Klotho alleviate contrast-induced acute kidney injury by suppressing oxidative stress, inflammation and NF-κB/NLRP3-mediated pyroptosis

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Research Article

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Abstract

Background

Contrast-induced acute kidney injury (CI-AKI) is a common complication following percutaneous coronary intervention in coronary artery disease (CAD) patients with more than 30% incidence. Klotho is a multifunctional protein that inhibits oxidative stress and inflammation, but its role in CI-AKI is poorly understood. The present study aimed to explore the effects of klohto in CI-AKI.

Methods

Six-week-old mice and HK-2 were divided into control group, contrast medium (CM) group, CM + klohto group, and klohto group. Kidney injury was evaluated by HE staining. And the renal function was shown by Scr and BUN. Cell viability and damage were determined by CCK-8 assay and lactate dehydrogenase (LDH) activity assay. Oxidative stress related indicators including intracellular reactive oxygen species (ROS), superoxidase dismutase (SOD), and malondialdehyde (MDA) were tested by fluorescent probe dichloro-dihydro-fluorescein diacetate (DCFH-DA) and enzyme-linked immunosorbent assay (ELISA) assay. IL-6, TNF-α, IL-1β, and IL-18 in the cell supernatant were tested by ELISA assay and used to reflect inflammation responses. Propidium iodide (PI) staining showed the cell death of HK-2. The expression of NF-κB, phosphorylated NF-κB (p-NF-κB), and pyroptosis-related protein levels of NLRP3, caspase-1, GSDMD, and cleaved-GSDMD were detected by western blot.

Results

Exogenous Klotho administration reduced kidney histopathological alterations and improved renal function in vivo. In vitro, Klotho significantly inhibited CM-induced oxidative stress and productions of both IL-6 and TNF-α. Moreover, we found klohto inhibited the activation of p-NF-κB and down-regulated pyroptosis-related protein (NLRP3, caspase-1, GSDMD, and cleaved-GSDMD).

Conclusion

The findings suggested that klohto had a protective effect on CI-AKI via suppressing oxidative stress, inflammation and NF-κB/NLRP3-mediated pyroptosis, contributing to the potential therapy of CI-AKI.

1. Introduction

Contrast-induced acute kidney injury (CI-AKI) was a common complication following percutaneous coronary intervention in coronary artery disease (CAD) patients [1, 2]. Inflammation was an important role in CI-AKI [3]. Studies had shown that inflammatory cytokines were elevated both in humans [4] and in CI-AKI model animals [5]. Anti-inflammatory drugs could reduce contrast-induced inflammation response [6,
On the other hand, oxidative stress was a damaging process of overproduction of reactive oxygen species (ROS) and oxidative-free radicals in intracellular [8]. Researchers had verified that the functions of tubular could be impaired by excess ROS production [9]. Finally, pyroptosis was a new programmed cell death and had been demonstrated to be involved in kidney injury [10]. Moreover, pyroptosis was a vital mechanism that mediated the development of CI-AKI [5]. As a consequence, alleviating oxidative stress, inflammatory response and pyroptosis was the goal of renal protection.

Klotho protein was defined as an aging-suppressor gene and was detectable in the circulation and acts as a humoral factor [11]. Recent studies had demonstrated that klotho had diverse activities, including antioxidant, anti-inflammatory, anti-apoptosis, and anti-cell senescence [12]. Increasing evidence had shown that early event in acute kidney injury was associated with klotho deficiency and exacerbated the progression of renal injury, contributing to long-term adverse outcomes [13, 14]. In addition, it had been shown that high serum levels of klotho could protect cardiovascular and non-cardiovascular disease by anti-inflammation and antioxidative activities [15, 16]. However, the underlying mechanisms that klotho improves CI-AKI remain unclear. Furthermore, the specific mechanism of klotho to alleviate cell pyroptosis also needs to be verified. Thus, the purpose of this study was to explore the protective effects of klotho on CI-AKI.

2. Method

2.1. Animal preparation

All operating procedures were in accordance with the Laboratory Animal Welfare Act, the National Institutes of Health Guide for the Care of Laboratory Animals, and the guidelines and policies for rodent experiments provided by the Animal Care and Use Committee of Guangdong Provincial People's Hospital. Six-week-old C57BL/6 male mice weighing 20–25 g purchased from GemPharmatech Co., Ltd. The CI-AKI animal model was previously proposed by Chen et al [17]. And the similar approach was used to build up the CI-AKI model. First, ketorolac tromethamine (Macklin, China) and \( \text{N}^2\)-nitro-L-arginine methyl ester hydrochloride (L-NAME, MedChemExpress) were given by intraperitoneal injection. Ten minutes after ketorolac tromethamine and L-NAME injection, contrast medium (CM) ioversol (10 µl/g body mass, which was equal to 7.4 mgI/g body mass) was administrated via the tail vein (CM group; \( n = 6 \)). Moreover, klotho (0.25 µg/kg body mass) was also injected via intraperitoneal injection (klotho group; \( n = 6 \)) and 10 minutes after ioversol was injected (CM + klotho group; \( n = 6 \)). The control group (\( n = 6 \)) was administered saline via intraperitoneal injection. Animals were euthanized 24 h after these agents were administered.

2.2. Assessment of Renal Function and Histopathology

Blood urea nitrogen (BUN) and serum creatinine (SCr) levels were measured by an automatic biochemical analyzer (Chemray 240, China). Kidney tissue was fixed and embedded, and tissue sections (4 µm thick) were then stained with hematoxylin-eosin (HE) for histopathological analysis. For semiquantitative analysis of the changes of kidney tissue, 10 high-magnification (× 200) fields of the cortex and outer medulla were selected randomly. The percentage of renal damage such as tubular epithelial swelling,
vacuolar degeneration, necrosis, and desquamation was accessed, and the score of tubular damage was determined as follows: no damage (score 0); 10% (score 1); 11–25% (score 2); 26–45% (score 3); 46–75% (score 4); >75% (score 5) [18].

2.3. Cell culture

The HK-2 human proximal renal tubular epithelial cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). They were grown at 37 °C in a humidified atmosphere containing 5% carbon dioxide, and cultured in DMEM/F12 medium (Corning, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 1 × penicillin/streptomycin (Gibco, USA). 0.25% trypsin-EDTA was used to digest cells. Cell medium was replaced every 3 days.

CM (Ioversol, 350 mgI/mL, Jiangsu Hengrui, China) was selected to build the model of CI-AKI. Ioversol was dissolved in sterile phosphate buffer saline (PBS) to obtain a concentration of 25 mgI/mL, 50 mgI/mL, 100 mgI/mL and 150 mgI/mL, respectively. Finally, stimulating with 2 h as Andreucci et al. did [19] and 100 mgI/mL were selected as the final interventional condition. After CM intervention, Recombinant human klotho protein (R&D systems, USA) was treated for 24 h. The cells were divided into control group, CM group, CM + klotho group and klotho group.

2.4. Cell viability assays

According to manual or standard operating procedure (SOP), cells (1×10^4 cells/well) were plated in 96-well plates after the serum starvation treatment, and cell viability was detected with a cell counting kit 8 (CCK-8, Bestbio, China). In short, after 24 h treatment, each well was added in 10 µL CCK-8 solution for incubation of 2 h at 37 °C. After that, the absorbance was detected at 450 nm in Multiskan GO (Thermo Fisher Scientific, USA) and cooperated with SkanIt software (version 3.2).

2.5. Lactate dehydrogenase (LDH) activity assay

Cytotoxicity was measured by the LDH release assay kit (Jiancheng, China). Once the cytomembrane damaged, LDH was rapidly released into the supernatant of cell culture. LDH activity was tested spectrophotometrically at 450 nm, using a microplate reader Multiskan GO (Thermo Fisher Scientific, USA).

2.6 ROS generation test

The level of intracellular ROS was determined by the dichloro-dihydro-fluorescein diacetate (DCFH-DA, Beyotime, China) dye method. In brief, after treatment, HK-2 cells were incubated with probe for 30 min in 37 °C and out of light. The ROS-associated fluorescence intensity was determined with a fluorescence spectrophotometer (Thermo Fisher Scientific, USA) using excitation and emission wave lengths at 450 nm.

2.7. Propidium iodide (PI) staining
After intervention, the cells were washed 3 times with PBS. DAPI (Sigma, USA) was used to stain cells for 5 minutes and cell death was assessed by staining with PI (P4170, Sigma-Aldrich, Germany) for 15 minutes in the dark followed by fluorescence microscopy (Nikon TI-S, Japan). Cell death was quantitated as the percentage of PI-positive cells relative to the total cell number (DAPI-positive cells). All experiments were performed at least three times.

2.8. Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-18, IL-6, TNF-α, superoxide dismutase (SOD), and malondialdehyde (MDA) concentrations were measured using an ELISA kit (Jiangsu Meimian Industrial Co., Ltd). The culture supernatant of HK-2 cells (50 µL) and standards were pipetted in triplicate into appropriate microtiter wells and the assay was performed according to the ELISA kit's instructions. The absorbance was measured at 450 nm and the correction wavelength was 650 nm. (Infinite F50, Sunrise, Switzerland)

2.9. Western blot

HK-2 cells were lysed with total protein extraction kit (Bestbio Company, China). The supernatant was collected after the lysates were centrifuged for 15 min at 12000 rpm in 4 °C atmosphere. Protein concentrations were tested using Pierce BCA protein assay kit (Thermo Fisher, USA). Mixing the protein SDS PAGE loading buffer (Takara, Japan) and total protein (30 µg) were boiled, separated on 12% SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Minipore, USA). Membranes were blocked with 5% nonfat milk in TBS with 0.1% tween for 1 h at room temperature. Then, the membranes were probed with primary antibodies against rabbit monoclonal anti-NLRP3 (1:1000, NOVUS, USA), mouse monoclonal anti-caspase-1 (1:1000, NOVUS, USA), rabbit monoclonal anti-GSDMD (1:1000, Abcam, USA), rabbit monoclonal anti-NF-κB (1:1000, Abcam, USA), rabbit monoclonal anti-phosphorylated-NF-κB (1:1000, Abcam, USA) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1:2000, Abcam, USA) overnight at 4 °C. The next day, the membranes were incubated with anti-rabbit IgG H&L (1:5000, Abcam, U.S.A.) or anti-mouse IgG H&L (1:5000, Cell Signaling Technology, USA) for 1 h at room temperature. After TBST (TBS containing 0.05% tween-20) washes, the blots were visualized with the enhanced chemiluminescence kit (Thermo Scientific, USA). Using ImageJ software (National Institute of Health, Bethesda, USA) to analyze densitometry and the data were normalized with GAPDH.

2.10. Statistical analysis

All data were presented as the mean ± standard deviation (SD). The statistical analyses were performed with GraphPad Prism 8.4.2 (GraphPad Software, USA). The differences among experimental groups were assessed with one-way ANOVA or two-way ANOVA followed by Tukey's test. P values < 0.05 were considered statistically significant.

3. Result

3.1. Klotho alleviated kidney injury in CI-AKI mice.
CI-AKI mice models were established by following steps (Fig. 1A). According to the HE staining results, CM-challenged mice exhibited remarkable pathological changes, including cell swelling, cell necrosis, severe dilatation, epithelial cell exfoliation, and inflammatory cell infiltration in renal tubules. The animals in the CM + klotho group showed significantly lower renal tubular injury scores \((P < 0.001)\) than those in the CM group (Fig. 1B). The levels of BUN and SCr were significantly \((P < 0.001)\) increased in the CM group compared with the control group. Administration of klotho significantly decreased the levels of BUN and SCr \((P < 0.001)\) as compared with CM group (Fig. 1C and D).

3.2. Effects of Various Concentrations of CM on Oxidative Stress and inflammation in vitro.

In order to explore the role of ioversol in HK-2 cells and determine the optimal intervention concentration, we cultured HK-2 cells and treated with different doses of CM for 2 h (Fig. 2A). Compared to control group (0 mg/I/mL of CM), cell viability was measured by CCK-8 assay and was found to decrease in a dose-dependent manner (Fig. 2B). The CM concentration for peak LDH releasing was at 100 mg/I/ mL (Fig. 2C). In the CM concentration of 100 mg/I/mL, HK-2 cells had the lowest SOD activity and the highest MDA and ROS levels (Fig. 2D, E and F). Simultaneously, it was found that the expression of inflammatory cytokines (IL-6 and TNF-\(\alpha\)) in cell supernatants was dramatically increased in the concentration of 100 mg/I/mL CM (Fig. 2G and H). We speculated that most HK-2 cells died when exposed to 150 mg/I/mL CM. Therefore, CM at 100 mg/I/mL was shown to be the significant injury, oxidative stress and inflammation.

3.3. CM exposure stimulated NF-\(\kappa\)B/NLRP3 signaling activation in HK-2 cells.

Pyroptosis was another type of programmed cell death, different from apoptosis, and had been proven to be involved in AKI [20]. Here, the cultured HK-2 cells were treated with different concentrations of CM (0, 25, 50, 100 or 150 mg/L) for 2 h. It was shown that 100 mg/I/mL ioversol treated HK-2 cells significantly increased PI/DAPI staining-positive cells (Fig. 3A). The expression level of IL-18 and IL-1\(\beta\) in the supernatant of HK-2 cells were highest in 100 mg/I/mL ioversol (Fig. 3B and C). Moreover, the expression of pyroptosis-related proteins (NLRP3, caspase-1, GSDMD, and cleaved-GSDMD) were up-regulated after treating with 100 mg/I/mL CM (Fig. 3D). We then found that NF-\(\kappa\)B, an important activator of NLRP3 inflammasome, was significantly activated in HK-2 cells following CM treatment. With the concentration of 150 mg/I/mL CM, we believed that a large number of HK-2 cells death or loss and led to undetectable. In conclusion, the mechanism of NF-\(\kappa\)B/NLRP3-mediated pyroptosis might be involved in the injury of CM-treated HK-2 cells.

3.4. The effect of different doses of klotho on HK-2 cells.

HK-2 cells were treated with klotho for 24 h. There was no significant change in cell viability and LDH release (Fig. 4A and B). Therefore, as an exogenous substance, klotho did not cause damage to HK-2 cells.

3.5. The effect of different doses of klotho on CM-induced HK-2 cells.
Subsequently, we evaluated the effect of different doses of klotho on CM-induced HK-2 cells. After 24 h treatment, klotho significantly protected cells from CM-induced injury. Cell viability gradually recovered and LDH decreased compared to the CM group. Statistically, a significant inhibitory effect of klotho on cytotoxicity commenced at 0.2 µg/mL (Fig. 4C and D).

3.6. Klotho prevented CM-induced oxidative stress and inflammation in HK-2 cells.

According to the above experiments, HK-2 cells were treated with the optimal drug concentration (Fig. 5A). The administration of klotho showed a significant increase in cell viability in HK-2 cells when compared with the CM group (Fig. 5B). CM exposure increased LDH leakage in HK-2 cells, whereas klotho treatment decreased the level of LDH leakage in those cells (Fig. 5C). Oxidative stress usually involved in the damage of CI-AKI. Klotho successfully reduced ROS production, MDA level, and SOD activity along with the antioxidant defence restoration in CM-induced HK-2 cells (Fig. 5D, E and F). Investigating the effect of inflammatory cytokines in CM-induced HK-2 cells, we found that klotho treatment successfully reduced upregulation of TNF-α and IL-1β in CM group (Figs. 5G and H).


CM exposure brought about significant pyroptosis. Fluorescence study showed elevated in PI/DAPI rate in CM-treated HK-2 cells. However, klotho treatment significantly mitigated PI/DAPI rate (Fig. 6A). Klotho decreased the secretion of inflammation cytokines IL-18 and IL-1β in CM-induced HK-2 cells (Fig. 6B and C). In CM + klotho group, there was upregulated expression of phosphorylated NF-κB and pyroptosis-related proteins (NLRP3, caspase-1, GSDMD and cleaved-GSDMD) (Fig. 6D). Therefore, Klotho supplementation significantly reduced pyroptosis reaction.

4. Discussion

In clinical practice, iodinated CM is the most commonly used in clinical angiography, it is excreted by the kidney and had strong side effects on the kidney [21]. CM induced renal tubular injury causes persistent irreversible chronic kidney disease (CKD) or renal failure [22]. However, the underlying mechanisms of CI-AKI were unknown, and there were not the effective drugs to prevent CI-AKI [23]. Therefore, the novel strategies for protecting against CI-AKI remains a crucial and a challenging work.

In this study, we elucidated the protective effects of klotho on renal histological abnormalities, oxidative stress, inflammatory responses and pyroptosis pathways using CM-induced mice model and HK-2 cells. Our study found that administration of ioversol lead to AKI, as shown by injury of renal tubular and deterioration of renal function. Ioversol injection can significantly increase the serum levels of BUN and Scr, which were alleviate by the kltho treatment. Moreover, results of histopathology also suggested that klotho could reduce the necrosis of tubular epithelial cells, vacuolization, and loss of brush borders induced by ioversol. In HK-2 cells, klotho also ameliorated cell injury by increasing cell viability and reducing LDH released. Thus, Klotho treatment offered protective effects by lessening histopathological injury and improving tubular cell injury for the kidney and HK-2 cells.
Accumulating literature had demonstrated that oxidative stress properties lead to CI-AKI [24, 25]. The production of oxidative stress may induce apoptosis and inflammation, therefore aggravating oxidative injury and renal hypoxia [26, 27]. The treatment of CM also increased ROS and lipid peroxidation levels and reduced antioxidant enzyme activities, which resulted in cytotoxic damage. Klotho, for its antioxidative functions and free radical scavenging, has been used effectively to ameliorate ischemia and perfusion injury [28]. In present research, the results showed that ROS levels in the CM group were prominent decreased compared with those in CM + klotho group. In addition, an increase in SOD activity and a reduce in MDA content were detected in CM + klotho group. Previous studies had reported that klotho exhibited a protective effect by reducing lipid oxidation in Diabetic Kidney Disease [29]. In our study, the beneficial effects of klotho against CI-AKI were verified by the reduction of ROS production and lipid peroxidation and the elevation in SOD activities in vitro. Therefore, klotho exerted a significant nephroprotective effect on CI-AKI by inhibiting oxidative stress.

The specific mechanisms underlying CI-AKI had not been fully demonstrated, but previous researches had shown that inflammatory response plays a vital role in CI-AKI [3, 30]. In our study, the IL-6 and TNF-α level were remarkably increased after CM treatment in HK-2 cells. Studies had verified that alleviating inflammatory response and reducing inflammatory indicators, like IL-6 and TNF-α, can alleviate the occurrence of CI-AKI [6]. In the present study, our findings found that klotho administration decreased the inflammatory indicators. Therefore, we thought that klotho showed its anti-inflammatory activity under CM stimuli.

Pyroptosis is a lytic programmed cell death initiated by inammasomes [31]. It has been demonstrated for the pathogenesis of CI-AKI [17]. CM can activate the NLRP3 inammasome to induce pyroptosis [32, 33]. NLRP3 inammasome is a multimeric protein complex, which could activate caspase-1. Activated caspase-1 cleaved GSDMD and inflammatory cytokines (IL-1β and IL-18). Cleaved-GSDMD induced pore formation on the cell membrane and releases mature IL-1β and IL-18 out of the cell, which was a critical process of pyroptosis [31]. Multiple studies have shown that NF-κB plays an important role in cellular inflammatory response and NLRP3 activation [34]. Many harmful substances activated NF-κB subsequently up-regulated NLRP3 and IL-1β [35, 36]. NF-κB binded to a 1.3-kbp fragment of the upstream transcription start site of the NLRP3 gene to activate the NLRP3 inammasome [37]. In our study, we demonstrated that the exposure of ioversol to HK-2 cells accompanied with increase in the PI/DDAPI rate, IL-1β and IL-18 levels and the expression of p-NF-κB, NLRP3, caspase-1, GSDMD, and cleaved-GSDMD. However, klotho administration blocked the up-regulation of IL-1β and IL-18 levels, and the relative expression of p-NF-κB, NLRP3, caspase-1, GSDMD, and cleaved-GSDMD induced by ioversol in HK-2 cells. These data suggested that the NLRP3 inammasome was activated by the p-NF-κB, and suppression of the NF-κB/NLRP3 inammasome helps to protect against CI-AKI. Thus, we describe a new mechanism that klotho reduced NF-κB/NLRP3 inflammation activation to mitigate CI-AKI.

Our study reveals that supplementary klotho protected kidney function by inhibiting renal pyroptosis. However, there is no evidence on whether endogenous Klotho affects NF-κB/NLRP3-mediated pyroptosis in CI-AKI. Future studies will focus on the in vivo mechanisms of CM-induced tubular pyroptosis.
establishment of Klotho deficient mice or conditional knockout of Klotho in the kidney is necessary for studying epigenetic mechanisms and relating signaling in CI-AKI.

5. Conclusion

In this study, we showed evidence that exposure to CM can result in renal injury through increasing oxidative stress and inflammatory reaction, and activating NF-κB/NLRP3 inflammasome-dependent pyroptosis. Klotho can attenuate CM-induced activation of oxidative stress, inflammation, and NF-κB/NLRP3 inflammasome-dependent pyroptosis. These findings provide insight into the therapeutic mechanisms of klotho on CI-AKI.

Declarations

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Guangdong Provincial People’s Hospital(No.KY2020-538-01). All operating procedures were in accordance with the Laboratory Animal Welfare Act, the National Institutes of Health Guide for the Care of Laboratory Animals.

Consent for publication

No applicable.

Competing interests

The authors declare that they have no conflicts of interest.

Availability of data and materials

All data can be obtained from corresponding author Danqing Yu or the first author Yanbin Fu.

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Authors’ Contributions:

(1) Conception and design: Yanbin Fu, Dan-Qing Yu, and Ze-Da-Zhong Su; (2) Administrative support: Danqing Yu, Jianfeng Cao, and Yanzhi Ge; (3) Provision of study materials: Yanbin Fu, Zedazhong Su, and Yanzhi Ge; (4) Collection and assembly of data: Ze-Da-Zhong Su, Yan-Bin Fu, Xue-Biao Wei, and Jianfeng Cao; (5) Data analysis and interpretation: Yan-Bin Fu, Ze-Da-Zhong Su, and Xue-Biao Wei; (6) Manuscript writing: All authors; (7) Final approval of manuscript: All authors.
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References


Figures

**Figure 1**

Klotho alleviated kidney injury in CI-AKI mice.

(A) The process of establishment of CI-AKI mouse model and treatment with klotho. (B) Representative images for H&E staining of kidney tissues and renal tubular injury score (scale bar: 100µm). (C) BUN levels in each group. (D) SCr levels in each group. (ns P > 0.05, ### P < 0.001 vs. the control group, ** P < 0.001 vs. the CM group).

**Figure 2**

Effects of Various Concentrations of CM on Oxidative Stress and inflammation in vitro.
(A) Treatment of HK-2 cells with different doses of ioversol. (B) Cell viability of HK-2 cell after CM exposure. (C) The LDH release of HK-2 cell after CM treatment. (D and E) The activity of SOD and the content of MDA (F) The levels of intracellular ROS were determined by fluorescent probe DCFH-DA (scale bar: 100µm). (G and H) The levels of inflammatory cytokines, including IL-6 and TNF-α. (nsP > 0.05, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the ctrl group). CM, contrast medium; LDH, Lactate dehydrogenase; SOD, superoxide dismutase; MDA, malondialdehyde; DCFH-DA, dichloro-dihydro-fluorescein diacetate.

**Figure 3**

CM promotes pyroptosis by activation of NF-κB/NLRP3 signaling in HK-2 cells.

(A) Representative PI staining images in HK-2 cells (scale bar: 100µm. 200× magnification). (B and C) The supernatant levels of inflammatory cytokines, including IL-18 and IL-1β. (D) The cellular extracts were collected and NF-κB, p-NF-κB and pyroptosis-related protein was measured by western blot analysis. (nsP > 0.05, #P < 0.05, ##P < 0.01, ###P < 0.001 vs. the control group).

**Figure 4**

Dose dependent effects on cell viability and toxicity after CM and klotho exposure on HK-2 cells.

(A) Effects of klotho in different concentrations ranging from 0 to 0.4 µg/mL, on cells viability by CCK-8. (B) The effect of klotho on cellular toxicity as tested by LDH assay. (C) Ameliorative effect of different concentrations of klotho ranging from 0 to 0.4 µg/mL, after CM (100mgI/mL) exposing. (D) Protective effect of different concentrations of klotho ranging from 0 to 0.4 µg/mL, on cellular toxicity after CM (100mgI/mL) exposing. (nsP > 0.05, ###P < 0.001 vs. the control group; ***P < 0.001 vs. the CM group).

**Figure 5**

Klotho prevented CM-induced oxidative stress and inflammation in HK-2 cells.

(A) The procession of experiment. (B) The cell viability of klotho (0.2µg/mL) on CM (100mgI/mL) induced HK-2 cells by CCK-8 assay. (C) The cytotoxicity of klotho (0.2µg/mL) on CM (100mgI/mL) induced HK-2 cells by LDH assay. (D) Effect of CM (100mgI/mL) and klotho (0.2µg/mL) on the activity of antioxidant enzymes SOD in HK-2 cells. (E) Effect of CM (100mgI/mL) and klotho (0.2µg/mL) on the level of MDA in HK-2 cells. (F) Fluorescence micrograph of intracellular ROS were detected by DCFH-DA (scale bar: 100µm.). (G and H) The levels of inflammatory cytokines (IL-6 and TNF-α) in the experimental groups.
(nsP > 0.05, ##P < 0.01, ###P < 0.001 vs. the control group; *P < 0.05, **P < 0.01, ***P < 0.001 vs. the CM group).

Figure 6

Klotho alleviated NF-κB/NLRP3-mediated pyroptosis in HK-2 cells.

(A) Representative PI staining images in HK-2 cells (scale bar: 100 µm). (B and C) The secretion levels of IL-18 and IL-1β. (D) Western blot results for levels of NF-κB, p-NF-κB, NLRP3 and caspase-1, GSDMD and cleaved-GSDMD. (nsP > 0.05, ##P < 0.01, ###P < 0.001 vs. the control group; *P < 0.05, ***P < 0.001 vs. the CM group).