Original article

Platelet-rich plasma pretreatment protects anterior cruciate ligament fibroblasts correlated with PI3K-Akt-mTOR pathway under hypoxia condition

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ABSTRACT

Background: /Objective: Biological factors such as platelet-rich plasma (PRP) combined with anterior cruciate ligament (ACL) primary repair technology are used to treat ACL injury. However, the protective mechanism of PRP for ACL fibroblasts under hypoxia condition is still unknown. The aim of this study was to investigate the protective effect of PRP on ACL fibroblasts under hypoxia condition and illustrate the mechanism of PRP regulating the ACL fibroblasts under hypoxia condition.

Methods: The cells were divided into three groups: control group, hypoxia group and PRP pretreatment group. Lethal dose (LD) 50 for hypoxia induction time and the maximum efficacy of PRP concentration were confirmed by CCK-8 assay. The ability of cell apoptosis, cell proliferation, and cell migration were tested by flow cytometry, scratch assay and transwell assay, respectively. Extracellular matrix (ECM) synthesis and hypoxia-inducible factor 1α (HIF-1α) were identified by immunofluorescence staining, Masson's staining and transmission electron microscope analysis. Inflammatory cell infiltration was assessed by hematoxylin and eosin staining as well as immunofluorescence staining. Western blot analysis and real-time PCR were performed to assess the associated gene and protein expression, respectively. The ratio of phosphorylated/total PI3K, Akt and mTOR were also assessed by western blot analysis.

Results: ① LD 50 of hypoxia was 48 h and the maximum efficacy of PRP concentration was 600×10^9/L. ② ANNEXIN V-FITC/PI flow cytometry showed that the hypoxia condition significantly increased the apoptosis of cells ($P < 0.001$) whereas PRP pretreatment significantly decreased the apoptosis of cells under hypoxia ($P < 0.001$). The expressions of gene and protein of Bax, Bcl-2, cleaved-caspase 3 were consistent with the results of flow cytometric analysis. ③ Cell cycle analysis for flow cytometry showed the inhibitory effect of hypoxia and promotive effect of PRP pretreatment. ④ Immunofluorescence staining (HIF-1α, collagen I and III) showed the positive effect of hypoxia and negative effect of PRP on these parameters. Real-time PCR showed that type I and III collagen were 2.1 folds and 2.5 folds higher after 48 h hypoxia induction compared to the control group. PRP pretreatment significantly reduced the type I and III collagen mRNA expression of the hypoxia induced ACL fibroblasts to 78.5% and 77.7% at 48 h compared to hypoxia group ($P < 0.001$), respectively. ⑤ Cell migration assay showed that hypoxia condition significantly restrained cell migration compared with the control group. PRP could alleviate the inhibitory effect of hypoxia on fibroblasts. ⑥ Western blot analysis showed the ratio of phosphorylated/total PI3K, Akt and mTOR in hypoxia group increased to 31%, 20% and 44/% compared to control group, respectively. ⑦ The results of in vivo analysis was in accordance with the results of in vitro analysis.

Conclusion: PRP can protect ACL fibroblasts via decreasing apoptosis and increasing cell viability, cell migration and cell proliferation under hypoxia condition. And such PRP protective effect was correlated with PI3K/Akt/mTOR pathway.

The translational potential of this article: PRP can be used to treat patients with ACL tear by injection under arthroscopy or ultrasound guiding.

1. Introduction

Anterior cruciate ligament (ACL) is the primary structure that restricts the anterior translation of the tibia [1,2]. Anterior instability of the knee joint has been proved to be related to meniscal tears, cartilage degradation and development of osteoarthritis (OA) [3]. Although ACL reconstruction (ACLR) was the gold standard for ACL complete tear, the non-negligible disadvantages such as invasive surgical trauma, loss of ACL proprioceptive nerve fibers [4], and progression of OA [3] have motivated researchers to look back on the primary repair with bio-enhancement methods to treat ACL injury, such as the application platelet-rich plasma (PRP). As a result, several regenerative methods including the application of PRP have been proposed to promote ACL healing.

PRP, produced from centrifugation of whole blood, has been widely used to promote tissue repair and wound healing. PRP contains plasma proteins and lots of bioactive factors such as platelet derived growth factor (PDGF), transforming growth factor beta (TGF-β), fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF) [5]. These bio-active factors released by PRP can clinically encourage tissues healing by serving as stimulators for cell proliferation after ACLR [6–8]. At the same time, many studies found that PRP significantly facilitated healing process in animal model of ACL injury [9–11]. In addition, the use of PRP combined with advanced ACL primary repair technology also achieved satisfactory clinical outcomes in ACL injured patients [12].

Previous studies demonstrated that PRP stimulated ACL healing, which appeared to increase type I and type III collagen gene expression, enhance ACL fibroblasts metabolism and reduce cell apoptosis [13,14]. Although PRP played an important role in promoting ACL healing and repairing, the protective effect on hypoxia-induced apoptosis after ACL blood vessel tear was still unknown and needed to be explored.

The blood supply of ACL is derived from the small blood vessels of the middle knee artery ligament branch and the sub-patellar fat pad [15]. During the ACL injury, hypoxia microenvironmental condition rapidly occurred after blood vessel rupture of ACL [16,17]. Microenvironment with low concentration of oxygen (3%) hindered ACL fibroblasts proliferation and survival [18]. The hypoxia microenvironmental condition is generally considered as a trigger leading to hypoxia-inducible factor-1α (HIF-1α) accumulation in ACL fibroblasts [19]. And many studies demonstrated that HIF-1α mediated the progress of cell apoptosis via increasing the stability of p53 [20] and the overexpression of the Bcl-2 [21]. Increased HIF-1α expression induced ACL fibroblasts apoptosis via increasing proapoptotic proteins expression levels such as Bax and cleaved-caspase 3, but decreasing antiapoptotic proteins expression levels like Bcl-2 [19]. Meanwhile, mTOR was an important cell signal protein, which was involved in regulating cell growth, apoptosis and metabolism [22]. The PI3K/Akt/mTOR pathway also correlated with cell apoptosis, proliferation and migration, as the upstream cell signaling pathway of HIF-1α [23].

The purpose of this study is 1) to investigate the protective effect of PRP on ACL fibroblasts under hypoxia condition; 2) to illustrate the potential mechanism involved in PRP regulating the ACL fibroblasts. We hypothesize that PRP could inhibit cell apoptosis, enhance cell viability, promote cell proliferation, stimulate type I and type III collagen production and increase cell migration capacity of ACL fibroblasts under hypoxia condition and PI3K/Akt/mTOR cell signaling pathway is involved in this process.

2. Materials and method

This study was approved by the Experimentation Ethics Committee in Beijing Jishuitan Hospital (approval number: 201912-04, 202011-01, Beijing, China).

2.1. Tissue harvest and cell culture

ACL fibroblasts used in this study were supplied by the Sports Medicine Service of Beijing Jishuitan Hospital. The stump of ACL ligament tissues was harvested from 4 patients (3 males and 1 female; age, 20–23 years) who underwent ACL reconstruction after non-contact ACL injury in our institute. The time from injury to surgery was limited within 7 days. ACL tissue was chosen from tibia or femur attachment site instead of the tear site.

The samples were immediately immersed in $1 \times$ phosphate-buffered saline (PBS) with $5 \times$ penicillin, streptomycin sulfate and washed out by $1 \times$ PBS three times after harvest. The ACL tissues were cultured in 5% high glucose-Dulbecco's modified eagle medium (DMEM) with 10% fetal bovine serum (FBS), incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air after cut into $2 \times 2 \times 2$ mm pieces. These pieces were transferred to a new Corning® 25 cm^2 rectangular canted neck cell culture flask once ACL fibroblasts migrated out from ACL tissues to reach 30–40% confluence.

Some cells were stored in 10% dimethyl sulfoxide (DMSO) in FBS in −80 °C refrigerator until use. The remaining cells were cultured and maintained in 10% FBS medium (high glucose DMEM, 1% L-glutamine and antibiotics) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. The cells used in this experiment were from the passage 4. All data were collected from cells of every patient.

2.2. PRP preparation

The amount of blood drawn from each patient was 20 ml. The PRP was prepared according to the method described by Yoshida et al. [24]. The blood was transferred into an anticoagulation tube containing sodium citrate. Then the whole blood was centrifuged for 6 min at $150 \times g$. The upper layer of plasma was collected and centrifuged at $1000 \times g$ for 10 min. Then the upper layer of plasma with low platelet content was removed. 20 ml whole blood was finally concentrated to obtain 2 ml of PRP. PRP was stored in 5% dimethyl sulfoxide (DMSO) in FBS in −80 °C refrigerator until use. PRP from different patients were mixed in order to remove confounding factors. The platelet concentration of the blood was 211×10^9/mL and PRP was 1048×10^9/mL (enrichment of 4.97 folds). In this study, PRP was collected from all patients that provided cell samples. Each part of the experiment used the same batch of PRP.

2.3. Cell treatment

ACL fibroblasts were seeded (1×10^6) on 6-well plates and 2 ml 10% FBS DMEM medium was added to each well. After the cell confluence reached 80–90%, cell culture medium was replaced by 2 ml of 2% DMEM medium for serum starvation to achieve cell synchronization.

The cells were divided into three groups: control group (5% CO_2 and 95%), hypoxia group (3.0% O_2 without PRP pretreatment) [18] and PRP pretreatment group (3.0% O_2 with PRP pretreatment). Hypoxia condition (3% O_2) was realized by Tri-gas Incubators (Shanghai Huikang Hydrogen Medical Center). The cells were incubated in hypoxia condition for

different time to evaluate the lethal dose (LD) 50 of hypoxia induction. Subsequently, different concentration of PRP (adding 100 μl of PRP to each 6-well plate) was used to pretreat cells before the culture in hypoxia condition to confirm the maximum efficacy of PRP concentration. HIF-1α expression, cell viability, cell apoptosis, cell proliferation, cell migration ability, extracellular matrix (ECM) synthesis and PI3K-Akt-mTOR pathways were identified for different groups.

2.4. ACL fibroblasts identification

Immunofluorescence and flow cytometry were used to identify the source and immunophenotype of ACL fibroblasts. For immunofluorescence, the ACL fibroblasts were washed out with precooled PBS and fixed with 4% paraformaldehyde for 10 min. Then, the ACL fibroblasts were permeabilized with 0.25% Triton-X100 for 15 min at room temperature (RT). Later, the ACL fibroblasts were washed out with precooled PBS for three times (3 min each time) and were blocked with PBS-tween (PBST) containing 1% bovine serum albumin for 60 min at RT. Subsequently, the ACL fibroblasts were incubated with primary antibody of α-smooth muscle (α-SMA, Abcam, Ab7871, Cambridge, UK), CD31(Abcam, Ab9498, Cambridge, UK), CD34 (Abcam, Ab81289, Cambridge, UK), CD44 (Abcam, Ab189524, Cambridge, UK), Collagen I (Abcam, Ab260043, Cambridge, UK), Collagen III (Abcam, Ab184993, Cambridge, UK) at 4 °C overnight, and with FITC-conjugated secondary antibody (Sangon Biotech, D111086, Shanghai, China) for α-SMA, CD31 and CD34, and with APC-conjugated secondary antibody (Solarbio, K0068R-APC, Beijing, China) for CD44, Collagen I and Collagen III 1 h at RT. After PBS washing, the ACL fibroblasts were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) and dissolved into PBS for 15 min. Finally, the samples were examined by fluorescence microcopy (Olympus, Tokyo, Japan).

Isolated human ACL fibroblasts were incubated with fluorescently labelled antibodies against α-SMA, CD31, CD44, Collagen I and III with flow cytometry used for detection. For flow cytometry, the ACL fibroblasts were digested using trypsin after the cell confluence reached 70%. The ACL fibroblasts were suspended with 1 ml 1 × PBS and the solution was centrifuged at $300 \times g$ for 10 min. Then, the supernatant was discarded and the ACL fibroblasts were resuspended with 1 ml 1 × PBS to adjust the cell density at 1×10^6 cells/ml. 100 μl of cell suspension (1×10^5 cells) was put into each tube and added 5 μl of antibody. The cells were incubated for 15min at RT in the dark after gentle mix. Then the suspension was centrifuged at $300 \times g$ for 5 min and the supernatant was discarded. Finally, 500ul 1 × PBS buffer was added into the tube and the examples were tested on flow cytometer (Beckman, FC500).

2.5. LD50 for hypoxia induction

ACL fibroblasts were seeded in a 96-well plate with 5000 cells/well and pre-incubated for 24 h in an incubator at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air after cell recovery. Then the ACL fibroblasts were divided into three groups as mentioned above and induced for 12 h, 24 h, 36 h, 48 h, 60 h and 72 h, respectively. Cell viability was assessed by a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Gaithersburg, MD, USA) according to manufacturer's instructions. Briefly, after treatment, 10μ CCK-8 solution was added to the culture medium and incubated at 37 °C for 2 h. The absorbance was read at 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Cell viability was calculated by (hypoxia group absorbance value/control group absorbance value) × 100%.

2.6. Maximum efficacy of PRP concentration

The method of ACL fibroblasts hypoxia induction was the same as the abovementioned. Then the ACL fibroblasts were treated with the following conditions: 37 °C in a humidified atmosphere of 5% CO_2 and 95% air with 20 μl 5% high glucose-DMEM with FBS (control group), 37 °C in a hypoxia induction with 20 μl 5% high glucose-DMEM with 10% FBS (hypoxia group) and 37 °C in a hypoxia induced condition with 10 μl 5% complete medium combined with 10 μl PRP (100, 200, 400, 600 and 800×10^9/L) for LD50 of hypoxia induction.

2.7. Cell apoptosis

Cell apoptosis of ACL fibroblasts under different conditions as mentioned before was analyzed through Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double-staining. Cell apoptosis was assessed by Annexin V-FITC Apoptosis Detection Kit (ANNEXIN V-FITC/PI; CA1020, Solarbio, Beijing, China) according to manufacturer's instructions. The cells were resuspended in 1 ml 1 × Binding Buffer and adjusted to 1×10^6 cells/ml. 100 μl cell suspension (1×10^5 cells) was added into each tube. Then 5 μl Annexin V-FITC was added into each tube and the examples were incubated for 10min at RT in the dark. Subsequently, 5 μl propidium iodide (PI) was added into each tube and the examples were incubated for 5min in the dark at RT. Finally, the samples were added by PBS to 500 μl and analyzed using a flow cytometer (Beckman, FC500).

2.8. Cell proliferation

Cell proliferation of ACL fibroblasts under different conditions was assessed through flow cytometry. The number of cells was controlled to 1×10^5-1×10^6. The cells were digested using trypsin and collected to prepare a single cell suspension. The cells were centrifuged at $1000 \times g$ for 5 min and resuspended using pre-cooled PBS. The cells were mixed gently with 1 ml of pre-cooled 70% ethanol, and fixed at 4 °C for more than 2 h 10ul PI solution was added into the tube and 10ul RNase A solution was added into 0.5 ml staining buffer. Then, 0.5 ml of prepared PI staining solution was added into each cell sample and mixed to resuspend the cells. Finally, the cells were incubated in the dark at 37 °C for 30 min. Then flow cytometry using Beckman flow cytometer can be performed.

2.9. Migration assay

2.9.1. Scratch assay

For the scratch wound assay, 2×10^5 cells/well were seeded into a 6-well plate and incubated to reach 90% confluence. Then, the medium was changed to serum-free medium and the cells were starved for 24 h. The monolayer was scratched using a 200ul plastic pipette tip and washed with 1 × PBS to remove detached cells. Then the ACL fibroblasts were treated as mentioned before. ACL fibroblasts were photographed at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, post-scratchily. The closure distance of each 12 h was also calculated.

2.9.2. Transwell chamber

For the transwell assay, 1×10^4 cells/well were suspended in no serum medium and seeded into the upper chamber with 0.22 μm pore filters. Then complete medium or PRP was added into the lower chamber according to the above grouping method. The ACL fibroblasts attached on the upper surface of the filter membranes. Then the cells were cleaned and stained with 0.5% crystal violet for 20 min. The level of migration was observed and photographed with an optical microscope (Olympus IX71, Japan) at 0 h and 24 h.

2.10. Quantitative real-time PCR

Total RNA was extracted using RNA simple total RNA kit (DP419, TianGen, Beijing, China) according to the manufacturer's instructions. Isolated RNA was dissolved in RNase-free water and quantified by measuring the absorbance at 260 nm with a spectrophotometer. The RNA samples were then reverse-transcribed into complementary DNA (cDNA) with a HiFiScript gDNA Removal cDNA Synthesis Kit (CWBIO,

CW2582M, Beijing, China) according to the manufacturer's instructions. The primers of Bax, Bcl-2, cleaved-caspase 3, collagen I, collagen III, HIF-1α, and β-actin were shown in Table 1. Quantitative real-time PCR was performed in Bio-rad CFX connect™ (CFX96TM) Real-Time PCR system (Bio-Rad, Hercules, CA) using PCR Master Mix Kit (Promega, Madison, WI). For each sample, standard curves were prepared for each target gene and β-actin. Taqman results were standardized to the levels of β-actin and reaction specificity was confirmed with melting curves. The relative mRNA expression levels of the genes of interests were calculated by the $2-\Delta\Delta Ct$ method.

2.11. Western blot analysis

Western blot analysis was performed to determine the translation of different proteins involved in apoptosis under different culture conditions. The ACL fibroblasts were harvested, washed with cold PBS (pH 7.4), and lysed with RIPA Lysis Solution (R0020, Solarbio, Beijing, China) on ice for 30 min. The lysates were centrifuged at $12,000 \times g$ at 4 °C for 10 min and the protein concentrations in the supernatants were determined by the method of Bradford. For Western blot analysis, equal amounts of protein were subjected to 12% SDS polyacrylamide (PAGE) gel electrophoresis and electrophoretically transferred onto a PVDF membrane (IPVH00010, Millipore, Massachusetts, the USA). The membrane was blocked with 5% powdered non-fat milk for 2 h to block non-specific protein binding, and then incubated with the primary antibodies against cleaved-caspase-3 (Affinity, AF1009, Jiangsu, China), Bax (Proteintech, 60267-1-ag, Wuhan, China), Bcl-2 (Proteintech, 26593-1-AP, Wuhan, China), collagen I (Abcam, Ab260043, Cambridge, UK), collagen III (Abcam, Ab184993, Cambridge, UK), HIF-1α (Affinity, AF1009, Jiangsu, China), PI3K (Affinity, AF6241, Jiangsu, China), p-PI3K (Affinity, AF3241, Jiangsu, China), Akt (Proteintech, 10176-2-AP, Jiangsu, China), p-Akt (Proteintech, 66444-1-Ig, Jiangsu, China), mTOR (Proteintech, 66888-1-Ig, Jiangsu, China), p-mTOR (Proteintech, 67778-1-Ig, Jiangsu, China) and β-actin (ZSGB-bio, TA-09, Beijing, China) overnight at 4 °C. After washing with TBS-Tween 10min (TBST) thrice, the blots were incubated with secondary antibody (ZSGB-bio, ZB2301, ZB2305, Beijing, China) for 90min at RT in blocking solution, and washed thrice with TBST. The detection of protein bands was visualized by enhanced chemiluminescence kit and the gray value of band was calculated by Tanon 1600.

2.12. Extracellular matrix synthesis

The ACL fibroblasts were divided into different groups as mentioned before. The cells were incubated under different conditions. Type I and III collagen were synthesized by ACL fibroblasts in different groups and were analyzed using quantitative real-time PCR and western blot as mentioned above.

Table 1
List of primer sequences used for real-time PCR.

Gene	Primer sequence
Bax	F: 5′-TTTGCTTCAGGGTTTCATC-3′
	R: 5′-ACACTCGCTCAGCTTCTTG-3′
Bcl-2	F: 5′-TCGCCGAGATGTCCAGC-3′
	R: 5′-CCCACCGAACTCAAAGAAGG-3′
Collagen I	F: 5′-GGTGTTGTGCGATGACGT-3′
	R: 5′-AGCTGGGGAGCAAAGTTT-3′
Collagen III	F: 5′-GTCCTTGCTGTGGTGGTGT-3′
	R: 5′-TTCTAGCGGGGTTTTTACG-3′
HIF-1α	F: 5′-AAACAGAGCAGGAAAAGGAG-3′
	R: 5′-TCAAAGCGACAGATAACACG-3′
Caspase-3	F: 5′-GTTCATCCAGTCGCTTTG-3′
	R: 5′-TTCTGTTGCCACCTTTCG-3′
β-actin	F: 5′-AAGGCCAACCGCGAGAA-3′
	R: 5′-ATGGGGGAGGGCATACC-3′

2.13. mTOR-PI3K-Akt pathways

ACL fibroblasts were seeded on 6-well plates at a cell density of 1×10^4 cells/cm^2 in high-glucose DMEM medium with 10% FBS. The medium was changed into DMEM medium with 1% FBS after cell starvation for 12 h. Then, the cells were cultured in different conditions and treated with 10 μl 600×10^9/L PRP. The expression level of phosphorylated (p)-PI3K/total (t)-PI3K, p-Akt/t-Akt, p-mTOR/t-mTOR were detected by western bolt analysis as mentioned above.

2.14. Experimental animals and surgical protocol

Ninety 12-week-old male Sprague–Dawley rats were utilized in the in vivo experiments to generate an ACL injury model. Their mean body weight was 412.6 g (range, 393–442 g). All the animals were randomized to one of three groups, control group (cut articular capsule only), hypoxia group (ACL partial tear) or PRP treatment group (PRP treatment after ACL partial tear). Each group would be analyzed 6 weeks after surgical intervention. The anesthesia and surgical technique were used according to our previous study [11].

2.15. Paraffin embedding, hematoxylin and eosin (H & E) staining, immunofluorescence staining, masson staining analysis

For H & E staining, immunofluorescence staining and Masson's staining, samples were fixed in 4% paraformaldehyde for 48 h. Subsequently, samples were dehydrated, embedded in paraffin and cut into 4-μm-thick sections using cryostat (Leica, Wetzlar, Germany). Immunofluorescence staining was performed according to the method described by Li et al. [11]. The sections were incubated with the primary antibodies of HIF-1α (Affinity, AF1009, China), cleaved-caspase 3 (CUSABIO, CSB-PA000004, Wuhan, China), type I (Abcam, Ab260043, Cambridge, UK) and III (BOSTER, A00788-3, California, USA), TNF-α (Proteintech, 60291-1-Ig, Jiangsu, China), IL-1β (Abcam, Ab283818, Cambridge, UK), collagen at 4 °C overnight and with Alexa Fluor 488-conjugated secondary antibody (ZSGB-BIO, ZF-0511, Beijing, China) 1 h at RT. Finally, the sections were detected by fluorescence microcopy (Olympus, Tokyo, Japan).

2.16. Transmission electron microscope (TEM) analysis

Samples were cut into 1 mm^3 tissue using a sharp blade. Then the samples were transferred into an EP tube with fresh TEM fixative for further fixation. The tissues were washed using 0.1 M PB (pH 7.4) for 3 times. Then ethanol of different concentration was used to dehydrate samples. The pure EMBed 812 was poured into the embedding models and the tissues were inserted into the pure EMBed 812. The embedding models with resin and samples were moved into 65 °C oven to be polymerized for more than 48 h. The blocks were cut to 60–80 nm on the ultra microtome. Finally, the samples were examined by TEM (HT7800, HITACHI, Japan) and calculated by the number of collagen fibers in the same size area.

2.17. Statistical analysis

All experiments were performed three times. Continuous variables were expressed as mean ± standard deviation. One-way ANOVA was used to determine the significant differences between groups and Tukey HSD post-hoc test was used to distinguish which group differed from one another at significant level of $P < 0.05$.

3. Results

3.1. ACL fibroblasts identification

The outcomes of immunofluorescence staining (Fig. 1A) and flow cytometry (Fig. 1B) showed that ACL fibroblasts were strongly positive
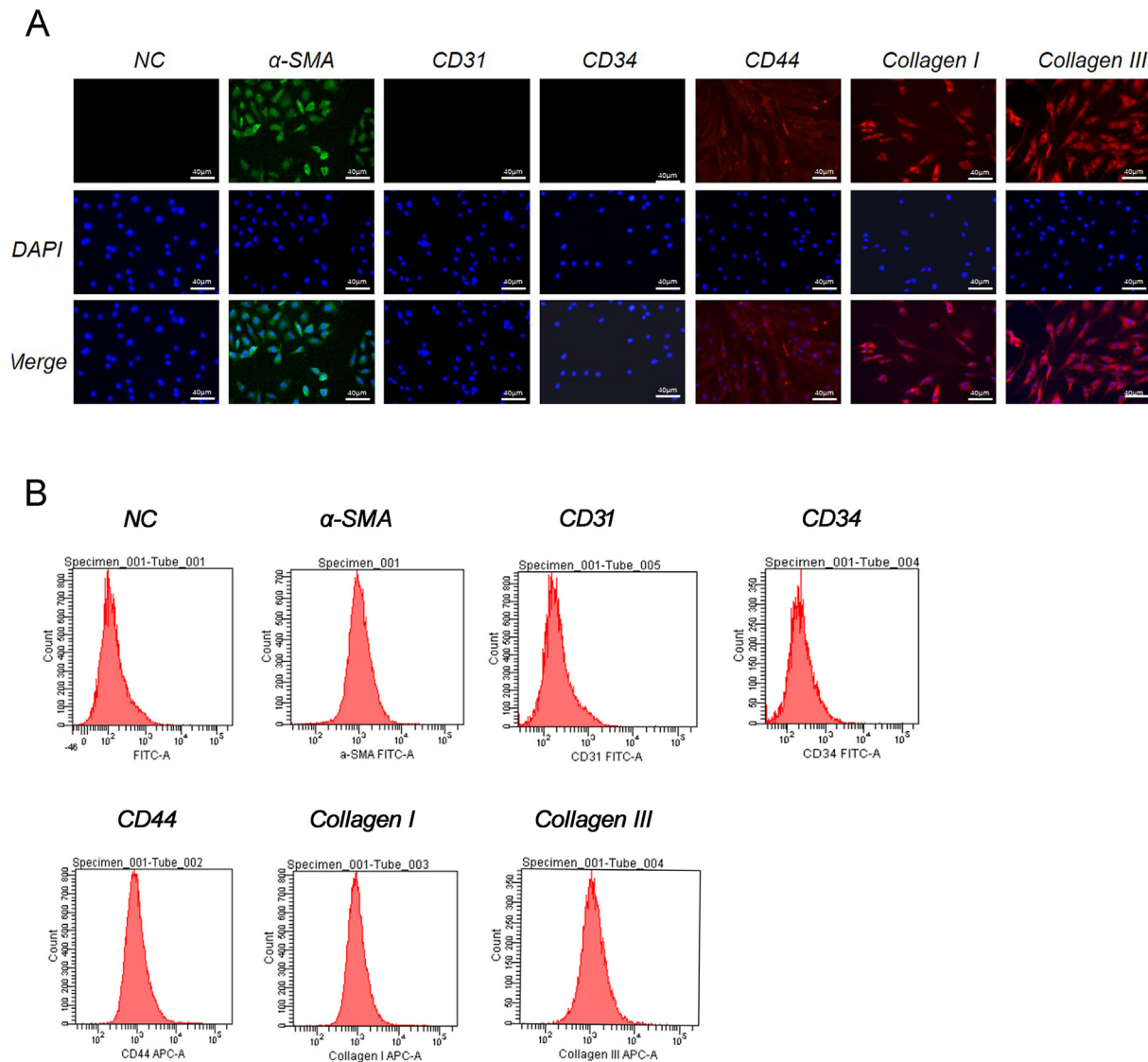
Fig. 1. A: Immunofluorescences of α-SMA, CD34, CD44, Collagen I and III were detected in human ACL fibroblasts by immunofluorescence staining. Bar = 40 μm. B: Human ACL fibroblasts identification using flow cytometry.

for CD44, α-SMA, collagen I and III, but negative for CD31 and CD 34. The positive expression rate was close to 100% and the immunophenotype was in accordance with ligament-forming fibroblasts in ACL tissues [25].

3.2. LD 50 of hypoxia induction was 48 h and the maximum efficacy of PRP concentration was $600 \times 10^9/L$

This study found that the time to achieve 50% hypoxia-induced cell death in cultured ACL fibroblasts was 48 h (Fig. 2A); and the effective dose of PRP to restore cell viability was $600 \times 10^9/L$, equivalent to 3-fold normal platelets concentration of whole blood (Fig. 2B).

3.3. PRP inhibited cell apoptosis under hypoxia condition

Fig. 3A showed the effects of apoptosis rate of ACL fibroblasts analyzed by flow cytometry assays. Compared to the control group, hypoxia condition significantly induced cell apoptosis for ANNEXIN V-FITC/PI flow cytometry (P < 0.001, Fig. 3B). PRP pretreatment significantly decreased the cell apoptosis of ACL fibroblasts under hypoxia (P < 0.001, Fig. 3B). The expression of gene and protein of

Bax, Bcl-2, cleaved-caspase 3 were consistent with the results from flow cytometric analysis (Fig. 3C–E). For example, Bax (P < 0.05 for western bolt analysis, P < 0.001 for real-time PCR) and cleaved-caspase 3 (P < 0.001 for western bolt analysis and P < 0.001 real-time PCR) were significantly higher in hypoxia group compared to the control group. The expression of Bax (P < 0.05 for western bolt analysis, P < 0.001 for real-time PCR) and cleaved-caspase 3 (P < 0.05 for western bolt analysis, P < 0.001 for real-time PCR) in PRP pretreatment group were significantly lower than that in hypoxia group.

3.4. PRP promoted cell proliferation under hypoxia condition

There was statistically significant difference between control group and hypoxia group in population doubling (G2 phase) for ACL fibroblasts (P < 0.001). The level of G2 phase varied between PRP pretreatment group (3.7%) and control group (19.2%).

PRP pretreatment caused a significant G2 phase arrest. Results showed the inhibitory effects of hypoxia on ACL fibroblasts proliferation and PRP pretreatment significantly improved cell proliferation compared to the control group (Fig. 4A–D).
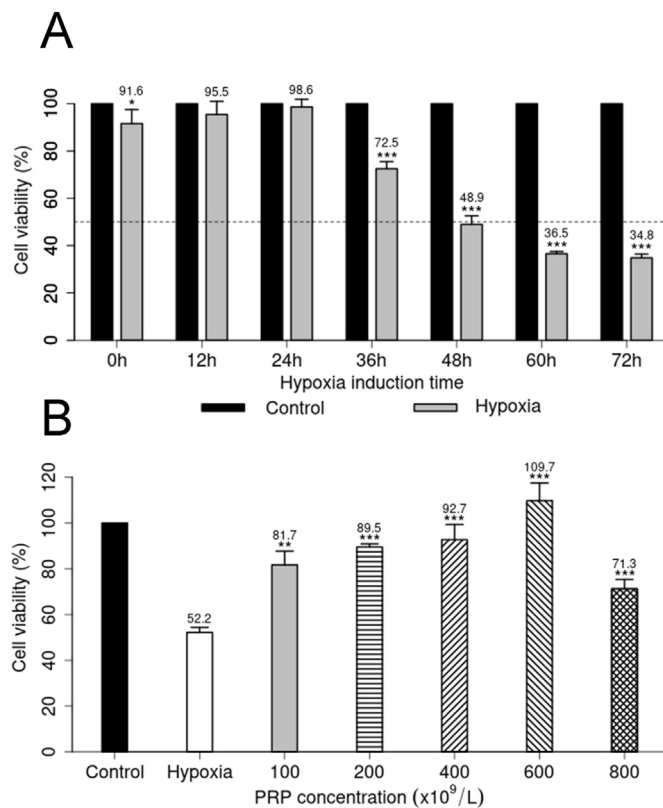
Fig. 2. A: Time matched control of cell viability between different hypoxia induction time and normal condition using CCK-8 assay. 48 h after hypoxia induction, cell viability is the closest to 50%. B: Effected dose of PRP pretreatment at 48 h hypoxia induction time using CCK-8 assay. 600×10^9/L PRP has the strongest protective effect on ACL fibroblasts under hypoxia. The data were shown as mean $\pm$ SD. *P < 0.05, **P < 0.01, ***P < 0.001.

3.5. PRP decreased ECM synthesis under hypoxia condition

Type I and III collagen were the main component ECM of ACL tissues. Therefore, collagen synthesis played an important part during the repair process after ACL injury. In our experiments, type I and III collagen mRNA expression were 2.1 folds and 2.5 folds higher after 48 h hypoxia induction compared to the control group, respectively. In addition, 600×10^9/L PRP pretreatment significantly reduced the type I and III collagen mRNA expression of the hypoxia induced ACL fibroblasts to 78.5% and 77.7%, respectively, at 48 h compared to hypoxia group (P < 0.001) (Fig. 5A–C). In PRP pretreatment group, however, type I and III collagen mRNA expression were still significantly higher than control group (P < 0.001). The expression of collagen I and III were consistent with HIF-1α. The outcomes of western blot assay were similar to real-time PCR (P < 0.001).

The outcomes of immunofluorescence staining (Fig. 5D) showed that HIF-1α, collagen I and III were strongly positive in hypoxia group, but negative in control group. The expression of HIF-1α, collagen I and III in PRP pretreatment group were between control group and hypoxia group.

3.6. PRP promoted cell migration under hypoxia condition

To explore the effect of hypoxia condition and PRP protective effect under hypoxia on the motility of ACL fibroblasts, cell migration ability was measured using scratch assay (Fig. 6A and B) and transwell assay (Fig. 6C). According to the results of scratch assay, PRP pretreatment significantly improved the migration ability compared to hypoxia group especially after 24 h of incubation.

As shown in Fig. 6C, this study found that hypoxia condition

significantly restrained cell migration compared with the control group (p < 0.05). PRP could alleviate the inhibitory effect of hypoxia on fibroblasts, but the intensity of this alleviation cannot balance out the inhibitory effect of hypoxia on cell migration (p < 0.05).

3.7. PI3K-Akt-mTOR pathway was correlated with protective effect of PRP under hypoxia condition

The underlying mechanism of PRP pretreatment regulating cell viability, proliferation, apoptosis and ECM synthesis of ACL fibroblasts under hypoxia was verified through western blotting. Activating PI3K-Akt-mTOR pathway could change cell viability, motility, proliferation and apoptosis. mTOR was the downstream target of PI3K-Akt pathway and HIF-1α was one of the downstream transcription factors of mTOR. Thus, PI3K-Akt-mTOR pathway was examined in this study. Firstly, western blotting assay showed that the protein expression of phosphorylated PI3K, AKT and mTOR was greatly activated by hypoxia, and the ratio of phosphorylated/total PI3K, Akt and mTOR increased to 31%, 20% and 44/% compared to control group, respectively. The expression of phosphorylated PI3K, AKT and mTOR was also significantly inhibited by PRP pretreatment compared to hypoxia group, and the ratio of phosphorylated/total of PI3K, Akt and mTOR decreased by 23%, 14% and 10%, respectively (Fig. 7A–D).

3.8. PRP reduced inflammatory cell infiltration as well as cell apoptosis and enhanced collagen synthesis in rats after ACL injury

ACL partial tear disturbed the blood vessels of ligament and created a hypoxia condition. Six weeks later, inflammatory cell infiltration, cell apoptosis (cleaved-caspase 3 expression) and HIF-1α expression increased due to hypoxia condition after ACL injury but were inhibited by PRP treatment (Fig. 8A, B, C, D and G). Meanwhile, the outcomes of Masson's staining (Fig. 8E and F) were in line with the collagen expression of ACL fibroblasts. The collagen fiber content significantly increased in hypoxia group compared with control group (93.3% VS 50.3%, P < 0.001). The proportion of collagen fiber decreased to 71.3% in PRP group but was still significantly higher than control group (P < 0.001). The immunofluorescence staining of collagen I and III also confirmed this effect (Fig. 8H and I). In addition, TEM found the number of collagen fibers in the same size area was higher in hypoxia group. The number of collagen fibers in PRP treatment group was between control group and hypoxia group (Fig. 8J and K).

4. Discussion

The blood vessels are inevitably compromised due to trauma, resulting in an ischemic and hypoxia microenvironment after ACL injury [16,17]. The current gold standard for treating ACL injuries is ACLR, although it cannot be neglected that ACLR may result in OA, graft failure and invasive trauma during procedure [26,27]. Recently, in the light of regenerative medicine, the ACL primary repair gradually becomes a treatment for ACL injuries [28].

However, the healing ability of ACL tissue was limited by its intrinsic characteristic and the function of ACL fibroblasts after ACL injury. In this study, the outcomes of cell identification were α-SMA^pos, CD31^neg, CD34^neg, CD44^pos, collagen I^pos and collagen III^pos, which was consistent with previous literature that reported [25] and demonstrated that cells migrated from ACL tissue was ligament-forming fibroblasts rather than ligament perivascular cells or ligament interstitial cells. In order to minimize the impact of hypoxia on the result, we controlled the time from injury to surgery within 7 days and chose tibia or femur attachment site of ACL remnant. Although we cannot ignore the influence of exposure to hypoxia environment on ACL fibroblasts, we have minimized the influence as much as possible. Harvesting an intact ACL tissue for young people was not ethically approved.

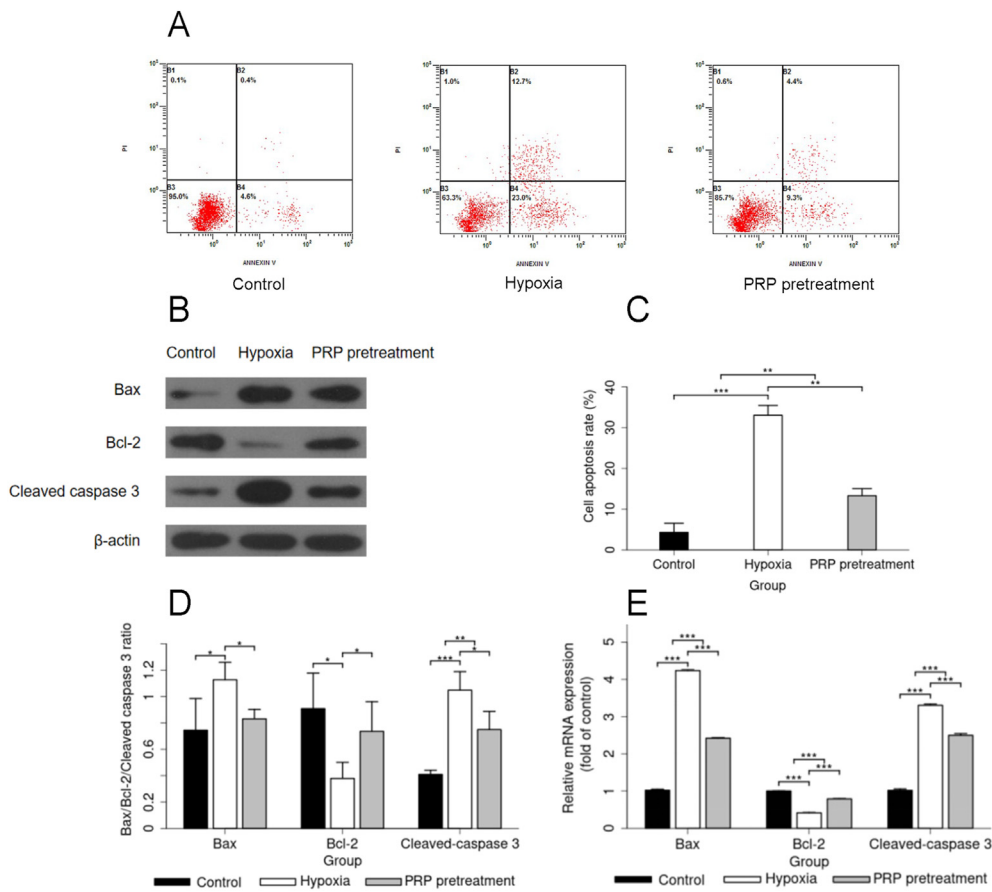ACL ligament-forming fibroblasts accounted for 60–70% of all cells in

Fig. 3. A–B:The effects of apoptosis rate of ACL fibroblasts determined by flow cytometry assays. C–D: Effects of hypoxia and PRP pretreatment on Bax, Bcl-2, cleaved-caspase 3 protein expression levels in ACL fibroblasts were detected by western blot analysis and the histogram showed these quantitative results, which was normalized by β-actin. E: Effects of hypoxia and PRP pretreatment on Bax, Bcl-2, cleaved-caspase 3 RNA expression levels in ACL fibroblasts were detected by real-time PCR analysis and the histogram showed these quantitative results.The data were shown as mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001.
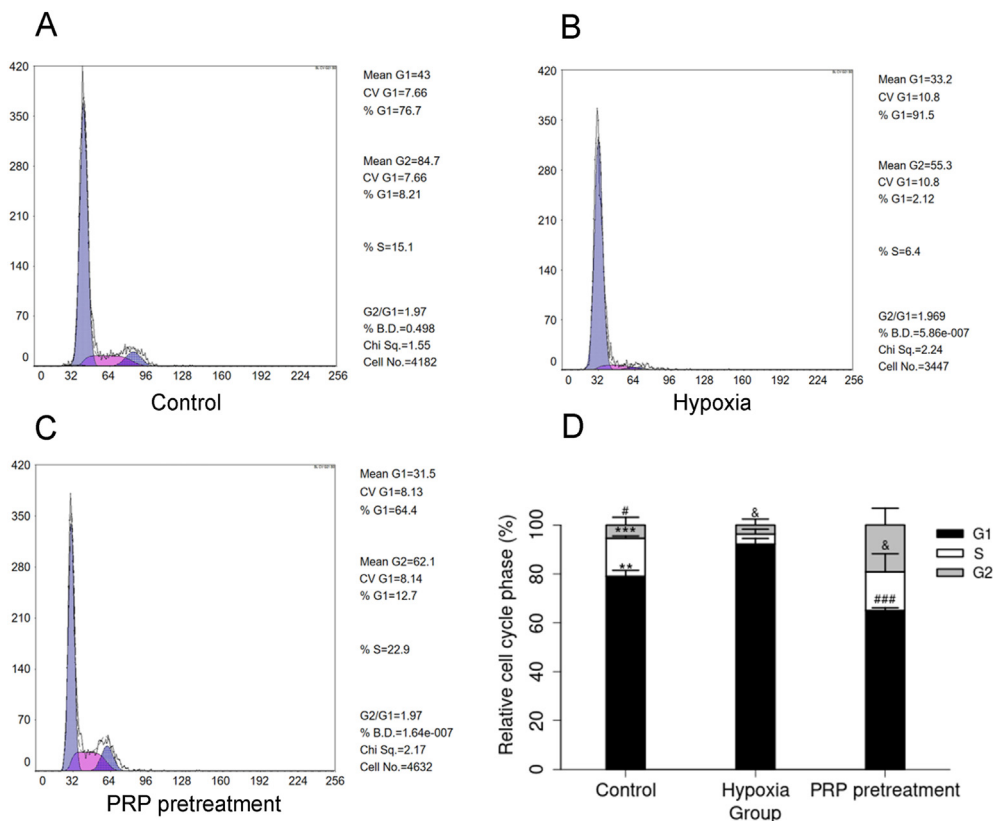

Fig. 4. A–C: Flow cytometry analysis of cell cycle for different groups. D: Flow cytometry assay of cell cycle. */#/&P < 0.05, **/##/&&P < 0.01, ***/###/&&&P < 0.001. *Comparison between control and hypoxia group, #comparison between hypoxia and PRP pretreatment group, &comparison between control and PRP pretreatment group.
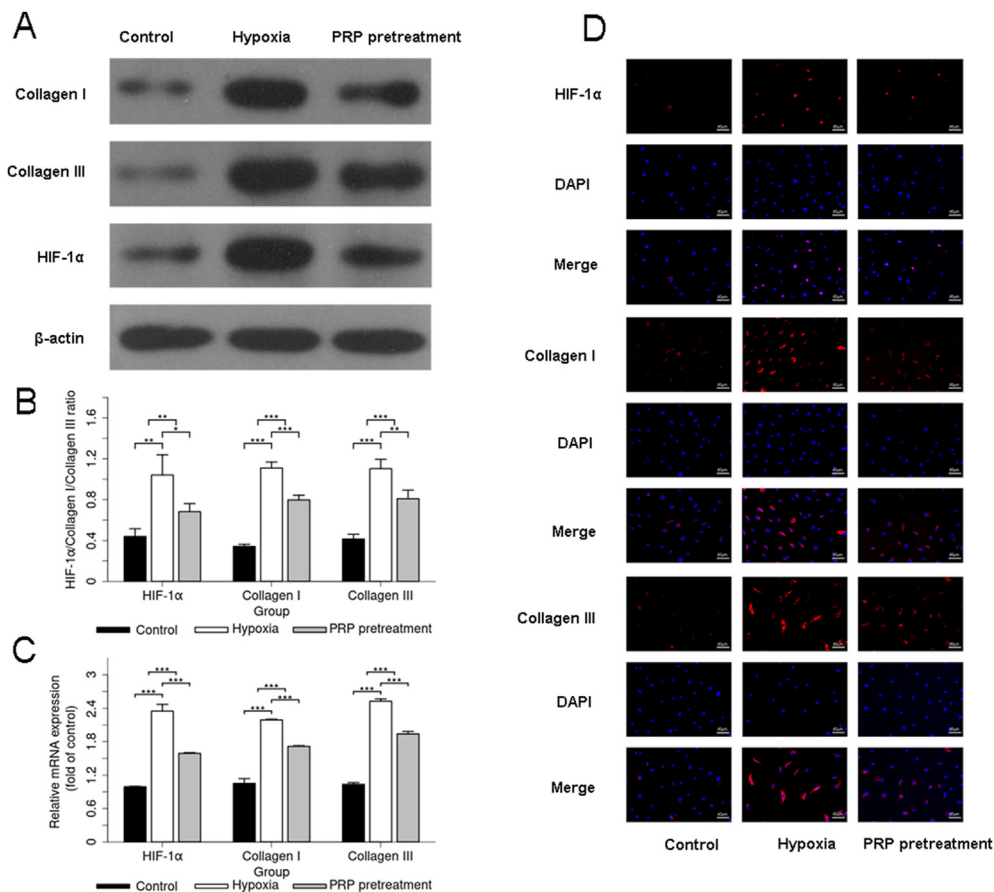
Fig. 5. A: Hypoxia condition increased the HIF-1α, collagen I and III expression and PRP pretreatment decreased their expression. These outcomes were detected by western blot (A–B), real-time PCR (C) and immunofluorescence staining (D). The data were shown as mean ± SD.*p < 0.05, **p < 0.01, and ***p < 0.001. Bar = 40 μm.

ACL tissue. The survival and migration of intrinsic ACL fibroblasts have been considered as a prerequisite for ACL healing [25,29]. In this study, we found hypoxia induced condition significantly inhibited cell viability and promoted cell apoptosis. After 48 h hypoxia induction, the cell viability of ACL fibroblasts decreased by 51.1%. Meanwhile, the cell apoptosis of ACL fibroblasts increased by 28.8% compared to control group. Similarly, Sha et al. [18] used 3% O_2 to induce ACL fibroblasts, and they found the apoptosis rate of cells increased significantly. Compared to 0 h group, the apoptosis rate of cells increased from 2.8% to 13.2% at 12 h and 11.9% at 24 h, respectively. Nevertheless, this damage effect to ACL fibroblasts can be reversed by PRP. Cell viability was improved by 57.5%, but cell apoptosis rate was suppressed by 19.8% with PRP pretreatment compared to hypoxia group. As shown in Fig. 8B, cleaved-caspase 3 expression in ACL tissue also confirmed the promotion of hypoxia and the inhibition of PRP on cell apoptosis. Adequate ACL healing required not only adequate blood supply but also extracellular matrix production and cellular proliferation [30]. In this study, PRP pretreatment significantly increased the proportion of cells at G2 phase compared to control group (3.7% VS 19.2%).

The massive ACL fibroblasts synthesized ECM contained collagen I and III, and they provided mechanical strength for ACL tissue. Cheng et al. [13] found PRP had positive effect on ACL fibroblasts. According to their findings, PRP significantly increased the gene expression of type I and III collagen, cell viability and metabolism of ACL fibroblasts. Surprisingly, the effect of PRP on synthesizing ECM of ACL fibroblasts was contrary to our expectation. The induction of hypoxia obviously stimulated ACL fibroblasts to synthesize more type I and III collagen (3.3 and 2.7 folds) compared to control group. This result was consistent with previous research. Kowalski et al. [25] used 1% O_2 to induce human

ligament-forming fibroblasts. Their study demonstrated that hypoxia condition enabled higher expression of collagen I and III. The gene expression of type I and III collagen were 3 and 3.5 folds higher compared to normoxic condition. However, PRP pretreatment did not stimulate ACL fibroblasts to synthesize type I and III collagen. On the contrary, PRP pretreatment significantly inhibited cells to produce collagen I and III compared to hypoxia group. The reason for this result may be the period of hypoxia induction and PRP may weaken scarring response after ACL injury. In this study, we adopted 48 h hypoxia induction in order to simulate hypoxia condition after ACL injury. And our in vivo model showed that hypoxia condition increased collagen expression via the number of fibers in the same size area (Fig. 8J and K). We speculated that in the early stage of hypoxia, there was enough adenosine triphosphate (ATP) in the cells to support the synthesis of collagen. PRP may stimulate cell proliferation and migration, which consumed a lot of ATP in ACL fibroblasts. PRP increased the expression of VEGF in ACL tissue [11]. This represented that PRP increased angiogenesis in ACL tissue and relieved hypoxia condition around ACL fibroblast. Therefore, PRP partly offsets the effect of hypoxia on the promotion of ACL fibroblasts collagen synthesis.

It was important that ACL fibroblasts migrated to tear site and formed fibrovascular scar tissue. If ACL fibroblasts did not migrate to tear site in time, it would affect the formation of tissue bridging and the process of tissue remodeling, resulting in the weakness of tissue strength and the failure in repair process. In this study, hypoxia microenvironmental condition significantly inhibited the ability of migration of ACL fibroblasts. While the bio-active factors of PRP can decrease the negative effect of cell migration ability induced by hypoxia. Zheng et al. [31] found that PRP could improve the migration abilities of ACL fibroblasts significantly, especially at 72 h after
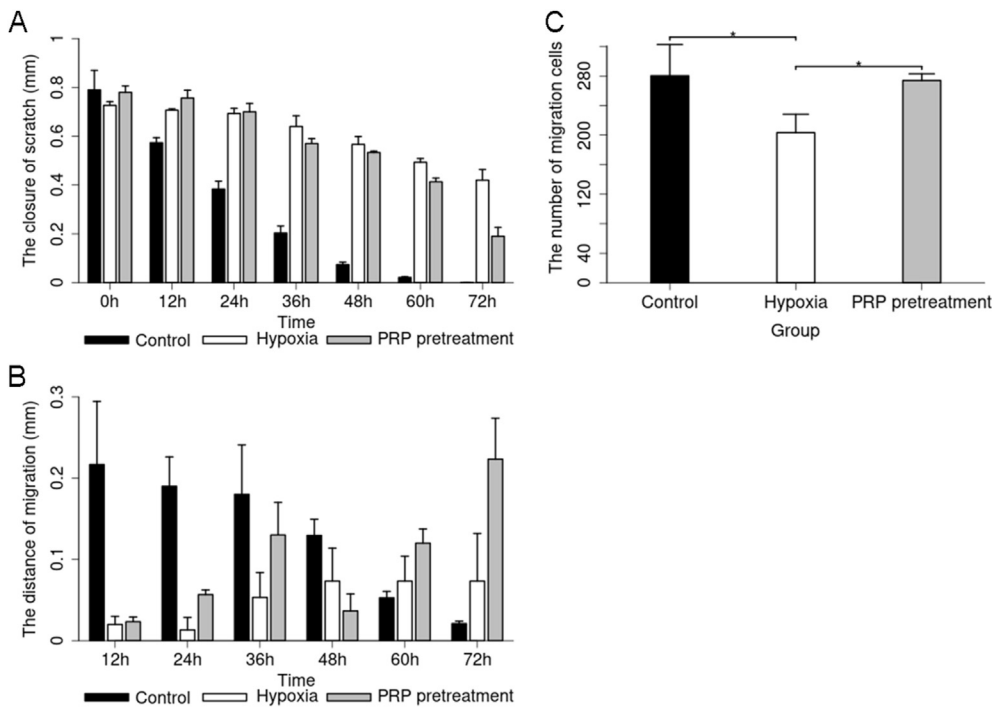
Fig. 6. A: Comparison of cell migration ability expressed as the closure of the scratch in each group. B: Comparison of cell migration ability expressed as the distance of migration in each group. Experimental images in each group used the scratch assay at 0 h, 12 h, 24 h, 36 h, 48 h, 60 h and 72 h respectively. C: Effects of hypoxia and PRP pretreatment on migration ability of ACL fibroblasts for transwell assay. The number of ACL fibroblasts passing through the chamber was recorded. The data were shown as mean $\pm$ SD.*p < 0.05, **p < 0.01, and ***p < 0.001.

cell seeded. The growth factors like TGF-β and FGF, as the component of PRP, have been proved to stimulate cell growth and migration [32,33]. As an important active factor in PRP, PDGF can bind to PDGF receptors and activate several signaling pathways such as mitogen-activated protein kinase (MAPK) and PI3K/Akt [34]. These pathways were involved in cell proliferation and apoptosis. Similarly, FGF-2 has been demonstrated to regulate many cellular functions including cell proliferation and migration of ligament tissues [35,36].
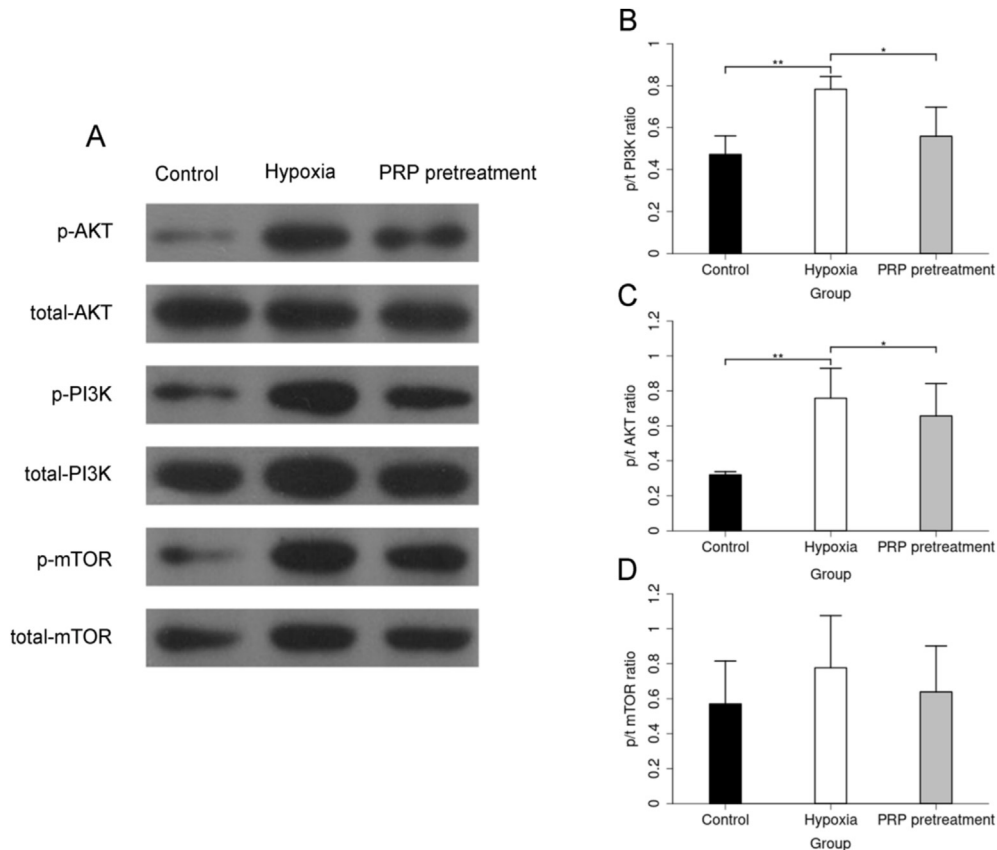

Fig. 7. A: Western blot analysis of phosphorylated and total PI3K, AKT and mTOR. B–D: The ratio of phosphorylated/total PI3K, Akt and mTOR expression among three groups.The data were shown as mean $\pm$ SD.*p < 0.05, **p < 0.01, and ***p < 0.001.
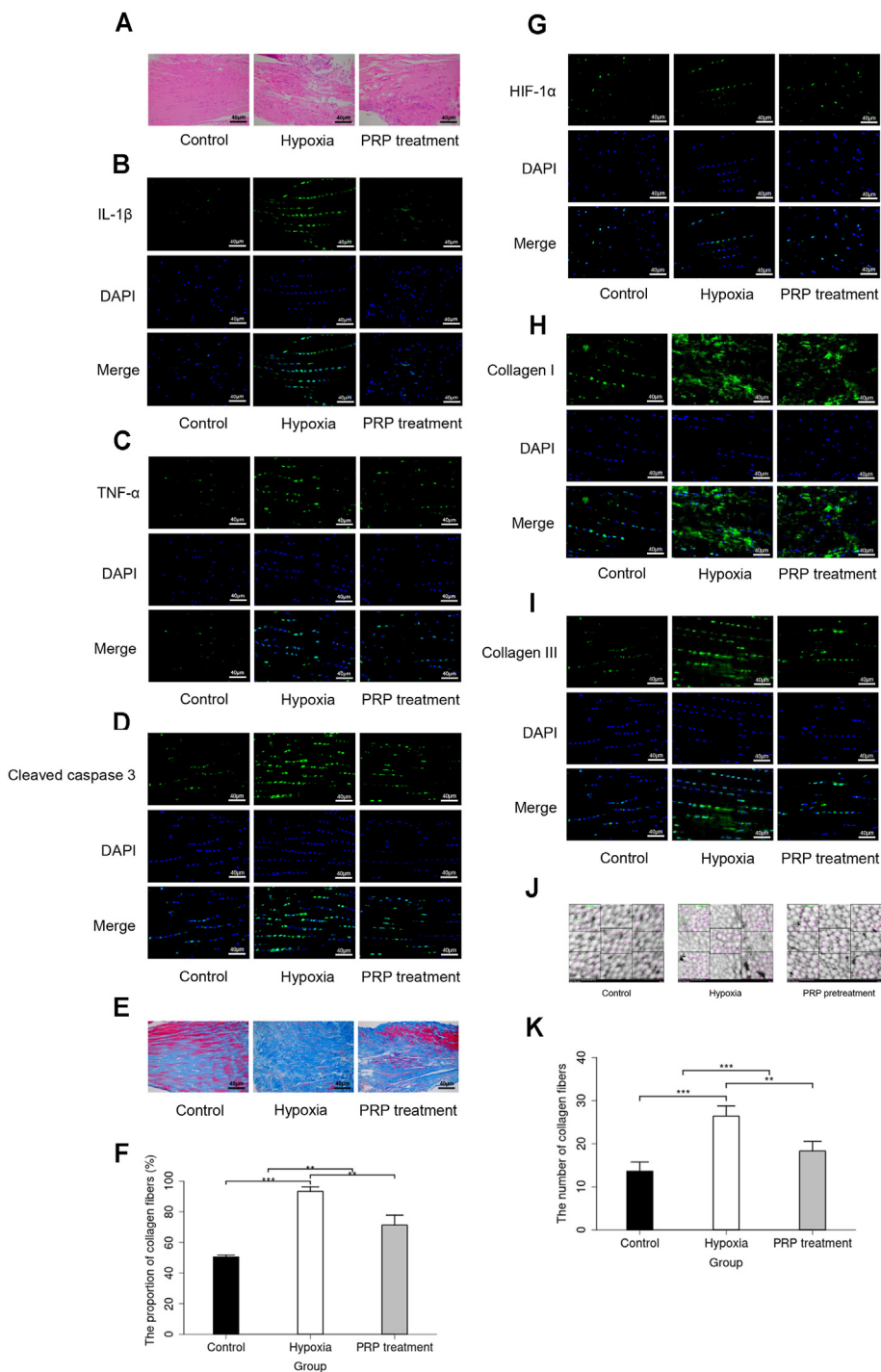
Fig. 8. A: H & E staining for each group. B: IL-1β immunofluorescence staining for each group. C: TNF-α immunofluorescence staining for each group. D: Cleaved caspase 3 immunofluorescence staining for each group. E: Masson's staining for each group. F: Comparison of collagen fibers proportion in each group by Masson's staining. G: HIF-1α immunofluorescence staining for each group. H: Collagen I immunofluorescence staining for each group. I: Collagen III immunofluorescence staining for each group. J: The number of collagen fibers analysis by transmission electron microscope. K: Comparison of collagen fibers number in each group by transmission electron microscope. The data were shown as mean ± SD.*p < 0.05, **p < 0.01, and ***p < 0.001. Bar = 40 μm.

Both hypoxia and the consumption of ATP were required to induce cell apoptosis. Sha et al. [19] found hypoxia condition increased HIF-1α expression and induced cell apoptosis via increasing proapoptotic proteins expression levels such as cleaved-caspase 3 and bax, but decreasing antiapoptotic proteins expression levels like bcl-2. Pore et al. [23] used the inhibitor of mTOR, and they found that HIF-1α was the downstream signaling molecule of PI3K/Akt/mTOR pathway. HIF-1α was the main regulator of cells in hypoxia microenvironment, controlling the expression of genes associated with metabolic, proliferation and apoptosis [19]. In this study, hypoxia significantly increased the HIF-1α expression compared to control group. PRP pretreatment had the opposite effect on HIF-1α compared to hypoxia group. Meanwhile, p-PI3K/t-PI3K,

p-Akt/t-Akt and p-mTOR/t-mTOR ratio had statistically significant difference between hypoxia group and PRP pretreatment group.

PRP in this study contained lymphocytes rather than pure PRP. The presence of lymphocytes may not be favorable for those treatments for chronic inflammation, but our data showed that it works well for acute injury like ACL tear. And PRP was not activated by $CaCl_2$ because PRP would become jelly-like substance form that may have undesirable influence on the results of migration assay. However, PRP contained many kinds of bioactive factors such as PDGF, FGF and VEGF. Lacking identification and measurement of bioactive factors were the major weakness in this study. However, we adopt two-step centrifugation to obtain PRP, which is a mature and commonly used method. Meanwhile, VEGF was

essential for new angiogenesis to increase blood supply at the damaged site and provide adequate nutrition for tissue healing. FGF stimulated the proliferation and migration of cells in ligament tissues. The above-mentioned mechanisms required further investigation.

5. Conclusion

The results from the present study demonstrated that PRP can protect ACL fibroblasts via decreasing the apoptosis and increasing the cell viability, migration and proliferation of ACL under hypoxia condition. PRP had no positive effect on the synthesis of type I and III collagen under hypoxia condition. And PRP protective effect was correlated with PI3K/Akt/mTOR pathway.

Funding

Conflict of interest

The author(s) have no conflicts of interest relevant to this article.

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