Original Article
Effects of amygdalin on alcohol-induced adipogenic differentiation of rabbit bone marrow mesenchymal stem cells

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Abstract: The present study aimed to investigate the effects of amygdalin on alcohol-induced adipogenic differentiation of rabbit bone marrow mesenchymal stem cells. Methods: New Zealand white rabbit adipocytes were extracted and cultured, and the rabbits were divided into five groups: the normal group, the model group, and the amygdalin low-middle-high concentration groups. Adipocytes were stained by oil-red O staining method. Triglycerides (TG) contents were measured. Osteocalcin (OCN) and Type I collagen were detected using Immunohistochemical staining method. Alkaline phosphatase cells were stained by improved Gomori chemical staining assay. PPARγ and cbfαl mRNA gene expressions were measured through PCR assay. Results: Amygdalin in different doses can reduce the occurrence and numbers of lipid droplets, in a positively proportional way. The model groups exhibited the most obvious increase of lipid droplets along with time. TG contents were reduced, proportionally to doses. Immunohistochemistry found that OCN expression increased and Type I collagen expression decreased. Alkaline phosphatase cells increased, PPARγ expression decreased, and cbfαl expression increased. Conclusions: Alcohol can facilitate the hypertrophy, proliferation and differentiation of marrow adipocytes, so that the bone marrow adipose tissue increased, internal bone pressure increased, blood flow reduced, leading to ischemia, which incurred femoral head necrosis. However, amygdalin is capable of inhibiting differentiation of adipocytes, which is of clinical application and innovation.

Keywords: Amygdalin, rabbit bone marrow mesenchymal stem cells, adipogenic differentiation, PPARγ, cbfαl mRNA

Introduction

Osteonecrosis of the femoral head (ONFH) is the death and subsequent repair of some energetic ingredients, including bone cells, adipocytes, and bone marrow cells, caused by femoral head blood supply interruption, which in turn leads to structural changes and collapse of femoral heads, plus joint dysfunction [1-3]. It is considered that ONFH is not a single process, but a combined result of some genic factors and one or more risk factors [4].

In recent years, scholars of the femoral head necrosis had proposed several in-depth theories of its pathogenesis, including dyslipidemia doctrine, the doctrine of mechanical damage to the blood circulation, intravascular coagulation theory, increased intraosseous pressure doctrine, cytotoxic and cell damage theory, mesen-chymal stem cell osteogenic and adipogenic differentiation theory and so on. Of all these theories, the lipid metabolism disorder is a relatively sophisticated theory [5-7].

Amygdalin is the main active ingredient of almonds, featuring anti-inflammatory and analgesic. In present study, rabbit femoral intramedullary adipocytes were cultured in vitro, and the effects of amygdalin on femoral head adipocytes were investigated, the increase of adipocytes and the pathogenesis of femoral head necrosis was also clarified.

Materials and methods

Cell culture

One New Zealand white rabbit (2.2 kg), under aseptic conditions, from which femoral head

marrow adipose tissues were obtained, and fibrous tissues and blood vessels were removed. Blood clots were washed away with PBS. The remained tissues were cut into pieces sizing 0.5 mm with ophthalmic scissors. Collagenase I (Ig/L) was added. Following this, the tissues were digested for 60-90 min in 37°C water bath oscillator (100 r/min), until the formation of chylous thick liquid. The digested liquid was added with 4 ml PBS, and agitated. Subsequently, the cells were filtered through a 250 μm diameter nylon mesh into a centrifuge tube, then washed with 10 ml PBS for 3 times. The tube was centrifuged at 250 g/min for 5 min, then the supernatant was decanted to another tube. The cells were washed with 10 ml PBS, and centrifuged again. The supernatant was discarded. The process was repeated for twice. The cells were washed with 10 ml DMEM/F12 culture containing 20% fetal bovine serum, and re-suspended. The cell density was adjusted to 2×10^5/ml. A total of 50 μl cells were obtained and added to a 6-well plate with 2 ml medium in each well. Subsequently, the plate was covered with 20 mm × 20 mm sterile coverslips treated by poly lysine. The plate was placed in a 37°C humidified incubator containing 5% CO_2. Cell morphology was observed daily under an inverted microscope.

Special staining of adipocytes

Intramedullary adipocytes stable passaged were obtained and planted into a 24-well culture plate, covered with 1 cm × 1 cm coverslips. The adipocytes were randomly divided into five groups. A: The Normal group, normal medium, which was given saline. B: The Model group, which was given primary medium, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 10 mg/L insulin, 100 mg/L indomethacin, 0.15 mol/L alcohol, and reacted for 4 d. C: Amygdalin Low dose group, which was given primary culture medium, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 10 mg/L insulin, 100 mg/L indomethacin, 0.15 mol/L alcohol, plus 50 μm amygdalin, and reacted for 4 d. D: Amygdalin Middle dose group, which was given primary culture medium, 0.5 mmol/L 3-isobutyl-1-methylxanthine, 10 mg/L insulin, 100 mg/L indomethacin, 0.15 mol/L alcohol, plus 100 μm amygdalin, and reacted for 4 d. E: Amygdalin High dose group, which was given primary culture medium, 0.5 mmol/L 3-isobutyl-1-methylxanthine,

10 mg/L insulin, 100 mg/L indomethacin, 0.15 mol/L alcohol, plus 200 μm amygdalin, and reacted for 4 d. The coverslips were removed after 4 days. Adipocyte morphology was observed under a microscopy after being oil-red O stained.

Measuring triglyceride (TG) contents

Cells were collected from all groups. Microtiter plates were coated with purified rabbit TG antibody, and made into solid-phase. The wells were added with TG, combined with HRP-labeled TG antibody. The antibody-antigen-enzyme labeled antibody complex was made, thoroughly washed, and colored with TMB substrate. As catalyzed by HRP enzyme, TM turned to blue, and then to final yellow due to acidification. The color depth is positively correlated with TG contents. Color absorbance was measured using a microplate reader at wavelength 450 nm, and then TG contents were calculated.

Detecting OCN and type I collagen using immunohistochemistry

Cells were collected from all groups, and dewaxed with xylene for 2 times, 10 min each time, then washed with gradient 100%, 95%, 85%, 75%, and 50% alcohol, following by pure water, 5 min each time. Subsequently, the cells were washed with PBS for 5 min, then incubated in a closed humid box with 3% H_2O_2 for 30 min under room temperature. Again, the cells were washed with PBS for 3 times, 5 min each time, then closed in a humid box with 10% normal goat serum at room temperature for 30 min, following by OCN monoclonal antibodies incubation overnight at 4°C. Once more, the cells were washed with PBS for 3 times, 5 min each time, and placed in goat-anti-mouse secondary antibody 37°C for 1 h. The cells were washed with PBS for 3 times, 5 min each time, and colored with DAB. The cells were washed with PBS for 3 times, 5 min each time, stained with hematoxylin, washed with water, and turned to blue. Following this, the cells were dehydrated by gradient alcohol at density 50%, 75%, 85%, 95% and 100%, vitrified by xylene for 2 times, 5 min each time. At last, the cells were mounted with neutral gum, osteocalcin (OCN) and Type I collagen contents were measured.

Determination of alkaline phosphatase through chemical staining

Modified Gomori method was applied. Working solutions include 2% sodium glycerol 25 ml, 2% sodium pentobarbital 25 ml, 50 ml distilled water, 2% calcium chloride 5 ml, 2% magnesium sulfate 2 ml, and 5 drops of chloroform. Cell climbing film was incubated in 37°C working solution for 3 h, then washed immediately, processed with 2% cobalt nitrate for 2 min, washed with water for 3 times, treated with yellow ammonium sulfide for 1 min, washed with water, dehydrated in gradient alcohol, vitrified, and mounted. Alkaline phosphatase was measured.

Detecting PPARγ and cbfαl mRNA gene expression levels using real-time quantitative PCR assay

The above cells were cultured. The cells were added with 1 ml Trizol Reagent, sufficiently agitated, 0.2 ml chloroform, and then ice-bathed standing for 5 min. The solution was centrifuged at 12000 rpm under 4°C for 20 min. The supernatant was discarded and replaced with an equal volume of isopropanol. After being ice-bathed standing for 5 min, the solution was centrifuged at 12000 rpm under 4°C for 20 min. The supernatant was abandoned, and 1 ml 75% ethanol was added into the solution, which was sufficiently agitated to dissolve RNA. Again, the solution was centrifuged at 10000 rpm under 4°C for 5 min. The supernatant was discarded, and the EP tube was turned upside down, dried for 15 min under room temperature. After the precipitate dried, 20 μl RNase-free water was added to dissolve it. 1 μl solution was drawn with an Eppendorf, and the rest was stored in a -70°C refrigerator. The 1 μl solution was diluted into 80 μl. OD 260 and OD 280 values were measured under a spectrophotometer, and the total RNA was calculated. The synthesis of cDNA and the reverse transcription experiment were conducted through PrimeScript RT Reagent Kit assay. According to the Kit instructions, 5 times PrimeScript Buffer 2 μl, PrimeScript RT Enzyme Mix 0.5 μl, Total RNA 2 μl, RNase Free dH$_2$O 5 μl, and Total 10 μl, were put under 37°C water bathing for 15 min, then under 85°C for 15 seconds. An appropriate amount of synthesized cDNA was obtained to do fluorescent

real-time quantitative PCR detection, and the rest samples were stored under -20°C standby. PCR detection was based on the Kit instructions of SYBR Premix Ex Taq TM II (Perfect Real Time): SYBR Premix Ex Taq 12.5 μl, PCR Forward Primer 1 μl, PCR Reverse Primer 1 μl, DNA template 2 μl, d H$_2$0 8.5 μl, and Total 25 μl. Real-time PCR is set as follows. Amplification profile is set to initial denaturation at 95°C for 5 min, denaturation at 95°C for 20 s, annealing at 60°C for 30 s, and extending at 72°C for 20 s. This stage is for the acquisition of fluorescence signals, a total of 40 cycles. Dissolution profile is set at 60°C~95°C, an increment of 0.5°C for 20 s each time, 71 cycles in total, for fluorescence signal acquisition. The size of PCR products were verified through electrophoresis in agarose gel at density 1%, and the sample volume is 10 μl.

PCR primers were as follows: PPARγ: CCAAC-ACGTAGTCCGTTGAAGAC, TCCTGCCGCAGCATT-ACACCG; cbfαl mRNA: TTGCCGCCGAACTGTC-TTTCCT, AGAAGGGCAATTGCCACTTGT; GAPDH: TACTGGTCTGGGCTGCCAG, CACGGAAGGTCAC-AATGTTT.

Statistical analysis

All data are represented by mean and standard deviation. In the Normal group, the Control group and the Drug group were compared, repeated measurement data were analyzed by repeated measures. Each experiment was repeated for three times, using the One-way ANOVA Bonferroni Test in SPSS13.0 to do pairwise comparison in multiple samples, and P<0.05 means a significant difference in results.

Results

Oil-red staining cells to observe the effects of amygdalin on adipocytes under a light microscope

In the model group, there emerged lipid droplets in the first place, while there were fewer lipid droplets in the amygdalin group, an also in later time. The amount and emergence of lipid droplets were positively correlated with the dose and density of amygdalin. As time passing by, the lipid droplets of all groups became more and larger, but still with the most significance in the model group. Oil-red O stain-
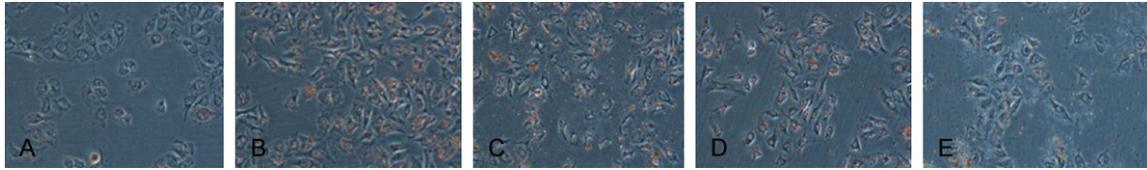
Figure 1. Oil-Red O staining of adipocytes (× 200). A. Normal group; B. Model group; C. Amygdalin; D. Amygdalin; E. Amygdalin. Low dose group Middle dose group High dose group.

Table 1. Effects of amygdalin on the contents of TG

TG contents detection	Average absorbance	Calibrated absorbance	Stock solution (mg/ml)
Normal group	0.65±0.03	0.55±0.01	1.40±0.06
Model group	1.29±0.01**,##,&	1.18±0.02**,##	3.01±0.01**,##,&
Amygdalin Low dose group	1.06±0.03**,##	0.97±0.05**,#	2.33±0.05**,#
Amygdalin Middle dose group	0.92±0.01*,#	0.84±0.00**,#	1.84±0.03*
Amygdalin High dose group	0.75±0.02	0.63±0.01	1.66±0.04

**P<0.01 vs Normal group; *P<0.05 vs Normal group; ##P<0.01 vs Amygdalin Low dose group; #P<0.05 vs Amygdalin Middle dose group; &P<0.05 vs Amygdalin High dose group.

Figure 2. Measuring the effects of amygdalin on OCN contents using Immunohistochemical assay (× 200). A. Normal group; B. Model group; C. Amygdalin; D. Amygdalin; E. Amygdalin. Low dose group Middle dose group High dose group.

ing results showed that, the lipid droplets were stained into orange color under the light microscope **Figure 1**.

Triglyceride (TG) content determination

Results showed that, the absorbance in the normal group is the lowest, while the model group exhibited the highest absorbance. However, after medicated by amygdalin, TG contents declined, which is proportionally correlated with the dose of amygdalin, and with higher dose comes more decline, as shown in **Table 1**.

Measurement of OCN and type I collagen contents using immunohistochemical assay

The results displayed that, OCN expressions in the normal group exhibited normally, but decreased in the model group. The dose of amygdalin can increase the OCN expression, in a positively proportional way. However, the expression of Type I collagen showed the cont-

rary way, which can be decreased by the medication of amygdalin. See **Figures 2** and **3**.

Alkaline phosphatase staining

The staining results showed that, the expression of alkaline phosphatase exhibited normally in the normal group, decreased in the model group, which can be increased by amygdalin medication, in a dose-dependent manner. As shown in **Figure 4**.

Detection of PPARγ and cbfαl mRNA gene expression using PCR assay

PPARγ expression increased, and cbfαl mRNA expression decreased, with which the model group showed significant difference from the normal group (P<0.001). However, after medicated with amygdalin, the PPARγ expression significantly increased, and the cbfαl mRNA expression significantly decreased (P<0.01). The effect is enhanced with the increasing concentration of amygdalin. As shown in **Figure 5**.
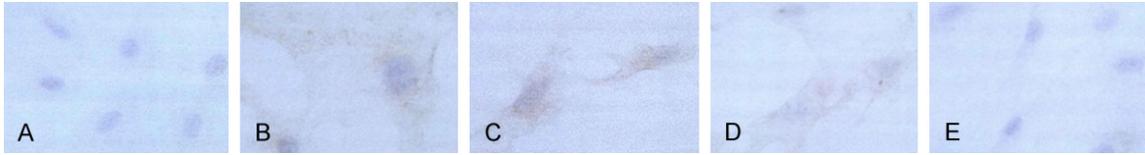
Figure 3. Measuring the effects of amygdalin on Type I collagen using Immunohistochemical assy (× 200). A. Normal group; B. Model group; C. Amygdalin; D. Amygdalin; E. Amygdalin. Low dose group Middle dose group High dose group.
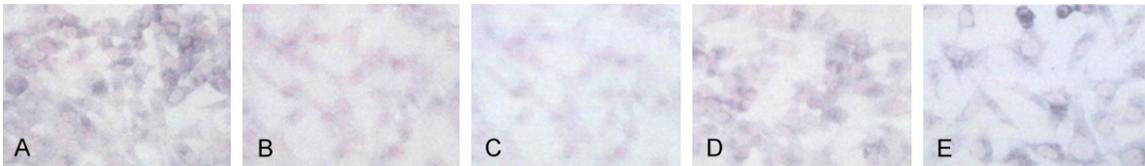
Figure 4. The staining of alkaline phosphatase. A. Normal group; B. Model group; C. Amygdalin; D. Amygdalin; E. Amygdalin. Low dose group Middle dose group High dose group.
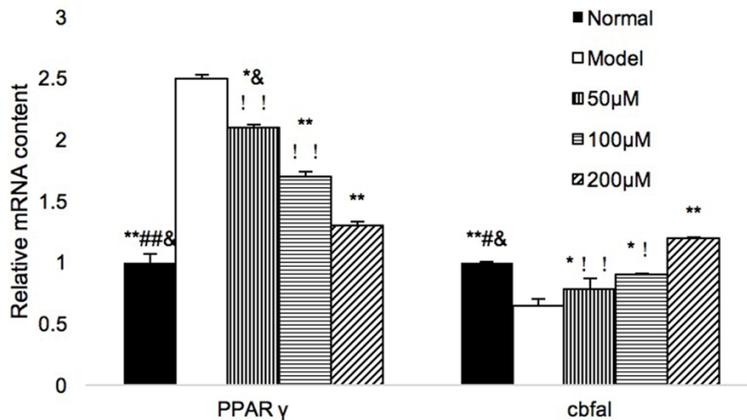
Figure 5. PCR assay to detect the expressions of PPARγ and cbfαl mRNA genes. **$P<0.01$ vs Model group; *$P<0.05$ vs Model group; ##$P<0.01$ vs 50 µM; #$P<0.05$ vs 100 µM; &$P<0.05$ vs 100 µM; !!$P<0.05$ vs 200 µM; !$P<0.05$ vs 200 µM.

sed fatty substances accumulate in blood circulation, gather into fat balls, reducing the blood flow speed, which clogs capillaries, causes microvascular thrombosis in femoral heads, and leads to femoral head ischemia eventually [8].

Studies have shown that the bone marrow mesenchymal stem cell adipogenic differentiation plays an important role in the process of alcohol osteonecrosis. There are reports comparing the osteogenic differentiation and adipogenic differentiation of bone marrow mesenchymal stem cells proximal to the femur from patients of alcohol-induced femoral head necrosis and femoral neck fracture, found that adipogenic differentiation of patients with alcohol-induced avascular necrosis is higher than that of the femoral neck fracture patients, but the former is with lower osteogenic differentiation than the latter [9]. This further evidences that the differentiation change of bone marrow mesenchymal stem cells may be one of the pathogeneses of alcoholic femoral head necrosis.

The PPAR (Peroxisome Proliferators Activated Receptor) is a nuclear receptor family that is closely related to thyroid hormone and reti-

Discussion

Alcoholic femoral head avascular necrosis is a disease with high incidence in the young and middle-aged, and progressively highly disabling in the course. Without effective early treatment, 80% the patients in 4 years will suffer femoral head collapse, joint space narrowing, and eventually necrosis osteoarthritis and hip joint dysfunction. Alcoholic avascular necrosis is caused by excessive secretion of glucocorticoids from the adrenal hormones stimulated by alcohol, suggesting that there may be a common pathway to bone necrosis caused jointly by alcohol and hormones. Alcoholism can cause hyperlipidemia. Increa-

noic acid receptor, with peroxisome proliferator, prostaglandin metabolite and insulin-sensitizing agents as ligands, mainly involves in the regulation of lipid metabolism [10-12]. The effects of PPARγ on bones have been a hot research topic in recent years [13]. Many studies have reported that, PPARγ activated by ligands can act on the bone marrow to facilitate the adipogenic differentiation and proliferation of pluripotent stem cells, and inhibit osteogenic differentiation, thus leading to reduction of bone mass. The capability of PPARγ facilitating adipocyte differentiation is closely correlated with bone metabolism. With age, marrow fat contents increase, while osteoblast metabolites decline [14-16].

Osteogenic differentiation of bone marrow mesenchymal stem cells is facilitated by the core binding factor (cbfαl), which can activate the expressions of osteoblast-specific genes such as bone sialoprotein and Type I collagen. Osteoblasts reduced in rabbits lack of these genes, with diminishing bone mineralization processes. PPARγ plays an important role in mesenchymal cell differentiation, suppresses expressions of cbfαl and thus inhibiting osteoblast-specific gene expressions. But this is not the crucial factor to mesenchymal stem cell differentiation [17]. The present experiment observed whether amygdalin can suppress adipogenic differentiation in alcohol-induced bone marrow mesenchymal stem cells, thus preventing the occurrence of alcoholic femoral head necrosis [18, 19]. The cytological results showed that, TG contents and PPARγ mRNA expressions in groups medicated by amygdalin were lower than those in the model group, as well as the alkaline phosphatase activities.

In summary, alcohol can facilitate intramedullary adipocyte hypertrophy and proliferation, which may be a main cause of the femoral bone marrow adipose tissue proliferation after long-term drinking, increased pressure within the bone, reduced blood flow, and ischemia, which leads to femoral head necrosis. Amygdalin can improve the situation of adipocyte differentiation, with a certain treating effect on alcoholic femoral head necrosis, which may be of some clinical application.

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Disclosure of conflict of interest

None.

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