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Review Article

Study on the regulatory mechanism of ICA on osteogenic differentiation of rabbit bone marrow mesenchymal stem cells

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Abstract: To explore the regulatory mechanism of icariin (ICA) on osteogenic differentiation of rabbit bone marrow mesenchymal stem cells (BMSCs). A total of 20 healthy adult New Zealand white rabbits were randomly divided into an ICA group and a control group (CG). In the ICA group, rabbits were given icariin at 0.25 g/kg by oral gavage. After the first intragastric gavage, the drug was administered a second time at an interval of 20 hours and the third time after 4 hours. After the third administration in the first hour, blood was taken by cardiac puncture. In the CG, rabbits were given with equal volume of 0.9% sodium chloride solution by oral gavage. The rabbits' serum in the two groups was obtained, and the effects of the different concentrations of drug-containing serum on BMSC proliferation and ALP activity were compared. After ICA induction, the secretion of osteocalcin at different time points, calcium deposition level, and mRNA expression of PI3K and mTOR were determined. The A570 value of the ICA group was higher than that of the CG ($P<0.01$), and the promotion effect of the 10% ICA group was the most robust. Except for the 10% ICA group, the ALP activity of the other two groups was significantly higher than that of the corresponding control group, and the 5% ICA group showed the highest ALP activity. In the two groups, the osteocalcin secretion from cells reached the highest value at 9 days. The osteocalcin secretion in the ICA group was significantly higher than that in the CG at different time points. In the two groups, the calcium deposition level increased with the prolonged induction of culture time. ICA may promote osteogenic differentiation of rabbit BMSCs through the mTOR pathway.

Keywords: Icariin, rabbit bone marrow mesenchymal stem cells, osteogenic differentiation

Introduction

Repair of bone is an intractable problem in the clinical treatment of bone defects, it often has a high disability rate, which results in many social problems. At present, autologous and allogeneic bone transplantation is the common treatment. Because bone transplantation is limited with many complications, a growing number of scholars have focused their research on bone repair in recent years [1]. Osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) after induction for the treatment of bone defects is currently a hot topic in bone repair research [2, 3]. Icariin (ICA) is the most abundant flavonoid glycoside compound in the epimedium, which can promote the proliferation and osteogenic differentiation of BMSCs [4]. Having the advantages of a simple extraction process, many sources and low cost, icariin is a traditional Chinese medicine monomer with great application prospects for promoting bone defect repairs [5]. Recent research results have confirmed that ICA can induce the osteogenic differentiation of mesenchymal stem cells and can promote the repair of bone defects in bone tissue engineering [6]. Existing studies have shown that BMSCs can differentiate into chondrocytes, adipocytes, nerve cells, and other cells, which have multidirectional differentiation and strong proliferation potential. BMSCs can differentiate into osteoblasts under

specific induction conditions [7-9]. Therefore, BMSCs are very suitable as seed cells for bone tissue engineering, and have a good application prospect in bone tissue engineering [10].

However, the mechanism of ICA promoting osteogenic differentiation of BMSCs has been rarely reported in the literature, which limits further research. The study of its mechanism of action is conducive to the development of more accurate bone repair materials and possible solutions to the problems of clinical bone repair. In this experiment, Real-timePCR was used to detect the mTOR mRNA from bone cells of the experimental group and CG. ICA was explored to regulate the osteogenic differentiation of BMSCs, with the goal of providing some reference for the basic research of bone repair.

Methods

Materials and methods

A total of 20 healthy adult New Zealand white rabbits, half male and half female, with body mass of (2.5 ± 0.2) kg, were raised in a SPF grade laboratory. All rabbits were allowed to eat and drink water freely, and the light period was 12 h. The animal model was prepared according to the principle of similar body weight and the rabbits were randomly divided into an ICA group and a CG. In the ICA group, rabbits were given icariin at a dose of 0.25 g/kg by oral gavage. After the first intragastric gavage, the drug was administered a second time at an interval of 20 hours and a third time after 4 hours. After the third administration in the first hour, blood was extracted by cardiac puncture. In the CG, rabbits were given an equal volume of 0.9% sodium chloride solution by oral gavage. The blood was centrifuged at 3000 r/min for 10 min after standing and coagulation to obtain ICA serum and control serum, it was then filtered, sterilized, subpackaged and stored at -20°C for later use. This experiment was approved by the Ethics Committee of Guangzhou Red Cross Hospital, and the experimental process followed the guiding principles for the protection and use of experimental animals {Poirier, 2015 #3165}.

BMSCs extraction

The BMSCs were separated by density gradient centrifugation combined with an adherence screening method. The operation steps were as follows: (1) Scissors, tweezers, EP tubes, gauze and other test instruments were sterilized by high pressure steam and dried for later use. A super clean bench was irradiated with ultraviolet for 30 min before being used. (2) One rabbit at the age of one week was taken, and put into the benzalkonium bromide for sterilization for 0.5 h. Afterwards, the rabbits were killed by air embolization of ear vein. The femur and tibia with muscle were removed aseptically and put into the 75% ethanol solution on the super clean bench. (3) After the muscle was removed from the bone surface, a 5 ml sterile syringe needle was used to drill holes from the distal and proximal articular surfaces of the bone to the marrow cavity, and then the culture medium was removed and inserted into the hole and washed repeatedly until the femoral shaft was clear and transparent. The cells in the petri dish were aspirated into a 15 mL BD tube. (4) The collected cells were diluted twice with PBS and mixed evenly with lymphocyte separation liquid at a ratio of 1:1, and then centrifuged at 450 g for 20 min at normal temperature. (5) A pipette was used to carefully suck up the separating fluid layer and lymphocyte layer into another new tube. Then, it was centrifuged at 250 g for 20 min and the supernatant was discarded. Culture medium (5 mL) without serum was aspirated to resuspend cells. Subsequently, it was centrifuged at 250 g for 10 min and the supernatant was discarded. (6) After being washed with L-DMEM culture solution twice, the cells were suspended in complete culture medium. After the cells were counted, 5×10^6 cells were put into a 25 cm^2 culture flask for culture.

Detection Indexes

(1) Determination of calcium deposition level: On the 3rd, 6th, 9th, 12th and 15th day of osteogenic induction culture, the medium was discarded, and then washed twice with PBS. Then, 1 mol/LHCl 1 mL was added, oscillated overnight, and centrifuged the next day at 1000 r/m for 10 min. The Ca^{2+} content in the supernatant was quantified by a calcium colorimetric asssay kit (BioVision, K380-250), and the result was expressed in μg/well. (2) Determination of osteocalcin secretion: Specimens were sectioned and stained with methylene blue/fuchsin. New bone formation was evaluated under a

Table 1. mTOR and U6 primer sequences

Genes	Forward primer	Reverse primer
PI3K	5'-GATGGCATGGCTTTAGATTG-3'	5'-TCCCTGTTCTAGGTCCCTGG-3'
mTOR	5'-ATTCAGATCGCTGGCAGCCT-3'	5'-CCCTGTGTTCAGCACCTCCA-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'

Table 2. Effect of different concentrations of drug-containing serum on BMSCs proliferation

Category	2.50%	5.00%	10.00%
ICA group	0.489±0.025	0.471±0.023	0.517±0.016
CG	0.373±0.018	0.354±0.026	0.369±0.014
T	6.522	5.835	12.060
P	0.003	0.004	0.000

microscope. Five histological sections with complete central sections of specimens were randomly taken from each group. After osteogenesis induction culture, the fluid was changed once every 3 days, and 1 mL original culture solution was reserved during each change and stored at -20°C. Since the samples at each time point were collected completely, the secretion of osteocalcin was detected by ELISA and expressed as n g/mL culture solution. (3) Detection of ALP activity: Extraction and detection of ALP activity samples in cells: When the fusion rate of cell adherent growth reached more than 90%, the number of digestive cells was counted at a concentration of 5×10^4 cells/mL, and 3 multiple wells were used in each group of 96-well plates. Since the cells adhered to the wall on the second day, ICA drugs with concentrations of 2.5%, 5% and 10% were added, and cultured continuously for 48 h, and the culture solution was discarded, and then washed twice with PBS. The serum-free DMEM/F12 medium containing 0.5% MTT was changed and cultured for 4 hours. The culture solution was discarded. DMSO (100 μL) was added to each well, shaken and dissolved for 10 min. Next, the absorbance value (A570) was determined at 570 nm on an enzyme reader. Each group had 6 samples in parallel.

Detection of qRT-PCR

Total RNA was extracted by TRIzol one-step method at 12, 24, 48 and 96 hours after osteogenesis induction, respectively. Cell RNA and reverse transcription were carried out on the collected cells with reference to the RNA extraction and reverse transcription kit, and the obtained cDNA was amplified with the qRT-PCR kit. The reaction conditions were 95°C for 15 s, 60°C for 1 min, 72°C for 30 s, with a total of 40 cycles. The internal reference was set to U6. mTOR expression level was calculated according to Ct value of each reaction, and gene relative quantitative expression analysis was performed according to 2-Δ Δ Δ CQ algorithm. The primer sequence was synthesized by Shanghai Shenggong Bioengineering Co., Ltd (**Table 1**).

Statistical methods

SPSS 22.0 (IBM, SPSS, Chicago, IL, USA) was used for data analysis of measurement results. The measurement data were expressed as the mean number ± standard deviation ($\bar{x}$ ± s) and analyzed by one-way ANOVA. The least significant difference test (LSD) was used for the comparison between groups. $P<0.05$ indicates a significant difference.

Results

Effect of different concentrations of drug-containing serum on BMSCs proliferation

Cell proliferation was detected by MTT assay. The A570 value of the ICA group was higher than that of CG ($P<0.01$), and the promotion effect of the 10% ICA group was the most robust (**Table 2**).

Effect of different concentrations of drug-containing serum on ALP activity

Comparison of the effects of different concentrations of drug-containing serum on ALP activity showed that the ALP activity of the other two groups was significantly higher than that of the corresponding CG except for the 10% ICA group, of which the ALP activity was the highest in the 5% ICA group. The ALP activity of the 10% ICA group was significantly lower than that of other groups due to cell floating death. Therefore, 5% ICA was used for intervention in the follow-up tests (**Table 3**).

Table 3. Effect of different concentrations of drug-containing serum on ALP activity

Category	2.50%	5.00%	10.00%
CG	1.54±0.085	1.673±0.057	1.534±0.112
ICA group	2.209±0.117	2.684±0.113	0.081±0.045
T	8.013	13.840	20.850
P	0.001	0.000	<0.0001

Table 4. Osteocalcin secretion at different time points after induction

Category	3 d	6 d	9 d	12 d	15 d
CG	135.14±11.564	215.36±28.71	328.45±37.92	303.43±34.17	287.675±17.746
ICA group	179.41±16.77	318.54±32.26	471.27±16.53	454.18±30.23	407.51±24.41
T	3.764	1269.000	0.004	5.723	6.878
P	0.020	<0.0001	5.898	0.005	0.002

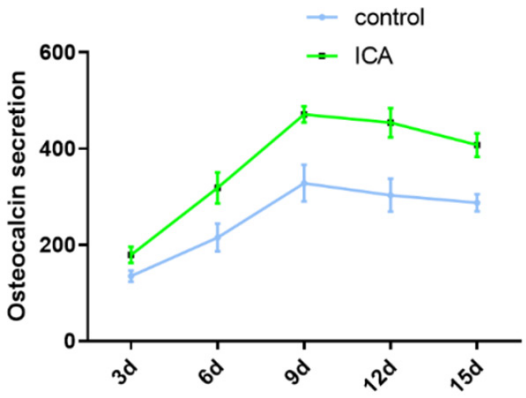

Figure 1. Detection of osteocalcin secretion at different time points after ICA induction. The secretion of osteocalcin increased with prolonged culture time, and reached the highest value at 9 days, and then decreased. The secretion of osteocalcin in the ICA group at different time points was significantly higher than that in CG (P<0.05).

Detection of osteocalcin secretion at different time points after ICA induction

The secretion of osteocalcin increased with the prolongation of culture time, and reached the highest value at 9 days, and then decreased. The secretion of osteocalcin in the ICA group at different time points was significantly higher than that in CG (P<0.05) (**Table 4**) (**Figure 1**).

Effect of ICA on calcium deposition level

Comparison of the calcium deposition level of cells in the two groups showed that the calcium deposition level increased with prolonged induction culture time in the two groups. Compared with the CG, calcium salt deposition was significantly promoted at all time points in ICA group (P<0.05) (**Table 5**) (**Figure 2**).

Analysis on the mRNA expression of PI3K

Comparison of the mRNA expression of PI3 in cells between the two groups showed that the mRNA expression of PI3 reached the highest level at 24 h after induced culture, and then decreased slowly. Compared with the control group, ICA in the ICA group significantly promoted the expression of the PI3K gene at different time points (P<0.05) **Table 6**.

Analysis of mTOR mRNA expression

Comparison of mTOR mRNA expression of cells between the two groups showed that mTOR mRNA increased with prolonged osteogenic induction culture time. Compared with the CG, the level of mTOR mRNA was significantly increased in the ICA group (**Table 7**) (**Figure 3**).

Discussion

ICA is an active component of the epimedium herb. Modern pharmacological studies have verified that ICA has the functions of immune regulation, anti-tumorigenic, osteogenesis, endocrine and cardiovascular system regulation, decompression and qi regulation, etc. It is a kind of traditional Chinese medicine monomer with medicinal value and relatively high health care function. However, the regulatory mechanism of epimedium control on BMSCs has been unknown [11-13].

Table 5. Determination of calcium deposition level at different time points after induction

Category	3 d	6 d	9 d	12 d	15 d
CG	27.75±1.135	33.59±1.951	337.51±32.86	48.41±2.17	58.73±3.169
ICA group	31.58±1.79	49.26±3.167	483.27±16.89	62.43±3.56	73.41±3.73
T	3.130	7.297	6.833	5.824	5.195
P	0.035	0.002	0.002	0.004	0.007

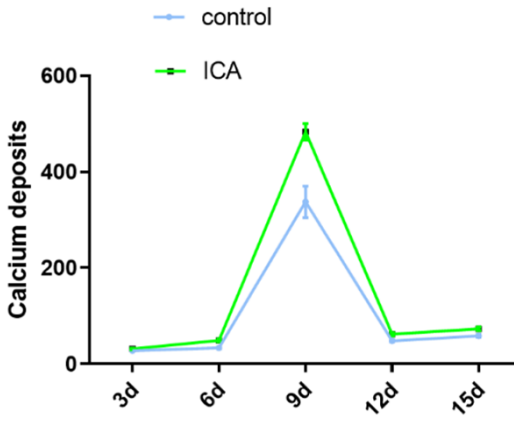

Figure 2. Effect of ICA on calcium deposition level. In the two groups, the calcium deposition level increased with prolonged induction culture time. Compared with the CG, calcium salt deposition was significantly promoted at all time points in the ICA group (P<0.05).

BMSCs are stem cells with multi-directional differentiation potential. It is a hot topic to study the mechanism of drug action from the perspective of BMSCs in recent years. The culture of BMSCs cells *in vitro* mainly undergoes a cell transformation phase, proliferation phase, aggregation phase and cell matrix calcification phase. In the initial stage of the transformation period, some cells will die during the adaptation process. With the extension of the culture time, the cells form a single fiber for transformation. When the cells enter the proliferation period, the number of cells increases rapidly, the boundaries between cells becomes blurred, and then enter the secretion period [8, 14]. One study by Chen et al. [15] has shown that the monomer icariin not only promotes BMSCs to differentiate into osteoblasts, but also inhibits BMSCs from differentiating into osteoclasts and inhibits its bone resorption activity. In this study, BMSCs cells were cultured *in vitro*, and the effects of different concentrations of drug-containing serum on BMSCs proliferation were compared. It was found that the A570 value of the ICA group was higher than that of the corresponding CG (P<0.01), and the promotional effect of 10% ICA group was the most robust, which indicated that ICA played a role in promoting the proliferation of BMSCs. However, detection of the secretion of osteocalcin at different time points after ICA induction showed that the secretion of osteocalcin increased with prolonged culture time, and reached the highest value at 9 days, and then decreased. The secretion of osteocalcin in the ICA group was significantly higher than that in CG at different time points. The calcium deposition level increased with prolonged induction culture time in the two groups. Compared with the CG, calcium salt deposition was significantly promoted at all time points in the ICA group (P<0.05), which indicated that ICA can promote the improvement of bone differentiation and osteocalcin expression. ALP is a representative enzyme for osteogenesis induction of BMSCs cells. It mainly distributes in cell membranes and can promote the proliferation and calcification of BMSCs cells. The enhanced activity of BMSCs shows that BMSCs cells differentiate significantly into bone [8, 16, 17]. In this study, the best dosage was determined by measuring the ALP activity of BMSCs cells treated with different concentrations of icariin. The ALP activity increased significantly after treatment with icariin, indicating that icariin could promote BMSCs cells to secrete and express ALP after treatment with the above concentration range, thus accelerating BMSCs cells to differentiate into osteoblasts. The ALP activity was the highest after treatment with 5.00% icariin, indicating that icariin had the most robust effect on BMSCs cells to differentiate into osteoblasts.

Mammalian target of rapamycin (mTOR) is an atypical serine/threonine protein kinase, which can regulate cell growth by converging and integrating stimulation signals from nutrition, growth factors, energy and environmental stress to the cells. It is the hub of many impor-

Table 6. Analysis on the mRNA expression of PI3K

Item	12 h	24 h	48 h	96 h
The control group	0.841±0.192	2.157±0.223	1.412±0.165	0.457±0.089
The ICA group	1.534±0.213	4.173±0.348	2.339±0.312	1.045±0.226
T	4.186	8.448	4.549	4.193
P-value	0.014	0.001	0.010	0.014

Table 7. Analysis of mTOR mRNA expression

Category	12 h	24 h	48 h	96 h
CG	0.543±0.065	0.934±0.048	1.421±0.165	2.772±0.165
ICA group	0.826±0.108	1.272±0.164	1.912±0.154	3.841±0.196
T	3.889	3.426	3.768	7.227
P	0.018	0.027	0.020	0.002

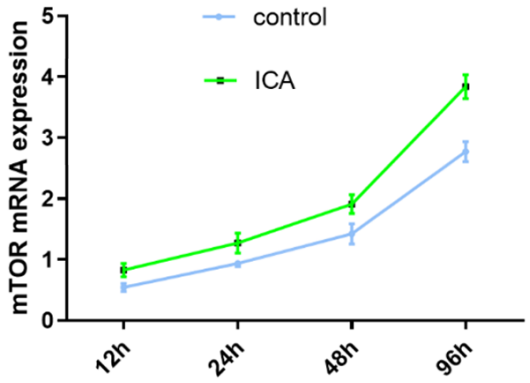

Figure 3. Analysis of mTOR mRNA expression. mTOR mRNA increased with prolonged osteogenic induction culture time. Compared with the CG, the level of mTOR mRNA was significantly increased in the ICA group.

tant signal transduction pathways in cells, participating in various biological behaviors such as gene transcription, protein translation, ribosome synthesis and cell apoptosis [18-20]. mTOR can regulate cell growth, cell cycle and other physiological functions by responding to various stimuli [21]. Studies have confirmed that the mTOR signaling pathway is involved in tumor cell proliferation and migration, as well as bone transformation after bone metastasis of tumor cells [25]. Further research has found that osteoclast differentiation-related factors promote the differentiation of osteoclast and inhibit the apoptosis of osteoclast through the mTOR RC1/S6K pathway [26]. This experiment showed that mTOR mRNA increased with the extension of osteogenic induction culture time. Compared with the CG, the level of PI3K and mTOR mRNA was significantly improved in the ICA group. Based on the above research results, we speculated that mTOR played a crucial role in bone repair. ICA may regulate the proliferation and differentiation of BMSCs by regulating mTOR, but the specific mechanism has not been studied.

Although this study has confirmed the effect of ICA on osteogenic differentiation of rabbit BMSCs, this experiment is only designed to use rabbits as the research subjects, and we did not conduct experiments on other animals, so there are deficiencies. In addition, although mTOR has been found to play an important role in osteogenic differentiation of rabbit BMSCs, its specific regulatory relationship needs to be further explored in future studies.

Conclusion

To sum up, ICA may promote osteogenic differentiation of rabbit BMSCs through the mTOR pathway.

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Disclosure of conflict of interest

None.

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