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Original Article

Secreted klotho from exosomes alleviates inflammation and apoptosis in acute pancreatitis

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Abstract: Acute pancreatitis is a potentially lethal disorder characterized by inflammation and apoptosis of parenchymal cells. Repeated acute pancreatitis results in chronic pancreatitis and is the major risk factor for pancreatic cancer. Current therapeutic approaches focus on anti-inflammatory and anti-apoptotic. However, the molecular mechanisms that lead to apoptosis and pathogenesis in acute pancreatitis remain unclear. Here, in the current study, we developed a novel approach that using exosomes from mesenchymal stem cells that overexpress Klotho reversed apoptosis, nuclear factor-kB activation in caerulein-stimulated AR42J cells. Klotho attenuated the severity of pancreatic inflammation after caerulein treatment. In conclusion, our results provide evidence that Klotho is a potential therapeutic target for clinical interventions towards acute pancreatitis.

Keywords: Acute pancreatitis, klotho, inflammation, apoptosis, exosome

Introduction

Acute pancreatitis is a life-threatening disease with high mortality rate, one of the most prevalent gastrointestinal conditions [1]. Acute pancreatitis is characterized by systemic inflammatory and pancreatic necrosis/apoptosis [2]. The relationship between molecular signaling after pancreatic injury and subsequent systemic response are not fully explored. However, accumulating evidence suggests that cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL-6) are correlated with the severity of pancreatic injuries [3].

Fibroblast growth factor (FGF21) is a hormone that controls metabolic and cellular stresses. FGF21 acts through FGF receptor with tyrosine kinase activity in complex with Klotho. Klotho is an anti-aging gene, a single-pass transmembrane protein. Both FGF21 and Klotho are co-expressed in pancreases, essential for digestive enzyme secretion from pancreatic acinar cells. Recent research identified that FGF21 is elevated in acute pancreatitis patients [4]. However, the roles of Klotho itself in pancreases are largely unexplored. It is possible that, as a co-receptor, similar to FGF21, Klotho plays key roles in acute pancreatitis. Here, we set to validate the hypothesis that Klotho is beneficial to acute pancreatitis by reducing apoptosis and inflammation.

Exosomes are small membrane vesicles derived from the fusion of multivesicular endosomes/lysosomes with the plasma membranes and extracellular release of the intraluminal vesicles [5, 6]. Exosomes released from mesenchymal stem cells (MSCs) exhibit anti-inflammatory effects and are able to repair tissue injuries. Therefore, we utilized exosomes from MSCs to secrete klotho on a caerulein-induced model of acute pancreatitis.

Materials and methods

Isolation and culture of MSCs

Rats (4-5 weeks) were sacrificed by cervical dislocation. Under sterile condition, femurs and tibias were taken and washed by PBS (2% mix of penicillin and streptomycin) for 3 times. The epiphyses were removed and then the bone marrow was flushed out into a normal medium by DMEM/F12 supplemented with 1% mix of

penicillin and streptomycin and 10% FBS. The single cell suspension was obtained through drawing the marrow into syringes repeatedly. The single cell suspension was centrifuged at 500 rpm for 5 min, and removed the supernatant. The obtained suspension was cultivated in normal medium at 37°C in 5% CO_2. The medium was replaced every 48 h.

Plasmid construction and transfection

The pLVX-Puro vector (Clontech) was utilized to generate lentiviral constructs. Klotho (No. NM_031336.1) was inserted into the the pLVX-Puro vector by EcoR I and BamH I digestion. The sequence information as follows: F: 5'-CGGAATTCATGCCAGCCCGCG-3' (EcoR I); R: 5'-CGGGATCCTTATTTATAACGTCTCCGGCC-3' (BamH I). All plasmids were validated by sequencing. The plasmid was extracted using E.Z.NA. Endo-free Plasmid Mini Kit I (OMGEA). The MSCs were infected for 1 week, no mismatch, deletion and reverse ligation were observed, indicating that the klotho recombinant eukaryotic overexpression vector was successfully constructed. The empty vector served as the negative control.

Exosome extraction and identification

The exosomes were extracted from the empty vector and klotho overexpressing mesenchymal stem cells. The morphology of the exosomes was observed by transmission electron microscopy. The expression of exosome surface markers (CD63, CD9 and CD81) was identified by Western blot [7].

Cell culture and caerulein-induced acute pancreatitis model

AR42J cells were cultured in F-12K medium containing 20% fetal bovine serum and 1% penn/strep in an incubator at 37°C with 5% CO_2 a. The cells were observed as adherent cells under the microscope, and the live cell rate of trypan blue staining was over 95%. The cells in the logarithmic growth phase were trypsinized and counted to prepare a cell suspension (8×10^5 cells/ml). 0, 5, 10, 20 nM caerulein was treated for 48 h before protein harvest.

Flow cytometry

Cells were suspended in PBS. FITC anti-rat CD90 Antibody, FITC anti-rat CD44H Antibody, FITC anti-rat CD11b/c Antibody and ITC anti-rat CD45 Antibody were added in the cell suspension according to the proportion of 1:40, and cultivated overnight at 4°C. On the next day, the cells were observed through the Accuri C6 flow cytometer (BD biosciences, CA, USA) according to the manufacturer's instructions.

Q-PCR

Total RNA was extracted by Trizol (Invitrogen, CA, USA) according to the manufacturer's instructions. And then, the RNA isolates were transformed into cDNA through reverse transcription reagent kit (Fermentas, MA, USA). cDNA was amplified by using the SYBR Green PCR kit (Thermo, MA, USA). The Real-Time PCR detection system (Thermo, MA, USA) was used for observation. The primer sequences were: Klotho (forward: 5' TCCCTCCTTTACCTGAGAAC 3'; reverse: 5' CGGATGGCAGAGAAATCAAC 3') and GAPDH (forward: 5' GGAGTCTACTGGCGTCTTCAC 3'; reverse: 5' ATGAGCCCTTCCACGATGC 3').

Western blot analysis

The cells were washed twice with PBS and lysed by lysis buffer with protease inhibitors and phosphatase inhibitors. The cell lysates were centrifuged at 12000 g for 10 min. The supernatants were collected and the protein contents were evaluated. 10% and 15% SDS-PAGE gels were used for isolation of protein. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4°C. The bands were detected by the enhanced chemiluminescence (ECL) system (Tanon, Shanghai, China).

The enzyme-linked immunosorbent assay (ELISA)

TNF-α and IL-6 content were determined using TNF-α ELISA kit and IL-6 ELISA kit (Thermo, MA, USA) in accordance with manufacturer's instructions. Briefly, purified rat TNF-α or IL-6 antibody was used to coat wells. TMB was added for color developing. Absorbance (optical density value) was measured at 450 nm. Quantification of TNF-α or IL-6 concentration was determined through the standard curve.

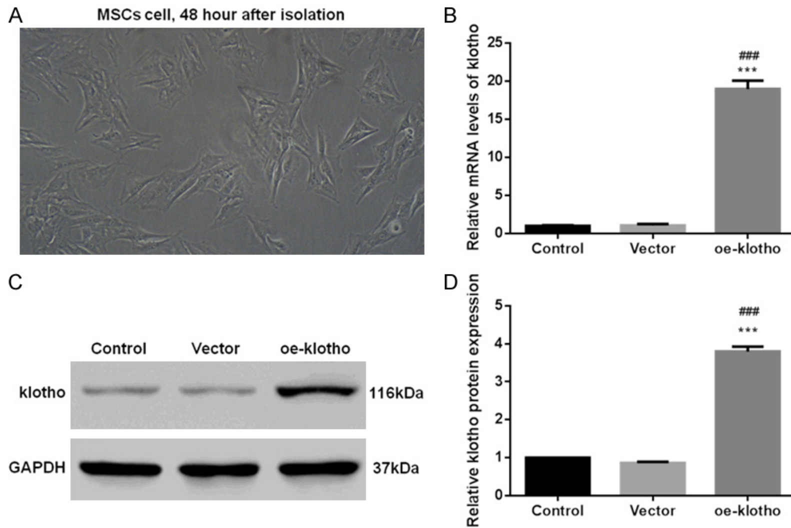

Figure 1. Overexpressed klotho was secreted from MSCs. A. Representative images of MSCs cell at 48 hour after isolation. B. Quantification of mRNA after MSCs infection. C. Klotho protein was significantly upregulated after lentiviral infection. D. Quantification of protein expression.

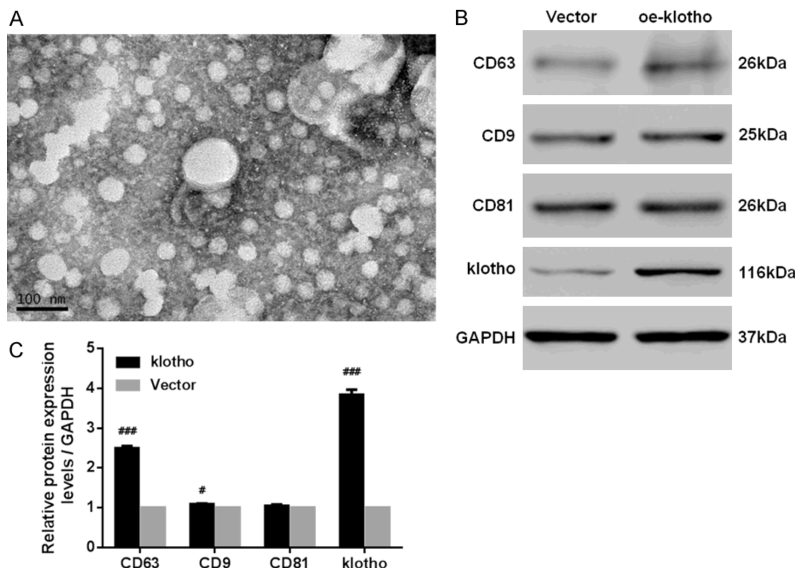

Figure 2. Klotho overexpression up-regulated selective exosome markers. A. Representative of electron microscopy images of exosomes. Scale bar: 500 nm. B. Exosome markers CD63 and CD9 were significantly elevated after klotho overexpression. C. Quantification of protein expression. ##P < 0.01, #P < 0.05.

TUNEL assay

TUNEL assay was performed using the Apoptosis Detection Kit and cell nuclei were stained with DAPI.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical differences between groups were evaluated by one-way ANOVA analysis, and the Bonferroni post hoc test was used for multiple comparisons. P value of < 0.05 was considered statistically significant.

Results

Morphological observation of MSCs

We first observed the morphology of the cultured rat MSCs before infection, we found that most of the cells were attached at 48 hours, some of the cells began to spread out, and most of the cells were round or polygonal (**Figure 1A**).

Lentiviral infection MSCs

Overexpression of klotho (oe-klotho) significantly elevated the expression of klotho levels in cells. Real-time PCR and immunoblotting (WB) were used to detect and analyze at transcription and translation levels. We found that klotho mRNA expression increased dramatically ($P < 0.001$, **Figure 1B**). Klotho protein was increased in overexpression group compared to the control and empty vector ($P < 0.001$, **Figure 1C**, **1D**).

Identification of lentiviral overexpressing in MSCs exosomes

Transmission electron microscopy analysis showed that the exosomes were round or elliptical bilayer lipid membrane vesicles with a diameter of 40-100 nm. The average diameter of exosomes was 94.3±5 nm (**Figure 2A**). The expression of exosome markers CD9, CD63 and CD81 was detected by Western blot (**Figure 2B**, **2C**), and the expression of CD63 and Klotho in Klotho overexpression group was significantly higher than the vector group ($P < 0.001$).

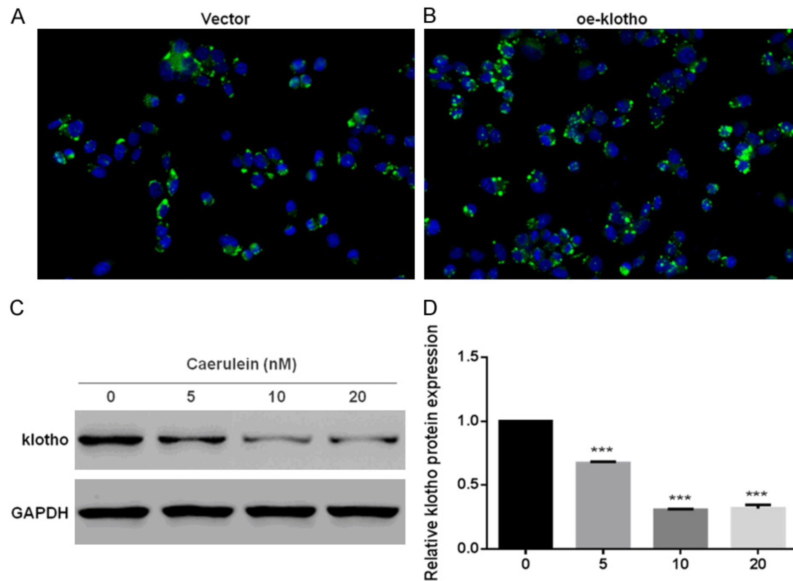

Figure 3. Representative images of PKH-67 tracer of AR42J engulfed exosomes from empty vector (A) or klotho overexpression (B) infection. (C) Caerulein inhibited klotho expression. (D) Quantification of western blot. ***P < 0.001.

Exosome treatment in AR42J cells

The empty vector and the klotho overexpressing exosomes from MSCs were co-cultured with AR42J cells, and the PKH-67 tracer exosomes were engulfed by AR42J cells (**Figure 3A**, **3B**).

Caerulein-induced pancreatitis model in AR42J cells

Next, we wondered whether klotho level changes during pancreatitis. AR42J cells were treated with different concentrations (0, 5, 10, 20 nM) of caerulein for 48 h. We examined the protein expression. The results showed that caerulein inhibited the expression of klotho protein in a dose-dependent manner ($P < 0.001$, **Figure 3C**, **3D**).

Effects of klotho overexpressing MSCs exosomes on the expression of apoptosis-related genes and NF-kB translocation

The exosomes extracted from klotho overexpressing MSCs were co-cultured with AR42J cells in the supplemented with 10 nM caerulein. We used ELISA to examine the expression of IL-6 and TNF-α in the supernatants. Our results showed that caeurlein increased IL-6 and TNF-α, whereas the expression of IL-6 and TNF-α was decreased in klotho overexpressing and the control group, and the decrease in klotho overexpressing exosomes was more significant ($P < 0.001$, **Figure 4A**, **4B**). Flow cytometry showed that compared with the caerulein treatment, the apoptotic rate was significantly decreased in the presence of exosomes ($P < 0.001$, **Figure 4C**, **4D**), with more reduction in the klotho overexpression. Western blot was used to detect the expression of klotho, Bax, bcl-2, NF-kB (nuclear protein and plasma protein). Compared with the caerulein group, the expression of klotho and bcl-2 protein in the exosomes of empty vector and klotho overexpressing MSCs were detected. Exosomes decreased Bax expression, suggesting that apoptosis was inhibited. Meanwhile, NF-kB was decreased in nucleoprotein expression, plasma protein expression increased, with more significant changes in klotho overexpressed exosomes (**Figure 4E**).

Effects of klotho overexpressing MSCs exosomes on the expression of apoptosis-related genes and NF-kB translation

To further explore the roles of klotho and NF-kB. Klotho overexpressing MSCs exosomes were co-cultured with AR42J cells in the supplement with 10 nM caerulein, respectively, and were treated with Klotho antibody and NF-kB activator. The results showed that compared with klotho overexpressing MSCs exosomes, the expression of IL-6 and TNF-α was increased in the presence of Klotho antibody and NF-kB activator, and the apoptosis was significantly increased (**Figure 5**). The changes in IL-6 and TNF-α in mRNA and protein levels were confirmed by qPCR and western (**Figures 6**, **7A**, **7B**). The expression of Bax is increased, and the expression of NF-kB is increased in nucleoprotein and decreased in plasma protein (**Figure 5**). The apoptosis alteration was confirmed by TUNEL staining (**Figure 7C**). These results confirmed that klotho is anti-apoptotic and anti-inflammatory.

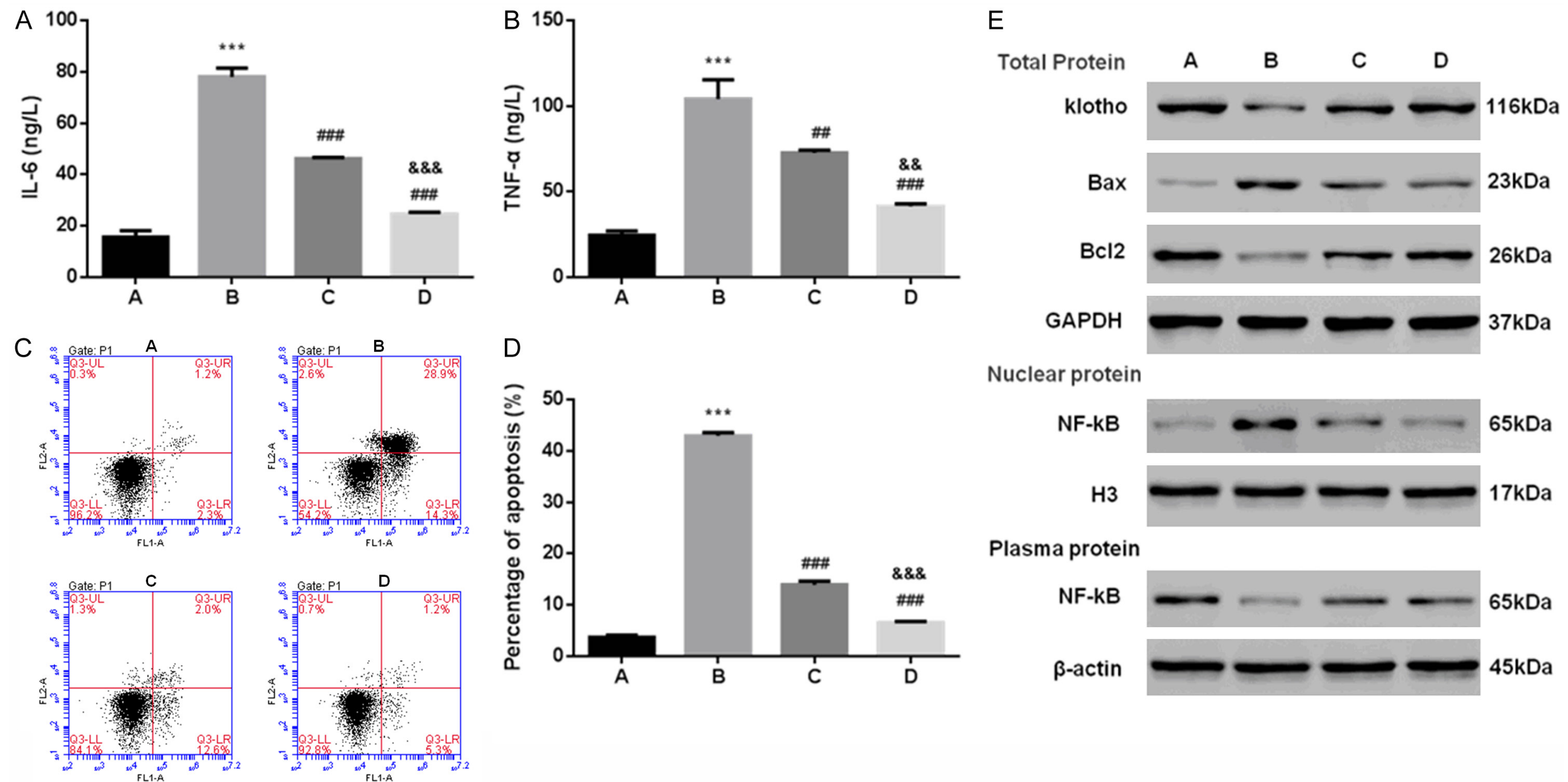

Figure 4. IL-6 and TNF-α content was significantly reduced in the presence of exosomes. A, B. Quantification of ELISA from AR42J supernatants. Caerulein-induced apoptosis was inhibited in the presence of exosomes. C. Representative images from flow cytometry. D. Quantifcation of cell apoptotic rate. E. Representative images of western blots of Bax, Bcl2 and NF-kB translation. A: control, B: caerulein, C: caerulein + empty vector, D: caerulein + klotho overexpression. ***P < 0.001, ###P < 0.005, ##P < 0.01, &&P < 0.05 compared to empty vector, &&&P < 0.01 compared to empty vector.

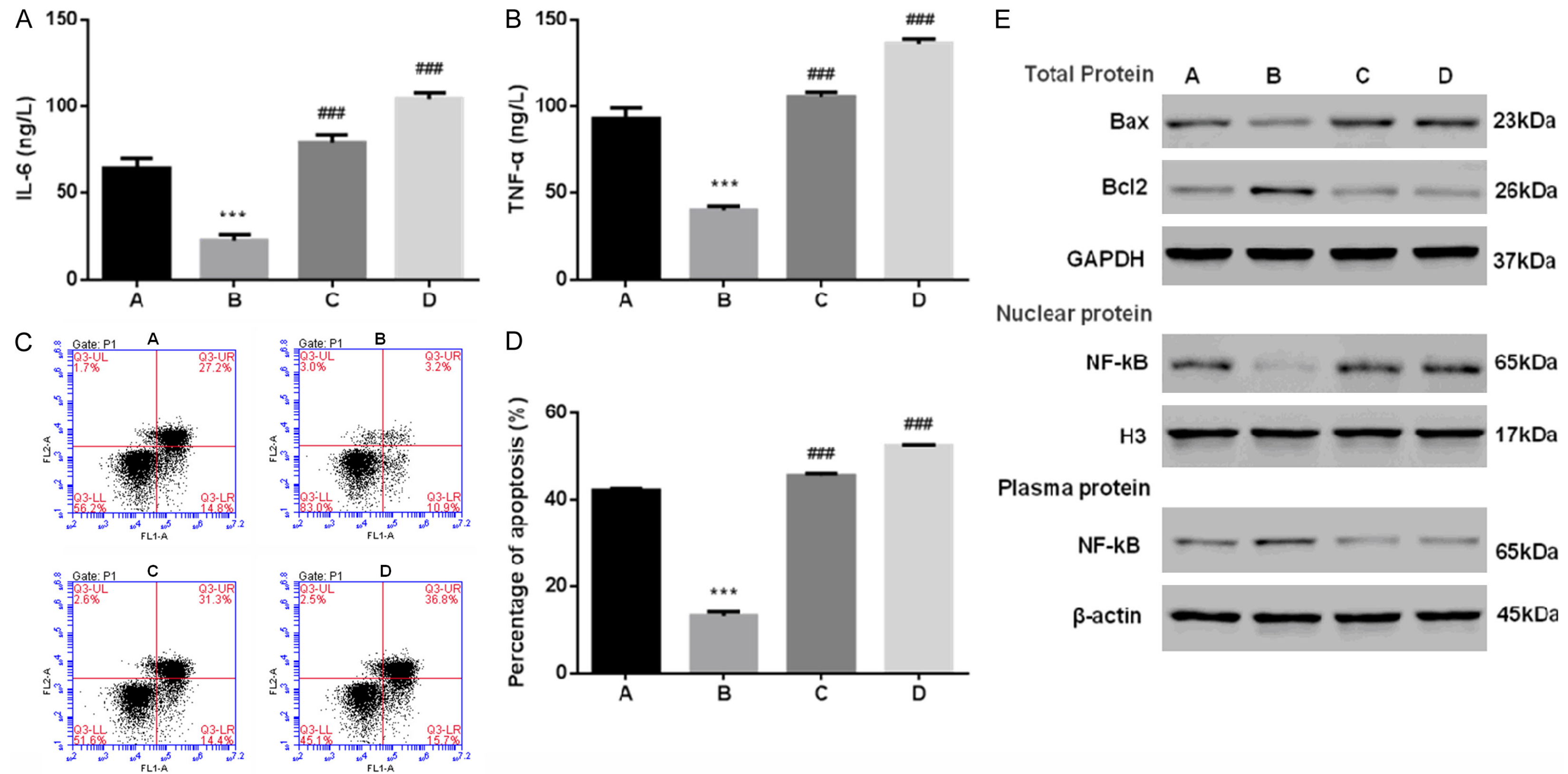

Figure 5. Klotho antibody and NF-kB activator confirmed kloth anti-apoptotic and anti-inflammatory effects. A, B. Quantification of ELISA from AR42J cell supernatants. Caerulein-induced apoptosis was inhibited in the presence of exosomes. C. Representative images from flow cytometry. D. Quantifcation of cell apoptotic rate. E. Representative images of western blots of Bax, Bcl2 and NF-kB translation. A: control, B: caerulein + exosomes, C: caerulein + klotho antibody, D: caerulein + NF-kB activator. ***P < 0.001, ###P < 0.005, ##P < 0.01.

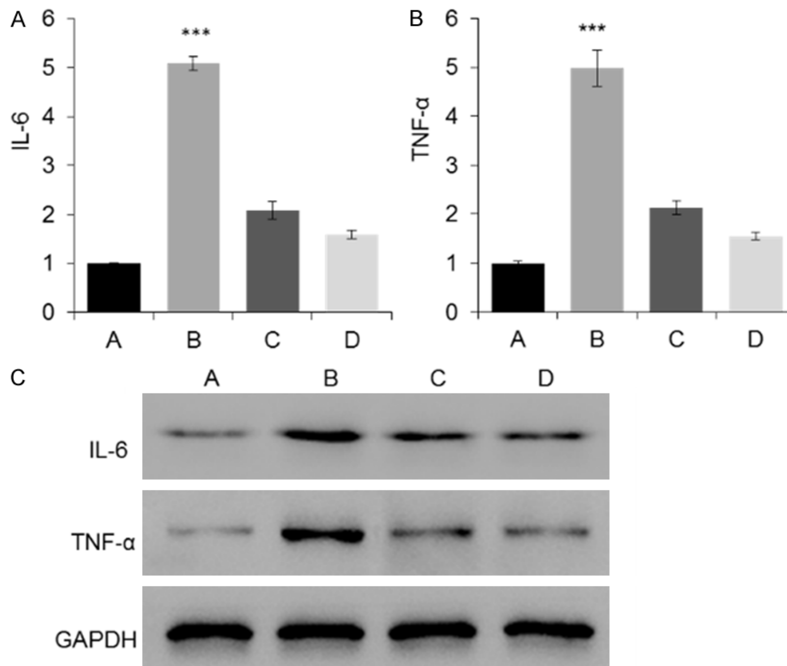

Figure 6. IL-6 and TNF-α content was significantly reduced in the presence of exosomes. A, B. QPCR quantifcation of IL-6 and TNF-α. C. Representative images of western blots. A: control, B: caerulein, C: caerulein + empty vector, D: caerulein + klotho overexpression. ***P < 0.001.

Discussion

Acute pancreatitis, especially severe acute pancreatitis, has a high mortality rate [8]. The systematic inflammation is due to the crosstalk between cytokines and oxidative stress during local inflammatory process. The other major impact during acute pancreatitis is local pancreatic damages due to cell death and necrosis [9] in the pancreatic exocrine tissues [10]. Therefore, treatments that neutralize cytokines to reduce inflammation and protect exocrine tissues are beneficial. To improve the outcomes of patients with acute pancreatitis, we focused on the molecular mechanisms underlying disease progression, inflammation and apoptosis.

Exosomes derived from MSCs exert a therapeutic effect in various physiological and pathological conditions. Among these roles, MSCs are involved in tissue remodeling and immunomodulatory potentials through some of the soluble factors [11]. MSCs cultures are well established and the easy and straight-forward culture method can be easily achieved in the laboratory platforms [11]. Here we have demonstrated a reasonable MSC culture and exosomes extraction *in vitro*. In addition, the immunosuppressive and anti-inflammatory properties of MSCs [12] are ideal candidates in the treatment of pancreatitis.

Caerulein was used to induce acute pancreatitis in animals. Caerulein induced the generation of inflammatory mediators of acute pancreatitis, several reagents including melatonin to reduce pro-inflammatory molecules can be beneficial [13]. We found the significant elevation of IL-6 and TNF-α in the model cells, suggesting that the inflammation responses were accelerated. In addition, TNF-α has recently been reported to exhibit dual role in the involvement of apoptosis during acute pancreatitis [14]. In sharp contrast, klotho reduced the IL-6 and TNF-α elevation, suggesting that klotho protects against inflammation. On the other hand, transcription factor NF-kB was reported as a therapeutic target for caerulein-induced acute pancreatitis, if NF-kB activation is blunted [15]. Selectively inhibiting NF-kB signaling significantly attenuated the injury severity of caerulein-induced acute pancreatitis [16]. In our study, we found that the klotho inhibits NF-kB translocation to the nuclei, confirming its anti-inflammatory effects. Similar to klotho, deletion of XIAP resulted in the decrease of NF-kB activation and less release of IL-6 and TNF-α [2]. Klotho and XIAP antagonize each other, and future studies are warranted to explore the critical roles of these two molecular interactions.

To verify that klotho can affect the apoptosis during acute pancreatitis, we examined apoptotic protein levels (Bcl-2 and Bax) after caerulein administration. Increased Bax and decreased Bcl-2 promotes apoptosis. The application of klotho from exosomes elevated Bcl-2 levels and reduced Bax levels, resulting in the increased Bcl2/Bax ratio. Since klotho was decreased in the presence of caerulein at a

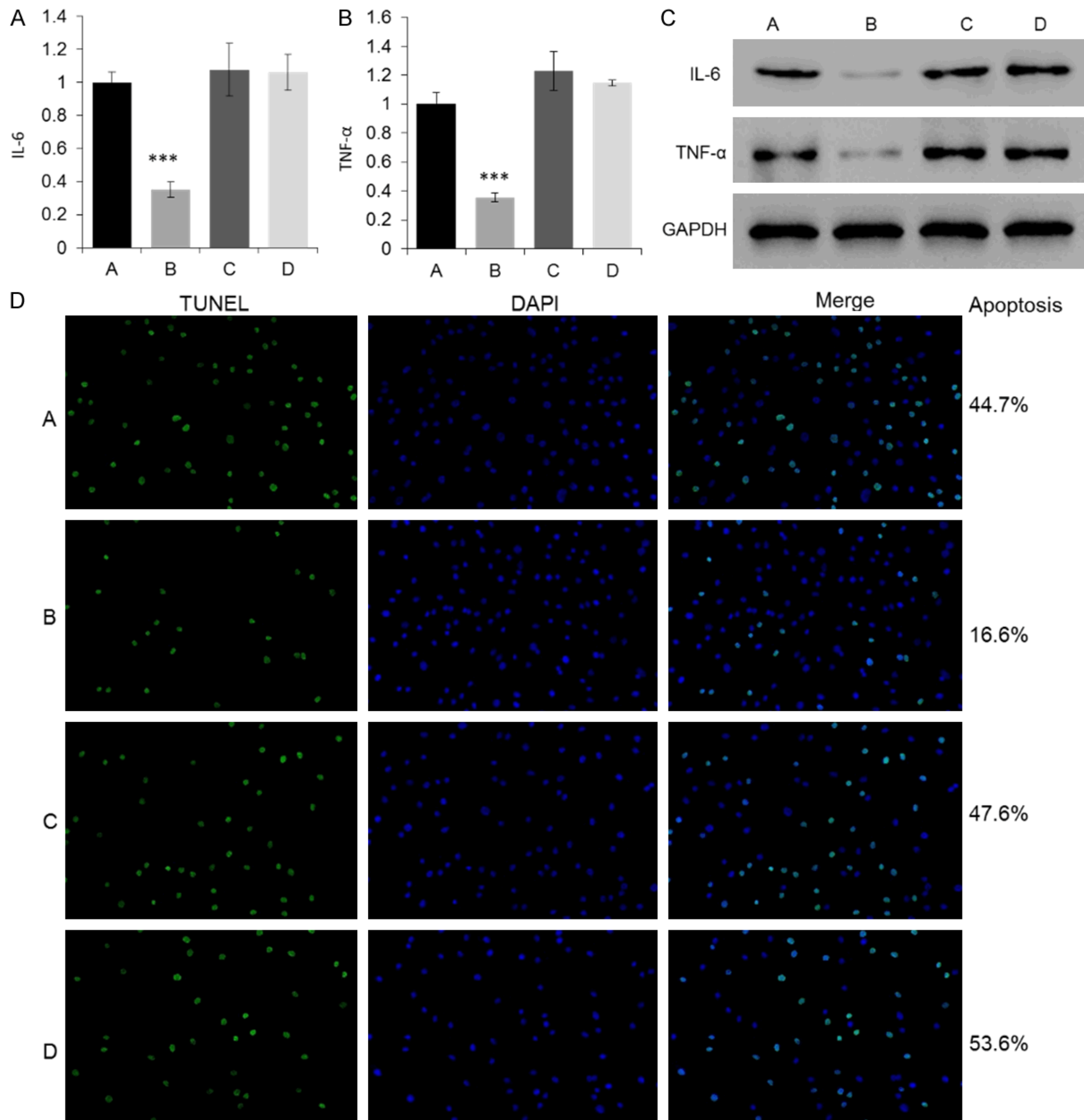

Figure 7. Klotho antibody and NF-kB activator confirmed kloth anti-apoptotic and anti-inflammatory effects. A, B. QPCR quantifcation of IL-6 and TNF-α. C. Representative images of western blots. D. Representative images of TUNEL staining. A: control, B: caerulein + exosomes, C: caerulein + klotho antibody, D: caerulein + NF-kB activator. ***P < 0.001.

dose responses manner. In addition, the administration of kloth antibody and NF-kB activator TNF-α promoted apoptosis. Future studies will focus on activation of caspases and programmed necrosis. Interestingly, consistent with our results, klotho can be induced by GABA therapy, and NF-kB activation is suppressed by klotho in pancreatic beta cells [17]. Taken together, these results indicated that klotho regulates apoptosis during acute pancreatitis. In summary, klotho provides a novel platform for future therapeutic approaches towards acute pancreatitis.

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Disclosure of conflict of interest

None.

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