1. Introduction

Osteoarthritis (OA), one of the most prevalent joint diseases that mainly damage articular cartilage and involves the whole joint tissue, is commonly found among the middle-aged and elderly and is a main cause of disability in the elderly (1). With the increasingly serious global aging, the incidence of OA is also increasing year by year (2). According to the statistics of the World Health Organization, the global incidence of OA is about 10-17% in people aged 40-60 years and 50% in people over 60 years of age in 2020 (3). OA typically presents with severe joint pain and limitation of motion, which can cause joint weakness, muscle atrophy and other serious diseases and eventually lead to limb disability as the disease progresses (4). Clinically, there is no plan to completely cure OA, and lifelong remission treatment is needed once patients develop the disease (4). Clinically, it is considered that a thorough understanding of the pathogenesis of OA and molecularly targeted therapies are breakthroughs for future diagnosis and treatment of OA (5).

The Semaphorin family is composed of a large number of secretory or membrane-bound proteins called axon-guiding molecules during nervous system development, which plays a role in various processes such as immunomodulation, angiogenesis, bone remodeling, apoptosis, cell migration, and invasion (6). Of them, Semaphorin 3A (Sema3A) is a secretory member of the Semaphorin family, which has been confirmed by a large number of studies to affect the pathogenesis of rheumatic immune diseases by participating in the immunoregulation and angiogenesis processes, and plays a role as a regulator in the occurrence and development of rheumatic immune diseases (7, 8). Recent evidence has demonstrated that inflammatory cytokines promote chondrocyte apoptosis in OA through Sema3A signal transduction (9). In addition, Sema3A can also alleviate lipopolysaccharids-induced chondrocyte inflammatory responses by binding to neuropilin 1 (NRP1), a downstream receptor of Sema3A (10). Therefore, there may be an important potential relationship between Sema3A and OA, which is expected to be the key to future diagnosis and treatment of OA. However, there is still little research confirming the direct regulation of Sema3A expression on OA, and its pathways of action remain unknown, lacking reference for the realization of...
molecular targeted therapy through Sema3A.

Therefore, by analyzing the effect of Sema3A on mitochondrial damage of chondrocytes in OA, this study provides reference and guidance for future clinical research on new diagnosis and treatment protocols of OA.

2. Materials and methods

2.1. Animal data

Ten SPF healthy male SD rats, 7 weeks old and weighing 200-220 g, were ordered from Pizhou Dongfang Breeding Co. Ltd., Jiangsu (SCXK (Su) 2019-0005). These animals were allowed to eat and drink at random in a controlled environment (room temperature: 20±2 °C, humidity: 55±15 %, and 12h/12h light/dark irradiation cycle). This study was conducted with the approval of the Animal Ethics Committee of our hospital, strictly following the 3R principle (refining, reducing and replacing the use of animals) of animal experiments. The animals were randomized into normal group (n=5) and model group (n=5) after adaptive feeding for one week.

2.2. Modeling

Rats in normal group were fed conventionally without any treatment. In the model group, an OS model of left hind limb was prepared by injection of papain (11): The rats were fasted for 12h before modeling. After anesthesia via 3% sevoflurane inhalation, the rats were placed on the operating table on their side, the knee hair was shaved and disinfected, and the knee joint of the left hind limb was bent by 45°. A needle was then inserted from the outer edge of the patellar tendon at the lower pole of the patella to the intercondylar area, and 0.15 mL of papain mixture (4% papain:0.03 mol/L L-cysteine = 2:1) was injected when there was a feeling of emptiness under the needle and no blood was drawn back. The injection was given on the 1st, 4th and 7th day of each week for 3 consecutive weeks. After the modeling was completed, the Lequesne MG index (12) (Table 1) was used for assessment of behavioral activities, with a score ≥6 considered as successful modeling.

2.3. Tissue staining

All rats were sacrificed with their necks severed under anesthesia. The knee cartilage tissues of model and normal group rats were collected, paraffin-embedded, and prepared as 5-μm sections for hematoxylin-eosin (HE) staining, and the pathological changes were observed under microscopy. Another portion of tissue sections was treated with clear bony markings and the pathological changes were observed under microscopy, (room temperature: 20±2 °C, humidity: 55±15 %, and 12h/12h light/dark irradiation cycle). This study was conducted with the approval of the Animal Ethics Committee of our hospital, strictly following the 3R principle (refining, reducing and replacing the use of animals) of animal experiments. The animals were randomized into normal group (n=5) and model group (n=5) after adaptive feeding for one week.

2.4. Western Blot

Total proteins were extracted from cartilage tissues of each group and their concentrations were determined using bicinchoninic acid (BCA) assays. After sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotting onto polyvinylidene fluoride (PVDF) membrane, the proteins were blocked by 5% skimmed milk powder for 2 hours and immersed in Sema3A, NRPI, TRAIL, OSCAR, OPG and β-actin primary antibodies all diluted at 1:1, 000. Following membrane washing with TBST, the proteins were added with an HRP-coupled secondary antibody (1:2, 000) and were allowed to stand for 1.5 h at room temperature. They were then subjected to ECL reaction and gel imaging. With β-actin as an internal reference, the relative expression level of each protein was calculated by Image J software.

2.5. Detection of inflammatory factors by enzyme-linked immunosorbent assay (ELISA)

5 mL of blood was drawn from the rat abdominal aorta in both groups, and centrifuged for 10 min (3, 000 rpm/ min) after standing for 1 hour to obtain serum. Serum tumor necrosis factor-α (TNF-α), interleukin-1β/6 (IL-1β/6), and matrix metalloproteinase-13 (MMP-13) were measured with reference to the ELISA kit instructions.

2.6. Chondrocyte extraction

The cartilage of the knee and hip joints of the model group was quickly collected under aseptic conditions, washed with aseptic PBS 2-3 times, cut into 1-3 mm³ pieces and transferred to a 25-mL aseptic culture bottle. After adding 10 mL of 0.2% trypsin, the pieces were incubated in a 5% CO₂ incubator at 37°C and oscillated every 15 min. Forty-five minutes later, the trypsin was removed, and 10 mL of 0.2% collagenase type II (Cls II) was added. Following 90 min of culture, the digestive fluid was collected, and 0.2% Cls II was repeatedly used until all the cartilage fragments were digested. Finally, the digestive fluid was collected, filtered with a 200-mesh gauze to remove impurities, and centrifuged for 10 min (1000 rpm/ min). The lower cell layer was diluted with the prepared

<table>
<thead>
<tr>
<th>Score (of student's work)</th>
<th>Knee joint localized pressure pain manifestation</th>
<th>Joint mobility</th>
<th>Joint swelling</th>
<th>Change in step</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 points</td>
<td>Unresponsive</td>
<td>&gt;90°</td>
<td>Clear bony markings</td>
<td>Normalcy</td>
</tr>
<tr>
<td>1 point</td>
<td>Contraction of the affected limb</td>
<td>45-90°</td>
<td>Bone markings are blurred</td>
<td>Can run with a mild limp</td>
</tr>
<tr>
<td>2 points</td>
<td>Mild systemic reaction</td>
<td>15-44°</td>
<td>Loss of bony markings</td>
<td>Walking, with marked lameness</td>
</tr>
<tr>
<td>3 points</td>
<td>Twitch</td>
<td>&lt;15°</td>
<td>-</td>
<td>unable walk</td>
</tr>
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Tab.1. Lequesne MG scoring criteria.
dulbecco's modified eagle medium (DMEM) and seeded in flasks at a density of $2 \times 10^5$, into which 15% foetal bovine serum (FBS) was added. After 3-5 days when the cells adhered to the wall, the culture medium was changed and the cells were allowed to grow until they were 80% fused, after which identification and cell passage were initiated.

2.7. Cell transfection

Hanbio Biotechnology Co. Ltd., Shanghai, was entrusted to design and construct lentivirus vectors that can overexpress or silence Sema3A expression and an empty vector. Subsequently, following the Lipofectamine 3000 transfection kit instructions, the abnormal expression vectors of Sema3A were transfected into chondrocytes, which were labeled as Sema3A-ov group, Sema3A-si group, and Sema3A-nc group, respectively. Western blot was carried out to detect Sema3A and NRPI expression to verify the transfection success rate. The expression of autophagy-associated proteins LC3-II and Beclin1 was also detected, using the same method as above.

2.8. Cell-counting kit (CCK)-8 and cell cloning assays for cell proliferation

Cells of each group were inoculated onto 96-well plates (3×10^5/well), and CCK-8 solution was added to each well (10 μL/well) at 24, 48, 72, and 96 h, respectively. After another 2 h of culture, the optical density (OD) was detected at 450 nm wavelength by microplate reader, and cell growth curves were drawn. In addition, the cells of each group were inoculated into 6-well plates (500 cells per well) for further culture, with the culture medium changed every 2 days. The culture was terminated when there were visible clonal cell clusters in the 6-well plates. After immobilization with 4% parafomaldehyde for 30 min, 1% crystal violet staining for 30 min, and drying, the cells were photographed to count the number of cell colonies.

2.9. Detection of cell apoptosis by flow cytometry

Cells were detached by digestion with a digestive solution containing 0.25% trypsin, followed by centrifugation and supernatant removal to collect the precipitate, which was then rinsed with PBS three times and added with 500 μL of binding buffer to suspend the cells. After mixing with AnnexinV-FITC (5 μL), propidium iodide (5 μL) was further added and mixed, and the culture was carried out away from light at room temperature for 5-15 min. Cell apoptosis in each group was analyzed by flow cytometry.

2.10. MitoSOX Red dye detection of reactive oxygen species (ROS) content

Cells were added with MitoSOX with a final concentration of 5 μmol/L for 30 min of culture at 37°C in the dark and then washed. The MitoSOX fluorescence intensity, which reflected the amount of superoxide produced by mitochondria (green fluorescence), was detected by fluorescence spectrophotometer, with the excitation and emission wavelength being 488 nm and 580 nm, respectively.

2.11. ELISA detection of superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA)

Cells were inoculated into a 12-well plate (1×10^5/well), lysed on ice, and centrifuged for 5 min to extract supernatant (12, 000 rpm/min), for ELISA determination of the expression of SOD, CAT and MDA.

2.12. JC-1 dye detection of mitochondrial membrane potential

Cells were inoculated into a 12-well plate at 1×10^5/well and added with JC-1 dye as a fluorescent probe (prepared with dimethyl sulfoxide with a concentration of 1 g/mL) for 20 min of incubation in a 37°C incubator. After three rinses with sterile PBS, the cells were observed using an inverted fluorescence microscope, and the average fluorescence intensity was analyzed by Image J software. The ratio of mitochondrial depolarization was measured by the ratio of mean red-green fluorescence intensity.

2.13 Statistical analyses

All assays in the study were run in triplicate, and the results were expressed as ($\bar{x} \pm s$). Inter- and intra-group comparisons were made using the independent sample t-test and repeated measures ANOVA plus Bonferroni post-hoc test, respectively. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. OS modeling results

The Lequesne MG score of each rat in the model group was ≥6 points (mean: 8.20±0.84), higher than that of the normal group (P<0.05, Fig 1A). According to HE staining, the cartilage tissue structure of normal control rats was normal, with distinct layers and orderly arrangement of chondrocytes. In the model group, the cartilage tissue structure was destroyed, the cartilage layer became thin and rough, and the chondrocyte arrangement was disordered, with considerable inflammatory cell infiltration and increased small vessels (Fig 1B). TUNEL staining showed that the apoptosis rate of chondrocytes in the model group was (7.67±0.77)%, higher than that of (0%) in the normal group (P<0.05, Fig 1C).

3.2. Comparison of Sema3A expression and inflammatory reaction

The levels of TNF-α, IL-1β, IL-6, and MMP-13 in the model group were (246.04±5.13)ng/L, (122.73±9.70)ng/L, (25.72±3.39)ng/L, and (100.41±10.90)μg/L, respectively, all of which were significantly higher compared with the normal group (P<0.05, Fig 2A), confirming the presence of serious inflammation in the model group. In addition, the model group showed lower Sema3A, NRPI, and OPG expression.
protein levels than the normal group, and higher TRAIL and OSCAR protein expression (P<0.05, Fig 2B).

3.3. Intervention effect of Sema3A abnormal expression vectors

The rat chondrocytes in the model group were extracted for transfection with Sema3A abnormal expression vectors. Sema3A and NRP1 were found to be higher in Sema3A-ov group than in Sema3A-si and Sema3A-nc groups, while those in Sema3A-si group were lower compared with Sema3A-nc group (P<0.05, Fig 3), confirming successful transfection.

3.4. Effect of Sema3A on chondrocyte activity

The OD value and cloning rate of Sema3A-ov group were the highest among the three groups, while those of the Sema3A-si group were lower compared with the Sema3A-nc group (P<0.05, Fig 4A, B). In addition, the apoptosis rate of Sema3A-ov group was (5.21±0.54)%, which was the lowest among the three groups; the apoptosis rate of Sema3A-si group was (11.59±0.89)%, higher versus the Sema3A-nc group (P<0.05, Fig 4C).

3.5. Impact of Sema3A on mitochondrial damage of chondrocytes

After detection, SOD and CAT in Sema3A-ov group were found to be the highest among the three groups, while MDA was the lowest (P<0.05). Sema3A-si group had lower SOD and CAT and higher MDA than Sema3A-nc group (P<0.05, Fig 5A). The results of JC-1 fluorescence staining indicated that the red/green fluorescence ratio of Sema3A-ov group was the highest, while that of Sema3A-si group was the lowest (P<0.05, Fig 5C).

3.6. Influence of Sema3A on chondrocyte autophagy

Finally, detection of autophagy-associated proteins showed that the expression of LC3-II and Beclin1 in Sema3A-ov group was the lowest among the three groups, while that in Sema3A-si group was the highest (P<0.05, Fig 6), indicating that silencing Sema3A expression can promote chondrocyte autophagy.

4. Discussion

OS, as one of the high-incidence diseases among middle-aged and elderly people, is also one of the major diseases that disable patients (13). A thorough understanding of the pathogenesis of OS is helpful for future clinical searches for new OS diagnosis and treatment schemes. In this study, we found that Sema3A presented low expression in OS and affected OS progression by modulating the biological behavior of chondrocytes and mitochondrial damage, which lays a foundation for the targeted treatment of OS through Sema3A in the future.

Although some studies have initially confirmed the potential link between Sema3A and OS (14, 15), no studies have yet verified the exact expression of Sema3A in OS. Therefore, we first confirmed Sema3A expression in OS. We established a rat model of OS by injection of papain, an effective, safe, rapid, and robust in vitro model of OS most commonly used in clinical research (16). The modeling results showed that the cartilage tissue structure of the model group was destroyed, the cartilage layer became thin and rough, and the arrangement of chondrocytes was...
disordered, with a large number of inflammatory cells infiltrated and increased chondrocyte apoptosis, which is in line with the pathological manifestations of OS. In addition, the OPG of OS rats decreased, and the levels of TRAIL, OSCAR and inflammatory factors increased, indicating that the model rats have obvious skeletal dysfunction and increased inflammatory reaction, which confirms the success of the modeling (17). Subsequently, both Sema3A and its downstream protein NRP1 were found to be low-expressed in OS rats, suggesting that Sema3A may be involved in the occurrence and development of OS, consistent with the research results of Sun J et al. (9).

Sema3A contains 771 amino acids, and its structure includes a semaphorin domain, a plexin-semaphorin-integrin domain, an immunoglobulin-like domain, and a C-terminal, in which the N-terminal semaphorin domain is a conserved extracellular domain shared by members of the semaphorin family, containing about 500 amino acids that can bind to different receptors to perform different biological functions (18). Current studies have confirmed the mechanism of action of Sema3A in diseases such as cardiovascular diseases and ankylosing spondylitis (19, 20), but the specific impact on OS is still unclear. For clarification, we extracted chondrocytes from OS rats and constructed abnormal expression vectors of Sema3A for intervention. After increasing Sema3A expression, the proliferation of chondrocytes was enhanced and the apoptosis was reduced, while silencing Sema3A inhibited chondrocyte proliferation and promoted apoptosis. It can be seen that under-regulating Sema3A in OS can accelerate the apoptosis of chondrocytes, which may be one of the mechanisms by which Sema3A affects the progression of OS. Similarly, Lin et al. mentioned that Sema3A accelerated the malignant progression of viral myocarditis by inhibiting the proliferation of cardiomyocytes (21), which can support our findings. However, it has also been reported that overexpressing Sema3A can inhibit the growth of oral cancer cells (22), contrary to our research results, which is speculated to be related to the fact that Sema3A may bind to different receptors in different diseases to exert different biological effects.

On the other hand, mitochondrial damage during Sema3A modulation of cell activity changes has been widely discussed (23, 24). For OS, mitochondrial damage of chondrocytes is also one of the most important pathological changes (25). Through detection, we found that silencing Sema3A expression significantly intensified the oxidative stress response of chondrocytes in OS, with significantly enhanced green fluorescence after JC-1 staining, indicating serious mitochondrial damage. At the same time, LC3-II and Beclin1 protein expressions were increased, which further confirms the enhancement of mitochondrial autophagy, indicating the important influence of Sema3A silencing on mitochondrial damage of chondrocytes. However, the above pathological changes were completely reversed after increasing Sema3A expression, suggesting that the targeted increase of Sema3A expression may be a potential therapeutic scheme for repairing mitochondrial damage in OS chondrocytes. In the study of Guo Z et al., they also found that silencing Sema3A could induce mitochondrial apoptosis of RAW264.7 (26), which is consistent with our view. These results lay a foundation for the future clinical development of molecularly targeted therapy regimens for OS.

However, due to limited conditions, this study has not yet monitored the actual clinical expression of Sema3A in clinical OS cases. Meanwhile, more experiments are needed to further analyze the influence mechanism and action pathway of Sema3A on OS, so as to provide more comprehensive clinical reference and guidance.

5. Conclusion

Sema3A is under-expressed in OS, and is involved in the pathological progression of OS by promoting mitochondrial damage and autophagy of chondrocytes and inhibiting their proliferation. The above pathological changes can be reversed by Sema3A expression. In the future, Sema3A is expected to be a new direction for the diagnosis and treatment of OS, providing a more reliable guarantee for the prognosis of OS patients.

Conflict of Interests

The author has no conflicts with any step of the article preparation.

Consent for publications

The author read and approved the final manuscript for publication.

Ethics approval and consent to participate

No human or animals were used in the present research.

Availability of data and material

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Authors’ contributions

Samir. J. Bilal did all the steps in the research work.

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None.

References

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