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Original Research Article

Klotho protects chondrocyte viability via FOXO1/3 in osteoarthritis

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Abstract

Purpose: *To investigate the effect of Klotho and FOXO1/3 on the CH viability in OA.*
Methods: *The survival rate of CHs, Klotho and FOXO1/3 protein expression, and ROS production were measured in the OA cartilages of different degenerative phases. H_2O_2 was also used to injure CHs, and the cell viability, Klotho and FOXO1/3 expressions, as well as ROS levels were investigated to clarify the effect of exogenic Klotho on the injured CHs. Additionally, in order to verify the role of FOXO1/3 in Klotho-treated CHs, SOD2, GPX1, inflammatory factors, collagen I/II, SOX9, and Runx-2 levels were analyzed by silencing FOXO1 and FOXO3 expression via siRNA transfection.*
Results: *Klotho and FOXO1/3 expressions significantly decreased, and ROS production increased in severely human OA cartilage ($p < 0.05$). Besides, H_2O_2 affected CHs viability with the suppression of Klotho and FOXO1/3 expression but ROS production was elevated. Exogenic Klotho application partly reversed the injury caused by H_2O_2. Furthermore, Klotho treatment of the injured CHs contributed to SOD2 and GPX1 expressions, and suppressed IL-1β, IL-6, TNF-α and MMP-13 production, resulting in the upregulation of collagen II and SOX9 as well as downregulation of collagen I and Runx-2. However, the protective effect of Klotho was weakened by FOXO1 and FOXO3 gene silencing.*
Conclusion: *Klotho protects CHs viability by suppressing oxidative stress and inflammation, which is associated with the mediation of FOXO1 and FOXO3. These findings provide new insights into the treatment of OA.*

Keywords: *Klotho, FOXO, Chondrocyte, Osteoarthritis, Oxidative stress*

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INTRODUCTION

Osteoarthritis (OA), is one of the most common types of joint disease. It is evidenced by lesions on cartilage tissue, joint synovium, subchondral bone, and other tissues. Cartilage degeneration is the result of a combination of factors, and is the most important pathological change in OA, which has a specific relationship with the viability of chondrocytes (CHs), inflammatory cytokines, and matrix metalloproteinases (MMPs) [1]. In the early stage of OA, the cartilage tissue swelling is caused by permeation. CHs then secrete inflammatory mediators such as interleukin-1 (IL-1) and MMPs, and the extracellular matrix (ECM) components begin to change [2]. During the development of OA, the increase of the inflammatory factors lead to the decline of CH

function, a further degradation of the ECM, and the weakening of the mechanical properties of the cartilage [3] At the late OA stage, the cartilage becomes thin and vascularized, a large number of CHs die, and the ECM is severely damaged [4]. Therefore, the accumulation of the disabled CHs contributes to cartilage loss and gradually aggravates OA progression. Thus, maintaining CH activity is of considerable significance in delaying the progression of OA [5].

Klotho is an aging-related gene discovered by Kuro-o *et al* [6] in a mouse aging model in 1997, and named after the goddess Klotho in ancient Greek mythology. Klotho is divided into membrane-bound Klotho and secretory Klotho, and they perform a variety of biological activities. On the one hand, membrane-bound Klotho acts as a receptor for fibroblast growth factor receptor 23 (FGF23), and mediates the biological activity of FGF23 in regulating the homeostasis of phosphorus metabolism [7]; and on the other hand, secreted Klotho is resistant to oxidative stress and inhibits inflammation and apoptosis in multiple cell types [8]. Klotho is involved in the occurrence and development of various diseases, such as vascular calcification, hypertension, kidney injury, diabetes, and tumors [9,10]. The association between Klotho and OA has already been confirmed in several studies. Gu et al. [11] found that Klotho deficiency causes cartilage damage in OA mice, and Klotho overexpression suppresses CH apoptosis. In addition, Chuchana *et al* [12] discovered that secreted Klotho balances cartilage homeostasis by inhibiting the catabolic process.

The mechanism behind the protection of CHs from Klotho still remains unclear. The previous study suggests that Klotho protein strengthens the resistance to oxidative stress at both cellular and organismal levels, which is related to Forkhead box O (FOXO) activation. Therefore, Klotho and FOXO might have a similar effect on CHs. This study aims to elucidate the role of secreted Klotho and its downstream target, FOXO1/3, in the viability mediation of CHs, with the primary focus being on the anti-oxidative and anti-inflammatory manners.

METHODS

OA tissue collection

Articular cartilage tissue was collected from five patients (2 females, 3 males; average age: 61 years) with advanced OA and who have undergone knee arthroplasty. According to the location, the specimen was divided into three groups: the lateral epicondyle with lighter OA as the control area, the medial condyle with mild OA as the mild degeneration area, and the articular surface with severe OA as the severe degeneration area. This study was approved by the Ethics Committee of Shaoxing Shangyu People's Hospital, and also followed international guidelines for human studies. Written, signed and informed consent was obtained from all participants before the study.

Calcein AM/PI/Hoechst staining

The surviving population of CHs was determined by Calcein AM and PI staining the ViaStain™ Calcein AM/PI/Hoechst Viability Kit (CSK-0118, Nexcelom Bioscience. Merrimack St, MA, USA). The living CHs were visible as green fluorescent, and dead CHs as red fluorescent. Besides, CHs were counterstained with Hoechst to enumerate the total number of nucleated CHs.

Western blot (WB) analysis

For protein isolation, the cartilage tissue was grinded with protein extraction buffer radioimmunoprecipitation assay (RIPA) and protease inhibitor phenylmethylsulfonyl fluoride (PMSF) (Beyotime, Shanghai, China) in order to extract the protein. Protein concentration was determined by utilizing the bicinchoninic acid (BCA) method (Beyotime, Shanghai, China). The protein was transferred by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membrane was blocked with milk, incubated with the primary antibodies at 4°C overnight, and the secondary antibody at room temperature for 1 h. These antibodies were purchased from Abcam (Cambridge, MA, USA). The membranes were finally imaged by an Odyssey machine, and grayscale analysis and quantification of scanned images were ascertained using the ImageJ software (NIH, Bethesda, MD, USA).

CHs isolation and treatment

The CHs from the three areas were cut into fragments and digested with 0.25% trypsin and type XI collagenase (Gibco, Waltham, MA, USA) for 12 h. The Cell pellets were filtered and re-suspended in Dulbecco's modified eagle medium/F12 (DMEM/F12) containing 10 % fetal bovine serum (FBS) and 1% penicillin-streptomycin (Gibco, Waltham, MA, USA). CHs of the first generation were treated with H_2O_2 with or without recombinant human Klotho protein (ab84072, Abcam, Cambridge, MA, USA).

Immunofluorescence (IF)

The protein levels of SOD2 and GPX1 was ascertained using the IF method. Before staining, CHs were fixed with 4 % paraformaldehyde and permeabilized with 0.1% Triton-X for 15 min., and were then treated with 5% bovine serum albumin (BSA) for 1 h at room temperature. Afterwards, CHs were washed with phosphate buffered saline (PBS), incubated with primary antibodies overnight at 4 °C and the secondary antibody at room temperature for 1 h. The first antibodies were purchased from Abcam (Cambridge, MA, USA), and the secondary antibody were from Invitrogen (Carlsbad, California, USA). The staining intensity was worked out using the ImageJ software.

siRNA transfection

The blocking of FOXO1 and FOXO3 gene expressions were performed using the siRNA transfection method, according to the Lipofectamine 2000 (Invitrogen, Carlsbad, California, USA) product instructions. CHs were incubated with the siRNA-transfection reagent mixture for 24 h, and then the medium was changed. siRNA targeting FOXO1 and FOXO3 was purchased from Thermo Fisher Scientific (106652; 115209. Waltham, MA, USA).

Real-time polymerase chain reaction (RT-PCR)

The RNA of CHs was extracted with TRIzol (Invitrogen, Carlsbad, California, USA) using conventional methods, and the purity of RNA was detected by NanoDrop; 1 μg of total RNA was taken for reverse-transcription into complementary deoxyribose nucleic acid (cDNA) using one-step reverse transcription according to the manufacturer's instructions, and then performed in RT-PCR using SYBR Green Master (TOYOBO, Osaka, Japan). The primers used are shown in Table 1. Three replicates were made for each template. Glyceraldheyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference to calculate the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

The levels of Klotho, FOXO1 and FOXO3 in CHs were determined using ELISA method (MBS2886510; MBS023752; MBS265005, San Diego, CA, USA) according to the manufacturer's instructions.

Cell viability assay

CHs viability was ascertained using the cell counting kit 8 (CCK8) method (Beyotime, Shanghai, China) according to the manufacturer's specifications. The intensity of the CCK8 product was measured at 450 nm using a microplate reader. Cell viability was presented as a percentage relative to the non-treated value.

Reactive oxygen species (ROS) assay

The total ROS in cartilage tissue and CHs was worked out using the ROS Detection Kit (PK-CA577-K936, Heidelberg, Germany), according to the kit protocol. The suspension of single-CH was analyzed on a plate reader for 3 replicates per condition. Relative fluorescence units (RFU) were detected using flow cytometry.

Statistical Analysis

The experimental data were statistically analyzed using Statistical Product and Service Solutions (SPSS) 18.0 (SPSS Inc., Chicago, IL, USA) and expressed as mean ± standard deviation (SD). A comparison amongst the various groups was carried out using the One-way ANOVA test, followed by Post Hoc Test (Least Significant Difference). $P < 0.05$ was considered statistically significant.

Table 1: Primer sequences for RT-PCR

Gene name	Forward (5'>3')	Reverse (5'>3')
Collagen I	GAGGGCCAAGACGAAGACATC	CAGATCACGTCATCGCACAAC
Collagen II	TGGACGATCAGGCGAAACC	GCTGCGGATGCTCTCAATCT
SOX9	AGCGAACGCACATCAAGAC	CTGTAGGCGATCTGTTGGGG
Runx-2	TGGTTACTGTCATGGCGGGTA	TCTCAGATCGTTGAACCTTGCTA
IL-1β	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA
TNF-α	CCTCTCTCTAATCAGCCCTCTG	GAGGACCTGGGAGTAGATGAG
IL-6	ACTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGTTG
MMP-13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

RESULTS

Klotho and FOXO1/3 decreased in OA cartilage

In order to clarify the relationship between Klotho and OA, OA cartilage was collected and separated into three groups based on their surfaces. As shown in Figure 1 A, a cartilage with a flat, uniform, and transparent surface indicated a normal or slightly degenerate condition; the cartilage with a somewhat rough surface indicated a mildly degenerate state; and the cartilage with an extremely rough surface indicated a severely degenerated state. Furthermore, calcein AM/PI staining was used to determine the living CHs in the three states.

The result showed the surviving CHs significantly reduced as the cartilage got worse OA (Figure 1 B and C). Klotho, FOXO1, and FOXO3 protein were observed by WB, and it was found that all three proteins reduced gradually with the development of OA, especially the Klotho (Figure 1 D and E). As Klotho and FOXO have been stated to be capable of suppressing oxidative stress, the ROS production in the cartilage was measured by flow cytometry. As expected, the result indicated a higher ROS accumulation in the severely degenerated cartilage compared to the mild group, and much more than the control (Figure 1 F). Therefore, the findings suggest that the Klotho, FOXO1, and FOXO3 expressions were obviously reduced in the severely degraded cartilage. Further studies however need to be carried out on how klotho aids the survival of CHs.

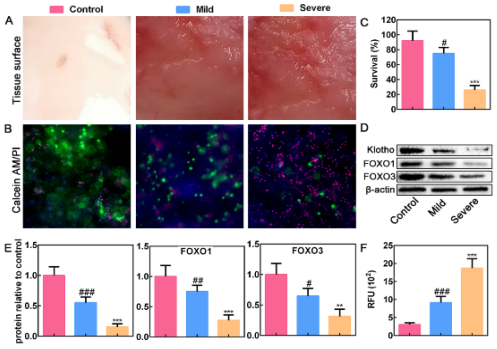

Figure 1: Klotho and FOXO1/3 decrease in the OA cartilage. (A) Images of the surface of the cartilage. (B) Representative images of Calcein AM/PI/Hoechst staining (magnification: 200×), and (C) quantification analysis. (D) Western blot analysis of Klotho, FOXO1, and FOXO3, and (E) quantification analysis. (F) Flow cytometry of ROS assay. #*P*< 0.05, ##*p* < 0.01, ###*p* < 0.001, compared to Control; **p < 0.01, ***p < 0.001, compared to mild)

Klotho and FOXO1/3 decreases in H_2O_2-treated CHs

To confirm that the expressions of Klotho and FOXO1/3 decreased with the CHs degeneration *in vitro*, H_2O_2 was used to trigger the injury of CHs. We cultured the CHs with different concentrations of H_2O_2 (100 to 400 μM) for 24 h, and harvested for analysis. As shown in the data in Figure 2 A, the Klotho content decreased under the H_2O_2 stimulation in a dose-dependent manner compared to the control. This was also the case with FOXO1 and the FOXO3 expression. The viability of CHs also significantly decreased due to the effects of H_2O_2 (Figure 2 B). As an inducer of oxidative stress, the ROS level was upregulated with the presence of H_2O_2, compared to the control group (Figure 2 C). The data here is convincing evidence that the content of Klotho and FOXO1/3 is suppressed in the degraded CHs.

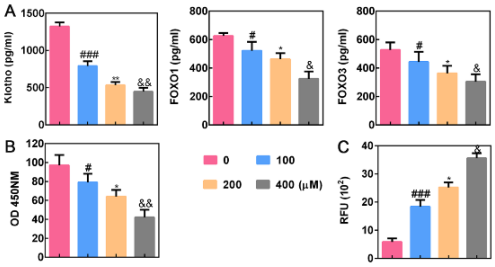

Figure 2: Klotho and FOXO1/3 decrease in H_2O_2-treated CHs. (A) Levels of Klotho, FOXO1, and FOXO3 determined by ELISA. (B) CCK8 assay of CHs viability. (C) Flow cytometry of ROS assay. (# p < 0.05, ### p < 0.001 compared to non- H_2O_2; * p < 0.05, ** p < 0.01 compared to 100 H_2O_2; & p < 0.05, && p < 0.01 compared to 400 H_2O_2)

Klotho protects H_2O_2-treated CHs survival and promotes FOXO1/3 expression

To determine whether Klotho could protect the CHs under the H_2O_2 stimulation, the CHs were treated with 200 μM H_2O_2 for 24 h and then the medium was exchanged with human-recombined Klotho protein (200 or 400 pM) for another 24 h treatment. The contents of Klotho, FOXO1, and FOXO3 were also measured using the ELISA method. These proteins were suppressed by H_2O_2, but the exogenic Klotho supplements reversed the effect of H_2O_2, which was very significant under a concentration of p400 pM (Figure 3 A). The stimulation of Klotho was also linked to the viability of CHs based on the result of the CCK assay, which was directly proportional to the number of living cells (Figure 3 B). In addition, the increased Klotho expression also played a role in the inhibition of ROS production when compared to the H_2O_2 treated

group, which also depended on the dose (Figure 3 C). The results above indicated that the H_2O_2 affected the viability of the CH, triggering the accumulation of ROS and the downregulation of Klotho, and FOXO1/3. However, the exogenic supplement of Klotho protein promoted the FOXO1 and FOXO3 expressions, which also protected the survival of CHs and suppressed the ROS production.

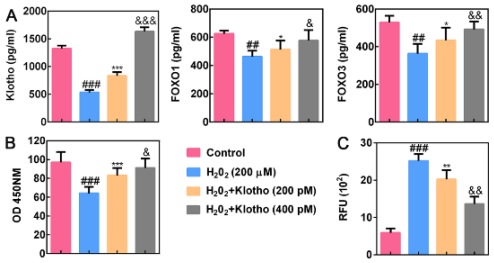

Figure 3: Klotho protects H_2O_2-treated CHs survival and promotes FOXO1/3 expression. (A) ELISA assay of Klotho, FOXO1, and FOXO3 level. (B) CCK8 assay of CHs viability. (C) Flow cytometry of ROS assay. (##P < 0.01, ###p < 0.001 compared to Control; *p < 0.05, **p < 0.01, ***p < 0.001 compared to H_2O_2; &p < 0.05, &&p < 0.01, &&&p < 0.001, compared to H_2O_2+200 Klotho)

FOXO1/3 deficiency weakens the protection of Klotho on H_2O_2-treated CHs

Klotho significantly upregulated FOXO1 and FOXO3 expression. Hence, the FOXO1 and FOXO3 gene was silenced with siRNA transfection. After 24 h incubation with siRNA, the FOXO1 or FOXO3-silenced CHs were treated with H_2O_2 (100 to 400 μM) for another 24 h. As shown in Figure 4 A, the silencing of FOXO1 or FOXO3 did not weaken the viability of CHs in standard culture conditions. But the ability to withstand the damage by H_2O_2 was reduced in the silenced CHs. A similar situation happened in ROS production (Figure 4B). Compared to the control, the silenced CHs produced more ROS under the stimulation of H_2O_2, which was also dose-dependent.

Furthermore, Klotho protein was added in the FOXO1, or FOXO3-silenced CHs after the treatment of H_2O_2 (200 μM). The Klotho protein supplement upregulated the cellular Klotho content, increased FOXO3 levels in FOXO1 silenced CHs, and also elevated the FOXO1 levels in FOXO3-silenced CHs. However, the FOXO1 and FOXO3 levels were still low in each corresponding silenced-CHs (Figure 4C). As a result of FOXO1/3 silencing, the viable protection of Klotho in the H_2O_2-treated CHs decreased (Figure 4 D). Besides, FOXO1/3 deficiency also weakened the anti-ROS effect of Klotho, which was also significant when compared to the H_2O_2 alone group (Figure 4E). Therefore, the blocking of FOXO1 or FOXO3 affected the influence of Klotho with regards to the protection of cell viability and ROS suppression of CHs.

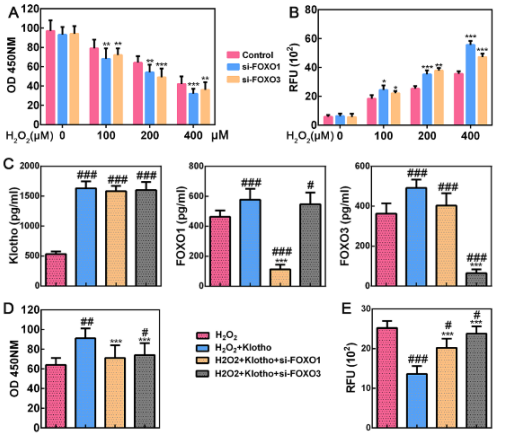

Figure 4: FOXO1/3 deficiency weakens the protection of Klotho. (A) CCK8 assay of CHs viability. (B) Flow cytometry of ROS assay. (*P < 0.05, **p < 0.01, ***p < 0.001 compared to Control) (C) ELISA assay of Klotho, FOXO1, and FOXO3 level. (D) CCK8 assay of CHs viability. (E) Flow cytometry of ROS assay. ## P < 0.01, ###p < 0.001 compared to H_2O_2; ***p < 0.001 compared to H_2O_2+Klotho)

Klotho suppresses ROS inflammation and production of H_2O_2-treated CHs via FOXO1/3

To further clarify the mechanism underlying the use of Klotho in the treatment of degradative CHs, the anti-oxidative enzymes, SOD2 and GPX1, the inflammation level, and the ECM metabolism-related gene mRNA expression were all analyzed. Compared to the control, H_2O_2 suppressed the SOD2 and GPX1 protein expression, which was partly recalled by Klotho stimuli. However, the silencing of FOXO1 or FOXO3 weakened the effect of Klotho again (Figure 5 A and B). H_2O_2 induced the upregulation of IL-1β, IL-6, TNF-α, and MMP-13, and Klotho played an inhibitory role in the inflammatory factor expression, which was also partly rejected by the deficiency of FOXO1 or FOXO3 (Figure 5 C). Besides this, the mRNAs of collagen II and SOX9 were upregulated, but collagen I and Runx-2 was downregulated due to Klotho stimuli applied to the H_2O_2 group. The silencing of FOXO1/3 also alleviates the function of Klotho in this gene expression. Therefore, it can be concluded that Klotho prevent H_2O_2 induced CHs injury by increasing the anti-oxidative stress and anti-inflammation ability with the mediation of ECM balance, which was partly based on the presence of FOXO1 and FOXO3.

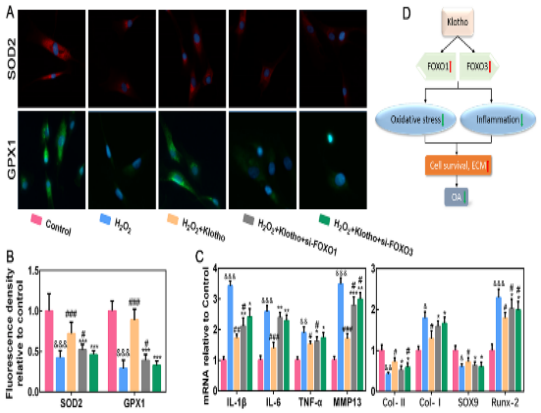

Figure 5: Klotho suppresses ROS and inflammation production *via* FOXO1/3. (A) IF staining of SOD2 and GPX1 (magnification: 400×), and (B) quantification analysis. (C) RT-PCR analysis of mRNA expression. (D) Graphical abstract of this study. &P < 0.05, &&p < 0.01, &&&p < 0.001 compared to Control; #p < 0.05, ##p < 0.01, ###p < 0.001 compared to H_2O_2; *p < 0.05, **p < 0.01, ***p < 0.001 compared to H_2O_2+Klotho)

DISCUSSION

Chondrocytes (CHs)s are terminally differentiated cells with weak proliferation abilities When the CH viability decreases, the anabolic capacity gradually decreases, and the ability to respond to growth factors, such as anabolic factors, gradually declines. It lacks the ability to synthesize sufficient cartilage matrix needed to maintain cartilage volume and cartilage structure [13]. In a healthy cartilage, the metabolism of the matrix is strictly regulated to maintain a balance between synthesis and degradation, which is generally believed to be broken when OA occurs [14]. During OA, degradation and synthesis usually increase together. Through anabolic metabolism, CHs attempt to repair the damaged ECM. Due to the effects of various matrix MMPs and specific cytokines, the cartilage matrix is degraded, the synthesis is inhibited, and then the cartilage destruction is intensified. In some areas, the rate of biosynthetic metabolism cannot keep up with the speed of catabolic metabolism, resulting in cartilage degeneration [15].

The decrease in functional CHs may be one of the reasons leading to reduced synthesis of cartilage matrix, increased degradation, and finally cartilage degeneration. In this study, Klotho is deemed adequate in supporting the viability of CHs in the oxidative injury condition. In the meantime, it also plays a role in anti-inflammatory action. Therefore, Klotho enhances the population of functional CHs as well as ECM stability, which is reflected in the maintenance of type II collagen and SOX9, and reduction of collagen I and Runx-2. The healthy cartilage matrix is mainly composed of collagen II, water, and proteoglycans. SOX9 is also known as a transcription factor that is vital for CH differentiation and cartilage formation [16]. Whereas, the different or degraded CHs express more type I collagen and Runx-2 [17].

Oxidative stress not only damages the cartilage matrix, it also injures the biofilm, proteins, and nucleic acids of CH, resulting in changes to the morphology, growth status, and function of the CHs. Redundant ROS leads to a considerable reduction of CHs survival, leading to the atrophy and thinning of articular cartilage which are essential mechanisms of OA [18,19]. The relationship between Klotho gene and oxidative stress has been studied for a long time. Padanilam *et al* [20] found that in H_2O_2-induced oxidative stress in a mouse's kidney; the expression of Klotho was reduced, and the apoptosis of renal collecting duct cells increased, which makes Klotho fit for use as an indicator of tissue apoptosis under oxidative stress. In addition, Klotho is efficient in the removal of ROS and confers oxidative stress resistance [21]. FOXO belongs to a class of transcription factors involved in the regulation of oxidative stress, as well as the modulation of the downstream gene targets in CHs or CHs' activity. Amongst the three principal membranes, FOXO1 and FOXO3 are found in both bone and cartilage [22]. Klotho can suppress oxidative stress by up-regulating FOXO. Lim *et al* [23] reported that Klotho protects oxidative stress by enhancing FOXO3-mediated MnSOD expression. Similar to what this study discovered, Akasaki *et al* [24] proposed that the silencing of FOXO1/3 affects CHs viability, accompanied by increased apoptosis. However, whether Klotho prevents CHs from ROS-induced injury by the upregulation of FOXO1 and FOXO3 has not been previously reported. Experimental findings so far provide convincing evidence that Klotho and its downstream target FOXO1/3 play a crucial role in the OA process.

CONCLUSION

Klotho has shown remarkable protective effects on CHs, oxidative injuries and inflammation, due to the upregulation of FOXO1 and FOXO3. The findings also show that the secreted Klotho can be a target that interrupts the disability of CHs, through the application of a deep understanding of Klotho and FOXO in OA treatment. These findings provide new insights into the treatment of OA.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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