

# Menadione (vitamin K3) inhibits hydrogen sulfide and substance P via NF- $\kappa$ B pathway in caerulein-induced acute pancreatitis and associated lung injury in mice

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## ABSTRACT

**Objective:** We aim to study the protective effect of menadione on caerulein-induced acute pancreatitis (AP) and associated lung injury and to explore the possible mechanism.

**Methods:** Male Swiss mice randomized into control and different experimental groups. AP was induced in mice by six hourly intraperitoneal (i.p) injections of caerulein (50  $\mu$ g/kg at 1 h interval). Menadione (10 mg/kg) was administered one hour (i.p, 10 mg/kg) after the first caerulein injection and control animals were given hourly intraperitoneal (i.p) injection of isotonic sodium chloride solution for 6 hours.

**Results:** Administration of menadione attenuated the severity of AP and associated lung injury as shown by the histopathology, reduced MPO and serum amylase activity. Further, the anti-inflammatory effect of menadione was associated with a reduction of pancreatic and pulmonary proinflammatory cytokine interleukin 1 $\beta$  (IL-1 $\beta$ ) and hydrogen sulfide (H<sub>2</sub>S). Moreover, menadione inhibited caerulein-induced cystathionine- $\gamma$ -lyase, preprotachykinin-A (PPTA) and neurokinin-1 receptor (NK-1R) expression in pancreas and lungs. Also menadione further enhances the beneficial effect by reducing caerulein-induced nuclear factor (NF) - $\kappa$ B activation in both pancreas and lung.

**Conclusion:** The present findings show for the first time that in AP, menadione may exhibit an anti-inflammatory effect by down-regulating substance-P and H<sub>2</sub>S signaling via the NF- $\kappa$ B pathway.

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## Introduction

Acute pancreatitis (AP) is a common inflammatory condition of the pancreas and is associated with the impairment of lung function. With the limited target-specific therapeutic options, AP carries a high mortality rate. Alcohol consumption and biliary tract disorders are the common aetiological factors involved in the AP pathogenesis [1]. Premature activation of the intracellular digestive enzymes and the subsequent auto-digestion of pancreas induces tissue injury and eventual multiple organ failures [2]. AP involves a complex cascade of events that starts with release of inflammatory signals from the pancreatic acinar cells leading to the release of proinflammatory cytokines, chemokines, and gaseous mediators.

Recent studies have identified the proinflammatory role of H<sub>2</sub>S in AP and associated lung injury which remains unexplored so far. In pancreas and lungs, H<sub>2</sub>S is synthesized by the pyridoxal-5-

phosphate-dependent enzyme, cystathionine  $\gamma$ -lyase (CSE) which is found to be overexpressed in AP [3]. Inhibition of H<sub>2</sub>S biosynthesis with DL-propargylglycine (PAG) [4] and CSE gene knockout [5] alleviates the severity of AP through the down-regulation of IL-1 $\beta$  [6,7], suggesting that endogenous H<sub>2</sub>S has an important role in the pathophysiology of inflammation. It has been previously proven that the proinflammatory cytokine IL-1 $\beta$  plays a primary role in determining the severity of AP. Blocking this cytokine ameliorates the lung and pancreatic injury and provides various survival benefits during pancreatitis [8,9]. In addition, H<sub>2</sub>S mediates the signaling of proinflammatory mediator Substance P (SP) and the activation of transcription factor NF- $\kappa$ B, which is responsible for the activation of several proinflammatory mediators in inflammation [5]. Substance P is a neuropeptide derived from preprotachykinin A (PPTA) gene and is linked with the inflammation associated both during clinical and experimental pathogenesis.

Substance P acting through neurokinin-1-receptor (NK-1R) is essential in regulating the severity of caerulein-induced AP and associated lung injury [10–12], in sodium hydrosulphide (H<sub>2</sub>S donor) stimulated mouse pancreatic acinar cells [13], and in NaHS-

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induced lung inflammation [14]. Unfortunately, anti-inflammatory therapy directed against a single inflammatory factor are not effective in the treatment of AP. Recent studies have demonstrated that H<sub>2</sub>S mediates the release of various inflammatory mediators [15]. Hence, blocking H<sub>2</sub>S could control the release of various proinflammatory mediators and may be viewed as a molecular target for developing therapeutic strategies for AP and lung injury.

Menadione (Vitamin K3, 2-Methyl-1, 4-naphthoquinone) a synthetic analogue of vitamin K, acts as a provitamin that is converted into a vitamin in the body. Recently, it was reported that menadione has anti-cancer activity, against human cancer cells [16]. Menadione showed an inhibitory effect on angiogenesis in a rat aortic ring model [17]. In addition, menadione attenuates AP by inducing apoptosis [18] and inhibition of autophagy [19] in pancreatic acinar cells. These studies indicate that menadione could be an effective therapeutic agent against various inflammatory conditions. For a better insight into the protective effect of menadione, we have examined the inhibitory effect of menadione towards SP and H<sub>2</sub>S synthesis in caerulein-induced AP and associated lung injury.

## Materials and Methods

### Chemicals and reagents

Menadione, caerulein, NaHS, zinc acetate, trichloroacetic acid, ferric chloride, tritramethylbenzidine, hexadecyltrimethylammonium, and NNDP sulphate were purchased from Sigma-Aldrich (Bangalore, India). Amylase Activity Assay Kit and TRI Reagent<sup>®</sup> from Sigma-Aldrich (Bangalore, India). Enzyme-Linked Immunosorbent Assay (ELISA) kits from R&D system (Bangalore, India). Real-time polymerase chain reaction (PCR) kit was ordered from Takara (Bangalore, India). For transcription factor activity purpose, Cayman's Nuclear Extraction kit (Cayman chemicals, Bangalore, India) was used to prepare the nuclear extracts and NF- $\kappa$ B transcription factor activity Cayman's Transcription Factor Assay kit (Cayman chemicals, Bangalore, India). Other chemicals were from HiMedia Laboratories (Bangalore, India).

### Experimental procedures

All the animal experiments were approved by the Institutional Animal Ethical Committee of Vellore Institute of Technology (Approval no. VIT/IAEC/13/FEB/25) and were carried out according to established international guiding principles for animal research. Swiss albino mice (male, 25–35 g) were maintained in the animal housing unit in an environment with controlled temperature (21–24 °C) and lighting (12:12 h light-darkness cycle). Standard laboratory chow and drinking water were provided. Animals were acclimatized for a period of at least 48 h before beginning the experimental procedures.

### Induction of acute pancreatitis

Male Swiss mice (25–30 gm) were selected and randomly divided into control and experimental groups of 6 animals in each. The control animals were given hourly intraperitoneal (i.p) injections of isotonic sodium chloride solution for 6 h. Experimental animals were given hourly i.p injections of saline-containing caerulein (50  $\mu$ g/kg) for 6 h [20]. In the present study, menadione (10, 25, 50 and 100 mg/kg) was administered i.p one hour after the first injection of caerulein. It was found that all the dosages (25, 50 and 100 mg/kg) except 10 mg/kg of menadione were lethal. Therefore, for further studies 10 mg/kg was used. After one hour of the last injection of caerulein, mice were euthanized with the

recommended i.p dose of ketamine (80 mg/kg) and xylazine (4 mg/kg). Blood, pancreas and lungs tissue were harvested for subsequent assays.

### Amylase estimation

After anesthetization, blood was collected from the experimental animals by the cardiac puncture method. The collected blood was allowed to stand still in a slanted position for 20 to 25 minutes at room temperature followed by centrifugation at 900 g for 10 m. The resulting supernatant was immediately transferred to the fresh tube and used for determination of amylase levels. The Serum amylase level was measured using the amylase activity assay kit (Sigma-Aldrich, Bangalore, India) and reported as units per litre (U/L).

### Myeloperoxidase (MPO) estimation

MPO activity of tissue was measured to quantify the neutrophil sequestration in both pancreas and lung tissue. Briefly, samples were thawed followed by homogenization in 20 mM phosphate buffer (pH 7.4). The homogenized tissue was centrifuged (13,000 rpm, 10min, 4 °C) and resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% w/v hexadecyltrimethylammoniumbromide. The suspension was subjected to four cycles of freezing and thawing and further disrupted by sonication (40sec). The sample was then centrifuged (13,000 rpm, 5 min, 4 °C), and the supernatant was used for MPO assay. The reaction mixture consisted of the supernatant (50  $\mu$ l), 1.6 mM tetramethylbenzidine, 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide (reagent volume: 50  $\mu$ l). This reaction mixture was incubated at 37 °C for 110 s and, the reaction was terminated by adding 50  $\mu$ l of 0.18 M H<sub>2</sub>SO<sub>4</sub>, and the absorbance measured at 450 nm. Tissue absorbance was then corrected for the protein concentration of the sample using a Bradford assay. The results were expressed as the fold change over corresponding control as enzyme activity.

### Histopathological examination

Paraffin-embedded lung and pancreas tissues were sectioned, slices of 5  $\mu$ m thickness were stained with hematoxylin/eosin (H and E), and were examined under the light microscopy.

### Measurement of H<sub>2</sub>S levels

The H<sub>2</sub>S levels of pancreas and lung tissues of mice were measured as described previously [20]. Briefly, tissues were homogenized in potassium phosphate buffer (pH6.8) to trap H<sub>2</sub>S; the homogenate was then added in eppendorf tube consisting of 150  $\mu$ l of zinc acetate (1% w/v). To this reaction mixture, 100  $\mu$ l of NNDP sulphate (light sensitive, 20 nM in 7.2 M HCl) and 100  $\mu$ l of FeCl<sub>3</sub> (30 mM in 1.2 M HCl) were added to terminate the reaction. Further 300  $\mu$ l of TCA (10%w/v) was added and kept in the dark for 20 min. Finally, the mixture (methylene blue) was centrifuged at 4000 rpm for 10 min, and the absorbance was measured at 670 nm. Results were then corrected for the protein content of the tissue homogenates and were expressed as  $\mu$ mol H<sub>2</sub>S/mg protein.

### Real Time PCR

Real-time polymerase chain reaction (RT-PCR) used to analyze the mRNA expression of target genes. Total mRNA from tissue samples was extracted using TRI Reagent<sup>®</sup> (Sigma-Aldrich, Bangalore, India) followed by synthesis of cDNA using Takara cDNA

synthesis kit. SyBr Green I master mix (Takara, Bangalore, India) was used for RT-PCR. A total of 100 ng of RNA was used for each real-time PCR. It was amplified by Light Cycler real-time PCR machine (Applied Biosystems® StepOne RT-PCR system, India). Gene expression was calculated relative to r18S levels by the comparative  $\Delta\text{CT}$  values method. The primers r18S, CSE, IL-1 $\beta$ , PPTA, and NK1R, obtained from Sigma Aldrich, Bangalore, India and the primer sequence used for the mRNA expression study are listed in Table 1.

#### Enzyme-linked immunosorbent assay (ELISA)

The level of pro-inflammatory cytokine IL-1 $\beta$  in experimental mice pancreas and lungs were measured by DuoSet® ELISA Kit (R&D systems) according to its manufacturer's instruction. Briefly, approximately 50 mg of tissue was homogenized in 20 mM sodium phosphate buffer (pH 7.4) on ice. Homogenates were spun at 15,000 g for 15 min at 4 °C, and the clear supernatant was used for performing the assay. Colour development was measured at 405 nm. Results were then corrected for the protein content of the tissue homogenates and were expressed as picograms per microgram of protein.

#### Nuclear extraction and NF- $\kappa$ B binding activity

Cayman's nuclear extraction kit (Cayman chemicals, Bangalore, India) was used to extract nuclear extracts from the pancreatic and pulmonary tissue. For further assay the isolated extract was stored in -80 °C. The DNA binding activity of NF- $\kappa$ Bp65 was estimated using NF- $\kappa$ B transcription factor activity Cayman's Transcription Factor Assay kit (Cayman chemicals, Bangalore, India). Results are expressed as absorbance at 405 nm.

#### Statistical analysis

Results were represented as Mean (Standard deviation [SD]) with P value 0.001 being significant using GraphPad Prism 5 (Software, Inc. La Jolla, CA, USA). One-way ANOVA with Bonferroni's test was used to analyse the differences among the groups.

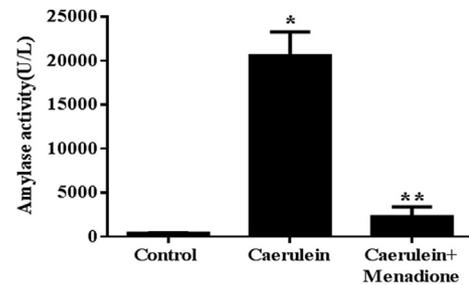
## Results

#### Effect of menadione on serum amylase in caerulein-induced AP

As shown in Fig. 1, after caerulein treatment, pancreatitis was manifested by a significant increase in serum amylase activity when compared with control ( $P < 0.05$ ). However, the serum amylase activity in menadione treated group following caerulein administration was markedly reduced when compared with mice treated with caerulein alone ( $P < 0.05$ ).

**Table 1**  
PCR primer Sequence.

Gene	Primer Sequence	Optimal Conditions
r18S	5'-GTAACCCGTTGAACCCATT-3'	22 cycles
	5'-CCATCCAATCGGTAGTAGCG-3'	Annealing 59 °C
IL-1 $\beta$	5'-TCCAGCTTCAAATCTCGCAGCAGACA-3'	22 cycles
	5'-CTGCCACAGCTTCTCCACAGCCACA-3'	Annealing 60 °C
CSE	5'-GCAATGGAATTCTCGTGCCG-3'	22 cycles
	5'-ATGCAAAGGCCAAACTGTGC-3'	Annealing 60 °C
PPTA	5'-CGCGATGCAACTACGAAA-3'	22 cycles
	5'-GCTTGGACAGCTCCTTCATC-3'	Annealing 60 °C
NK1R	5'-CTTGCTTTGGAACCGTGTG-3'	22 cycles
	5'-CACTGTCTCAITCTCTTGTTGGG-3'	Annealing 60 °C



**Fig. 1.** Treatment with menadione attenuated caerulein-induced increase in serum amylase activity. Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. Data are represented as mean  $\pm$  SD (n = 6 in each group). \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.

#### Effect of menadione on MPO activity and histopathological changes in caerulein-induced AP

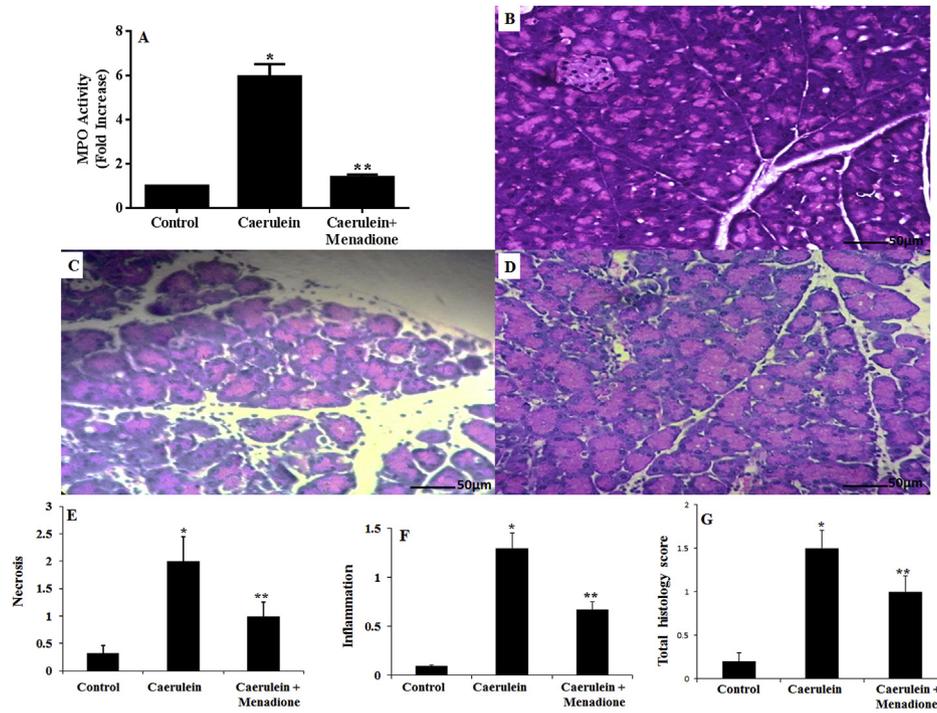
As shown in Figs. 2 and 3, the pancreatic and pulmonary injury was assessed by measuring MPO activity and histological changes. MPO measurement indicates the degree of neutrophil infiltration into the tissues. AP induction by caerulein significantly increased the MPO activity (pancreas and lungs) ( $P < 0.05$ ) suggesting that caerulein increased neutrophil infiltration and induced tissue injury. Therapeutic administration of menadione showed significant inhibition ( $P < 0.05$ ) of caerulein-induced MPO activity in both pancreas and lung (Figs. 2A and 3A). In agreement with the results of tissue MPO, menadione treatment decreased pancreatic (Fig. 2B–D) and pulmonary injury (Fig. 3B–D) induced by caerulein. Histological examination of pancreas sections of caerulein treated mice showed clear tissue damage evidenced from the infiltration of inflammatory cells, oedema formation, destruction of the architecture of acini and necrosis (Fig. 2C) and reflected in marked increase of overall histological score (Fig. 2E–G). However the menadione treatment protected and thus displayed very less damage in AP mice (Fig. 2D). Histological examination of the lung sections revealed lung injury in AP as evidenced by alveolar thickening and infiltrating inflammatory cells into the tissue (Fig. 3C), and represented in noticeable increase in the acute lung injury pathological score (Fig. 3E) whereas AP mice treated with menadione showed a clear reduction in cellular infiltration and accordingly less effective tissue injury (Fig. 3D). Thus, treatment with menadione resulted in a substantial reduction in the severity of pancreatitis as well as associated lung injury.

#### Menadione decreased caerulein-induced IL-1 $\beta$ expression in pancreas and lungs

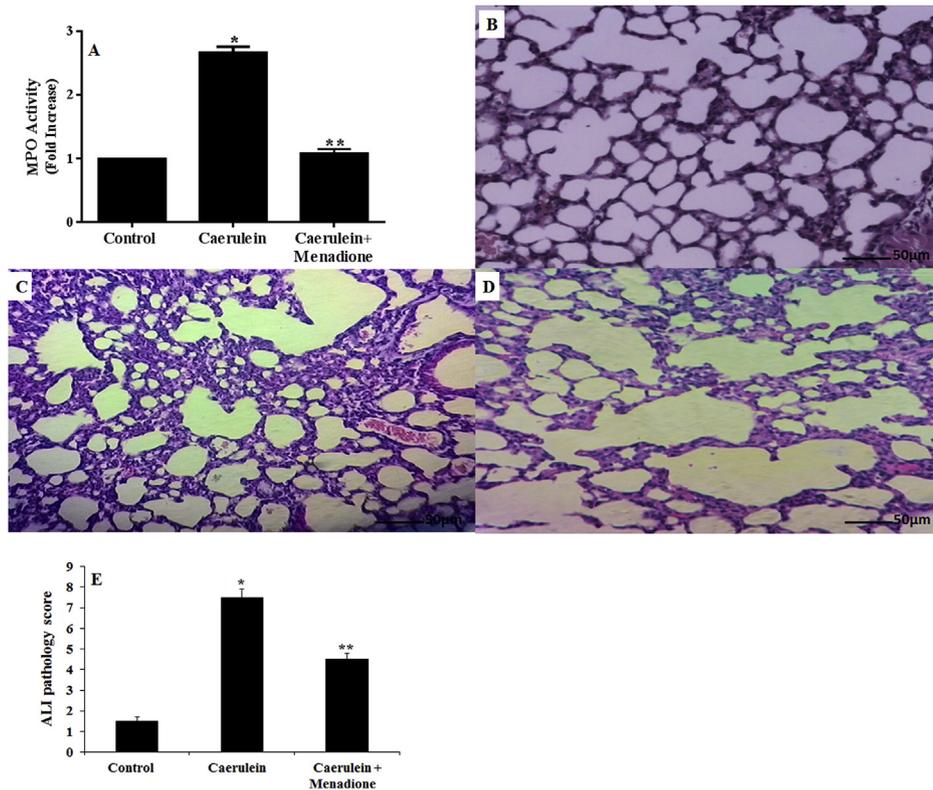
In our caerulein treated mice model, as reported before the inflammatory marker IL-1 $\beta$  acts as a primary agent in mediating pancreatitis [6]. In both pancreas and lung, caerulein induction resulted in a significant increase in IL-1 $\beta$  production (Fig. 4A and B) ( $P < 0.05$ ) and the mRNA expression (Fig. 4C and D) ( $P < 0.05$ ) when compared with control mice. We next examined whether menadione blocked the IL-1 $\beta$  expression, in pancreas and lungs during AP. Therapeutic administration of menadione attenuated caerulein-induced IL-1 $\beta$  protein level (Fig. 4A and B) ( $P < 0.05$ ) and mRNA expression (Fig. 4C and D) ( $P < 0.05$ ) in pancreas and lungs. These results clearly indicate the anti-inflammatory effect of menadione in AP and associated lung injury.

#### Menadione inhibited caerulein-induced H<sub>2</sub>S production and CSE mRNA expression in pancreas and lungs

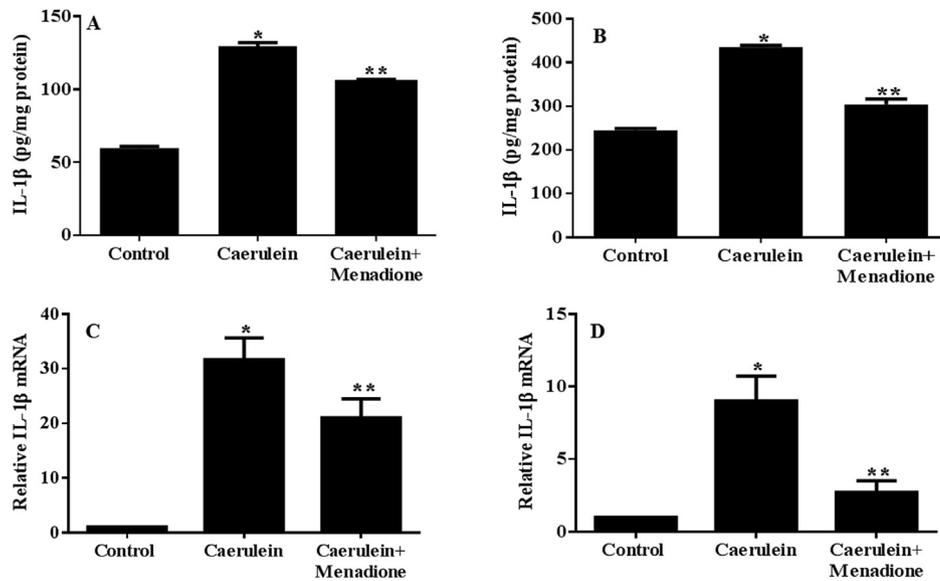
To understand the molecular mechanism of menadione in



**Fig. 2. Treatment with menadione attenuated the caerulein-induced increase in MPO activity as well as histopathology in the pancreas.** Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. (A) MPO activity was measured as indicated in the experimental protocol. ± Representative hematoxylin-eosin stained images for sections (X20) (B–D) Pancreas histology. (B) Control; (C) Caerulein; (D) Caerulein + menadione; (E) Necrosis; (F) Inflammation; (G) Total histology score. Data are represented as mean ± SD for 6 animals in each group. \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.



**Fig. 3. Treatment with menadione attenuated the caerulein-induced increase in MPO activity in the lung.** Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. (A) MPO activity was measured as indicated in the experimental protocol. ± Representative hematoxylin-eosin stained images (X20) for sections of Lung histology (B–D). (B) Control; (C) Caerulein; (D) Caerulein + menadione; (E) acute lung injury score. Data are represented as mean ± SD for 6 animals in each group. \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.



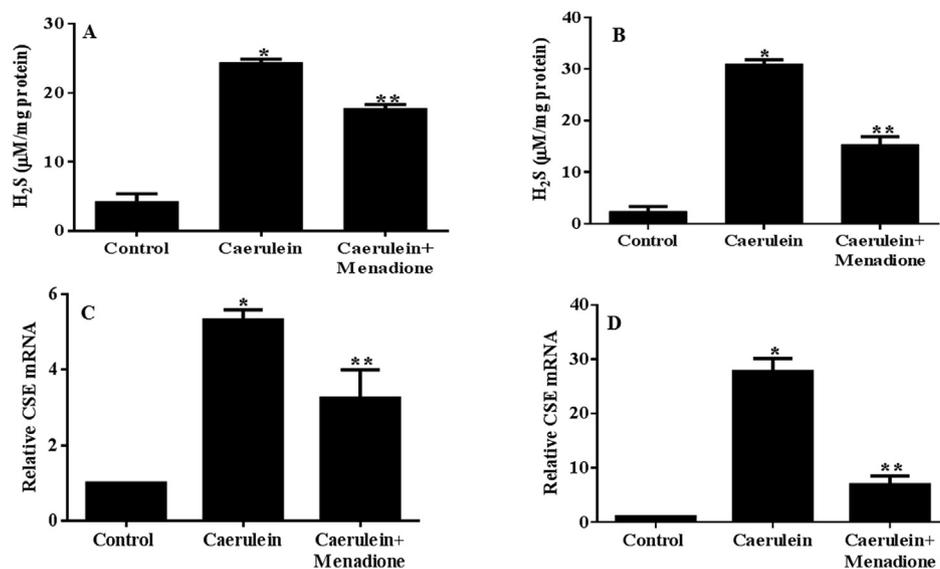
**Fig. 4. Treatment with menadione attenuated the caerulein-induced increase in IL-1 $\beta$  in pancreas and lung.** Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. IL-1 $\beta$  mRNA expression was detected by Real Time-PCR, and protein levels were measured by ELISA as described in Materials and Methods. (A, B) caerulein hyper stimulation and menadione treatment on IL-1 $\beta$  protein levels in the pancreas (A) and lung (B). Values are expressed as pg/mg protein and represent means  $\pm$  SD for 6 animals in each group. (C, D) Real Time-PCR detection of IL-1 $\beta$  mRNA expression in the pancreas (C) and lung (D). Sample loading was normalized with 18S ribosomal RNA internal control. Values represent means  $\pm$  SD for 6 animals in each group; \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.

caerulein-induced AP, we have assessed whether the menadione regulated H<sub>2</sub>S/CSE signaling involved in AP. Fig. 5A and B shows a significant increase (P < 0.05) in H<sub>2</sub>S levels in both pancreas and lungs following caerulein administration when compared with the control. However, the tissue H<sub>2</sub>S levels decreased in menadione treated animals when compared with the caerulein only treated mice (P < 0.05). As CSE mRNA increased in caerulein-induced pancreas and lung, we decided to investigate whether menadione inhibited CSE expression and to further confirm the anti-inflammatory activity of menadione over H<sub>2</sub>S signaling. In the caerulein treated inflammatory animals there was significant upregulation of pancreatic (Fig. 5C) and pulmonary (Fig. 5D) CSE

expression, whereas the menadione administration resulted in a significant decrease in caerulein-induced CSE gene expression both in pancreas and lung (P < 0.05; Fig. 5C and D).

#### Menadione blocked caerulein-induced PPTA and NK-1R gene expression in pancreas and lungs

To understand further the molecular mechanism of menadione in caerulein-induced AP, we assessed whether menadione regulated the SP/NK-1R pathway involved in AP. In both pancreas and lung, after caerulein administration in mice resulted in a significant increase in PPTA and NK1R mRNA expression. Therapeutic



**Fig. 5. Effect of menadione on H<sub>2</sub>S production and cystathionine-gamma-lyase (CSE) mRNA expression in pancreas and Lungs.** Mice were given 6 hourly i.p injections of caerulein. SC was administered 1 h after the first caerulein injection. CSE mRNA was detected by Real Time-PCR and H<sub>2</sub>S concentration was measured as described in Materials and Methods. (A, B) H<sub>2</sub>S concentration in the pancreas (A) and lungs (B). (C, D) CSE mRNA expression in the pancreas (C) and lungs (D). Sample loading was normalized with 18S ribosomal RNA internal control. Values represent means  $\pm$  SD for 6 animals in each group. \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.

administration of menadione attenuated caerulein-induced PPTA (Fig. 6A and B;  $P < 0.05$ ) and NK1R (Fig. 6C and D;  $P < 0.05$ ) gene expression in the pancreas and lung.

#### Menadione inhibited caerulein-induced translocation of NF- $\kappa$ B in pancreas and lungs

To investigate the effect of menadione on the transcription factor NF- $\kappa$ B in the lung and pancreas of AP mice, nuclear fractions from the pancreas (Fig. 7A) and lungs (Fig. 7B) were extracted and assayed for NF- $\kappa$ B p65 DNA-binding activity. In line with the anti-inflammatory role, the increase in the activity of NF- $\kappa$ B after caerulein administration ( $P < 0.05$ ) was substantially inhibited by therapeutic administration of menadione (Fig. 7A and B;  $P < 0.05$ ). This further supports the anti-inflammatory mechanism behind menadione action substantiated with other inflammatory disease [21,22].

#### Discussion

In this study, we have investigated the effect of menadione in a well-established caerulein-induced mice acute pancreatitis (AP) model. Mice treated with menadione significantly inhibited caerulein-induced levels of serum amylase, MPO activity, IL-1 $\beta$  mRNA and protein in the pancreas and lung. In addition, menadione inhibited H<sub>2</sub>S production, CSE, PPTA and NK1R mRNA expression in the pancreas and lung. Furthermore, menadione inhibited the caerulein-induced NF- $\kappa$ B activation in both pancreas and lung. These findings for the first time suggest that menadione ameliorates the inflammatory process in caerulein-induced AP and act as an anti-inflammatory agent through inhibiting H<sub>2</sub>S/CSE and SP/NK1R signaling, both of which requires NF- $\kappa$ B activation.

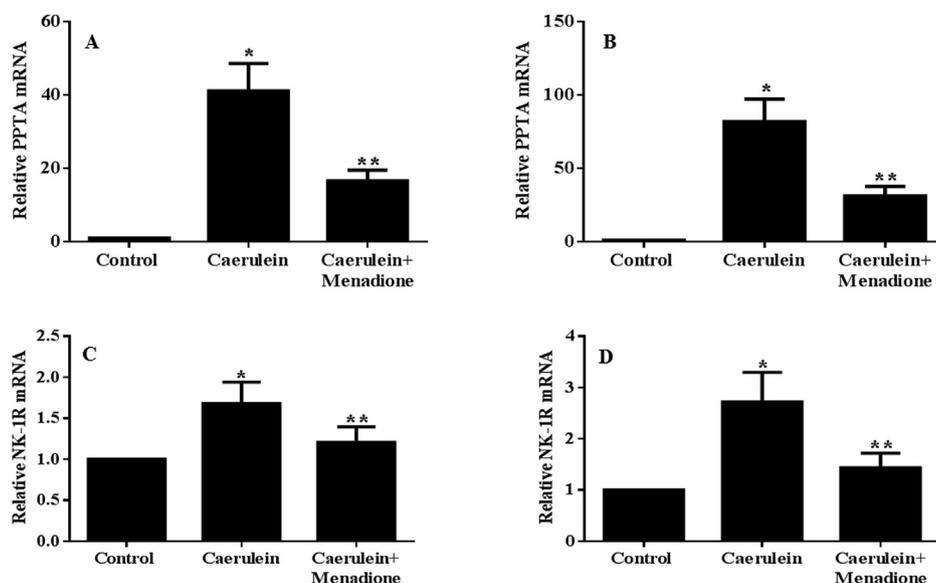
Menadione was shown to suppress inflammatory conditions in different experimental set-ups. For instance, menadione was shown to have anti-inflammatory effects in rabbit antigen-induced chronic immune arthritis [23]. Also, the previous studies have implied that menadione suppressed NF- $\kappa$ B nuclear translocation and the production of a pro-inflammatory cytokine in LPS-induced lung injury; TNF- $\alpha$  produced from murine macrophage (RAW

264.7 cells) [22]. Menadione also attenuates inflammation through the induction of apoptosis in the pancreatic acinar cells [18] and inhibition of the autophagy pathway in AP [19]. Although the anti-inflammatory mechanism is partly unraveled, still anti-inflammatory molecular mechanism in AP needs to be further clarified. AP, being a severe inflammatory condition without proper treatment options attracts a lot of attention nowadays. Thus in the present study, we have investigated the therapeutic potential of menadione (2-methyl-1, 4-naphthoquinone) in caerulein-induced AP.

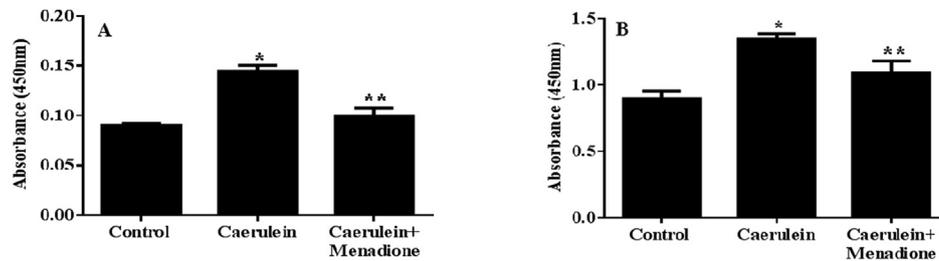
The serum amylase activity rapidly increases and the quantification of which is commonly used to detect the extent of AP in patients [24]. The release of this enzyme in the pancreas leads to the damage of pancreatic acinar cells and contributes to the inflammatory response during the onset of AP [24,25]. Thus, measuring the serum amylase activity plays a key role in determining the severity of AP. In our model, caerulein significantly increased serum amylase levels. Moreover, this increase was inhibited by menadione suggesting the protective effect of menadione against AP (Fig. 1). Chinzei et al. recently reported that menadione inhibits amylase activity and would attenuate caerulein-induced AP [19] which is in concordance with our results. However, the mechanism by which the H<sub>2</sub>S signaling is regulated by menadione is not clear till date.

Sequestration of neutrophils into the inflammatory site is one of the initial steps in the pathogenesis of AP. MPO abundantly present in the azurophilic granules of neutrophils promotes tissue damage in AP. The progression of pancreatitis and associated lung injury was accompanied by increased MPO activity in the tissues. The present study showed a clear increase in MPO both in pancreas and lung tissue. However, menadione treatment reduced MPO activity both in the pancreas (Fig. 2A) and the lung tissue (Fig. 3A). Therefore, menadione reduced infiltration of macrophage and neutrophil to the pancreas and lung in AP. Thus aiming to block the recruitment and inhibiting the activity of neutrophils could be used as a potential therapeutic method for AP [26].

Pancreatic acinar cells are the sources of cytokine (IL-1 $\beta$ ) expression in caerulein-induced AP which contribute to the severity of the disease [27]. Activation of pancreatic acinar cells that



**Fig. 6. Treatment with menadione attenuated caerulein-induced PPTA and NK1R in Pancreas and Lungs.** Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. (A, B) PPTA expression in the pancreas (A) and lungs (B). (C, D) NK1R expression in the pancreas (C) and lungs (D). Data are represented as mean  $\pm$  SD for 6 animals in each group. \* $P < 0.05$  versus control, \*\* $P < 0.05$  versus caerulein.



**Fig. 7. Effect of menadione on caerulein-induced activation of transcription factor NF- $\kappa$ B in pancreas and lungs.** Mice were given 6 hourly i.p injections of caerulein. Menadione was administered 1 h after the first caerulein injection. Nuclear extracts were prepared from pancreas and lungs for NF- $\kappa$ B DNA binding assay. (A) Pancreas and (B) Lungs. Data are represented as mean  $\pm$  SD for 6 animals in each group. \*P < 0.05 versus control, \*\*P < 0.05 versus caerulein.

release cytokines play a detrimental role in the progression of AP (as multi-organ disorder), and for instance by attracting leukocytes to the pulmonary interstitium contributing to lung injury [28,29]. Furthermore, IL-1 $\beta$  produced by pancreatic acinar cells contribute to the severity of the disease [27]. Blocking this proinflammatory cytokine production reduced the severity of both AP and lung injury [30]. In tune with these earlier results, the anti-inflammatory effect of menadione is effected through the significant decrease in IL-1 $\beta$  production (Fig. 4).

Increasing evidence indicates that the neurogenic proinflammatory mediator SP, play an essential role in regulating the inflammatory response in AP and associated lung injury [12]. The previous study showed that SP and its receptor NK1R are upregulated in pancreatic acinar cells [13] and lung during experimental pancreatitis in mice [14]. Further study revealed that the NK1R antagonist treatment reduced inflammation both in pancreas and lung [31]. Similarly proinflammatory effect of H<sub>2</sub>S together with increased CSE gene expression has also been reported in caerulein-induced AP and lung injury whereas inhibition of CSE expression by DL-propargylglycine [4] and siRNA-mediated gene silencing reduced the inflammatory responses [32] thus, confirming the pathogenic role played by H<sub>2</sub>S signaling [3].

In this present study we have also found that menadione administration reduces the caerulein-induced proinflammatory mediators SP and H<sub>2</sub>S both in pancreas and lung. There is the possible interplay between H<sub>2</sub>S and SP, as genetic deletion of PPTA and NK1R resulted in the inhibition of caerulein-induced H<sub>2</sub>S production. It is reported that the inhibition of H<sub>2</sub>S with CSE specific inhibitor (propargylglycine) suppressed caerulein-induced SP release thereby signifying the causative role played by SP in the severity of the disease [13,33]. These results suggest the interaction between the two proinflammatory mediators H<sub>2</sub>S and SP is involved in determining the graveness of the disease.

Results of reduced expression of SP and NK1R indicate the possibility that menadione protects against AP by inhibiting SP/NK1R and H<sub>2</sub>S/CSE signaling axis. Involvement of H<sub>2</sub>S signaling is not explored in many of the inflammatory and pathogenic situations, and revealing the complete picture could bring in new approaches in targeting AP.

Effectively by inhibiting SP and H<sub>2</sub>S-induced inflammation, menadione protects against the development of AP, though we could not exclude the involvement of other inflammatory mediators such as IL-1 $\beta$  at this juncture.

In the present study, we have demonstrated the novel role of menadione in regulating the SP-NK1R and H<sub>2</sub>S-CSE pathways in AP mice model. Furthermore, previous studies have also revealed that overexpression of CSE and PPTA led to significant activation of NF- $\kappa$ B whereas inhibition of SP and H<sub>2</sub>S caused suppression of NF- $\kappa$ B activation and attenuation of caerulein-induced AP in mice [34]. It is well established that proinflammatory genes are highly regulated

by NF- $\kappa$ B activation [35] and that inhibition of NF- $\kappa$ B is a preventive and therapeutic means of AP treatment [36]. Administration of menadione suppressed the caerulein-induced activation of NF- $\kappa$ B and hence reduced the severity of AP. Thus our study highlights the importance specifically of the interlinked SP-NK1R and H<sub>2</sub>S-CSE system in disease progression during stages of AP and the combative role played by menadione. As the phyto-supplementation, menadione could also be included in the treatment regimen.

Taken together, we have demonstrated the protective effect of menadione on caerulein-induced AP in mice. Therapeutic intake of menadione inhibited the development of caerulein-induced AP by inhibiting SP/NK1R and H<sub>2</sub>S/CSE signaling through inhibition of NF- $\kappa$ B activation. Our data supports the use of menadione as a potential novel strategy for preventing AP and acute lung injury.

#### Conflicts of interest

The authors have no conflicts of interest.

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