Osteogenic Induction of Low-dose Ipriflavone on Bone Marrow Mesenchymal Stem Cells Extracted from Osteoporosis Rats

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Objective: *To investigate the effects of ipriflavone (IPF), a synthetic isoflavone plant oestrogen with a structure similar to that of oestradiol, on the osteogenic differentiation of bone mesenchymal stem cells (BMSCs).*
Methods: *BMSCs were derived from ovariectomised rats (rBMSCs-OVX) and then induced with or without IPF. Cell cytotoxicity, mineralisation in vitro and osteoblast-specific gene expression of BMSCs were studied.*
Results: *IPF at a concentration of 10^{-8}, 10^{-7} and 10^{-6} mol/l exhibited no cytotoxic effect on the proliferation of BMSCs but increased alkaline phosphatase activity and osteoblast-specific gene expression.*
Conclusion: *IPF enhances osteogenic differentiation of rBMSCs-OVX partly in vitro, thus its use offers a potential strategy for the treatment of osteoporosis.*
Key words: *ipriflavone, mesenchymal stem cells, osteogenic differentiation, osteoporosis*
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In 1993, osteoporosis was defined as a systematic bone disease characterised by decreased bone mass, microstructural degeneration, fragility and increased risk of fracture[1]. Bone loss accelerates after menopause[2] and affects the quality of life of middle-aged and elderly women; however, because its specific mechanism is unclear, it is difficult to prevent and treat osteoporosis. As yet, no ideal drug or method has been proposed to treat bone loss[3]. At present, the drugs for the treatment of osteoporosis include oestrogen, calcitonin and bisphosphate; however, due to safety and price, the clinical application is limited. We are therefore eager to establish new healthier and safer agents that actively promote bone formation as alternatives.

Bone tissue is a special kind of calcified tissue. Its occurrence and calcification are mainly due to osteoblasts and regulated by many factors in vivo[4,5]. When pathological changes in bone tissue occur, osteoblasts also undergo corresponding changes. According to previous research, osteoblasts in bone marrow are derived from bone mesenchymal stem cells (BMSCs)[6,7]. Some reports have found that in the bone marrow of postmenopausal osteoporosis patients, the number of adipocytes increased as the number of osteoblasts decreased[8,9]. Interestingly, there is a balance between the adipogenic and osteogenic differentiation of BMSCs[10,11]. This suggests that promotion of the differentiation of bone marrow stem cells could be a new strategy to treat osteoporosis.

At present, in the treatment of postmenopausal osteoporosis, oestrogen has been used in clinical practice as an alternative treatment for many years[12,13]; however, studies have shown that its long-term application has many side effects on the body, thus its application has been limited[14,15]. As such, the question of how to use oestrogen replacement therapy safely, effectively and reasonably is one we are eager to explore. Flavonoids

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show promise in enhancing bone mass; their molecular structure is similar to that of oestradiol and they have an oestrogen-like effect in oestrogen deficiency, which can reduce bone loss caused by this deficiency[16,17]. Among other drugs that are available to treat osteoporosis, ipriflavone (IPF) has been used as a synthetic phytoestrogen in many countries as a prescription drug, and studies have shown its effectiveness and fewer side effects in the treatment of systemic and jaw osteoporosis[18,19]. IPF can treat osteoporosis through direct and indirect actions. It can stimulate the thyroid gland to release calcitonin and then play the therapeutic role of oestrogen and calcitonin, and has no side effects; some studies have shown that in the treatment of osteoporosis, IPF serves mainly to directly inhibit bone resorption. It therefore has a broad application prospect in the prevention of osteoporosis. Low doses of IPF have not yet been investigated regarding their effects on the osteogenesis of BMSCs derived from ovariectomised rats (rBMSCs-OVX).

The present study observed the ontogenetic effect of IPF in rBMSCs-OVX in vitro. Cell proliferation and osteogenic experiments including alkaline phosphatase (ALP) activity analysis and polymerase chain reaction (PCR) assays were performed to evaluate the differentiation potential of rBMSCs-OVX in vitro. The purpose of the present study was to provide a framework for further investigation of IPF treatment for bone defects in osteoporosis patients.

Materials and methods

Preparation of drugs

IPF (purity 99.9%; Sigma, St Louis, MO, USA) was dissolved in anhydrous ethanol, then diluted with a culture medium to obtain the concentration required for the experiments: 0, 10^{-8}, 10^{-7} and 10^{-6} mol/l, respectively.

Isolation and culture of rBMSCs-OVX

All experiments concerning animals were approved by the Animal Care and Experiment Committee of the 9th People's Hospital Affiliated to Shanghai Jiao Tong University School of Medicine. Employing the same methodological approach as in our previous study[20], a bilateral ovariectomy was performed on 6-week-old Sprague-Dawley rats and the OVX rats were sacrificed after 3 months by an overdose of pentobarbital sodium. The rBMSCs-OVX were flushed out with 10 ml Dulbecco's modified Eagle's medium (DMEM) (Gibco, Waltham, MA, USA) and supplemented with 10% foetal bovine serum (FBS) (Gibco) and antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml) from rat femurs after both ends were cut off. After 24 hours of culture, the solution was changed. The cells were subcultured after growing to 80% to 90% fusion. In the present study, cells at passages 2 to 3 were used.

Cell proliferation

In the present study, the effect of IPF on the proliferation of rBMSCs-OVX at different concentrations was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The cells in the logarithmic growth phase were digested with trypsin and gently pipetted into a single cell suspension. The cells were counted and seeded in a 96-well culture plate at a density of 1×10^4 cells/well. After 24 hours of culture, the original culture solution was discarded, and the culture medium containing different concentrations of IPF was added at concentrations of 0, 10^{-8}, 10^{-7} and 10^{-6} mol/l, respectively, and the pure DMEM was used as a control, with five replicates made for each concentration. After incubation for 1, 4 or 7 days, 30 µl newly prepared MTT solution was added to each well and incubated in a 37°C CO_2 incubator for 4 hours. The supernatant was discarded carefully and then 100 µl dimethyl sulfoxide (DMSO) was added. The plate was placed in an incubator for 10 minutes to completely dissolve the crystals, and the absorbance optical density (OD) value was measured immediately at 490 nm using an enzyme-linked immunosorbent assay.

ALP activity

rBMSCs-OVX were seeded in 24-well plates (1×10^4 cells/well). After 24 hours of culture, the cells were completely adherent and the medium was changed. 2 ml DMEM culture medium with different IPF concentrations (0, 10^{-8}, 10^{-7}, 10^{-6} mol/l) were added, while the pure DMEM medium was used as a control. After 4 or 7 days of culture, an ALP activity assay was performed.

To qualitatively characterise ALP activity, immunohistochemical staining was used. The cultured cells were first fixed with 4% paraformaldehyde for 15 minutes, then washed with phosphate-buffered saline (PBS) and stained with a 5-Bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase chromogenic kit (Biyuntian Biotechnology, Shanghai, China) for 15 minutes. After staining, an inverted microscope (TE2000-U, Nikon, Tokyo, Japan) was used for observations and photographs.

Quantitative real-time PCR (RT-PCR) analysis

To evaluate the effect of IPF on expression of osteogenic markers, PCR assays were carried out. rBMSCs-OVX were seeded in 6-well plates (1×10^5 cells/well). After 24 hours of culture, the cells were completely adherent and the medium was changed. 3 ml DMEM culture medium with different IPF concentrations (0, 10^{-8}, 10^{-7}, 10^{-6} mol/l) were added, while the pure DMEM medium was used as a control. After 4 and 7 days of culture, the total ribonucleic acid (RNA) was isolated with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA concentrations were determined using a NanoDrop spectrophotometer (ND-1000; Thermo Fisher Scientific, Waltham, MA, USA). Complementary DNA (cDNA) was synthesised using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Tokyo, Japan) and the real-time PCR was performed using a Real-time PCR kit (Takara). Quantitative real-time PCR analysis was conducted using a LightCycler Real-time PCR system (Roche, Rotkreuz, Switzerland). β-Actin was used as an internal control. The data were analysed using the comparative Ct (2-ΔΔCt) method and expressed as fold changes with respect to the control. All reactions were run in triplicate for each sample and gene. The primer sequences used in the present study were synthesised commercially (Shengong, Shanghai, China), and the specific primer sets are listed in Table 1.

Statistical analysis of data

The experimental data were expressed as mean ± standard deviation and analysed using Origin 8.0 software (OriginLab, Northampton, MA, USA). Statistical differences between the data sets were compared using analysis of variance (ANOVA). Statistics were obtained with confidence levels greater than 95% ($P < 0.05$).

Results

Effect of IPF on cell proliferation of rBMSCs-OVX

The effects of different concentrations of IPF on the proliferation of rBMSCs-OVX were investigated. rBMSCs-OVX were cultured for 1, 4 and 7 days in a medium containing a low (0,10^{-8},10^{-7} mol/l) and high concentration (10^{-6} mol/l) of IPF. As shown in Fig 1, after 4 and 7 days of culture, the relative activity of cells changed significantly with the increase in IPF concentration, and there was a significant difference between the 10^{-7} mol/l group and the other groups.

Table 1 Primer sequences used in the present study, including ALP, BMP-2, OCN, OPG, RANKL and β-actin.

ALP	Forward	5′-GGGGTCAAAGCCAACTACAA-3′
	Reverse	5′-CTTCCCTGCTTTCTTTGCAC-3′
BMP-2	Forward	5′-GGGGTCAAAGCCAACTACAA-3′
	Reverse	5′-CTTCCCTGCTTTCTTTGCAC-3′
OCN	Forward	5′-GCCGGGAATGATGAGAACTA-3′
	Reverse	5′-GGACCGTCCACTGTCACTTT-3′
RANKL	Forward	5′-AATGGTGCTCCTGGTATTGC-3′
	Reverse	5′-GGTTCACCACTGTTGCCTTT-3′
OPG	Forward	5′-GATCGATAGTGCCGAGAAGC-3′
	Reverse	5′-TGAAACTCGTGGCTCTGATG-3′
β-actin	Forward	5′-CTAAGGCCAACCGTGAAAAG-3′
	Reverse	5′-TACATGGCTGGGGTGTTGA-3′

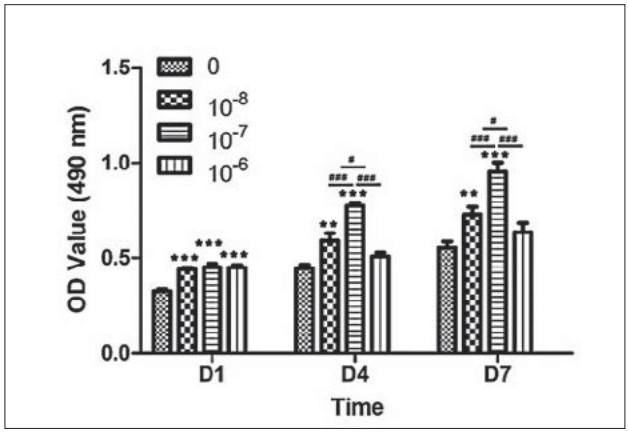

Fig 1 Cell viability of rBMSCs-OVX. MTT assays of rBMSCs-OVX were cultured for 1, 4 and 7 days with 0, 10^{-8}, 10^{-7} and 10^{-6} mol/l IPF, respectively. Results were displayed as relative cell viability compared with each other. $^{*}P < 0.05$, statistically significant difference as compared with the 0 mol/l group; $^{\#}P < 0.05$, statistically significant difference as compared with other groups (n = 5).

Effect of IPF on ALP activity of rBMSCs-OVX

ALP activity was examined to investigate the effect of IPF on osteogenic differentiation ability. Fig 2 shows that within the concentration range used (10^{-8}, 10^{-7}, 10^{-6} mol/l), IPF significantly promoted ALP activity. When the concentration of IPF was 10^{-7} mol/l, ALP activity reached its peak, which was around twice that in the 0 mol/l group after 7 days. In the concentrations of 0, 10^{-8} and 10^{-7} mol/l, ALP activity increased as IPF concentration increased; in 10^{-6} mol/l, ALP activity decreased; it was dose-dependent on IPF.

To further determine the extent to which IPF promotes ALP activity, ALP expression in rBMSCs-OVX

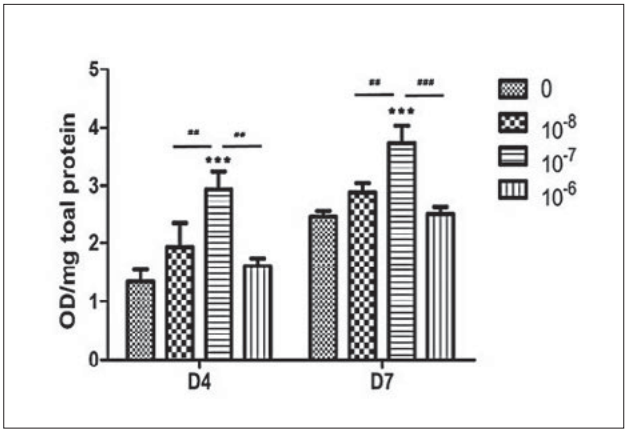

Fig 2 ALP activity assay. Relative ALP activity was detected after 4 and 7 days of culture of rBMSCs-OVX with different concentrations of IPF. $^{*}P < 0.05$, statistically significant difference as compared with the 0 mol/l group; $^{\#}P < 0.05$, statistically significant difference as compared with other groups (n = 5).

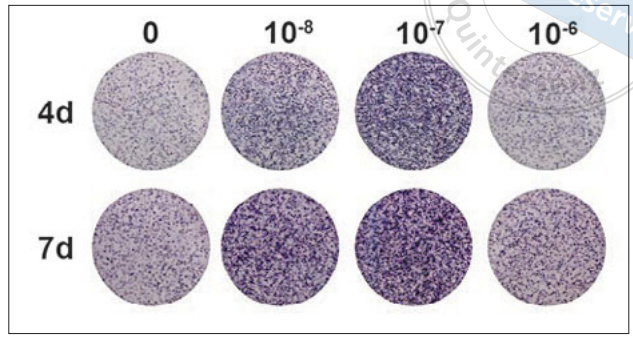

Fig 3 ALP immunohistochemical staining. rBMSCs-OVX were respectively cultured with different mediums for 4 and 7 days before fixation and staining.

was stained using a BCIP/NBT alkaline phosphatase chromogenic kit (Fig 3). As shown in Fig 3, when rBMSCs-OVX were cultured with 10^{-7} mol/l IPF, a large number of differentiated cells were stained (blue-violet area). The rBMSCs-OVX cultured in the original DMEM medium were significantly less stained. When the IPF concentration was increased to 10^{-6} mol/l, the blue ALP staining area was reduced.

Effect of IPF on rBMSCs-OVX osteogenic differentiation

The effect of osteogenic differentiation of rBMSCs-OVX cultured in different conditions was evaluated by RT-PCR. As shown in Fig 4, ALP, osteocalcin and bone morphogenetic protein 2, as markers of osteogenesis, had the highest relative expression level on day 4, whereas osteoprotegerin/receptor activator of NF-kB-ligand (OPG/RANKL) was the late marker of osteogenesis, and the relative expression level increased continuously. The results demonstrated that 10^{-7} mol/l IPF had a greater ability to upregulate the expression of osteogenic related genes in rBMSCs-OVX.

Discussion

The present authors found that the effect of IPF on rBMSCs-OVX was related to drug concentration, indicating that this drug showed low cytotoxicity and could therefore be used safely within its effective range. In terms of the effect on cell proliferation, the 10^{-7} mol/l concentration of IPF may affect differentiation of rBMSCs-OVX.

ALP is one of the commonly used indicators for evaluating early osteogenic differentiation in cells in vitro[21]. The results of the immunohistochemical staining were consistent with those of the previous quantitative detection of ALP activity. It could therefore be inferred that in a certain range, the effect of IPF on biological activity is concentration-dependent.

Osteogenic differentiation of BMSCs is crucial for the repair of bone tissue defects[22,23]. Within a certain range of concentrations, IPF could promote the osteogenic differentiation of rBMSCs-OVX into osteoblasts, which would be of great significance for biomedical applications.

Conclusion

In the present study, we prepared an OVX-rat model and cultured rBMSCs-OVX. We found that IPF could promote the adhesion of cells without side effects, which suggests that IPF has good biocompatibility. The bioactivity of IPF on osteogenesis and angiogenesis was investigated and the results demonstrated that IPF could enhance the osteogenic differentiation of rBMSCs-OVX at a proper concentration. The results of the repair of skull defects in the rats in vivo also showed the potential for IPF to promote bone regeneration in bone tissue engineering by promoting osteogenesis of BMSCs in the OVX-rat.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

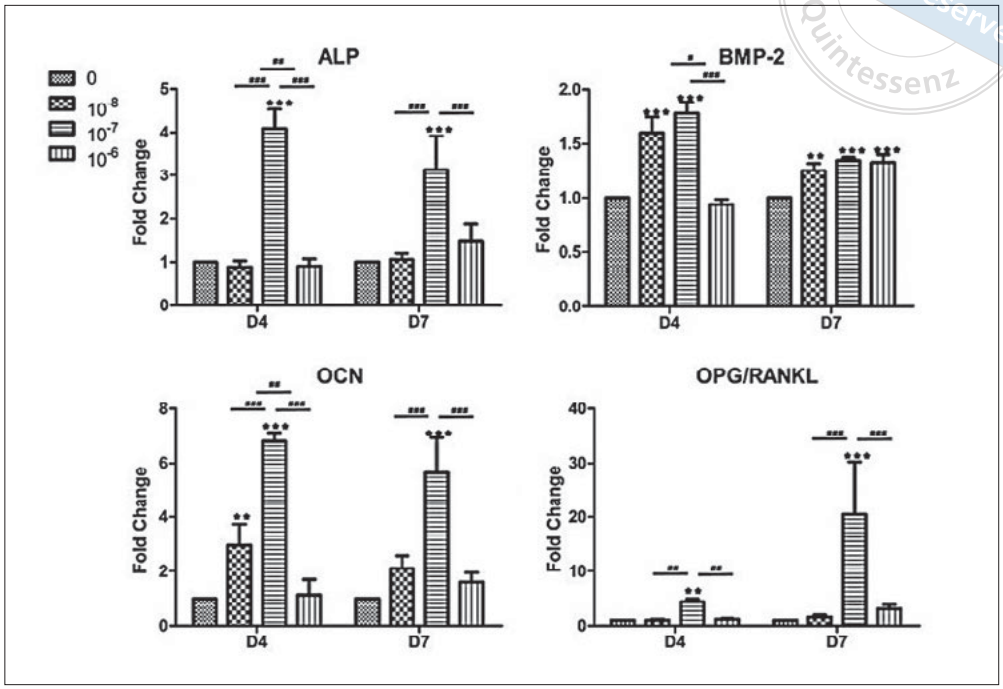

Fig 4 RT-PCR analysis. Relative gene expression was detected after 4 and 7 days of culture of rBMSCs-OVX with 0, 10^{-8}, 10^{-7} and 10^{-6} mol/l IPF. $^{*}P < 0.05$, statistically significant difference as compared with the 0 mol/l group; $^{\#}P < 0.05$, statistically significant difference as compared with other groups (n = 5).

Author contribution

Drs Xiao WANG and Ao Zheng contributed to the conception, data collection, investigation, methodology and writing of the original draft; Dr Xian Zhen XIN contributed to the data statistics; Drs Ling Jie PENG and Jie Wang contributed to the methodology and validation; Drs Ling Yan CAO and Xin Quan JIANG contributed to the conception, funding acquisition, project administration, text review and supervision.

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