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ORIGINAL ARTICLE

Biliary antibiotics irrigation for *E. coli*-induced chronic proliferative cholangitis and hepatolithiasis: A pathophysiological study in rabbits

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KEYWORDS
Hepatolithiasis;
Chronic proliferative cholangitis;
Local antibiotic irrigation

Summary
Background: The gram-negative bacteria secreted endotoxin, Lipopolysaccharide (LPS), plays important roles in the formation and recurrence of hepatolithiasis and chronic biliary inflammation in patients of Southeast Asia. We aimed to elucidate the anti-inflammatory effect and mechanism of local antibiotics irrigation on chronic proliferative cholangitis (CPC) and hepatolithiasis.
Methods: Escherichia coli was injected into rabbit bile ducts to induce CPC. Rabbits were divided into sham operation (SO), povidone-iodine, Metronidazole plus chlorhexidine, ofloxacin, furacillin, Neosporin® G.U., and CPC groups. Local irrigation was performed for 28 days after CPC was established. Residual *E. coli* and LPS, and the expression of MCP-1, CD14, COX-2, VEGF, IL-6, NF-κB, TNF-α, Fas, TGF-β1, α-SMA, Collagen-I, β-glucuronidase, PKC, C-myc, and Mucin 5AC were assessed in bile duct tissues.
Results: The residual *E. coli* and LPS, and expression of MCP-1, CD14, COX-2, IL-6, NF-κB, TNF-α, Fas, TGF-β1, α-SMA, β-glucuronidase, PKC, C-myc, and Mucin 5AC in the SO, povidone-iodine, Metronidazole plus chlorhexidine, ofloxacin, and Neosporin® G.U. groups were significantly lower than those in the furacillin and CPC groups ($P < 0.05$). VEGF and Collagen-I levels in the SO, povidone-iodine, metronidazole plus chlorhexidine, and ofloxacin groups were significantly lower than those in the furacillin, Neosporin® G.U., and CPC groups ($P < 0.05$).

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Conclusions: LPS affects the pathophysiology of *E. coli* caused chronic proliferative cholangitis and hepatolithiasis recurrence. Local antibiotics irrigation could prevent chronic proliferative cholangitis and stones formation by decreasing LPS-induced proinflammatory and profibrotic cytokines release. Povidone iodine, metronidazole plus chlorhexidine, and ofloxacin were more effective than Neosporin® G.U. and furacillin.

Introduction

Hepatolithiasis, or primary intrahepatic stones, is a common benign biliary disease in Southeast Asia [1]. However, it is regarded as an intractable disease because of its characteristics of easy recurrence [2]. Recently, the role played by chronic proliferative cholangitis (CPC) in the recurrence of hepatolithiasis has been recognized [3]. The residual CPC would lead to the recurrence of stones even after the stones are removed. Therefore, follow-up treatment of diseased bile ducts should be adopted to prevent postoperative recurrence of stones and biliary restenosis.

Gram-negative bacteria are the most common bacteria isolated from the bile of patients with hepatolithiasis in Southeast Asia [4]. By interacting with Toll-like receptor 4 (TLR4), the bacteria could promote CPC, stone formation and recurrence through lipopolysaccharide (LPS) induced release of a variety of pro-inflammatory and pro-fibrotic factors, including Cyclooxygenase-2 (COX-2), interleukin (IL)-6, vascular endothelial growth factor (VEGF), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta 1 (TGF-β1), β-glucuronidase (β-G), and mucin 5AC (MUC5AC) [5—8].

Tabibian et al. [9] found that oral use of antibiotics had a certain inhibitory effect on biliary stenosis caused by chronic biliary inflammation. However, the mechanism remains unclear. CPC is a chronic inflammatory disease caused by gram-negative bacteria, but the effectiveness of traditional intravenous antibiotics is poor. Irrigation treatments have been used to treat severe urinary tract infections [10—12]. In view of the above factors, we hypothesized that local irrigation using strong, tissue-penetrating antibiotics might achieve a better outcome in CPC treatment. Therefore, the present study aimed to investigate the efficacy and potential mechanisms of local antibiotics irrigation in a rabbit model of CPC.

Materials and Methods

Study design and surgical procedure

All animal experiments were approved by the Animal Care and Use Committee of Sichuan University. A total of 66 New Zealand White Rabbits weighing 2.2—2.5 kg were randomly divided into seven groups. (a) CPC group ($n = 10$): in which a 5-0 nylon thread was inserted into the common bile duct (CBD) through the duodenal papilla (Fig. 1A) [13,14]. To create a biliary stricture, a transverse incision on the anterior wall of the CBD was made with a length of one-third perimeter of the CBD. The incision was anastomosed using non-invasive sutures (Fig. 1B). Then, a total of 1×10^5 plaque-forming units (PFU) of *Escherichia coli* were infused into bile duct. Irrigation was not performed; (b) Povidone-iodine group (PI group, $n = 10$): On the basis of the above CPC model, an epidural catheter was left in the bile duct through the cystic duct (Fig. 1C). Daily local irrigation using 20 ml 5% povidone-iodine (Yong'an Pharmaceutical Co., Ltd., Chengdu, China) through the epidural catheter was performed after the CPC model was established 7 days (Fig. 1D); (c) Metronidazole plus chlorhexidine group (MC group, $n = 10$): 20 ml of 0.02% metronidazole plus 0.12% chlorhexidine (Guojing Pharmaceutical Co., Ltd., Hangzhou, China) was used daily as a local irrigation reagent for the CPC model (Fig. 1D); (d) Ofloxacin group (OF group, $n = 10$): 20 ml of 0.5% ofloxacin (Xinyijinzhu Pharmaceutical Co., Ltd., Shanghai, China) was used daily as a local irrigation reagent for the CPC model (Fig. 1D); (e) Furacillin group (FU group, $n = 10$): 20 ml of 0.02% furacillin (Meilun Biotechnology Co., LTD., Dalian, China) was used daily as a local irrigation reagent for the CPC model (Fig. 1D); (f) Neosporin® G.U. group (NE group, $n = 10$): 20 ml of Neosporin® G.U. (Pharmacia & Upjohn Co., Sweden) was used daily used as a local irrigation reagent for the CPC model (Fig. 1D); (g) Sham operation group (SO group; $n = 6$), in which the common bile duct was dissected only. The efficiency of each group was detected after 28 d of irrigation. All the rabbits were sacrificed and their common bile ducts were collected (Fig. 1D) and fixed in liquid nitrogen and/or 10% formaldehyde for further tests.

Immunohistochemistry for *Escherichia coli*, and α-SMA, and mucin 5AC expression

The avidin-biotin-peroxidase complex method was used to detect *Escherichia coli*, and the expression of α-SMA and mucin 5AC. Briefly, tissue sections were incubated overnight at 4 °C with primary antibodies (ABcam Co., Cambridge, MA, USA), followed by incubation with biotinylated secondary antibody for 1 h at 37 °C. The *Escherichia coli* and the expression of α-SMA, and mucin 5AC were observed under a microscope simultaneously.

TUNEL analysis

The retina tissues isolated from rabbit bile ducts were fixed with 4% paraformaldehyde (pH 7.4) for 15 min at 4 °C, embedded with paraffin wax and cut into 3—5 μm sections. Tissue sections were digested with proteinase K for 30 min at room temperature. After being washed with phosphate-buffered saline (PBS) three times, slides

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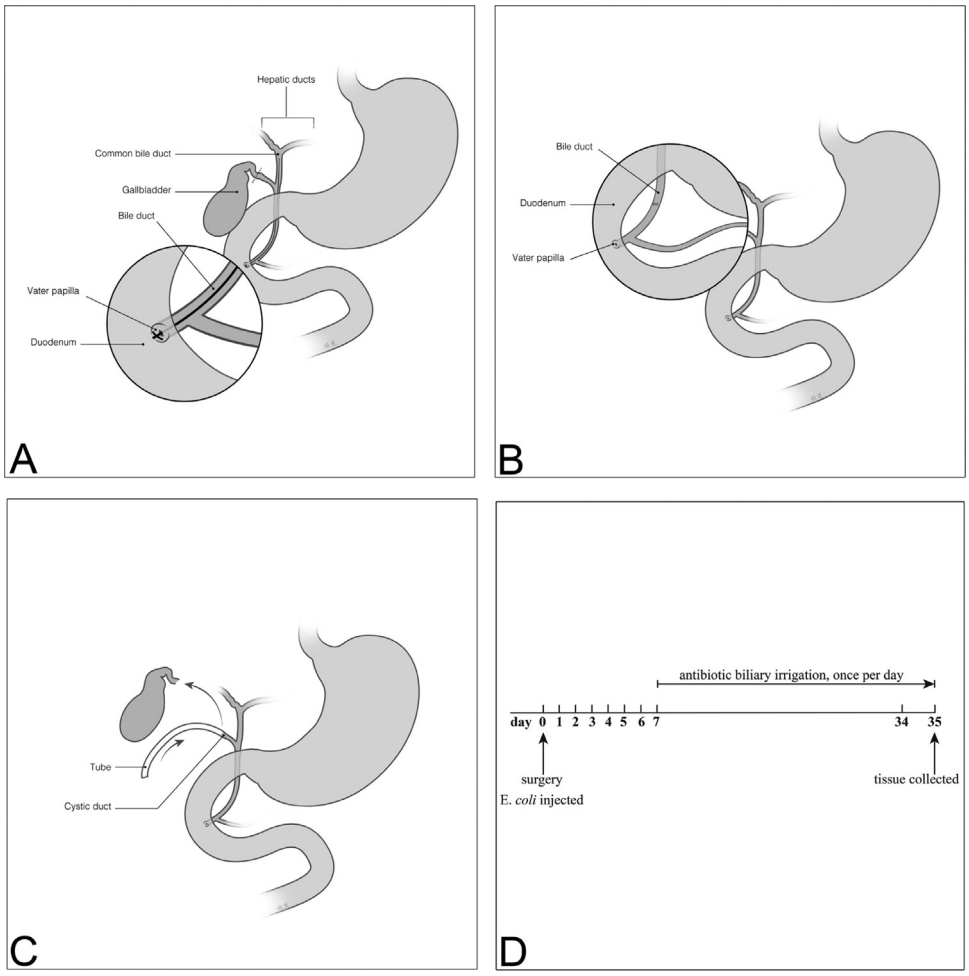

Figure 1 Schematic diagram of establishing the chronic proliferative cholangitis (CPC) rabbit model and the time-line of the experiment. A. A 5—0 nylon thread was inserted into the bile duct across the papilla of Vater to induce retrograde infection. B. A transverse incision on the anterior wall of the common bile duct was made to induce biliary stricture. C. The irrigation tube was inserted into the common bile duct through the stump of the cystic duct after cholecystectomy. D. The surgical procedure was performed on the first day. Biliary irrigation with 10 ml of solution was performed (except for the sham-operation group) regularly from the 7th to the 28th day. All rabbits were euthanized and the common bile ducts were collected for further analysis.

were incubated with 50 μL of terminal deoxynulceotidyl transferase nick-end-labeling (TUNEL) detection solution (2 μL of TdT enzyme and digoxigenin-dUTP reaction buffer, Roche Co., Basel, Switzerland) for 60 min at 37 °C. After being washed with PBS, the sections were incubated with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (1 mg/mL) for nuclear staining at room temperature for 10 min. TUNEL positive cells were stained green, and normal cells were blue. In the experiments, five non-overlapping fields were captured under a fluorescence inverted microscope (Leica, German).

Enzyme-linked immunosorbent assay (ELISA) of LPS, IL-6, and β-glucuronidase

Concentrations of LPS, IL-6, and β-glucuronidase in the bile duct tissues were measured using a commercial ELISA kit following the manufacturer's instructions (CUSABIO, Wuhan, PR China). Each sample was determined in duplicate, and the average was used for data analysis.

Western Blotting analysis of MCP-1, CD14, COX-2, VEGF, TNF-α, TGF-β1, Collagen-I, and C-myc

Proteins extracted from bile duct tissues were separated by SDS-PAGE, and then transferred to a polyvinylidene fluoride membrane. Specific proteins were detected using the following antibodies: anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000, Bioss Antibodies, Beijing, China), anti-MCP-1 (1:1000, Bioss Antibodies), anti-CD14 (1:1000, Bioss Antibodies), anti-COX-2 (1:1000, ABcam), anti-VEGF (1:1000, Bioss Antibodies), anti-TNF-α (1:1000, Bioss Antibodies), anti-TGF-β1 (1:1000, ABcam), anti-Collagen-I (1:1000, ABcam), and anti-C-myc (1:1000, Bioss Antibodies). Tris-buffered saline with Tween20 was used as the blocking and washing solution. Horseradish peroxidase-conjugated secondary antibodies were incubated with the membrane, and antibody complexes were detected using an

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electrochemiluminescence kit (4A Biotech Co., Ltd, Beijing, China)

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) analysis of NF-κB, Fas, PKC, and MUC5AC

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Bremen, Germany). CDNAs were synthesized from the mRNAs using a RevertAid First Strand cDNA synthesis Kit (ThermoFisher Scientific, Bremen, Germany). QRT-PCR was performed in a Bio-Rad CFX-96 System (Bio-Rad, Foster City, CA, USA) using SsoFast™ EvaGreen® Supermix (Bio-Rad). The specific forward (F) and reverse (R) primers used were: NF-κB, F 5′-TGGTGTTACAGCTTTGTTGTGT-3′ and R 5′-GGAATGGGCC ATCTGCTGTT-3′; Fas, F 5′-CCTCCCGGCACGAAGAAAAA-3′ and R 5′-TGCACTTGGTATTCT GGATCGT-3′; PKC, F 5′-CCATGGTGGAGAAGCGGGTT-3′ and R 5′-TGCATAGAATACTGCTTGTGGCT-3′; MUC5AC, F 5′-GGGGGTATCCGAGCAAACAT-3′ and R 5′-TCTTTGGCA TCGTCGGAGAC-3′; and GAPDH F 5′-CCCGAGACACGATGG TGAA-3′ and R 5′-ATGTAGACCATGTAGTGGAGGTC-3′. Each sample was analyzed in three replicate wells. Relative mRNA expression levels were normalized to the levels of the endogenous reference gene, *GAPDH*.

Statistical analysis

All data are presented as the mean ± SD and were analyzed using the SPSS 24.0 software. Statistical analysis was conducted using one-way analysis of variance (ANOVA) to evaluate the variance among the seven groups. $P < 0.05$ was considered statistically significant.

Results

Local antibiotics irrigation could decrease the counts of residual *E. coli* and the local recruitment of $CD14^+$ macrophages.

Immunohistochemistry was used to evaluate qualitatively the antibacterial effect of local antibiotics irrigation on CPC. Fewer residual *E. coli* cells were observed in bile ducts tissues of the SO, PI, MC, OF, and NE groups compared with those in the FU, and CPC groups. However, the counts of residual *E. coli* in the PI, MC, OF, and NE groups were still more than those in the SO group under microscopy (Fig. 2A).

The blood cell counting method was used to further quantitatively assess the antibiotic effect of each irrigation reagent on residual *E. coli* after culture. The amount of residual *E. coli* in the SO, PI, MC, OF, and NE group was significantly less than that in the CPC group ($P < 0.001$). The amount of residual *E. coli* in the FU group was lower than that in the CPC group but without significant difference ($P > 0.05$). The amount of *E. coli* in the FU group was significantly higher than that in the SO, PI, MC, OF, and NE groups ($P < 0.01$). The numbers of residual *E. coli* in the PI, MC, OF, and NE groups were a little higher than those in the SO group ($P > 0.05$) (Fig. 2B).

E. coli can synthesize LPS, which plays an important role in inducing cholangiocytes hyperplasia, fibrosis of the bile duct, and the formation of hepatolithiasis [15—19]. Therefore, we further detected the concentration of LPS in bile duct tissue of each group using ELISA. The amount of residual LPS in the SO, PI, MC, OF, and NE groups was significantly lower than that in the CPC group ($P < 0.001$), but not in the FU group. In addition, the residual LPS in the PI, MC, OF, and NE group was slightly higher than that in the SO group ($P > 0.05$). The difference in the amount of LPS among PI, MC, OF, and NE group was not significant ($P > 0.05$) (Fig. 2C).

Changes in the concentration of LPS would impact chemokine production, such as the MCP-1 released by biliary epithelial cells and the subsequent localization of $CD14^+$ macrophages; therefore, we further examined the effect of local antibiotic irrigation on local recruitment of $CD14^+$ macrophages using western blotting. The levels of MCP-1 in the SO, PI, MC, OF, FU, and NE group were significantly lower than those in the CPC group ($P < 0.001$). Meanwhile, the level of MCP-1 in the SO, PI, MC, OF, and NE group was significantly lower than that in the FU group ($P < 0.01$). The level of MCP-1 in the SO group was also significantly lower than that in the PI, MC, OF, and NE groups ($P < 0.001$). However, there was no significant difference in MCP-1 levels among the PI, MC, OF, and NE groups ($P > 0.05$) (Fig. 2D). The level of CD14 in the SO, PI, MC, OF, and NE group was significantly lower than that in the FU and CPC groups ($P < 0.01$). There was no significant difference in CD14 levels between the FU and CPC groups. In addition, the CD14 levels in the PI, MC, OF, and NE groups were a little higher than that in the SO group ($P > 0.05$) (Fig. 2D).

Local antibiotics irrigation could decrease the formation of biliary stones

Next, we observed the formation of biliary stones in each group. No obvious stone formation was observed in the SO, PI, MC, OF, and NE groups. However, a number of stones formed in the FU and CPC groups (Fig. 3A).

β-glucuronidase can hydrolyze conjugated bilirubin to non-conjugated bilirubin, and the latter can combine with calcium ions to produce calcium bilirubinate stones [7]. ELISA was used to detect the concentration of β-glucuronidase in bile duct tissues in each group. The concentration of β-glucuronidase in the SO, PI, MC, OF, and NE groups was significantly lower than that in the FU and CPC groups ($P < 0.001$). Additionally, the concentration of β-glucuronidase in the PI, MC, OF, and NE groups was significantly higher than that in the SO group ($P < 0.01$). However, no significant difference was observed for the secretion of β-glucuronidase among the PI, MC, OF, and NE groups ($P > 0.05$) (Fig. 3B).

C-myc could regulate the proliferation of cholangiocytes and the expression of endogenous β-glucuronidase; [7,20] therefore, the expression of C-myc was detected by western blotting. The C-myc levels in the SO, PI, MC, OF, and NE groups were significantly lower than those in the FU and CPC groups ($P < 0.001$). Furthermore, the levels of C-myc in the PI, MC, OF, and NE groups were significantly higher than that

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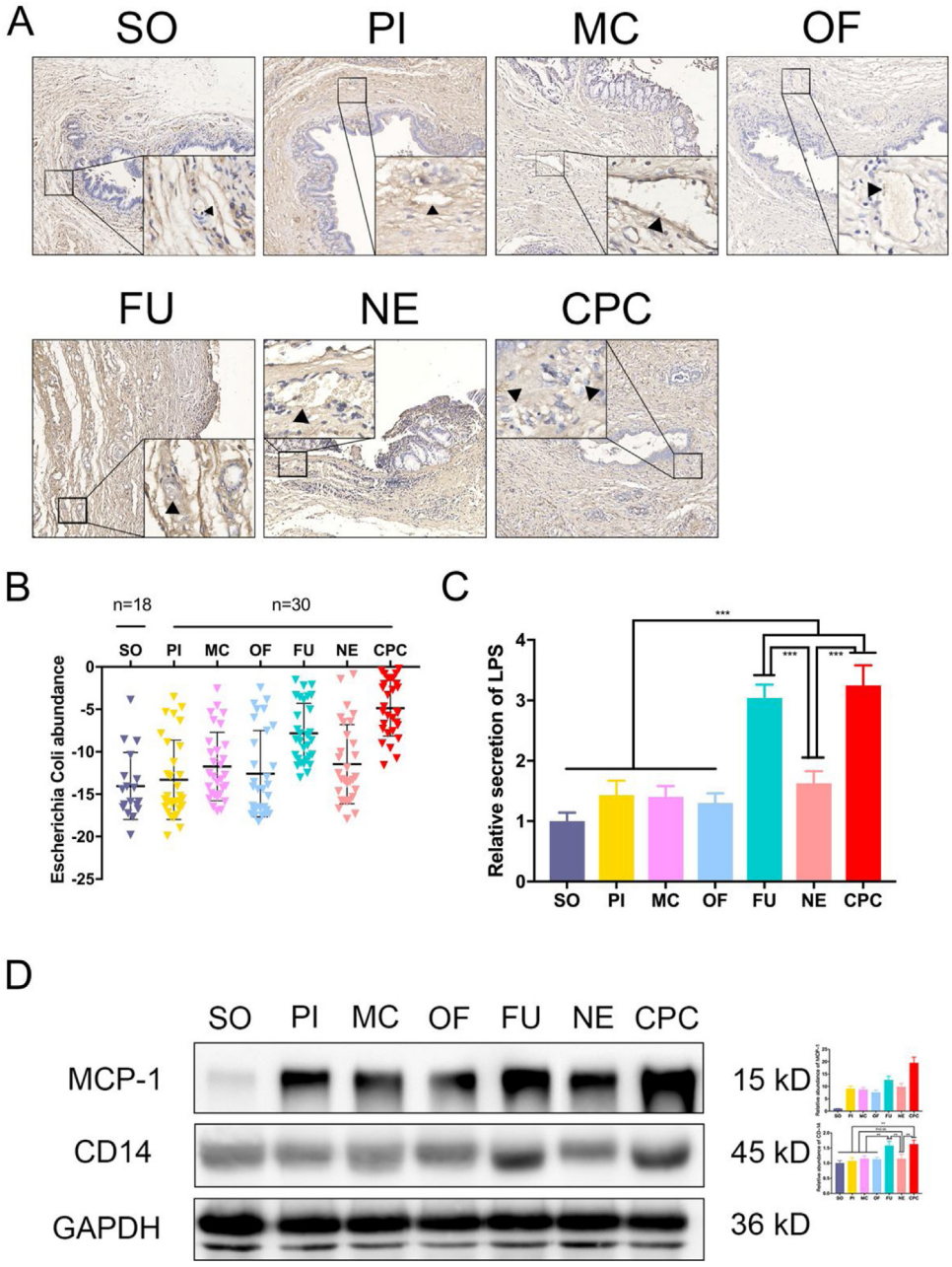

Figure 2 Local antibiotic irrigation could decrease the counts of residual *Escherichia coli* and the local recruitment of CD14+ macrophages. A. Immunohistochemistry of residual *E. coli* in the sham-operation (SO) ($n = 6$), povidone-iodine (PI), metronidazole plus chlorhexidine (MC), ofloxacin (OF), furacillin (FU), Neosporin® G.U. (NE), and chronic proliferative cholangitis (CPC) groups ($n = 10$). B. Residual *E. coli* culture of the SO ($n = 18$), PI, MC, OF, FU, NE, and CPC groups ($n = 30$). C. Enzyme-linked immunosorbent assay (ELISA) of lipopolysaccharide (LPS) in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$). D. Western blot of monocyte chemoattractant protein-1 (MCP-1) and CD14 in the SO, PI, MC, OF, FU, NE, and CPC groups.

in the SO group ($P < 0.01$). However, a significant difference was not observed for the expression of C-myc among the PI, MC, OF, and NE groups ($P > 0.05$) (Fig. 3C).

QRT-PCR was used to evaluate the expression of *PKC*, which plays important role in regulating cholangiocytes proliferation, C-myc expression, and endogenous β-glucuronidase secretion [7]. The expression of *PKC* in the PI, MC, OF, and NE groups was significantly lower than that in the FU and CPC groups ($P < 0.001$). In addition, the expression of *PKC* in the PI, MC, OF, and NE groups was still significantly higher than that in the SO group ($P < 0.001$). No significant difference was observed for the expression of *PKC* among the PI, MC, OF, and NE groups ($P > 0.05$) (Fig. 3D).

The expression of MUC5AC was also tested because it is a component of biliary stones [21]. Immunohistochemistry of MUC5AC showed that there was no obvious MUC5AC expression in the submucosal and submucosal glands of the bile duct in the SO group. A certain level of MUC5AC expression was observed in the submucosa of the bile duct in the PI, MC, OF, and NE groups. However, a large amount of MUC5AC

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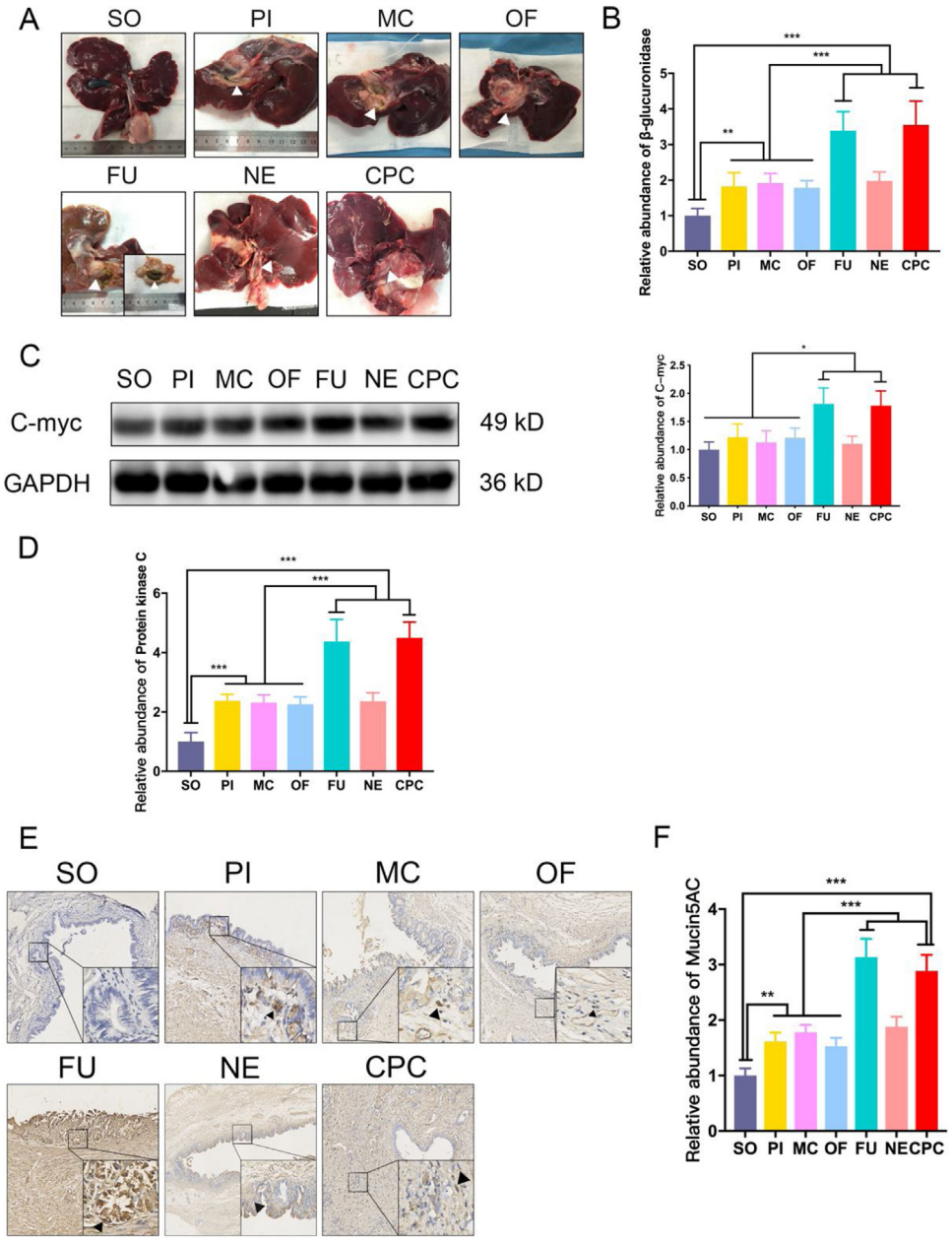

Figure 3 Local antibiotics irrigation could decrease the formation of biliary stones. A. Gross observation of the formation of biliary stones in the sham-operation (SO), povidone-iodine (PI), metronidazole plus chlorhexidine (MC), ofloxacin (OF), furacillin (FU), Neosporin® G.U. (NE), and chronic proliferative cholangitis (CPC) groups. B. Enzyme-linked immunosorbent assay (ELISA) of β-glucuronidase in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$). C. Western blot of C-myc in the SO, PI, MC, OF, FU, NE, and CPC group. D Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) of protein kinase C in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$). E. Immunohistochemistry of Mucin5AC in the SO, PI, MC, OF, FU, NE, and CPC groups. F. qRT-PCR of Mucin5AC in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC group ($n = 10$).

expression was observed in the mucosa and submucosa of the bile duct in the FU and CPC groups (Fig. 3E). QRT-PCR was used to further quantify the expression of *MUC5AC* in the bile duct tissue in each group. Similar to the expression of PKC and C-myc, the expression of *MUC5AC* in the PI, MC, OF, and NE groups were significantly lower than that in the FU and CPC groups ($P < 0.001$). At the same time, the expression of *MUC5AC* in the PI, MC, OF, and NE groups was still significantly higher than that in the SO group ($P < 0.001$). No significant difference was observed for the expression of *MUC5AC* among the PI, MC, OF, and NE groups ($P > 0.05$) (Fig. 3F).

Local antibiotics irrigation could inhibit the proliferation of cholangiocytes in CPC

Hematoxylin and eosin (HE) staining was used to qualitatively evaluate the proliferation of cholangiocytes in each group. The results suggested increased proliferation of cholangiocytes and the submucosa in the PI,

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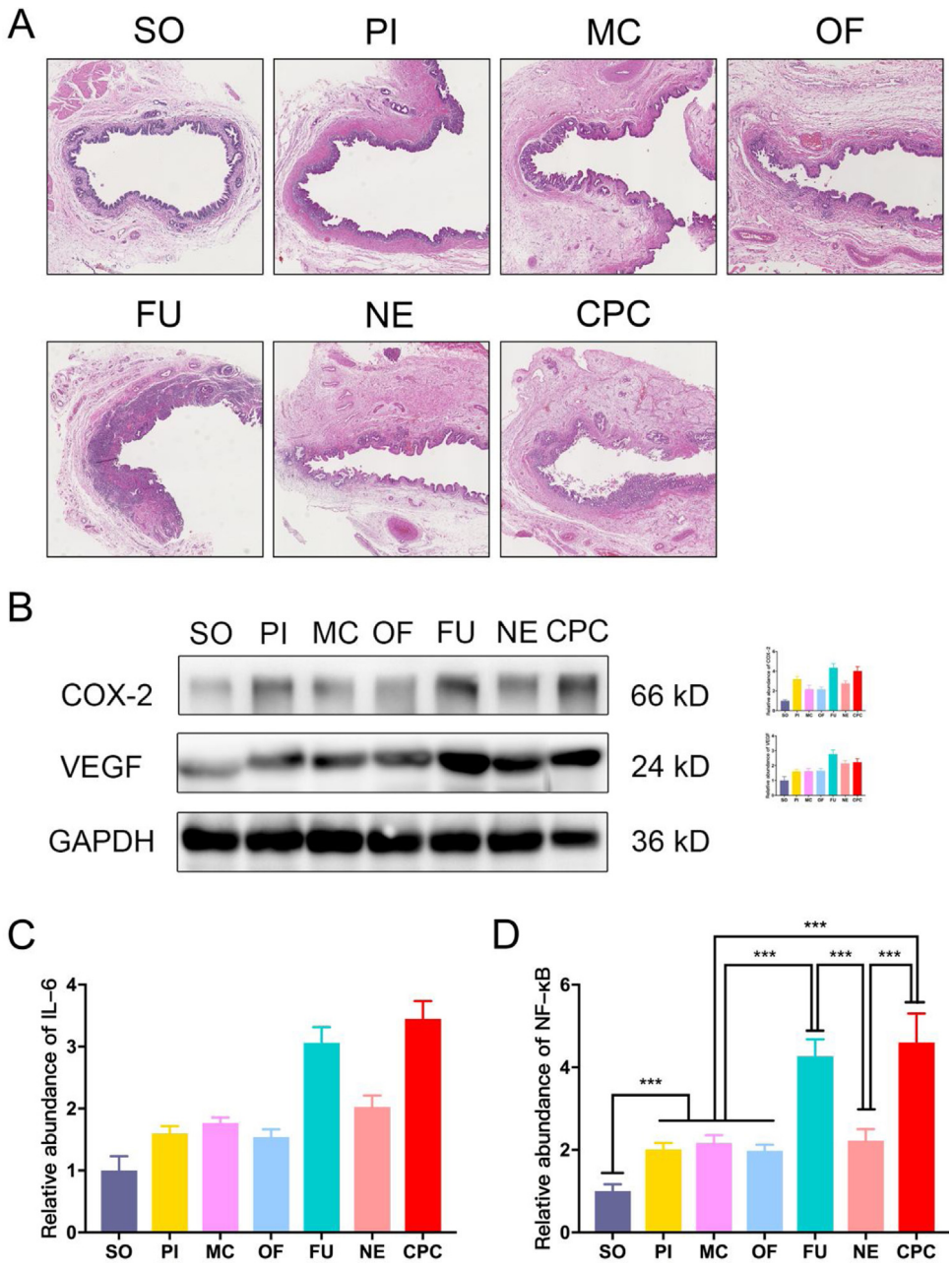

Figure 4 Local antibiotics irrigation could inhibit the proliferation of cholangiocytes in chronic proliferative cholangitis (CPC). A. Hematoxylin and eosin (HE) staining of the bile duct in the sham-operation (SO), povidone-iodine (PI), metronidazole plus chlorhexidine (MC), ofloxacin (OF), furacillin (FU), Neosporin® G.U. (NE), and CPC groups. B. Western blot of Cyclooxygenase-2 (COX2) and vascular endothelial growth factor (VEGF) in the SO, PI, MC, OF, FU, NE, and CPC groups. C. Enzyme-linked immunosorbent assay (ELISA) of interleukin-6 (IL-6) in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$). D. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) of nuclear factor kappa B (NF-κB) in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$).

MC, OF, FU, NE, and CPC groups compared with that in the SO group. However, the proliferation of cholangiocytes and submucosa in the PI, MC, OF, and NE group was weaker than that in the FU and CPC groups (Fig. 4A).

In view of the important roles that COX-2, VEGF, and IL-6 play in cholangiocyte proliferation, we examine their expression levels after irrigation using western blotting for COX-2, and VEGF, and ELISA for IL-6. Compared with the samples from the CPC group, we observed a significant decrease COX-2, and IL-6 levels in the PI, MC, OF, and NE groups ($P < 0.05$). However, the levels of COX-2 and IL-6 in the PI, MC, OF, and NE groups were still significantly higher than those in the SO group ($P < 0.05$) (Fig. 4B, C). VEGF levels in the PI, MC, and OF groups were significantly lower than those in the FU, NE, and CPC groups, but were significantly higher than that in the SO group ($P < 0.05$) (Fig. 4B).

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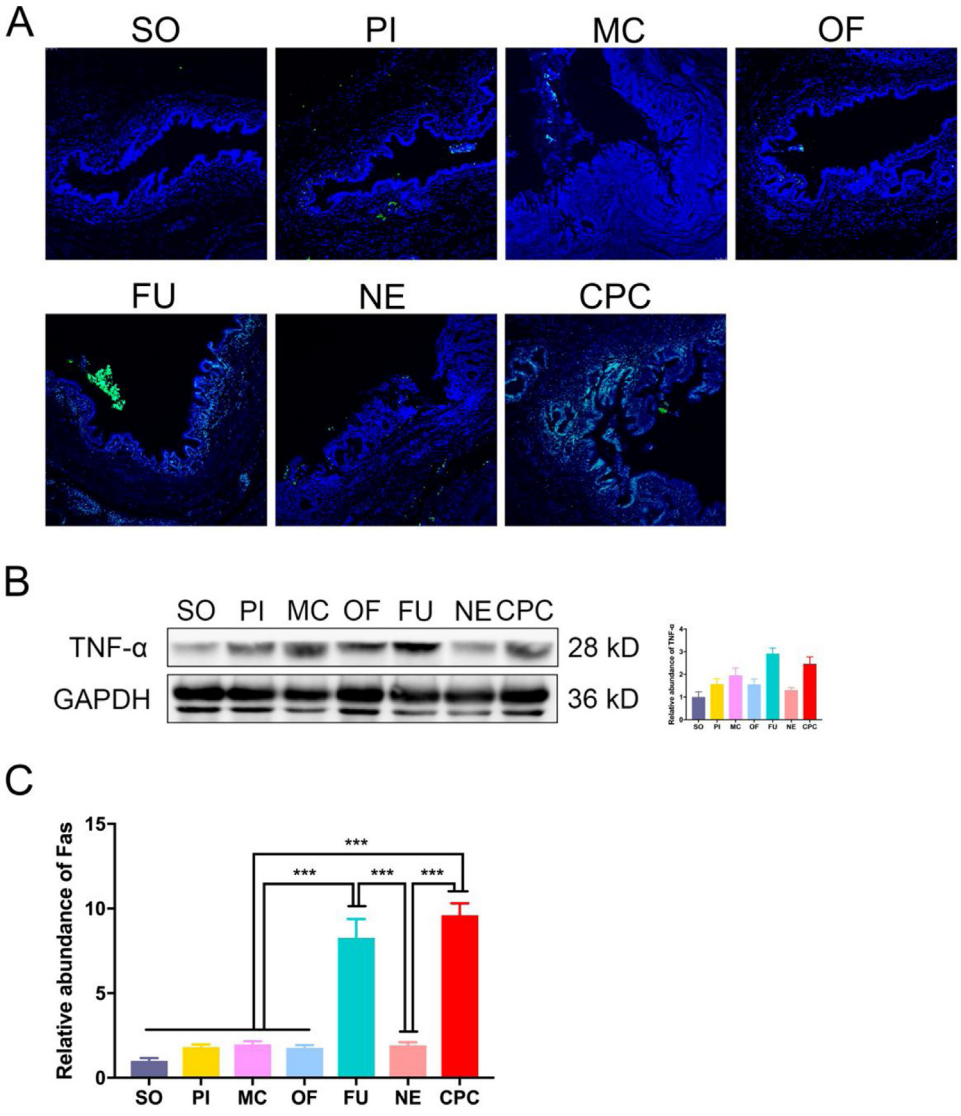

Figure 5 Local antibiotics irrigation could protect cholangiocytes from apoptosis in chronic proliferative cholangitis (CPC). A. Terminal deoxynulceotidyl transferase nick-end-labeling (TUNEL) staining of the bile duct in the sham-operation (SO), povidone-iodine (PI), metronidazole plus chlorhexidine (MC), ofloxacin (OF), furacillin (FU), Neosporin® G.U. (NE), and chronic proliferative cholangitis (CPC) groups. B. Western blot of tumor necrosis factor alpha (TNF-α) in the SO, PI, MC, OF, FU, NE, and CPC groups. C. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) of Fas cell surface death receptor (Fas) in the SO ($n = 6$), PI, MC, OF, FU, NE, and CPC groups ($n = 10$).

LPS stimulates cholangiocytes to secret COX-2, IL-6, TNF-α, Fas, TGF-β1, and other cytokines by activating the NF-κB pathway; therefore, we used qRT-PCR to detect *NFKB1* (NF-κB) expression in each group. We observed a significant decrease in *NFKB1* expression in the PI, MC, OF, and NE groups when compared with that in the FU and CPC groups ($P < 0.05$, Fig. 4D). Taken together, these data showed a decreased inflammatory response of cholangiocytes to residual *E. coli* and LPS in the PI, MC, and OF groups following local antibiotics irrigation of CPC.

Local antibiotics irrigation could protect cholangiocytes from apoptosis in CPC

Next, we sought to determine if local antibiotics irrigation would affect LPS-induced cholangiocytes apoptosis. First, we used TUNEL staining to detect the apoptosis of cholangiocytes after irrigation for 28 days. No obvious apoptosis factors were observed in the bile duct mucosa in the SO group. A small number of apoptotic factors were expressed in the bile duct mucosa of PI, MC, OF, and NE group. A large number of apoptotic factors were observed in the bile duct mucosa of the FU and CPC groups (Fig. 5A).

LPS induces apoptosis of infected cholangiocytes by stimulating cholangiocytes to secrete TNF-α; therefore, we examined the levels of TNF-α in each group. We observed a significant decrease of TNF-α levels in the PI, MC, OF, and NE groups compared with those in the FU and CPC groups ($P < 0.05$, Fig. 5B). Additionally, Fas is a transmembrane protein of the TNF family that induces apoptosis of cells by binding to its receptor FasR or CD95 [5]. Therefore, we further investigated the expression of *FAS* using

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qRT-PCR. Similar results to those for TNF-α were obtained. We also observed that the expression of *FAS* in the PI, MC, OF, and NE groups was significantly lower than that in the FU and CPC groups ($P < 0.001$, Fig. 5C). These data demonstrated that after antibiotics irrigation, there is a decrease in cholangiocyte apoptosis in CPC.

Local antibiotics irrigation could inhibit fibrosis of the bile duct in CPC

Masson staining was used to qualitatively evaluate fibrosis of the bile duct in each group. There were different degrees of fibrosis in the PI, MC, OF, FU, NE, and CPC groups; however, they all showed more fibrosis than that observed in the SO group. There was most obvious fibrosis was observed in the FU and CPC groups (Fig. 6A).

The overexpression of α-SMA is a hallmark of hepatic stellate cells activation, which can convert into fibroblasts to promote fibrosis of bile ducts in CPC [22]. We used immunohistochemistry and positive cell ratio analysis to quantitatively analyze the expression of α-SMA in each group. The positive cell ratios of α-SMA expression in all groups were significantly higher than that of the SO group, the ratios in the PI, MC, OF, and NE group were significantly lower than those in the FU and CPC groups ($P < 0.05$) (Fig. 6B).

Given the importance of TGF-β1 in regulating the activation of hepatic stellate cells, the secretion of extracellular matrix and collagen, we examined the effects of antibiotics irrigation might influence the levels of TGF-β1. We observed a significant increase in the levels of TGF-β1 in all irrigation groups and the CPC group ($P < 0.05$). The levels of TGF-β1 in the FU and CPC groups were significantly higher than those in the PI, MC, OF, and NE groups ($P < 0.05$, Fig. 6C).

Collagen secretion plays a critical role in fibrosis of CPC. Therefore, we quantified the degree of fibrosis in each group using western blot analysis of Collagen I, and confirmed a significantly reduced expression of Collagen-I in the PI, MC, and OF groups compared with that in the FU, NE, and CPC groups ($P < 0.05$, Fig. 6C).

Discussion

The recurrence of hepatolithiasis is a sequential and continuous process involving biliary tract infection, chronic proliferative cholangitis, biliary stenosis, and stones formation [3,7]. LPS, the major endotoxin of gram-negative bacteria, is associated with chronic biliary inflammation, such as primary sclerosis cholangitis (PSC), and primary biliary cholangitis (PBC). Both are characterized by chronic cholangiocyte proliferation, and bile duct fibrosis, [6,23] which are similar to the pathological features of CPC. Unfortunately, patients with CPC generally do not respond effectively to traditional intravenous antibacterial treatment [3,20]. Thus, developing a new method to treat CPC is essential to optimize current therapeutic strategies to prevent the recurrence of hepatolithiasis.

Application of antibiotic irrigation has been used to reduce urinary tract infections [10,24]. Additionally, recent studies have shown that oral antibiotics administration can reduce liver damage in chronic biliary diseases to some extent [9,25]. Tabibian et al. hypothesized that the antibiotics may relieve stricture of the bile duct in primary sclerosis cholangitis by decreasing the bacterially derived immunoactive molecules and thus decreasing hepatobiliary immune responses.

In vitro experiments demonstrated that the LPS could stimulate the proliferation of cholangiocytes and the secretion of CCL2 (MCP-1) to recruit $CD14^+$ macrophages [26,27]. CD14 can also be expressed on the surface of cholangiocytes [28]. The main function of CD14 on cholangiocytes is to bind to LPS/LBP (LPS binding protein) complexes or directly binding to LPS, thereby activating cholangiocytes to secrete cytokines such as TNF-α and IL-6, eventually mediating cytotoxicity [5,28]. We demonstrated that local antibiotics irrigation could reduce the counts of residual *E. coli*, the concentration of LPS, and the expression of MCP-1, therefore reducing the level CD14. In this respect, the effects of povidone iodine, metronidazole plus chlorhexidine, ofloxacin, and Neosporin® G.U. irrigation were found to superior to those of furacillin.

Gram-negative bacteria, especially *E. coli*, are involved in the onset of hepatolithiasis [7,26,28,29]. *E. coli* promotes hepatolithiasis by secreting exogenous β-G, and stimulating cholangiocytes to secret endogenous β-G and MUC5AC [7,8,29]. The interaction between β-G-derived calcium bilirubinate and MUC5AC facilitates hepatolithiasis formation. Yao et al. reported that LPS induces increased secretion of endogenous β-G via the PKC/NF-κB/C-myc pathway even after the infected bile has been removed, [7] which may also be an important reason for the recurrence of hepatolithiasis after stones removal. In the present study, the increased expression of PKC, NF-κB, and C-myc in the CPC group were also found (Figs. 3 and 4). Furthermore, local antibiotics irrigation could decrease the expression of PKC, NF-κB, C-myc, and MUC5AC in CPC. In addition, biliary stone formation was not found in the samples from the PI, MC, OF, and NE groups. However, *E. coli* also can secret exogenous β-G to promote stones formation. Thus, we concluded that local antibiotic treatment could inhibit biliary stone formation partly by reducing the amount of LPS in CPC by controlling the number of residual *E. coli*, therefore inhibiting the PKC/NF-κB/C-myc/endogenous β-glucuronidase pathway and the expression of MUC5AC. In this screen of antibiotics, povidone iodine, metronidazole plus chlorhexidine, ofloxacin, and Neosporin® G.U. were superior to furacillin.

LPS from gram-negative bacteria induces cholangiocytes proliferation, the apoptosis of cholangiocytes, and fibrosis of the bile duct by activating NF-κB, and therefore induces cholangiocytes to release a variety of cytokines, including proinflammatory cytokines like IL-6, COX-2, TNF-α, Fas, and profibrotic factor TGF-β1 [5,6,23]. The expression of VEGF is also increased when the bile duct is dilated [5]. IL-6, COX-2, and VEGF are majorly associated with cholangiocyte proliferation in chronic biliary inflammation [16,30,31]. In the present study, we observed increased expression of NF-κB, IL-6, COX-2, and VEGF in the CPC group, and local antibiotics irrigation (PI, MC, and OF group) could decrease the proliferation of cholangiocytes and submucosa, and the expression of NF-κB, IL-6, COX-2, and VEGF in CPC (Fig. 4). Thus, local antibiotics irrigation inhibits cholangiocyte proliferation partly by decreasing LPS-induced IL-6, COX-2, and VEGF release. In this respect, the effectiveness

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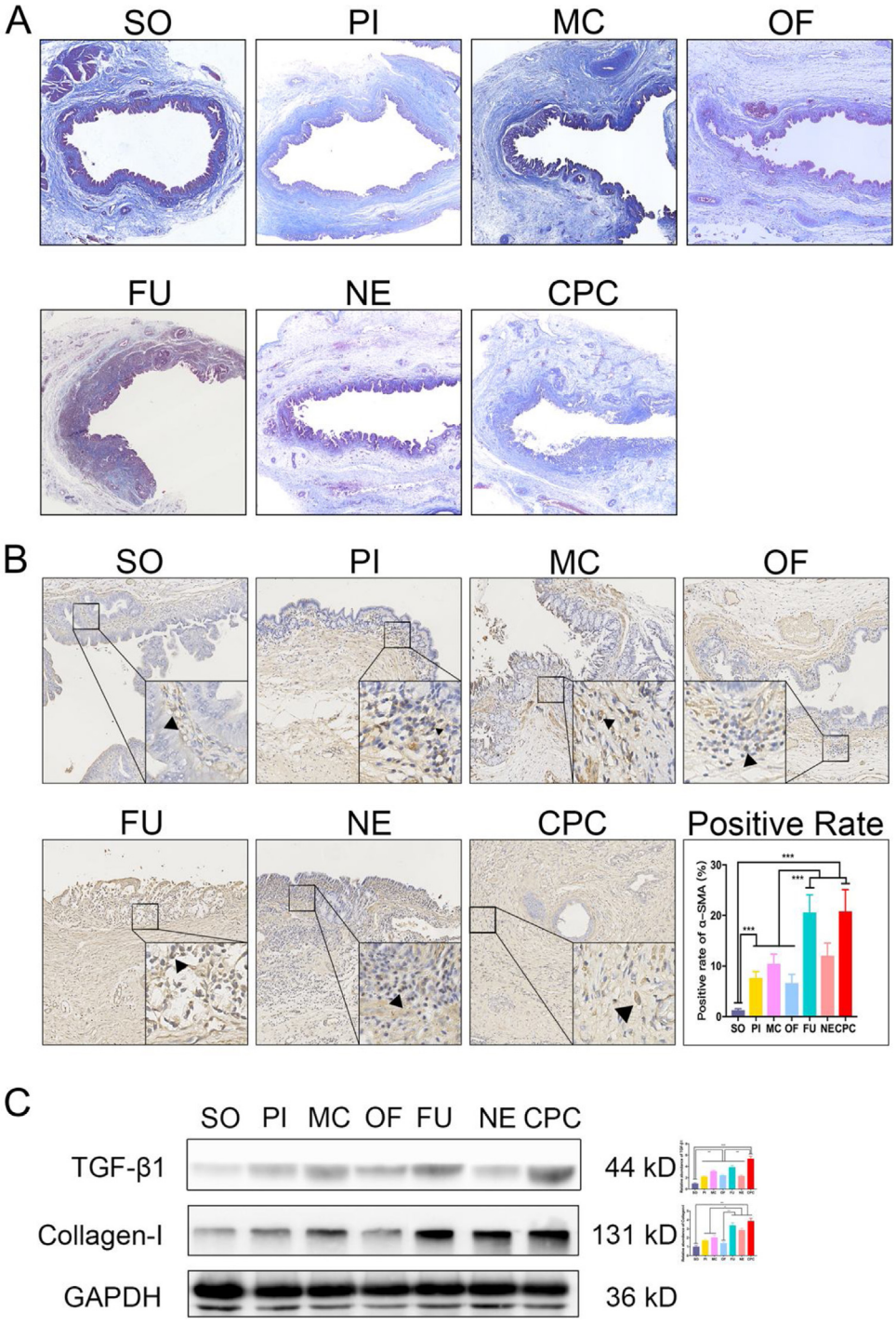

Figure 6 Local antibiotics irrigation could inhibit the fibrosis of bile duct in chronic proliferative cholangitis (CPC). A. MASSON staining of bile duct in the sham-operation (SO), povidone-iodine (PI), metronidazole plus chlorhexidine (MC), ofloxacin (OF), furacillin (FU), Neosporin® G.U. (NE), and chronic proliferative cholangitis (CPC) groups. B. Immunohistochemistry and positive cell ratio analysis of alpha smooth muscle actin (α-SMA) in the SO, PI, MC, OF, FU, NE, and CPC groups. C. Western blot of transforming growth factor beta 1 (TGF-β1) and Collagen-I in the SO, PI, MC, OF, FU, NE, and CPC groups.

of povidone iodine, metronidazole plus chlorhexidine, and ofloxacin were superior to Neosporin® G.U., and furacillin.

Given that LPS also induces cholangiocyte apoptosis via the NF-κB/TNF-α/Fas pathway, we also investigated whether local antibiotics irrigation could inhibit cholangiocyte apoptosis in CPC [5]. In patients with primary biliary cholangitis induced by LPS, increased expression of Fas was also found in the cholangiocytes, [5] which suggested that Fas participates in the apoptosis of cholangiocytes and bile duct injury induced by LPS. After injury of bile ducts, the periductular stroma and the basolateral plasma membrane domains of hepatocytes and cholangiocytes are

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exposed to toxic bile salts. In turn, this exacerbates immune-mediated bile duct injury and causes periductal inflammation in CPC [6]. In our study, NF-κB did induce high expression of TNF-α and Fas, resulting in increased apoptosis of cholangiocytes in CPC. However, local antibiotics irrigation (PI, MC, OF, and NE group) significantly decreased the LPS-induced NF-κB/TNF-α/Fas and apoptosis factors expression. This would relieve the apoptosis of cholangiocytes and protect the integrity of the bile duct wall in CPC, thereby avoiding toxic bile salts entering into the periductular stroma and the basolateral plasma membrane domains of hepatocytes and cholangiocytes, which would further protect cholangiocytes from apoptosis and bile duct fibrosis. The anti-apoptosis effectiveness of povidone iodine, metronidazole plus chlorhexidine, ofloxacin, and Neosporin® G.U. were observed to be superior to furacillin (Fig. 5).

In addition to regulating proinflammatory factors such as MCP-1, COX-2, IL-6, TNF-α, and Fas, the LPS/NF-κB pathway also regulates TGF-β1, a profibrotic factor [5]. The TGF-β1 signaling pathway promotes the activation of hepatic stellate cells (HSCs), and their transformation into myofibroblasts (MFs) or cholangiocytes. MFs in turn secrete more collagen and growth factors involved in fibrosis and regeneration [32—34]. Bile duct fibrosis and extracellular matrix secretion are the most important characteristics of CPC [3]. Alpha-SMA is the hallmark of HSCs activation [35]. In our study, increased expression of TGF-β1, α-SMA, and Collagen-I were found in CPC. Local antibiotics irrigation significantly decreased the expression of TGF-β1, thus decreasing the activation of HSCs, and inhibiting the fibrosis of bile ducts in CPC. Furthermore, in this respect, the effectiveness of povidone iodine, metronidazole plus chlorhexidine, and ofloxacin were superior to those of Neosporin® G.U. and furacillin. (Fig. 6) However, although the expression levels of COX-2, IL-6, and α-SMA in the Neosporin® G.U. group were slightly higher than those in the povidone iodine, metronidazole plus chlorhexidine, and ofloxacin groups, these eventually led to significantly higher fibrosis of the bile duct in the Neosporin® G.U. than in the above three groups, resulting in a higher expression of VEGF in the Neosporin® G.U. group.

In summary, the results suggested that *E. coli* secreted LPS has a crucial role in mediating the pathophysiological process of the recurrence of *E. coli* caused chronic proliferative cholangitis and hepatolithiasis. Local antibiotics irrigation could inhibit chronic proliferative cholangitis and stones formation by decreasing the release of LPS-induced proinflammatory and profibrotic cytokines. The effectiveness of povidone iodine, metronidazole plus chlorhexidine, and ofloxacin are superior to that of Neosporin® G.U. and furacillin.

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Author contributions

Wen-Jie Ma and Zhen-Ru Wu performed the majority of experiments; Qin Yang, Hai-Jie Hu, and Jun-Ke Wang collected the material and were involved in editing the manuscript; Yu-Jun Shi, Fu-Yu Li, and Nan-Sheng Cheng revised the manuscript critically and gave the final approval of the version to be published.

Disclosure of interest

The author declares that he has no competing interest.

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