Research Article

PRP from Personal Blood Relieves Joint Fluid-Inducing Synovial Injury through NF-κB Pathway and Mitochondrial Apoptosis in Human Synovial Fibroblast Cells

Li Xie, Wanchang Lin, Lingqi Zhu, Liang Zhou, Dasheng Lin, Lianshui Huang, Wei Xie, and Wenliang Zhai

1Orthopaedic Center of People’s Liberation Army, Xiamen University Affiliated Southeast Hospital, Zhanghua Road 269, Zhangzhou 363000, China
2School of Medicine, Xiamen University, Xiamen University Xiang’an Campus, Xiang’an South Road, Xiamen 361102, China
3Affiliated Renhe Hospital of China Three Gorges University, Yiling Road 410, Yichang 443000, China
4The Second Hospital of Longyan, Shuangyang West Road 8, Longyan 364000, China

Correspondence should be addressed to Wenliang Zhai; wlzhai1971@xmu.edu.cn

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1. Introduction

Osteoarthritis, commonly occurring in the elderly patients, is recognized as a degenerative musculoskeletal disease characterized by osteoproliferation and the degeneration of cartilage articularis and synovium [1–3]. According to the statistics of Global Burden of Disease Study 2019 by Lancet, there is approximately 350 million incidences meeting with osteoarthritis around the earth [4], and the amount of osteoarthritis patients remain to be greatly rising with the growing aging population [5, 6]. The quietness of the early symptoms and the lack of the effective early screening strategy in osteoarthritis make the delayed treatment and contribute to the osteoarthritis-associated cellular injury, which lead to the undesired disability incidence [7]. Recently, many strategies including surgery and pharmacological treatment such as nonsteroidal anti-inflammatory drugs (NSAIIDs) have been reported and used for treating osteoarthritis and relieving osteoarthritis-associated cellular injury [8, 9]. However, the effective treatment strategy for osteoarthritis-associated cellular injury and disability incidence was still facing a challenge clinically. Therefore, it is
an urgent issue to explore and understand the novel and effective treatment manner to fight against osteoarthritis.

Platelet-rich plasma (PRP) therapy is a new kind of biological therapy to retune the plasma concentrator into the patient’s body for the treatment of diseases, which have been developed in the recent few years [10, 11]. Because it is an autologous biological agents prepared through extracting autologous blood, centrifuging at low speed and enriching high-concentration platelets, PRP exhibited a lower rejection and the side effect in body [12]. Currently, PRP therapy has been widely applied to the treatment of various diseases such as ophthalmology [13, 14], orthopedics, and dermatology [15, 16]. Our previous treatment in clinic found that autologous PRP therapy could effectively treat knee osteoarthritis of patients with level II and III according to Kellgren-Lawrence grading standard via accelerating the absorption of effusion of knee joint [17] and repairing the injury of synovial fibroblast cells. The accumulation of joint effusion and the injury of osteoarthritis-associated cells are two of the major symptoms of osteoarthritis. However, the mechanism of PRP therapy alleviating the two symptoms of osteoarthritis remains rarely reported up to now. Therefore, elucidating the mechanism of PRP therapy would be significant for understanding the effect of PRP and further developing PRP application in clinic.

Inflammation is considered as a common pathological event characterized by the symptoms of redden, swollen, hot, and painful, which occurs in nearly all of the acute and chronic diseases response to the physical injury and infection. For the response, the inflammation event initiates the expression of both proinflammation cytokines such as tumor necrosis factor α (TNFα), and in turn regulates, especially aggravate the localized tissue and cellular injury. Plenty of evidences have been confirmed that inflammation is an important indicator of osteoarthritis. Inflammation factors greatly aggregate in the joint effusion of patient with osteoarthritis and stimulate the tissues including synovium directly contacting joint effusion, which aggravate the damage of synovial tissue and fibroblast cells, consequently leading to the disability, and the loss of working capability for osteoarthritis patients. These implied that alleviating inflammation would be an effective strategy to treat the damage symptoms of osteoarthritis. However, it was rarely reported that whether PRP therapy alleviating damage of tissue and cells in osteoarthritis patients was associated with the inflammation. Therefore, elucidating the association of PRP therapy and inflammation would be a feasible signifi cate for understanding the mechanism of PRP therapy alleviating osteoarthritis.

The present research aims to elucidate the roles of NFκB-associated inflammation and mitochondrial apoptosis pathway in PRP therapy alleviating osteoarthritis. First, human synovial fibroblast cells (HSF cells) and synovial fluid were employed to construct the injury model of synovial fibroblast cells. The protective effect of PRP therapy was assessed by MTT assay. Second, the protective effect of apoptosis by PRP was examined by flow cytometry of dual-staining of FITC-Annexin V/PI, and the mitochondrial potential depolarization was detected via JC-1 staining. Following, the inflammation factors induced by synovial fluid and suppressed by PRP were assessed by ELISA and western blotting assays.

2. Materials and Methods

2.1. Chemicals and Reagents. Dulbecco’s modified Eagle medium (DMEM) and RPMI-1640 medium were purchased from HyClone. Foetal bovine serum (FBS) was obtained from Gibco. The primary antibody of cleaved PARP, cleaved caspase3, caspase3, Bax, Bad, Bcl-2, COX2, iNOS, p-IKK, IKK, IkB, p-p65, and GAPDH were obtained from abcam (USA). Annexin V-FITC apoptosis kit was supplied from Becton and Dickinson Company (BD, USA). JC-1 dye was purchased from SolarBio (China).

2.2. Cell Culture. The human synovial fibroblast cells (HSF cells) were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% FBS in a 5% CO2 atmosphere at 37°C. When the amounts of HSF cells in the logarithmic phase reached to 80-90% confluency in 10 cm cell culture plates, they were digested with 0.25% trypsin solution, resuspended into 5 mL EP tubes with PBS, and centrifuged at 1000 rpm for 5 minutes, following the precipitated cells were seeded into 6-wells plate and treated with synovial fluid, TNFα, and/or PRP as necessary. And then, the cells were collected and detected by the following experiments.

2.3. The Construction of Cellular Injury Model in HSF Cells. HSF cells were seeded into 96-wells cultured plate and maintained for 12 hours. And then, the seeded cells were treated with TNFα with different doses of 20, 40, and 60 ng/mL or synovial fluid with different dilution ratio of 1:5, 1:10, and 1:20 for 48 hours. After treating, the cellular morphology was imaged using microscope, and the cellular proliferation was detected by MTT assay. Finally, the condition under 1:5 dilution ratio of synovial fluid, displayed the abnormal morphology and the lower proliferation ratio in HSF cells, was chosen as the model condition for the research.

2.4. MTT Assay. HSF cells were seeded into the 96-wells plate and cultured for 12 hours. And then, the seeded cells were treated with synovial fluid, TNFα, and/or PRP at the response concentration for the specific time. After treating, the cells were added with 20 μL MTT (5 mg/mL), cultured for another 3 hours, diluted with 100 μL DMSO, and read the OD value using microplate reader (Peijing, China). The proliferation rate was calculated using Microsoft Excel software following as:

\[
\text{Proliferation Rate} = \left( \frac{\text{OD}_{\text{Sample}} - \text{OD}_{\text{Blank}}}{\text{OD}_{\text{Control}} - \text{OD}_{\text{Blank}}} \right) \times 100\%. \tag{1}
\]

2.5. Flow Cytometry of Dual-Staining of FITC-Annexin V/PI Detecting the Cellular Apoptosis. The HSF cells treated by synovial fluid, TNFα and/or PRP were digested into single cells with 0.25% trypsin and transferred into 1.5 mL EP tubes. The digested cells were washed with precold PBS.
and centrifuged at 1500 rpm and 4°C for 5 minutes. The precipitate of cells was resuspended with 300 μL 1× binding buffer, following 5 μL Annexin V-FITC was added to incubate at room temperature away from light for 15 minutes, and then 5 μL of PI was added to stain. At last, the stained cells were supplied with another 200 μL 1× binding buffer and detected by the flow cytometry (Beckman, USA).

2.6. Western Blotting. The HSF cells treated by synovial fluid, TNFa and/or PRP were harvested and lysed with RIPA buffer. The total protein concentration of cell lysates was detected using BCA kit, and then was denatured with loading buffer under the condition of boiling. 30 μg of the total denatured proteins were loaded and subjected to the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. The separated proteins were transferred to PVDF membrane. After blocking with 5% skim milk in Tris-buffered saline with tween20 (TBST) for 1 hour, the PVDF membranes were incubated with the primary antibodies against cleaved PARP, cleaved caspase3, caspase3, Bax, bad, bcl-2, cox2, iNOS, p-IKK, IKK, IkB, p-p65 at 4°C overnight, and the antibody of GAPDH was employed as an internal reference. The PVDF membranes were washed with TBST three times and further incubated with the corresponding secondary antibody at room temperature for 2 hours. Finally, the corresponding target on the PVDF membrane was probed using ECL reagent.

2.7. Flow Cytometry for Mitochondrial Potential by JC-1 Staining. HSF cells subjecting to with synovial fluid dilution, TNFa and/or PRP dilution were digested with 0.25% trypsin solution, washed with phosphate buffer solution, and then suspended with 1 mL fresh DMEM medium. The suspended cells were added with 1 mL JC-1 working solution and incubated at 37°C for 20 minutes. Following the incubated cells were centrifuged, washed twice using precold JC-1 washing buffer, and examined and analyzed using flow cytometry.

2.8. ELISA. HSF cells were seeded into 96-wells cultured plate and maintained for 12 hours. And then, the seeded cells were treated with synovial fluid of 1:5 dilution ratio for 24 hours and maintained with different doses of platelet-rich plasma for another 24 hours. After treatment, the cellular supernatant was collected, diluted with PBS for the corresponding proportion, and mixed with the buffer in ELISA kit according to 1:1. 100 μL of mixture solution, and the standard substance were added into the corresponding ELISA plate of IL-6, IL-1β, and TNFa, and incubation at 37°C for 30 minutes. The values were read using microreader, the standard curve was drawn, and the relative contents...
of the three inflammation factors were calculated according to the standard curve.

2.9. Statistical Analysis. SPSS software of version 19.0 (SPSS, USA) was employed for statistical analysis. The data were displayed as mean ± standard deviation (SD) and t-tests were used to compare the differences among the control, model, and synovial fluid/PRP groups. \( P < 0.05 \) represented significance of the statistical difference. The sign of * was for the comparison with the control group and the sign † was for the comparison with the model group.

### 3. Results

3.1. The Preparation Flow of Synovial Fluid and Platelet-Rich Plasma. The synovial fluid was drawn from the articular cavity of patients with level II and III knee osteoarthritis according to Kellgren-Lawrence grading standard and collected into the sterile tube stored at 20°C. When the cells were treated, the collected synovial fluid was diluted with the DMEM medium with FBS by the ratio 1:4, 1:9, and 1:19, and replaced the normal DMEM medium to culture cells for the needed time (Figure 1(a)). The peripheral blood from individuals were harvested into the blood-collecting tube with anticoagulant and gently shake upside down for mixing uniformity, and centrifuged at 2500 rpm for 10 min. The supernatant serum was collected and transferred into another blood-collecting tube, secondly centrifuged at 3200 rpm for 8 min, and the supernatant was collected as the original PRP. Then, the original PRP was added with activating agent following the volume ratio of 9:1 for activation. The mixture was shocked and centrifuged at 1500 rpm for 10 min, incubated at 4°C for 16 hours, following centrifuged at 3000 rpm for another 20 min, and collected the supernatant to obtain the activated PRP (Figure 1(b)).

3.2. Synovial Fluid Induces the Cellular Injury in Human Synovial Fibroblast Cells. The number and morphology changes of cells subjecting to the inappropriate agents reflect the toxicity/injury effect of the agents on cells. The cellular...
Figure 3: The protection effect of PRP on synovial fibroblast injury.

Figure 4: Effect of PRP on cellular apoptosis in the damaged HSFC.
The outline of HSF cells subjected to higher doses of 40 and 60 ng/mL TNFα displayed a dispersive and ambiguous state compared with that in the control group, and the cellular amounts of higher doses TNFα were declined. MTT assay showed that HSF cells subjected to the indicated concentrations of TNFα displayed a dose-dependent decrease of proliferation ratio (Figure 2(b)), reversely; the ALP level in HSF cells subjected to the indicated concentrations of TNFα displayed an obvious increase (Figure 2(c)). Meanwhile, we employed the preprepared synovial fluid to treat HSF cells, and the cellular number and morphology was microscopic imaged. Figure 2(d) shows that HSF cells treated with 1:10 and 1:20 diluent of synovial fluid displayed a dispersive state which was similar to the cells subjected to TNFα. Furthermore, HSF cells subjected to the indicated dilute ratio of synovial fluid displayed a dose-dependent decrease of proliferation ratio (Figure 2(e)) and increase of ALP level (Figure 2(f)). These results demonstrated that synovial fluid could induce the cellular injury in HSF cells similar with the effect of TNFα, and the condition of 1:5 dilution was used to construct the injury model of synovial fibroblast cells for the following research.

3.3. PRP Protects the Synovial Fluid-Inducing Cellular Injury in HSF Cells. In order to confirm the protection effect of PRP against synovial fibroblast cells injury, cell morphology was observed and the proliferation ratio was analyzed. Figure 3(a) shows that HSF cells in model group displayed a dispersive morphology and decreased amounts compared to that in control group, excitingly, PRP with the dilution ratio of 1:20 and 1:10 relieved the cellular injury induced by synovial fluid. MTT assay revealed that the low dose of PRP (1:50) slightly relieved synovial fluid-inducing the decrease of cellular proliferation ratio in HSF cells, and the middle and high doses of PRP obviously ease the decrease of cellular proliferation ratio induced by synovial fluid (Figure 3(b)). Furthermore, ALP level analysis showed that the middle and high doses of PRP inhibited the induction of ALP content by synovial fluid (Figure 3(c)). These results demonstrated that PRP could protect the synovial fluid-

![Figure 5: Effect of PRP on mitochondrial apoptosis in the damaged HSFC.](image)
inducing cellular injury in HSF cells, which was consistent with the protective effect of PRP on damaged synovial tissues in patients with osteoarthritis.

3.4. PRP Relieves the Cellular Apoptosis in the Damaged HSF Cells. Cellular apoptosis is one of the common events for evaluating the normal cell injury. In order to further evaluate the protective effect of PRP against synovial fibroblast cells injury, dual-staining experiments of Annexin V-FITC and PI were performed, and the apoptosis proteins were detected by western blotting to evaluate the relieving effect of PRP on cell apoptosis. The scatter diagram of dual-staining experiments by flow cytometry showed that HSF cells in model group had an increase of apoptosis ratio from 1.41% to 22.1% compared to that in control group. Meanwhile, the damaged cells were treated with the higher dose of PRP (1:10) and had an obvious decrease of apoptosis ratio from 22.1% to 11.36% compared to that in model group, and the middle dose of PRP displayed a decrease of apoptosis ratio from 22.1% to 16.83% (Figure 4(a)). Furthermore, western blot assay showed that HSF cells in model group had an obvious cleavage induction of PARP and caspase 3. However, the higher dose of PRP (1:10) inhibited the cleavage induction of PARP and caspase 3 mediated by synovial fluid (Figure 4(b)). These results demonstrated that PRP could relieve the cellular apoptosis in the damaged HSF cells induced by synovial fluid.

3.5. PRP Relieves the Mitochondrial Apoptosis in the Damaged HSF Cells. Mitochondrial was considered as a central organelle to mediate the respiratory chain for almost all of the cellular physiopathology, and exerted an important role in cellular apoptosis. In order to confirm whether mitochondrial was associated with the relieving effect of PRP on cellular apoptosis, mitochondrial membrane potential and related apoptosis proteins of Bax, Bcl-2, and Bad were studied. JC-1 staining data from flow cytometry analysis showed that the fluorescence ratio of aggregate/monomer in the HSF cells of model group was obviously increased compared to that in control group, and the treatment of PRP relieved the change trend of aggregate/monomer ratio (Figure 5(a)), demonstrating that synovial fluid induced the mitochondrial membrane potential depolarization and PRP reserved the depolarization induced by synovial fluid. Furthermore, western blot assay showed that HSF cells in model group had an obvious induction of Bax and Bad expression and inhibition of Bcl-2 expression. However, different doses of PRP relieved these change trends (Figure 5(b)). The results demonstrated that PRP could relieve the cellular apoptosis via
mitochondrial pathway in the damaged HSF cells induced by synovial fluid.

3.6. PRP Inhibits the Inflammation in the Damaged HSF Cells. Inflammation is considered as one of the main causes for chronic disease such as cancer, diabetes, and degenerative disease of the joint, which mediates the physiological and pathological processes of the diseases. In order to confirm whether the inflammation was involved into the protective effect of PRP on cellular injury induced by synovial fluid, ELISA assay and western blot analysis were performed for detecting the inflammation factors TNFα, IL-1β, IL-6, COX2, and iNOS. Elisa assay revealed that HSF cells subjected to synovial fluid had a content increase of TNFα, IL-1β, and IL-6, and PRP inhibited the increases in a dose-dependent manner (Figures 6(a)–6(c)). Furthermore, western blot analysis showed that HSF cells in model group displayed the upregulation expression of COX2 and iNOS, and high-dose PRP inhibited the expression of COX2 and iNOS (Figure 6(d)). These results demonstrated that inflammation may take part in the cellular injury effect induced by synovial fluid and PRP could inhibit the inflammation in the damaged HSF cells.

3.7. PRP Inhibits the Activation of NF-κB Pathway Induced by Synovial Fluid in the HSF Cells. NF-κB (Nuclear Factor kappa-B) is an important pathway of inflammation, which is associated with cell proliferation, apoptosis, and injury. In order to further confirm the relationship between NF-κB pathway-related inflammation and the protection effect of PRP on cellular injury, the NF-κB pathway-related indicators were detected by western blot. Figure 7 shows that the phosphorylation level of IKK and p65 was obviously induced, and the IκB expression was inhibited during the inflammation induction by synovial fluid in HSF cells. However, HSF cells subjected to synovial fluid were treated with PRP, and then displayed the inhibition of IKK and p65 phosphorylation and the reversion of IκB expression inhibition. These results demonstrated that PRP could protect the cellular injury via clearing away the inflammation mediated by NF-κB pathway.

4. Discussion

PRP therapy has been widely applied to the treatment of various diseases such as ophthalmology, orthopedics, and dermatology. Our previous treatment in clinic found that PRP therapy could effectively alleviate knee osteoarthritis caused by exercise. However, the mechanism of PRP therapy alleviating osteoarthritis remains rarely reported up to now. Although it was in the vague understanding that how PRP therapy alleviating osteoarthritis, some shreds of evidence have shown that the inflammatory pathway of NF-κB is associated with the injury effect of osteoarthritis. Additionally, the mitochondrial apoptosis pathway plays a vital role in the cellular injury. In the present study, we found that PRP could alleviate the cellular apoptosis and mitochondrial depolarization during synovial fluid-inducing injury in HSF...
cells, and regulate the mitochondrial-associated apoptosis protein expression. Furthermore, the inflammation factors and the NF-κB pathway-associated proteins p-IKK, IκB and p65 were also mediated by PRP treated. These results demonstrated that PRP therapy might relieve the synovial fluid-induced injury via the mitochondrial apoptosis and NF-κB pathway-related inflammation.

Apoptosis is a kind of physiological process controlling the programmed cell death to maintain body homeostasis. It exerts essential roles throughout the life cycle including the differentiation, developments, growth, and aging. However, several pathological stimuli, such as inflammation factors, toxic substances, and physical events could also induce the abnormal cellular apoptosis leading to the tissue injury. Caspase 3, an important member of cysteiny1 aspartate-specific protease family, is commonly recognized as an executioner of apoptosis events. In the apoptosis process, caspase 3 is activated by other caspases initiators via its cleavage, induce the hydrolysis/cleavage of target proteins including poly ADP-ribose polymerase (PARP), and eventually lead to the cellular apoptosis and tissue injury. In the present research, we found that synovial fluid could induce the cellular apoptosis and trigger the cleavage of caspase 3 and PARP, meanwhile PRP treatment relieved the change trends of apoptotic. These results demonstrate that PRP could protect against synovial fluid-inducing cellular injury characterized by apoptosis.

It is commonly considered that apoptosis is divided into two pathways of intrinsic apoptosis and extrinsic apoptosis, and intrinsic pathway is the major manner. In the intrinsic apoptosis process, the mitochondria response to the apoptosis stimuli, cause the change of mitochondrial membrane potential, depolarization of mitochondrial, the expression changes of mitochondrial-associated protein Bax, Bad, and Bcl-2, and eventually lead to the cellular apoptosis [18]. In the present research, we found that synovial fluid could induce the change of mitochondrial membrane potential and depolarization of mitochondrial, which have been confirmed by JC-1 staining, furthermore, it had also been observed that synovial fluid could induce the expression of Bax and Bad, and inhibit the expression of Bcl-2. Reversely, PRP treatment relieved the change of mitochondrial membrane potential and the expression of mitochondrial-associated proteins Bax, Bad, and Bcl-2, demonstrating that PRP could protect against synovial fluid-inducing cellular injury via intrinsic apoptosis pathway.

Inflammation is a typical characteristic symptom of many chronic disease, which is not only the complication of disease, its occurrence would also aggravate the disease progress. Inflammation usually triggers the release of proinflammation including IL-6, TNFα, and IL-1β, and promotes the cellular apoptosis and tissue injury. The nuclear factor κB pathway (NF-κB pathway) is considered as an important inflammation pathway. In NF-κB pathway, IKK is initatively phosphorylated response to the inflammation stimuli, following the degradation of IκB and phosphorylation of p65 are triggered, and then the proinflammation genes are activated. In the present research, we found the cells subjecting to synovial fluid displayed an obvious phosphorylation of IKK, degradation of IκB and phosphorylation of p65, meanwhile the cells treated with PRP had a reverse trend changes of the above NF-κB pathway indicators, demonstrating that PRP could protect against synovial fluid-inducing cellular injury via the NF-κB inflammation pathway.

Data Availability

The datasets used during the current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Li Xie and Wanchang Lin contributed equally to this research.

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