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ORIGINAL RESEARCH

Sulfasalazine inhibits inflammation and fibrogenesis in pancreas via NF-κB signaling pathway in rats with oxidative stress-induced pancreatic injury

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Background: Pathogenesis and effective therapeutics of chronic pancreatic inflammation and fibrosis remain uncertain.

Purpose: To investigate the effects of sulfasalazine (SF) on pancreatic inflammation and fibrogenesis.

Methods: Chronic pancreatic injury in rats was induced by diethyldithiocarbamate (DDC) and interfered by SF through intraperitoneal injection. The rats were divided into five groups: group N, normal control group, rats were treated with distilled water only; group DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC only. Pancreatic inflammation and fibrosis were detected by hematoxylin and eosin staining and Sirius red staining. The genes and proteins related to NF-κB pathway and fibrogenesis including *NF-κB/p65*, *TNF-α*, *ICAM-1*, *α-SMA*, and *Con 1* were detected by immunohistochemical staining, reverse transcription polymerase chain reaction, and Western blotting.

Results: Rats in the DDC and DS1 groups showed the highest histological scores after DDC treatment, but the scores of DS2 and DS3 groups decreased significantly when compared with the DDC group. Sirius red staining showed collagen formation clearly in DDC and DS1 rats rather than in DS2 and DS3 rats. *NF-κB/p65*, *ICAM-1*, and *α-SMA* were strongly expressed in DDC and DS1 rats, while DS2 and DS3 rats showed mild to moderate expression by immunohistochemistry. Reverse transcription polymerase chain reaction showed increased levels of *NF-κB/p65*, *ICAM-1*, *TNF-α*, *α-SMA*, and *Con 1* mRNA in DDC and DS1 rats in comparison to normal controls. The mRNA levels of these molecules in DS2 and DS3 rats were significantly lower than those in DS1 and DDC rats. Western blotting demonstrated that the *NF-κB/p65*, *ICAM-1*, and *α-SMA* expressions in pancreatic tissues of the rats of the DDC group were more clear than those of the normal control, DS2, and DS3 rats.

Conclusion: SF inhibits pancreatic inflammation and fibrogenesis via NF-κB signaling pathway.

Keywords: sulfasalazine, pancreatic injury, inflammation, fibrogenesis, NF-κB

Introduction

Acute or chronic inflammatory cells infiltration in pancreas tissues is a typical pathological characteristic in acute or chronic pancreatitis.[1,2] Fibrogenesis is temporary in acute pancreatitis but persistent in chronic pancreatitis. The causes that induce these pathological alterations in pancreas are bile duct stones, alcohol abuse, severe hyperlipidemia, and others. No matter what the etiological factors are, one of

the important underlying pathogenesis is oxidative stress, which would incite inflammatory cells activation, promote proinflammatory cytokines release, and therefore lead to pancreatic damage.[3–6] NF-κB signaling pathway is important in the development of inflammation process, and recurrent or chronic inflammation could induce transfer growth factor beta activation and then lead to pancreatic stellate cells (PSCs) activation and pancreatic fibrosis. Previous studies demonstrate the close relationship between oxidative damage and NF-κB activation; overactivated NF-κB signaling incites upregulation of a series of inflammatory molecules, PSCs activation, and then contributes to development of pancreatic lesions.[7,8] Therefore, NF-κB signaling could be a therapeutic target to ameliorate inflammation and fibrogenesis within pancreas tissues.

It is reported that sulfasalazine (SF) is an inhibitor of NF-κB signaling pathway, which can inhibit NF-κB translocation and activation and downregulate inflammatory cytokines release and expression of some adhesion molecules, such as *TNF-α* and *ICAM-1*.[9–11] We speculate that SF may inhibit PSCs activation and prevent pancreatic fibrogenesis on the basis of previous studies which reported its antifibrogenesis effect on experimental liver fibrosis.[10,12] Diethyldithiocarbamate (DDC) is a superoxide dismutase inhibitor, which can induce pancreatitis in rats.[13] In this study, we induced pancreatic damage by DDC and intervened by SF in rats, observed the pancreatic histological alterations and molecules expressions, and investigated whether SF prevents or ameliorates oxidative stress-induced pancreatic injuries.

Materials and methods

Animals and reagents

This animal study was approved by the Ethics Committee of Shandong University and the experiments were performed in accordance with the Laboratory Animal Care and Use Regulations of Shandong University. All Wistar rats, weighing 160–185 g, were obtained from The Laboratory Animal Center of Shandong University and housed in a temperature and humidity controlled room for 1 week. The rats were then divided into five groups (15 rats per group) on the basis of comparable mean body weight as follows: group N, normal control group, rats were treated with dilated water only (intraperitoneal [ip], twice a week) for 10 weeks; group DS1, rats received SF treatment (ip, 10 mg/kg) 2 hours before DDC treatment (ip, 750 mg/kg, twice a week) for 10 weeks; group DS2, rats were treated with DDC (ip, 750 mg/kg, twice a week) first and then SF (ip, 100 mg/kg, twice a week) for 10 weeks; group DS3, rats were treated with DDC (ip, 750 mg/kg, twice a week) for 10 weeks, then SF (ip, 100 mg/kg, thrice a week) for 2 weeks; and group DDC, rats were treated with DDC only (ip, 750 mg/kg, twice a week) for 10 weeks. DDC and SF were purchased from Sigma-Aldrich Co. (St Louis, MO, USA).

H&E staining

Each formalin-fixed and paraffin-embedded pancreas specimen was cut into 5 μm thick sections. Hematoxylin and eosin (H&E) staining was performed for routine histologic observations.

Histological inflammation in pancreas was evaluated quantitatively based on three aspects: acinar cell atrophy, vacuolization, and inflammatory cell infiltration, and was presented as histological score.

Sirius red staining

Slides were deparaffinized and immersed for 25 minutes in saturated aqueous picric acid containing 0.5% Sirius red. All the Sirius red-stained sections were observed and photographed under both common light and polarization microscopes. Under polarization microscope, collagen appears bright orange-red and/or bright green. The images were digitized using ImageJ software (Version 1.50g, National Institutes of Health, USA).

Pancreas collagen deposition was presented by a fibrosis index (%) that indicates the ratio of the mean collagen stained area to the mean whole area of the section.

Immunohistochemistry staining

Immunohistochemistry staining for *NF-κB/p65*, *ICAM-1*, and *α-SMA* was performed as follows: the sections were deparaffinized, immersed in 3% H_2O_2 (v/v) to quench endogenous peroxidase activity, and microwaved in 10 mM sodium citrate (pH 6.0) for 15 minutes for antigen retrieval. Then, the avidin and biotin were applied to eliminate endogenous biotin-related background staining. The sections were then incubated with primary antibodies (1:150) (Santa Cruz Biotechnology Inc., Dallas, TX, USA) at 4°C overnight and incubated, respectively, with biotinylated goat anti-mouse antibodies and horseradish peroxidase-conjugated streptavidin (Santa Cruz Biotechnology Inc.) for 15 minutes at room temperature. The slides were washed and the chromogen was developed for 5 minutes with liquid 3,3′-diaminobenzidine before observation. Distilled water with 0.4% Tween-20 was used as a rinsing solution. Positive staining areas were measured by ImageJ software and expressed as integrated optical density.

All histological samples were evaluated blindly by the same pathologist. To evaluate the histological changes, three sections were randomly selected from each rat, and five non-overlapping fields per section were captured for observation.

RT-PCR assay for mRNA levels

mRNA levels were determined by reverse transcription polymerase chain reaction (RT-PCR). Pancreatic samples were rapidly immersed in RNAlater (Sigma-Aldrich Co.) for RNA protection and stored at −20°C before assay. Total RNA was extracted from pancreatic tissues using a TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) and was reverse-transcribed using oligo (dT) as a primer. The sequences used as an internal standard control, housekeeping gene β-actin, and target genes are listed in Table 1.

PCR amplification cycles were carried out under the following conditions: initial activation of 95°C for 5 minutes followed by 35 cycles of 45 seconds of denaturation at 95°C, 45 seconds of annealing at (58°C for *β-actin*, *ICAM-1*, and *α-SMA*; 60°C for *NF-κB/p65*; 50°C for *TNF-α*), 45 seconds of extension at 72°C followed by one final extension at 72°C for 7 minutes. Completed reactions were held at 4°C.

PCR products were separated by gel electrophoresis (1.5% agarose stained with ethidium bromide). Specific bands were visualized with an image system (FluorChem 9900; Alpha Innotech, San Leandro, CA, USA). The detection of each gene was repeated for three times. The optical density (OD) values of the bands were analyzed using Image J software and standardized to the β-actin signal.

Western blotting for *NF-κB/p65*, *ICAM-1*, and *α-SMA* expression

Briefly, frozen tissues were lysed in lysis buffer. The lysates were centrifuged at 12,000 × *g* and supernatants were immediately stored at −70°C until use. Protein concentration was determined using the Lowery method, and 50 μg of protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Nonspecific binding was blocked by preincubation of the nitrocellulose membrane in Tris-buffered saline containing 5% nonfat milk for 1 hour. The nitrocellulose membrane was incubated overnight at 4°C with anti-*NF-κB/p65*, *ICAM-1*, and *α-SMA* antibodies (Santa Cruz Biotechnology Inc.). Bound primary antibody was detected using a peroxidase-conjugated secondary antibody (Boshide, Wuhan, People's Republic of China) and enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA). The detection of each protein was repeated for three times. The OD values of the bands were quantified using ImageJ software and standardized to the signal of the control.

Statistical analysis

Data were expressed as mean ± standard deviation. The statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. $P<0.05$ was considered significant.

Results

General information

Rats demonstrated discomfort after DDC injection but recovered soon. The rats' urine turned deep yellow after SF treatment, but became normal in a day. All rats survived well during the experimental period.

H&E staining

After DDC treatment, acinar cells atrophy and vacuolization, enlarged space between lobules, and inflammatory cells infiltration were demonstrated. Rats in DDC and DS1

Table 1 Target genes primers and products

Rat genes	Primers	Product (bp)
β-actin	Forward: 5′-AAG,ATC,CTG,ACC,GAG,CGT,GG-3′ Reverse: 5′-CAG,CAC,TGT,GTT,GGC,ATA,GAG,G-3′	327
NF-κB/p65	Forward: 5′-ATG,GAC,GAT,CTG,TTT,CCC-3′ Reverse: 5′-GTC,TTA,GTG,GTA,TCT,GTG,CT-3′	170
ICAM-1	Forward: 5′-AGC,CTC,AGG,CCT,AAG,AGG,AC-3′ Reverse: 5′-AGG,GGT,CCC,AGA,GAG,GTC,TA-3′	496
TNF-α	Forward: 5′-TCG,TAG,CAA,ACC,ACC,AAG-3′ Reverse: 5′-CTG,ACG,GTG,TGG,GTG,A-3′	193
α-SMA	Forward: 5′-AGT,CGC,CAT,CAG,GAA,CCT,CGA,G-3′ Reverse: 5′-ATC,TTT,TCC,ATG,TCG,TCC,CAG,TTG-3′	296
Con 1	Forward: 5′-CTT,CGT,GTA, AAC,TCC,CTC,C-3′ Reverse: 5′-CAC,TTT,TGG,TTT,TTG,GTC,AC-3′	221

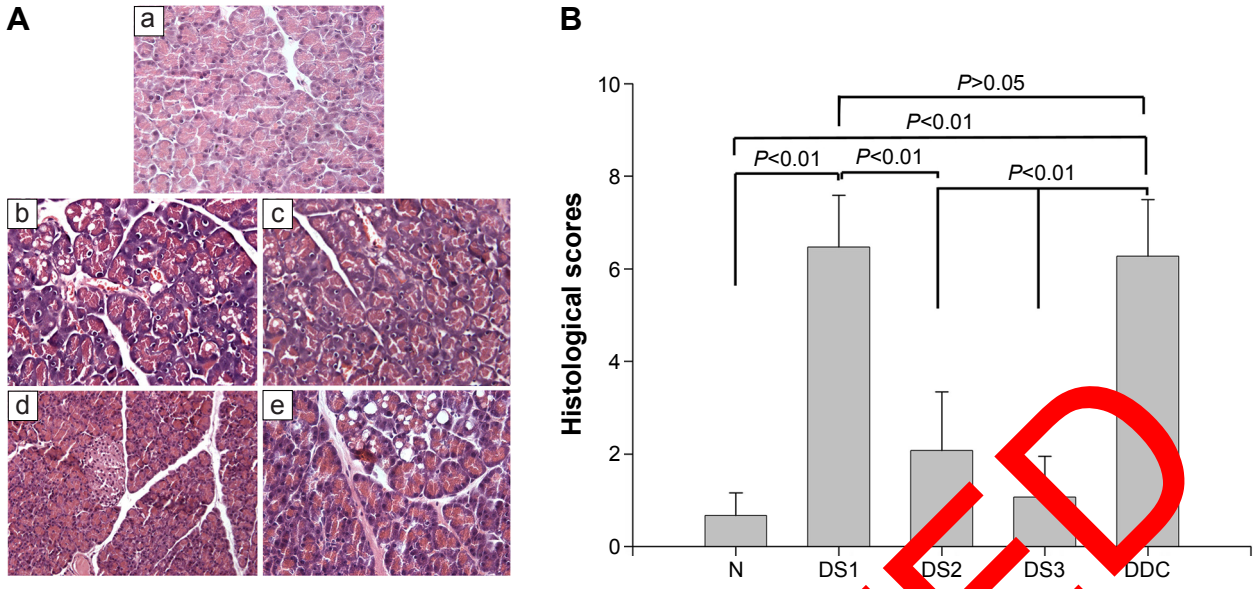

Figure 1 Pancreatic histological alterations (**A**) and histological scores (**B**).
Notes: (**A**, H&E staining, original magnification 200×) Representative histological changes in pancreas in normal group (a), group DS1 (b), DS2 (c), DS3 (d), and DDC (e). Quantitative analysis (**B**) demonstrates different pancreatic histological alterations in rats with different silibilalate (SF) treatments in different groups. Group N, normal control group, rats were treated with dilated water only; DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC only.
Abbreviations: H&E, hematoxylin and eosin; DDC, diethyldithiocarbamate.

groups showed the highest histological scores; DS2 and DS3 groups had all aforementioned presentations, but the histological scores decreased significantly when compared with the DDC group (Figure 1).

Sirius red staining for fibrogenesis

Pancreatic fibrogenesis was detected by Sirius red staining, and observed under common light and polarized microscopes. Collagen formation was clearly observed in DDC and DS1 rats, but in DS2 and DS3 rats, collagen presentation was less in comparison to that in DDC rats (Figure 2).

Immunohistochemistry staining

Inflammation and fibrosis-associated molecules were detected by immunohistochemistry staining. As a result, *NF-κB/p65*, *ICAM-1*, and *α-SMA* were strongly expressed in DDC and DS1 rats, while DS2 and DS3 rats showed mild-to-moderate expression. *ICAM-1* was expressed in endothelial cells and in a small amount of acinar cells, while *α-SMA* was expressed around vessels and between acinar cells (Figure 3).

RT-PCR assay

The results of PCR showed increased mRNA levels of *NF-κB/p65*, *ICAM-1*, *TNF-α*, *α-SMA*, and *Con 1* in DDC and DS1 rats in comparison to normal controls. The mRNA levels of these molecules in DS2 and DS3 rats were significantly lower than those of DS1 and DDC rats (Figure 4).

Western blotting

Western blotting demonstrated that the *NF-κB/p65*, *ICAM-1*, and *α-SMA* expressions in pancreatic tissues of DDC rats were significantly higher than those of normal control rats. The differences in the expression of these molecules between DS2 and DDC and DS3 and DDC rats were all significant (Figure 5).

Discussion

Pancreatitis is a complex disorder the exact mechanism of which remains controversial. Recurrent acute pancreatitis or chronic pancreatitis of various origins damage pancreatic parenchymal cells, including acinar and islet cells, and result in exocrine or/and endocrine insufficiency. So far, effective and widely accepted therapeutic methods for pancreatic injuries are not well established because of the vague mechanisms underlying this pathophysiological process. Although the exact pathogenesis of pancreatitis remains uncertain, several mechanisms related to oxidative and inflammatory stress are implicated. Injuries to the pancreatic cells cause a complex cascade of events that includes increased production of reactive oxygen species (ROS), which leads to oxidation of lipids and proteins and disruption of the cell membrane.

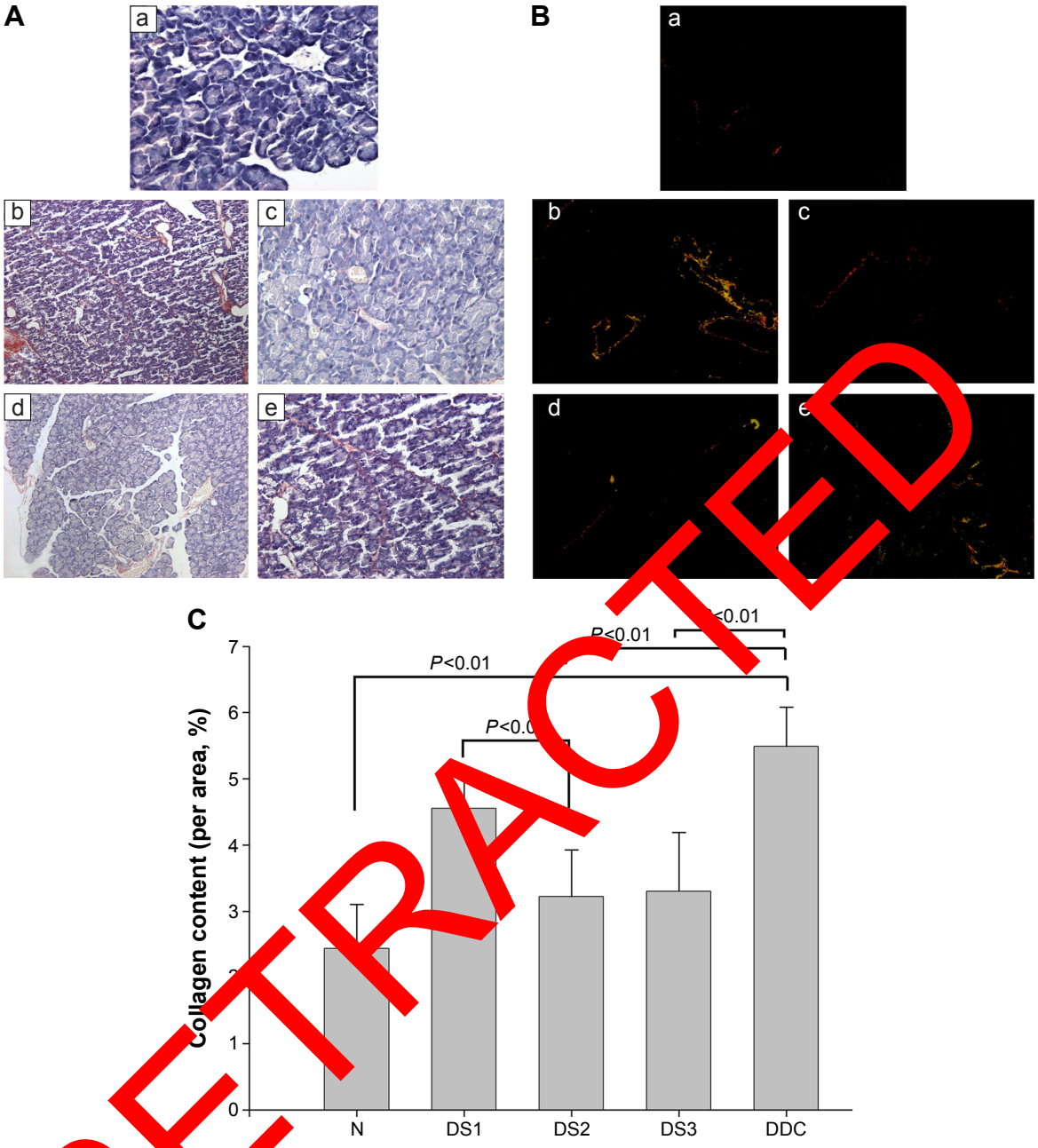

Figure 2 Fibrogenesis in pancreas and the corresponding statistical analysis.
Notes: Collagen deposition was demonstrated by Sirus red staining and observed under common light (**A**, original magnification 200×) and polarized microscopes (**B**, original magnification 200×) in group (a), group DS1 (b), DS2 (c), DS3 (d), and DDC (e). Under polarization microscope, collagen appears bright orange-red and/or bright green. Quantitative analysis (**C**) demonstrates different levels of pancreatic fibrogenesis in rats with different sulfasalazine (SF) treatments in different groups. Group N, normal control group, rats were treated with dilated water only; DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC only.
Abbreviation: DDC, diethyldithiocarbamate.

Thus, oxidative stress is considered to play a key role in the development of chronic pancreatic injury.[14,15] We found high-fat diet induced vascular disturbances and oxidative stress in pancreas and led to pancreatic injury in our previous study.[16] In our present study, rats were treated with DDC, a reagent which could incite oxidative stress and lead to cell damage.[17] As a result, inflammation, acinar atrophy, and fibrogenesis were observed inside the pancreatic tissues after DDC administration, suggesting development of oxidative stress-induced pancreatic injuries. In consideration of the role of oxidative stress in development of pancreatic injury, antioxidant is believed to be an effective therapy.

Actually, in the past decade, experimental and clinical investigations have demonstrated that antioxidants

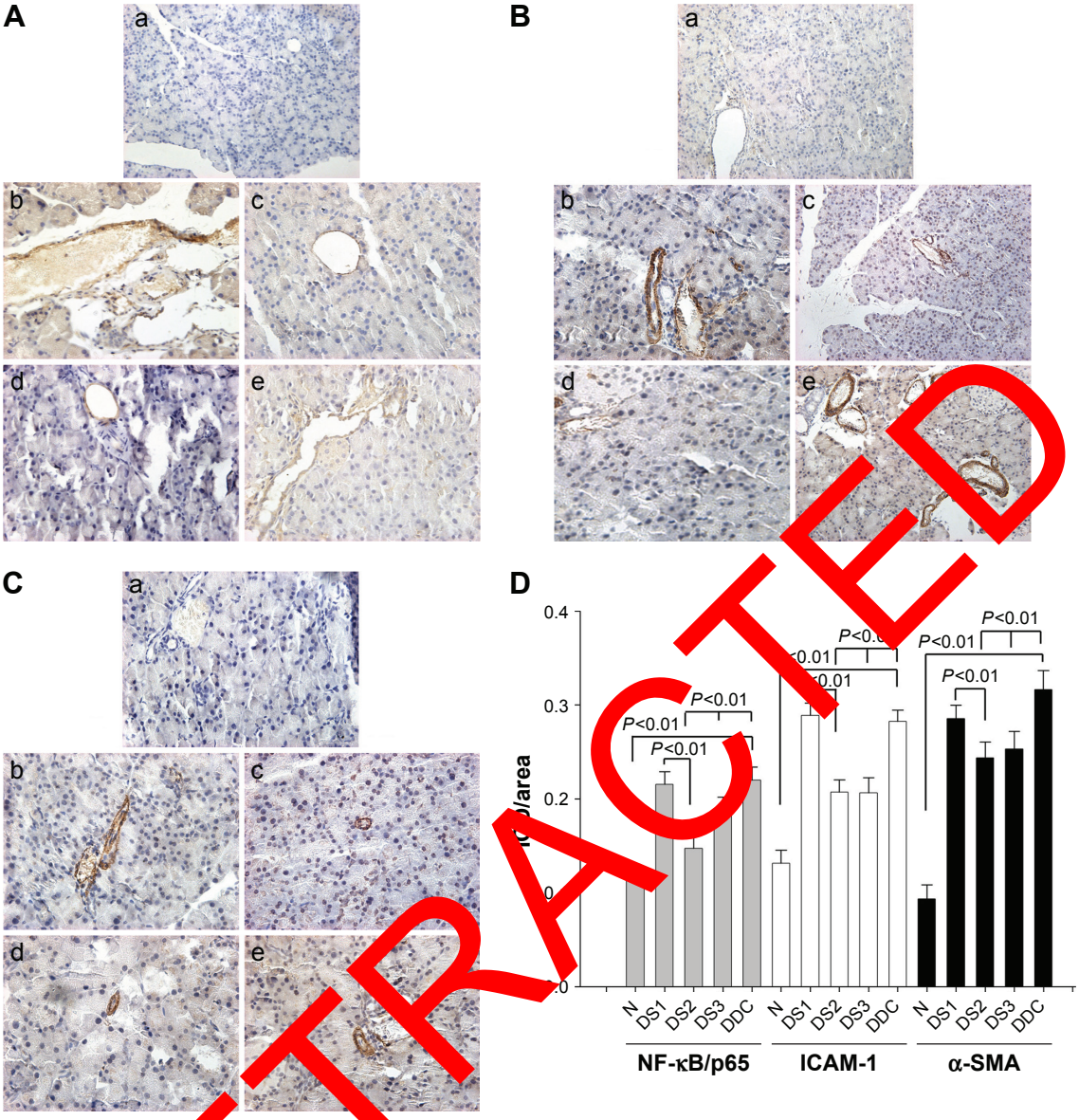

Figure 3 Immunohistochemistry staining for *NF-κB/p65*, *ICAM-1*, and *α-SMA* and the corresponding statistical analysis.
Notes: *NF-κB/p65* (**A**, original magnification 200×), *ICAM-1* (**B**, original magnification 200×), and *α-SMA* (**C**, original magnification 200×) expression in normal group (a), group DS1 (b), DS2 (c), DS3 (d), and DDC (e). Quantitative analysis (**D**) demonstrates different levels of pancreatic fibrogenesis in rats with different sulfasalazine (SF) treatment in different groups. Group N, normal control group, rats were treated with dilated water only; DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC only.
Abbreviations: DDC, diethyldithiocarbamate; IOD, integrated optical density.

have protective effects on acute[8,18–20] and chronic[5,21–23] pancreatitis. SF is used comprehensively for treatment of inflammatory bowel disease and arthritis, but recent studies have demonstrated its antioxidant properties based on experimental researches. It is reported that SF, especially its metabolite 5-aminosalicylic acid, scavenges ROS, inhibits oxidative stress, and therefore has therapeutic effects on inflammation.[11,24,25] It is also reported that a new drug, which was developed from SF, ameliorates amyotrophic lateral sclerosis because of its antioxidant property.[26] Additionally, in an experimental study of CCl_4-induced liver fibrosis, SF showed antifibrotic effects because of its antioxidant ability and its ability to inhibit NF-κB nuclear translocation.[10] Accordingly, we treated the rats with SF before and after DDC stimulation in our study in order to investigate whether SF could ameliorate pancreatic injuries. The results show that SF treatment decreases inflammatory cells infiltration, inhibits PSCs activation and fibrogenesis in pancreas tissues, suggesting the protective and therapeutic effects of SF on oxidative stress-induced pancreatic damage.

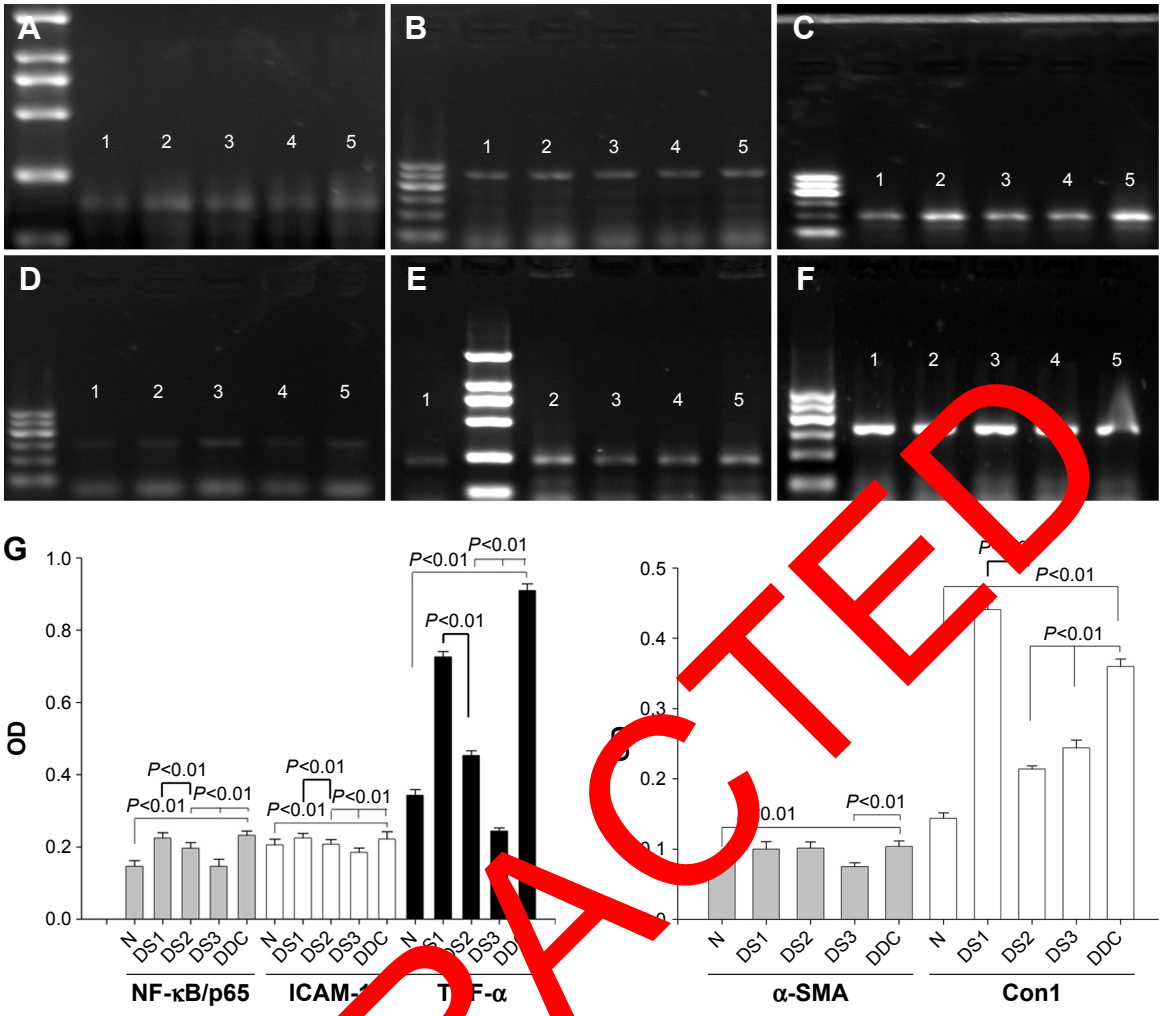

Figure 4 Agarose gel electrophoresis for PCR product[s] and the [corresp]onding statistical analysis.
Notes: Genes (**A**) *NF-κB/p65* (mark: 100, 250–[2,]000 bp); ([**B**]) *ICAM-1* (mark: 100–600 bp); (**C**) *TNF-α* (mark: 100–600 bp); (**D**) *α-SMA* (mark: 100–600 bp); (**E**), *Con 1* (mark: 100, 250–2,000 bp); (**F**) actin (mark: 100–60[0] bp); groups 1, [N]; 2, DS1; 3, DS2; 4, DS3; 5, DDC; and (**G**) the statistical analyses of mRNA levels between different groups with different sulfasalazine (SF) treatment[s]. N, normal control group, rats were treated with dilated water only; DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC only.
Abbreviations: PCR, polymerase chain reaction; DDC, diethyldithiocarbamate; OD, optical density; mark, marker.

It is known that inflammatory transcription factor NF-κB signal pathway is important during development of inflammation. Activation of NF-κB has been shown to elicit acute pancreatitis as an early event, together with trypsinogen activation.[27,28] Previous investigations have shown that NF-κB and its modulated molecules, such as *TNF-α* and *ICAM-1*, have close relations with oxidative damage and oxidants' protective effects.[3] Lv et al found that "lycopene" protects pancreatic acinar cells against necrosis and apoptosis through NF-κB/JNK pathway.[18] In another experimental study, Gulcubuk et al found "resveratrol" can reduce oxidative damage, prevent IκB degradation, and decrease the levels of NF-κB, *TNF-α*, and IL-6.[20] "Gallic acid", which is a strong antioxidant, upregulates the expression of Nrf2 and attenuates experimental colitis.[29] In our previous study, we also demonstrated the role of NF-κB signal pathway in high-fat diet-induced pancreatic injury.[7] SF is reported to be a potent inhibitor of NF-κB activation, which partly explains its pharmacological effects as an immunomodulatory agent in chronic inflammation.[9] In an animal study of CCl_4-induced liver fibrosis, SF was found to have antifibrotic effects because of its antioxidant property and its ability to inhibit NF-κB nuclear translocation and TGF-β expression.[10] According to our current study, we found elevated *NF-κB/p65* expression after DDC stimulation, and its corresponding regulatory molecules including *TNF-α* and *ICAM-1* were upregulated simultaneously in pancreatic tissues in comparison to controls. The genes of these inflammatory molecules

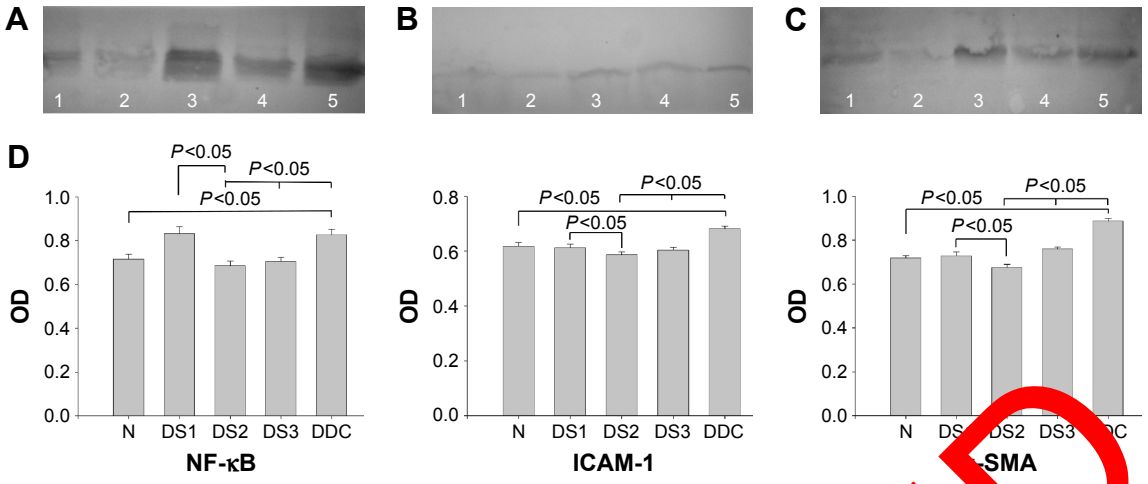

Figure 5 Western Blotting for detection of protein expression and the corresponding statistical analyses.
Notes: Proteins (**A**) *NF-κB/p65* (65 kDa); (**B**) *ICAM-1* (85 kDa); (**C**) *α-SMA* (42 kDa); group 1, DS3; 2, DS2; 3, DS1; 4, N; 5, DDC. (**D**) Statistical analysis to show differences in the expression of *NF-κB/p65*, *ICAM-1*, and *α-SMA* in different groups with different sulfasalazine (SF) treatments. Group N, normal control group; rats were treated with dilated water only; DS1, rats received SF (10 mg/kg) 2 hours before DDC treatment; group DS2, rats were treated with DDC, and then SF (100 mg/kg, twice a week); group DS3, rats were treated with DDC, then SF (100 mg/kg, thrice a week); and group DDC, rats were treated with DDC.
Abbreviations: DDC, diethyldithiocarbamate; OD, optical density.

changed in the same way. These findings indicate the role of NF-κB signal pathway in the ameliorative effects of SF on pancreatic injuries.

Conclusion

Our study suggests that SF might be a potential candidate for the treatment of pancreatic inflammatory disease. But, the adverse effects of SF should also be considered. It is reported that SF itself could induce oxidative stress and react with ROS, and these reactions might be a possible mechanism of male infertility, hepatotoxity, and nephrotoxicity.[24,30] SF-associated pancreatitis is also reported during treatment of inflammatory bowel disease. But there is a possibility that therapeutic rather than adverse effects might be dominant when treated with SF during the pathophysiological process of chronic pancreatic injuries. Whether SF is suitable for clinical treatment of patients with acute or chronic pancreatitis needs further experimental and clinical investigations and should be well assessed in the future.

Acknowledgments

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Disclosure

The authors report no conflicts of interest in this work.

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