Activating Mitochondrial Sirtuin 3 in Chondrocytes Alleviates Aging-Induced Fibrocartilage Layer Degeneration and Promotes Healing of Degenerative Rotator Cuff Injury

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The present study aimed to examine the impact of mitochondrial sirtuin 3 (SIRT3) on the degenerative rotator cuff injury, which is a prevalent issue among the elderly population primarily due to aging-related tissue degeneration. The study hypothesized that SIRT3, as a major deacetylase in mitochondria, is a significant factor in controlling the quality of mitochondria and the deterioration of fibrocartilage, a crucial component of the rotator cuff. Results showed that the aging process led to weakened biomechanical properties and degeneration of the fibrocartilage layer in mice, accompanied by a decrease in SIRT3 expression. SIRT3 activation ameliorated the aging-related disruption of chondrocyte phenotype and fibrocartilage degradation. SIRT3 activator honokiol improved the phenotype of senescent chondrocytes and promoted rotator cuff healing in aged mice through SIRT3 activation. In conclusion, the findings suggested that the decline in SIRT3 levels with age contributes to rotator cuff degeneration and chondrocyte senescence, and that SIRT3 activation through the use of honokiol is an effective approach for promoting rotator cuff healing in the elderly population. (Am J Pathol 2023, 181: 1–11; https://doi.org/10.1016/j.ajpath.2023.03.013)

Degenerative rotator cuff injury is a disease caused primarily by age-related tissue degeneration and occurs in the elderly population.1 Epidemiologic data showed that rotator cuff injury occurs in about 62% of people aged >80 years, and the retear occurs in 25% of people aged >70 years.2 The incidence of degenerative rotator cuff injury in the elderly population poses a significant economic burden and requires the development of an effective prevention and treatment approach. The difficulty in repairing the injury, which occurs at the bone-tendon interface, is primarily due to the slow and incomplete regeneration of the native fibrocartilage layer, which is associated with its relative avascularity. So far, fibrocartilage regeneration occurs primarily through the modulation of biological or biomechanical environment, as well as tissue engineering, which promotes rotator cuff repair, but is undertreated in the elderly.3–5 To address this challenge, it is necessary to identify the underlying mechanisms that contribute to the difficulty in regenerating the aging fibrocartilage layer.

Fibrocartilage consists of chondrocytes and their extracellular matrix. The function of chondrocytes is crucial for the maintenance of fibrocartilage properties and regeneration after injury.2,9 Chondrocyte function, which requires energy support, can be regulated by controlling the quality of mitochondria, which are the production sites of energy. Sirtuin 3
(SIRT3), a major deacetylase in mitochondria, is involved in regulating mitochondrial function by removing acetylation modifications after protein translation and activating enzymes in mitochondria. The expression level of SIRT3 decreases during aging and is linked to aging-associated degeneration and disease. Honokiol (HK) is a pharmacologic activator of SIRT3. It can activate SIRT3 to block cardiac hypertrophy, ameliorate cognitive decline induced by surgery/anesthesia, protect hepatocytes, or inhibit atrial metabolic remodeling in atrial fibrillation. Herein, HK as a SIRT3 activator was used to treat rotator cuff injury in aged mice.

This study examined the role of mitochondrial SIRT3 in aging-induced fibrotic cartilage layer degeneration and evaluated the therapeutic potential of SIRT3 activator HK in promoting the healing of rotator cuff injury in aged mice.

Materials and Methods

This study was approved by the Medical Ethics Committee of Central South University (number 201703222). All animal experiments were performed in the Department of Experimental Animal of Central South University.

Animal Model Establishment and Group

In this study, a total of 130 male C57BL/6 mice at the age of 18 months underwent surgical repair of the detached supraspinatus tendon in the left shoulder. The mice were randomly divided into seven groups, including the control group, fibrin gel group and HK/fibrin gel group (n = 30), as well as the positive control group, SIRT3 siRNA group, SIRT3 siRNA + HK group, and HK group (n = 10). The surgical procedure followed a previously established protocol and involved aseptic preparation of the upper limb, anesthetization with pentobarbital sodium, and incision of the skin and deltoid muscle to expose the supraspinatus tendon. The tendon was reattached with a suture and ligated the skin and deltoid muscle to expose the supraspinatus tendon in the left shoulder. The mice were anesthetized with 0.3% pentobarbital sodium (0.6 mL/20 g), and after making a longitudinal skin incision on the lateral side of the left shoulder, a transverse cut was made on the deltoid muscle to expose the supraspinatus tendon; then, the mice were injected with 5 mL of AAV2-SIRT3 (https://www.ncbi.nlm.nih.gov/gene; accession number NM_001177804) into the enthesis of the left shoulder, using a 10-μL microinjector, to deliver the SIRT3 gene. The virus was injected over 60 seconds each time, after which the incision was closed.

Preparation of Fibrin Gel Drug Delivery System

The preparation of fibrin gel for drug delivery was based on a previously published method. To prepare the fibrin gel, 10 mg/mL fibrinogen solution and 50 IU/mL thrombin solution were prepared, as per the manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO). Then, 10 μL of the fibrinogen solution and 1 μL of the thrombin solution containing 1 μL of HK were mixed in a petri dish and allowed to form fibrin gel for HK delivery during rotator cuff repair. The control mixture consisted of the same amount of fibrinogen solution and thrombin solution containing the solvent dimethyl sulfoxide.

AAV2-Mediated Short Hairpin SIRT3 Transfection

AAV2 vectors were used to establish an SIRT3 gene knockdown model in vivo. The AAV2-U6-CMV-EGFP-SV40 vector was provided by Starfish Biotechnology Co (Hunan, China) with a titer of 1 × 10^{13} vector genomes/mL. The short hairpin SIRT3 was delivered via the AAV2 vectors in a fibrin gel and positioned at the enthesis during the animal modeling procedure. That is, the fibrin gel drug delivery system and AAV2 vectors were sequentially placed at the enthesis after exposing the supraspinatus tendon, and the dose and method of the virus delivery are the same as before.

Evaluation

Biomechanical Analysis

The biomechanical properties of the supraspinatus tendon-humerus complex of the treated animals were evaluated. Fresh samples of supraspinatus tendon-humerus from mice of 3, 10, and 18 months of age were also obtained and subjected to mechanical testing using a biomechanical machine (Instron, Norwood, MA). The samples were wrapped at both ends with sand cloth before testing, which was performed at a constant rate of 0.03 mm/second with a preload of 0.1 N. The load (N) and stiffness (N/mm) at which the tendon failed were recorded, and data were discarded if the tendon did not break at the insertion site or slipped out during testing.
Histomorphologic Analysis
The tissue specimens were processed for histomorphologic analysis as follows. The samples were fixed in 4% paraformaldehyde for 48 hours and then decalcified in 10% EDTA until the humeri were softened. Subsequently, the specimens were embedded in paraffin, cut into sections (5 μm thick), and stained with hematoxylin-eosin and toluidine blue. The maturity of the tissue specimens was quantified using a modified maturing scoring system, as described in previous studies. The thickness of the fibrocartilage layer was determined using a previously published protocol. The fibrocartilage layer area was defined as the region between the proximal humerus boundary and the distal supraspinatus tendon boundary, whereas the fibrocartilage layer length was the distance between the upper and lower points. The thickness of the fibrocartilage layer was calculated as the ratio of the area/length.

Immunohistochemical Analysis
For immunohistochemical analysis, the tissue sections were first treated with xylene to remove wax and then hydrated using a gradient series of alcohols. The antigen repair was performed using gastric enzyme repair solution, followed by blocking of endogenous peroxidase with 3% hydrogen peroxide (H2O2). After blocking with 3% bovine serum albumin, the sections were incubated overnight with primary antibodies specific to SIRT3 (1:100; ab86671; Abcam, Cambridge, UK), collagen II (1:400; ab34712; Abcam), and p16INK4a (1:100; ab211542; Abcam) at 4°C overnight. The sections were then washed with phosphate-buffered saline three times and incubated with horseradish peroxidase–conjugated goat polyclonal anti-rabbit IgG secondary antibody (1:200; GB23303; Servicebio, Wuhan, China) for 1 hour at room temperature. The sections were then colored using diaminobenzidine and counterstained with hematoxylin. The images were observed using a microscope (CX31; Olympus, Tokyo, Japan), and semi-quantitative assessments were performed using Image-Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD).

Primary Chondrocyte Culture
The primary chondrocyte culture was performed using 5- to 6-day-old mice, according to a previously described method. To briefly summarize the procedure, the articular cartilage was isolated from the knee joint, then digested with a 10-mL collagenase D solution at a concentration of 10 mg/mL, and incubated overnight at 37°C. The cell suspension was filtered through a 70-μm sterile cell strainer and centrifuged for 5 minutes at 161 × g. The resulting pellet was resuspended in Dulbecco’s modified Eagle’s medium, low glucose, containing 10% fetal bovine serum and 1% penicillin/streptomycin, and cultured in a T25-cm² flask at 37°C in a 5% CO2 humidified atmosphere, with the culture medium changed every 3 days. The phenotype of the primary chondrocytes was verified using Alcian blue staining and immunofluorescence staining for collagen II (Supplemental Figure S1).

Treatment of Chondrocytes in Vitro
To induce senescence in primary chondrocytes, they were seeded into a 12-well plate at a density of 2 × 10⁴ cells per well and treated with various concentrations of H2O2 (0, 250, 500, and 1000 μmol/L) for 48 hours. This was based on previous published methods, and the optimal induction concentration was determined on the basis of cell viability and senescence-associated β-galactosidase staining. The SIRT3 plasmid (https://www.ncbi.nlm.nih.gov/nuccore; accession NM_001177804.1) and control plasmid, as well as the SIRT3 siRNA (5′-CCACGGGACCTTTGTAACA-3′) and scramble siRNA, were constructed by Ribobio (Guangzhou, China). The SIRT3 plasmid and SIRT3 siRNA, along with the corresponding controls, were transfected into chondrocytes using lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s protocol. As an activator of SIRT3, HK was applied to chondrocytes at different concentrations (0, 10, 30, and 50 μmol/L) and incubated at 37°C for 24 hours.

Alcian Blue Staining
For the purposes of Alcian blue staining, the previously treated cells were fixed with 4% paraformaldehyde at 4°C for 30 minutes. The cells were then washed three times with phosphate-buffered saline and stained with Alcian blue solution for 30 minutes, and the images were captured under a microscope (CX31). The percentage of Alcian blue staining was quantitatively analyzed using Image-Pro Plus 6.0.

Senescence-Associated β-Galactosidase Staining
Senescence was determined using a Cell Senescence β-Galactosidase Staining Kit (Yeasen, Shanghai, China) following the manufacturer’s protocol. The cells that were treated with H2O2 were fixed with fixation solution at room temperature for 15 minutes, washed with phosphate-buffered saline, and incubated with the staining mixture containing X-Gal at 37°C overnight. The images were observed under a microscope (CX31), and the percentage of senescence-associated β-galactosidase–positive cells was analyzed semiquantitatively using Image-Pro Plus 6.0 in a blinded manner.

Cell Viability Detection
The impact of HK or H2O2 on cell viability was determined using the cell counting kit-8 (Biosharp, Hefei, China; BS350B) according to the manufacturer’s protocol. Briefly, 2000 cells per well of P1 chondrocyte suspension were seeded into a 96-well plate and allowed to incubate overnight. Then, the chondrocytes were treated with varying
concentrations of HK or H2O2, and 10 μL of cell counting kit-8 solution was added to each well and incubated for 1 hour. The absorbance value of each well was then measured at 450 nm using a microplate analyzer (Thermo Fisher Scientific).

Immunofluorescence Assay
The effect of different treatments on chondrocytes was analyzed using an immunofluorescence assay. The chondrocytes were first fixed with 4% paraformaldehyde at 4°C for 30 minutes, and then permeabilized with 0.1% Triton X-100 for 30 minutes. After blocking with 4% bovine serum albumin at room temperature for 1 hour, the samples were incubated with primary antibodies specific to SIRT3 (5 μg/mL; ab86671; Abcam), collagen II (1:200; ab34712; Abcam), and γ-histone H2A.X (γ-H2AX; 1:500; ab11174; Abcam) at 4°C overnight. The samples were then washed with phosphate-buffered saline three times and incubated with donkey anti-rabbit IgG H&L (Alexa Fluor 594; 1:500; ab150076; Abcam) at 37°C for 1 hour. After washing with 0.1% Tween-20 three times, the samples were counterstained with DAPI and observed under a fluorescence microscope (Zeiss, Oberkochen, Germany). Semiquantitative assessments were performed using Image-Pro Plus 6.0.

Western Blot Assay
The protein levels in treated chondrocytes were analyzed using a Western blot assay. The protein was extracted from the chondrocytes using a total protein extraction kit (Solarbio, Beijing, China; BC3711), and the protein concentration was quantified using a bicinchoninic acid protein colorimetric assay kit (Elabscience, Wuhan, China). The protein was separated on SDS-PAGE and transferred to a polyvinylidene fluoride membrane, then blocked in 5% nonfat milk and incubated with primary antibodies speciﬁc to SIRT3 (5 μg/mL; ab86671; Abcam), collagen II (1:200; ab34712; Abcam), and P16 (Cdkn2a): forward, 5'-ATCCGGGACCTTCAGATCCC-3' and reverse, 5'-ACAATGAAAAAGGGCCTTGGG-3'; SOX9: forward, 5'-CGTCGCAATACGACTACGC-3' and reverse, 5'-TAGAGCCCTGACCCCTGGC-3'; GAG (aggrecan): forward, 5'-GGGAAGCAGTACAACATAGG-3', and reverse, 5'-ATACCCCCCACCACGCGGG-3'; P16 (Cdkn2a): forward, 5'-GAACATCTCAGGGCCGAAA-3', and reverse, 5'-GCGCTTGGAGTGATAGAAATC-3'; and GAPDH: forward, 5'-TAGTCGCTGGATGATAAGAAC-3', and reverse, 5'-ACTGTGCCCGTTGAATTTGCC-3'.

Statistical Analysis
All quantitative data were expressed as means ± SD, and analyzed by t test (comparison between two groups) or one-way analysis of variance (analysis of variance, comparison among three groups and above) followed by the Bonferroni post hoc test for comparison between different groups, using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

Results
Aging-Related Degeneration of Rotator Cuff in Mice
This study investigated the aging-related degeneration of rotator cuff in mice by comparing 3-month-old (young), 10-month-old (middle-aged), and 18-month-old (old) populations. Biomechanically, the failure load and stiffness in 18-month-old mice were significantly weaker than those in 3-month-old mice (Supplemental Figure S2A). Histologically, the maturing score, fibrocartilage thickness, and mean OD for collagen II in 18-month-old mice were also significantly lower than those in 3-month-old mice (Supplemental Figure S2, B and C). The above results indicated that the 18-month-old mice can be used to represent the aged mice, which showed weakened biomechanical properties and histologic features. Meanwhile, the increased expression of p16InK4a, a biomarker of cellular senescence, was observed in the fibrocartilage layer of the 18-month-old mice (Supplemental Figure S2, B and C), which indicated that individual aging of mice is accompanied by chondrocyte senescence.

To further study the changes in senescent chondrocytes, a cellular senescence model was established by exposing chondrocytes to H2O2. As shown in Supplemental Figure S3, A and B, senescence-associated β-galactosidase
staining result indicated that H$_2$O$_2$ at 250 and 500 μmol/L effectively induced chondrocyte senescence, and cell counting kit-8 assay indicated that cell viability dramatically decreased with H$_2$O$_2$ at 500 and 1000 μmol/L (Supplemental Figure S3C). Considering the above results, 250 μmol/L of H$_2$O$_2$ was chosen as the best concentration to induce cellular senescence, which was used in the following experiments. In addition, the senescent status of chondrocytes was identified by detecting senescence-related markers. As shown in Supplemental Figure S4, higher expression levels of senescence-related mRNA and proteins P16, P21, and P53, as well as stronger immunofluorescence intensity for γ-H2AX, were observed in H$_2$O$_2$-treated group, which further evidenced the senescent status of chondrocytes.

The phenotype of senescent chondrocytes was impaired. As shown in Supplemental Figure S2, D–G, the percentage of Alcian blue staining and the intensity for collagen II in senescent group were both significantly lower than that in young group. RT-qPCR analysis demonstrated that the mRNA levels of chondrocytes biomarkers (COL2A1, GAG, and SOX9) were significantly down-regulated in senescent chondrocytes. Western blot analysis also showed that the expression level of collagen II decreased in senescent chondrocytes, but there was no significant change in the expression level of SOX9. The role of mitochondrial SIRT3 in aging-induced fibrocartilage layer degeneration was investigated next.

SIRT3 Is Down-Regulated at Fibrocartilage Layer of Rotator Cuff in Aged Mice

The expression level of SIRT3 was observed in the fibrocartilage layer of rotator cuff in mice of different ages: 3 months (young), 10 months (middle-aged), and 18 months (old). As shown in Figure 1A, there was a significant decrease in the expression level of SIRT3 in the fibrocartilage layer of rotator cuff in the middle-aged and old mice, compared with that in the young mice. Furthermore, the induction of chondrocyte senescence in vitro also resulted in a decreased expression of SIRT3. As shown in Figure 1, B–D, the intensity of SIRT3 was significantly lower in the senescent group compared with that in the young group, and both the mRNA and protein levels of SIRT3 showed a significant decrease in senescent chondrocytes. All above results indicated that the expression level of SIRT3 was reduced in senescent chondrocytes.

SIRT3 Alleviates Fibrocartilage Layer Degeneration of Rotator Cuff in Aged Mice

To examine the potential of SIRT3 in mitigating degeneration of the fibrocartilage layer in the rotator cuff of aged mice, SIRT3 plasmid was transfected into senescent chondrocytes. Transfection efficiency was confirmed by RT-qPCR analysis (Supplemental Figure S5A). SIRT3 significantly increased the Alcian blue staining percentage and
the intensity of collagen II (Figure 2, A and D). In addition, SIRT3 elevated both mRNA and protein levels of chondrocyte-related markers (Figure 2, F and G), indicating a promotion of proteoglycan and collagen synthesis and secretion by chondrocytes. Delivery of SIRT3 to the bone tendon junction of the rotator cuff improved the maturing score, fibrocartilage thickness, mean OD of collagen II, and the expression level of SIRT3 (Figure 2, B, C, E, H, and I). These results suggested that activation of mitochondrial SIRT3 in chondrocytes can ameliorate the impairment and degeneration of the fibrocartilage layer induced by aging.

HK Improves Phenotype of Senescent Chondrocytes by Activating SIRT3

On the basis of prior research, HK was used as an activator of SIRT3. Herein, HK increased the expression of SIRT3 in senescent chondrocytes in a dose-dependent manner (Figure 3, A and C). To evaluate the efficacy of HK in treating rotator cuff injuries in aged mice, its impact on senescent chondrocytes was first examined in vitro. As demonstrated in Figure 3A, HK at 50 μmol/L significantly elevated the protein level of collagen II. Furthermore, dose-dependent increases in collagen II intensity and Alcian blue staining were observed (Figure 3, B, E, and F). RT-qPCR analysis also indicated a significant up-regulation of the chondrocyte biomarker COL2A1 after treatment with HK at 50 μmol/L (Figure 3D). On the basis of the cell counting kit-8 result (Supplemental Figure S6), a concentration of 30 μmol/L was selected as the optimal dose for HK treatment on senescent chondrocytes. To test whether the improvement in senescent chondrocyte phenotype by HK was through the activation of SIRT3, HK was applied to senescent chondrocytes treated with SIRT3 siRNA. The efficiency of SIRT3 siRNA transfection was verified by RT-qPCR analysis (Supplemental Figure S5B). Treatment with HK increased the mRNA levels...
of GAG, but this effect was neutralized by SIRT3 knockdown (Figure 3G). Furthermore, the effect of HK on increasing the intensity of collagen II and Alcian blue staining percentage was also neutralized by SIRT3 knockdown (Figure 3, H–J). These results indicate that the effect of HK on senescent chondrocytes is through the activation of SIRT3.

HK Promotes Healing of Rotator Cuff Injury through SIRT3 in Aged Mice

The effect of HK on the healing of rotator cuff injury in aged mice was evaluated, and HK was delivered via fibrin gel. HK treatment significantly increased the failure load and stiffness of the rotator cuff at 4 and 8 weeks after operation (Figure 4A). Histologic analysis demonstrated that HK treatment significantly improved the maturing score of rotator cuff at 4 weeks after operation, increased the thickness of fibrocartilage layer at 2 and 4 weeks after operation, and enhanced the mean OD of collagen II at 4 and 8 weeks after operation (Figure 4, B–E).

To examine the role of SIRT3 in the effect of HK on rotator cuff healing, HK was applied to SIRT3 knockdown mice. HK treatment increased the biomechanical properties of the rotator cuff, including the failure load and stiffness,
and this effect was neutralized by SIRT3 knockdown (Figure 5, A and B). Furthermore, HK treatment increased the maturing score, fibrocartilage thickness, and mean OD of collagen II in the repaired rotator cuff, and these effects were also neutralized by SIRT3 knockdown (Figure 5, C–F). All above findings suggested that SIRT3 played a crucial role in the effect of HK on the healing of rotator cuff injury.

Discussion

The rotator cuff is an important structure of the shoulder, and its injury is common, particularly among the elderly population. Previous studies have shown that advanced age leads to rotator cuff degeneration, resulting in tears in the muscles, tendons, and fibrocartilage, and reducing the ability to heal rotator cuff injuries. The challenge in repairing rotator cuff injuries is the slow and incomplete regeneration of the native fibrocartilage layer, which has been addressed in previous studies through tissue engineering and regulation of the regenerative environment. However, these methods are limited in their efficacy for elderly patients. Therefore, the purpose of this study was to explore strategies to promote the regeneration of degenerative fibrocartilage.

Mitochondrial function is known to play a central role in aging, and aging process is accompanied by a decline in mitochondrial quality. SIRT3, a major deacetylase in the mitochondria, is crucial in maintaining mitochondrial quality. This study, for the first time, demonstrated the role of SIRT3 in aging-induced fibrocartilage degeneration. The role of SIRT3 was investigated at the cellular level, in chondrocytes, which are the main components of fibrocartilage. Previous research has shown a decrease in SIRT3 expression in aged hematopoietic stem cells, joint cartilage, ovaries, and age-related diseases. In addition, overexpression of SIRT3 could alleviate the cell senescence and progression of diseases of aging. The current study
found that the expression level of SIRT3 was downregulated at the fibrocartilage layer of rotator cuff in aged mice, and in senescent chondrocytes in vitro. In addition, activating SIRT3 alleviated the impaired phenotype of senescent chondrocytes and degenerative fibrocartilage in aged mice.

In young individuals, rotator cuff injuries are usually repaired through surgery and supplementary physical therapy. In elderly individuals, conservative treatment methods, including the use of anti-inflammatory, analgesic, and swelling medications, are primarily utilized. HK, an activator of SIRT3, has several beneficial effects, including anti-inflammatory, antioxidant, antibiotic, antitumor, and anti-aging properties.40-44 Its safety and lack of toxic effects have been established through prior evaluations.45 In addition, HK can protect against liver and heart damage by activating SIRT3.17,19 In this study, rotator cuff injuries in aged mice were treated with HK. This was found to play a protective role in senescent chondrocytes by activating SIRT3, leading to an improvement in rotator cuff healing. This provided a novel approach for the treatment of rotator cuff injuries in elderly individuals.

There are some limitations to this study. The first is that the induction of senescence in chondrocytes through H2O2 in vitro may not fully simulate the senescence of cells in vivo. However, it is currently the best method for constructing a model of senescent chondrocytes in vitro. The second limitation is the non-specific delivery of HK. The development of a chondrocyte-specific delivery system for more precise therapy will be a focus of future research.

In conclusion, this study demonstrated that the expression level of SIRT3 significantly decreased at degenerative fibrocartilage layer of rotator cuff in aged mice. The current data indicate that activating SIRT3 in chondrocytes can alleviate aging-induced fibrocartilage degeneration and promote healing of degenerative rotator cuff injury.

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Author Contributions

H.L. and D.S. conceived and designed the study; S.X. wrote the manuscript; and S.X., C.G., T.H., G.Y., and J.H. performed the experiments, analyzed the data, and prepared the figures. All authors have reviewed the final manuscript and approved the submission to this journal.

Supplemental Data

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