the APN functions is rapidly increasing, we expect that effective therapy measures might be available for ischemic cholangiopathy in the near future. In addition, APN and its related receptors may became potential molecular targets for ischemic cholangiopathy therapy.

Methods

Ethics statement. Every possible effort was made to minimize animal suffering. All animals were housed and handled according to the guidelines of China Council on Animal Care and Use(GB14925-2010) and all animal procedures carried out in this study were reviewed, approved, and supervised by the Institutional Animal Care and Use Committee of the Ethics Committee of Chongqing Medical University, China. (Permit Number: 195).

Animals and Materials. One hundred and twenty-eight male Sprague-Dawley rats (200–250 g) were obtained from Chongqing Medical University Animal Center,

Chongqing, China. All animals received humane care in compliance with institutional animal care policies with ad-libitum food and water in a temperature controlled room on a 12 h light/12 h dark cycle.

Recombinant rat globular adiponectin expressed in Escherichia coli was purchased from Alexis Biochemicals (San Diego, CA, USA). Serum TNF-α and IL-6 were measured by ELISA kits supplied by R&D systems (Minneapolis, Minnesota, USA). MPO and Caspase-3 activity was measured by using MPO and Caspase-3 Activity Assay kit (Beyotime, Haimen, China). Anti-NF-κB (p65) and anti-Fas antibody was purchased by Cell Signaling Technology (Beverly, Massachusetts, USA). Detection of apoptotic cells was based on the terminal UDP nick end labeling (TUNEL) assay using the In Situ Death Detection Kit (Roche Molecular Biochemicals, Germany).

Experimental Groups. The rats were randomly divided into three experimental groups:(1) a sham group (n=24), which underwent laparotomy without autologous liver transplantation; (2) a APN group (n=32),which was pretreated with APN (100 μg/kg in 10 mL saline per animal) before orthotopic autologous liver transplantation; and (3) a IRI group (n=32), which was administered an equivalent volume of 0.9% normal saline before orthotopic autologous liver transplantation. APN was injected intravenously into the tail before surgery. The rats were kept in a controlled environment at a temperature of 22°C under 12 h/12 h light/dark cycles. Before the experiments, the rats were fasted overnight with free access to water. We observed eight animals in each group for 14 days after surgery to assess survival. Plasma and bile duct samples were obtained at 3, 6, 12 and 24 h after surgery.

Surgical Procedures. The orthotopic autologous liver transplantation model was induced according to methods previously described[36]. Under sodium pentobarbital anesthesia, the abdomen of rats was opened through an inverted T-incision, and the left diaphragm vein, hepatoesophageal ligament vein and right adrenal vein were separated, ligated and severed. The suprahepatic and infrahepatic inferior vena cava was anatomized, and then the common bile duct, portal vein, and hepatic artery were anatomized over the margin of the duodenal bulb. The infrahepatic vena cava was dissociated downwards about 6–8 mm. 2 ml heparin 5 U/ml about 0–4°C was
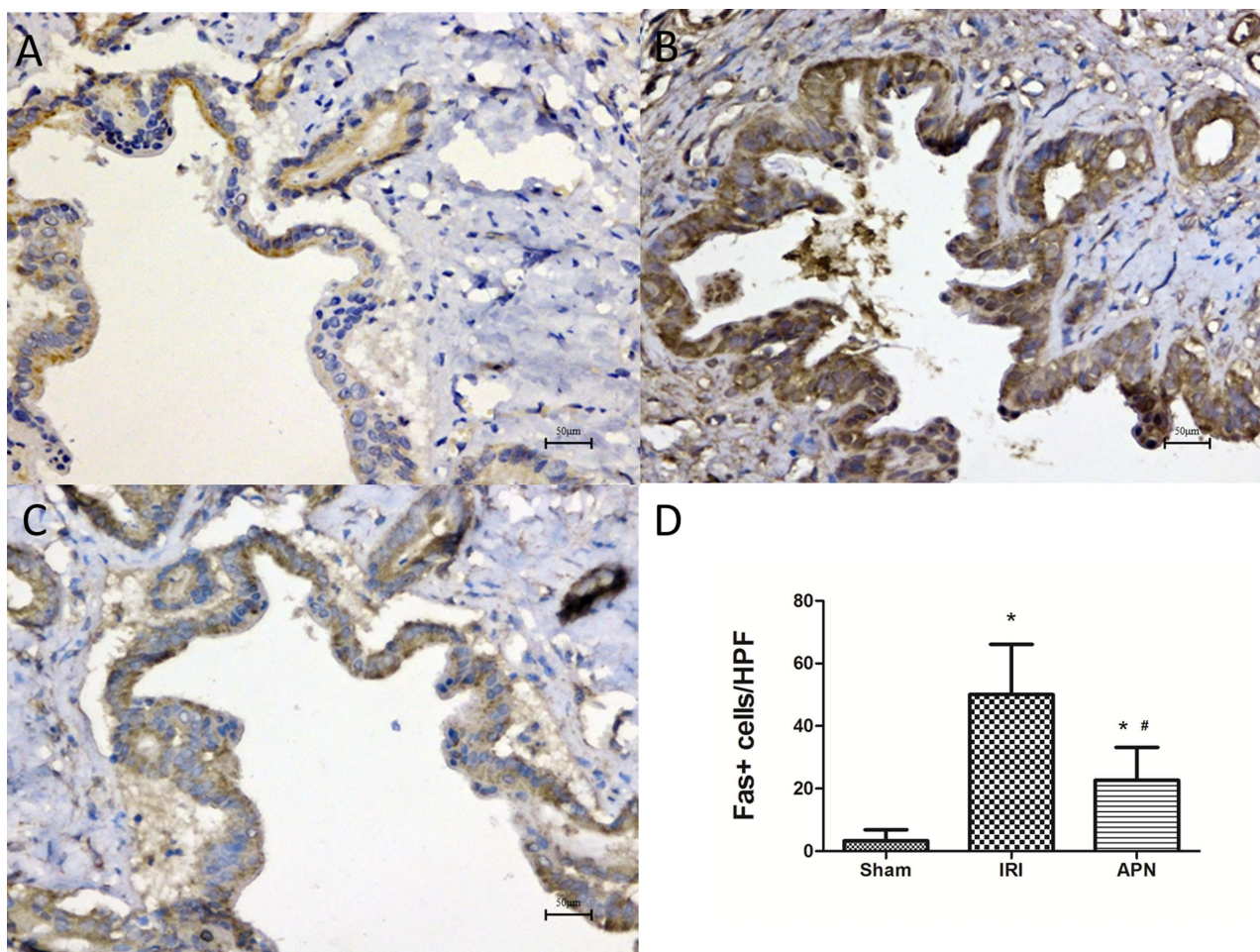
Figure 8 | Protein expression of FAS in liver tissue at 24 h after reperfusion (magnification, ×400). (A) Sham group (n=4) (B) IRI group (n=6) (C) APN group (n=6). (D) Immunostaining score as described in the methods. Sham: The sham group. IRI: Ischemia-reperfusion group. APN: Adiponectin group. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, # p<0.05 vs. IRI group.
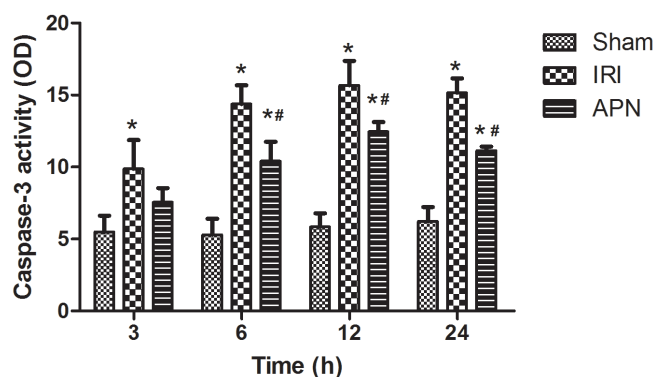
injected into portal vein to prevent blood clotting. After clipping the distal portal vein and hepatic artery with clamps, the liver was irrigated with lactated Ringer's solution of 0–4°C at the rate of 150 ml/h to perform cold perfusion with transfixion pins. The liver surface was covered with 4°C normal saline solution to lower the temperature. Subsequently, the clamps were placed on the suprahepatic and infrahepatic inferior vena cava, and an incision was placed above the clipped area in the infrahepatic inferior vena cava as an outflow tract. The cold perfusion phase was terminated when the normal liver color faded. After finishing irrigation, the portal vein, hepatic artery, and infrahepatic inferior vena cava outflow tract were then repaired. Blockages in the portal vein and inferior vena cava were relieved, followed by the blockage of the hepatic artery for 30 min to induce warm ischemia-reperfusion injury. At the same time, the surface of the liver was irrigated with 20 mL normal saline solution of 38°C for rapidly rewarming the liver. After this period of occlusion, the clamp was removed. Thus, the bile duct ischemia-reperfusion injury model was established.

Serum Analysis. Blood was collected from the abdominal aorta via the diaphragm for the measurement of alanine aminotransferase (ALT), alkaline phosphatase (ALP), direct bilirubin (DBIL), TNF-α and IL-6 levels. ALT, ALP and DBIL were determined using commercial kits, with the automatic biochemical analyzer (Olympus-AU5400, Japan) provided by the Laboratory of the Second Affiliated Hospital of Chongqing Medical University. Serum TNF-α and IL-6 were measured using ELISA according to the manufacturer's instructions.

Real-time reverse transcriptase-polymerase chain reaction. Bile duct tissues removed from each animal at different time points were immediately frozen in liquid nitrogen. Total RNA was isolated and messenger RNA (mRNA) expression was quantified, as previously described[37], by Fast SYBR Green real-time reverse-transcription polymerase chain reaction (RT-PCR) with an ABI- Prism 7700 Fast Sequence Detection System (ABI Japan, Co., Ltd., Tokyo, Japan) and with appropriate primers (all from Invitrogen, Carlsbad, CA) in triplicate. The primer sequences (Invitrogen, Shanghai, China) were as follows: TNF-α (X 66539): 5′-TCG TAG CAA ACC ACC AAG CA-3′ and 5′-CCC TTG AAG AGA ACC TGG GAG TA-3′. IL-6 (NM012589): 5′-TAT GAA CAG CGA TGA TGC ACT G-3′ and 5′ -TTG CTC TGA ATG ACT CTG GCT T-3′. β–actin (V 01217): 5′-TCC TCC TGA GCG CAA GTA CTC T-3′ and 5′-GCT CAG TAA CAG TCC GCC TAG AA-3′. β-actin was used as an endogenous control.

Figure 9 | The Caspase-3 activity in the three groups after surgery. Sham: The sham group (n=16). IRI: Ischemia-reperfusion group (n=24). APN: Adiponectin group (n=24). There are three rats in each group at single time point. Values are expressed as the mean ± SD. *p<0.05 vs. sham group, #p<0.05 vs. IRI group.

MPO Activity Assay. 12 h after surgery, rats in each group were sacrificed and the protein of bile duct was obtained from the injured bile duct tissues. The content of MPO activity was measured using colorimetric assay kit for MPO chlorination activity according to the manufacturer's instruction. MPO activity in the supernatant

was determined by measuring the changes in absorbance (460 nm). Results are expressed as units per g tissue.

Caspase-3 Activity Assay. 3,6,12 and 24 h after surgery, bile duct samples were collected and rinsed with PBS, lysed in lysis buffer for 5 min on ice, then centrifuged at 20,000 × g for 10 min at 4°C. Caspase-3 activity in the supernatant was assayed according to the manufacturer's instructions. All experiments were carried out in triplicate. Results are expressed as optical density.

Pathological Examination. The tissues of bile duct were fixed with 10% formalin and then embedded in paraffin. Specimens were collected at 24 h after reperfusion in each group. Edema of bile duct cells, infiltration of inflammatory cells were observed with high power field microscope. The sections of bile duct were assessed for pyknosis, cell necrosis and cell shedding. Histological indicators were analyzed by these semi-quantitative assessment by calculating a bile duct injury severity score (BDISS)[38]:bile duct damage (graded as 0, absent; 1, mild; 2, moderate; and 3, severe); infiltration of inflammatory cells (graded 0–3, using a similar scale as stated earlier); and cholestasis (graded 0–3, using a similar scale as stated earlier).

Immunohistochemistry. We performed immunohistochemical analysis of anti-NF-κB(p65) and anti-Fas staining. Monoclonal rabbit antibody was at a dilution of 1 : 100 at 4°C. A PolinI-2 Plus HRP rabbit/mouse kit (Zhongshan Goldenbridge Biotech Co., Ltd, China) was used according to the manufacturer's instructions. Immunoperoxidase staining was performed with the diaminobenzidine substrate kit (Zhongshan Golden brideg Biotech Co., Ltd). Non-specific rabbit IgG were used as negative control slides while still performed all the other steps. The selection criteria was based on brown and blue colors. Blue cells that were also brown were scored as positive, and cells that were blue only were scored as negative. The number of positive cells was counted in 5 random microscopic fields of each tissue section. The percentage of positive cells over total bile duct cells per high-power field indicated the expression levels of NF-κB(p65) and Fas protein at a magnification of ×400.

TUNEL Staining. Apoptosis of biliary tract epithelia was identified by TUNEL staining according to the manufacturer's instructions. Following TUNEL staining, TUNEL-positive cholangiocytes displayed a characteristic morphology of apoptosis, such as chromatin condensation, cell fragmentation and apoptotic bodies. Apoptotic cells were examined at original magnification ×400 in 5 randomly blindly chosen fields per section. The apoptotic index (AI) was calculated as percentage of apoptotic cells related to the total number of cholangiocytes.

Western blot analysis. Western blot was carried out as described previously[22]. The antibodies used here were anti-NF-κB(p65), anti-p-p65, anti-IκBα and anti-βactin antibodies. (Cell Signaling, MA, USA).

Statistical Analysis. All data are presented as mean ± standard deviation. The statistical analysis was performed by one-way analysis of variance (ANOVA). Values were analyzed using the statistical package SPSS for Windows version 18.0 (SPSS Inc, Chicago, IL, USA). P values<0.05 were considered statistically significant.

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Author contributions

Y.X. supervised the project and wrote the manuscript. Y.X. designed experiments and performed experiments. Y.X analyzed the data and prepared all figure. J.G. gave constructive comments on the results and provided the research fundings. All authors reviewed the manuscript.

Additional information