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Platelet-rich plasma affects the proliferation and collagen production of mesenchymal stem cells

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Abstract

BACKGROUND: Mesenchymal stem cells are the seed cells for tendon tissue engineering which can be obtained in large quantities, but how to induce *in vitro* is a key technology.

OBJECTIVE: To explore the effect of platelet-rich plasma on the proliferation and collagen production of *in vitro* cultured mesenchymal stem cells.

METHODS: The rabbit mesenchymal stem cells were separated ad cultured. The high-dose platelet-rich plasma group, middle-dose platelet-rich plasma group and low-dose platelet-rich plasma group were set to induce the mesenchymal stem cells, and the blank control group was set as control.

RESULTS AND CONCLUSION: The proliferation of mesenchymal stem cells in the high-dose platelet-rich plasma group, middle-dose platelet-rich plasma group and low-dose platelet-rich plasma group was high, and in rapid growth with big increase amplitude, and there was no significant difference in the proliferation when compared with the blank control group ($P < 0.05$). The effect was positively correlated with the culture time, and after cultured for a certain time, the effect was in dose-dependent manner, as higher dose platelet-rich plasma had more significant effect on the proliferation of the cells. The results indicate that platelet-rich plasma can significantly promote the synthesis of collagen type Ⅰ and Ⅲ of mesenchymal stem cells, the higher the dose, the more significant the effect on the collagen.

Subject headings: blood platelets; plasma; mesenchymal stem cells; collagen type Ⅰ; collagen type Ⅱ

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INTRODUCTION

The premise of tendon tissue engineering is to obtain a large amount of seed cells, but how to efficiently amplify the cells *in vitro* is the key to the technique. The mesenchymal stem cells can differentiate into various mature body cells in a certain condition with good plasticity, and have the potential to differentiate into some specific tissues, such as bone, cartilage, fat, tendon, muscle and bone marrow stroma[1-3]. The studies have shown that *in vitro* cultured mesenchymal stem cells have the ability to secrete collage type Ⅰ before induction, and the main functions are similar to those of tendon cells, which meet the requirement to the seed cells during the construction of tendon tissue engineering[4-6]. The various differentiation potential of mesenchymal stem cells have been given high differentiation capacity, but there is no specific induction mechanism to obtain sufficient number of functional tendon-like cells. The direct induced differentiation efficiency is not ideal, so the further in-depth studies are needed. Growth factors are large class of biological media, which have important role in the proliferation and differentiation of cells. Due to the disadvantages of source limitation, expensive, certain immune rejection and risk of disease transmission, the clinical application of exogenous growth factors is limited. In this experiment, modified Appel method was used to obtain the platelet-rich plasma, and set the platelet-rich plasma groups with different concentrations to induce the *in vitro* cultured rabbit mesenchymal stem cells, in order to understand the effect of platelet-rich plasma on the proliferation and collagen production of *in vitro* cultured mesenchymal stem cells, thus finding an

ideal method to obtain the tendon tissue engineering seed cells.

MATERIALS AND METHODS

Design

A comparative cytology experiment.

Time and setting

The experiment was completed in the Experimental Animal Center, Medical College of Qingdao University from October 2010 to May 2011.

Materials

Thirty-two adult New Zealand white rabbits were collected without limitation in gender, and the body mass was 4.0−4.5 kg. The rabbits were provided by the Experimental Animal Center of Qingdao. Certificate of conformity: Scxk(Lu)2010-12.

Methods

Collection and primary culture of mesenchymal stem cells

The rabbit ear vein was injected with 30 g/L sodium pentobarbital of 1.0 mL/kg for anesthesia, in order to shear the hair on the iliac crest and obtain the skin for preparation. The skin was disinfected with 1.0% povidone iodine, and then the No. 16 bone marrow biopsy needle was connected with 10 mL syringe to puncture into the bone marrow cavity. The fluid in the syringe contained 1.0 mL diluted heparin with the concentration of 3 000 U/mL. A total of 5.0 mL bone marrow fluid was obtained. The specific culture method was operated according to the method described in the literatures[7-8].

Cell passage and culture

The culture medium was sucked out from the flask. Then, 5.0 mL PBS with the concentration of 0.01 mol/L was added into the medium to wash the cells for twice. After that, the fluid was absorbed; 2.0 mL trypsin with the concentration of 2.5 g/L was added into the flask and absorbed immediately. Then, 2.5 g/L trypsin (3.0 mL) was added into the flask and cultured with the cells in the incubator under 37 ℃ for 2−3 minutes, and the cell morphology was observed under inverted microscope (×200). When the cytoplasmic retraction, cells elongating and cell gap increasing appeared, as well as small amount of round cells that took off the wall observed, the digestion could be stopped, and the general digestion time was 3−5 minutes. After stopping the digestion, the fluid in the flask was took off or absorbed, and then added with serum-containing L-Dulbecco's modified Eagle's medium. The fluid in the flask was absorbed gently with a straw, and then pipetting the sidewall repetitively in order to make the cells took off from the wall of the bottle, and thus forming the single cell suspension. The cells suspension was moved to the centrifuge tube, 500 r/min centrifugation for 5 minutes. Then the suspension was washed with PBS once and 500 r/min centrifugation for 5 minutes again. Slightly blow the cell mass to make it loose, and then cultured with L-Dulbecco's modified Eagle's medium completely, after that the cells were incubated into the plastic flask for culture with the concentration of 1.0×10^8/L.

Identification of mesenchymal stem cells

The growth of the mesenchymal stem cells was observed under inverted microscope irregularly (×200). Two bottles of cells growth in good conditions were collected, and digested with 2.5 g/L trypsin, 800 r/min centrifugation for 5 minutes, and abandoned the supernatant, then the cells were added with 1.5 mL L-Dulbecco's modified Eagle's medium to re-suspend the cells. The cells were moved to the 1.5 mL Ep tube, 1 500 r/min centrifugation for 10 minutes and abandoned the supernatant. A gray clump with the size of sesame seed was observed under the bottom of the tube, then 1.5 mL glutaraldehyde with the concentration of 25 g/L was added into the tube along with the wall and fixed for 2 hours. After that, the gray clump was fixed with 10 g/L osmic acid for for 2 hours, and dehydration with graded ethanol and acetone, embedded with epoxy resin, and made into thin slice, and then received sodium acetate and lead citrate double staining. The ultrastructure of the cells were observed under transmission electron microscope (×2 500). Identification of the cell surface markers: the passage 3 cells that grew well were collected and then subcultured in the six-well plate with creep plate. Immunohistochemical ABC method was used to detect the expressions of CD34, CD44 and CD90 antibodies. The specific method was operated according to the method described in the literatures[9-12].

Preparation of platelet-rich plasma

Modified Appel method was used to obtain the platelet-rich plasma under sterile conditions: 5.0 mL femoral venous blood were collected from the New Zealand white rabbits, and then added with 10% sodium citrate anticoagulant, 1 000 r/min centrifugation for 15 minutes with the centrifugal radius of 8 cm; absorbed the plasma and platelet on the upper layer, then 3 000 r/min centrifugation for 8 minutes with the centrifugal radius of 8 cm, abandoned the upper plasma, and the remaining liquid was mixed with the

activator (mixture of thrombin and 10% $CaCl_2$) with the ratio of 9:1, then shaking and kept in the 4℃refrigerator overnight; after blood clot fully contraction, 1 000 r/min centrifugation for 10 minutes with the centrifugal radius of 8 cm, absorbed all the supernatant about 0.5 mL (platelet-rich plasma) for preparation.

Effect of platelet-rich plasma on the proliferation of mesenchymal stem cells

Grouping of the experiment: the experiment was divided into four groups according to the content of platelet-rich plasma in 1.0 mL L-Dulbecco's modified Eagle's medium, eight rabbits in each group. High-dose group: 1.0 mL L-Dulbecco's modified Eagle's medium contained 0.2 mL platelet-rich plasma. Middle-dose group: 1.0 mL L-Dulbecco's modified Eagle's medium contained 0.1 mL platelet-rich plasma. Low-dose group: 1.0 mL L-Dulbecco's modified Eagle's medium contained 0.05 mL platelet-rich plasma. Blank control group: no platelet-rich plasma contained in 1.0 mL L-Dulbecco's modified Eagle's medium Growth curve of the mesenchymal stem cells in each group: the passage 3 mesenchymal stem cells were collected and seeded in seven 24-well plates with 1.0×10^4 cells per hole (1.0 mL per hole), and then cultured with the serum-free L-Dulbecco's modified Eagle's medium for 24 hours, discarded the medium and nonadherent cells, and the cells in each plate were divided into four groups, 6 holes in each group; the L-Dulbecco's modified Eagle's medium containing corresponding concentration of platelet-rich plasma were added into the cells according to different groups, the cells on the plate was counted, one plate every day, continuously observed for 7 days (from the 2nd day to the 8th day); the growth curve was draw with the ordinate of cell number and the abscissa of days, and the doubling time was detected.

Survival and proliferation ability of mesenchymal stem cells in each group analyzed with methylthiazolyldiphenyl-tetrazolium bromide assay

The passage 3 mesenchymal stem cells were inoculated into five 96-well plates respectively with 2.0×10^3 cells per hole, and then cultured with the serum-free L-Dulbecco's modified Eagle's medium for 24 hours, discarded the medium and the nonadherent cells, and the cells in each plate were divided into four groups, 6 holes in each group (totally 24 holes), the L-Dulbecco's modified Eagle's medium containing corresponding concentration of platelet-rich plasma were added into the cells according to different groups; after cultured for 2, 3, 4, 6 and 8 days, 20 μL methylthiazolyldiphenyl-tetrazolium bromide medium with the concentration of 50 g/L were added into each hole, and then continuously cultured for 4 hours and discarded the methylthiazolyldiphenyl-tetrazolium bromide medium; then 200 μL dimethyl sulfoxide was added into each well and shocked for 5 minutes. The absorbance value ($A_{450\ nm}$) was measured with enzyme-linked immunosorbent assay, and the results were compared.

Effect of platelet-rich plasma on the collagen production of mesenchymal stem cells

The collagen production of mesenchymal stem cells was quantitatively measured with enzyme-linked immunosorbent assay. The passage 3 mesenchymal stem cells were collected and cultured in the 48-well plate overnight with the concentration of 2×10^4 per well, and then cultured with the serum-free L-Dulbecco's modified Eagle's medium for 24 hours, discarded the medium and the nonadherent cells, and the wells in each plate were divided into four groups, 6 wells in each group (totally 24 wells), the L-Dulbecco's modified Eagle's medium containing corresponding concentration of platelet-rich plasma were added into the cells according to different groups, and after cultured for 3 days, the cells were washed with PBS and fixed with 2% formaldehyde; 0.3% hydrogen peroxide was added to neutralization the endogenous peroxidase, then Ⅰ and Ⅲ collagen antibodies were added and incubated at room temperature for 60 minutes; peroxidase-labeled secondary antibodies were added, and after 30 minutes, the reaction was stopped with 0.5 N sulfuric acid. The samples about 100 μL in each well were moved into the 96-well plate, and the collagen production was measured using a microplate reader with optical density of 450 nm ($A_{450\ nm}$), six times of each specimen.

Expression of collagen type Ⅰ gene by reverse transcription-PCR

The expressions of platelet-rich plasma and collagen type Ⅰ gene of various cells were detected with reverse transcription-PCR according to the methods proposed by Xu *et al* [10]. The glyceraldehyde 3-phosphate dehydrogenase was used as the control to detect the expressions of target genes. The primers of rabbit collagen type Ⅰ and glyceraldehyde 3-phosphate dehydrogenase were synthesized with DNA/RNA automatic synthesizer, the primer sequences of collagen type Ⅰ were left primer: 5'-CCA GAT TGA GAC CCT CCT CA-3', right primer: 5'-ATG CAA TGC TGT TCT TGC AG-3', and the expected amplification product was 381 bp; the primer sequences of glyceraldehyde 3-phosphate dehydrogenase were left primer: 5'-TCA CCA TCT

TCC AGG AGC GA-3', right primer: 5'-CAC AAT GCC GAA GTG GTC GT-3', and the expected amplification product was 293 bp. The RNA of all the cells were extracted through TRIzol method, and then 1 μL RNA specimens of different groups were collected to synthesize cDNA according to procedures of reverse transcription kit (Sigma). The collagens type Ⅰ were amplified on the PCR amplification, and the response procedures were 94 ℃ denaturation for 3 minutes, 94℃ denaturation for 45 seconds, 59 ℃ denaturation for 45 seconds, 72 ℃ denaturation for 45 seconds, and repeated for 30 times. The glyceraldehyde 3-phosphate dehydrogenase was used as the internal reference, and each tube was added with 1 μL internal reference glyceraldehyde 3-phosphate dehydrogenase primer s+as (10 pmol of each primer), then the amplification products of collagens type Ⅰ were detected with 1.5% agarose gel electrophoresis.
The gel pieces were put into the camera obscura of the ultraviolet projection instrument with gene band image processing system after electrophoresis, the concentration of glyceraldehyde 3-phosphate dehydrogenase was 1, and the relative density of target gene was the ratio between band density of target gene and glyceraldehyde 3-phosphate dehydrogenase; after measured three times, the mean value was calculated for analysis.

Main outcome measures

The proliferation and collagen production of mesenchymal stem cells in each group were measured.

Statistical analysis

The measurement data were presented as mean±SD, and statistical analyses were undertaken using SPSS 12.0 software. The intergroup comparison of the mean value of multiple samples was done using analysis of variance and *q* test.

RESULTS

Culture of mesenchymal stem cells

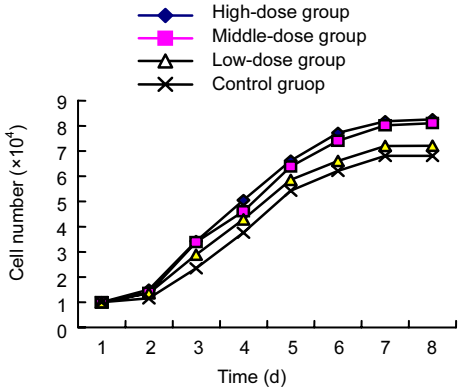
After primary cultured for 12 hours, most of the cells were adherent; after 2−3 days, the morphology of the cells was in polygons and fusiform; after 3−5 days, distinct colony formation was observed with rapid proliferation, and the clonies were close to each other; after 10−12 days, cell integration for about 90%, at this time, the cells were arranged in relatively uniform helical or spiral shape. The passaged mesenchymal stem cells adhered rapidly, and after 3 hours, most of the cells were adherent and evenly distributed in fusiform; that of the morphology of the passaged cells was more even than that of the primary cultured cells, the passaged cell growth well with rapid proliferation, obvious nucleus, clear nucleolus and a large proportion of nucleoplasm. Generally, after cultured for 7 days, the cells could reach 90% integration with even morphology and arranged in parallel or swirling spiral. There was no significant difference after continuously passaged to the 5th generation. However, with the increased frequency of passage, the cells appeared diversity; after passaged to the 10th generation, the cells were gradually aging, presented as increased cell debris, flattened cell morphology and decreased proliferation rate till stop.

Expressions of surface antigens of mesenchymal stem cells

Positive immunocytochemical staining of CD44 and CD90 of passage 3 cells was observed, the cytoplasm was brown or chocolate brown, and the CD34 staining was negative.

Growth curve of the cells in each group

The number of the incubated cells in each group was counted (mean *n*=6), then the data were recorded in the EXCEL table to generate the curve (growth curve) automatically. The growth curves of the cells in each group were in "S" shape (Figure 1).



The growth curves of the cells in each group were in "S" shape, the rapid growth period appeared at 3 d after intervention, and reached peak at 6 d and formed plateau phase. The cell level in the platelet-rich plasma groups was maintained in a high level after reach to plateau phase, and the number of the cells in the platelet-rich plasma groups was larger than that in the control group.

Figure 1 Growth curve of rabbit bone marrow mesenchymal stem cells in different groups at different time points after platelet-rich plasma intervention

At day 1 after incubation, the cells growth in each group was relatively flat, and presented as the latent growth curve. At day 2 after incubation, the cells in the platelet-rich plasma groups had high proliferative activity and grew rapidly, and the growth curve was

significantly increased, especially in the high-dose platelet-rich plasma group; then the rapid growth period of the growth curve was constructed. The proliferation of the cells was most significant in the high-dose platelet-rich plasma group with the shortest doubling time about 30 hours, and then reached to peak at 6 days, and formed plateau phase. The cells in the control group were grew rapidly at day 3, and showed the longest doubling time about 46 hours, and after 3 days, the rapid growth period of growth curve was constructed. The cell level in the platelet-rich plasma groups was maintained in a high level after reach to plateau phase, and the number of the cells in the platelet-rich plasma groups was larger than that in the control group.

Survival and proliferation ability of mesenchymal stem cells in each group

The survival and proliferation ability of mesenchymal stem cells in each group were showed in Tables 1 and 2. Methylthiazolyldiphenyl-tetrazolium bromide assay showed the absorbance values in the high-dose, middle-dose and low-dose platelet-rich plasma groups and the control group at 2 days after intervention were (0.171±0.026), (0.169±0.030), (0.165±0.034) and (0.161±0.029) respectively, and there was no significant difference ($P > 0.05$), that may be because of the cells growth in the stationary state, there was no significant difference in proliferation of cells. At day 3, there was significant difference in the absorbance value between platelet-rich plasma groups and the control group ($P < 0.01$), while there was no significant difference between platelet-rich plasma groups ($P > 0.05$), indicating that the platelet-rich plasma has certain effect in the proliferation of cells at 3 days after intervention without dose-dependent manner. There was significant difference in the absorbance value between high-dose platelet-rich plasma group and middle- and low-dose platelet-rich plasma groups at day 4 ($P < 0.05$ or $P < 0.01$), but there was no significant difference between middle-dose platelet-rich plasma group and the low-dose platelet-rich plasma group ($P > 0.05$), the results indicated that high-dose platelet-rich plasma had significant effect on cell proliferation, the low-dose platelet-rich plasma could also promote cell proliferation but not significant. At day 6, the platelet-rich plasma had significant promotion effect on cell proliferation and showed dose-dependent manner. At day 8, the platelet-rich plasma had significant promotion effect on cell proliferation and showed dose-dependent manner, but also found that the promotion effect was decreased when compared with that in the previous few day, and that may be saturated cell density.

Table 1 Proliferation of mesenchymal stem cells in the high-dose, middle-dose and low-dose platelet-rich plasma groups and the control group measured with methylthiazolyldiphenyl-tetrazolium bromide assay ($\bar{x}\pm s$, absorbance value)

Time	High-dose group	Middle-dose group	Low-dose group	Control gruop
The 2nd d	0.171±0.026	0.169±0.030	0.165±0.034	0.161±0.029
The 3rd d	0.591±0.031	0.584±0.017	0.566±0.021	0.451±0.023
The 4th d	0.681±0.038	0.634±0.021	0.606±0.031	0.481±0.027
The 6th d	0.751±0.025	0.684±0.031	0.646±0.027	0.511±0.034
The 8th d	0.681±0.026	0.597±0.032	0.485±0.031	0.353±0.029

The high-dose platelet-rich plasma had significant promotion effect on cell prolifetration, and low-dose platelet-rich plasma could also promote cell proliferation, but not significant.

Statistical results showed that platelet-rich plasma had significant promotion effect on the proliferation of mesenchymal stem cells (since day 3), and the effect was in time-dependent manner. After cultured for several days, the effect had dose-dependent manner (since day 6), and the high-dose platelet-rich plasma had the most significant effect on cell proliferation.

Effect of platelet-rich plasma on collagen production of mesenchymal stem cells

Immunochemical staining results and enzyme-linked immunosorbent assay for quantitative determination of collagen showed that mesenchymal stem cells could produce the collagen type Ⅰ and Ⅲ platelet-rich plasma which could significantly promote the collagen production of mesenchymal stem cells, and there was significant difference when compared with the control group ($P < 0.05$). The effect of platelet-rich plasma on the collagen production showed a dose-dependent manner (Table 2).

Table 2 Effects of platelet-rich plasma on collagen production of mesenchymal stem cells ($\bar{x}\pm s$, absorbance value)

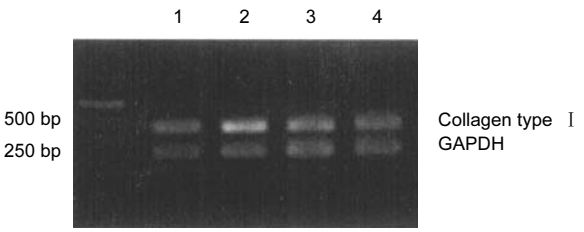
Group	Collagen	
	Ⅰ	Ⅲ
Control	0.54±0.10	0.35±0.02
High-dose	0.82±0.04	0.67±0.03
Middle-dose	0.98±0.02	0.81±0.05
Low-dose	1.32±0.02	0.96±0.02

The effect of platelet-rich plasma on the collagen production showed a dose-dependent manner.

Gene expression of collagen type Ⅰ

The relative ratio of collagen type Ⅰ density is

showed in Table 3. The Tband location of collagen type Ⅰ on the electropherogram was corresponded to the designed amplification length (Figure 2). The expression of collagen type Ⅰ of mesenchymal stem cells in each platelet-rich plasma group was increased when compared with that in the control group, and there was no significant difference between groups ($P < 0.01$).



1: Control group; 2: High-dose platelet-rich plasma group; 3: Middle-dose platelet-rich plasma group; 4: Low-dose platelet-rich plasma group; the expression of collagen type Ⅰ of mesenchymal stem cells in each platelet-rich plasma group was higher than that in the control group

Figure 2 Gene expression electrophoresis of collagen type Ⅰ of mesenchymal stem cells in the high-dose, middle-dose and low-dose platelet-rich plasma groups and the control group

Table 3 Relative ratio of electrophoretic bands density of collagen type Ⅰ gene in the mesenchymal stem cells of the control group and platelet-rich plasma groups ($\bar{x}\pm s$, n=8)

Group	Collagen type Ⅰ	P
Control	2.2±1.1	
High-dose	5.1±1.4	< 0.01
Middle-dose	3.9±1.0	< 0.01
Low-dose	2.8±1.3	< 0.01

The expression of collagen type Ⅰ of mesenchymal stem cells in each platelet-rich plasma group was higher than that in the control group.

DISCUSSION

The premise of cell replacement therapy is to obtain a large amount of seed cells, and how to amplify the cells effectively is the key of the therapy. Growth factors are large class of biological media, which have important role in the proliferation and differentiation of cells, as well as the repair of various traumas (fracture)[13-14]. The international research of autologous platelet-rich plasma is just beginning, and it is the research hot spot. The autologous platelet-rich plasma is isolated from the plasma containing platelet on the upper layer of leukocyte after autologous blood centrifugation stratification. After platelet activation, α particle will release a variety of growth factors. The enzyme-linked immunosorbent assay identified that platelet-rich plasma contains a large amount of platelet-derived growth factor, transforming growth factor, vascular endothelial growth factor, epidermal growth factor and insulin-like growth factor 1. These growth factors have a higher concentration which is about 17 times to the whole blood, and the activity can sustain for 5–8 days[15-16], which plays an important role in regulating cell proliferation. Most of the scholars believe that the cell biological effect of platelet-rich plasma is derived from the high-concentration growth factors, such as platelet-derived growth factor, transforming growth factor, insulin-like growth factor and fibroblast growth factor. These factors can affect cell chemotaxis, proliferation and differentiation through the combined effect and the synergistic effect[17]. The experimental results indicated that platelet-rich plasma can significantly promote the proliferation of the *in vitro* cultured mesenchymal stem cells, and showed a dose-dependent manner, the higher that concentration of platelet-rich plasma, the more significant the effect is.

Collagen type Ⅰ and Ⅲ are the most abundant collagens in the tendon tissues. Collagen type Ⅰ is mainly isolated from the normal tendon, and collagen type Ⅲ often appears after tendon injury, and finally form scar and replaced with collagen type Ⅰ[18]. The cell culture results showed that the mesenchymal stem cells can produce collagen type Ⅰ and Ⅲ, but the volume was low. Platelet-rich plasma can significantly promote the collagen production, indicating that mesenchymal stem cells have the basic characteristics of tendon tissue engineering seed cells, and platelet-rich plasma can promote the mesenchymal stem cells to transform into tendon cells.

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富血小板血浆与间充质干细胞增殖和胶原的产生

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文章亮点：

课题设计的创新之处在于使用富血小板血浆诱导间充质干细胞向腱样细胞分化，并取得初步效果。实验发现富血小板血浆对体外培养的间充质干细胞具有明显的促增殖作用。其作用具有剂量依赖性，较高剂量的富血小板血浆对细胞的增殖作用更为明显。

关键词：
干细胞；干细胞培养与分化；富血小板血浆；间充质干细胞；增殖；Ⅰ型胶原；Ⅲ型胶原；细胞培养；细胞分化；肌腱细胞
主题词：
血小板；血浆；间质干细胞；胶原Ⅰ型；胶原Ⅱ型

摘要

背景：间充质干细胞是可大量获取的肌腱组织工程种子细胞，但如何体外诱导成为此项技术的关键。

目的：观察富血小板血浆对体外培养间充质干细胞分裂增殖和胶原产生的影响。

方法：分离培养兔间充质干细胞，设立富血小板血浆高、中、低剂量组干预间充质干细胞，并设空白组做对照。

结果与结论：富血小板血浆高、中、低组间充质干细胞均保持较高的增殖活性，呈快速生长，曲线上升幅度大，与空白对照组比较差异有显著性意义($P < 0.05$)。培养时间越长作用越明显，培养一定时间后其作用具有剂量依赖性，较高剂量的富血小板血浆对细胞的增殖作用较为明显。提示富血小板血浆能明显促进间充质干细胞的Ⅰ和Ⅲ型胶原合成，剂量越大，刺激胶原产生的作用越明显。

作者贡献：设计、实施、评估均为本文作者，均经过正规培训，未采用盲法评估。

利益冲突：课题未涉及任何厂家及相关雇主或其他经济组织直接或间接的经济或利益的赞助。

伦理要求：实验过程中对动物的处置符合 2009 年《Ethical issues in animal experimentation》相关动物伦理学标准的条例。

作者声明：文章为原创作品，数据准确，内容不涉及泄密，无一稿两投，无抄袭，无内容剽窃，无作者署名争议，无与他人课题以及专利技术的争执，内容真实，文责自负。

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