Therapeutic effect of platelet-rich plasma on glucocorticoid-induced rat bone marrow mesenchymal stem cells in vitro

Yanxue Wang†, Shuo Luan†, Ze Yuan, Caina Lin, Shengnuo Fan, Shaoling Wang, Chao Ma* and Shaoling Wu*

Abstract

Background: Glucocorticoid-induced osteonecrosis of the femoral head (GIONFH) is a progressive and disabling disease caused by long-term or high-dose glucocorticoid use. Decreased osteogenesis and proliferation of bone marrow mesenchymal stem cells (BMSCs) are the main pathogenesis of GIONFH. Platelet-rich plasma (PRP) has been shown to play a promising role in bone regeneration. However, the effects of PRP on glucocorticoid-induced BMSCs inhibition remains elusive. The objective of this study was to explore whether PRP could improve the in vitro biological activities of BMSCs inhibited by high-dose glucocorticoid in vitro.

Methods: In this study, a dexamethasone (Dex)-induced in vitro cell model was established. The effects of PRP on proliferation, migration, cell cycle and apoptosis of rat BMSCs induced with high-dose Dex compared to BMSCCTRL, using CCK-8 assay, transwell, flow cytometry and TUNEL assay, respectively. We further performed the alkaline phosphatase (ALP) and alizarin red (ALR) staining to explore the influence of PRP on osteogenic differentiation. Western Blot was used to detect the expression of Bcl-2, Caspase-3, RUNX2 apoptosis, and osteogenic-related proteins.

Results: We observed increased apoptosis rate and Caspase-3 expression, and the decreased migration and osteogenic differentiation, and down-regulation of RUNX-2 and Bcl-2 expression in Dex-induced BMSCs. PRP could reverse these inhibitory effects of Dex, and enhance the BMSCs proliferation, migration, and osteogenic ability in vitro.

Conclusion: Our in vitro study showed that PRP significantly protected BMSCs from Dex-induced apoptosis, and further promoted BMSCs proliferation, migration, and osteogenic differentiation. This study provides a scientific basis for the prevention and treatment of GIONFH with PRP. Meanwhile, it also lays the foundation for the application of PRP in other musculoskeletal diseases.

Keywords: Platelet-rich plasma, Glucocorticoid, Bone marrow mesenchymal stem cells

Background

Glucocorticoid-induced osteonecrosis of the femoral head (GIONFH) is a common disease after long-term or high-dose glucocorticoid use. Generally, the incidence of GIONFH is related to dose and administration routes of glucocorticoid, even though there is no available exact definition of dose and duration of use. In addition, the individual susceptibility varies in different diseases. Based on existing clinical data, cumulative methylprednisolone (> 2 g for more than three months) significantly increased the risk for GIONFH [1]. As ONFH progresses, the femoral head eventually collapses, resulting in pain and dysfunction [2–4]. Studies have shown that high-dose of glucocorticoid decreases the proliferation and osteogenic capacity and increases the apoptosis of bone marrow mesenchymal stem cells (BMSCs), which aggravates...
The purpose of this study was to explore whether PRP could improve the inhibited biological activities of BMSCs induced by high-dose of glucocorticoid. Our results provide important insights into the potential of PRP on glucocorticoid-induced BMSCs activities in vitro. Therefore, it provides a theoretical basis for the future clinical exploration of PRP in the treatment of ONFH.

Methods
Study design and ethics statement
All procedures were carried out according to the guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University. Male Sprague-Dawley (SD) rats were purchased from Sun Yat-sen University Animal Experimental Center (Guangzhou, China). Rats were maintained under standard laboratory conditions, with free access to food and water, and housed prior to experiments in an animal room under standard conditions (23 ± 2 °C; 60 ± 10% humidity; 12 h light/dark cycle).

Preparation and composition analysis of platelet-rich plasma
Ten 12-week-old SD rats were anesthetized by intraperitoneal injection of 2.5% pentobarbital sodium (40–45 mg/kg). The preparation of PRP was strictly aseptic. Ten milliliter whole blood was drawn from each rat via intracardiac puncture. A total of 9 ml of blood sample was drawn from each rat into 10-ml syringes containing 1 ml sodium citrate anticoagulant, of which 0.5 mL was used for blood count (quantitative analysis of platelet and leukocyte counts). The remaining 9.5 ml was transferred into a sterile test tube and centrifuged twice to obtain fresh PRP [19]. After the first centrifugation (400 g, 10 min), the blood was divided into three layers, with the plasma in the supernatant, a buffy coat layer in the middle, and red blood cells at the bottom. Then, the supernatant was pipetted into another sterile test tube for second centrifugation (800 g, 10 min). The 3/4 upper fraction of the plasma (platelet-poor plasma) was discarded, and the remaining liquid was platelet-rich plasma (about 1.5 mL). 10% CaCl₂ (C5670, Sigma-Aldrich) plus 1000 U/ml thrombin from bovine plasma (T8021, Solarbio, Beijing, China) was used for activation of the alpha granules in platelets and was added into the apheresis platelets at a volume ratio of 1:10. The activated PRP-containing supernatant was collected and filtered through 0.22-µm aseptic membrane filter and then aliquoted and stored at −80 °C to avoid repeated freezing and thawing. A 0.2 mL sample of PRP was reserved for the detection of the growth factors.

As mentioned above, the platelets and leukocyte concentrations in the samples of whole blood (0.5 mL) and PRP were measured with the Mindray BC-5000Vet analyzer. ELISA kits were utilized to quantify the concentrations of rat transforming growth factor-beta (TGF-β, LER822–1, Laizee, Shanghai, China) based on the manufacturer’s protocol.

BMSCs isolation and culture
To investigate the effects of PRP, the BMSCs were induced by Dex (D4902, Sigma-Aldrich). Different interventions were used to assess a series of functional assays, including the control group, the Dex group, and the Dex + PRP group. In cell proliferation assay, migration assay, and osteogenic differentiation assay, the concentration of Dex was 10 µM. The concentration of Dex was 100 µM in the apoptosis experiment.

Bone marrow stem cells (BMSCs) of 4-week-old SD rats were obtained from the femur and tibia according to the method described by Shen [6] and were cultured with complete medium: Dulbecco's modified Eagle's medium (DMEM) (Gibco, MA, USA) containing 10% fetal bovine serum (FBS, Gibco), 1% penicillin-streptomycin (Gibco) in an incubator at 37 °C and 5% CO₂. After 24 h, the medium was changed and the attached cells were washed by phosphate-buffered saline (PBS, Gibco). The culture medium was refreshed every 2 days until adherent cells reach 80–90% confluence, then the cells were passaged for three to five passages for all further experiment use.

Cell proliferation assay
Effect of PRP on Dex-induced BMSC proliferation
To determine the optimal PRP concentration that could promote the proliferation of BMSCs, we first carried out
Flow cytometry

The cell cycle was measured by flow cytometry. The cells were inoculated in a 6-well plate with $1.5 \times 10^5$ cells/well. After 24 h of complete adherence, 100 μL complete medium containing 0, 2.5, 5, 10, and 20% PRP was added. The proliferation of BMSC was measured with a CCK-8 assay kit (K1018–1, ApexBio, MA, USA) at 24, 48, and 72 h. The optimum PRP concentration obtained in this experiment was used in all subsequent experiments.

The CCK-8 kit was used to explore the proliferation rate of BMSCs treated with Dex. A total of 3000/well BMSCs were inoculated into 96-well plates. The cells were divided into three groups: (1) control group, (2) Dex group, and (3) Dex + PRP group. The absorbance was measured at 450 nm using an enzyme-linked immunosorbent assay reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA).

BMSCs migration assay

BMSCs were inoculated into 96-well plates with 3 replicate wells in each group of $3 \times 10^3$/well. After 24 h of complete adherence, 100 μL complete medium containing 0, 2.5, 5, 10, and 20% PRP was added. The proliferation of BMSC was measured with a CCK-8 assay kit (K1018–1, ApexBio, MA, USA) at 24, 48, and 72 h. The optimum PRP concentration obtained in this experiment was used in all subsequent experiments.

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TUNEL assay

The terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labeling (TUNEL) assay was used to estimate the effect of PRP on BMSCs cell apoptosis according to the manual [21]. After 48 h of culture with the serum-free medium in the presence or absence of PRP, the cells were fixed with 4% paraformaldehyde for 25 min at 37 °C, incubated with 0.3% Triton X-100 for 5 min, and washed with PBS twice between each step. The cells were incubated with TUNEL reagent (C1086, Beyotime, Shanghai, China) according to the manufacturer’s instructions, and the nuclei were counterstained with DAPI (C1002, Beyotime) for five minutes. The samples were observed and imaged under a fluorescence microscope (Olympus IX 71).

Alkaline Phosphatase (ALP) and Alizarin Red (ALR) staining

Bone marrow mesenchymal stem cells were seeded on a 24-well plate at a density of $1 \times 10^5$ cells/well. After 7 days of using osteogenic differentiation induction medium (RAXMX-90021, Cyagen, Guangzhou, China), ALP staining (Beyotime) was performed. 14 days later, alizarin red staining (Cyagen) was performed according to the manufacturer’s protocol. The cells were washed twice with PBS, fixed with 4% paraformaldehyde for half an hour, and then stained. Before taking pictures, each well was washed 3 times with double distilled water.

Western blotting

The protein was extracted from the cells using radio immunoprecipitation assay (PC101, RIPA) lysis buffer (EpiZyme, Shanghai, China). The protein concentration was determined using Thermo Scientific Pierce BCA (23227). An equal amount of protein was separated by 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). After blocking with protein-free fast blocking buffer (PS108, EpiZyme), it was incubated with primary antibodies against Runx2 (Affinity, AF5186), Bcl-2 (Affinity, AF6139), Caspase-3 (Huabio, ET1608–64), and GAPDH (EpiZyme, LF205) at 4 °C overnight, and then incubated with the secondary antibody at 37 °C for 1 h. Thereafter, the proteins were visualized using Omni ECL reagent (SQ201, ECL; EpiZyme) under e-Blot (Touch Imager, Shanghai, China). The gray densitometric analysis was analyzed using Image J (USA).

Statistical analysis

All of these experiments were repeated three times. Data were shown as mean ± standard deviation (SD) and analyzed with the statistical software GraphPad Prism 8 (GraphPad Software, Inc., USA).
multiple groups were compared by one-way analysis of variance (ANOVA). $P<0.05$ was considered statistically significant.

**Results**

**PRP alleviated the inhibitory effects of Dex on proliferation and migration of BMSCs while protecting cells from apoptosis**

The platelet concentration was $(2875 \pm 236) \times 10^9 / L$, about four times that of the whole blood $(650 \pm 89) \times 10^9 / L$. After the PRP purification process, the presence of white blood cells in the PRP sample $(0.007 \pm 0.0065) \times 10^9 / L$ was drastically reduced, as the whole blood sample showed a raw white blood cell count of $7.2 \times 10^9 / L$. Therefore, the samples obtained should be considered as leucocyte-poor platelet-rich plasma (P-PRP). The concentration of TGF-β was $(139 \pm 21.3) \mu g / L$. As shown in Fig. 1A, compared with no addition of PRP, PRP significantly increased the growth of BMSCs. The proliferation of BMSC at 20% PRP is weaker than that at 10% PRP. From the above result, we can conclude that the treatment of BMSCs with 10% PRP caused maximum

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**Fig. 1** PRP alleviated the inhibitory effects of Dex on the proliferation and migration of BMSCs. **A** Effect of different concentrations of PRP on cell proliferation. The treatment of BMSCs with PRP caused maximum extent proliferation. **B** After treatment of Dex and Dex supplemented with PRP, the proliferation of BMSCs was detected by CCK-8. **C** Flow cytometry analysis of cell cycle after treatment with PRP for 24 h. The number ratio of cells in the $(S + G_2/M)$ phase was significantly increased in the Dex + PRP group compared with that in the Dex group, demonstrating the state of cell proliferation. **D** Transwell assay was used to detect the migration capacity changes of BMSCs after treatment. (*$p < 0.05$ vs. Dex group)*
extent proliferation. Accordingly, all further experiments were conducted with 10% PRP. The CCK-8 assay and the transwell migration assay showed that BMSCs proliferation and migration capacities were significantly suppressed by Dex, while this inhibition was antagonized by PRP (Fig. 1B and D). After 24 h treatment with PRP, the number ratio of cells in the (S + G2/M) phase was significantly increased compared with that in the Dex group (Fig. 1C). In addition, compared to the control group, 48 h of Dex treatment significantly promoted the apoptosis of BMSCs, and this effect could be ameliorated after PRP treatment (Fig. 2A). We further detected the expression of apoptosis-related proteins by Western Blot. After PRP treatment, the apoptosis marker Caspase-3, was significantly down-regulated while the expression of anti-apoptosis marker Bcl-2, was significantly up-regulated (Fig. 2B).

**PRP alleviated the inhibitory effects of Dex on osteogenic differentiation of BMSCs**

ALP staining and alizarin red staining results showed that Dex could inhibit the process of osteogenesis while
PRP treatment mitigated this effect of Dex (Fig. 3A and B). The protein level the osteogenic marker Runx2 was significantly up-regulated in the Dex + PRP group.

Discussion
In recent years, with the wide application of glucocorticoid in clinical practice, GIONFH has become a major cause of non-traumatic necrosis of the femoral head [1, 2]. Although there have been many treatment attempts to prevent the progression of GIONFH, the potential effective strategy is still under investigation [2]. In this study, we found PRP could enhance the proliferation and anti-apoptotic activities of Dex-induced BMSCs. Furthermore, Dex-induced suppression of cell osteogenic differentiation was rescued by PRP in vitro.

Previous studies have clearly shown that activated PRP promotes the proliferation of multiple MSCs lines in vitro in a concentration-dependent manner [22–25]. Consistent with these findings, our research showed that higher concentrations of activated PRP had a stronger effect on BMSCs. In our study, 10% PRP was found as the optimal concentration of BMSCs proliferation, which was similar to the results of previous studies [23, 26]. Interestingly, however, the effect of increased PRP concentration (20% PRP) did not show similar or enhance BMSC proliferation effect, indicating that there is an optimal dosage of PRP. Further research may be needed to study whether PRP can improve the activity of BMSCs induced by high-dose glucocorticoids.

In this study, Dex reduced the proportion of S + G2/M (proliferation index, PrI) phase cells and increased cell apoptosis, while PRP could reverse the glucocorticoid-induced inhibition. S + G2M phase represents the percentage of proliferating cells in the population, reflecting
In conclusion, our studies showed that PRP significantly protected BMSCs from Dex-induced apoptosis, promoted BMSCs proliferation, migration, and osteogenic differentiation. This study provides a promising strategy for the treatment of GIONFH with PRP. It also indicates that PRP is a promising drug that may be used to prevent ONFH, which also lays the foundation for the application of PRP in other musculoskeletal diseases.

Abbreviations
GIONFH: Glucocorticoid-induced osteonecrosis of the femoral head; PRP: platelet-rich plasma; P-PRP: leucocyte-poor platelet-rich plasma; BMSCs: bone marrow mesenchymal stem cells; Dex: dexamethasone; ALP: alkaline phosphatase; ALR: alizarin red staining.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12891-022-05094-2.

Additional file 1. Western blot original images.

Acknowledgments
Not applicable.

Authors’ contributions
YXW and SL performed the experiments and wrote the manuscript. ZY and CNL participated in executing the experiment’s statistical analysis. SNF and Shaoling Wang participated in cell culture. SNF also participated in revising the manuscript. CM and Shaoling Wu participated in the design of the study and the interpretation of the data and supervised all the experiments. All authors read and approved the final manuscript.

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Availability of data and materials
The data analyzed during the current study are available from the corresponding author on reasonable request.

Declarations
Ethics approval and consent to participate
All the experimental procedures were carried out according to the guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee (IACUC approval: SYSU-IACUC-2020-000483). The study was carried out in compliance with the ARRIVE guidelines.

Consent for publication
Not applicable.

Competing interests
The authors have no conflict of interest to disclose.
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