International Immunopharmacology 142 (2024) 113113

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Bufalin alleviates inflammatory response and oxidative stress in experimental severe acute pancreatitis through activating Keap1-Nrf2/HO-1 and inhibiting NF-κB pathways

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ARTICLE INFO

Keywords:
Bufalin
Acute pancreatitis
Inflammatory
Oxidative stress
Nrf2
NF-κB

ABSTRACT

Severe acute pancreatitis (SAP) is a prevalent acute inflammatory disease that is clinically manifested by systemic inflammation dysregulation, resulting in a significantly elevated mortality rate. Bufalin has been verified to have potent pharmacological properties, including analgesic, anti-tumor and anti-inflammatory effects. However, it remains unclear whether bufalin inhibits SAP. Thus, we aim to explore the impact of bufalin in SAP rats and to evaluate the potential mechanisms of action. In addition to analyzing serum biochemistry and pancreatic tissue pathology, we elucidated its mechanisms of action through enzyme-linked immunosorbent assay (ELISA), immunohistochemical analysis, Western blot, and quantitative real-time PCR. The results demonstrated that bufalin dose-dependently reversed the elevation of serum Amylase (Amy) and Lipase (LPS) levels in SAP rats, alleviating pancreatic tissue pathological damage. Bufalin exhibited potent antioxidant effects by reducing malondialdehyde (MDA) levels, decreasing Superoxide dismutase (SOD) and glutathione(GSH) consumption, inhibiting the interaction of Keap1-Nrf2, and increasing HO-1 expression. Furthermore, bufalin inhibited TNF-α, IL-6, IL-1β, p-NF-κB-p65, p-IκBα, and NF-κB-p65 expression, while enhancing IκBα expression, ultimately confirming its anti-inflammatory effects on SAP. In summary, our findings suggest that bufalin exerts anti-inflammatory and antioxidant actions in NaT-SAP rats by inhibiting NF-κB and activating the Keap1-Nrf2/HO-1 pathway. This study represents the inaugural application of bufalin in NaT-induced SAP rats, indicating its potential as an effective therapeutic agent for SAP patients.

1. Introduction

SAP is a serious and fatal inflammatory disease of the pancreas [1]. It is characterized by local or systemic inflammatory reactions and presents with various clinical manifestations such as abdominal pain, bloating, and elevated amylase levels [2,3]. The vast majority of patients with AP present with mild acute pancreatitis (MAP), which generally disappears in less than a week [4]. However, Approximately 15–20 % of patients develop severe acute pancreatitis (SAP), which is the primary cause of systemic inflammatory response and multi-organ failure, with a mortality rate as high as 20–40 % [5,6]. Therefore, it is critical for the clinical management of SAP to explore new treatment modalities.

Increasing evidence suggests that excessive inflammation and oxidative stress are critical events in developing AP [7–9]. As two interacting systems that regulate cellular redox balance, the Nrf2/HO-1 and NF-κB pathways may indeed regulate inflammatory response and oxidative stress [10,11]. Nuclear factor-erythroid 2-related factor 2 (Nrf2) Nrf2 is involved in various biological processes such as anti-oxidation and anti-apoptosis in the body [12]. Under normal conditions, Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) associate in the cytoplasm. When the body experiences oxidative stress or inflammation, Nrf2 dissociates from Keap1 and translocates to the nucleus

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https://doi.org/10.1016/j.intimp.2024.113113
Received 24 April 2024; Received in revised form 2 August 2024; Accepted 5 September 2024
Available online 13 September 2024

where it binds to the antioxidant response element (ARE) to regulate various downstream antioxidants, such as SOD and heme oxygenase-1 (HO-1), thereby reducing oxidative stress damage [13,14].

Oxidative stress can activate NF-κB and trigger the activation of inflammatory signaling pathways [15]. In its inactive state, NF-κB typically binds to an inhibitor protein called Inhibitor of κB (IκB) in the cytoplasm. When cells are exposed to oxidative stress, IκB is degraded, releasing NF-κB to enter the nucleus and activate the transcription of genes encoding proteins like Interleukin (IL-6), tumour necrosis factor-α(TNF-α), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which play crucial roles in the inflammatory response [16,17]. Currently, there is abundant evidence that the Keap1-Nrf2/HO-1 and NF-κB pathways are closely correlating with the severity of AP. Therefore, reducing inflammation and oxidative stress represent feasible strategies for alleviating AP [18,19].

Bufalin (BF) is derived from the skin and parotid venom of the Chinese toad [20]. It has received widespread attention due to its various pharmacological activity, including analgesic, anti-tumor and anti-inflammatory properties [21–23]. Research reports have shown that bufalin inhibits inflammation in asthmatic mice by blocking NF-κB activity [24]. It has also been shown to have powerful anti-inflammatory and analgesic activity in rats with carrageenan-induced paw edema [25]. However, it remains unclear whether bufalin affects NaT-induced SAP rats. Therefore, further studies are necessary for the clarification of the potential impacts of bufalin in NaT-SAP rats.

Based on this background, the objective of this study was to explore the effects of bufalin in NaT-SAP rats and evaluate its potential mechanisms of action. The results indicate that bufalin reduces the inflammatory response through inhibiting the NF-κB activity and ameliorates pancreatic injury by activating the Keap1-Nrf2/HO-1 pathway. This is the first application of bufalin in NaT-SAP rats, These results provide new ideas for the clinical treatment of SAP.

2. Materials and methods

2.1. Reagents and antibodies

Bufalin(B20152, HPLC Grade, Purity ≥ 98 %) and DMSO(S24295, AR Grade, Purity ≥ 99 %): Purchased from Yuan Ye Biological Technology Co.Ltd(Shanghai,China). Bufalin was dissolved in DMSO at a concentration of 30 mmol/L and stored at 4 °C. Prior to use, it was diluted to final concentration with saline for injection. The final concentration of DMSO in each sample was kept below 0.5 % (v/v), which did not significantly affect the experimental data. Sodium Taurocholate (#01024356, Purity > 97 %):Purchased from Abcam Ltd.(Basel, Switzerland). Isoflurane(S10010533): Purchased from Yuyan Instruments Co.Ltd(Shanghai,China). Synthesis SuperMix for qPCR (G3337), 2 × SYBR Green qPCR Master Mix (G3320), Trizol, BCA Protein Quantitation Assay Kit (G2026), Ultra-sensitive ECL Chemiluminescence Kit (G2020-25ML): Obtained from Servicebio (Wuhan, China).Primary Antibodies: NF-κB p65 (GB11997), p-NF-κB p65 (GB113388), Keap1 (GB113747), Nrf2, HO-1 (GB12104), COX-2 (GB11077-1), TNF-α (GB13452), ACTIN (GB12001), purchased from Servicebio (Wuhan, China); p-IκBα (#2859) from Cell Signaling Technology (Massachusetts, USA); IκBα (10268–1-AP) from Proteintech Group, Inc. (Wuhan, China); Nrf2 (AF7006) from Affinity Biosciences.

2.2. Animals

28 Male Sprague-Dawley (SD) rats (8 weeks old, 200 ± 20 g) were provided by Sippr-BK laboratory animals Co.Ltd.(Shanghai, China, animal License: NO. SCXK(Shanghai)2020–0009). Rats were individually housed in the SPF-level breeding room with controlled environmental conditions: The temperature is maintained at 20–26 °C, relative humidity at 50 %-70 %, and a 12-hour light/dark cycle. Normal chow diet and sterile water are provided. All animal procedures were reviewed and approved by the experimental animal center of Shanghai university of traditional Chinese Medicine(Registration number: PZSHUTCM2306150003)

2.3. Animal experiments and treatment

To study the effects of bufalin on NaT-SAP rats, all rats were randomly divided into four groups.

(A)The Sham group(Sham, n = 7), in which the rats were given an intraperitoneal injection of the vehicle control;

(B)NaT-SAP group(NaT-SAP, n = 7), in which the rats were treated with the same amount of vehicle;

(C)BF+NaT group(0.1 mg/kg.BW, n = 7), where the rats were intraperitoneal injection of 0.1 mg/kg solution of bufalin;

(D)BF+NaT group (0.2 mg/kg.BW,n = 7), where the rats were intraperitoneal injection of 0.2 mg/kg solution of bufalin.The NaT-SAP models have been set up as described previously [26]. 3.5 % NaT (1 ml/kg.BW) was retrogradely injected into the biliary-pancreatic duct at a rate of 100 μL/min to induce severe acute pancreatitis (SAP) models. Refer to Supplementary Fig. S1 for a schematic diagram of the model induction process. The experimental design is shown in G 1B and involved intraperitoneal injections administered once daily for 7 consecutive days. Sham and NaT-SAP groups were administered the same quantity of vehicle solution. (containing less than 0.5 % v/v DMSO), while the treatment groups were given BF (0.1 mg/kg or 0.2 mg/kg.BW) solution. Tissue histology and biochemical analysis were conducted 24 h post-surgery, with pancreatic and blood samples collected.

2.4. Pancreatic function and inflammatory factors analysis

Blood samples were collected from the abdominal aorta after surgery and centrifuged at 4 °C and 4000 rpm for 10 min. The colorimetric method was used to determine the serum concentrations of Amy (CO16-1), LPS (A045-1), MDA (A003-1–2), GSH (A006-2–1), and SOD (A001-3–2) (Nanjing Jiancheng Bioengineering Institute, Nanjing, China), this were espressed as U/dL, U/L, nmol/mL, μmol/mL, U/mL. TNF-α(ER330103), IL-6(ER330219) and IL-1β(ER330206) level were using the commercially available immunoassay ELISA kits(Shanghai Weiao Biotechnology, Shanghai, China). The results are expressed as pg/mL.

2.5. Histopathology staining

Remove the pancreatic head and immediately immerse it in 10 % formalin solution for fixation. Allow it to fix for 72 h.After fixation, the tissues were embedded in paraffin according to standard procedures, and serial sections with a thickness of 40 μm were prepared. Perform dehydration and clearing of the paraffin-embedded tissue sections using xylene to remove wax and prepare them for staining. Stain the tissue sections with hematoxylin and eosin for visualization under the optical microscope. This staining helps to distinguish cellular structures and assess tissue morphology. Mount the stained tissue sections on slides and examine them under an optical microscope at 200 × magnification. Images of pancreatic tissue were captured and examined blind by 2 morphologists for grading the histological alterations. According to the semiquantitative scoring system [27] the pancreatic tissue damage score is shown in Table 1 were as follows:

2.6. Immunohistochemitry for TNF-α and COX-2

The immunohistochemical analysis of the expression of TNF-α and COX-2 was performed according to the instructions of the manufacturer. After extracting the antigen with citric acid antigen retrieval buffer (pH 6.0), incubate in 3 % H2O2 solution for 25 min at room temperature in the dark, and wash three times with PBS (pH 7.4). The samples were incubated with goat serum for 30 min at room temperature. Add TNF-a

Table 1
Detailed rules for pathological scoring of pancreatic tissue.

scores	edema	inflammation	necrosis
0	no edema	no inflammation	no necrosis
1	confined to the lobules	Infiltration area < 25 %	localized parenchymal necrosis
2	diffuse among the lobules	Infiltration area 25 %-50 %	< 20 % necrosis
3	widely separated lobules	Infiltration area 51 %-75 %	20 %-50 % necrosis
4	gross lobular separation	Infiltration area > 75 %	> 50 % necrosis

primary antibody (1:100 dilution) and incubate samples overnight at 4 °C. Wash samples with PBS three times, then stain with HRP-goat anti-rabbit (1:200) for 50 min. For staining, after adding diaminobenzidine solution, hematoxylin solution was counterstained, after dehydration, the slides were mounted and examined through microscope.(CIC, XSP-C204). Analyzing the percentage of positive cells by TNF-α and COX-2 immunohistochemical staining in pancreatic tissue using Aipathwell software.

2.7. Western blot

Take pancreatic tissue stored at −80 °C, cut into pieces with ophthalmic scissors, homogenise, add RIPA lysis buffer, centrifuge, extract and quantify protein by BCA method. Take protein sample, mix with loading buffer, denature in metal bath, centrifuge and electrophoreze, transfer to PVDF membrane, add blocking solution to block for 1 h, wash membrane with TBST and seed with rat NF-κB p65, p-NF-κB p65, IκBα, Keap1, Nrf2, HO-1 antibodies (1:1000), refrigerate at 4℃ overnight, wash membrane with TBST, mix secondary antibody (1:5000), incubate at room temperature for 2 h and and then ECL Western blotting reagent was used to detect the protein strips. Protein bands were visualized with a gel imager, and protein intensity was quantified with AIWBwellTM analysis software and corrected with β-actin measurements.

2.8. RNA isolation and quantitative Real-Time PCR

Take 100 mg of pancreatic tissue and grind it into powder in a pre-cooled grinding bowl. Trizol, chloroform, isopropanol, and 75 % ethanol were added in sequence to extract total RNA. A spectrophotometer was used to measure RNA concentration and purity. Then, Synthesis SuperMix for qPCR and 2 × SYBR Green qPCR Master Mix were used to measure various mRNAs using GAPDH as an internal control. Relative mRNA expression was evaluated using the $2^{-\Delta\Delta Cq}$ method. The primers used are shown in Table 2.

2.9. Statistical analysis

IBM SPSS Statistics 25.0, Adobe Illustrator 2022 and GraphPad Prism 9.0 and were used for data analysis and image processing. The results of each group are expressed as mean ± SEM. The data that satisfied the normal distribution of each group were subjected to a test for homogeneity of variances. If the variances are homogeneous, One-Way ANOVA is employed, with pairwise comparisons carried out via the LSD method. If the variances are not homogeneous, Welch's ANOVA is used and pairwise comparison is performed by Dunnett's T3 test. $P<0.05$ indicates that the difference is statistically significant.

3. Results

3.1. Bufalin concentration-dependently ameliorated pancreatic damage in NaT-SAP rats

We investigated the therapeutic efficacy of BF in NaT-SAP rats.This model is a commonly used to study biliary acute pancreatitis and associated multi-organ damage. It has high success rate and good stability, and its pancreatic tissue lesions more closely resemble clinical features [28]. Aftering NaT injection, we observed severe congestion and edema of the pancreas (Fig. 1C). Gross morphological observation showed that the pancreatic tissue in the NaT-SAP had obvious necrotic, dedma, and hemorrhage accompanied by saponification spots, which was significantly reduced in the BF+NaT group (Fig. 1D). To further study the effect of BF on pancreatic tissue damage, we performed HE staining on pancreatic tissue. The pancreatic acini in NaT-SAP group were significantly more edematous, massive patchy necrosis and mixed inflammatory cell infiltrates (Fig. 1F). Histopathological scoring for edema, necrosis, and inflammatory infiltration indicated a significant exacerbation of pancreatic tissue injury in NaT-SAP rats. However, BF treatment dose-dependently reduced pancreatic damage (Fig. 1F, Fi).

Serum Amy and LPS levels exceeding 3 times the upper limit of normal are important indicators for diagnosing acute pancreatitis clinically [29,30]. In this study, compared with the Sham group, NaT-SAP led to a 3.2-fold increase in serum Amy and a 7.7-fold increase in LPS. However, BF (0.1 mg/kg, BW) reduced Amy and LPS levels by 24 % and 25 % respectively; BF (0.2 mg/kg, BW) reduced Amy and LPS levels by 47 % and 65 % respectively.It can be seen that BF treatment concentration-dependently reversed the increase in thses indicators (Fig. 1E).

3.2. Bufalin treatment alleviated inflammatory responses in NaT-SAP rats

Inflammatory mediators and cytokines are key effectors of AP that aggravate tissue damage and mediate systemic inflammation [31]. we used immunohistochemistry to detect TNF-α and COX-2 expression in pancreatic tissue. Compared with the Sham group, the NaT-SAP group had a large accumulation of TNF-α and COX-2, and the number of positive cells increased significantly (Fig. 2.B,C,Bi,Ci).

In addition, we used ELISA to further determine the production of inflammatory cytokines TNF-α, IL-6, and IL-1β in serum. The NaT-SAP group had a great increase in serum TNF-α, IL-6, and IL-1β levels in comparison with the sham group. (Fig. 2.A). The number of TNF-α and COX-2 positive cells in pancreatic tissue decreased significantly after BF treatment. Consistently, serum levels of TNF-α, IL-6 and IL-1β were also significantly reduced. These results confirm that BF can attenuate the inflammatory response in NaT-SAP rats.

Table 2
Primer sequences for qRT-PCR.

Gene	Forward	Reverse
Keap1	GAGATATGAGCCAGATCGAGACG	GGTGTAATCATCCGCCACTCAT
Nrf2	GACATCCTTTGGAGGCAAGACAT	TGGGAATGTGGGCAACCTG
HO-1	CAGCATGTCCCAGGATTTGTC	CCTGACCCTTCTGAAAGTTCCTC
IκBα	TCGTGGAGCACTTGGTGACTT	GTAGCCCTGGTAGGTTACTCTGTTG
GAPDH	CTGGAGAAACCTGCCAAGTATG	GGTGGAAGAATGGGAGTTGCT
Keap1	GAGATATGAGCCAGATCGAGACG	GGTGTAATCATCCGCCACTCAT

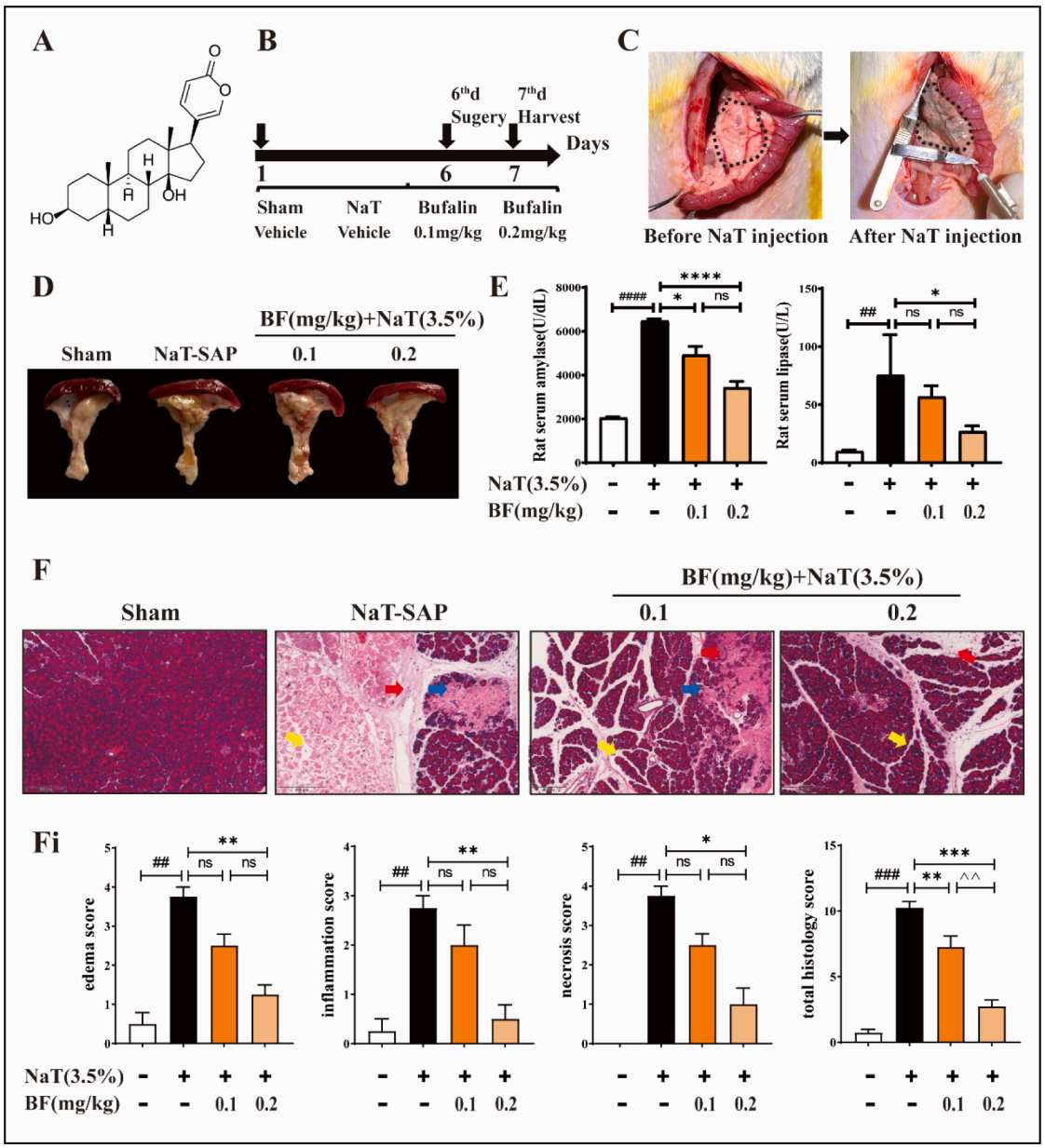

Fig. 1. BF attenuates serum biochemical and pancreatic histopathologic changes in NaT-SAP rats. (A) Chemical structure of bufalin.(B) The experimental procedure. (C) Changes in the pancreas before and after NaT injection. The black area indicates pancreatic edema. (D) Gross morphological changes of freshly harvested pancreatic tissue. (E) Changes in serum Amy and LPS activities of rats in each group. (F) H&E images of pancreas. Scale bar (H&E) = 200 μm, red arrow indicates inflammation, yellow arrow indicates edema, and blue arrow indicates necrosis. (Fi) Histopathologic scores of pancreatic tissue. The results of each group are expressed as mean ± SEM (n = 6–7). $p < 0.01$(##), $p < 0.001$(###) and $p < 0.0001$(####) vs Sham group; $p < 0.05$(*), $p < 0.01$(**), $p < 0.001$(***) and $p < 0.0001$ (****) vs NaT-SAP group; $p < 0.001$({\,}^^) vs BF (0.1 mg/kg) group; $p > 0.05$(ns).

3.3. Bufalin treatment alleviated oxidative strress in NaT-SAP rats

Many previous works have shown that there is excessive oxidative stress in SAP rats. MDA is usually taken as an indicator of the extent of oxidative injury, with elevated levels indicating insufficient antioxidant defense capacity in the body. GSH and SOD play crucial roles in combating oxidative stress, contributing to maintaining oxidative balance within cells [32,33]. Therefore, we assessed changes in oxidative stress parameters in rat serum. In comparison with the sham group, GSH and SOD levels were greatly reduced in the NaT-SAP group, and MDA levels were greatly increased. BF pretreatment significantly ameliorated the oxidative stress state, mainly manifested by reducing the consumption of SOD and GSH, and reducing the production of MDA (Fig. 3A).

3.4. The effects of bufalin pre-treatment on the Keap1-Nrf2/HO-1 pathway in NaT-SAP rats

To evaluate the regulatory actions of BF on oxidative stress, we focused on the Nrf2/HO-1 pathway. The Nrf2/HO-1 pathway is regarded as the major endogenous antioxidant pathway in the body [34]. Further evidence shows that the activation of Nrf2/HO-1 contributes to the alleviation of damage caused by oxidative stress [35–37]. Therefore, we investigated the potential mechanism of BF in regulating the Nrf2/HO-1 pathway in NaT-SAP rats. We found that Keap1, Nrf2, and HO-1 mRNA expression levels in pancreatic tissue of NaT-SAP group were slightly higher than that of Sham group. Compared with the NaT-SAP group, BF (0.2 mg/kg.BW) pretreatment greatly up-regulated Nrf2 and HO-1 mRNA expression and down-regulated Keap1 mRNA expression

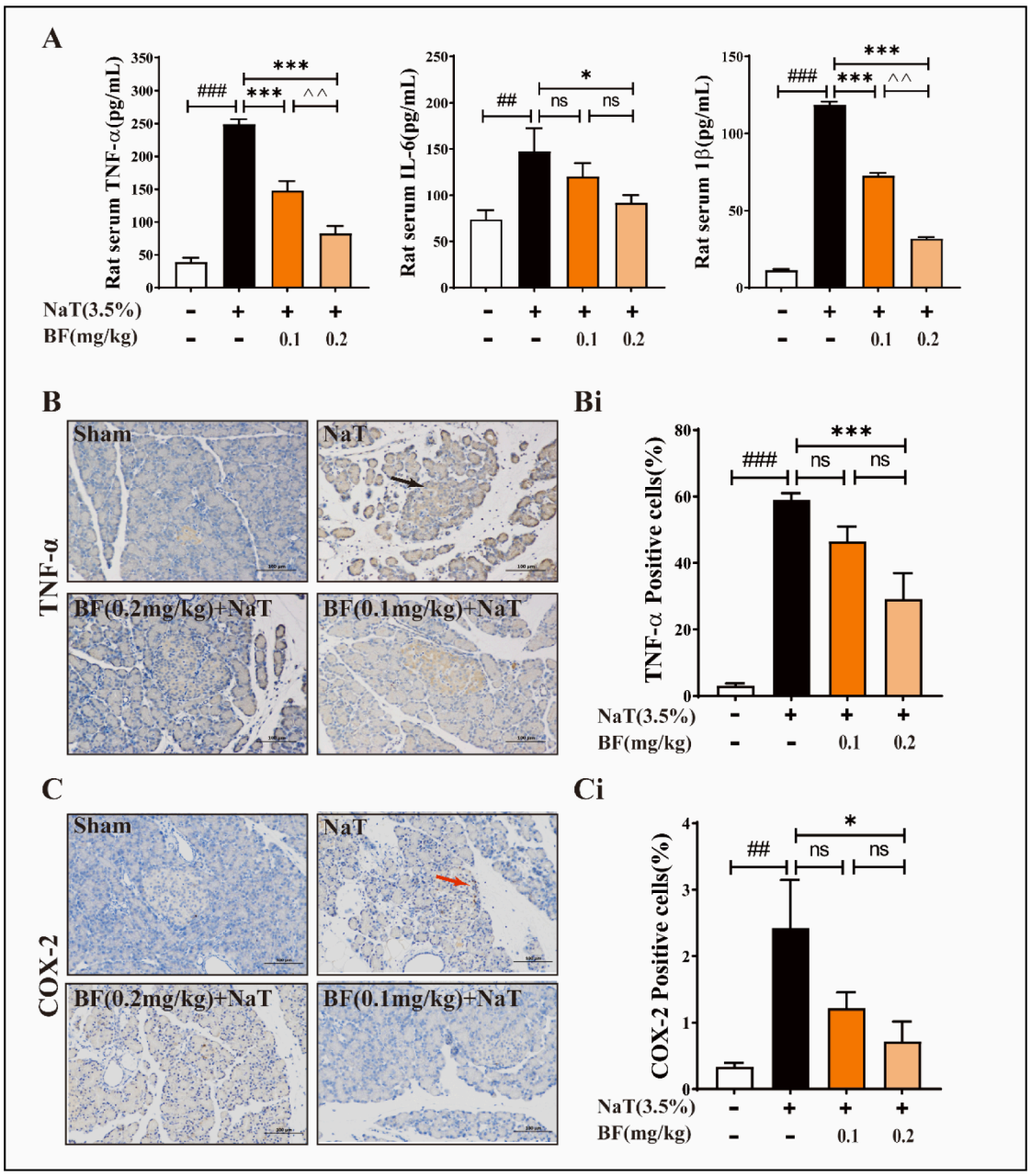

Fig. 2. BF reduces inflammatory response in NaT-SAP rats. (A) Changes in serum inflammatory factors of rats in each group. (B-C)Immunohistochemical analysis of TNF-α and COX-2 expression in pancreatic tissues. Magnification = 200 × . Scale bar = 100 μm. (B-I) Percentage of TNF-α and COX-2 Positive Cells in Pancreatic Tissue. The results of each group are expressed as mean ± SEM (n = 6–7). $p < 0.01$(##), $p < 0.001$(###) vs Sham group; $p < 0.05$(*), $p < 0.001$(***) vs NaT-SAP group; $p < 0.01$({\,}^^), $p < 0.001$({\,}^^^) vs BF (0.1 mg/kg) group; $p > 0.05$(ns).

(Fig. 4.A). In addition, similar to the qRT-PCR results, Western blot analysis showed that BF (0.2 mg/kg.BW) pretreatment greatly increased Nrf2 and HO-1 expression, whereas Keap1 expression was inhibited (G 4B,Bi). The data demonstrate that BF may mitigate oxidative stress-induced damage in NaT-SAP rats through activating the Keap1-Nrf2/HO-1 pathway.

3.5. The effects of bufalin pre-treatment on the NF-κB pathway in NaT-SAP rats

To determine the potential mechanism of BF on the inflammatory response in NaT-SAP rats, we used qRT-PCR and Western blot to evaluate the expression of NF-κB pathway-related inflammatory factors in pancreatic tissue. Compared to the sham group, NaT-SAP led to a great increase in NF-κB p65 and a great decrease in IκBα within rat pancreatic tissue. BF pretreatment reversed the above mRNA expression (Fig. 5.A). p-IκBα controls NF-κB p65 activity and nuclear translocation. Therefore, We examined p-IκBα at the protein level and found that bufalin pre-treatment pretreatment greatly suppressed the p-NF-κB p65 and IκBα expression (Fig. 5.B, Bi, C, Ci). In conclusion, these results further confirm that bufalin may exert anti-inflammatory effects on NaT-SAP rats by inhibiting the NF-κB pathway.

4. Discussion

In this study, we found that BF treatment reduced serum levels of Amy and LPS in NaT-SAP rats, restored the pro-inflammatory/anti-inflammatory balance, alleviated oxidative stress state and

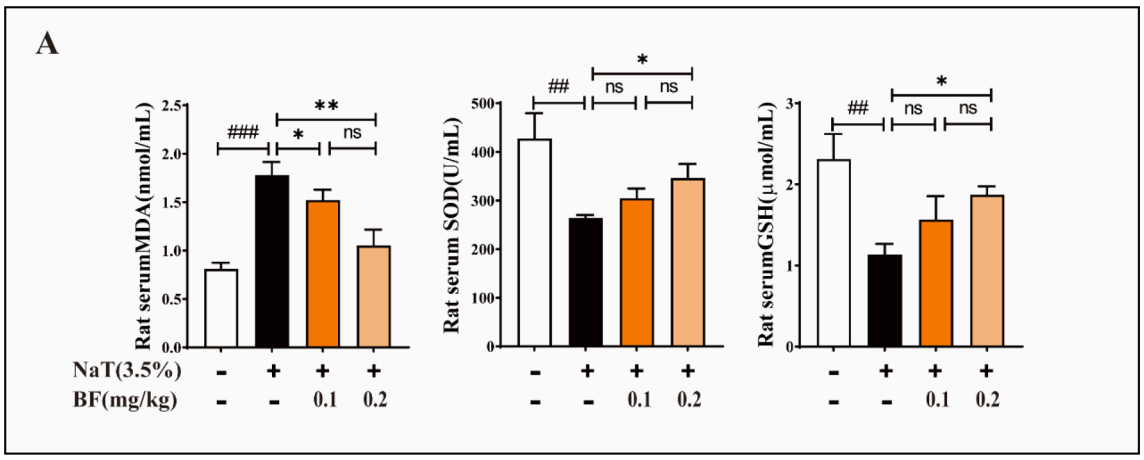

Fig. 3. Effect of BF on serum oxidative parameters in SAP rats. (A) Changes in serum MDA, SOD and GSH levels of rats. The results of each group are expressed as mean ± SEM(n = 6–7). $p < 0.01$(##), $p < 0.001$(###) vs Sham group; $p < 0.05$(*), $p < 0.01$(**) vs NaT-SAP group; $p > 0.05$(ns).

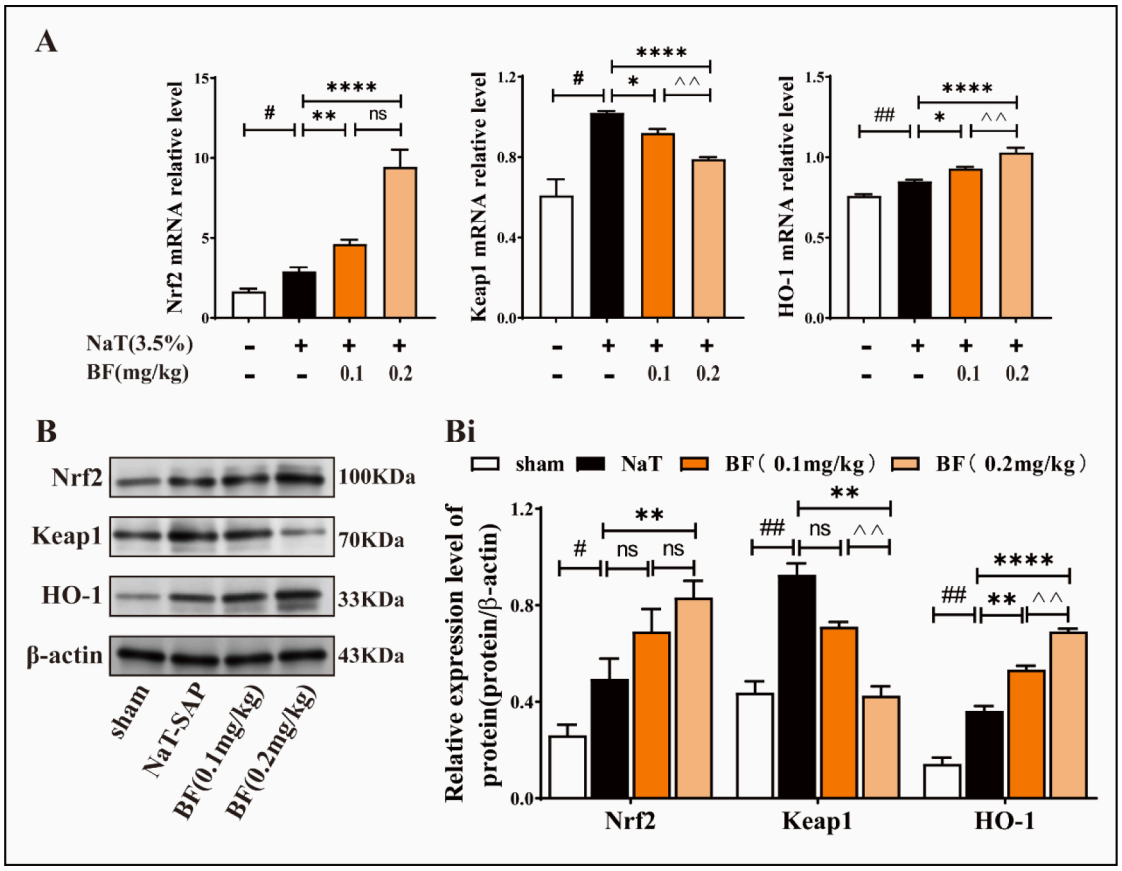

Fig. 4. The Effects of Bufalin pre-administration on the Nrf2/HO-1 Pathway in NaT-SAP rats. (A) The mRNA expressions of Keap1, Nrf2 and HO-1 in pancreatic tissues were measured by using qRT-PCR. (B) Protein levels of Keap1, Nrf2 and HO-1 in pancreatic tissue were analyzed by Western blot. β-Actin used as internal reference. (Bi)Protein quantification of Keap1, Nrf2 and HO-1 by using ImageJ. The results of each group are expressed as mean ± SEM (n = 4). $p < 0.05$(#) and $p < 0.01$(##) vs Sham group; $p < 0.05$(*), $p < 0.01$(**) and $p < 0.001$(***) vs NaT-SAP group; $p < 0.01$({\,}^^) vs BF (0.1 mg/kg) group; $p > 0.05$(ns).

pathological damage in pancreatic tissue. Its protective effect against NaT-SAP may be associated with the inhibition of NF-κB and activation of the Keap1-Nrf2/HO-1 signaling pathway (Fig. 6.).

The retrograde injection method into the bile-pancreatic duct was first proposed by Aho [38]. This model simulates the reflux of bile and pancreatic fluid into the pancreatic duct when the distal common bile duct is blocked. It primarily simulates the pathogenesis of gallstone-induced acute pancreatitis, which accounts for over 50 % of acute pancreatitis cases, so this method is widely used [28]. In our study, NaT caused edema, necrosis, and inflammatory cell infiltration in pancreatic tissue, which is consistent with the results of the current study [39]. BF can alleviate the pathological features of pancreatic tissue, reduced the pathological scores, and alleviated pancreatic injury in NaT-SAP rats.

Serum Amy and LPS are classical markers for diagnosing AP [40]. This is because pancreatic cells are attacked by inflammatory cytokines and digestive enzymes in the early stages of SAP, leading to acinar cell

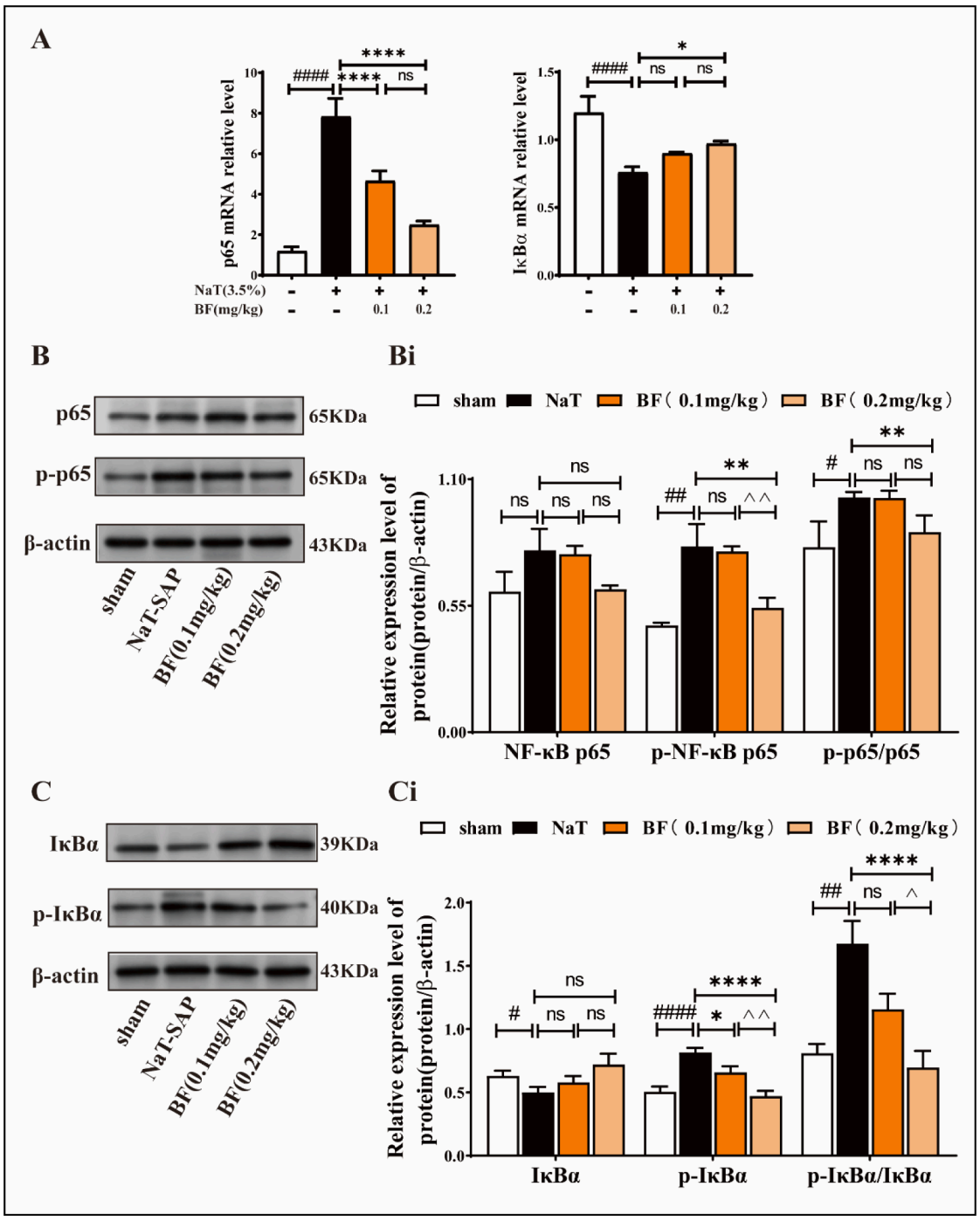

Fig. 5. The Effects of Bufalin pre-treatment on the NF-κB Pathway in NaT-SAP rats. (A) The mRNA expressions of IκBα, NF-κB p65 in pancreatic tissues were measured by using qRT-PCR. (B-C) Protein levels of NF-κB p65, p-NF-κB p65, IκBα and p-IκBα in pancreatic tissue were analysed by Western blot. β-Actin was used as an internal reference. (Bi-Ci) Protein quantification of NF-κB p65, p-NF-κB p65, IκBα and p-IκBα by using ImageJ. β-Actin used as internal reference. The results of each group are expressed as mean ± SEM (n = 4). $p < 0.05$(#) and $p < 0.01$(##) vs Sham group; $p < 0.05$(*), $p < 0.01$(**) and $p < 0.001$(***) vs NaT-SAP group; $p < 0.01$({\,}^^) vs BF (0.1 mg/kg) group; $p > 0.05$(ns).

injury and disruption of cell membranes. This results in the release of damage-associated molecular patterns (DAMPs), promoting the release of chemokines and cytokines [41]. More basic experiments and clinical studies have shown that serum Amy and LPS levels increase by at least three times during AP [30]. In our study, NaT caused a 3.2-fold increase in serum Amy and a 6.8-fold increase in LPS in NaT-SAP rats. However, this change was significantly reversed by BF, which means that BF may exert its effects in treating SAP by reducing these enzyme levels.

In studies of the pathogenesis of AP, regardless of the initiating factors, pancreatic acinar damage is accompanied by overexpression of inflammatory factors such as TNF-α, COX-2, and platelet activating factor (PAF) [42,43]. the release of these inflammatory factors is the beginning of local inflammatory response. In the absence of intervention, the inflammatory cascade will eventually lead to a multiple organ dysfunction syndrome or organ failure [44]. Therefore, blocking the inflammatory cascade as early as possible is crucial for the prognosis of AP [45]. Previous studies have shown that BF has impressive anti-inflammatory properties in a gentamicin (GM)-induced acute kidney

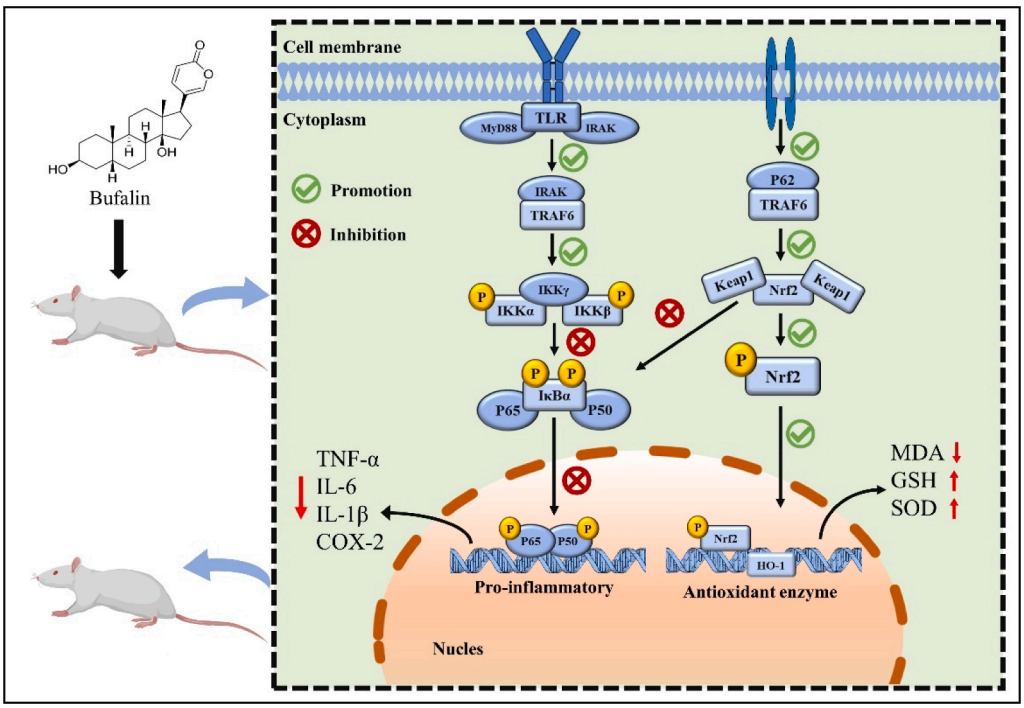

Fig. 6. BF alleviates experimental acute pancreatitis by regulating the NF-κB and Keap1-Nrf2/HO-1 signalling pathways.

injury model and an ovalbumin (OVA)-induced mouse asthma model [23,24]. In our study, BF also exerted a remarkable anti-inflammatory property in NaT-SAP rats, as evidenced by the reduction of TNF-α, IL-6, IL-1β, and COX-2 levels. The molecular mechanisms underlying the anti-inflammatory activity of BF were initially identified in mouse models as mediated by the NF-κB pathway. NF-κB is one of the key signaling pathways that determines the inflammatory response. In this study, we observed that BF remarkably downregulated NF-κB p65 expression. Further studies showed that BF pretreatment greatly suppressed the p-NF-κB p65 and IκBα expression. In sum, these findings confirm that the inhibitory actions of BF on the inflammatory response in NaT-SAP rats are achieved through blocking the NF-κB pathway and key cytokines.

Oxidative stress is a key factor in the pathogenesis of AP, and its reactive oxygen species can exacerbate inflammation and damage surrounding tissues. MDA is the most commonly used indicator for the detection of lipid peroxides in cells and tissues, and plasma MDA levels are considered an important indicator for assessing the severity of AP [46]. GSH and SOD can be used as markers to evaluate anti-oxidative stress. Our research results demonstrate that the application of BF reduces MDA content and decreases the consumption of GSH and SOD. To further elucidate the upstream mechanism of BF regulation of oxidative stress, we focused on Nrf2. Nrf2 is the key player in regulating the antioxidant stress response. During oxidative stress, Nrf2 separates from Keap1 and adjusts the redox state through the expression of the downstream antioxidant enzyme HO-1 [47]. Therefore, Nrf2/HO-1 is considered to be the most crucial endogenous antioxidant pathway [34]. More evidence suggests that Nrf2/HO-1 activation contributes to the alleviation of oxidative stress damage [35,36]. In fact, in our study, BF pretreatment further promoted Nrf2 and HO-1 expression and downregulated Keap1 expression. Therefore, these findings reveal that BF contributes to enhance the ability of cells to resist oxidative stress and reduce tissue inflammatory response by activating the Keap1-Nrf2/HO-1 signalling pathway.

Currently, it is well established that maintaining the balance of cellular Nrf2 and NF-κB is essential for resisting inflammation and oxidative stress damage. Dysregulation of the Nrf2 and NF-κB pathways has been associated with several diseases, including neurodegenerative, autoimmune, and cancer [48]. In this work, we report that BF exerts anti-inflammatory and antioxidant effects in NaT-SAP rats through downregulation of Keap1 expression, upregulation of Nrf2 and HO-1 expression, and inhibition p-NF-κB p65 and p-IκBα expression.

To the best of our knowledge, this is the first investigation of the therapeutic effect of BF in NaT SAP rats. The results indicated that BF may be a prospective therapeutic agent for SAP patients. However, the study still has certain limitations. BF is a highly active toxic ligand extracted from the traditional Chinese medicine Chan Su, and the effect of the drug itself on rats has not yet been studied. Therefore, in addition to paying attention to effectiveness, we should also pay attention to its safety issues. According to the information we currently have, there is still a lack of systematic drug safety evaluation. In the future, we will further carry out the drug safety evaluation of BF.

5. Conclusion

In conclusion, we used BF to study its effect in NaT-SAP rats for the first time, and found that BF inhibited inflammation and oxidative stress and alleviated the disease progress in NaT-SAP rats by inhibiting NF-κB and activating the Keap1-Nrf2/HO-1 pathway. Based on these findings, BF may become new clinical option for SAP patients.

Author contributions

The experiment was conceived and designed by GTY and XLN. XLN, WS, JLC, HQZ, and GMY conducted experiments. XLN, HXT, HQZ, and GMY performed result processing and statistical analysis. XLN wrote the manuscript and GTY reviewed it. All authors agree to be responsible for all aspects of the work and to ensure its completeness and accuracy.

Funding

This work was supported by the Science and Technology Commission of Shanghai Municipality(Shanghai, China)(NO. 22S21901300).

CRediT authorship contribution statement

Xiaolong Niu: Writing – original draft, Visualization, Validation, Supervision. **Wei Sun:** . **Xiaohang Tang:** . **Jialiang Chen:** . **Huaqun Zheng:** . **Guimei Yang:** . **Guangtao Yao:** Writing – review & editing, Visualization, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data involved in this study are available in the supplementary material; further requests can be directed to the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2024.113113.

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