

Original Article

Quantitation of nuclear factor kappa B activation in pancreatic acinar cells during rat acute pancreatitis by flow cytometry

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Abstract: This study aimed to develop a specific and sensitive method for the rapid detection of NF- κ B activity in pancreatic tissue. Male Wistar rats were randomly divided into two groups: (1) 16 rats in the acute pancreatitis (AP) group received retrograde injection of 5% sodium taurocholate (STC) into the biliopancreatic duct, and (2) 16 rats in the Control group received saline. NF- κ B activation in rat pancreatic acinar cells was assessed by flow cytometry (FCM). We found that the NF- κ B activity in the AP group significantly increased at 1.5 h (29.80% \pm 7.83), had a peak at 3 h (65.17% \pm 13.22), and then decreased gradually to 12 h time point, close to the level after 1.5 h stimulation of STC. The NF- κ B activity of the Control group did not significantly vary at different time points ($P>0.05$). FCM is a specific and sensitive assay for the rapid detection of NF- κ B activity in pancreatic tissue.

Keywords: Acute pancreatitis, nuclear factor kappa B, flow cytometry

Introduction

Nuclear factor kappa B (NF- κ B) is a ubiquitous transcription factor involved in the regulation of genes encoding many pro-inflammatory mediators such as cytokines, cell adhesion molecules, and acute phase proteins [1, 2]. This transcription factor plays important roles in sepsis, cancer, reperfusion injury, and autoimmune disease [3, 4]. NF- κ B is dimeric complex family, composed of five members of the Rel/NF- κ B family, including NF- κ B1 (p50/p105), NF- κ B2 (p52/p100), Rel A (p65), Rel B, and c-Rel. It is very notable that selective binding sites for Rel A/c-Rel heterodimers are found in the promoters of several inducible genes [5]. The most obvious characteristic of NF- κ B transcription factors are that, under most circumstances, NF- κ B lies dormant in the cytoplasm of inactive cells, combined with an inhibitory protein termed I κ B [6]. After the inhibitor I κ B has been inactivated by one of many extracellular signals (e.g., oxygen free radical, lipopoly-

saccharide, TNF- α), NF- κ B translocates rapidly from the cytoplasm to the nucleus, thereby binding to *cis*-acting κ B sites in promoters and enhancers of defense-related genes bearing the conserved consensus sequence 5'-GGGRNNYYCC-3', where N is any base, R is a purine, and Y is a pyrimidine. These transcriptionally regulated genes are involved in inflammation, embryonic development, tissue injury, and repair [7-9].

Acute pancreatitis (AP) is a common inflammatory disease of the pancreas and ranges from mild to severe. In recent years, studies have demonstrated that NF- κ B is involved in the onset of AP, and has captured the focus of many scientists [10-14]. Intense efforts have been devoted to elucidate the role of NF- κ B and I κ B during development and progression of AP [15, 16]. With a rapid, objective, and multi-parameter analytic features, flow cytometry (FCM) offers the prospect of rapid detection of NF- κ B activity in cells from the body's organs [17]. This

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study aimed to develop a specific and sensitive method which can be used to quantify NF- κ B DNA binding capacity in rat pancreatic acinar cells.

Materials and methods

Reagents

Sodium pentobarbital, sodium taurocholate (STC), Triton X-100, and fluorescein isothiocyanate (FITC)-conjugated anti-human monoclonal antibody were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Cycletest™ Plus DNA Reagent Kit was purchased from Becton Dickinson (BD Bioscience, MD, USA). Rabbit anti-rat monoclonal NF- κ B p65 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Additionally, all other reagents were purchased from Nanjing Jiancheng Biological Engineering Co, Ltd. (Zhejiang, China).

Animals

Male specific pathogen-free Wistar rats (weight, 255 ± 20 g) were obtained from the Experimental Animal Center of Shanxi Medical University (Shanxi, China). All animal procedures were approved by the ethics committee of Shanxi Medical University (Shanxi, China) and performed in compliance with the EC regulations and the NIH standards (Guide for the Care and Use of Laboratory Animals, NIH publication, 85-23, revised 1996).

Animal grouping

Thirty two animals were randomly divided into two groups, the AP group ($n=16$) and the control group ($n=16$).

Induction of AP

The AP model was induced according to Gulbenk et al. [18], with improvements. Twelve hours prior to the start of the experiment, rats were deprived of food but allowed access to water ad libitum. The rats were anesthetized by intraperitoneal injection of 3% sodium pentobarbital (40 mg/kg body weight). The biliopancreatic duct was reached and cannulated by penetrating into the duodenum with a 24-gauge catheter after clamping the hepatic duct with a noninvasive Angiocath. The sixteen animals in the AP

group were laparotomized, and 5% sodium STC (1 ml/kg body weight) was injected at a rate of 0.07 ml/min into the common biliopancreatic duct. After the abdomen was closed, the rats recovered from the anesthetic and were allowed access to water. The control group was injected with saline instead of sodium STC. Four rats from each group were randomly sacrificed at 1.5 h, 3 h, 6 h and 12 h. The head of the pancreatic tissues were obtained carefully from the rats. Blood samples were obtained by direct intracardiac puncture.

Serum amylase assay

Serum amylase (AMY) activity was measured using an automatic biochemistry analyzer (Olympus Optical Co., Ltd., Tokyo, Japan).

Histopathological examination

Microscopic pancreatic damage was pronounced in 6 h after the induction of AP. A portion of the pancreatic head tissue was removed gently and fixed with 10% buffered formalin. Four continuous sections of paraffin-embedded pancreatic tissue were stained with hematoxylin and eosin (HE). Using a light microscope (Olympus Optical Co., Ltd., Tokyo, Japan), a single pathologist blinded to the group evaluated pancreatic injury using the Schmidt Scoring Criterion [19].

Single nuclear suspension preparation

Nuclear suspension was prepared as previously described [20]. Briefly, Fresh pancreatic tissue (0.5 g) was cut approximately into 1.0 mm^3 fragments with a scissors, and put into Falcon tubes ($12 \times 75 \text{ mm}$). After adding Tris-HCl buffer solution (1 M Tris Base, pH 7.6) immediately, samples were centrifuged 300-400 g for 5 min. The supernatant was discarded, and 10 mL of 1% Triton X-100 detergent solution was added to pellet, and the pieces were stored at 4°C refrigerator for 8 h [20]. The resulting homogenized suspension contained free-floating nuclei, were gently removed to the Cell Strainers. The Cell strainers were made of a strong nylon mesh with 40 micron pores. The primary fragments were disassociated, and the single nuclear suspension from pancreatic tissues was obtained. The total suspension volumes based on the sample density, were between 2 ml and 4 ml [21]. Cell nuclei were

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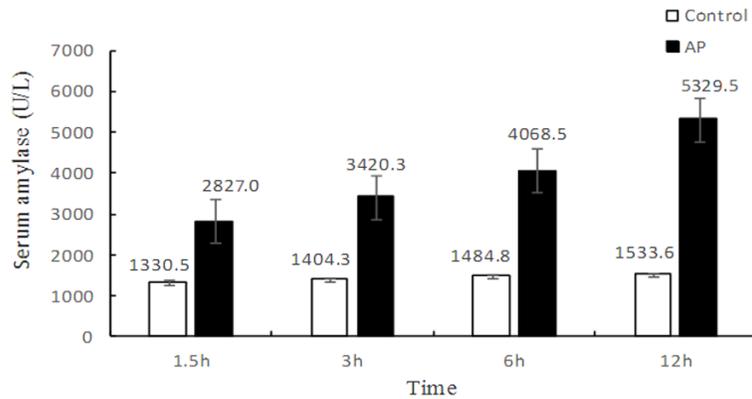


Figure 1. Time course of serum amylase in rats. Serum amylase level was markedly higher in the acute pancreatitis group compared to the control group, $P < 0.01$.

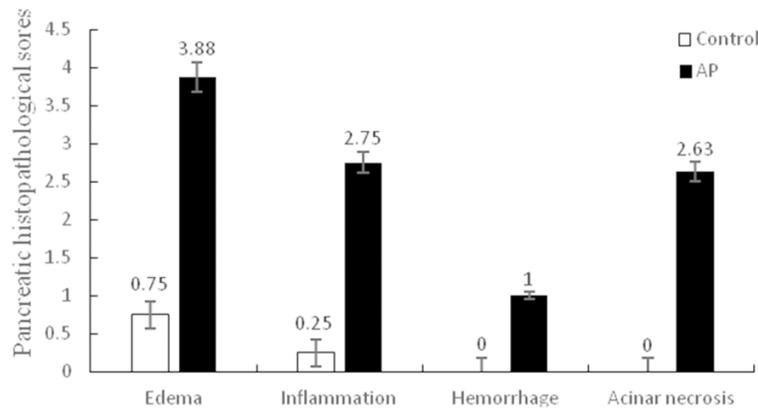


Figure 2. Histopathological scores of pancreatic tissue in two groups. Scores were based on edema, inflammation, hemorrhage and necrosis. $P < 0.01$ compared with the control group.

counted in phosphate-buffered saline (PBS) containing 5 g/L bovine serum albumin (BSA) at pH 7.2 after Giemsa staining and their number was adjusted to 1×10^6 /ml.

Immunofluorescent staining of NF- κ B p65

The nuclei (2×10^6) were stained using reagents contained in the Cycletest™ Plus DNA Reagent Kit according to the manufacturer's instructions with the modification of using 40 μ L rabbit anti-rat monoclonal NF- κ B p65 antibody or rabbit control antibody for a 20 min incubation at room temperature. Fluorescein isothiocyanate (FITC)-conjugated anti-human monoclonal antibody (2 μ L, 1:300) was added to the single nuclei suspension, incubated for 20 min at room temperature, and the nuclei were rinsed with washing buffer [9]. Propidium iodide (PI, 20 μ L, 5 μ g/ml) was then added and the sam-

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ple was incubated for 30 min at room temperature in dark room, then rinsed with washing buffer [22].

Flow cytometry requirements

Single nuclear sample was performed on Beckman-Coulter Elite ESP (Beckman Coulter, Fullerton, CA, USA). The instrument was equipped with four lasers and provided emission wave lengths ranging from 350 to 647 nm. PI fluorescence was detected through a 488 nm dichroic long/band pass filters. Prior to analysis, the instrument was checked for linearity with Flow-Check fluorospheres (Beckman Coulter, Fullerton, CA, USA). The droplet cell-sorting function was used to separate fluorescence positive cells from fluorescence negative cells. Each assay was done at least three times.

Flow cytometric dot plot setup

Preparation for single stained nuclear sample was detected by using the above flow cytometry. Each assay recorded 20,000 events, including the

Forward Scatter (FSC) information and the Side Scatter (SSC).

Statistical analysis

Each experiment was performed in triplicates. All results were presented as mean \pm standard deviation. Using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA), statistical analyses of data were performed by one-way ANOVA, and P values less than 0.05 were considered as significant difference.

Results

Amylase level in serum

The level of serum amylase in acute pancreatitis rats had increased significantly by 1.5 h after the induction (2827.0 ± 708.2 ; $P <$

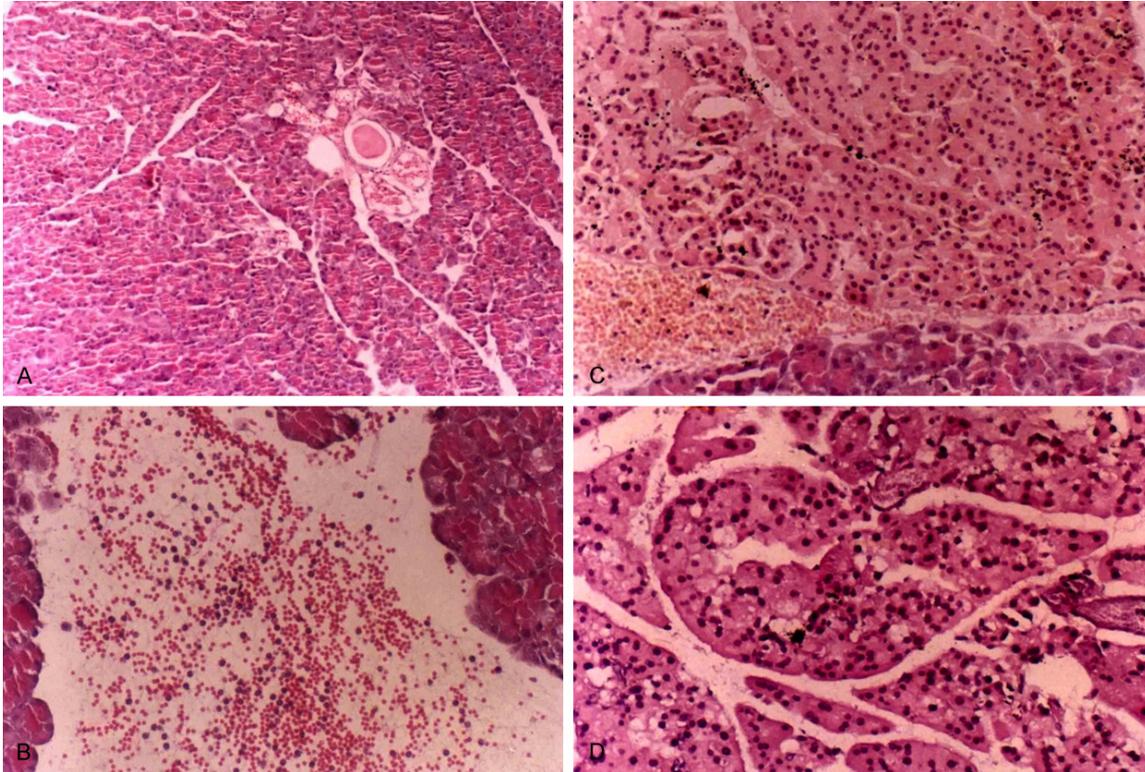


Figure 3. Morphological changes of AP. A: The control group at 6 h (HE, $\times 100$); B: STC-induced AP at 6 h (HE, $\times 200$), pancreatic interstitial and hemorrhage inflammatory cell infiltration; C: STC-induced AP at 6 h (HE, $\times 200$), pancreatic acinar degeneration; D: STC-induced AP at 6 h (HE, $\times 200$), vacuolization of acinar cells, occasionally parenchymal necrosis.

0.01), and continued to increase at 12 h (5329.5 ± 1138.5 ; $P < 0.01$) compared to that of the control group (**Figure 1**).

Pancreatic tissue histopathology

Histomorphology after STC-induced AP was analyzed using the Schmidt scoring criteria [19]. Acute pancreatitis was characterized by severe interstitial edema formation, inflammation, hemorrhage and necrosis changes. In this study, the control group showed almost normal glandular architecture, while the AP group showed obvious inflammatory cell infiltration, hemorrhage, and acinar necrosis in the pancreas tissues. The total score of the AP group was significantly higher at any time point compared with that of the control group ($P < 0.01$). Typical histological sections of pancreatic tissue are shown in **Figures 2** and **3**.

Confirmation of single pancreatic acinar nuclei

By using Giemsa staining, the dilute pancreatic acinar nuclei suspension was examined and confirmed under a light microscope (**Figure 4**).

Detection of NF- κ B p65 activity in pancreatic acinar nuclei

All nuclei were visualized on a FSC/SSC dot plot. Gate A in **Figure 5** contained typical pancreatic acinar nuclei domain and DNA in nuclei were visualized by PI staining. Gate B in **Figure 5** contained nuclei with strong PI fluorescence intensity. Gate C in **Figure 5** indicated the percentage and the peak of NF- κ B p65 positive nuclei in single pancreatic acinar nuclei. On behalf of double stained pancreatic acinar nuclei (PI and NF- κ B p65), Gate D, 2nd region in **Figure 5** indicated the presence of NF- κ B DNA binding activity. Each sample was also repeatedly measured to assess variation associated with FCM.

NF- κ B p65 activity of pancreatic acinar nuclei in rat acute pancreatitis

The percentage of pancreatic double stained positive acinar nuclei (PI fluorescence and NF- κ B p65) indicated NF- κ B DNA binding activity. FCM analysis showed that the expression of

NF-κB in pancreatic acinar cells during rat acute pancreatitis

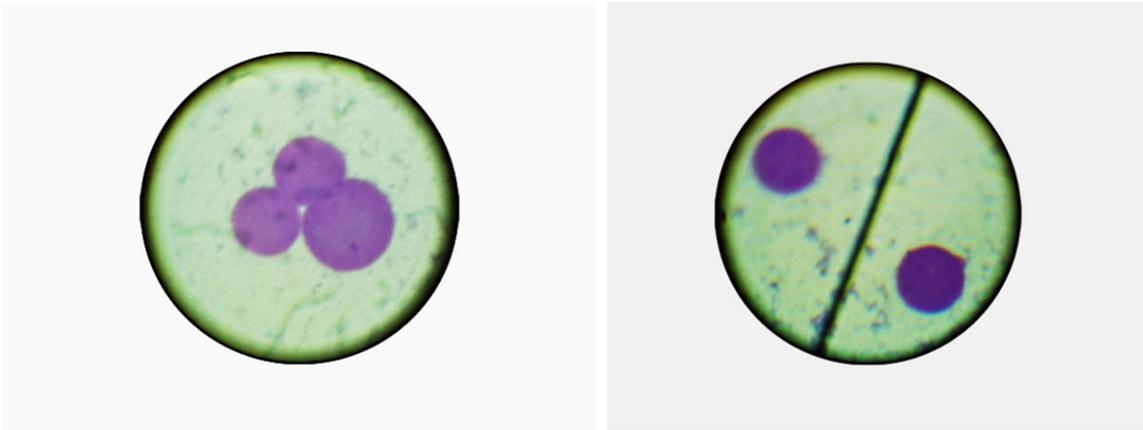
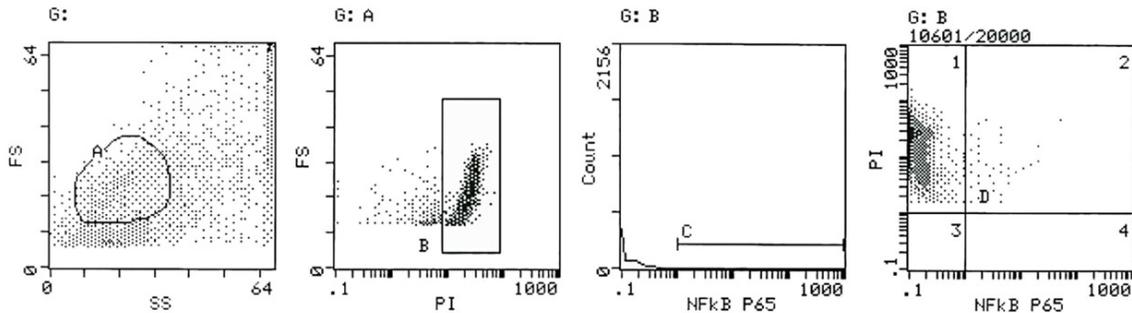


Figure 4. The dilute pancreatic acinar nuclei suspension was stained with Giemsa and was examined under a light microscope (original magnification, $\times 1000$).

Control group



AP group

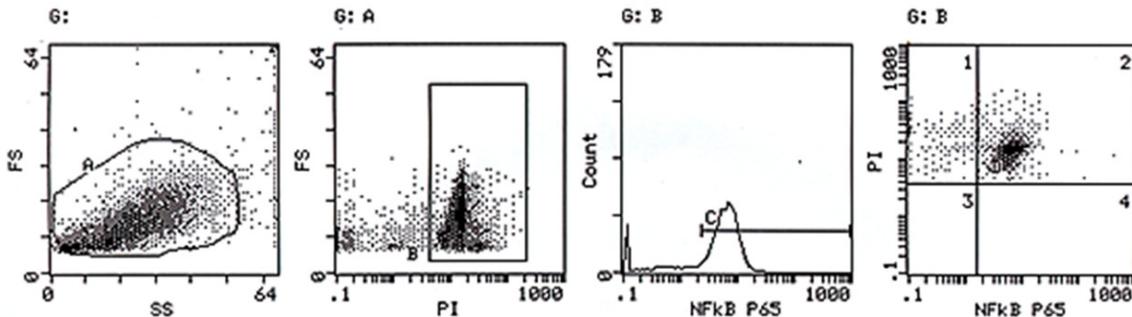


Figure 5. Flow cytometric FSC/SSC dot-blot of pancreatic acinar nuclei from rats. A: Gate A stands for typical pancreatic acinar nuclei domain. B: Gate B has strong PI fluorescence intensity. C: Gate C stands for the percentage and the peak of NF-κB p65 positive nuclei in single pancreatic acinar nuclei. D: Gate D region 2 contained the double stained pancreatic acinar nuclei (PI fluorescent markers and NF-κB p65 fluorescent markers).

NF-κB DNA binding activity was significantly higher in the AP group than that in the control group (**Figure 6**; $P < 0.01$). The pancreas of the control group showed very low DNA binding activity of NF-κB at all time points (1.5 h, 3 h, 6 h, 12 h), with no significant difference among them ($P > 0.05$). The specificity of NF-κB DNA

binding activation displayed a prominent time course in the AP group. The percentage of double positive cells increased several fold by 1.5 h ($29.80\% \pm 7.83$) after STC-induced AP, and peaked at 3 h ($65.17\% \pm 13.22$) and then decreased gradually to 12 h, close to the level after 1.5 h stimulation of STC (**Figure 7**).

NF-κB in pancreatic acinar cells during rat acute pancreatitis

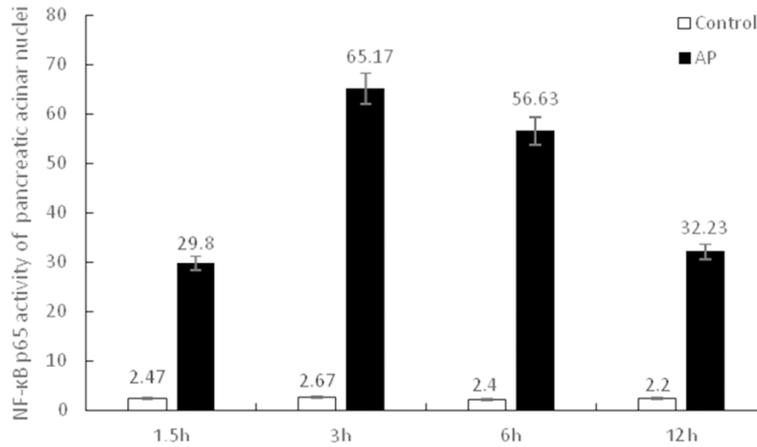


Figure 6. Percentage of pancreatic double stained positive acinar nuclei (PI and NF-κB p65 fluorescence). Data are expressed as means ± standard deviation. For statistical evaluation, $P < 0.01$, compared with the control group.

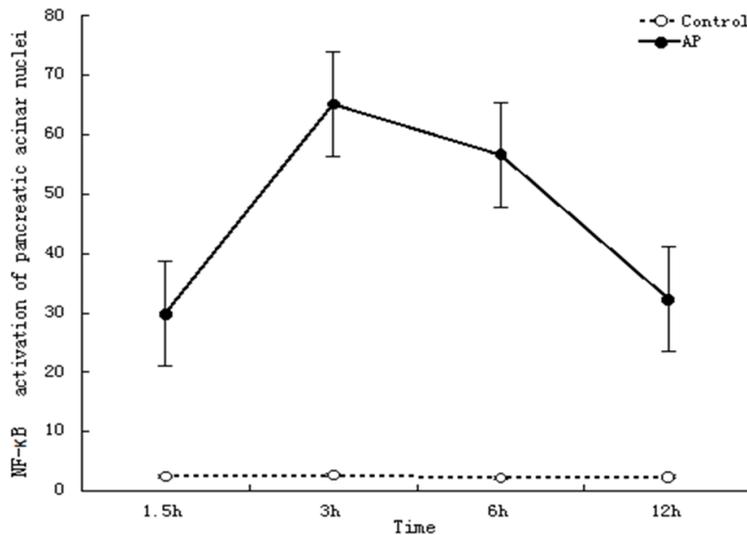


Figure 7. Time course of NF-κB activation of pancreatic acinar nuclei in rats.

Discussion

The transcription factor NF-κB, as one of the central coordinating regulators, plays a key role in myriad physiological and pathological scenarios. NF-κB is transported to the nucleus in response to many extracellular stimuli in many diverse cell types. These stimuli may trigger inflammation, innate immune responses, and/or adaptive immune responses [23]. Many studies have indicated that NF-κB is involved in the pathogenesis of AP [24]. However, more than twenty years after its discovery, the role of NF-κB and IκB in the pathogenesis of AP field

remains a lively arena for research [25, 26].

Four major methods are used to detect NF-κB DNA binding capacity currently. First, activated NF-κB is determined by electrophoretic mobility shift assays (EMSA), also known as gel shift assay. This popular and efficient technique can be utilized to analyze the affinity of proteins to a specific DNA or RNA sequence. One disadvantage of this assay is the radio labeling of the probe, which creates radioactive waste and reagents, has a relatively high cost, and needs specific infrastructures. Moreover, the short half-life of ^{32}P necessitates the use of the labeled probe within a short time [27, 28]. Second, cytoplasmic IκB degradation can be assessed using western blot with antibodies against IκB [29]. This method is time consuming, highly expensive, and is less amenable to high throughput analysis. Third, the reporter gene method indirectly detects NF-κB DNA binding capacity. A common reporter gene is luciferase since luciferase expression is easily measured and has been engineered to be under the control of NF-κB [30]. The Signal Lenti NF-κB Reporter is widely used; it provides a preparation of ready-to-transduce lenti viral particles for monitoring the activity of NF-κB signaling pathways in virtually any mammalian cell type. Fourth, a recently reported novel method is a multi-well colorimetric assay for active NF-κB [31]. In comparison, this flow cytometry method offers several advantages over conventional methods: more simultaneously assayed parameters on higher cell numbers, greater sensitivity, fewer required fluorescent molecules, longer stability of fluorescent probes than that of radioactive counterparts, and less expensive [32]. In this study, we assessed the NF-κB DNA binding capacity

of rat pancreatic acinar cells during AP in order to investigate the role of NF- κ B activation in acute pancreatitis.

Acute pancreatitis is a common clinical disease [33]. About 25% of patients with AP progress to systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS) and multi-organ dysfunction syndrome (MODS), and consequently develop severe acute pancreatitis [34]. The results obtained in this study demonstrated that NF- κ B activation in inactivated pancreatic tissue using flow cytometry during the initial 12 hrs, is tiny (<5%). As Vaquero et al. described, this minute change could not be detected by EMSA [35]. In rat STC induced pancreatitis, the high expression of NF- κ B activation occurred early in the onset of AP, which lead to acinar cell injury and AP. The NF- κ B DNA binding activity increased several fold at 1.5 h after STC stimulation, peaked at 3 h, and declined to the 1.5 hr level by 12 h post STC administration. We observed that the high expression of NF- κ B activation was typical at 1.5 h STC stimulation. This kinetics correlated with the degradation of different inhibitory I κ B proteins, and the rapid translocation of NF- κ B from the cytoplasm to the nucleus. Notably, the monophasic NF- κ B activation observed in this study was distinct from the biphasic kinetics described by Gukovsky et al. [36]. He described that the NF- κ B DNA binding capacity initially increased after cerulein hyperstimulation, peaked at 30min, then decreased gradually, and secondly elevated at 3 h, remained at 6 h at a level three times that of the control.

As shown in the recent research, NF- κ B activation of inflammatory mediators is the potential early intracellular event of AP [34, 36]. Activated NF- κ B triggers a waterfall-like cascade reaction of inflammatory mediators, tissue damage and fulminant inflammatory reaction [12]. Blocking NF-kappa B activation and subsequent prevention of transcription of pro-inflammatory molecules may reduce the generation, activation and release of NO, IL-1, IL-6, TNF-alpha [37], reducing tissue damage and inflammatory reaction [38, 39]. Interestingly, although most researchers agree that inhibiting NF- κ B activation is beneficial in acute experimental pancreatitis, the opposite point was reported in recent years. Steinle et al. indicated that blocking NF- κ B activation with pyrrolidine dithiocarba-

mate (PDTC) or N-acetyl-L-cysteine (NAC) was associated with worse morphological alterations, serum amylase, and lactate dehydrogenase levels in rat cerulein-induced pancreatitis model [40]. They assumed that NF- κ B was involved in preventing apoptosis, so the inhibition of NF- κ B activation could result in increased apoptosis and necrosis. Surprisingly, Algül et al, reported that pancreatic specific deletion of the Rel A/p65 gene encoding the Rel homology domain increased the susceptibility of inflammation-associated cell death in mouse cerulein induced pancreatitis [41]. All in all, the role of NF- κ B activation in acute pancreatitis has been protective, detrimental, or inconsequential, and thus remains still under intense scrutiny [42, 43].

In this paper, we developed a new sensitive and specific method to assess the amount of activated NF- κ B in cell extracts from solid tissue. Once activated, NF- κ B moved rapidly from cytoplasm to nucleus and combined with the DNA consensus sequence. Consequently, double staining using PI fluorescence probe conjugated DNA and a primary anti-NF- κ B antibody followed by a secondary antibody conjugated fluorescence probe quantified the percentage of acinar nuclei with both characteristics. However, there were some limitations to this study. The method relies on the specificity of the antibody for NF- κ B. As we all know, the Rel/NF- κ B family has several homodimers and heterodimers and RelA/p65 is the main trans activating subunit. Here, we have only detected NF- κ B p65, but not NF- κ B, so it is feasible that this method may underestimate the quantity of NF- κ B DNA binding. In view of this experimental acute pancreatitis, further studies are required to confirm the role of NF- κ B activation and the trend of NF- κ B activation in the pathogenesis of adult acute pancreatitis. Flow cytometry is a specific and sensitive assay for the rapid detection of NF- κ B activity in acute pancreatitis.

Disclosure of conflict of interest

None.

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