Catalytic anti-oxidative stress for osteoarthritis treatment by few-layered phosphorene

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\textbf{ABSTRACT}

As one of the most common representations of articular cartilage damage, osteoarthritis (OA) is characterized by the apoptosis and dysfunction of chondrocytes as well as the progressive degradation of extracellular matrix, of which the main components are glycosaminoglycan and type II collagen. Few-layered phosphorene (FLP) has been attracting great attentions in biomedical fields owing to the excellent capability of in-situ catalysis for scavenging oxidate-associated molecules, especially the reactive oxygen species (ROS) and reactive nitrogen species (RNS). Herein, FLP has been fabricated and employed for articular cartilage protection by means of deleting oxidate-associated molecules. The \textit{in vitro} results show that as low as 200 $\mu$g/mL FLP is capable of diminishing oxidative damages on the osteoarthritic chondrocytes through the efficient elimination of ROS, H$_2$O$_2$ and NO. Meanwhile, the cartilage matrix protection has also been achieved at 200 $\mu$g/mL FLP by the uniform restoration of glycosaminoglycan and type II collagen. FLP enables the nanocatalytic treatment for the overloaded oxidative stress in the injured articular cartilage and represents a promising alternative for osteoarthritis therapy.

1. Introduction

Articular cartilage lacks blood vessels, nerves and lymph supplies, which would lead to intrinsic limitations of self-repair and regeneration in the aging process and joint injury, including various common pathological conditions like osteoarthritis (OA), rheumatoid arthritis and local cartilage defect [1]. Although multiple treatments such as conventional drug administrations, microfracture technique, cartilage transplantation surgery and stem cell-based therapies have been performed in surgical clinical trials, the optimal solution in repair and regeneration of articular cartilage is still vague \cite{2,3}. Injured cartilage would not only induce DNA damage and protein denaturation, but accelerate the degradation of the extracellular matrix due to the considerable generation of reactive oxygen species (ROS) that responds to the inflammatory-related signals and chemicals, among which hydrogen peroxide (H$_2$O$_2$) features the most common and abundant category \cite{4-6}. In addition, the over-production of nitric oxide (NO) surrounding the cartilage and synovial tissues during the progressive stage of arthritis conversely promotes the destruction of cartilage and synovium in the injured joint \cite{7-9}. Therefore, it is of great importance to efficiently restrict the production of these oxidate-associated molecules and repair the damaged articular cartilage.

The rapid development of clinical biomedicine and nanobiotechnology has furnished the emergence of diverse inorganic nano-systems, which offers multiple therapeutic routines as potential alternatives in combating various pathological abnormalities, especially in orthopedic diseases \cite{10,11}. Currently, great efforts for cross-disciplinary research frontier have been focused on biomedical applications of two-dimensional (2D) nanomaterials, a newly emerging...
subtypes of nanomaterials with ultrathin layer-structured topology [12–15]. As a new family of 2D nanomaterials, few-layered phosphorene (or black phosphorus) has been arousing much interest in tremendous biomedicine fields such as drug nanocarriers [16,17], tumor theranostics [18–20], biosensors [20], and bone formation [16,20–22]. For instance, phosphorene nanosheet was previously applied in specific drug delivery for depression therapy, photothermal and photodynamic treatments for tumor immunotherapy as well as biomineralization and repair for bone defect [23–26]. Nevertheless, the therapeutic practice of phosphorus-based nanomaterials for articular cartilage restoration and injury repair is rarely demonstrated in the previous studies [27]. Taken together, 2D structural phosphorene featuring high surface-to-volume ratio, flexibility of modification, and ample physicochemical properties [22], and it marks a promising alternative for cartilage protection by eliminating the oxidate-associated molecules.

Herein, few-layered phosphorene (FLP) was synthesized for the injured cartilage tissue with a rat model of osteoarthritis, and further investigated of its efficacy for cartilage protection. The injured chondrocytes were firstly prepared through the stimulation of inflammatory factors, and co-incubated with FLP to determine the appropriate working concentrations. After the dosages of 50 μg/mL and 200 μg/mL FLP were determined, the survival analysis and measurements of oxidate-associated molecules of the injured chondrocytes were conducted by co-incubation with FLP for 48 h. Afterwards, by using in vivo rat model of osteoarthritic injured cartilage, the cartilage protection ability of FLP was validated through the staining of extracellular matrix consisting of glycosaminoglycan and type II collagen (Scheme 1). Therefore, this study provides convincing evidences of FLP-based cartilage protection in the joint diseases.

2. Results and discussion

2.1. Synthesis and characterization of FLP

In this study, the few-layered phosphorene (FLP) was synthesized by an ultrasonication-assisted mechanical exfoliation method using bulk black phosphorus as a precursor. TEM images display freestanding few-layered or monolayer nanosheets featuring the planar morphology with lateral size of hundreds of nanometers after ultrasonication treatment for 18 h (Fig. 1a and b). The crystalline FLP was further investigated by scanning transmission electron microscopy with high-angle annular dark field (HAADF-STEM). It could be found that a clear crystal lattice spacing of 0.17 and 0.26 nm corresponded to lattice planes of (0 6 0) and (0 4 0) of as-prepared FLP (Fig. 1c). The elemental mapping of FLP suggests a typical monoelemental layered microstructure (Fig. 1e and f). AFM results match the few-layered or monolayered structure of FLP with a thickness of 1.0–1.3 nm (Fig. 1g and h). Raman scattering spectrum shows three characteristic Raman peaks at ~356.7 cm⁻¹, ~430.1 cm⁻¹, and ~456.7 cm⁻¹, in accord with the three vibrational modes of A₁g, B₂g, and A₃g, respectively (Fig. 1d). The above characterization results demonstrated the successful preparation of FLP.

2.2. Antioxidative efficiency evaluation

As one of the cytotoxic ROS, hydroxyl radicals (⋅OH) were generated by Fenton reaction and captured by DMPO, which generated a typical 1:2:2:1 signal peak in ESR spectroscopy. Obviously, the significantly attenuated signal of ⋅OH peaks could be seen upon the addition of FLP, demonstrating the high ⋅OH scavenging capability (Fig. 1i). Additionally, the O₂⁻ scavenging capability was assessed by color reaction of WST-1, for the reaction product featuring the characteristic absorption at 450 nm. It is found that the inhibition rate of O₂⁻ significantly increased with elevated FLP concentration and reached almost 100% when the concentration up to 150 μg/mL, implying that the O₂⁻ can be effectively eliminated by FLP (Fig. 1j). Considering that the excessive oxidative stress is closely related to cartilage injury [28], the radicals oxidized from 2'-azinobis (3-ethylbenzthiazoline-6-sulfonic acid), was suppressed by the increment of FLP while producing the characteristic ABTS decolorization assay. As depicted in Fig. 1k, dose-dependent antioxidative activity was demonstrated, where the efficacy of 100 μg/mL FLP was equivalent to 1.4 mM Trolox. Notably, the antioxidant capacity ratio of Trolox/glutathione/ascorbic acid was 1/1.09/1 through the
ABTS assay. It has been known that H$_2$O$_2$ features another primary source of cellular ROS [29]. In general, the elimination capacity of H$_2$O$_2$ was assessed via detecting its characteristic absorption at 240 nm. Fig. 1l shows that the absorbance of mixture at 240 nm gradually declined over time, indicating a time-dependent increase of H$_2$O$_2$-inhibiting effect. It is expected that such FLP possessed the superb ROS scavenging ability, which could be employed as an emerging antioxidative nanoagent.

2.3. Chondrocyte activation and FLP concentration screening

To investigate the therapeutic efficacy of FLP on cartilage restoration and osteoarthritis treatment, the osteoarthritic injured chondrocytes were prepared with the stimulation of IL-1β according to the previous studies [30–33]. Fig. S1a shows that the expression of CD54 (red fluorescence) in the normal chondrocytes was lower than that in the IL-1β-induced injured chondrocytes (Fig. S1b). Meanwhile, the augmented ratio of CD54/DAPI (red/blue) fluorescence intensity of the injured chondrocytes also revealed a rising expression of CD54 with the stimulation of IL-1β when compared with the normal cells (Fig. S1c). The previous study showed that CD54 expression would be activated and elevated in cartilage injury and inflammatory arthritis [34], thus the over-expression of CD54 in this study suggested the activation of the chondrocytes and the early-stage damage of the cartilage extracellular matrix. The above results implied the successful establishment of the in vitro model of the injured chondrocytes.

Afterwards, the appropriate working concentrations of FLP for further practices were determined by a gradient analysis. An elevated concentration series of FLP from 0.01 to 200 μg/mL was co-cultured with the injured chondrocytes and examined with the standard Cell Counting Kit-8 (CCK-8) assay. There were no significant inhibition effects on the cell viabilities when the concentrations were less than 0.40 mg/mL, and 50 as well as 200 μg/mL FLP exhibited the highest viabilities among these examined concentrations (Fig. S1d). Therefore, two FLP concentrations (50 μg/mL and 200 μg/mL) were selected and applied for following therapeutic experiments.

2.4. Survival analysis of FLP-treated injured chondrocytes

Survived chondrocytes in osteoarthritis are necessary to maintain the degenerated cartilage extracellular matrix, and they will encounter injury, degradation and apoptosis [35]. Therefore, in this work, the survival of injured chondrocytes after incubation with FLP was stained with Calcein-AM and PI for cell live/dead observation. As shown in Fig. 2a, with the treatment of 200 μg/mL FLP, the green fluorescence of the survived injured chondrocytes gradually increased over time, while the fluorescence of the injured cells without FLP treatment significantly decreased. Furthermore, the green fluorescence of 200 μg/mL FLP-treated chondrocytes was also higher than 50 μg/mL FLP-treated cells. Moreover, based on the quantitative analysis, the mean fluorescence of the chondrocytes was 8.23 ± 4.09 as FLP of 50 μg/mL versus 18.01 ± 3.03 as FLP of 200 μg/mL in 72 h (P < 0.001) (Fig. 2d), when Fig. 2b showed no differences of fluorescence among these groups in 24 h and Fig. 2c showed that 200 μg/mL FLP-treated fluo-intensity has not resumed to that of the normal cells in 48 h. Hence, 200 μg/mL of FLP could promote the proliferation of the survived injured chondrocytes, and 50 μg/mL FLP just partially maintained the injured cells.

In addition, the dead chondrocytes after IL-1β stimulation were reported by the presence of red fluorescence. After incubation with 200 μg/mL of FLP for 72 h, the red fluorescence of the injured chondrocytes evidently reduced in comparison to the injured cells without FLP treatment and 50 μg/mL FLP-treated cells (Fig. 2a). The mean fluorescence quantification was significantly different between 50 μg/mL FLP-treated chondrocytes and the other groups (P < 0.001) (Fig. 2d), indicating the improved elimination of dead chondrocytes. Therefore, 200 μg/mL of FLP could be a potential candidate for the treatment of osteoarthritis.
and 200 μg/mL FLP-treated cells in 72 h (0.63 ± 0.22 versus 0.11 ± 0.06, \( P < 0.001 \)) (Fig. 2g), while 200 μg/mL FLP-treated fluro-intensity showed no differences with 50 μg/mL FLP-treated group in 24 and 48 h (Fig. 2e and f). The results confirm that 200 μg/mL of FLP ameliorated the survival of the IL-1β-induced chondrocyte injury instead of 50 μg/mL FLP.

2.5. Detection of intracellular oxidative stress

The free radical contents as ROS, H₂O₂ and NO after FLP co-culturing were further visualized by incubating with their specific probes. Compared with the normal chondrocytes, the ROS, H₂O₂ and NO markedly overexpressed in the injured chondrocytes. Importantly, high levels of these oxidative stress-associated substances not only result in cellular toxicity to the adjacent normal chondrocytes, but act as the pro-inflammatory factors that lead to various immune-related responses in the focal microenvironments [36]. Hence, it is of great significance to effectively prohibit free radical production. With treatment of 200 μg/mL FLP, the levels of ROS, H₂O₂ and NO were significantly attenuated in the

Fig. 2. The survival analysis of the injured chondrocytes after FLP treatment. a) Live and dead chondrocytes stained by Calcein/PI assay kit after 24 h, 48 h and 72 h incubation. Green fluorescence represented the live cells while red fluorescence represented the dead cells (Scale bars, 200 μm). b-d) Semiquantitative measurement for live cells at 24 h, 48 h and 72 h (n = 6). e-g) Semiquantitative measurement for dead cells at 24 h, 48 h and 72 h (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001 and ns meant no significance. All data were presented as mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
injured chondrocytes, which were respectively mitigated more than that in 50 μg/mL FLP-treated cells (Fig. 3a). Specifically, the mean ROS fluoro-intensity was 35.22 ± 4.45 for 200 μg/mL the FLP-treated chondrocytes, which significantly decreased by 26.04% of the injured chondrocytes (P < 0.001) and 24.31% of 50 μg/mL the FLP-treated cells (P < 0.01), respectively (Fig. 3b). Meanwhile, the mean fluoro-intensity of the H2O2 addition for 200 μg/mL FLP-treated chondrocytes was 8.95 ± 2.00, reducing by 54.52% of the injured chondrocytes (P < 0.001) and 51.65% of the 50 μg/mL FLP-treated cells (P < 0.01), respectively (Fig. 3c).

Similarly, the NO content also showed a marked suppression in the 200 μg/mL FLP-treated chondrocytes as 5.99 ± 0.96 in contrast to the other two groups of cells, of which the NO fluoro-intensity were 14.40 ± 2.67 (P < 0.001) and 11.00 ± 2.59 (P < 0.01), respectively (Fig. 3d). Additionally, the inhibition rates of \( \text{O}_2^- \) in the supernatant from the cracked injured chondrocytes elevated along with the increased concentration of FLP from 10 μg/mL to 200 μg/mL (Fig. S2), which further proved the \( \text{O}_2^- \) scavenging efficacy of FLP in vitro. Besides, the in vitro total antioxidant capacity reached equivalently 1.14 mM Trolox when the FLP

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Fig. 3. The detection of the intracellular contents of ROS, H2O2 and NO in the chondrocytes. a) ROS, H2O2 and NO that respectively stained with the specific probes (Scale bar for ROS, 200 μm; Scale bars for H2O2 and NO, 50 μm). b-d) Mean fluorescence intensity of ROS, H2O2 and NO (n = 6). ***P < 0.001, **P < 0.01, ns meant no significance. All data were presented as mean ± SD.
concentration was 200 μg/mL (Fig. S3), indicating that FLP was capable of clearing the excessive oxidative substances. Conclusively, 200 μg/mL of FLP massively depleted the production of ROS, H₂O₂, NO and O₂⁻, thus enabling the survival of the injured chondrocytes.

2.6. Analysis of inflammatory indicators by PCR

As shown in Fig. S4, the gene expressions of the inflammatory indicators including TNF-α, IL-1α and IL-1β significantly elevated in the injured chondrocytes than the normal cells. After treated with 200 μg/mL FLP, compared with the untreated injured cells, the expression fold changes of TNF-α, IL-1α and IL-1β returned to (14.88 ± 0.46), (2128.38 ± 134.36) and (9215.16 ± 776.59) from (22.83 ± 3.15), (3364.74 ± 414.65) and (11976.86 ± 522.02), respectively (all P < 0.01). The restraining of the inflammatory indicators in the cells treated by 50 μg/mL FLP was also inferior to 200 μg/mL FLP. These above results implied that the inflammatory response was relieved with the treatment of 200 μg/mL FLP.

2.7. Cartilage protection efficacy in vivo

In this study, a precisely-verified rat model of osteoarthritis was established with the intra-articularly injected sodium iodoacetate [37–39], of which the cartilage tissue was sectioned and stained. As depicted in Fig. 4a, the osteoarthritic injured cartilage induced by IL-1β notably degenerated, in which a distinguishable gap was marked by H&E staining. Besides, the Safranin O-stained section showed the reduced red pigmentation, suggesting the reduction of glycosaminoglycan. Compared with 50 μg/mL FLP-treated cartilage, there was a smoother cartilage surface and a more orderly layer of continuously-stained glycosaminoglycan that were close to the structure of the normal cartilage with the treatment of 200 μg/mL FLP. More precisely showed in Fig. 4b, the tissue thickness was measured from the surface to the tidemark of cartilage and the average intensity of the cartilage content was calculated. The thickness of 200 μg/mL FLP-treated cartilage was 219.80 ± 13.88 μm versus 207.00 ± 14.20 μm of the normal cartilage (P < 0.05), which implied that the injured cartilage thickness restored to normal size upon FLP treatment. Furthermore, the average cartilage content was 91.48% ± 5.48% of 200 μg/mL FLP, as opposed to 42.76% ± 8.92% (P < 0.001) of the injured cartilage and 77.51% ± 7.72% (P < 0.01) of 50 μg/mL FLP-treated cartilage (Fig. 4c). Moreover, for the histological analysis of Mankin score in Fig. 4d and 200 μg/mL FLP-treated cartilage exhibited a total score as 1.67 ± 0.82, showing significant differences to the injured cartilage (P < 0.001) and 50 μg/mL FLP-treated cartilage (P < 0.01). Above results verify that the superb protecting capability of 200 μg/mL FLP for the osteoarthritic injured cartilage, which was characterized by the preservation of the intact continuous layer of glycosaminoglycan.

Inspired by the encouraging results of glycosaminoglycan restoration in vivo, the efficacy of FLP for cartilage collagen protection was further measured, and immunohistochemical staining for type II collagen was also conducted. As displayed in Fig. 4a, the type II collagen staining was uniform distributed in the normal cartilage tissue, while the injured cartilage exhibited the rare staining. After the treatment of 50 μg/mL FLP, a small amount of type II collagen was disorderly preserved, of which the staining was apparently weakened than that of 200 μg/mL FLP-treated cartilage. More importantly, the even formation of collagen inside cartilage further revealed 200 μg/mL of FLP was superior for the protection of the injured cartilage. Besides, the biosafety of FLP for in vivo study was also evaluated by the H&E-staining of major organs including liver, kidney, spleen, intestine, heart, and lung, which demonstrated no significant pathological lesions in these groups (Fig. S5). Therefore, 200 μg/mL of FLP with approved biosafety would ameliorate the osteoarthritic injured cartilage by preserving the glycosaminoglycan and type II collagen, which were the main components of articular cartilage extracellular matrix.

3. Conclusions

In summary, the present study reports a novel therapeutic treatment for cartilage protection in osteoarthritis by intra-articular FLP delivery. 200 μg/mL FLP has been demonstrated to well-restore the cell viability of the injured chondrocytes to that of normal cells. Notably, this effect is achieved by diminishing oxidate-associated molecules such as ROS, H₂O₂ and NO. Furthermore, 200 μg/mL FLP can protect both glycosaminoglycan and type II collagen in the osteoarthritic cartilage tissue against obvious diminishing in vivo. Overall, the fabricated FLP possesses great potential for articular cartilage protection via mitigating the loss of extracellular matrix including glycosaminoglycan and type II collagen, which presents a promising opportunity for the articular cartilage repair and regeneration.

4. Material and methods

4.1. Synthesis and characterization of FLP

FLP was synthesized by the ultrasonication-assisted mechanical exfoliation method. In brief, 100 mg bulk black phosphorus crystal was dispersed in 100 mL 1-Methyl-2-pyrrolidinone (Sigma). The mixture was then put in an ultrasonicator (Scienitca) at 600 W for 18 h, while using an ice-water bath. The ultrasound probe worked 3 s with an interval of 2 s. After ultrasonication, the solution was centrifuged at 1, 000 rpm for 5 min to remove any non-exfoliated bulk black phosphorus. Then, the supernatant was centrifuged at 13, 000 rpm for 10 min to get the precipitate and washed with ethanol for 3 times.

The characterization of FLP were achieved with transmission electron microscopy (TEM), high-resolution scanning transmission electron microscopy (HR-STEM) and energy dispersive X-ray spectrometry (EDS), which were provided by the JEM-2100F field emission electron microscope at 200 kV (JEOL). Atomic force microscopy (AFM) was provided by the Dimension ICON (Bruker). The quantitative analysis of sample elements was measured by an inductively coupled plasma-optical emission spectrometry (ICP-OES, Agilent). UV–vis–NIR absorbance spectra were collected by a UV–vis–NIR spectrometer (Shimadzu). The electron spin resonance (ESR) spectra were obtained with the Bruker E500 electron paramagnetic resonance spectrometer.

4.2. Electron spin resonance (ESR) measurement

Hydroxyl radicals were produced through the Fenton reaction, 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Adamas) was used as the spin trapping agent. To study the ·OH-scavenging capacity of the product, FeSO₄, DMPO, 30% H₂O₂ and FLP (100 μg/mL) were mixed in sequence to monitor the change of the relative peak intensity in the ESR spectra of the DMPO-OH·.

4.3. O₂⁻ scavenging ability of FLP

The O₂⁻ scavenging effectiveness of FLP were evaluated by the commercial SOD assay kit. In brief, FLP with the concentrations of 10 μg/mL, 20 μg/mL, 40 μg/mL, 80 μg/mL, 100 μg/mL, 150 μg/mL and 200 μg/mL were added into the prepared detection reagent. After about 30 min incubation, the absorbance of samples at 450 nm were detected by microplate spectrophotometer.

4.4. ABTS radical scavenging assay

The antioxidant capacity of FLP was tested based on the reduction of *ABTS + radicals through the total antioxidant capacity assay kit (Beyotime). Briefly, ABTS was oxidized to generate ABTS radical cation (*ABTS•⁺). Then, FLP with the concentrations of 10 μg/mL, 20 μg/mL, 40 μg/mL, 60 μg/mL, 80 μg/mL and 100 μg/mL was added into the
detection reagent, while monitoring the absorbance at 734 nm after 15 min incubation. Trolox was set as the standard for evaluating the antioxidant levels of FLP.

4.5. H$_2$O$_2$ scavenging ability of FLP

H$_2$O$_2$ consumption capability of FLP was assessed by monitoring the change of characteristic absorption of H$_2$O$_2$ at 240 nm. Briefly, 100 μg/mL FLP and 20 mM H$_2$O$_2$ were mixed and reacted for period of time, while monitoring the change of absorption at 240 nm at varied time points.

4.6. Isolation of articular chondrocytes

Animals in this study were provided by Department of Laboratory Animal Science of Shanghai Tenth People's Hospital, Tongji University School of Medicine, and all experiments involving the animals were authorized by Animal Care and Use Committee. Chondrocytes were

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**Fig. 4.** In vivo evaluation of the cartilage protection capability of FLP. a) H&E and Safranin O staining for the cartilage tissue sections (Scale bars, 200 μm) and immunohistochemical staining for type II collagen of the representative cartilage tissue (Scale bar, 20 μm). b) Cartilage tissue thickness measurement (n = 6). c) Cartilage content estimation based on the Safranin O staining (n = 6). d) Mankin histological scoring for the structure and quality of the cartilage (n = 6). **P < 0.01, ***P < 0.001 and ns meant no significance. All data were presented as mean ± SD.
isolated and prepared by degrading the articular cartilage of a mature Sprague-Dawley (SD) rat, in which the cartilage tissue was digested with type II collagenase (Sigma) for 4 h at 37 °C. The second generation of the chondrocytes were used in the cellular experiments.

4.10. Intracellular changes of oxidative stress-associated compounds

4.11. In vitro SOD test

Solution was mixed with 2.5 mL dilution buffer. Twenty microliter was captured by a live/dead staining (Calcein/PI assay kit, Beyotime). The region of mean intensity of the diluted enzyme solution were mixed with 200 μL volume of physiological saline was injected into the articular cavity to produce osteoarthritic changes of the articular cartilage. For the therapeutic study of the injured cartilage, 50 μg/mL and 200 μg/mL FLP were then co-cultured with these chondrocytes for 48 h.

4.12. In vitro ABST test

The cells were prepared following the similar method in the previous SOD test. After treated with different concentrations of FLP, the supernatant was collected from the cracked cells. The ABTS working solution (200 μL) was mixed with 10 μL sample solution or Trolox standard solution to measure the A734 after incubation at room temperature for 5 min.

4.13. PCR analysis

The cells were treated with FLP, and then analyzed by quantitative Polymerase Chain Reaction (PCR) for the cytokine contents of Tumor Necrosis Factor-α (TNF-α), Interleukin-1α (IL-1α) and Interleukin-1β (IL-1β). The primers used in the PCR test were provided in the following Table 1.

4.14. The animal model of osteoarthritic injured cartilage

24 rats in total were selected to establish the animal model of osteoarthritis, and were randomly assigned into 4 experimental groups (6 rats per group). With the general anesthesia using intra-peritoneal pentobarbital sodium (5 mg/100 g), the skin surface of intra-articular injection site was carefully disinfected with medical iodophor. Then, 1 mg sodium iodoacetate (Sigma) in 50 μL volume of physiological saline was injected into the articular cavity to produce osteoarthritic changes of the articular cartilage. For the therapeutic study of the injured cartilage, 50 μg/mL and 200 μg/mL FLP were additionally injected at a volume of 50 μL.

4.15. In vivo cartilage protective efficacy evaluation

After FLP treatment for 6 weeks, the rats were sacrificed. The distal femurs were fixed with paraformaldehyde, decalcified with EDTA decalcification kit (Ribology) and sectioned into 5-μm slides. Then, hematoxylin and eosin (H&E), Safranin O and type II collagen immunohistochemical staining were used for the verification of the cartilage tissue structure and quality. The cartilage tissue thickness from the surface to the tidemark of the retained cartilage and the cartilage content of the injured cartilage referring to the normal tissue were separately measured. Besides, Mankin histological scoring system was utilized for the semiquantitative evaluation of the cartilage.

4.16. Statistical analysis

In this work, the experiments were duplicated for at least 3 times. All statistical data were showed as mean ± standard deviation (SD), and the significant differences were analyzed with GraghPad Prism software. Once the P value was less than 0.05, the difference between two groups was considered to be statistically significant (**P < 0.05, ***P < 0.01 and ****P < 0.001). Ns meant that there were no significances.

Authors’ contributions

XY Zhang, YL You and H Lin design this research. XY Zhang, YL You and YY Sun conducted the experiments. XY Zhang and YL You analyzed
the results. XY Zhang and YL You wrote the manuscript. H Lin, CM Zong and JL Shi reviewed the manuscript. All authors read and approved the final manuscript.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All animal studies were confirmed to the guidelines by the Animal Care Ethics Commission of Shanghai Tenth People’s Hospital, Tongji University School of Medicine (SHDSYY-2020-Z0026).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mtbio.2022.100462.

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