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The osteogenetic effect of astragaloside IV with centrifugating pressure on the OCT-1 cells

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Astragaloside IV (ASI), a pure compound derived from *Radix Astragali*, is commonly used in degenerative bone diseases such as osteoporosis. Our previous study identified *in vivo* the osteogenetic effect of *Fu Fang Qi She Pills* (FFQSP), a Chinese herbal formula containing *Radix Astragali* from which ASI was extracted. In this study, we investigated the osteogenetic effects of ASI under the conditions of centrifugating pressure on OCT-1 cells. These preosteoblasts were grown in 3D-culture, and treated with ASI at 50 μmol/l with centrifugation at 200 rpm, 500 rpm for 3 and 5 days. Morphocytological examination, morphometry of alkaline phosphatases (ALP) staining was performed. Expression of type I collagen (Col I) was detected by immunocytochemistry assays. ALP, Col1a2, Osteocalcin (OC), and runt-related transcription factor-2 (Runx2) mRNA expression were determined via real-time PCR. The results showed ASI plus 500 rpm for 3 days and ASI plus 200 rpm for 5 days significantly induced osteogenesis related protein and gene expression. We concluded that ASI would promote osteogenesis when cells of preosteoblast OCT-1 were subjected to proper centrifugating pressure and a pertinent period of time.

1. Introduction

Astragaloside IV (ASI) is one of the active components of Radix Astragali and usually used as standard substance in quality evaluation since its high correlation to various pharmacological actions of the herb. For example, it was effective in improving endothelial dysfunction, cardiomyocyte damage, memory function and delaying the aging, which was somewhat related to its antioxidant activity (Hu et al. 2008; Li et al. 2007; Qiu et al. 2010; Qu et al. 2009; Xu et al. 2008; Zhang 2007a etc.; Zhang 2010c etc.; Zhang 2006d etc.; Zhang 2006e etc.; Zhao et al. 2009). On the other hand, ASI prevented hepatic fibrosis, chronic experimental asthma, experimental Parkinson's disease, diabetic complications such as peripheral neuropathy since it acted as an aldose-reductase inhibitor (Chan et al. 2009; Chen et al. 2008; Du et al. 2008; Liu 2009a etc; Lv et al. 2010; Motomura et al. 2009; Yu et al. 2006; Zhu et al. 2008). It also elicited the activity of anticancer, anti hepatitis B and Coxsackie virus B3 (CVB3) (Qi et al. 2010; Wang 2009a etc.; Zhang 2006f etc.). Recently, it was reported that AST could promote bone mesenchymal stem cells (MSCs) proliferation and differentiation in rats, partly by increasing stem cell factor (SCF) mRNA and ALP excretion (Liu 2006b etc.; Tan et al. 2010).
However, the effects of ASI on osteogenesis, especially combined with mechanical pressure, an important factor of promoting bone formation, remain largely unknown. In our previous study, *Fu Fang Qi She Pills* (FFQSP) a Chinese herbal compound composed of Radix Astragali obviously promoted lumbar vertebral bone formation of rats in upright posture (Bian et al. 2010). The content of ASI in FFQSP was within the range of 5.175∼36.225 μg determined by HPLC-ELSD (Zhang 2008b etc.). In the present study, we focus on the effect of ASI on osteogenesis *in vitro* associated with overloading imposed by centrifugation, which mimics mechanical environment in upstanding posture *in vivo* (Wong and Rabie 2008).

2. Investigations, results and discussion

MTT is a common method for observing cellular proliferation. However, in our study, cells were cultured in a solid phase, which brought difficulties in MTT detection. So, we used frozen section and H&E staining method for morphological examination. It showed that cells were able to anchor in the agarose gel matrix which is hyaline and like branches of trees; little cells were located on the inner section of the matrix. In the 200 rpm groups and 500 rpm groups, more cells could be seen. Cells tended to be bigger when treated with ASI, but no significant changes in cellular shapes were seen. These results suggested that the preosteoblasts tended to differentiate into osteoblasts. Three ASI groups showed an increasing amount of cells after centrifugation for 3 d and 5 d, and the extra cellular matrix was augmented. These changes might acclimatize the cells in hypergravity condition.
Type I collagen (Col I) is expressed both in the committed preosteoblast and the mature osteoblast (Robling et al.

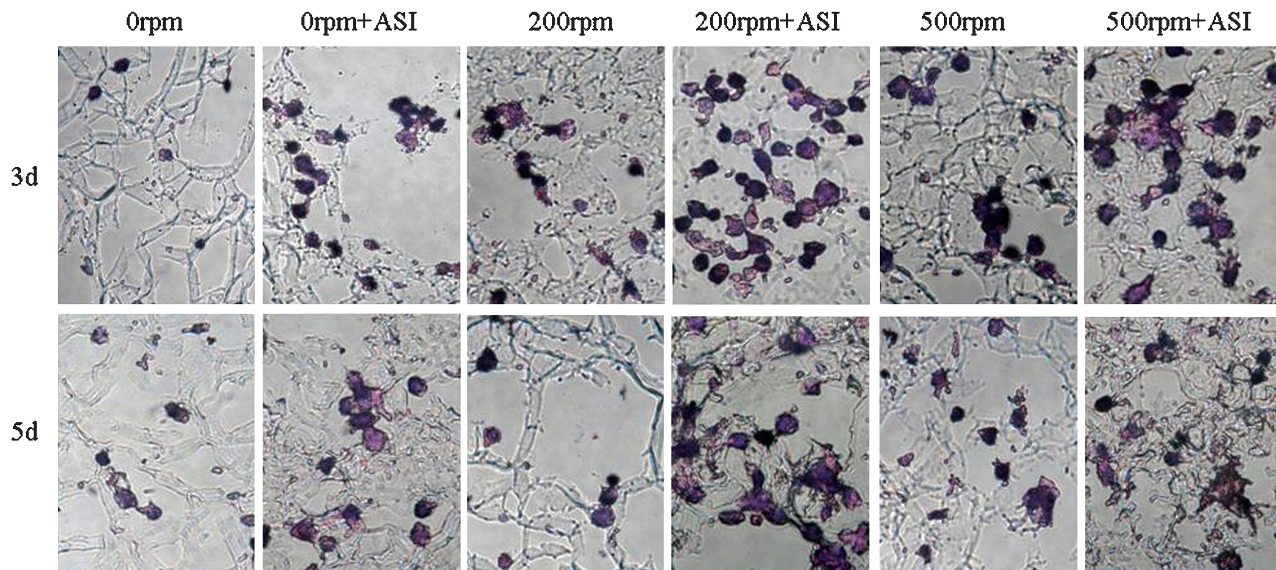

Fig. 1: H&E staining for OCT-1 cells cultured in agarose gel at 1% concentration. Three ASI groups showed an increasing amount of cells after centrifugation for 3d and 5d, and the extra cellular matrix was augmented (×200)

2006). It is reported that type I collagen was enhanced when osteoblasts were exposed to pressure (Nagatomi et al. 2003). In our study, the immunofluorescence study demonstrated OCT-1 cells expressed increased Col I protein when treated with ASI after centrifugation for 3 days. However, there were no significant changes between 200 rpm group and 200 rpm + ASI group. ASI, with or without centrifugation by 200 rpm for 5 days significantly promoted Col I expression while ASI with 500 rpm centrifugation slightly inhibited it. The result is consistent with the changes in Col I mRNA level. It indicated that ASI could facilitate the expression of Col I protein and mRNA, especially with the assistant of centrifugation at 500 rpm.

Alkaline phosphatase (ALP) expressed by mature osteoblasts, is a key enzyme in the mineralization process (Robling et al. 2006). Although ALP activity is an early indicator of bone formation and is transient, making it a less sensitive marker of osteogenesis, especially in response to mechanical stimuli (Lian and Stein 1995). Osteocalcin (OC) is one of abundant bone extracellular matrix proteins and molecular markers of osteoblast differentiation (Robling et al. 2006; Candeliere et al. 2001). We

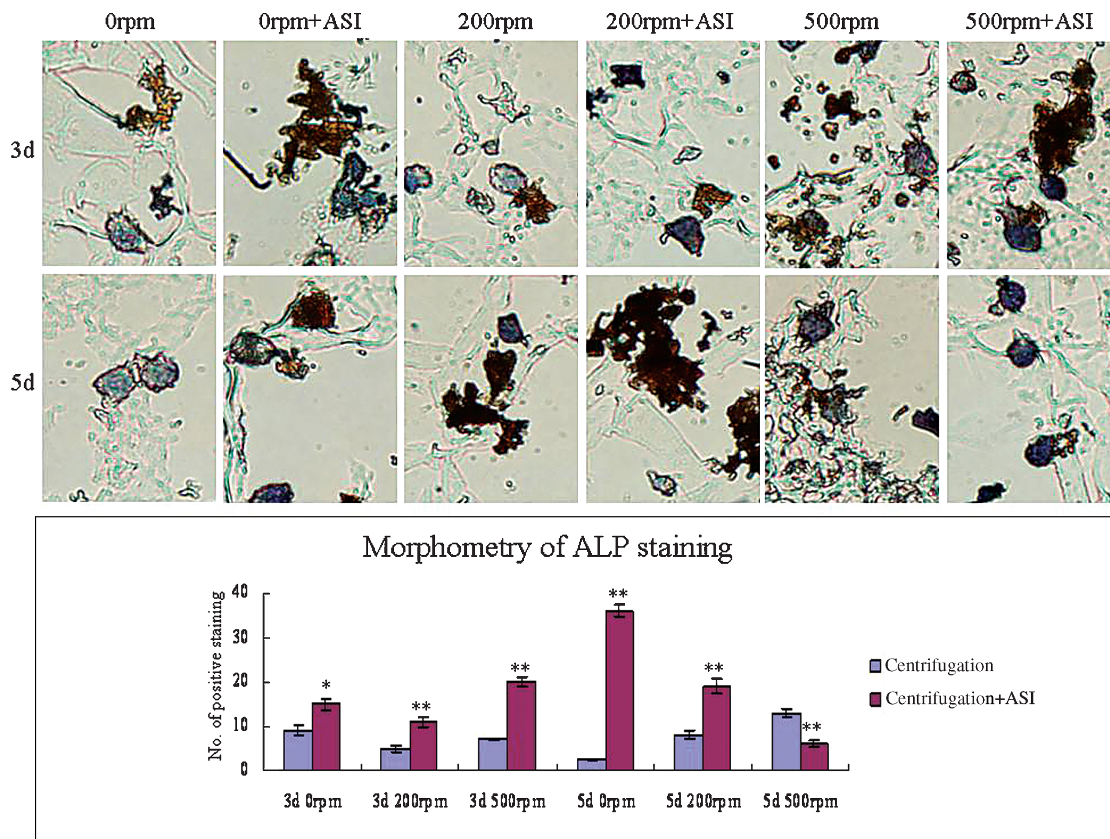

Fig. 2: ALP staining for 3D-cultured OCT-1 cells and Morphometry of ALP staining. OCT-1 cells were cultured in centrifugal condition at 0 rpm, 200 rpm, and 500 rpm with or without ASI for 3d and 5d. Brown sediments indicated the positive staining (×400). Histogram shows the morphometry of ALP staining ($n = 6$). *: $p < 0.05$, **: $p < 0.01$ ASI+centrifugation vs centrifugation at the same centrifugal force

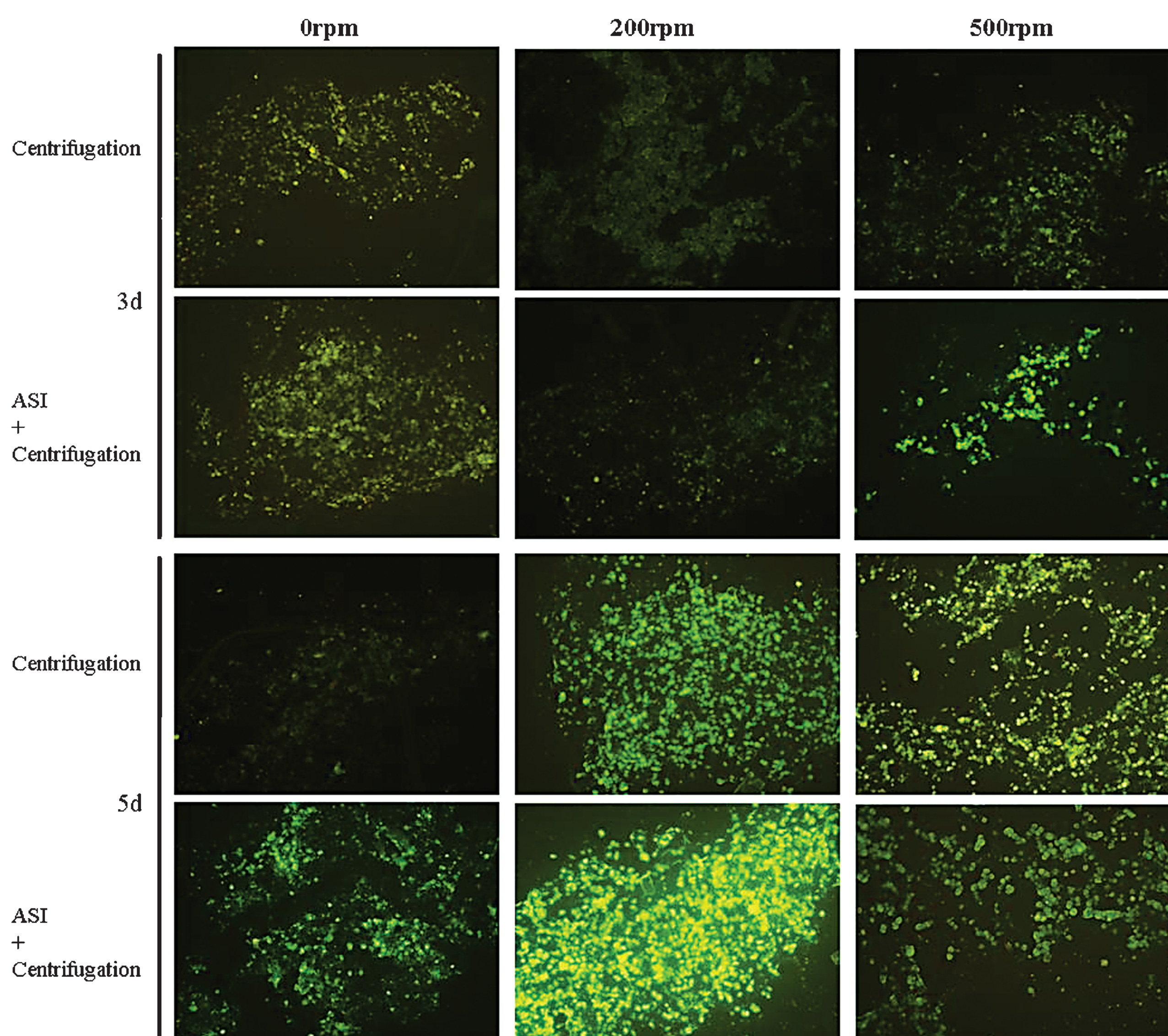

Fig. 3: Immunofluorescence study of CollI. OCT-1 cells expressed increased CollI protein when treated with ASI after centrifugation for 3 days, expecially in ASI+500 rmp group. ASI, with or without centrifugation by 200 rpm for 5 days significantly promoted CollI expression while ASI with 500 rpm centrifugation slightly inhibited it (×100)

found both ALP and OC mRNA up-regulated after three days. In 5 d groups, ASI raised both ALP and OC mRNA level at 0 rpm and 200 rpm. On the contrast, 500 rpm centrifugation inhibited the osteogenetic effect of ASI. The morphometry of ALP staining showed the accordant tendency with the changes in mRNA expression.

After centrifugation for three days, the up-regulation of ALP, Col1a2 and OC genes suggested an adaptation of ASI to OCT-1 cells in centrifugation condition, and 500 rpm+ASI showed the most effective in osteogenesis on this phase. Nevertheless, 500 rpm+ASI attenuated the osteogenetic effect on the fifth day by down-regulating the related gene mRNAs. It was supposed that ASI started to produce an effect on the third day, and this effect was enhanced by the support of high dose of pressure (500 rpm). However, this osteogenetic effect of ASI and centrifugation might become fatigued and over its threshold value at later period, leading to loss the action due to overconsumption.

The transcription factors runt-related transcription factor-2(Runx2) is considered a "master gene" that plays a critical role in the formation of the skeleton (Komori et al. 1997). In detail, expression of Runx2 is required to push the proliferating precursor cells toward the osteoblast lineage and away from other lineages also yielded by the mesechymal stem cell. Further differentiation of the preosteoblast into a mature, bone forming osteoblast phenotype requires the expression of Runx2 (Ducy et al. 1997). Our results showed that Runx2 mRNA increased only

Table: Sequences of primers used in the Real-time PCR

Genes	Forward primer	Reverse primer	Product length(bp)
ALP	TGACCTTCTCTCCTCCATCC	CTTCCTGGGAGTCTCATCCT	183
Beta-actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG	150
Col 1a2	TCCTGGCAATCGTGGTTCAA	ACCAGCTGGGCCAACATTTC	133
OC	TGCCCTCCTGGTTCATTTCT	TTCTGTTCCCTCCCTGCTGT	119
Runx2	CCATAACGGTCTTCACAAATCCT	TCTGTCTGTGCCTTCTTGGTTC	99

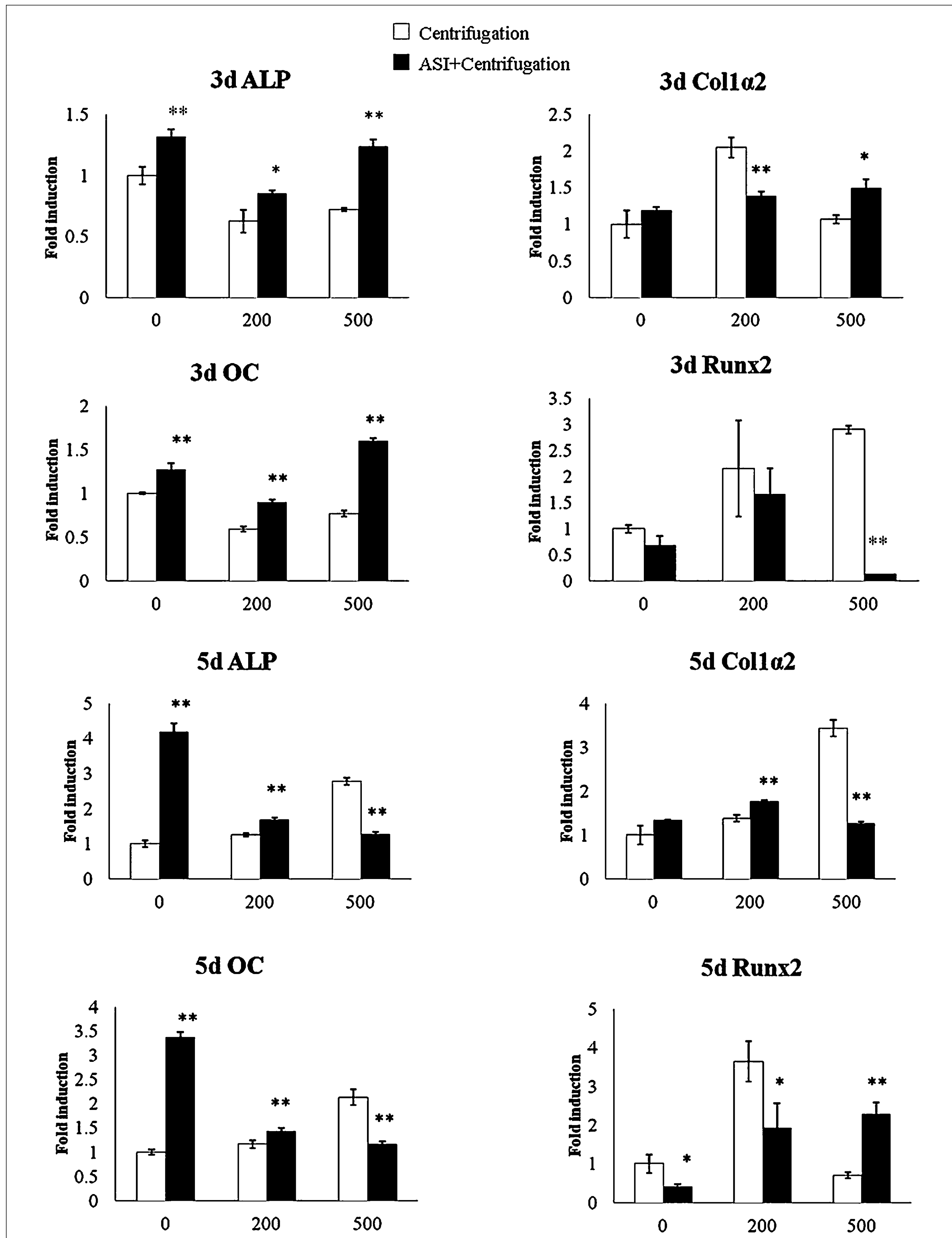

Fig. 4: The mRNA expression of ALP, Col1a2, OC and Runx2. The columns represent the mean ± SE of three independent experiments. *: $p < 0.05$, **: $p < 0.01$ ASI+centrifugation vs centrifugation at the same centrifugal force

after centrifugation at 200 rpm, but there was no significant difference at 500 rpm, which indicated a magnitude- but not a time-dependent induction by centrifugating pressure. However, ASI induced increased Runx2 gene expression merely in 5d 500 rpm group. These variations were different from those of other three genes, hinting that the upstream of Runx2 includes several other molecules besides ALP and Col I molecules (Alliston et al. 2001). And, the targets and effects were manifold of ASI with centrifugating pressure on osteogenesis, which caused discordant changes in Runx2 molecule and other three molecules.

We concluded that ASI would promote osteogenesis combined with proper centrifugating pressure and pertinent period of time in preosteoblast OCT-1.

3. Experimental

3.1. Materials

Astragaloside Ir (ASI) was purchased from Shanghai Pharmaceutical Company Ltd, China. The preosteoblast strain OCT-1 originates from the skull of osteocalcin driven element SV-40 transgenetic mice (OC-Tag), presented by Prof. Di Chen (University of Rochester, U.S.). High-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibico, US), fetal bovine serum (FBS) (HyClone, Logan, UT), antibiotic/antimycotic (Gibico, US), trypsin-EDTA (Gibico, US), agarose (Biowest, France), RNAprep pure Cell Kit (Tiangen Biotech Co., LTD, Beijing, China), Histostain-Plus Kit (Jingmei Biotech, Shenzhen, China), ALP staining kits (Jiancheng Biotech Institute, Nanjing, China), and Collagen I antibody (Abcam Ltd., Cambridge, UK).

3.2. Preparation of ASI

The molecular weight of ASI is 784.98 g/l. 50 nmol/l ASI was obtained by 39.24 mg ASI powders and 1 ml DMSO. Then, the above fluid was diluted into 100 μmol/l using double concentration of DMEM containing 4% FBS.

3.3. Grouping

There were two groups based on different durations: 3 days and 5 days. Each group was divided into six subgroups according to different centrifugal speed: 0 rpm, 200 rpm, and 500 rpm with or without ASI.

3.4. 3D culture

Cells were cultured to confluence at 8×10^6 mononuclear cells per cm^2 in medium consisting of high DMEM, 2% fetal bovine serum, 1% antibiotic/antimycotic, and then detached with 0.05% trypsin/1 mM EDTA. An equal volume of cells were suspended at 4×10^7 cells per millilitre in double concentration of high DMEM, fetal bovine serum and antibiotic/antimycotic, and then combined with the same volume of agarose solution, which were assembled by autoclaving 2% agarose in ultrapure water and then cooling the solution to 40 °C. The final concentration was 2×10^7 cells per millilitre in 3D culture system of 1% agarose and 50 μmol/l ASI, 2% FBS.

3.5. Calculation of centrifugal speed

It has been reported that human's L_4-L_5 disc produced a pressure of 0.1 MPa when lying prone. More value has been measured as followed: relaxed standing, 0.5 MPa; and lifting a 20-kg weight with round flexed back, 2.3 MPa. From the first formula: P = mg/S (mechanism pressure), P = ρgh (hydraulic pressure), it can be derived that regardless of what pressure the system is at, pressure is related to gravity "g". According to the above formula, we supposed that $G_{lying} = P/\rho h = 0.1/\rho h = 1g$, $G_{relaxed\ standing} = P/\rho h = 0.5/\rho h = 5g$, and $G_{flexed\ back} = P/\rho h = 2.3/\rho h = 23g$. The value shows that the two standing postures need to overcome a force of gravity of 5g and 23g respectively. For the second formula as follows, we put 5g and 23g as an RCF into the calculation. according to Eq. (1).

$$\text{RPM} = \sqrt{\frac{\text{RCF}}{1.118 \times 10^5 \times (\text{Radius})}}$$

$$\text{RCF : centrifugal force(g)}$$

$$\text{RPM : rotation speed per minute}$$

$$\text{Radius : length of shaft radius} \quad (1)$$

The highest rotation speed of the centrifuge (Allegra^{TM} X-22R, Beckman, USA) was 10000 rpm and the maximum centrifugal force was 10528 g. According to the second formula, the radius was worked out as 9.4168 cm. We put the value into the second formula as follows: $\sqrt{1/(1.118 \times 10^{-5})} \times 9.4168 = 97$ rpm; $\sqrt{5/(1.118 \times 10^{-5})} \times 9.4168 = 218$ rpm; $\sqrt{23/(1.118 \times 10^{-5})} \times 9.4168 = 467$ rpm. The values of 5g or 23g may be obtained using the centrifuge at 218 rpm or 467 rpm. In fact, the centrifuge can work at 200 rpm and 500 rpm limited by its accuracy. Furthermore, 5g and 23g correspond to physiological level of five ustrain and 23 ustrain respectively (Hughes-Fulford 2003). The 200 rpm groups and the 500 rpm groups were centrifuged for three hours once a day under room conditions for one day or five days. The control groups were located in the same environment, but without centrifugation. All the samples were put back into an incubator (37 °C, 5% CO_2) immediately after centrifugation or after three hours standing under room conditions.

3.6. H&E Staining

Cells were fixed in 4% paraformaldehyde for 20 min, and then washed with PBS. Frozen sections were stained with hematoxylin and eosin. A morphometric study was performed by an image auto-analysis system (Olympus BX50; Japan).

3.7. Morphometry of ALP

The substrates were dissolved by dimethylformamide. The buffer and solid violet B were then added and mixed to form the fluid. The fixed slides were put into the fluid, and cultured at 37 °C for 45 min. Positive staining of brown sediments could be seen and counted by image auto-analysis system (Olympus BX50; Japan) after counterstaining with hematoxylin.

3.8. Immunofluorescence analysis

Sections were deparaffinised and incubated in 3% H_2O_2 to block endogenous peroxidase activity for 15 min, washed with PBS for 2 min, followed by a digestion with protease K for 10 min. Sections were blocked with 5% BSA in which the secondary antibody was produced, and then washed with PBS three times for 2 min. Specific primary rabbit polyclonal antibodies against type I collagen (1:200) were incubated overnight at 4 °C. After extensive washing with PBS three times for 2 min. A fluorescence microscope was used for observation and a photograph was taken within one hour to prevent fluorescent from decreasing.

3.9. Real-time PCR detection

Cells were directly processed following RNA prep pure Cell Kit protocol. One microgram of total RNA was reversely transcribed using the Advantage RT-for-PCR kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Freshly transcribed cDNA (1 μl) was used for quantitative real-time PCR using SYBR Green (Bio-Bad, Hercules, CA) to monitor DNA synthesis using specific primers (Table) designed by TaKaRa Biotechnology Co. Ltd. The PCR was carried out in RotorGene real-time DNA amplification system (Corbett Research, Sydney, Australia) using the following cycling protocol: a 95 °C denaturation step for 15 min followed by 45 cycles of 95 °C denaturation (20 s), 56–62 °C annealing (30 s), and 72 °C extension (30 s). Gene expression was normalized to the housekeeping gene β-actin. PCR products were subjected to a melting curve analysis, and the data were analyzed and quantified with the RotorGene6.0 analysis software.

3.10. Statistical analysis

The data is expressed as mean ± SE and statistical significance was calculated using a Student t test and analysis of variance by SPSS software (SPSS Inc, Chicago, USA). The significance level was defined as $p < 0.05$.

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