Effect of Astragaloside IV On Precartilaginous Stem Cells to Promote Bone Development in Rats

Zhizhou Jiang
the second Clinical Medical College of Zhejiang Chinese Medical University

Hang Yin (feidao95@126.com)
Hangzhou Xiaoshan No 1 People's Hospital  https://orcid.org/0000-0002-9523-8913

Lei Zhao
the second Clinical Medical College of Zhejiang Chinese Medical University

Jianyong Jiang
the second Clinical Medical College of Zhejiang Chinese Medical University

Jinbo Ni
the second Clinical Medical College of Zhejiang Chinese Medical University

Jiewei Sun
the second Clinical Medical College of Zhejiang Chinese Medical University

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Abstract

Objective To explore the effect of astragaloside IV in promoting bone development by promoting the proliferation of precartilaginous stem cells.

Methods To co-cultured the cells from the resting chondrocyte of growth plate and LaCroix of 24-hours old rats, and identified by FGFR-3 staining. Choosing astragaloside IV induce precartilaginous stem cells cultured in vitro, using Collagen typeⅡ monoclonal antibody staining and MTT to test cell biological characteristics. Four 4 weeks old SD rats were selected and divided into an experimental group and control group, 24 rats in each group. The rats in the experimental group were injected with astragalus injection in a dose of 8.0g / kg once a day. The rats in the other group were injected with the same amount of normal saline. The 3rd and 5th week after feeding, 12 rats were killed, and the tibial length was measured by vernier caliper.

Results The FGFR-3 staining was positive, which proved that the cultured cells were precartilaginous stem cells. Collagen typeⅡ monoclonal antibody staining is positive and the OD value detected by MTT test was higher, after astragaloside IV induced the precartilaginous stem cells. After astragaloside IV injection, the tibial length of experimental group measured by vernier caliper was significantly higher than that of the control group.

Conclusion astragaloside IV can promote the proliferation and biological characteristics of precartilaginous stem cells, and then promote bone development.

Introduction

With the advancement of society and the improvement of people's living standards, people's attention to height is increasing, and height may even affect mental health. Under the current strategy of building a healthy China, and under the guidance of the policy of equal development of Chinese and Western medicine, explore the promotion of Chinese medicine The method of bone development is of great significance. In the developmental stage of bones, there are epiphyseal growth plates in bone tissue. When the epiphyseal growth plates are closed, the bones stop developing. Recent studies have found that there are Precartilaginous stem cells in the LaCroix ring and quiescent zone of the epiphyseal growth plates. Precartilaginous stem cells (PSCs) are the driving force of epiphyseal growth plate and bone development. This experiment found that astragaloside IV can promote the proliferation of epiphyseal stem cells through in vitro experiments. In vivo experiments have further confirmed that astragaloside IV has the effect of promoting bone development. The report is as follows.

Materials And Methods

1.1 The main reagent
The main reagent included 0.25% trypsin-0.53mmol EDTA (Invitrogen Company, USA), DMEM/F-12 (Invitrogen Company, USA), Rabbit Anti-FGFR-3 (Santa-cruz Company, USA), Fetal Bovine Serum (Gibco Company, USA), DAB chromogenic kit (Fuzhou Maixin Biotechnology Co., Ltd.), rabbit anti-mouse collagen type II monoclonal antibody (Fuzhou Maixin Biotechnology Co., Ltd.), endogenous peroxidase blocker (Fuzhou Maixin Biotechnology Co., Ltd.), animal non-immune serum (Fuzhou Maixin Biotechnology Co., Ltd.), Astragalus injection (10ml/bottle, each equivalent to 20g of original medicinal material, Zhengda Qingchunbao Pharmaceutical Co., Ltd.).

1.2 Experimental animals

Healthy newborn 24h SD rats, clean grade, not limited to males and females. Healthy 4-week-old SD rats, clean grade, not limited to males and females, come from the same closed group (provided by the Animal Center of Zhejiang University of Traditional Chinese Medicine).

2.1 Cultivation of PSCs

The method of co-cultivation of epiphyseal growth plate LaCroix ring and quiescent zone Precartilaginous stem cells[1]: Newborn 24h SD rats were selected and sacrificed by cervical dislocation. After disinfection, the knee joints were separated under a microscope (×10 times) under sterile conditions. Surrounding muscle tissue, exposing the distal femur and proximal tibia, cut out the epiphyseal growth plate LaCroix ring and resting area (Figure 1), cut to about 1mm cube, digested with 0.25% trypsin-0.53mmol EDTA for 20 minutes, 10% fetal bovine serum Digestion of the DMEM/F-12 medium was terminated for 2 minutes, filtered by a 40-mesh sieve, centrifuged for 5 minutes (1500 r/min), and then resuspended in DMEM/F-12 medium containing 10% fetal calf serum to culture in saturated humidity. Medium was changed every other day.

2.2 Identification of PSCs

Select the third-generation cells to be placed in DMEM/F-12 culture medium containing 10% fetal bovine serum to make a cell suspension, and inoculate them in the culture wells of a 6-well plate pre-installed with a cover glass and culture in saturated humidity. The cells covered approximately 60%, the culture medium was aspirated from the 6-well culture plates, were washed twice with PBS, followed by FGFR-3 immunohistochemistry, the specific operational steps performed according to the kit.

2.3 Astragaloside IV Intervenes PSCs

Set up the experimental group and the control group, select the 3rd generation cell to make a cell suspension and inoculate it on a 24-well culture plate, 1*10^4 cells per well, add 100ug/ml astragaloside IV to the experimental group, 3ml/d, Add culture medium to the control group, 3ml/d. After every 24 hours, 3 wells were taken from the control group and the experimental group, and the cells were digested with 0.25% trypsin for relevant tests.

2.4 MTT cell viability detection
Control group and experimental group as described above were prepared from 2d cells, 4d cells and 6d cells. Use ELX-800UV enzyme label detector to adjust the zero hole to zero, and Select light wave of 490nm to measure the absorbance of each hole (OD).

### 2.5 Monoclonal antibody staining of collagen type

Selects the third-generation cells into cell suspension, culture wells were seeded in 6 well plates previously cover glass placed in a humidity saturated culture, cells were grown until about 80%, 4% paraformaldehyde 4°C under overnight. At room temperature Triton-100 + 0.5% BSA (bovine fetal protein) for 30min. The rabbit anti-mouse type II collagen monoclonal antibody was cultured overnight at 4°C. FITC-labeled secondary antibody was incubated for 30 min at room temperature. Observe under a laser confocal microscope.

### 2.6 Astragaloside IV Intervention in SD Rats

A total of 48 4-week-old clean SD rats with similar body weight were randomly divided into 2 groups with 24 rats in each group. Experimental group: intraperitoneal injection of Astragalus injection, the amount of which was calculated at 8.0 g per kilogram of the rat, 10ml per kilogram, once a day. Control group: Inject the same amount of normal saline, and do related inspections with the materials taken in the 3rd and 5th weeks after feeding.

### 2.7 Measurement of tibia length

The animals were killed by cervical dislocation. The muscle tissues around the tibia and the upper and lower articular surfaces were cleaned, and the tibia was exposed and placed flat. Choose a vernier caliper (with an accuracy of 0.02mm) to measure the length of the tibia, so that the vernier caliper clamps the two ends of the tibia. Do not press too tightly to avoid bending the tibia and thus affecting the experimental data.

### Results

#### 3.1 FGFR-3 immunohistochemical staining

The detected cells were positive for FGFR-3 staining: the cell membrane and cell cytoplasm were stained brown after staining, indicating good cell viability and stable expression of FGFR-3 (Figure 2).

#### 3.2 MTT cell viability detection

On the second day after culture, the OD value of cell activity was measured. There was no significant increase between the control group and the experimental group, and there was no statistical significance (P>0.05). On the 4th and 6th day, the OD value of cell activity was measured. Compared with the control group, the experimental group had a significant increase, and the difference was statistically significant (P<0.05) (Table 1).
3.3 Monoclonal antibody staining of collagen type

The PSCs intervened by astragaloside IV showed green fluorescence under a laser confocal microscope stained with type II collagen monoclonal antibody, and the test result was positive (Figure 3).

3.4 Measurement of tibia length

After the intervention of astragaloside IV, the tibia was taken in the 3rd and 5th weeks after feeding and the length was measured with a vernier caliper (Figure 4, 5). The tibia length of the experimental group was significantly greater than that of the control group, and the difference between the two groups was significant and statistically significant (P<0.05)(Table 2).

Discussion

"Status Report China National Nutrition and Chronic Diseases (2020)," pointed out that currently China 18-44 year old men and women were average height 169.7 cm and 158 cm. The report reminds us that the problem of height has been paid more attention, and that height is related to people's physical and mental health. The height depends on the development of the bones, the development of the bones depends on the epiphysis, and the development of the epiphysis depends on the Precartilaginous stem cells of the epiphyseal growth plate.

The LaCroix ring is composed of Precartilaginous stem cells, covering the membranous soft tissue surrounding the epiphyseal growth plate. If it is removed, the epiphyseal growth plate will calcify and close the bone and stop developing. The quiescent zone of the epiphyseal growth plate is composed of Precartilaginous stem cells, and its destruction will also cause the calcification of the epiphyseal growth plate to close and stop the development of bones [2-4]. It can be seen that when the Precartilaginous stem cells no longer differentiate into chondrocytes, the calcification of the epiphyseal growth plate will stop the development of bone. On the contrary, when the Precartilaginous stem cells differentiate into chondrocytes, the calcification closure of the epiphyseal growth plate can be delayed, thereby promoting bone development and increasing the final height.

According to the above ideas, we first cultured Precartilaginous stem cells in vitro, and adopted the method of co-cultivation of Precartilaginous stem cells in the quiescent zone of the LaCroix ring and the epiphyseal growth plate in the previous experimental study. Fibroblast growth factor receptor-3 is a specific marker of Precartilaginous stem cells. The cultured cells in this experiment showed positive results by immunohistochemical FGFR-3 staining method, which confirmed that the cultured cells were Precartilaginous stem cells with the landmark antigen FGFR-3.

The second step is to promote the proliferation of Precartilaginous stem cells cultured in vitro. The differentiation and expansion of cells is an important indicator that reflects the number of cells, and the extracellular matrix is closely related to the functional state of cells, and is an important indicator of cell quality [5, 6]. It is significant that proliferation under the premise of maintaining good cell biological
In terms of promoting the proliferation of stem cells, research on cell co-culture [9], cytokines [10], cellular pathways [11], gene modification [12, 13] and other aspects have achieved relatively rich research results. Traditional Chinese medicine has unique advantages in disease prevention, treatment and rehabilitation. A large number of studies have found that Chinese medicine has the function of promoting neural stem cells, adipose stem cells, bone marrow mesenchymal stem cells and other stem cell functions [14, 15].

Astragalus is the sacred medicine for spleen and kidney meridian, the sacred medicine of nourishing Qi, the kidney governs the bones, which is the foundation of the innate, and the spleen governs the movement and transformation, and is the foundation of the acquired. Therefore, Astragalus can stimulate the spleen and kidney to strengthen the muscles and bones to promote bone development. Astragaloside IV is the main active component of Astragalus, and its role in inducing and promoting stem cell proliferation has been confirmed.

Some scholars [16-17] found that astragaloside IV can induce bone marrow mesenchymal stem cells to differentiate into nerve cells, and a middle-dose astragaloside IV can promote the expression of Notch to promote the proliferation of neural stem cells. In this experiment, astragaloside IV was used to intervene externally cultured Precartilaginous stem cells and reached similar conclusions.

The experiment found that the OD value of the astragaloside IV intervention group in the MTT cell activity test was significantly higher than that of the control group, indicating that the cell activity of the Precartilaginous stem cells was significantly increased and proliferated by the astragaloside IV intervention. In addition, type I collagen is a characteristic secretion of Precartilaginous stem cells, which is a sign of their normal differentiation and proliferation. The staining result of monoclonal antibody to type I collagen was positive, indicating that the Precartilaginous stem cells can still maintain stable biological characteristics through the intervention of astragaloside IV. Combining the above two points shows that astragaloside IV can promote the proliferation of Precartilaginous stem cells and maintain good biological performance.

In view of the fact that the role of stem cells in the field of disease prevention and treatment has been verified from many aspects such as severe lower limb ischemia [18], pancreatitis [19], cell targeting [20], and promotion of healing [21], we have further proved astragaloside IV to promote bone development through vivo experiments. The experiment found that the tibia length of adolescent rats injected with astragaloside IV was significantly higher than that of the control group, and the bone development was improved.

Through the above experiments, we preliminarily confirmed that astragaloside IV stimulates the differentiation and proliferation of Precartilaginous stem cells while maintaining stable biological properties, thereby promoting bone development. At present, we focus on gene research and cell conduction pathway research on the mechanism of promoting cell proliferation, and focus on target research on the mechanism of traditional Chinese medicine. In the next step, we plan to focus on the
above research hotspots and further explore the mechanism of astragaloside IV to promote the development of Precartilaginous stem cells and bones.

Declarations

Ethics approval and consent to participate

Studies involving animals must include a statement on ethics approval. (ZSLL-2011-25)

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

ZJ, JJ, and JS developed the idea of the study, participated in its design and coordination and helped to draft the manuscript. LZ and JN contributed to the acquisition and interpretation of data. HY provided critical review and substantially revised the manuscript. All authors read and approved the final manuscript.

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**Tables**

Table 1
Cell viability after intervention of astragaloside IV (OD, ±s)

| Group                | N  | 2d       | 4d       | 6d       |
|----------------------|----|----------|----------|----------|
| the control group    | 48 | 0.38 ± 0.10 | 0.42 ± 0.05 | 0.51 ± 0.10 |
| the experimental group | 48 | 0.39 ± 0.12# | 0.49 ± 0.09* | 0.61 ± 0.11* |
| T                    |    | -0.26    | -4.03    | -4.12    |
| P                    |    | 0.79     | 0.00     | 0.00     |

Note: Analysis and comparison between the experimental group and the control group on the 4th and 6th day, * P < 0.05; Analysis and comparison between the experimental group and the control group on the 2nd day, # P > 0.05
Table 2
Measured value of tibia length (mm, ±s)

| Group                | N  | 3rd week after feeding | 5th week after feeding |
|----------------------|----|------------------------|------------------------|
| the control group    | 12 | 34.4 ± 0.73            | 42.60 ± 1.18           |
| the experimental group| 12 | 37.4 ± 0.85*           | 47.9 ± 1.83*           |
| T                    |    | -9.30                  | -8.63                  |
| P                    |    | 0.00                   | 0.00                   |

Note: Analysis and comparison between the experimental group and the control group in the 3rd and 5th weeks, * P <0.05

Figures

Figure 1
The distal femur and proximal tibia, cut out the epiphyseal growth plate LaCroix ring and resting area.

**Figure 2**

FIG detection FGFR-3 cells were staining: After staining with both cell membrane and cytoplasmic brown, suggesting good cell viability stable expression of FGFR-3.
Figure 3

Type I collagen monoclonal antibody staining, green fluorescence under a laser confocal microscope
Figure 4

Tibia length of the control group at the 5th week after feeding
Figure 5

Tibia length of the experimental group in the 5th week after feeding