Osteogenic Traditional Chinese Medicine for Bone Tissue Engineering

Xin Zhang

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Osteogenic Traditional Chinese Medicine for Bone Tissue Engineering

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Abbreviations

α-MEM	α-Minimum essential medium
Akt	Serine/threonine kinase
ALP	Alkaline phosphatase
BioCaP	Biomimetic calcium phosphate
BMD	Bone mineral density
BMP-2	Bone morphogenic protein 2
BMP-4	Bone morphogenic protein 4
BMP2/7	Bone morphogenic protein 2/7 heterodimer
BMSCs	Bone marrow mesenchymal stem cells
BR	Bone resorption
BSP	Bone sialoprotein
BV/TV	Bone volume/total volume
Cbfa 1	Core-binding factor alpha 1
CCD	Charge coupled device
CD14/TLR4	Cluster of differentiation 14/toll-like receptor 4
CPC	Calcium phosphate cement
EGF-EGFR	Epidermal growth factor - epidermal growth factor receptor
ERK	Extracellular signal-regulated kinase
GAGs	Glycosaminoglycans
HU	Holzknecht’s unit
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinases

MEK	Mitogen-activated extracellular signal-regulated kinase
Micro-CT	Micro-computed tomograph
NO	Nitric oxide
NOS	Nitric oxide synthase
OCG	Osteoclastogensis
OCN	Osteocalcin
OPG	Osteoprotegerin
OSX	Osteoblast-specific gene osterix
PA	Proliferative activity
PGE2	Prostaglandin E2
PI3K	Phosphoinositide-3 kinase
RANKL	Receptor activator of nuclear factor-κB ligand
Runx2	Runt-related transcription factor 2
Smad 4	Drosophila mothers against decapentaplegic protein 4
SMC-PHBHHx	Siliceous mesostructured cellular foams-poly(3-hydroxybutyrate-co3-hydroxyhexanoate)
Sox9	SRY (sex determining region Y)-box 9
TBA	Trabecular bone area
TCP	Tertiary calcium phosphate
TGF-β	Transforming growth factor-β

Chapter 1

GENERAL INTRODUCTION

Objectives of the thesis

The objective of this dissertation is to investigate the osteogenic effect of different concentrations of BMP-2 mixed with Icariin *in vitro* and *in vivo.*

The general aim of this dissertation includes 4 aspects:

1. To determine the optimal concentration of Icariin and to evaluate the osteogenic differentiation of MC3T3-E1 under treatment of Icariin.
2. To compare the osteogenic differentiation of MC3T3-E1 under stimulation of BMP-2 alone and the administration of Icariin with BMP-2.
3. To explore the osteogenic effect of BioCaP granules alone or BioCaP internally -incorporated with Icariin *in vitro*.
4. To evaluate the therapeutic effect of BioCaP incoporated internally with Icariin or/and BMP-2 for the repair of calvarial critical-sized bone defect in SD rats.

Icariin

Icariin, which has the composition $C_{33}H_{40}O_{15}$ and a molecular weight of 676.67, was recorded in the Chinese pharmacopoeia as an anti-inflammation treatment of rheumatics , as a tonic to promote health, and as an aphrodisiac[1].

Through the development of modern separation techniques, Icariin has been extracted successfully as the active ingredient of Herba Epimedii[1, 2]. Herba Epimedii is a centuries old traditional medicine herb[3]. It is recorded in the Chinese pharmacopoeia as ‘yin yang huo’ and was used to cure bone diseases such as osteoporosis and bone fractures in ancient China[1].

The multiple function of Icariin and especially the induction of osteogenesis is remarkable. Loading Icariin onto calcium phosphate biomaterials provides a good alternative for delivering Icariin locally for bone repair since the calcium phosphate biomaterials have been used as osteoconductive scaffolds. The local use of Icariin has shown positive effects in bone formation at an early stage[4]. Several studies have tried to clarify the molecular mechanisms underlying the osteogenic effects. In summary, Icariin may exert its osteogenic effects through the induction of BMP-2 and NO synthesis and the BMP-2/Smad4 signal transduction pathway, by up-regulating the expression of BMP-2, BMP-4, Smad4, Cbfa1/Runx2, OPG, RANKL, and the OPG/RANKL ratio[1, 5]. Icariin can inhibit the LPS-induced osteoclastogenesis by suppressing the activation of the p38 and JNK pathway, which in turn strengthen the bone[6]. The positive effects of Icariin on a

potent chondrogenic effect might be the up-regulation of the expression of aggrecan, collagen II, and Sox9 genes and down-regulation of the expression of the collagen I gene of chondrocytes[7].

BMP-2 combined with bioactive agents

It is well known that BMPs induce a sequential cascade of events leading to chondrogenesis, osteogenesis, angiogenesis, and the controlled synthesis of extracellular matrix[8]. BMP-2 and BMP-7 have been the most extensively evaluated BMPs with a very high price[9]. Although BMPs have an outstanding performance in bone formation, they could also have some negative effects[10]. Research has developed a variety of methods in bone tissue engineering to reduce the use of BMPs and to improve the osteoinductive effects of BMPs by a slow delivery[11, 12]. But one of the simplest ways would be to search for an effective and low cost substitute for the expensive BMPs. The perspectives discussed in this article demonstrate the importance of exploiting an inexpensive osteoinductive drug.

The administration of exogenous bioactive agents, such as fibroblast growth factor-2 FGF-2[13] and vascular endothelial growth factor VEGF[14], with BMP-2 to increase the osteogenic effects has become a hot topic in the field of bone regeneration. However, the increased osteoinduction seemed to be not significant. The addition of FGF-2 did not increase the mineralization in young mouse calvarial cultures[13]. The combination with VEGF did not enhance the bone formation in the orthotopic defects compared with BMP-2 alone[14].

In this thesis, we explore the outcome of the combined application of Icariin with BMP-2 *in vitro* and *in vivo*.

Bone regeneration

Bone defects are quite common resulting from an inflammatory disease, tumor, trauma, or anatomical and congenital disorders, and they cause a quite large clinical and biomedical burden. When the defects cannot heal by themselves they require bone grafts or bone substitutes, which are of paramount importance in achieving a suitable functional and great aesthetic restoration[15, 16]. The application of allografts, xenografts or biosynthetic substitutes eliminates the disadvantages, such as the limited availability of bone, the morbidity of the donor site together with risk of infection, associated with autologous bone [17].

Bone regeneration in large bone defects site requires four essential elements:

(1) osteogenic cells such as progenitor cells or osteoblasts ;

(2) osteoinductive signals such as growth factors and bioactive agents;

(3) a biocompatible, biodegradable and osteoconductive matrix such as a scaffold;

(4) an adequate blood and nutrient supply[18].

Therefore, bone grafts are often associated with the terms biocompatibility, biodegradability, osteoconductivity and osteoinductivity.

Drug delivery system

The drug delivery system for bone regeneration needs to comprise osteoinductive and osteoconductive functions. The bioactive agents, such as a growth factor, an ingredient of Chinese medicine, an antibody, or a gene, contribute to the osteoinductive action. The material, which acts as a scaffold, forming the drug delivery system should contribute to the osteoconductivity.

Modern bioengineering has evolved from medical devices that imitate the architectural features and mechanical properties of tissues to be repaired or regenerated to biofunctional devices that aspire tointeract with the host and through their sustained and localized release of their cargo promote functional repair and regeneration. The dynamic and spontaneous of this naturally occurring process is influenced by various extrinsic conditions, such as the PH, temperature, light, ion nature, and concentration, redox potential, overexpression of a chemical or biological signal[19].

The drug release from a system can be manipulated by different immobilization techniques[20]. These can depend on covalentor non-covalent binding. Covalent binding uses a linker or direct coupling. Many growth factors possess for instance a heparin-binding domain, which can be used for the covalent coupling. Non-covalent binding relies on three different modes (Fig. 1):1. Entrapment; 2. Adsorption; 3. Complexation[21].

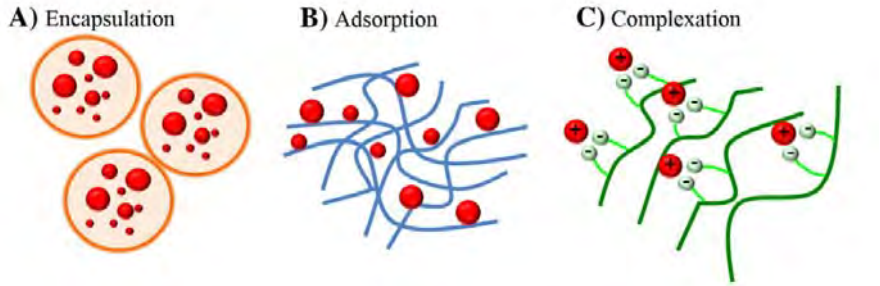

Fig. 1. Modes for non-covalent immobilization of drugs for bone regeneration.

Biomimetic calcium phosphate for bone regeneration

Recently, the biomimetic calcium phosphate (BioCaP) has been very well developed. The BioCaP with an internal depot of protein has the capacity to maintain a slow and sustained protein release in the presence of osteoclasts *in vitro*. The good biocompatiblility and osteoconductivity of BioCaP may be attributed to its biomimetic chemical property. The drug release system using BioCaP granules is a promising tool for an orderly delivery of several therapeutic agents, such as antibiotics, osteogenic agents, and anti-cancer drugs for different clinical applications[22].

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Chapter 2

Icariin: Does It Have An Osteoinductive Potential for Bone Tissue Engineering?

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ABSTRACT

Traditional Chinese Medicines (TCMs) have been recommended for bone regeneration and repair for thousands of years. Currently, the Herba Epimedii and its multi-component formulation are the attractive native herbs for the treatment of osteoporosis. Icariin, a typical flavonol glycoside, is considered to be the main active ingredient of the Herba Epimedii from which icariin has been successfully extracted. Most interestingly, it has been reported that icariin can be delivered locally by biomaterials and that it has an osteoinductive potential for bone tissue engineering. This review focuses on the performance of icariin in bone tissue engineering and on blending the information from icariin with the current knowledge relevant to molecular mechanisms and signal pathways. The osteoinductive potential of icariin could be attributed to its multiple functions in the musculoskeletal system which is involved in the regulation of multiple signaling pathways in anti-osteoporosis, osteogenesis, anti-osteoclastogenesis, chondrogenesis, angiogenesis, and anti-inflammation. The osteoinductive potential and the low price of icariin make it a very attractive candidate as a substitute of osteoinductive protein − bone morphogenetic proteins (BMPs), or as a promoter for enhancing the therapeutic effects of BMPs. However, the effectiveness of the local delivery of icariin needs to be investigated further.

Key words icariin, osteoinductive, BMPs, bone regeneration, bone tissue engineering

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Acknowledgment

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1. Introduction

1.1. Osteoinduction and osteoconduction in bone regeneration

The satisfactory bone regeneration of bone defects which are so large that they cannot heal by themselves remains a big problem for surgeons (Otto and Rao 2004). An ideal osteoinductive bone graft is always desired. The osteoinductive property has become the most important issue for bone substitutes. Autografts are the gold standard due to their osteoconductive and osteoinductive properties, while they are unfortunately associated with a limited availability as well as with pain and morbidity at the donor site (Ahlmann *et al.*, 2002). The use of allografts or xenografts can overcome these problems but they are associated with possible infections and immune responses (Bauer and Muschler 2000; Stevenson 1998; Donos *et al.*, 2004). At present, synthetic bone substitutes such as calcium phosphate based biomaterials have become widely used in clinics because of their high osteoconductivity (Dorozhkin 2010), but most of them lack an intrinsic osteoinductivity. Consequently, bone growth factors and mesenchymal stem cells are usually introduced into the system to render these synthetic biomaterials osteoinductive followed by protein or gene delivery (Cowan *et al.*, 2004; Franceschi *et al.*, 2004; Byers *et al.*, 2004; Yamamoto *et al.*, 2000). Osteoinductive growth factors such as bone morphogenetic proteins (BMPs) have been widely studied in bone tissue engineering (Langer 2009). In particular BMP-2 and BMP-7 were usually carried (delivered) by bone substitutes, and thus confer osteoinduction on the bone substitutes (Liu *et al.*, 2010b; Magin and Delling 2001). They have been applied in clinically. BMP-2 was used to expedite and augment spinal fusion (Shimer *et al.*, 2009), to heal open tibia fractures (Garrison *et al.* 2010; Alt *et al.*,2006), and to augment the alveolar bone (Casado *et al.*, 2010; Tonetti and Haemmerle *et al.*, 2008); BMP-7 was used to promote the healing of bony non-unions (Schmidmaier *et al.*, 2009).

1.2. Clinical and economic backgrounds

Nowadays the implantation of bone substitutes for bone repair and augmentation is fairly routine clinically and the postoperative healing follows a predictable course in most patients resulting in a good long-term functional outcome (Mordenfeld *et al.*, 2010; Ozkan *et al.*, 2011). However, the expectations of surgeons and patients are continually increasing and aspiring to a shortening of the recovery (Rustemeyer and Bremerich 2007). Large amounts of BMPs are required in some cases for osteoinduction and for a further improvement in bone formation (Seeherman *et al.*, 2006, Dickerman *et al.*, 2007). The

devices containing BMPs tend to fail in a certain percentage of cases, and thereby raise concerns about costs and safety (Geesink *et al.*, 1999; Lieberman *et al.*, 2002; Bridwell *et al.*, 2004). The high price and the rapid degradation of BMPs are its major shortcomings and limit its use clinically. (Urist 1965; Zhao *et al.*, 2006). Therefore, there is an impending need to develop alternative methods to overcome these limitations (Zhao *et al.*, 2008). Attempts have been made to reduce the dose of BMP and so raise the efficiency, such as the use of biomimetic calcium-phosphate coating (Liu *et al.*, 2010b) and polymers mixed with calcium-phosphate cements (Ruhe *et al.*, 2005) which give a sustained release of BMP. Most of these attempts have been effective but remain in a preclinical stage. There remains the need for improving the loading efficiency of BMP to reduce the amount of BMP used. All in all, this research is still on the way to developing a simple, efficient and cost effective method.

1.3. Traditional Chinese Medicine in bone regeneration

Traditional Chinese medicines (TCMs) are considered as good alternatives for bone regeneration (Shang *et al.*, 1987). It becomes of great interest to combine bone substitutes with TCMs used for bone regeneration (Zhao *et al.*, 2010). TCMs are divided into single component and multi-component formulations. One multi-component formulation contains many kinds of herbs, whereas one single component contains only one herb. Some of TCMs have been recommended for bone regeneration for hundreds of years (Putnam *et al.*, 2007). A variety of TCMs for bone regeneration have been widely studied (see Table 1). They have shown positive effects on the treatment of osteoporosis, and can stimulate the proliferative activity of osteoblasts, inhibit the formation of osteoclasts, prevent bone loss, and increase the bone mineral density (Zhu *et al.*, 2012; Qin *et al.*, 2005; Xu and Lawson 2004; Lee *et al.*, 2005).

Among these TCMs, the Herba Epimedii and its multi-component formulation ‘Xian Ling Gu Bao’ (XLGB) have icariin as their main ingredient. Recently, it was reported that icariin is safe, non-toxic, inexpensive and osteoinductive (Wu *et al.*, 2009b; Zhao *et al.*, 2010), and this makes it a very attractive potential agent for bone tissue engineering. It was demonstrated that icariin enhanced the osteogenic induction activity of BMP-2 in a fibroblastic cell line (Zhao *et al.*, 2010) and induced osteogenic differentiation of preosteoblastic cells (Zhao *et al.*, 2008). After the intramuscular implantation in the backs of rats for three months, new bone formation was observed in β- tricalcium phosphate (TCP)

ceramic loaded with icariin but not in the β-TCP ceramic alone (Zhang *et al.*, 2011a). All these studies indicate the highly positive effects of icariin on bone formation. Especially, the ectopic bone formation strongly proved the potential of osteoinduction of icariin (Zhang *et al.*, 2011a). Therefore, it is of great interest that icariin may be used as a substitute for BMP, or as a promoter to enhance the therapeutic effects of BMP and so reduce the dose of BMP. Potentially there is an application for icariin in bone tissue engineering.

Hereby, we review the performance of icariin for bone tissue engineering with the current knowledge relevant to molecular mechanisms and signal pathways. The aim of this review is to clarify whether icariin has osteoinductive potential. The publications in the regard of icariin and bone tissue regeneration were selected using following keywords: icariin AND (bone* OR osteoblasts* OR osteoclasts* OR chondrocytes*). Databases were searched from the earliest date available until 1 April 2013. The initial literature search resulted in 52 articles from PubMed, 104 from ISI, and 2 from Cochrane. After screening all titles and abstracts, 32 articles from PubMed and 41 from ISI were considered to be eligible for this study. The exclusion of 32 duplicates resulted in a total of 41 articles, as shown in Figure. 1. All references in the selected manuscripts were reviewed in order to ensure that no papers had beenmissed with the chosen search strategy.

2. What is icariin?

Icariin ($C_{33}H_{40}O_{15}$, molecular weight: 676.67) was recorded in the Chinese pharmacopoeia for the purpose of anti-rheumatics (anti-inflammation), tonics (health promotion), and aphrodisiacs (Hsieh *et al.*, 2010). It is a prenyl flavonoid glycoside with a glucosyl group on C-3; a rhamnosyl group on C-7; a methoxyl group on C-4; and a prenyl group on C-8 position (Figure. 2). This prenyl group on C-8 could be the active group that takes part in osteoblastic differentiation and explains its greater potency in osteogenesis and mechanisms of action (Ma *et al.*, 2011). The metabolites profiles in plasma revealed that glucuronide conjugates of isoflavonoids and flavonoid aglycones were the major circulating forms of icariin (Qian *et al.*, 2012).

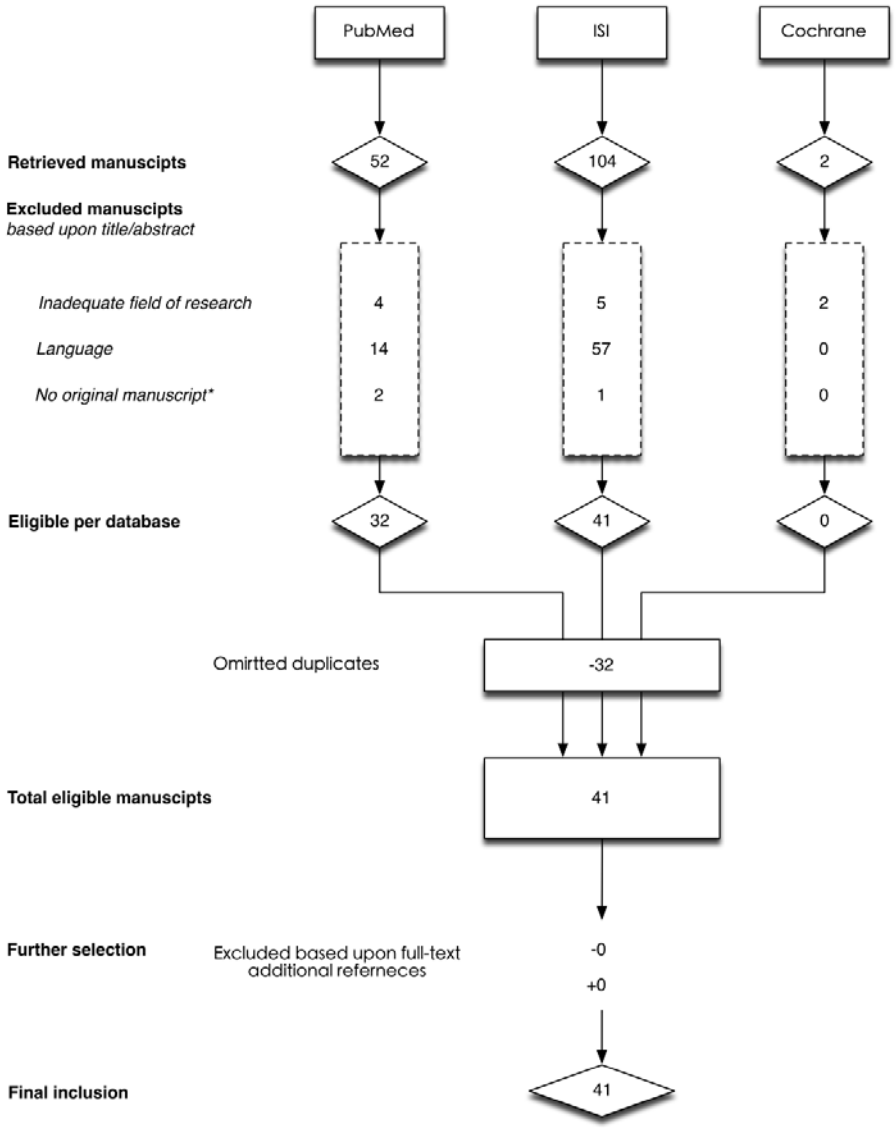

Figure. 1 Flow diagram of literature selection process. *E.g. reviews, letters.

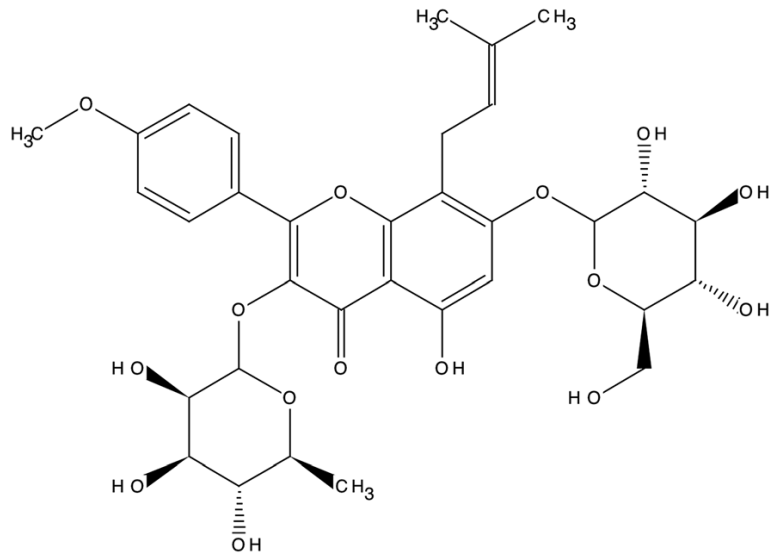

Figure 2. Chemical structure of icariin (Ma et al., 2011).

Through the development of modern separation techniques, Icariin has been extracted successfully as a bone active ingredient from Herba Epimedii (Nian *et al.*, 2009, Hsieh *et al.*, 2010). A rapid and accurate reversed-phase liquid chromatography-tandem mass spectrometry method has been developed and validated for the quantitative determination of the flavonoid glycosides in Herba Epimedii (Islam *et al.*, 2008). Icariin can also be extracted and purified by an ultrasonic technique (Zhang *et al.*, 2008a, Jia *et al.*, 2011) and by Dual-Mode HSCCC (Li and Chen 2009).

3. Icariin-based multi- or single- component formulation

3.1 Xian Ling Gu Bao

Xian Ling Gu Bao (XLGB) is a phytoestrogen-rich multi-component formulation containing Epimedin B, Epimedin C and icariin (Guan *et al.*, 2011). These three flavonoids were all from the Herba Epimedii. XLGB is one such herbal medication officially approved by the Chinese Food and Drug Administration and is orally administered intermittently in the treatment of osteoporosis (Zhu *et al.*, 2012). The ingredients of XLGB consist of six non-leguminous herbs with percentages in weight as follows: Herba Epimedii (70%), Radix Dipsaci (10%), Radix Salviae Miltiorrhizae (5%), Rhizoma Anemarrahenae (5%), Psoralea Corylifolia L. (5%), and Rehmannia Glutinosa (5%) (Guan *et al.* 2011). Recently, both

qualitative and quantitative methods were established for the comprehensive quality control of XLGB. Using high performance liquid chromatography coupled with diode array detection and electrospray ionization tandem mass spectrometry, a total of 47 compounds were identified from XLGB (Guan *et al.*, 2011). XLGB prevented a deterioration of musculoskeletal tissues induced by ovariectomy (OVX). (Qin *et al.*, 2005). The treatment over one year with the conventional dose of XLGB demonstrated a safe and a statistically significant increase in bone mineral density in the lumbar spine after 6 months in postmenopausal women (Zhu *et al.*, 2012).

3.2 Herba Epimedii

Icariin is the main pharmacological component of Herba Epimedii. Herba Epimedii is a centuries old traditional medicine herb and its formulation is one of the most frequently prescribed herbs (Pei and Guo 2007). It is recorded in the Chinese pharmacopoeia as ‘yin yang huo’ and was used to cure bone diseases such as osteoporosis and bone fracture in ancient China. Herba Epimedii can be considered as a complementary and alternative medicine for treatment of postmenopausal osteoporosis (Xie *et al.*, 2005; Zhang *et al.*, 2007). It was shown that the Herba Epimedii can promote the proliferation, the differentiation and the expression of osteoprotegerin (OPG) mRNA of the osteoblasts cultured *in vitro* (Liu *et al.*, 2006; Meng *et al.*, 2005a). Core binding factor alpha1 (Cbfa1) is a member of the runt family of transcription factors, which appears to play a pivotal role in regulating the differentiation of osteoblastic precursors and the activity of mature osteoblasts. Herba Epimedii could increase the expression of Cbfa1 mRNA in the bone of ovariectomized rats depending on the dose. Furthermore, a high dose of Herba Epimedii of 160 mg/kg administered for 12 weeks *in vivo* stimulated osteocalcin expression (Qian *et al.*, 2006).

4. Icariin applications in bone tissue engineering

The applications of icariin in bone tissue engineering are summarized in Table 2. In order to enhance bone formation for the repair of bone defects, icariin, was loaded into porous beta-tricalcium phosphate ceramic (ICA/beta-TCP) disks (Zhang *et al.*, 2011a). It was revealed that loading icariin in Ica/beta-TCP disks hardly affected the attachment and morphology of rat osteoblast-like (Ros17/28) cells, supporting the proliferation and differentiation of the cells at a higher level than the porous beta-TCP ceramic (beta-PTCP)

disks. After intramuscular implantation in the back of rats for three months, no obvious osteogenic evidence was detected in beta-PTCP disks, but new bone formation was observed in ICA/beta-TCP disks. These results indeed prove a potential of osteoinductive property of icariin.

More and more studies reported the application of icariin combined with calcium phosphate biomaterials. Calcium phosphate cement (CPC) loaded with Icariin filled in the mouse calvarial bone defect induced significant new bone formation and increased bone thickness (Zhao *et al.*, 2010). Obvious blood vessel formation was also observed in the icariin induced new bone in the calvarial bone defect. Moreover, by the thorough mixing of icariin and chitosan/hydroxyapatite (ICA-CS/HA) using a freeze-drying technique, a new bone repair scaffold was generated (Wu *et al.*, 2009b). The results showed that ICA-CS/HA had favorable cell compatibility and promoted osteogenic differentiation of human bone marrow stem cells (hBMSCs). The controlled release of icariin was satisfactory and the release retained after 90 days *in vitro*. Most interestingly, ICA-CS/HA scaffolds showed favorable osteoconduction and osteoinduction *in vivo*. They could fill bone segment defects and stimulate new born bone tissues formation at early stage. Recently, another icariin-loaded chitosan/nano-sized hydroxyapatite system was developed, which also controls the release kinetics of icariin to enhance bone repair (Fan *et al.*, 2012). The *in vitro* bioactivity assay revealed that the loaded icariin was biologically active.

Due to the development of the carrier as mentioned above, icariin administered locally can be more efficient for the local bone repair than a systemic administration. For example, the gastrointestine may reduce the therapeutic effect of icariin given orally. Therefore, the use of icariin for bone tissue engineering should concentrate on administration locally rather than systemically.

5. Underlying mechanisms of icariin for bone regeneration

5.1 Anti-osteoporosis

Icariin has a definite anti-osteoporotic effect which is similar to estrogen and it is especially effective for the prevention of bone fractures induced by an estrogen deficiency (Nian *et al.*, 2009; Liu *et al.*, 2012). The anabolic effects of icariin in bone possibly result from activating the estrogen receptor in a ligand-independent manner. Research delineates the mechanism by which icariin prevented bone loss after ovariectomy. Icariin suppressed the loss of bone mass and increased the strength in distal femur and the mRNA expression ratio

of OPG/RANKL in tibia (Mok *et al.*, 2010). Oral administration of icariin could promote bone formation during mandibular distraction osteogenesis and might be a promising method for shortening the course of distraction osteogenesis (Wei *et al.*, 2011). OVX rats treated orally with icariin could improve the degree of bone mineralization and bone strength and also prevent the suppression of serum calcium phosphorus and 17β-oestradiol (Nian *et al.*, 2009). The oral administration of icariin, limited the metabolism of the medicine due to the gastrointestine. Icariin propylene glycol-liposome suspension (ICA-PG-liposomes) injected intraperitoneally in mice changed the pharmacokinetic behavior (Yang *et al.*, 2012). With improved pharmacokinetics, ICA-PG-liposomes might be developed as promising carriers for icariin injection. Consequently, the use of icariin locally should be considered for future clinical applications.

5.2 Osteogenesis

The investigation of icariin on rat bone marrow stroma cells revealed an enhancement of the osteogenic differentiation of these cells. A higher concentration of icariin in the extract caused more mineralized bone nodules and higher levels of calcium deposition. The gene expression involved in osteogenesis was also improved, including alkaline phosphatase, bone matrix protein (osteocalcin, osteopontin, bone sialoprotein) and cytokines (TGF-β1 and IGF-I) (Chen *et al.*, 2007b). The effect of icariin on the proliferation of human marrow stroma cells was found to be dependent on the dose and it could also enhance the osteogenic differentiation of these cells in a suitable range of concentrations (Fan *et al.*, 2011). Icariin may strengthen the bone by enhancing the osteogenic differentiation of bone marrow stroma cells, which partially explains the anti-osteoporotic action of the Epimedium herb.

When icariin was added to osteoblasts, it promoted the proliferation of human osteoblast and MC3T3-E1 cell lines (Guo *et al.*, 2011; Cao *et al.*, 2012). However, a certain concentration of icariin showed no effect on the proliferation of rat osteoblasts (He *et al.*, 2009). Osteoprotegerin (OPG) plays an essential role in beneficial effects of icariin on bone (Zheng *et al.*, 2012). It was also reported that icariin significantly promoted the expression of type I collagen and osteopontin (OPN) mRNA in rat osteoblasts, and the expression was strengthened gradually with increasing concentration of Icariin (Xiao *et al.*, 2005). Icariin with final concentration of 1×10^{-5} mol/L, which was the best concentration, significantly enhanced the osteogenic differentiation and maturation of rat osteoblasts. It improved

significantly the secretion of collagen I, CFU-F(ALP) amounts and mineralized nodules and it also enhanced the mRNA level of Cbfa1 and Osterix (Zhai *et al.*, 2011; Ming *et al.*, 2011). Furthermore, the Cbfa1, BMP2, BMP4 and mRNA were significantly up-regulated after icariin treatment (He *et al.*, 2009). It was suggested that icariin exerts its potent osteogenic effect through the induction of Cbfa1 expression, the production of BMP-4 and the activation of BMP signaling (Zhao *et al.*, 2008). The osteogenic effect was inhibited by the introduction of Smad6 or dominant-negative Cbfa1, as well as Noggin treatment. It was demonstrated that icariin is a bone anabolic agent that may exert its osteogenic effects through the induction of BMP-2 and nitric oxide (NO) synthesis, subsequently regulating Cbfa1/Runx2, OPG, and RANKL gene expressions (Figure. 3) (Hsieh *et al.*, 2010). NO regulates the Cbfa1/Runx2 gene expression, and these effects may contribute to the induction of osteoblasts proliferation and differentiation. Meanwhile BMP-2/Smad suppresses capsase-3 activities and thus inhibits apoptosis of osteobalsts and hence improves the survival of osteoblasts. In a recent study, icariin up-regulated the expression of BMP-2, Smad4, Cbfa1/Runx2, OPG, RANKL and the OPG/RANKL ratio, indicating that icariin can modulate the process of bone formation via the BMP-2/Smad4 signal transduction pathway in human osteoblastic cell line (Liang *et al.*, 2012).

5.3 Anti-osteoclastogenesis

Icariin inhibited osteoclastic differentiation in both osteoblast-preosteoclast co-culture and osteoclast progenitor cell culture, and reduced the motility and bone resorption activity of isolated osteoclasts (Huang *et al.*, 2007a). It can be concluded that icariin has the ability to inhibit the formation and bone resorption activity of osteoclasts (Chen *et al.*, 2007a). This in turn, supports the use icariin as an effective component for strengthening bone. In a recent study, icariin decreased osteoclast numbers and activity levels, and increased OPG/RANKL expression ratios, evoking a reparative effect on rapid palatal expansion induced root resorption in rats (Wang *et al.*, 2012). The detail molecular mechanisms of icariin on anti-osteoclastogenesis were further examined (Hsieh *et al.*, 2011). It was demonstrated that a low dose of icariin inhibited LPS-induced osteoclastogenesis without losing cell viability. Icariin can also inhibit LPS-induced pro-inflammatory cytokines synthesis and scavenge LPS-induced RANKL up-regulation and OPG down-regulation. Icariin decreased LPS-mediated prostaglandin E2 (PGE2) production by inhibiting the cyclooxygenase-2 (COX-2) synthesis of osteoblasts and osteoclasts. In osteoclasts, icariin suppressed LPS-mediated activation of the IκB, Jun N-terminal kinase (JNK), extracellular

regulated protein kinases (ERK1/2), p38, and Hypoxia-inducible factor 1α (HIF-1α) pathways. While in osteoblasts, only IκB and ERK1/2 pathways were involved. It can be concluded that Icariin inhibited LPS-induced osteoclastogenesis by suppressing the activation of the p38 and JNK pathway (Figure. 4).

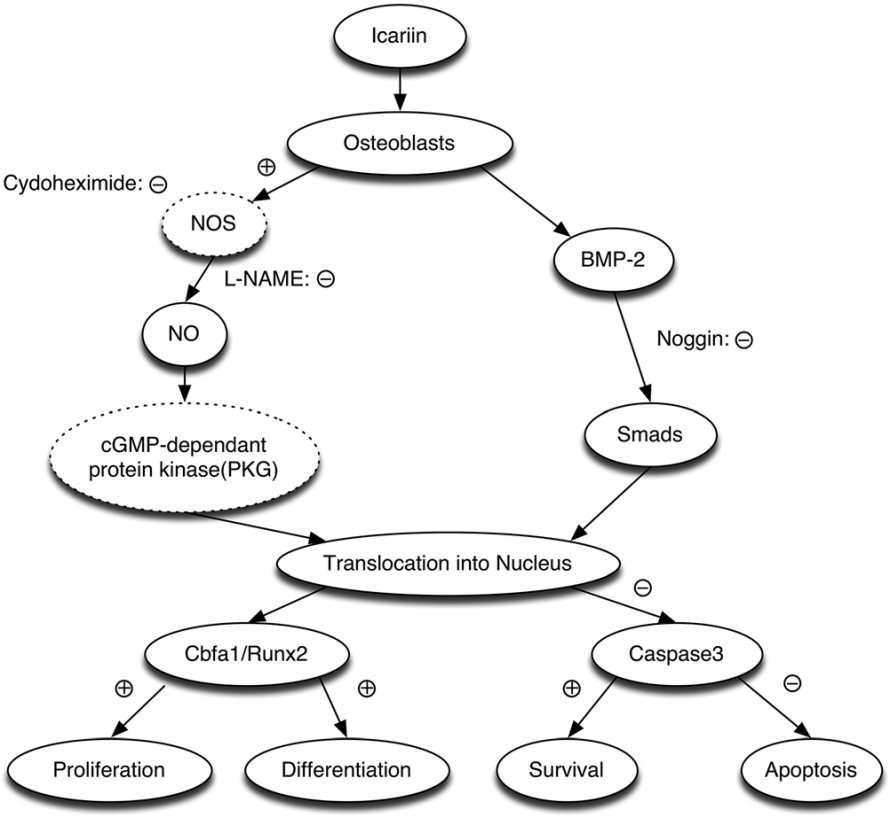

Figure 3. Molecular mechanism of the anabolic effect of icariin on osteoblasts (Hsieh et al., 2010).

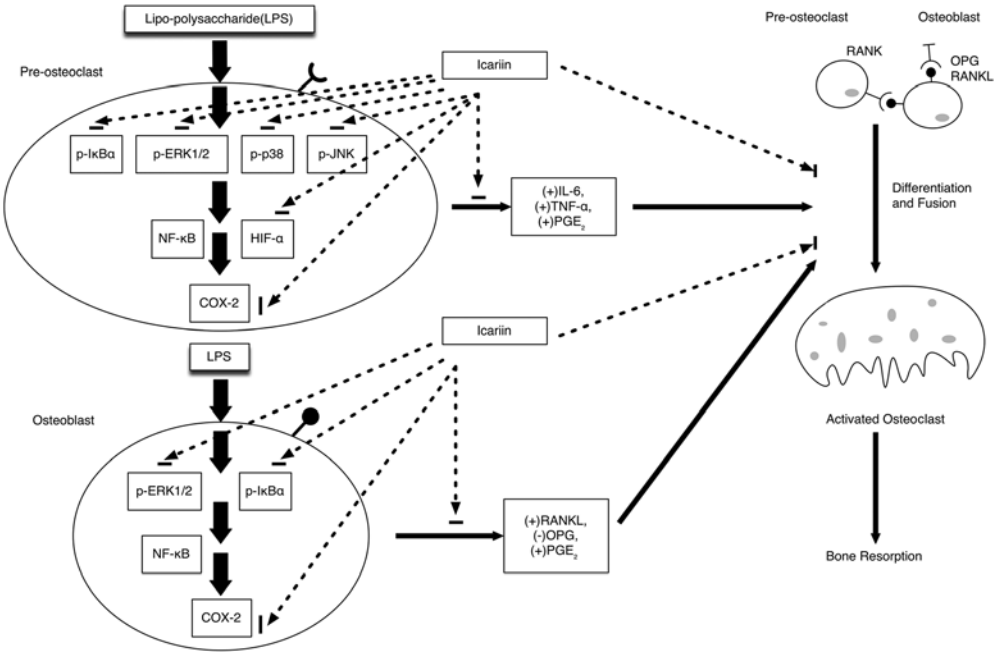

Figure 4. Molecular mechanism of icariin on the LPS-induced osteoclastogensis (Hsieh et al., 2011).

5.4 Chondrogenesis

Icariin is a safe anabolic agent for chondrogenesis (Liu *et al.*, 2010a). When rabbit chondrocytes isolated from articular cartilage were cultured *in vitro* with different concentrations of icariin, the higher concentration of icariin produced more extracellular matrix synthesis and expression of chondrogenesis genes of chondrocytes (Zhang *et al.*, 2012a). The effect of icariin on the synthesis of glycosaminoglycans (GAGs) and collagen of chondrocytes, and its potent chondrogenic effect, might be due to its ability to up-regulate the expression of aggrecan, collagen II and Sox9 genes and to down-regulate the expression of the collagen I gene of chondrocytes (Li *et al.*, 2012). It also improves the efficiency of restoring of supercritical-sized osteochondral defects in adult rabbit model, and enhances the integration of newly formed cartilage with subchondral bone (Li *et al.*, 2012). These preliminary studies imply that icariin might be an effective accelerant for chondrogenesis and a substitute for the use of some growth factors. The biomaterials loaded with icariin might have a potential in bone and cartilage tissue engineering.

It is known that there are two mechanisms for bone formation. They are

intramembranous, which is direct bone formation, and endochondral ossification which is indirect bone formation on a cartilage intermediate. (Einhorn TA, 1998). The potential different mechanisms between chondrogenic and osteogenic differentiation are associated with two transcription factors, Sox9 and Cbfa1. The transcription factor Sox9 acts during early chondrogenic differentiation (Bi et al., 1999), while Cbfa1 is essential for osteoblast differentiation (Qian et al., 2006). Icariin can up-regulate the expression of Sox9 and Cbfa1 in controlling osteogenesis and chondrogenesis. However, more mechanisms need to be investigated in detail.

5.5 Angiogenesis

Vascularization is considered to be a crucial step in bone formation (Wernike *et al.*, 2010). Icariin stimulated *in vitro* endothelial cell proliferation, migration, and tubulogenesis, as well as increasing *in vivo* angiogenesis (Chung *et al.*, 2008). It was shown that Icariin has the protective effect on injured vascular endothelial cells, which may be related to its anti-apoptosis effect (Ji *et al.*, 2005, Wang and Huang 2005). Icariin increases the endothelial nitric oxide synthase (eNOS) expression through activating the EGF-EGFR pathway in porcine aorta endothelial cells, by which the endothelial cell function could be regulated (Liu *et al.*, 2011). Moreover, icariin activated the angiogenic signal modulators, ERK, phosphatidylinositol 3-kinase (PI3K), Akt, and eNOS, and increased NO production, without affecting the expression of vascular endothelial growth factor. This indicates that icariin may stimulate angiogenesis directly (Xu and Huang 2007, Chung *et al.*, 2008). Therefore, it should be noted that Icariin stimulated angiogenesis by activating the MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways and it may also have a potential as a drug in angiogenic therapy (Koizumi *et al.*, 2010, Chung *et al.* 2008).

5.6 Anti-inflammatory

Anti-inflammation plays an important role in bone healing. For example, the treatment of bone defects in peri-implantitis in dentistry particularly needs anti-inflammation (Park 2011). Icariin has displayed its anti-inflammatory potential (Wu *et al.*, 2011). The partial mechanism could be the multiple link intervention on pro-inflammatory cytokines (TNF-α, IL-6), inflammatory mediators (NO) and adhesion molecules (CD11b) (Wu *et al.*, 2009a). Research on the anti-inflammatory effects of icariin on LPS-induced acute inflammatory and its molecular mechanism, suggests that activation of the PI3K/Akt pathway and the

inhibition of NF-kappaB are involved in the protective effects of icariin on lipopolysaccharide (LPS)-induced acute inflammatory responses (Xu *et al.*, 2010). Icariin may exert its protective effects through the inhibition of nitric oxide and matrix metalloproteinase (MMP) synthesis, and it may then reduce the destruction of the extracellular matrix (Liu *et al.*, 2010a). Recently, researchers have found an anti-inflammatory property of a novel derivative of icariin, 3, 5, 7-Trihydroxy-4'-methoxy-8 -(3-hydroxy-3-methylbutyl)-flavone (ICT) (Wu *et al.*, 2012). It was reported that Icariin and ICT exert anti-inflammatory and anti-tumor effects, and modulate myeloid derived suppressive cell (MDSC) functions (Zhou *et al.*, 2011). The anti-inflammatory effects of ICT were mediated, at least partially, via inhibition of the CD14/TLR4 signaling pathway. ICT reduced NO and PGE2 levels by inhibiting inducible NO synthase and cyclooxygenase-2 protein expression (Wu *et al.*, 2011). This icariin derivative inhibits tumor necrosis factor-alpha (TNF-α) production, inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA expression, and protein expression in LPS stimulated macrophages. Furthermore, ICT suppresses the activation of mitogen-activated protein kinase and inhibits translocation of nuclear factor (NF)-kappaB p65 to the nucleus through decreasing the phosphorylation of IkappaBalpha (Chen *et al.*, 2010). As a result of all these properties, icariin and its derivative can be considered as a potential drug for inflammatory diseases.

6. Toxicity of icariin

There was no cytotoxicity toward hBMSCs when the concentration of icariin was smaller than 10^{-6}M, whereas icariin can limit the cell viability when the concentration was larger than 10^{-5}M (Fan *et al.*, 2011). The cytotoxicity test of icariin on MC3T3-E1 cells (a pre-osteoblastic cell line) revealed that the cell viabilities varied from 88% to 98% on both days 1 and 3 after treating with different concentrations of icariin (range from 10^{-10}M to 10^{-5}M) for 72 hours (Zhao *et al.*, 2008). Icariin at a concentration of 5×10^{-5}M strongly inhibited the proliferation of osteoblast-like (Ros17/28) cells (Zhang *et al.*, 2011a). However, many studies demonstrated that icariin with concentration of 10^{-5}M had positive effect on the proliferation of UMR106 cell and human osteoblast (Meng *et al*., 2005a; Huang *et al.*, 2007a; Yin *et al*., 2005). Therefore, the optimal concentration of icariin with low cytotoxicity toward osteoblasts was equal to or less than 10^{-5} M (6.8 μg/ml) (Zhang *et al.*, 2011a). Additionally, more than 90% of murine macrophages (ANA-1) can survive at

concentrations up to 80 μg/ml icariin (Li *et al.*, 2011). In general, icariin is safe and non-toxic at low doses (Wu *et al.*, 2009b; Zhao *et al.*, 2010). At doses up to 120 mg/kg in rats given orally administration, icariin has low toxicity, but without overt toxic effects (Luo *et al.*, 2007).

7. Concluding remarks and perspectives

It is well known that BMPs induce a sequential cascade of events leading to chondrogenesis, osteogenesis, angiogenesis and the controlled synthesis of extracellular matrix (Kang *et al.*, 2004). BMP-2 and BMP-7 have been the most extensively evaluated BMPs with a very high price (Wang *et al.*, 2011). Although BMPs have an outstanding performance in bone formation, they also could result in some cases in negative effects (Kao *et al.*, 2012). Research has developed a variety of methods in bone tissue engineering to reduce the use of BMPs and to improve the osteoinductive effects of BMPs by a slow delivery (Liu *et al.*, 2010b; Ruhe *et al.*, 2005). However, one of the simplest ways could be to search for an effective and low cost substitute for the expensive BMPs. The perspectives discussed herein demonstrate the importance of exploiting an inexpensive osteoinductive drug.

More and more researches show that icariin has an osteoinductive potential, due to its properties of inducing osteogenesis, chondrogenesis and angiogenesis. The multiple function of icariin, especially the induction of osteogenesis, is remarkable. The loading of icariin in calcium phosphate biomaterials provides a good alternative to for delivering icariin locally for bone repair, since the calcium phosphate materials have been used as osteoconductive scaffolds. It has been known that the local use of icariin demonstrated positive effects in bone formation at an early stage (Wu *et al.*, 2009b). Several studies have tried to clarify the molecular mechanisms underlying the osteogenic effects. In summary, icariin may exert its osteogenic effects through the induction of BMP-2 and NO synthesis and the BMP-2/Smad4 signal transduction pathway, by up-regulating the expression of BMP-2, BMP-4, Smad4, Cbfa1/Runx2, OPG, RANKL and the OPG/RANKL ratio (Hsieh *et al.*, 2010; Liang *et al.*, 2012). Icariin can inhibit LPS-induced osteoclastogenesis by suppressing the activation of the p38 and JNK pathway (Hsieh *et al.*, 2011), which in turn contributes to strengthening the bone. The positive effects of icariin on a potent chondrogenic effect might be the up-regulation of the expression of aggrecan, collagen II and Sox9 genes and down-regulation the expression of the collagen I gene of chondrocytes (Zhang *et al.*, 2012a). However, the more detailed osteoinductive mechanisms and the

clinical applications of icariin need to be investigated further.

Compared with BMPs, icariin is cheaper and has low adverse effects (Zhao *et al.*, 2010; Wu *et al.*, 2009b). The extremely low cost and the high abundance of icariin and its excellent function for bone regeneration make it very appealing for clinical applications. Therefore, it could be candidate for an assistant of BMPs or as a substitution. Nevertheless, the effects of local use of icariin still need to be continually investigated and there is also a need for an appropriate carrier for the most effective delivery. According to the current studies and knowledge, it can be concluded that icariin can be a potential osteoinductive agent. We would like to prove that icariin indeed has a potential for bone tissue engineering. Several projects are running in our lab, both *in vitro* and *in vivo*. One of our studies was to use icariin that was incorporated into a biomimetic calcium phosphate bone substitute for the repair of critical-sized bone defects in the rat calvaria. On the whole, the developing techniques give us the confidence to believe that icariin might have a very bright future in bone tissue engineering.

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Table 1. Multi-component formulations and single components for bone health

Product Name	Multi-component formulations (M) or Single components (S)	Experimental subjects	Main effects	References
Xian ling gu bao	M	OVX rats; Postmenopausal women	BMD↑	Zhu *et al.*, 2012; Qin *et al.*, 2005
Shu di shan zha	M	Menopausal women	BR↓	Xu and Lawson 2004
Shen gu	M	Osteoporotic patients	BMD↑; BR↓	Mingyue *et al.*, 2005
Yang huo gu bao	M	Osteoporotic male rats	BMD↑	Liao *et al.*, 2001
Hachimi-jio-gan	M	OVX rats	BR↓	Hidaka *et al.*, 1997
Kami-kihi-to	M	OVX rats	BMD↑	Kanai *et al.*, 2005
Jian gu	M	OVX rats	BMD↑; TBA↑	Lin *et al.*, 2004
BushenNingxin	M	Osteoblasts; OVX mice	PA↑	Wang *et al.*, 2001
Dang-gui-ji-hwang-yeum	M	OVX rats	TBA↑; OCG↓	Chae *et al.*, 2004
Hochu-ekki-to	M	Rats	BMD↑	Sakamoto *et al.*, 2000
Herba Epimedii	S	Postmenopausal women; UMR-106 cells; Rat osteoblasts; OVX rats	BR↓; PA↑; mRNA of OPG↑; RANKL↓; cbfa1 mRNA↑; OCN↑	Zhang *et al.*, 2007; Meng *et al.*, 2005a; Liu *et al.*, 2005; Qian *et al.*, 2006
Sambucus williamsii	S	OVX rats; UMR106 cells	BMD↑; ALP↑; OCN↑; OCG↓	Xie *et al.*, 2005
Cistanche salsa	S	OVX rats	BR↓	Yamaguchi *et al.*, 1999
Red sage	S	Osteoclasts	OCG↓	Lee *et al.*, 2005
Drynariae rhizoma	S	Rats and mice; Human osteoprecursor cells	Cathepsins K and L↓	Jeong *et al.*, 2004; Jeong *et al.*, 2005
Puerariae radix	S	Castrated mice	BMD↑; TBA↑	Wang *et al.*, 2001
Astragalus membranaceous	S	OVX rats	BR↓	Kim *et al.*, 2003
Abelmoschus manihot (L.) Medik	S	OVX rats	BR↓	Shirwaikar *et al.*, 2003; Puel *et al.*, 2005
Wedelia calendulacea Less.	S	OVX rats	BR↓	Annie *et al.*, 2006
Sophorae fructus	S	OVX rats	BR↓	Joo *et al.*, 2004
Cimicifuga racemosa	S	OVX rats	BR↓	Nisslein and Freudenstein 2003

Table 2. Icariin in bone tissue engineering

Experimental subjects	Main effects	Carrier for loading icariin	References
Rabbits	BMD↑	Chitosan/hydroxyapatite	Wu et al. 2009b
	Cartilage formation↑	Cell-hydrogel constructs	Li et al. 2012
	BMD↑; Volumes of new bone↑; TBA↑; Trabecular separation↓	-	Wei et al. 2011
Rats	Bone formation↑	Porous â-TCP ceramic	Zhang et al. 2011a
	BMD↑; BR↓; Biomechanical strength↑; Serum estrogen, calcium and phosphorus↑; Root resorption index↓; Collagen↑; Osteoclast number and activity↓; OCN↑; OPG/RANKL↑; Cbfa1↑; Osteoblast-specific gene osterix (OSX)↑	-	Xue et al. 2012a; Xue et al. 2012b; Wang et al. 2012; Liu et al. 2012; Bian et al. 2012; Nian et al. 2009; Qin et al. 2008
Mice	Bone formation↑; Bone thickness↑	Calcium phosphate cement	Zhao et al. 2010
	TBA↑; OPG/RANKL↑	-	Zheng et al. 2012 Mok et al. 2010
BMSCs	ALP↑; Mineralized nodules↑; Proliferation↑	Chitosan/nano-size hydroxyapatite	Fan et al. 2012
	ALP↑; Proliferation↑	Chitosan/hydroxyapatite	Wu et al. 2009b
	Mineralized nodules↑; Proliferation↑; Differeatition↑; Calcium deposition↑; ALP↑; OCN↑; OPN↑; Bone sialoprotein↑; TGF-â1↑; IGF-I↑; Cbfa1↑; Collagen I↑	-	Fan et al. 2011 Chen et al. 2007b Chen et al. 2005 Bian et al. 2012
Osteoblasts	Proliferation↑	PHBV coatings	Dai et al. 2011
	ALP↑; Runx2↑; BSP↑; OCN↑; Mineralization↑	Calcium phosphate cement	Zhao et al., 2010
	Proliferation↑; Mineralization↑; Osteoblast colonies↑; Cell viability↑; Calcified nodules↑; ALP↑; Cbfa1↑; BSP↑; BMP-2↑; OPG↑; OPG/RANKL↑; RANKL↑; Smad4↑; NO↑; Collagen I↑; OCN↑; OSX↑;OPN↑; ERK1/2↓; IêBá↓; p38↑	-	Zhang et al. 2011a Zheng et al. 2012 Mok et al. 2010 Qin et al. 2008 Liang et al. 2012 Cao et al. 2012 Zhang et al. 2011b Ma et al. 2011 Hsieh et al. 2011 Hsieh et al. 2010 Zhao et al. 2008 Zhang et al. 2008b Zhang et al. 2008c Yin et al. 2007 Xiao et al. 2005 Meng et al. 2005a Meng et al. 2005b Huang et al.2007a Yang et al. 2013

Osteoclasts	Apoptosis and cell cycle arrest ↑; Osteoclastogenesis↓; Pit areas↓; Superoxide anion↓; TRAP↓; MMP-9↓; RANKL↓; OPG↑; IL-6↓; TNF-á↓; COX-2↓; PGE2↓; HIF-1á↓; p38↓; JNK↓	-	Zhang et al. 2012b Xue et al. 2012a Hsieh et al. 2011 Huang et al.2007a Huang et al.2007b Qin et al. 2008 Chen et al. 2007a
Chondrocytes	Viability↑; Extracellular matrix↑; NO↑; MMP-1,3,13↓; COX-2↓; iNOS↓; Aggrecan↑; Sox9↑; Collagen II↑; GAGs↑		Liu et al. 2010a Zhang et al. 2012a Li et al. 2012

Chapter 3

Osteogenic potential of icariin compared with recombinant human bone morphogenetic protein 2 *in vitro*: a preliminary study

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ABSTRACT

Icariin, the primary active ingredient of Herba Epimedii which has been used for decades to treat bone related maladies in China, has the ability to support bone regeneration. In this study, we investigated icariin's potential to stimulate osteogenesis using an *in vitro* study to compare icariin's ability as a function of time and dose to induce osteoblastogenesis in MC3T3-E1 cells with that of recombinant human bone morphogenetic protein 2 (rhBMP2).The optimal concentration of icariin in stimulating the proliferation of MC3T3-E1 was 10^{-5}M. There is no significant difference between icariin and rhBMP2 relative to cell proliferation, alkaline phosphatase (ALP) activity, and osteocalcin (OCN) expression. Icariin had better ALP activity after a 7-day culture and OCN expression after 4 and 10 day cultures compared with rhBMP2. Moreover, icariin produced more mineralized nodules of a larger area than rhBMP2. However, compared with rhBMP2, icariin only showed a significantly higher ALP gene expression on day 4, a 3-fold BMP2 expression on day7, and Col1 expression on day4. In conclusion, compared with rhBMP2, the results indicate that icariin could be a good option for bone regeneration clinically, since it is an inexpensive and safe drug with an excellent osteogenesis potential.

Key words: icariin; recombinant human bone morphogenetic protein 2; osteogenesis; alkaline phosphatase; ostocalcin; type Ⅰ collagen; runt-related transcription factor 2;

Introduction

Clinicians and patients alike are looking for a reduction of the period of recovery of bone related diseases [1]. Osteoinductive cytokines, such as bone morphogenetic proteins (BMPs), were applied to enhance bone formation [2]. However, the effective dose of recombinant human bone morphogenetic protein 2 (rhBMP2) is very high (milligram level) clinically [3] and it is quite expensive. So the cost is high and there is a series of potential side effects such as abnormal bone resorption [4] and unexpected bone formation in ectopic area [5]. Reducing the administered dose of rhBMP2 is a simple solution to the problem. But using a lower dose may compromise the therapeutic effectiveness of this agent.

In an attempt to find a cheaper alternative to rhBMP2, researchers have investigated a traditional Chinese medicine, icariin ($C_{33}H_{40}O_{15}$, molecular weight: 676.67), which has potential osteogenic properties for bone tissue engineering[6]. Icariin is one of the primary active ingredients of Herba Epimedii. Herba Epimedii, which is a centuries old traditional medicine herb, is recorded as “yin yang huo” in the Chinese pharmacopoeia and was used to cure bone diseases such as bone fracture and osteoporosis [7, 8]. Through the development of modern separation techniques, icariin has been extracted successfully from Herba Epimedii [9]. It has been recorded in the Chinese pharmacopoeia for the purpose of anti-rheumatics (anti-inflammation), tonics (health promotion), and aphrodisiacs [10]. The osteogenic potential and the low price of icariin make it an attractive candidate as a substitute of rhBMP2. Previous studies have demonstrated that icariin promoted osteoblast differentiation and mineralization *in vitro*[11, 12].It may exert its osteogenic effects through the induction of BMP2 and nitric oxide (NO) synthesis and the bone morphogenetic protein 2/drosophila mothers against decapentaplegic protein 4(BMP2/Smad4) signal transduction pathway [10, 13]. On the other hand, icariin is also able to reduce osteoclastogenesis [14] and enhancing the ratio of OPG/RANKL [15]. This effect of icariin may compromise a series of potential side-effects which were caused by the transiently high dosage of rhBMP2, such as the overstimulation of osteoclastic bone resorption [16]. Hitherto, the osteogenic effect of the icariin compared with rhBMP2 has been rarely investigated, and the detailed molecular mechanisms underlying the effects aren’t completely recognized.

Murine calvaria-derived MC3T3-E1 cell, a precursor of functionalized osteoblast which is established from the newborn mouse calvaria, is a useful model system to examine the potency of osteoinductive agents as it possesses ERs (ERα and ERβ) and it has been

reported that it retains the capacity to differentiate into osteoblasts [17, 18]

When MC3T3-E1 cells were stimulated by rhBMP2, the effective concentration range of rhBMP2 for DNA content was 5-50ng/ml. The initial effective dose of rhBMP2 concerning the ALP activity was 50ng/ml and OCN expression showed an increase depending on the dose from an initial concentration of 50ng/ml rhBMP2 [19]. Therefore, we identified 50ng/ml of rhBMP2 as the minimum concentration necessary for a significant osteogenic effect in MC3T3-E1 cells. Consequently, we used it as the basis for comparison in our experiment with icariin. In this study, we compared the osteogenic effects of icariin and rhBMP2 on MC3T3-E1 cells and the molecular mechanism using an *in vitro* culture system.

Materials and methods

1. Materials:

MC3T3-E1 cell line was acquired from Chinese Academy of Sciences (American Type Culture Collection (ATCC); Shanghai, China). Icariin (purity ≥99%) was purchased from Tauto Biotech (Shanghai, China). α-Minimal essential medium (α-MEM) was obtained from Genom Bio-medicine Co (Hangzhou, China). Fetal bovine serum (FBS) was the product of Hangzhou Tianhang Bio-technology Co (Hangzhou, China). Cell Counting Kit (CCK-8) was purchased from Donjindo (Kumamoto, Japan). LabAssayTM alkaline phosphatase (ALP) was from Wako (Osaka, Japan), osteocalcin (OCN) EIA kit was from Biomedical Technologies (Stoughton, MA). Bicinchoninic acid (BCA) Protein Assay kit was from Beyotime institute of biotechnology (Shanghai, China). RNeasy Mini Kit and RNase-Free DNase Set were from Giagen sample & assay technologies (Germany). Primescrip™ RT Reagent Kit and SYBR® Premix Ex Taq TM II were all from Takara Biotechnology (Dalian, China). Cell lysates, Alizarin red, L-ascorbic acid and β-glycerophosphate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

2. Study design:

This study has been divided into two parts. The first part was to choose the optimal concentration of icariin necessary to stimulate proliferation of MC3T3-E1 cells. The second part was to compare the osteogenic effects of icariin and rhBMP2 by examining cell proliferation, alkaline phosphatase (ALP) activity, and osteocalcin (OCN) expression. In

addition, ALP, BMP2, type Ⅰ collagen (Col1), OCN, runt-related transcription factor 2 (Runx2) mRNA expression assays were used. Three experimental groups were set up: 1) control group without rhBMP2 or icariin; 2) rhBMP2 group; 3) icariin group.

3. Method:

Cell culture

MC3T3-E1 cells were cultured in α-Minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). The medium was changed every 3 days. Exponentially growing cells were plated at 5×10^3 cells/well in 96-well plates for cell proliferation, at 2×10^5 cells/well in 6-well plates for ALP activity assay and OCN detection, or 1×10^4 in 48-well plates for alizarin red staining. After 24-h incubation, cells were subjected to a low-serum medium (2% FBS) for another 24 h and then followed by rhBMP2 treatment.

Icariin concentration selection test

To identify the optimal concentration for osteoblastic proliferation of icariin, a dose–response (10^{-4}M, 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M, 10^{-9}M, 0M) test of Cell Counting Kit-8 assay was performed. MC3T3-E1 cells were plated on 96-well plates (5000 cells/well). After preincubation for 24h in a humidified incubator, the cells were subjected to a low-serum medium (2% FBS) for another 24 h and then fresh media containing different concentrations of icariin were added. The cell numbers at 2 and 4 days were used as indicators of cell proliferation and determined using a CCK-8 assay. At predetermined times, the cells were washed three times with PBS to eliminate non-viable cells, and then incubated with 10 μl of CCK-8 solution for 1 h in the incubator. The optical density was then measured using a microplate reader at a wavelength of 450 nm. Three wells were tested in each group for each incubation time and the resulting absorbance for each of the wells was averaged. The optimal concentration producing the highest osteoblastic proliferation (absorbance measurement of the reaction product) was determined by comparing the absorbance readings of cultures with different icariin concentrations.

Cell proliferation assay

To investigate cell proliferation of MC3T3-E1 cells in response to icariin or rhBMP2, the number of cells was determined after stimulation for 2, 4 and 7 days using Cell Counting

Kit-8 as previously described.

ALP activity assay

To determine the early differentiation of preosteoblasts stimulated byicariin or rhBMP2, ALP activity and protein content were measured after icariin or rhBMP treatment on day 4, 7 and 10. ALP activity in the cell lysates was determined using the LabAssay ALP colorimetric assay kit. The cell number was estimated by determining total protein content measured at 570nm using a commercial BCA Protein Assay kit. The values representing ALP activity were expressed as mmol p-NP/mg total protein.

OCN expression assay

To assess the terminal differentiation of MC3T3-E1 cellsstimulated by icariin or rhBMP2, OCN secreted into the cell culture medium was determined. The cell supernatants were collected on days 4, 7 and 10 and were centrifuged (10,000 rpm, 4℃, 5 min) before detection. The supernatant OCN concentration was determined using the ELISA mouse OCN EIA kit.

Cell matrix mineralization

We compared the mineralization of MC3T3-E1 cells stimulated by either icariin or rhBMP2. Triplicate cell cultures were prepared in the same way as described previously and then treated with mineralizing medium (10% FBS, 50mg/mL L-ascorbic acid-2-phosphate (AsAP), and 10mM β-glycerophosphate (β-GP)) containing rhBMP2 or icariin. The medium was replaced every 3 days. After 27 days, mineralized nodules were determined by alizarin red staining. Culture plates were photographed by NIS-Elements F2.20 (Nikon Eclipse 80i, Tokyo, Japan), and the calcified area was quantified using Image-Pro Plus 6.0 analysis.

Real-time PCR Quantification of gene expression

Effects of icariin or rhBMP2 in stimulating osteogenic gene expression were also examined by quantitative RT-PCR. The messenger ribonucleic acid (mRNA) expression of ALP, BMP2, osteocalcin (OCN), collagen-I (Col I), and runt-related transcription factor 2 (Runx-2) was quantitatively determined after 1d, 4d and 7d of osteogenic induction. Total

RNA was extracted from the cells using RNeasy Mini Kit and RNase-Free DNase Set. Single stranded complementary deoxyribonucleic acid (cDNA) was synthesized from total ribonucleic acid (RNA) with a Primescrip™ RT Reagent Kit. Real time polymerase chain reaction (PCR) was performed using 1μl of cDNA product in a 25μl reaction volume with Mastercycler ep realplex Real Time PCR System (Eppendorf, Germany). SYBR® Premix Ex TaqTM II, specific primers (see below), and 1μl of cDNA were used in each PCR reaction (95℃ for 30 s, 40 cycles of denaturation at 95℃ for 5 s, and annealing and extension at 60℃ for 30 s). The sense and antisense primers (see Table1) were designed with the Primer Express 3.0 based on published mouse cDNA sequences. β-actin was used as an internal control gene. All real - time PCR reactions were performed in triplicate. The results were calculated using the comparative threshold cycle (DDCT) method - after calibration with β-actin expression - and are presented as fold increase relative to the non-stimulated control.

Statistical analysis

Statistical comparisons between results obtained with rhBMP2 or icariin were made by a one way analysis of variance. Post hoc comparisons were made using Bonferroni corrections. The level of significance was set at $p<0.05$. Statistics package for social science (SPSS) software (version 17, SPSS Inc., Chicago, IL) for a Windows computer system was employed for the statistical analysis.

Results

Selection of icariin concentration

The icariin concentration of 10^{-5}M resulted in the highest proliferation of cells compared with other concentrations (Fig. 1). Therefore, this concentration was used in subsequent experiments.

Cell proliferation

The effects of icariin (10^{-5}M) and rhBMP2 (50ng/ml) on cell proliferation were determined. Both drugs significantly enhanced the cell proliferation at all the three times compared with the control (* $P<0.05$, ** $P<0.01$, Fig. 2). There were no significant differences between icariin and rhBMP2.

ALP activity

Treatment with both icariin and rhBMP2 resulted in a significant higher ALP activity than the control group at all three times (* $P<0.05$, ** $P<0.01$). Moreover, ALP activity induced by icariin was significantly higher than the rhBMP2 after a 7-day culture ($P<0.01$), but there were no significant differences between icariin and rhBMP2 after 4 and 10 day culture (Fig. 3).

OCN expression

Icariin induced significantly higher OCN expression than the control at all three times, and significantly higher than rhBMP2 at 4 and 10 day times (* $P<0.05$, ** $P<0.01$, Fig.4). However, rhBMP2 did not induce a higher OCN expression than the control at the 4-day time.

Cell matrix mineralization

There were far more areas of mineralized nodules in the icariin group compared with the rhBMP2 and control groups after 27-day culture ($P<0.01$, Fig. 5). No significant differences were found between rhBMP2 and the control.

Real-time PCR quantification of gene expression

Icariin significantly increased ALP gene expression compared with the control on day 4 and 7, while rhBMP2 resulted in significantly higher ALP expression on day 1 and 7 (Fig. 6A). Icariin showed significantly higher ALP expression compared to rhBMP2 only on day 4.

Stimulation by icariin induced an almost 3 fold increased expression of BMP2 than the other two groups on day 7($P<0.01$, Fig. 6B). Stimulation by rhBMP2 resulted in a higher BMP2 expression compared with the control on day 4.

Both icariin and rhBMP2 induced significantly higher Col1 expression than the control on day 1 ($p<0.05$, Fig. 6C). Icariin produced significantly higher Col1 expression than rhBMP2 on day 4, but significantly lower expression than rhBMP2 on day 7.

The stimulation effect of icariin on OCN expression was about 3 times higher than the control on day 4 and 7 ($P<0.01$). However, stimulation by rhBMP2 resulted in higher expression of OCN than icariin for all three times. (Fig. 6D).

There were no statistical differences observed between icariin and rhBMP2 on Runx2 gene expression on day 1. Both of them significantly increased Runx2 expression compared

with the control ($P<0.01$). However, rhBMP2 showed significantly higher Runx2 expression than icariin on day 4. Afterwards, there were no significant differences among the three groups on day 7 (Fig. 6E).

Discussion

Many agents exert their promoting effect on osteogenesis through stimulating the endogenous BMPs, such as icariin [20], simvastatin and Vitamine D3 [21]. Among them, icariin has become a hot focus in the field of bone regeneration because of its low cost and osteogenic potential. .In the present study, we aimed to evaluate the osteogenic effects of icariin on MC3T3-E1 cells and to compare it with rhBMP2. Icariin of 10^{-5}M was found to be the optimal concentration for the induction of osteogenic proliferation. This is comparable to values derived in previous studies [22-24]. This concentration was reported as an optimal concentration of icariin for the up-regulation increased regulation of bone related gene expression, since a higher concentration may result in toxicity to cells [22, 23]. The toxicity of drugs is always the first concern for its clinical use. Previous studies have proved that icariin is safe and that it has no cytotoxicity at low doses ($\leq 10^{-5}$M) [22, 23, 25].

Icariin induced osteogenic differentiation in a Runx2, BMP2, NO, Smad4, osteoprotegerin (OPG) or receptor activator of nuclear factor-kB ligand (RANKL) dependent manner [10, 20, 22, 26]. Hsieh et al. [10] gave us a possible answer in that their result showed that Noggin (the bone morphogenetic proteins antagonist) blocked - cell proliferation, ALP secretion, and NO production in osteoblast cells when mediated with icariin. Consistent with previous findings, we found that icariin indeed has osteogenic effects. Mineralization is important for bone regeneration. Icariin alone increased the *in vitro* mineralization, surpassing even rhBMP2. Ohba et al [27] suggested two possible mechanisms for the involvement of BMP signaling in the effects of icariin. Firstly, icariin may activate BMP signaling indirectly through extracellular BMPs. Second, icariin may activate BMP signaling directly by interacting with Smads via unknown mechanisms. RhBMP2 promotes differentiation mainly through increased intracellular alkaline phosphatase activity, osteocalcin and collagen protein synthesis [28]. Our study further suggested that icariin did up-regulate the gene expression of BMP2; moreover, icariin produced more BMP2 mRNA than the rhBMP2 groups at the later time (Day 7). This indicates that icariin may activate BMP signaling indirectly through extracellular BMPs after BMPs are produced.

In our study there seemed to be no correlation between the protein and gene expressions in ALP or OCN. This could be partly attributed to the fundamental biological differences between the transcription and translation processes and partly to the experimental challenges [29]. The mRNA levels cannot be relied on to predict positively and consistently protein expression [30, 31].

Recently *in vitro* and *in vivo* studies showed that icariin/calcinated antler cancellous bone (CACB) composites (icariin at a concentration of 4.0×10^3 ng/ml) displayed positive effects on rBMSC attachment, proliferation, osteogenic differentiation and mineralization compared with rhBMP-2/CACB composites (rhBMP2 at a concentration of 100ng/ml). The quality and quantity of *in vivo* bone formation and neovascularization with the Icariin/CACB implant were superior compared with those of the rhBMP-2/CACB composites[32]. The author did not discuss the reason for these results in the article very much; our study may explain the results.

Bone regeneration is a delicate balance between bone formation and bone resorption. The effective doses of BMP homodimers for clinical use are high. The transiently high dosage of BMP-2 may lead to a series of potential side effects, such as the overstimulation of osteoclastic bone resorption [33]. On the other hand, icariin has been shown to inhibit lipopolysaccharide (LPS)-induced osteoclastogenesis by suppressing the activation of the p38 and Jun N-terminal kinase (JNK) pathway [14]. The anti-osteoclastogenesis effects of icariin may compromise the overstimulated osteoclastic bone resorption impact of BMP-2.
Dexamethasone (DEX) has been routinely used as the standard inducer of osteoblast differentiation [34-38]. However, high doses of DEX have been shown to inhibit osteoblast maturation and function[39]. In our study, icariin was found to support osteogenic differentiation and mineralization even in the absence of DEX. These findings are consistent with the article published recently [40]. This further indicates the osteogenic benefits of treatment with icariin alone.

Previous studies used different types of osteoblast to evaluate icariin. The viability of the primary osteoblasts, which were harvested from 8-month old female Imprinting Control Region mice, reached their maximum osteogenic benefit at 10^{-8} M icariin [10]. Cell viability in the presence of 10^{-9} M of icariin increased by $136.50\pm6.47\%$ in hFOB 1.19 human osteoblastic cell line cells compared to the control cells[20]. In the present study, we used MC3T3 cell line which has beenwidely used for evaluating osteoinductive agents. However, this could be a limitation of the study. The cell line might behave differently from

others such as bone marrow stromal cells or periosteal-derived cells [41]. Another limitation was that only one concentration of the agents was evaluated. More concentrations are going to be evaluated in the future studies *in vitro* or *in vivo*. In our ongoing studies, we are evaluating the combination of icariin and rhBMP2 at different concentrations to examine icariin's potential as a promoter or substitute for rhBMP2 *in vitro*. *In vivo* we also incorporated icariin into a novel biomimetic CaP bone substitute [42] for a significantly enhanced osteoindution by the slow release of the agent (unpublished data) .

Conclusions

In conclusion, icariin is a strong bone anabolic agent, which is comparable with rhBMP2 in terms of enhancing MC3T3-E1 cells proliferation and osteogenic differentiation. The low cost of icariin and its osteogenic potential make it very appealing for bone tissue engineering. It is proposed that Icariin may have potential as substitute of rhBMP2 in the future.

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Conflict of interests

The authors declared that there are no competing conflicts of interest.

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Table 1. Primer sequences for real-time quantitative polymerase chain reaction analysis of the gene expression.

Gene	Accession No.	Primers (F=forward; R=reverse)
ALP	NM_007431	F: 5'- TGCCTACTTGTGTGGCGTGAA -3'; R: 5'- TCACCCGAGTGGTAGTCACAATG -3'
BMP-2	NM_007553	5'-AAGAGACATGTGAGGATTAGCAGGT-3' and 5'-GCTTCCGCTGTTTGTGTTTG-3'
Collagen I	NM_007742	F: 5'- ATGCCGCGACCTCAAGATG -3'; R: 5'- TGAGGCACAGACGGCTGAGTA -3'
Osteocalcin (OCN)	NM_007541	F: 5'- AGCAGCTTGGCCCAGACCTA -3'; R: 5'- TAGCGCCGGAGTCTGTTCACTAC -3'
Runx2	NM_009820	F: 5'- CACTGGCGGTGCAACAAGA -3'; R: 5'- TTTCATAACAGCGGAGGCATTTC -3'
β-actin	NM_007393	F: 5'- AGGAGCAATGATCTTGATCTT -3'; R: 5'- TGCCAACACAGTGCTGTCT -3'

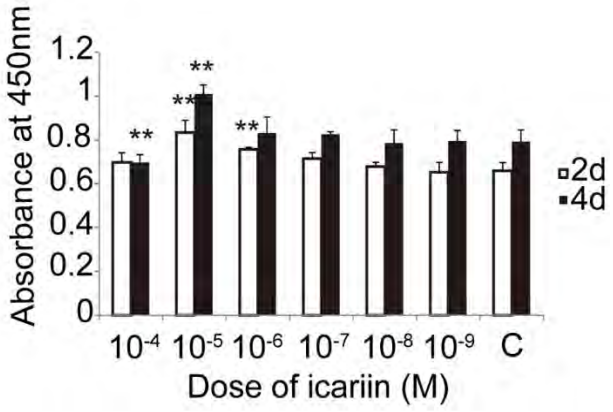

Fig.1. The dose-dependent effects of icariin in osteogenic poliferation of MC3T3-E1 grown in 96-well culture plates treated for 2 or 4 days with various concentrations of icariin (10^{-4}M, 10^{-5}M, 10^{-6}M, 10^{-7}M, 10^{-8}M, 10^{-9}M, 0M). Proliferation was measured, and mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05 **P <0.01. The significant differences were statistical calculated separately by the time.

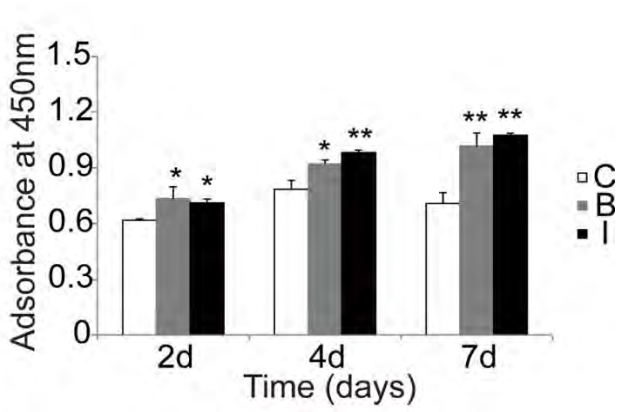

Fig.2. The proliferation of MC3T3-E1 cells stimulated by icariin or rhBMP2. The absorbance was measured on days 2, 4 and 7. Icariin (I) was added at 10-5M, and rhBMP2 (B) was added at 50 ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05 **P <0.01 versus control (C).

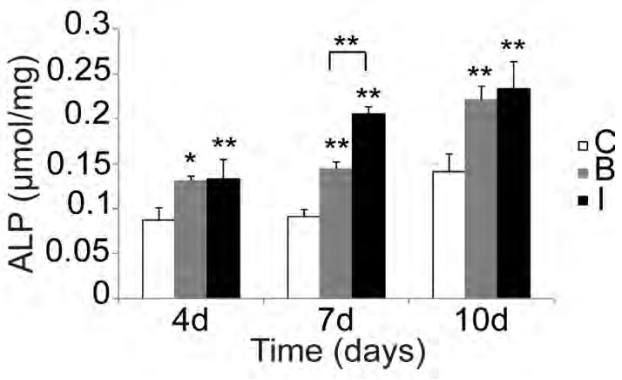

Fig.3. The ALP activities of MC3T3-E1 cells after treatment with icariin or rhBMP2. The ALP activities were determined by colorimetric assay on day 4, 7 and 10. The concentration of icariin (I) was 10-5M, and rhBMP2 (B) was 50 ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation normalized by total cellular protein. Error bars denote the standard deviation. *P<0.05, **P<0.01 without lines mean versus control (C).

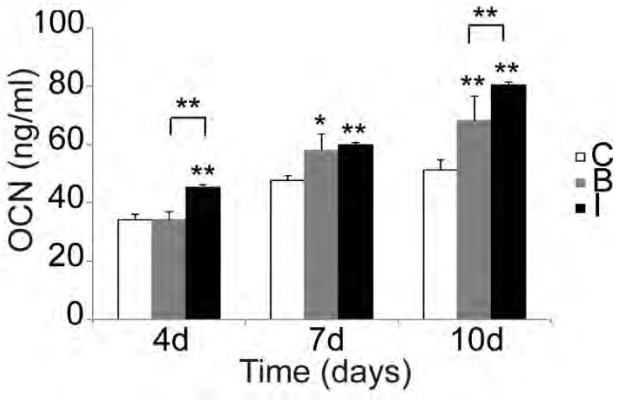

Fig.4. The OCN expressions of MC3T3-E1 cells after treatment with icariin or rhBMP2 treatments. ELISA was used to determine OCN expression on day 4, 7 and 10. The concentration of icariin (I) was 10^{-5}M, and rhBMP2 (B) was 50 ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01 without lines mean versus control (C).

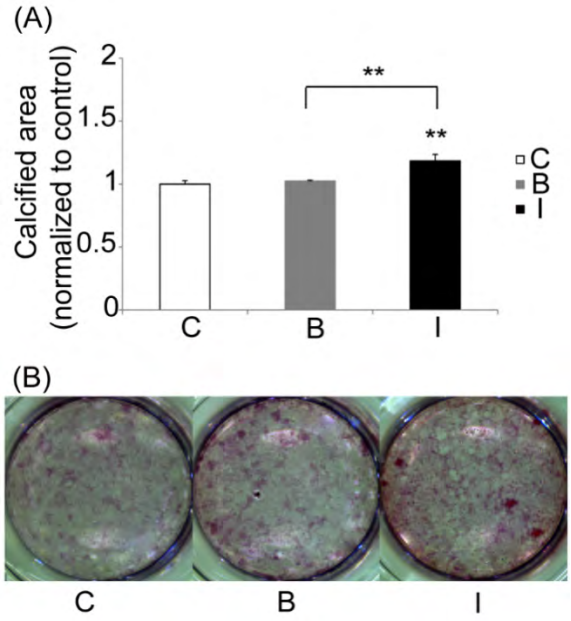

Fig. 5. (A) The quantitative calculation of calcified area in icariin or rhBMP2 on day 27. (B) Marocopic images of alizarin red stained 27 days after three groups treatment.The concentration of icariin (I) was 10^{-5}M, and rhBMP2 (B) was 50 ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01 without lines mean versus control (C).

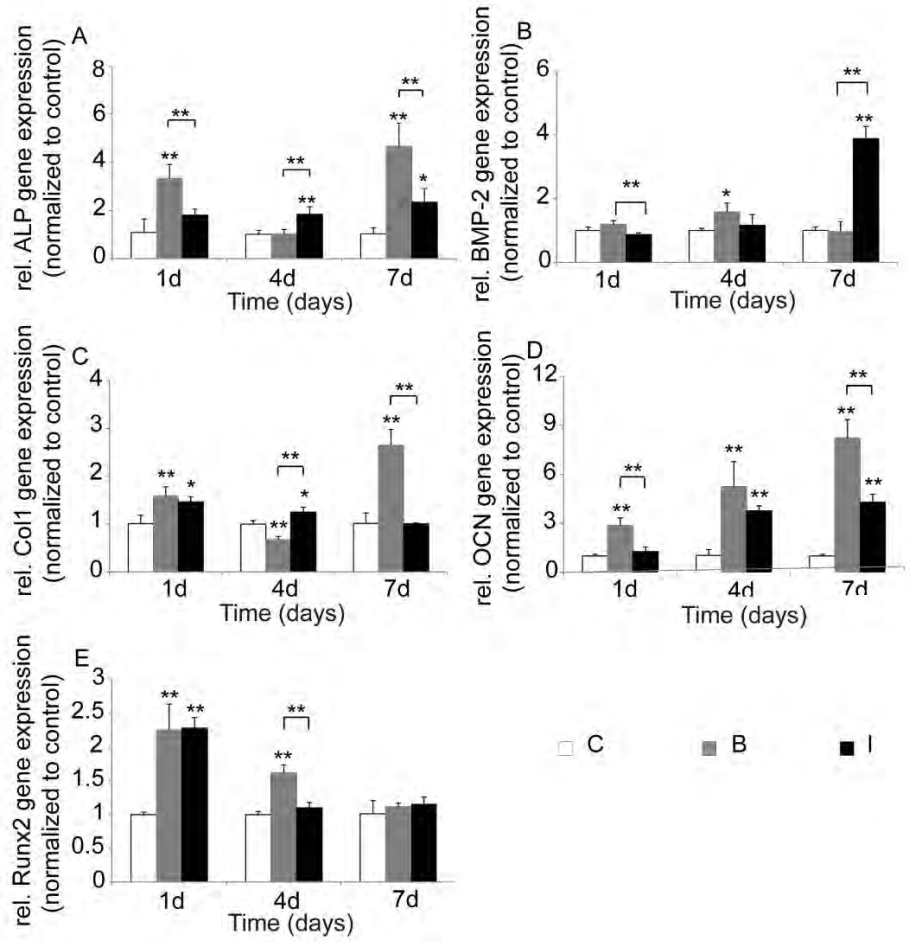

Fig.6. The time course changes in mRNA expression of (A) ALP, (B) rhBMP2 (C) Col1, (D) OCN, (E) Runx2 in MC3T3-E1 on day 1, 4 and 7. The concentration of icariin (I) was 10^{-5}M, and rhBMP2 (B) was 50 ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01 without lines mean versus control (C).

Chapter 4

Slowly delivered Icariin/allogeneic bone marrow-derived mesenchymal stem cells to promote the healing of calvarial critical-size bone defects

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Research Article

Slowly Delivered Icariin/Allogeneic Bone Marrow-Derived Mesenchymal Stem Cells to Promote the Healing of Calvarial Critical-Size Bone Defects

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Bone tissue engineering technique is a promising strategy to repair large-volume bone defects. In this study, we developed a 3-dimensional construct by combining icariin (a small-molecule Chinese medicine), allogeneic bone marrow-derived mesenchymal stem cells (BMSCs), and a siliceous mesostructured cellular foams-poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (SMC-PHBHHx) composite scaffold. We hypothesized that the slowly released icariin could significantly promote the efficacy of SMC-PHBHHx/allogeneic BMSCs for repairing critical-size bone defects in rats. In in vitro cellular experiments, icariin at optimal concentration (10^{-6} mol/L) could significantly upregulate the osteogenesis- and angiogenesis-related genes and proteins, such as Runx2, ALP, osteocalcin, vascular endothelial growth factors, and fibroblast growth factors, as well as the mineralization of BMSCs. Icariin that was adsorbed onto the SMC-PHBHHx scaffold showed a slow release profile within a 2-week monitoring span. Eight weeks after implantation in calvarial critical-size bone defects, the constructs with icariin were associated with significantly higher bone volume density, trabecular thickness, trabecular number, and significantly lower trabecular separation than the constructs without icariin. Histomorphometric analysis showed that icariin was also associated with a significantly higher density of newly formed blood vessels. These data suggested a promising application potential of the icariin/SMC-PHBHHx/allogeneic BMSCs constructs for repairing large-volume bone defects in clinic.

1. Introduction

Large-volume bone defects may result from congenital non-union, trauma, inflammation, and clinical treatments such as osteosarcoma-resection. The osseous repair of large-volume bone defects is still a challenge in the fields of orthopedics, maxillofacial surgery, and dental implantology. Although autologous bone grafts are routinely adopted to treat large-volume bone defects, the disadvantages of these grafts (e.g., limited quantity, donor site morbidity) have engendered tremendous efforts to develop alternatives [1], among which bone tissue engineering technique is highly promising [2, 3]. Bone tissue engineering technique is an interdisciplinary science that applies the principles of biology and engineering to develop viable substitutes for restoring, maintaining, or improving the function of bone tissue. A good combination of osteogenic cells, osteopromotive drugs, and osteoconductive biomaterials is critical for the success of this technique.

The accumulation, proliferation, and osteogenic differentiation of multipotent mesenchymal stem cells (MSCs) are indispensable for a proper fracture healing [4]. Thereby, bone marrow-derived MSCs- (BMSCs-) based strategies are

introduced to promote the healing of bone fractures and other bone metabolic diseases in clinics [5]. Their ability to modulate immune responses enables the application of allogeneic BMSCs without a substantial risk of immune rejection [5]. Continuous attempts have already been performed in preclinical models to apply allogeneic BMSCs in promoting the repair of bone defects [6]. In segmental critical-size bone defects, allogeneic BMSCs could significantly promote bone regeneration in a comparable level with autologous BMSCs [7]. The application of allogeneic BMSCs is also advantageous over autologous BMSCs due to their timeliness and sufficient availability [8].

Growth factors can be frequently adopted to further enhance and accelerate bone healing process. In the field of bone regeneration, bone morphogenetic proteins (BMPs) are the most important growth factors. Recombinant human BMP-2 and BMP-7 have been proved to significantly promote bone formation both in animal models and in clinical trials [9–11]. However, the effective doses of BMPs for current clinical use are always too high [12, 13], which results in a substantial economic burden to patients and healthcare system. Furthermore, the transiently high dosage may also lead to a series of potential side effects, such as the overstimulation of osteoclastic bone resorption [14], which may compromise its therapeutic effect. For promoting bone repair, one of the viable alternatives can be Chinese medicine, such as icariin. Icariin, a small-molecule drug extracted from a Chinese traditional medicine Herba Epimedii [15], shows a very promising potential. Icariin has been shown to enhance in vitro osteoblastogenesis [16, 17] through the induction of endogenous BMP-2 and nitric oxide (NO) [18, 19]. On the other hand, icariin is also able to reduce osteoclastogenesis through suppressing the signaling of MAPKs/NF-κB (mitogen-activated protein kinase/nuclear factor kappa-light-chain-enhancer of activated B cells) [20] and enhancing the ratio of OPG/RANKL [21]. In addition, icariin can promote angiogenesis, which may further facilitate the repair of large-volume bone defects. In comparison with BMPs, icariin, as a small-molecule drug, can also be easily synthesized, presenting an inexpensive drug to promote bone regeneration.

In the field of bone tissue engineering, an ideal scaffold should have a proper biodegradability and a good biocompatibility to accommodate osteogenic cells. Furthermore, a good slow-delivery capacity is also critical for prolonging the release profile of bioactive agents, thereby maximizing their biological effects. In this study, we adopted a novel composite SMC-PHBHHx (20 : 80) that incorporates biocompatible siliceous mesostructured cellular foams (SMC) [22] into high-toughness and easily moldable poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) [23] using a solvent casting and salt-leaching method [24]. The highly porous and well-interconnected structure conferred excellent physicochemical, biological, and drug-release properties on this novel composite scaffold.

Hitherto, it remains unclear whether icariin can promote the efficacy of allogeneic BMSCs-based tissue engineering technique in repairing large-volume bone defects. In this study, we first selected the best concentration of icariin by assessing its effect on promoting the proliferation and early differentiation of allogenic BMSCs. Thereafter, we evaluated the promoting effect of icariin on the osteogenesis- and angiogenesis-related genes and proteins. Finally, we evaluated the efficacy of slowly delivered icariin from the novel SMC-PHBHHx scaffold to treat in vivo calvarial critical-size bone defects in rats through radiographic and histomorphometric analysis. We hypothesized that the slowly released icariin could significantly promote the efficacy of SMC-PHBHHx/allogeneic BMSCs for repairing critical-size bone defects.

2. Materials and Methods

2.1. In Vitro Cellular Evaluation

2.1.1. Culture of BMSCs. BMSCs in passage 1 were purchased from ATCC and cultured with DMEM (Dulbecco's Modified Eagle Medium, Cyagen, Guangzhou, China) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C under the atmosphere of 5% CO_2 and 100% relative humidity. The passage was carried out when the cells were confluent to 80%. The cells of passage 4 were used in the following studies.

2.1.2. Multiple Differentiation. To identify the multipotency, the allogeneic BMSCs were induced to osteogenic, chondrogenic, and adipogenic differentiation using osteogenic medium, adipogenic medium, and chondrogenic medium (Cyagen, Guangzhou, China), respectively. The medium was replaced every 3 days. The osteogenic, chondrogenic, and adipogenic differentiation were examined at 14 days, 21 days, and 22 days, respectively. The staining was done using an alizarin red staining solution and oil red staining solution (Cyagen, Guangzhou, China) to check osteogenesis and adipogenesis, respectively. In order to check chondrogenesis, the chondrogenic cell pellets were fixed, embedded in paraffin, sectioned, and stained with alcian blue staining solution (Cyagen, Guangzhou, China) to detect the glycosaminoglycans. The images of various differentiations were captured using light microscope (Eclipse Ti-U, Nikon, Tokyo, Japan).

2.1.3. Concentration Selection Test. To identify the optimal concentration of icariin, cell proliferation assay and alkaline phosphatase (ALP) activities were performed with a time-course and dose-dependent setup. Cell proliferation was assessed using the cell counting kit-8 (CCK-8) (Yeasen, Shanghai, China). 2000 BMSCs per well were seeded in 96-well plates. After refreshing the medium 24 h after seeding, 100 μL of culture medium with different concentrations (0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, and 10^{-4} Mol/L) of icariin (Tauto Biotech, Shanghai, China) was added to each well. Six wells per group were measured. The treatment medium was refreshed every 3 days. After 1-, 3-, 5-, and 7-day incubation, the treatment medium was replaced with 100 μL CCK-8 working solution according to the manufacture's instruction. After a 40 min incubation, the OD (optical density) values were measured at 450 nm. ALP activity and total protein content were measured after the treatment for 1 day, 3 days, 5 days, and 7 days. ALP activity was determined using LabAssay ALP

Table 1: Primer sequences for real-time quantitative polymerase chain reaction analysis of the expression of osteogenic genes (Runx2, alkaline phosphatase (ALP), and osteocalcin (OCN)) and angiogenic genes (vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGF)).

Gene	Primers (F = forward; R = reverse)
ALP	F: 5′-GTC CCA CAA GAG CCC ACA AT-3′; R: 5′-CAA CGG CAG AGC CAG GAA T-3′
OCN	F: 5′-CAG TAA GGT GGT GAA TAG ACT CCG-3′; R: 5′-GGT GCC ATA GAT GCG CTT G-3′
Runx2	F: 5′-TCT TCC CAA AGC CAG AGC G-3′; R: 5′-TGC CAT TCG AGG TGG TCG-3′
VEGF	F: 5′-CTT GAG TTG GGA GGA GGA TG-3′; R: 5′-TGG CAG GCA AAC AGA CTT C-3′
FGF	F: 5′-CTC TGT CTC CCG CAC CCT AT-3′; R: 5′-CCT TCC ACC CAA AGC AGT AG-3′
GAPDH	F: 5′-GGC AAG TTC AAC GGC ACA GT-3′; R: 5′-GCC AGT AGA CTC CAC GAC AT-3′

colorimetric assay kit (Wako Pure Chemicals, Osaka, Japan). The total protein content was measured at 570 nm using a commercial BCA Protein Assay kit (Beyotime, Shanghai, China) to normalize the ALP activity.

2.1.4. The Expression of Osteogenesis and Angiogenesis-Related Genes and Proteins. To identify the effect of icariin on osteogenesis-related genes and proteins, BMSCs were cultured in an osteogenic medium as described above. For the angiogenesis-related genes and proteins, BMSCs were cultured in full culture medium without extra inductive agents. The effects of icariin on stimulating the expression of osteogenic and angiogenic genes were examined by quantitative RT-PCR 3 days, 5 days, 7 days, and 14 days after treatment. Total RNA was extracted from the cells using a Trizol Kit (Invitrogen, USA). The cDNA was synthesized from total RNA with a Primescrip™ RT Reagent Kit (Takara Biotechnology, Dalian, China). Real-time polymerase chain reaction (PCR) was performed using 1 μL of cDNA product in a 25 μL reaction volume with Mastercycler® ep realplex Real-Time PCR System (Eppendorf, Germany). In each PCR reaction, SYBR® Premix Ex Taq™ II (Takara Biotechnology), specific primers (Table 1), and 1 μL of cDNA were used according to the manufacturer's instructions. GAPDH was used as housekeeping gene. We calculated the folds of upregulation for each gene of interest using the following formula:

$$2^{-[(\text{CT gene of interest} - \text{CT internal control})\ \text{sample} - (\text{CT gene of interest} - \text{CT internal control})\ \text{control}]}. \quad (1)$$

Western blot analysis was used to assess the expression level of osteogenesis- and angiogenesis-related proteins such as ALP, osteocalcin (OCN), runt-related transcription factor 2 (Runx2), vascular endothelial growth factor (VEGF), and fibroblast growth factors (FGF). After 3 days, 5 days, 7 days, and 14 days, cells lysis was made using M-PER Mammalian Protein Extraction Reagent (ThermoFisher, USA) with a protease and phosphatase inhibitor cocktail (Sigma, USA). Anti-rat primary antibodies were used to detect osteogenesis- and angiogenesis-related proteins ALP, OCN, Runx2, FGF, and VEGF. Horseradish peroxidase-labeled secondary antibodies were then used to label detect the primary antibodies. Images were acquired using darkroom development techniques for chemiluminescence. Image-Pro Plus 6.0 software was adopted to analyze the Integral Optical Density (IOD).

2.1.5. Cell Matrix Mineralization. The effect of 10^{-6} mol/L icariin on the matrix mineralization of BMSCs was examined in osteogenic medium as described in Multilineage Differentiation of Allogeneic BMSCs with DMSO as control. After 14- and 21-day treatments, mineralized nodules were determined by alizarin red staining. Culture plates were photographed using NISElementsF2.20 (Eclipse 80i, Nikon, Tokyo, Japan), and the calcified area was quantified using Image-Pro Plus 6.0 software.

2.2. In Vivo Study

2.2.1. Preparation of SMC-PHBHHx Composite. The SMC-PHBHHx composite was fabricated as previously reported [24]. Briefly, to prepare SMC, 1.6 M HCl solution containing 0.53 mM Pluronic P123 (BASF, Frankfurt, Germany) and 0.23 M 1,3,5-triethylbenzene was stirred and kept in 40°C for 60 minutes. Thereafter, tetraethyl orthosilicate was added to reach the final concentration of 0.28 M and reacted for 20 hours at 40°C. The mixture was then subjected to an autoclave at 100°C for 24 hours under static conditions. After cooling at room temperature, the white precipitate was collected, dried, and calcined at 550°C for 6 hours to produce the SMC materials. To prepare SMC/PHBHHx composite, SMC was added to the chloroform containing PHBHHx (20 wt%) to a final concentration of 5 wt% with stirring for 24 hours. After adding NaCl particles with diameters ranging from 300 to 500 μm, the mixture was cast into cylindrical PTFE molds. The samples were then air-dried under flowing air for 24 hours and subsequently vacuum-dried at 40°C for 48 hours. The NaCl particles were then removed by immersing in deionized water for 72 hours with the water replaced every 6 hours. Thereafter, the samples were air-dried for 24 hours and vacuum-dried overnight to obtain the sponge-like scaffolds.

2.2.2. Preparing the Constructs of Icariin/SMC-PHBHHx/Allogeneic BMSCs. To prepare icariin/SMC-PHBHHx composite, we adopted SMC-PHBHHx (20 : 80) discs (5 mm in diameter and 1.5 mm in thickness) and 10^{-6} mol/L icariin suspension in DMEM. 200 μL of either icariin-containing or non-icariin-containing suspension was then adsorbed onto each SMC-PHBHHx disc with a mild shaking for 72 hours.

The icariin/SMC-PHBHHx discs were then freeze-dried under sterile condition for 48 hours. The discs were thereafter stored in 4°C for later use. Allogeneic BMSCs were seeded onto either icariin-containing or non-icariin-containing SMC-PHBHHx discs with a mild shaking for 72 hours at 37°C under 5% CO_2 before implantation.

2.2.3. Characterization of Icariin/SMC-PHBHHx Constructs

(1) Scanning Electronic Microscope. Scanning electron microscope (Phenom™ Pro, Eindhoven, The Netherlands) was adopted to reveal the influence of icariin adsorption on the surface morphologies of SMC-PHBHHx composite and BMSCs. For this purpose, either icariin-containing or non-icariin-containing SMC-PHBHHx discs with or without allogeneic BMSCs were mounted on aluminium stubs and sputtered with gold particles.

(2) Release Kinetics of BSA In Vitro. The release kinetics of icariin was monitored over a 15-day period in vitro using a high-performance liquid chromatography (BAS PM-80, West Lafayette, IN). Each sample ($n = 6$) was introduced into a 1.5 mL Eppendorf tube containing 1 mL of DMEM. The tubes were incubated for up to 14 days in a shaking water bath (60 agitations/minute), which was maintained at 37°C. Triplicate 200 μL aliquots of the medium (containing released FITC-BSA) were withdrawn for analysis after 12 hours, 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 8 days, 10 days, 12 days, and 14 days. The temporal release of icariin was expressed as a percentage of the total amount of adsorbed icariin.

2.2.4. Surgery. The calvarial critical-size bone defects in rats were established as previously described [25]. Briefly, 6 male Sprague-Dawley rats (5-week-old and weighing 180–220 g) were randomly assigned into 2 groups: either icariin-containing or non-icariin-containing SMC-PHBHHx/allogeneic BMSCs constructs. The animal care was performed in accordance with the guidelines of the Ethical Committee of Shanghai East Hospital Affiliated with Tongji University, Shanghai, China. All animal experiments were carried out according to the ethic laws and regulations of China. Critical-sized cranial defects (5 mm in diameter) were created in these rats. Briefly, the rats were anaesthetized with an intraperitoneal injection of pentobarbital (Nembutal 3.5 mg/100 g). A subcutaneous injection of 0.5 mL of 1% lidocaine as a local anesthetic was given along the sagittal midline of the skull. A sagittal incision was made over the scalp from the nasal bone to the middle sagittal crest and the periosteum was dissected. The 5 mm defects were created using a dental surgical drill with a trephine with a constant cooling rinse. Subsequently, the calvarial disk was carefully removed to avoid tearing the dura. After rinsing with physiological saline to wash out any bone fragments, samples from various groups were implanted randomly into these defects. Afterwards, the periosteum and the scalp were closed in layers with interrupted 4-0 Vicryl resorbable sutures.

2.2.5. Radiographic Evaluation. Eight weeks after operation, the rats were sacrificed by intramuscular injection of overdose of Sumianxin II. All the 6 calvarial blocks of the sacrificed animals were harvested and immediately immersed into the 10% neutrally buffered formalin for fixation. Radiographic analysis of bone regeneration within the defects was performed using an X-ray unit (Vario^{DG}, Sirona), with the exposure time set at 0.03 seconds. After a 2-day fixation, the specimens were scanned along the sagittal direction though by micro-CT (Inveon, Siemens) with a resolution of 18 μm followed by an off-line reconstruction. After scanning, the selection of the area of interest was performed manually. Our preliminary study showed that the grey value of SMC-PHBHHx material was around −217, which was lower than water. The thresholding of mineralized bone was set at 500.

The following morphometric parameters obtained in direct mode were adopted to estimate the bone regeneration within the defects using software Siemens Inveon:

(1) Relative bone volume (bone volume/tissue volume, BV/TV: %)

(2) Trabecular number (Tb.N: 1/mm)

(3) Trabecular separation (Tb.Sp: mm)

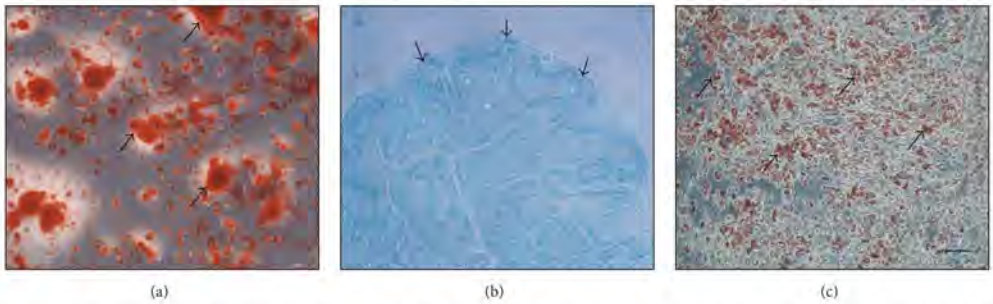
(4) Trabecular thickness (Tb.Th: mm).

2.2.6. Histomorphometric Analysis. The samples were then decalcified in 4.18% EDTA + 0.8% formalin at pH 7.2 for four weeks at 4°C, rinsed with phosphate buffer, and embedded in paraffin. Serial 6 μm thickness sections were stained with hematoxylin-eosin (HE). The numbers of blood vessels were evaluated under light microscopy. The final magnification was ×50.

2.3. Statistical Analysis. We first used both Kolmogorov-Smirnov test and D'Agostino and Pearson omnibus normality test to comprehensively check the normality of the data of each group. According to the results, we selected either parametric tests or nonparametric tests to analyze the data. The data in concentration selection test were statically analyzed using two-way ANOVA. For the other data, we used either unpaired t-test or Man–Whitney test to compare the effect of icariin with the corresponding control (no icariin). The level of significance was set at $p < 0.05$. SPSS software (version 20) for a Windows computer system was employed for the statistical analysis.

3. Results

3.1. Multilineage Differentiation of Allogeneic BMSCs. Multilineage differentiation assay showed that the allogeneic BMSCs could differentiate into osteogenesis, chondrogenesis, and adipogenesis (Figure 1). For the osteogenic differentiation, the mineralized nodules in cell matrix—the final osteogenic differentiation marker—were stained red (indicated by black arrows in Figure 1(a)). The chondrogenic differentiation was approved by the abundant presence of glycosaminoglycans that were a typical chondrogenic differentiation marker and were stained blue in the cell pellet by alcian blue (indicated by black arrows in Figure 1(b)). The adipogenic differentiation was characterized by the oil droplets within the cells that were stained red (indicated by black arrows in Figure 1(c)).



(a) (b) (c)

FIGURE 1: Light micrographs depicting the (a) osteogenic, (b) chondrogenic, and (c) adipogenic differentiation of multipotent BMSCs. The black arrows indicated the red-stained mineralized nodules in (a), blue-stained chondrogenic cell pellet in (b), and red-stained lipid droplets in cells in (c). Bar = 100 μm.

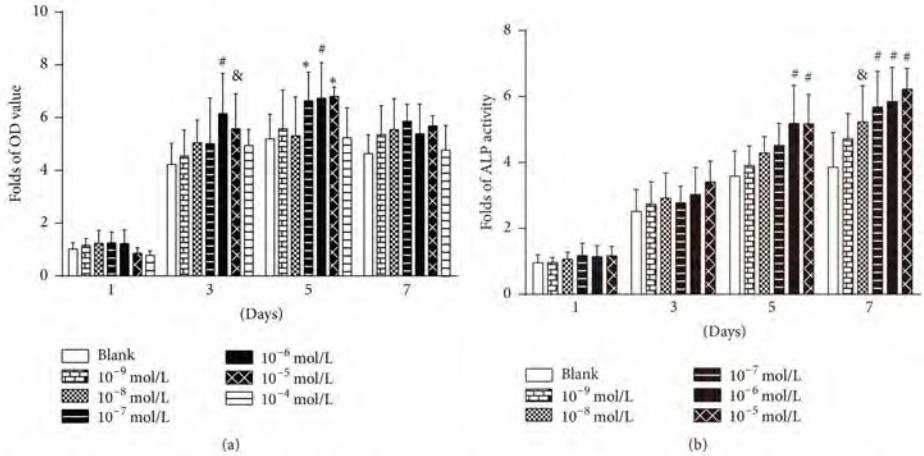


FIGURE 2: Time-course and dose-dependent tests to select the optimal concentration of icariin for inducing the osteogenic differentiation of BMSCs. Icariin of 0, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, or 10^{-4} mol/L was used to treat preosteoblasts for 1 day, 3 days, 5 days, and 7 days. (a) Cell proliferation assays; (b) alkaline phosphatase (ALP) activity assays. All data are presented as mean values together with the standard deviation (SD). $^{*}p < 0.05$; $^{\&}p < 0.01$; $^{\#}p < 0.001$ indicating the statistical difference between the indicated group and the control group (blank) at the same time point.

3.2. Concentration Selection through Cell Proliferation and ALP Activity Assays. Icariin at only 10^{-5} mol/L and 10^{-6} mol/L resulted in significantly higher OD value (indicator for cell proliferation) than the blank control after a 3-day treatment. On the 5th day, icariin at 10^{-5} mol/L, 10^{-6} mol/L, and 10^{-7} mol/L was associated with significantly higher OD value than the blank control. The average OD value under the induction of 10^{-6} mol/L icariin was the highest on all the selected time points (Figure 2(a)). Icariin at 10^{-5} mol/L and 10^{-6} mol/L resulted in a significantly higher ALP activity than the control after a 5-day treatment. Icariin ranging from 10^{-8} mol/L to 10^{-4} mol/L was associated with significantly higher OD value than the blank control on the 7th day. The value of ALP activity induced by 10^{-6} mol/L was the highest and second highest on the 5th day and 7th day, respectively (Figure 2(b)). Consequently, we selected 10^{-6} mol/L as the optimal concentration for icariin in the following tests.

3.3. Expression of Osteogenesis- and Angiogenesis-Related Genes. In comparison with the control (no icariin), 10^{-6} mol/L icariin could induce significantly higher expression of osteogenesis-related genes, such as Runx2 mRNA (at 3 days, 7 days, and 14 days) (Figure 3(a)), ALP mRNA (at 7 days and 14 days) (Figure 3(b)), and OCN mRNA (at 14 days) (Figure 3(c)), as well as angiogenesis-related genes,

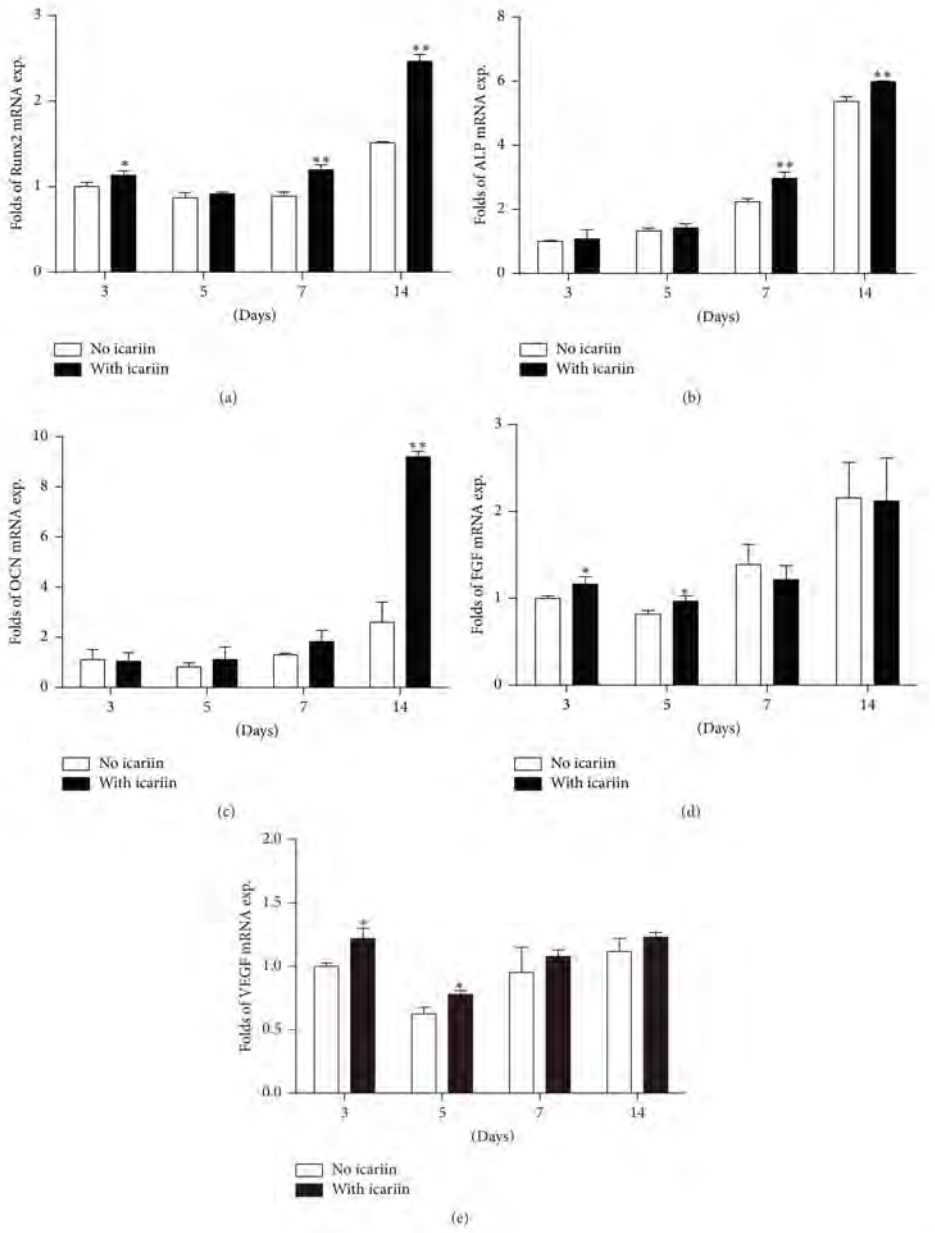


FIGURE 3: Graphs depicting the fold changes of the expression of osteogenesis-related and angiogenesis-related mRNA in BMSCs under the stimulation of icariin for 3 days, 5 days, 7 days, and 14 days. (a) Runx2, (b) alkaline phosphatase (ALP), (c) osteocalcin (OCN), (d) fibroblast growth factors (FGF), and (e) vascular endothelial growth factor (VEGF). All data are presented as mean values together with the standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$ indicating the statistical difference between the experimental group (with icariin) and the control group (no icariin) at the same time point.

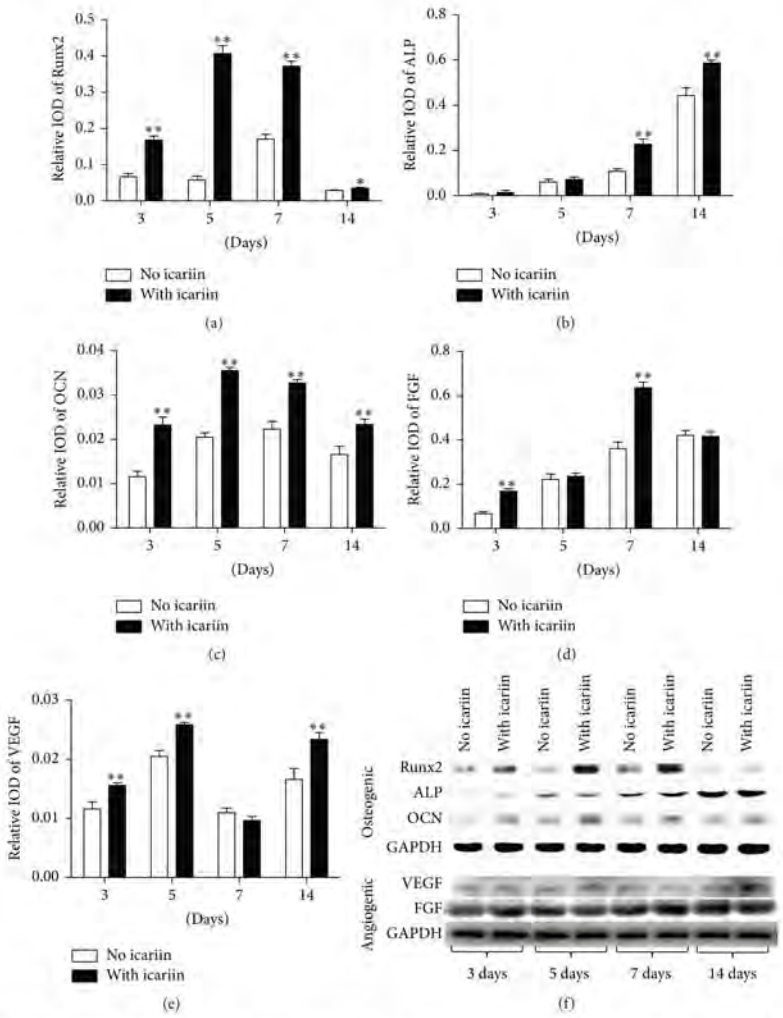


FIGURE 4: Graphs depicting the fold changes of the expression of osteogenesis-related and angiogenesis-related proteins in BMSCs under the stimulation of 10^{-6} mol/L icariin for 3 days, 5 days, 7 days, and 14 days. (a) Runx2, (b) alkaline phosphatase (ALP), (c) osteocalcin (OCN), (d) fibroblast growth factors (FGF), (e) vascular endothelial growth factor (VEGF), and (f) photographs of western blot analysis from a representative experiment. All data are presented as mean values together with the standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$ indicating the statistical difference between the experimental group (with icariin) and the control group (no icariin) at the same time point.

such as FGF mRNA (at 3 days and 5 days) (Figure 3(d)) and VEGF mRNA (at 3 days and 5 days) (Figure 3(e)), than the corresponding no-icariin treatments.

3.4. Expression of Osteogenesis- and Angiogenesis-Related Proteins. Western blot analysis showed that 10^{-6} mol/L icariin could induce significantly higher expression of osteogenesis-related proteins, such as Runx2 (at all the time points) (Figure 4(a)), ALP (at 7 days and 14 days) (Figure 4(b)), and OCN (at all the time points) (Figure 4(c)), as well as angiogenesis-related genes, such as FGF (at 3 days, 5 days, and 14 days) (Figure 4(d)) and VEGF (at 3 days and 7 days) (Figure 4(e)), than the corresponding no-icariin treatments.

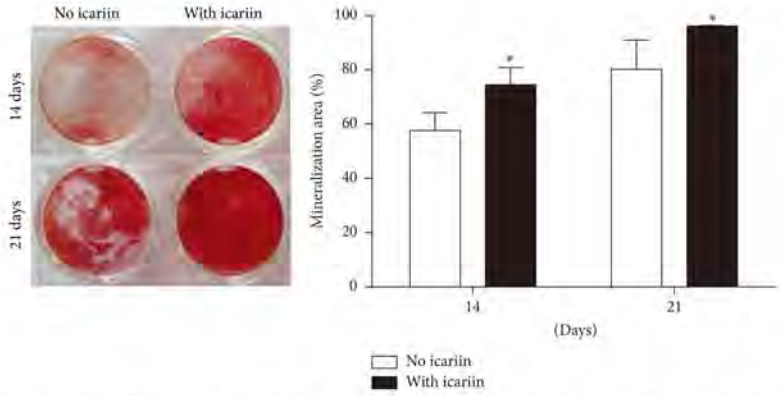


FIGURE 5: Mineralization assay of BMSCs with or without the stimulation of 10^{-6} mol/L icariin for 14 days and 21 days. All data are presented as mean values together with the standard deviation (SD). $^{*}p < 0.05$ indicating the statistical difference between the experimental group (with icariin) and the control group (no icariin) at the same time point.

3.5. In Vitro Mineralization of Allogeneic BMSCs Induced by Icariin. The mineralization area in the group either with or without 10^{-6} mol/L icariin increased with time. On both the 14th day and the 21st day, 10^{-6} mol/L icariin resulted in significantly higher mineralization area than the control (no icariin) (Figure 5).

3.6. SEM Characterization of Icariin Adsorption and Cell Adhesion. The SMC-PHBHHx composite scaffolds showed an interconnected porous structure (Figures 6(a) and 6(b)). The adsorption of icariin onto the scaffolds did not significantly change the structure and topography of the scaffold (Figures 6(c) and 6(d)). Qualitative observation showed that more BMSCs could be found on icariin-containing SMC-PHBHHx composite scaffolds than on non-icariin-containing ones (Figures 6(c) and 6(f)).

3.7. Release Kinetics of Icariin. The release kinetics of the adsorbed icariin from the SMC-PHBHHx composite followed a biphasic course: an initial (5 days) rapid phase and a subsequent slower phase (Figure 7). The adsorbed icariin showed a slow release profile with nearly 10% per day within the first 5 days. During the subsequent slower phase (days 5–14), the adsorbed icariin was released 2.2% per day with 70% depleted by the end of 14 days.

3.8. Micro-CT Analysis and Histological Observation of Newly Formed Bone Tissue. Micro-CT analysis showed that the constructs with icariin resulted in significantly higher BV/TV (Figure 8(a)), Tb.Th (Figure 8(b)), and Tb.N (Figure 8(c)) and significantly lower Tb.Sp (Figure 8(d)) than the constructs with no icariin. Histological observations showed significantly less new bone formation within the defects treated with SMC-PHBHHx/allogeneic BMSCs with no icariin (Figure 9(a)) than that with icariin (Figure 9(b)).

3.9. Histomorphometric Analysis of Blood Vessels. Histomorphometric analysis indicated that the numbers of blood vessels within the defects treated with SMC-PHBHHx with icariin were significantly higher than those within the defects treated with SMC-PHBHHx without icariin (Figure 9(c)).

4. Discussion

Critical-size bone defects, a standard experimental model for large-volume bone defects, are commonly used to evaluate the treatment efficacy of novel biomaterials. The lack of osteogenic cells, osteoinductive growth factors, and osteoconductive scaffolds always leads to a nonosseous repair in a critical-size bone defect. Bone tissue engineering is a technique to integrate various knowledge in osteogenic stem cells, osteoconductive scaffolds, and osteoinductive growth factors with an aim of significantly accelerating and promoting bone regeneration. In this study, we, for the first time, showed that the slowly released icariin could significantly promote the efficacy of SMC-PHBHHx/allogeneic BMSCs for repairing critical-size bone defects.

The selection of bioactive agents is critical for the effect of a tissue engineering technique. BMPs are still the most potent growth factors for bone tissue engineering. BMPs can bind their transmembrane serine/threonine kinase receptors [26] and trigger two main downstream signaling pathways: Smad-dependent and Smad-independent signaling pathways [27]. Activated BMP receptors phosphorylate Smad1/5/8, which assembles into a complex with Smad4 and translocates to the nucleus, regulating the transcription of target genes, such as Runx2 [26]. In addition to Smad-dependent signaling, a series of Smad-independent downstream signaling pathways, including MAPK pathways, such as p38, c-Jun N-terminal kinase (JNK), and extracellular signal-related kinase (ERK), are also activated [28]. Stimulating the expression of endogenous BMPs is the pathway to exert osteoinductive effects of many drugs, such as icariin [29]. Icariin could enhance the

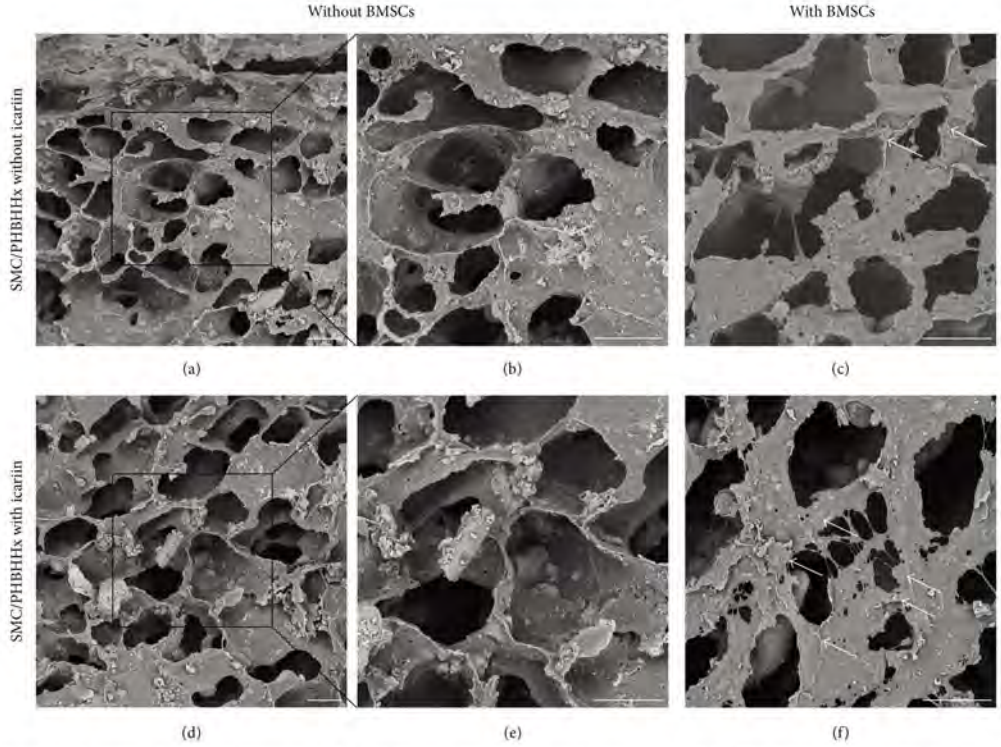


FIGURE 6: Scanning electron microscopy graphs depicting the morphology and topography of SMC-PHBHHx scaffolds without (a, b, d, e) or with (c, f) BMSCs in the absence (a, b, c) or presence (c, d, e) of 10^{-6} mol/L icariin. Bar = 30 μm.

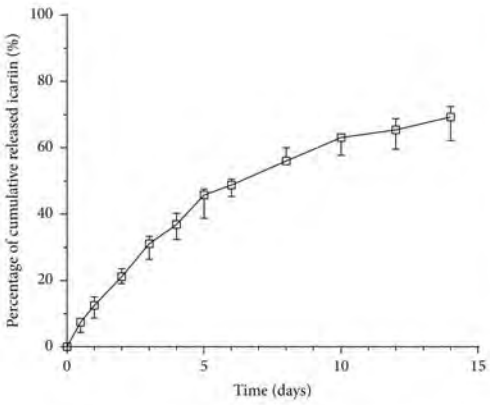


FIGURE 7: The in vitro release profile of icariin from a SMC-PHBHHx scaffold. All data are presented as mean values together with the standard deviation (SD).

expression of endogenous BMPs and subsequent osteogenic signaling pathways, such as Smad4, Runx2, and OPG [18, 29]. The induction of endogenous BMP-2 by icariin was, at least partially, mediated by the Wnt/β-Catenin-BMP signaling pathway [30]. Ohba et al. suggested two possible mechanisms for the involvement of BMP signaling in the effects of icariin [31]: (1) icariin indirectly activated BMP signaling through extracellular BMPs; (2) icariin directly activated BMP signaling by interacting with Smads via unknown mechanisms. The indispensability of endogenous BMPs for the effect of icariin was proved by the fact that noggin, an extracellular BMP antagonist, could diminish the icariin-induced enhancement of osteogenic differentiation (such as ALP, OCN, and mineralization) in osteogenic cells [18]. Consistently, the specific inhibitor for the Smad-independent ERK, JNK, and p38 MAPK signaling pathways could dramatically attenuate the promoting effect of icariin on the osteogenesis of BMSCs [32]. In addition to the BMP-associated signaling pathways, icariin could also stimulate the osteogenic differentiation of rat bone marrow stromal cells via activating the PI3K-AKT-eNOS-NO-cGMP-PKG [33]. More importantly, icariin

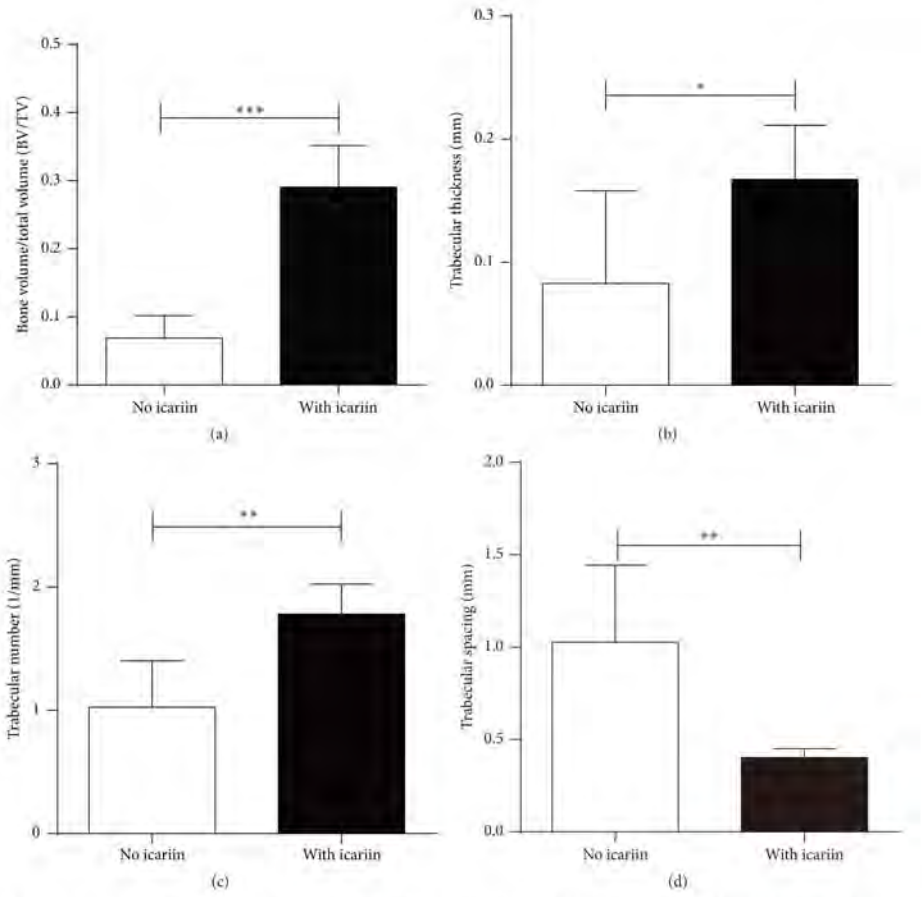


FIGURE 8: Graph depicting the micro-CT analysis of the BV/TV (a), Tb.N (b), Tb.Th (c), and Tb.Sp (d) of the newly formed bone within the calvarial critical-size bone defects that were treated with SMC-PHBHHx scaffolds/allogeneic BMSCs either without or with adsorbed icariin. All data are presented as mean values together with the standard deviation (SD). $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

could induce the osteogenic differentiation of BMSCs in many pathological conditions, such as osteoporosis [34, 35] and osteonecrosis [36]. Interestingly, icariin could activate different molecular cascades on BMSCs in corticosterone and ovariectomy induced osteoporotic rats [35]. Estrogen and epigenetic modulation were the newly found targets of icariin for its beneficial effect on osteogenesis in pathological conditions [34, 36]. In addition to the promoting effect on osteoblastic differentiation, icariin could also suppress osteoclastic activity, which was different from BMPs. Icariin inhibited osteoclastic differentiation in both its coculture with osteoblasts and single culture [21]. This effect was, at least partially, mediated by icariin-induced increase of OPG/RANKL expression ratios [37]. Consequently, although the potency of icariin in inducing bone formation is less than that of BMP-2, icariin is advantageous in balancing the osteoblastic and osteoclastic activity. This is especially important for the patients with osteoporosis. All these properties confer a very promising clinical application potential on icariin.

Although most of the previous reports indicated the promoting effect of icariin on osteogenesis of BMSCs, whether icariin can promote the repairing efficacy of allogeneic BMSCs in vivo is, hitherto, not known. In this study, we hypothesized that the slowly released icariin could significantly promote the efficacy of SMC-PHBHHx composite and allogeneic BMSCs for repairing critical-size bone defects. We tried to answer this question in a step-forward way. Firstly, we

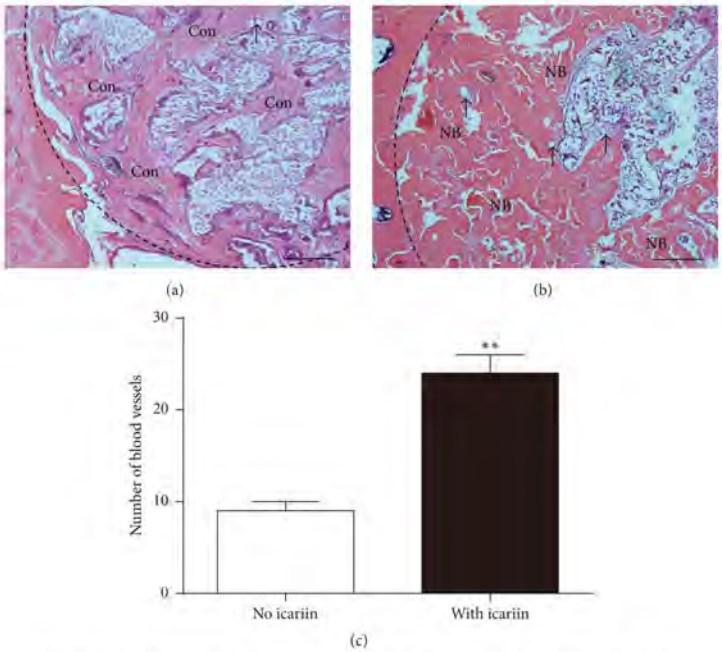


FIGURE 9: Light micrographs depicting the new bone formation within the calvarial critical-size bone defects that were treated with SMC-PHBHHx/allogeneic BMSCs either (a) without or (b) with icariin. Bar = 200 μm. Then all data are presented as mean values together with the standard deviation (SD). NB: new bone; Con: connective tissue; black arrow: blood vessels. (c) Graph depicting the number of vessels per section. All data are presented as mean values together with the standard deviation (SD). $^{**}p < 0.01$.

showed the multipotency (Figure 1) of the purchased BMSCs using well-established assays. Thereafter, we determined the optimal concentration of icariin at 10^{-6} mol/L in the time-course and dose-dependent proliferation assays (Figure 2(a)) and ALP assays (Figure 2(b)). The following RT-PCR and western blot analyses corroborated that icariin was associated with either equivalent or significantly higher level of osteogenesis-related genes and proteins, such as Runx2, ALP, and OCN (Figures 3 and 4) during the monitoring span (3–14 days). Interestingly, the icariin-induced upregulation magnitude of Runx2 and OCN proteins was much more significant than their genes on the 3rd, 5th, and 7th day, while the upregulation fold of OCN protein was much lower than OCN gene. These phenomena suggested a posttranscriptional modulation might also be involved in icariin-related effects. In the mineralization assay, we showed that 10^{-6} mol/L icariin could significantly promote the calcium nodule formation on both 14th day and 21st day (Figure 5).

A suitable scaffold is indispensable for the application of allogeneic BMSCs. In previous studies, SMC-PHBHHx composite materials showed good biocompatibility, proper stiffness, and, more importantly, the ability to carry and control release of bioactive agents [24]. Moreover, the radiolucency of this material is highly suitable for radiographic examination of new bone formation in clinic. In this study, we tried to functionalize SMC-PHBHHx with icariin in order to achieve enhanced efficacy in bone regeneration. Our data showed that the adsorbed icariin did not significantly influence the morphology of SMC-PHBHHx scaffold (Figures 6(a), 6(b), 6(d), and 6(e)). The adsorbed icariin showed a controlled slow release profile with 30% left by the end of 14 days in the in vitro condition (Figure 7). Moreover, qualitative scanning electron microscope observation showed that more BMSCs could be found on icariin-containing SMC-PHBHHx composite scaffolds than on non-icariin-containing ones (Figures 6(c) and 6(f)). These results indicated the feasibility to construct icariin/SMC-PHBHHx/allogeneic BMSCs constructs for bone tissue engineering.

Subsequently, we tested the effect of icariin on the efficacy of SMC-PHBHHx/allogeneic BMSCs in a calvarial critical-size bone defect. Eight weeks after implantation, micro-CT evaluation showed that the BV/TV, Tb.Th, and Tb.N of the new bone regenerated in the SMC-PHBHHx/allogeneic BMSCs with icariin were 4.2 times, 1.8 times, and 2.0 times higher than those in the SMC-PHBHHx/allogeneic BMSCs without icariin, respectively (Figures 8(a), 8(b), and 8(c)). The

presence of icariin was also associated with a significantly lower Tb.Sp (Figure 8(d)). Consistent with the radiographic analysis, histological observation also indicated the significantly promoting effect of icariin on bone regeneration (Figures 9(a) and 9(b)). These results clearly indicated that the slowly delivered icariin could promote bone regeneration of SMC-PHBHHx/allogeneic BMSCs in critical-size bone defects.

In addition to the direct promoting effect, icariin may also benefit bone regeneration through enhancing angiogenesis. Vascularization is a crucial step in bone regeneration, which brings mesenchymal stem cells and nutrition to wounds [38]. Icariin could promote not only in vitro endothelial tubulogenesis assay but also in vivo angiogenesis [39], possibly through activating EGF-EGFR pathway and thereafter endothelial NO synthase [40]. Moreover, icariin could directly stimulate angiogenesis through activating a series of angiogenic signals, such as ERK, PI3K, and Akt [41]. In our in vitro cellular experiments, icariin could significantly promote the angiogenesis-related genes and proteins, such as VEGF and FGF (Figures 3 and 4). Accordingly, our histomorphometric analysis indicated that the slowly released icariin resulted in significantly higher number of blood vessels (Figure 9(c)).

This study bears also some limitations, such as the limited group setup in the animal studies and no in vivo tracking of allogeneic BMSCs. Consequently, the contribution of allogeneic and autologous BMSCs could not be precisely determined. However, our findings clearly showed that the slowly delivered icariin could promote the efficacy of SMC-PHBHHx/allogeneic BMSCs for healing the critical-bone defects in rats. Such an effect may be mediated by icariin-induced upregulation of osteogenesis and angiogenesis. With the indication of the current study, we are trying to further explore these factors.

Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Chapter 5

BMP2/7 heterodimer can modulate all cellular events of the *in vitro* RANKL-Mediated Osteoclastogenesis, Respectively, in Different Dose Patterns

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BMP2/7 Heterodimer Can Modulate All Cellular Events of the *In Vitro* RANKL-Mediated Osteoclastogenesis, Respectively, in Different Dose Patterns

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Bone morphogenetic protein (BMP) heterodimers can trigger and sustain osteoblastic bone regeneration in significantly lower dosages than BMP homodimers. However, their effects on osteoclastic activity—a paramount coupling process with ostoblastic activity—remain undocumented. In this study, we delineated the functional characteristics of BMP2/7 heterodimer in inducing the *in vitro* osteoclastogenesis. We compared the dose-dependent effects of BMP2/7 heterodimer on the osteoclastogenesis of a preosteoclast cell line (RAW264.7) with those of BMP2 and BMP7 homodimers under the stimulation of 50 ng/mL receptor activator of nuclear factor-κB ligand. We quantitatively monitored the following parameters: cell proliferation, osteoclastic genes expression, morphological characteristics of osteoclasts, and calcium phosphate (CaP) resorption. BMP2/7 heterodimer could dose dependently modulate each osteoclastogenic event with different concentration patterns from the BMP homodimers. All BMPs of 10–150 ng/mL could increase the numbers of osteoclasts. Not BMP7 but 50–200 ng/mL BMP2 homodimer and 100–200 ng/mL BMP2/7 heterodimer could significantly enlarge the average surface-area of an osteoclast. BMP2/7 of 5–150 ng/mL could significantly enhance the osteoclastic CaP resorption to a similar level as the two homodimers. BMP2/7 heterodimer affects every osteoclastogenic event in a complicated dose-dependent manner. Low-concentration BMP2/7 heterodimer may favor a rapid and spontaneous remodeling of its induced bone and, thus, bear a promising potential in cytokine-based tissue engineering.

Introduction

THE OSSEOUS REPAIR OF CRITICAL-SIZED BONE DEFECTS (CSBDs) is still a challenge in the fields of orthopedics, maxillofacial surgery, and implantology. Although autologous bone grafts are routinely used to heal CSBDs, the disadvantages of the intervention (e.g., limited quantity, donor site morbidity) engender tremendous efforts to develop alternatives.[1] A promising strategy is the cytokine-based bone tissue engineering, which is advantageous in safety, feasibility, and potential for nearest clinical application over the gene- and cell-based ones.[2,3]

One of the most important classes of cytokines for promoting bone regeneration are bone morphogenetic proteins (BMPs), a group of disulfide-linked dimeric polypeptide growth factors under the superfamily of transforming growth factors-β. The classical role for BMPs is to induce *de novo* bone in an ectopic site.[4–7] Recombinant human (rh) BMP2 and BMP7 homodimers have been shown to significantly promote bone formation both in animal models and in clinical trials.[8–10] However, the effective doses of BMP homodimers for current clinical use are extremely high (e.g., up to milligrams),[11,12] which results in a substantial economic burden to patients and healthcare systems. Furthermore, the transiently high dosage may also lead to a series of potential side effects, such as the overstimulation of osteoclastic bone resorption.[13]

One approach to overcome this dilemma is to adopt the BMPs that bear higher osteoinductive potency and, thus, can be applied in lower dosages.[14] As a promising option, BMP heterodimers exhibited several- or dozen-fold higher effects than the respective homodimers in inducing *in vitro* osteoblastogenesis and *in vivo* bone formation.[15–18] On the other hand, the effects of BMP heterodimers on osteoclastogenesis,

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a paramount coupling process with osteoblastogenesis during osteogenesis, remain undocumented. Proper communication between osteoclasts and osteoblasts is essential for bone regeneration, maintenance, and remodeling.[19] For example, in a BMP-2-induced ectopic bone formation, osteoclast precursors appear before the bone matrix,[20] and osteoclastic resorption of hypertrophic cartilage is a prerequisite for the replacement by osteoblastic bone formation.[21] During bone remodeling, osteoclasts are also central regulators of bone formation.[22] Improper communication between osteoblastic and osteoclastic activities can lead to a series of bone diseases, for example, osteopenia and osteoporosis. The transient high dose of exogenous BMP homodimers that are applied for promoting osteogenesis could result in overstimulated osteoclastic resorption in surrounding cancellous bone and compromise the efficacy of bone regeneration.[13] We recently showed that BMP2/7 heterodimer induced osteoblastogenesis with a lower effective concentration and a similar (instead of higher) maximum effect than the respective BMP2 and BMP7 homodimers.[23] Whether BMP2/7 heterodimer in such a low effective concentration can also trigger proper osteoclastic activities remains unclear. Unveiling this knowledge is essential to optimize the osteoinductive efficacy of, and avoid the potential side effects of BMP heterodimers.

Hitherto, how exogeneous BMPs, particularly of BMP heterodimers, modulate osteoclastic activities remained to be elucidated. BMPs can indirectly influence osteoclastogenesis through the intermedium of osteoblasts and mesenchymal stromal cells.[24] These cells can secrete receptor activators of nuclear factor-κB ligand (RANKL), to modulate the osteoclastogenesis.[25,26] The osteoclastic activity stimulated through this pathway is well controlled by osteoblasts,[27] which may not contribute to the abnormal local osteoclastic activity. On the other hand, accumulating evidence indicates that BMPs can directly enhance *in vitro* osteoclastogenesis.[13,28–30] Furthermore, RANKL is a prerequisite for BMPs' effect on osteoclastogenesis.[30] An *in vitro* study also attempted to use BMP2/7 heterodimer and RANKL to stimulate bone formation and remodeling respectively.[31] However, the direct effect of BMP2/7 on osteoclastogenesis remains undocumented. A dose-response study is indispensable to obtain an optimal osteogenic effect and to delineate the target cellular event of BMP2/7 heterodimers.

In this article, we hereby unveiled the functional characteristics of BMP2/7 heterodimer in RANKL-induced osteoclastogenesis of RAW264.7 cells (a preosteoclast cell line[32,33]) in comparison to BMP2 and BMP7 homodimers.

Materials and Methods

Experimental design

We performed dose-response studies to compare the respective effects of purified BMP2/7 heterodimer and homodimers on the RANKL-induced osteoclastogenesis of RAW264.7 cells. Four groups were set up: (1) rhBMP2/7 heterodimer; (2) rhBMP2 homodimer; (3) rhBMP7 homodimer; and (4) the 1:1 mixture of the two homodimers. We examined the following parameters: cell proliferation of preosteoclasts, osteoclast formation, tartrate-resistant acid phosphatase (TRACP) activity, osteoclastic gene expression, and calcium phosphate (CaP) resorption.

Cell culture

Preosteoclasts, RAW264.7 cell line (ATCC, Cell Bank of Chinese Academy of Sciences), were cultured in Dulbecco's minimum essential medium (Gibco™ Invitrogen) containing 10% fetal bovine serum (FBS, Gibco Invitrogen Corp., Grand Island, NY), and the medium was changed every three days. Exponentially growing cells were seeded at a concentration of 2×10^4 cells/well in 48-well plates to examine the cell proliferation, TRACP activity assay, and TRACP-positive cell staining, or at 4×10^4 cells/well in 24-well Osteoclast Activity Assay Substrate plates (OAAS, OCT USA, Inc., Irvine, CA) for CaP resorption assay, or at 2×10^5 cells/well in six-well plates for real-time reverse transcription polymerase chain reaction (RT-PCR) assay. Soluble rhRANKL (PeproTech Inc., Rocky Hill, NJ) were added to stimulate the osteoclastogenesis of preosteoclasts. After 24 h of incubation, different concentrations of rhBMPs (R&D Systems Inc., Minneapolis, MN) were added into the cultures. The medium and the factors, both BMPs and RANKL, were replaced every 3 days. Triplicates of cell cultures were performed for the gene experiment, and quadruplicates of cell cultures were performed for other experiments. We repeated the experiments twice to confirm the results.

RANKL concentration selection test

To find the optimal concentration for the osteoclastogenesis-inducing effect of the selected RANKL, we tested the effects of RANKL at concentrations of 10-, 20-, and 50 ng/mL on the osteoclastic differentiation properties of RAW264.7 cells. After 5 days, the cells were subjected to TRACP staining using a commercial kit (Sigma-Aldrich, St. Louis, MO). The cells that were positively stained with more than three nuclei were regarded as osteoclasts. The area and the number of osteoclasts stimulated by 50 ng/mL RANKL were 5- to 10-fold more than those stimulated by 20 ng/mL RANKL. In contrast, osteoclasts were merely detected under the stimulation of 10 ng/mL RANKL. Consequently, we selected 50 ng/mL RANKL in this *in vitro* osteoclastogenesis-inducing model.

Effect of BMPs on the cell proliferation of preosteoclasts

After the stimulation of BMPs for 3 days, to estimate the proliferation of RAW264.7 cells, cellular DNA content was fluorometrically quantified by a fluorescence spectrometer (SPECTRAmax M2, Molecular Devices, Sunnyvale, CA, Ex 480 nm/Em 520 nm) using Quant-iT™ PicoGreen dsDNA reagent and kits (Invitrogen™, Molecular Probes™, Molecular Probes, Eugene, OR) as previously described.[34]

Effect of BMPs on the histomorphological characteristics of osteoclasts

To visualize the effect of BMPs on osteoclastic differentiation of RANKL-induced RAW264.7, the cells were processed to TRACP staining after 4 days of BMP-stimulation. Applying a random sampling protocol, we examined the culture plates using a Nikon microscope with NIS-Elements F2.20 (Nikon Eclipse 80i, Tokyo, Japan) at a final magnification of ×100, and collected ~24 photomicrographs per well using a systematic random-sampling strategy. The

photomicrographs were printed in color for histomorphometric analysis. The total surface area and the total number of osteoclasts per well were histomorphometrically determined on the prints using a point-counting technique.[35] Furthermore, the average surface area of an individual osteoclast was also determined by dividing the total surface area of osteoclasts per well by the total number of osteoclasts per well.

TRACP activity assay

After a 4-day stimulation of BMPs, the culturing medium was removed, and the cells were lysed with 200 mL/well of 0.2% Triton X-100. Relative TRACP activity compared with the control group in the cell lysate was determined using TRACP detection solution as previously described.[32] The total protein content was spectrometrically measured at 570 nm using a commercial BCA Protein Assay kit (Beyotime® Institute of Biotechnology, Shanghai, China). The relative TRACP activity was normalized by the level of total protein content.

Real-time RT-PCR analysis

To investigate the relative gene expression of osteoclast cells, total RNA was extracted from the cells using RNeasy Mini Kit and RNase-Free DNase Set (Giagen GmbH, Hilden, Germany) after a 4-day stimulation of BMPs in the presence of 50 ng/mL RANKL. Real-time RT-PCR analysis was performed using PrimeScript™RT Reagent Kit, SYBR Premix Ex Taq™ (TaKaRa Biotechnology, Dalian, China), and ABI PRISM 7900HT Fast Real-Time PCR System with 384-Well Block Module (Applied Biosystems, Foster City, CA). To assess the quality of the extracted RNA and as an internal Real-time PCR standard, *β-actin* was also amplified. The primers' sequences were described in Table 1 and synthesized by Haojia Biotechnology Co., Ltd. (Shanghai, China).

CaP resorption assay

To estimate the CaP resorption capability of the formed osteoclasts, RAW264.7 cells were seeded on OAAS plates. After a 7-day BMP stimulation (5-, 50-, and 150 ng/mL), the plates were washed with 6% sodium hypochlorite solution to remove the cells. The wells were examined in a Nikon microscope with NIS-Elements F2.20 and photographed in color at a final magnification of ×100. Approximately 24 photomicrographs were collected per well using a systematic random-sampling strategy. The photomicrographs were printed in color for histomorphometric analysis. The resorption areas that exhibited white color on the pale brown background were estimated using the point-counting technique.[35]

Statistical analysis

All data are presented as mean values ± the standard deviation (Mean ± SD). Data pertaining to each group were compared using a one-way analysis of variance. The level of significance was set at $p < 0.05$ (SPSS statistical software, version 15, SPSS Inc., Chicago, IL).

Results

Proliferation of preosteoclasts

After a 3-day stimulation, a biphasic pattern in DNA content was consistently detected for each BMP (Fig. 1). For BMP2/7 heterodimer, the DNA content showed a dose-dependent increase from 5–150 ng/mL and then decreased at 200 ng/mL. The turning point occurred at as low as 10 ng/mL for BMP2 and BMP7 homodimer, or at 100 ng/mL for the 1:1 mixture of the two homodimers. In comparison to the non-BMP (RANKL alone) control group, a significantly higher level in DNA content was found under the stimulation of either 10–200 ng/mL BMP2/7 heterodimer or 5–100 ng/mL BMP2, BMP7, or their 1:1 mixture. No significant difference could be found among the four highest levels of DNA contents achieved by different BMPs.

Morphological characteristics of osteoclasts

After a 4-day stimulation, in the control group (RANKL without BMPs), hundreds of osteoclasts exhibited different size, varied approximately from Ø20 μm to Ø500 μm; while in the RANKL-BMP-treated groups, the osteoclasts sized approximately from Ø20 μm to Ø1500 μm.

The total surface area of osteoclasts per well was significantly enhanced by either 10–200 ng/mL BMP2/7, BMP2 or the 1:1 homodimers mixture, or 10–150 ng/mL BMP7 in comparison with the control group (Fig. 2: A–E). A biphasic pattern in the total area of osteoclasts was consistently detected for each BMP. The increase of the total area of osteoclasts started at the concentration of 10 ng/mL. The turning point (the highest level) occurred either at 150 ng/mL for

TABLE 1. SEQUENCES OF PRIMERS TO DETECT THE GENES OF ACP5 (ACID PHOSPHATISE 5, TARTRATE RESISTANT), CTSK (CATHEPSIN K), CALCR (CALCITONIN RECEPTOR), AND B-ACTIN USING REAL-TIME–POLYMERASE CHAIN REACTION

Genes	*GeneBank accession number*	*Sequences of primers (F = forward; R = reverse)*
Acp5 (acid phosphatase 5, tartrate resistant)	NM_007388.2	F:5′-GGAAATGGCCAATGCCAAAG-3′; R:5′-ATCATGGTTTCCAGCCAGCAC-3′
Ctsk (cathepsin K)	NM_007802.3	F:5′-CAGCAGAACGGAGGCATTGA-3′; R:5′-CTTTGCCGTGGCGTTATACATACA-3′
Calcr (calcitonin receptor)	NM_007588.2	F:5′-TTACCGACGAGCAACGCCTAC-3′; R:5′-AGCAAGTGGGTTTCTGCACTCA-3′
β-actin	NM_007393	F:5′-AGGAGCAATGATCTTGATCTT-3′; R:5′-TGCCAACACAGTGCTGTCT-3′

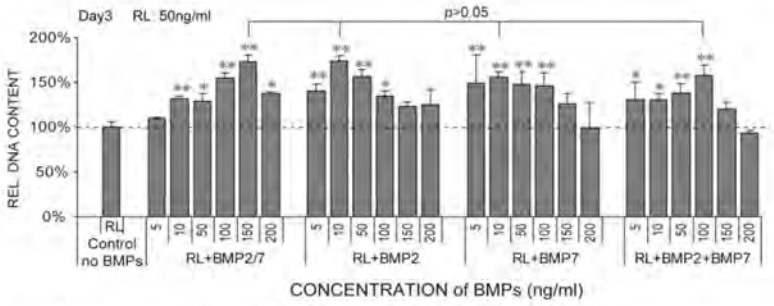

FIG. 1. Graph depicting the DNA contents of RAW264.7 cells after a 3-day stimulation of 50 ng/mL RANKL (RL) without or with BMP2/7 heterodimer, BMP2, BMP7, or 1:1 mixture of BMP2 and BMP7 homodimers of certain concentrations. Values are shown as means ± SD, $n = 4$, $^{**}p < 0.01$, $^{*}p < 0.05$ indicated the significant difference versus RL control group. BMP, Bone morphogenetic protein; RANKL, receptor activator of nuclear factor-κB ligand.

BMP2/7, BMP2, and the 1:1 homodimers mixture or at 100 ng/mL for BMP7. The highest level of the total area of osteoclasts under the stimulation of 150 ng/mL BMP2 was significantly higher than that under the stimulation of 150 ng/mL BMP2/7 or 100 ng/mL BMP7.

Consistent with the total surface area of osteoclasts, the total number of osteoclasts also exhibited an increasing trend within the lower concentration range and a subsequently decreasing trend in the higher concentration range. However, slightly different from the total area of osteoclasts, the highest level occurred at either 50 ng/mL for BMP2/7, or at 150 ng/mL for BMP2, or at 100 ng/mL for BMP7 and the 1:1 homodimers mixture. However, no significant difference in the highest level of osteoclast number was found among the four BMP groups (Fig. 2F).

Either 100–200 ng/mL BMP2/7, or 50–200 ng/mL BMP2, or the 1:1 homodimers mixture induced an increase in the average surface area of an individual osteoclast in a dose-dependent manner. There was no significant difference in the highest values of the average surface area of an individual osteoclast among these three different BMP groups. BMP7 did not show a promoting effect on the average surface of an individual osteoclast for all the monitoring concentration (Fig. 2G).

TRACP activity

After a 4-day stimulation, compared with the control group, significantly higher TRACP activity was found under the stimulation of 50–200 ng/mL BMP2/7, BMP2, and the 1:1 homodimers mixture or 50–150 ng/mL BMP7 (Fig. 3). The highest value of this parameter occurred at 150 ng/mL for BMP2/7 and BMP2 or at 100 ng/mL for BMP7 and the 1:1 homodimers mixture. No significant difference was found in the highest value of TRACP activity among the four groups (Fig. 3).

Relative mRNA expression of Acp5, Calcr, and Ctsk

The relative mRNA expressions of all three osteoclast-related genes, *Acp5*, *Calcr*, and *Ctsk*, were normalized by mRNA expression of *β-actin* (Fig. 4A). In comparison to the control group, the mRNA level of *Acp5* was significantly enhanced by 150–200 ng/mL BMP2. On the other hand, the mRNA level of *Acp5* was significantly decreased when stimulated by BMP2/7, BMP2, or the 1:1 homodimers mixture at 5 ng/mL.

Different from *Acp5*, the mRNA expression of *Calcr* when stimulated by each BMP in addition to RANKL was lower than that when stimulated by RANKL alone (Fig. 4B). The significant decrease in *Calcr* mRNA occurred at 5–10 and 200 ng/mL for BMP2/7, 5–50 ng/mL for BMP2, 5- and 50–200 ng/mL for BMP7, and all the selected concentration for the 1:1 homodimers mixture. The mRNA level of *Calcr* exhibited a biphasic pattern for BMP2/7, BMP7, and the 1:1 homodimers mixture. However, for BMP2, the *Calcr* mRNA expression increased with the increase of BMP2 concentration. There was no significant difference in the lowest level of *Calcr* gene expression among the four groups.

Referring to relative *Ctsk* mRNA expressions, although there were some variations depending on the concentrations of each BMP, no significant change could be observed when compared with RANKL control group (Fig. 4C).

CaP resorption assay

After 7-day stimulation, compared with the control group, BMPs significantly increased the CaP resorption area at the concentration of 5–150 ng/mL for BMP2/7, BMP2, and the 1:1 homodimers mixture or at 5–50 ng/mL for BMP7. The resorption area dose dependently increased in the presence of both BMP2/7 and BMP2. In contrast, the resorption area exhibited a dose-dependent decreasing trend for BMP7 and a biphasic pattern for the 1:1 homodimers mixture. BMP2/7 at 5 ng/mL induced significantly less resorption area than BMP7 at 5 ng/mL, while at 150 ng/mL, the relation between BMP2/7 and BMP7 was reversed. No significant difference could be found in the highest levels of CaP resorption among the four groups (Fig. 5).

Discussion

Bone regeneration is a delicate balance between bone formation and bone resorption, in which osteoblasts and osteoclasts play essential roles to couple these two processes functionally and quantitatively.[36] Although BMP heterodimers were repeatedly shown to induce osteogenesis both *in vivo* and *in vitro* in a significantly higher efficiency than

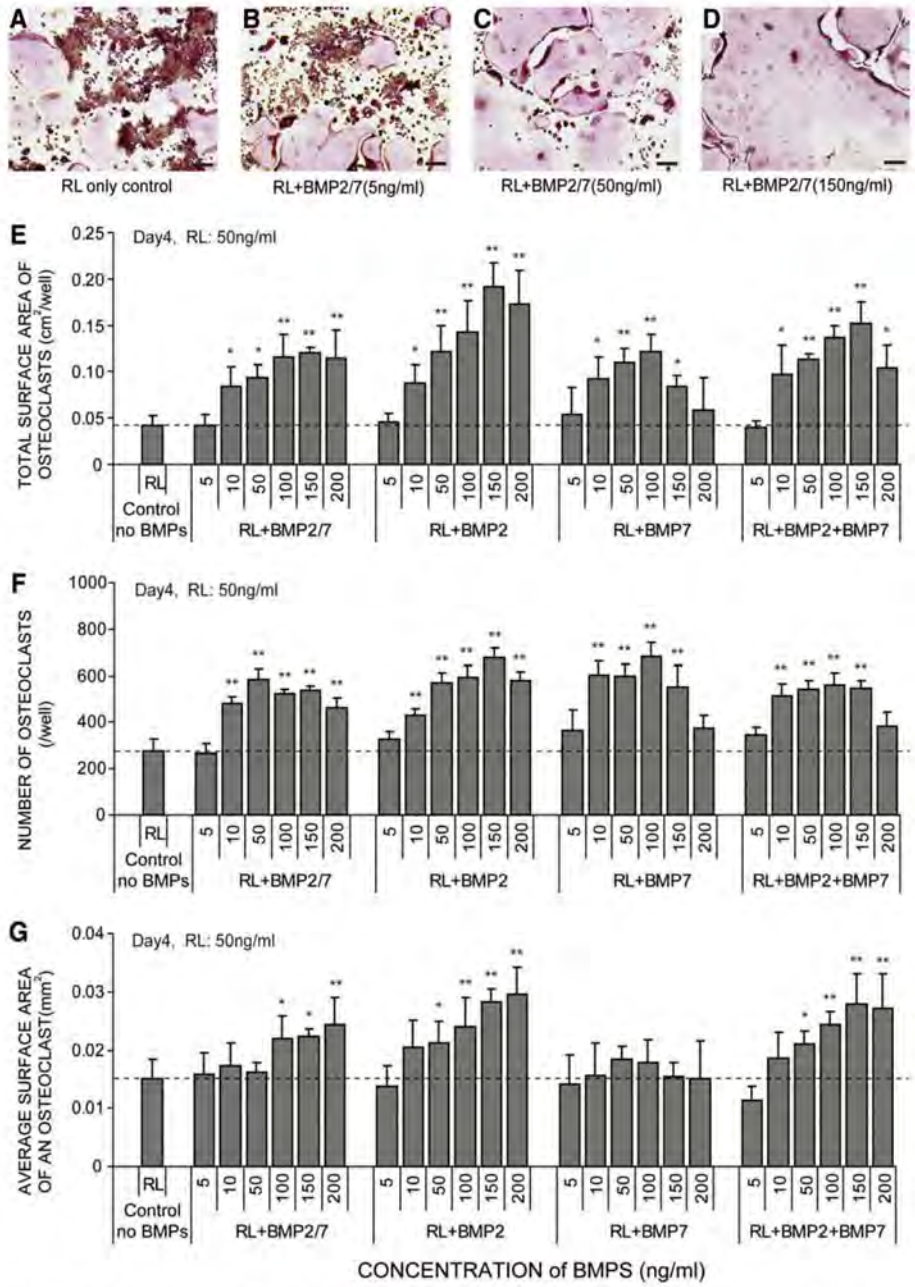

FIG. 2. Graph depicting the morphological characteristics of osteoclasts after a 4-day stimulation of BMP2/7 heterodimer, BMP2, BMP7, or 1:1 mixture of BMP2 and BMP7 homodimers of certain concentrations. **(A)** RANKL only control group (50 ng/mL RANKL without BMPs), RL; **(B)** BMP2/7 at 5 ng/mL had no significant effect on osteoclastic differentiation on the basis of 50 ng/mL RANKL stimulation; **(C–D)** BMP2/7 at 50 ng/mL, or 150 ng/mL significantly increased the osteoclastic differentiation on the basis of 50 ng/mL RANKL stimulation. **(E)** Total area of osteoclasts. **(F)** Total number of osteoclasts. **(G)** Average size of osteoclasts (total area/total number in each cell culture). Values shown are means ± SD, $n = 4$, $^{**}p < 0.01$, $^{*}p < 0.05$ indicated significant difference versus RL control group. Color images available online at www.liebertonline.com/tea

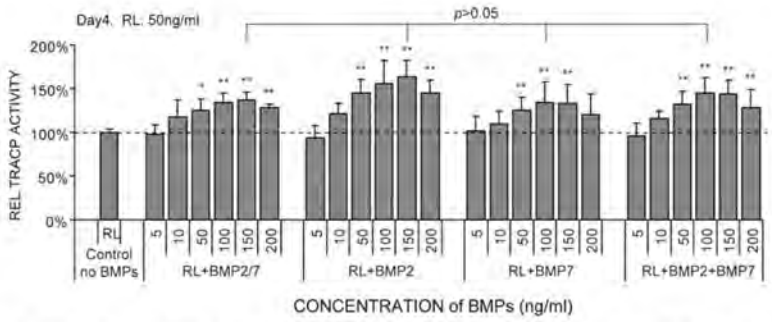

FIG. 3. Graph depicting the relative changes of TRACP activity under a 4-day stimulation of 50 ng/mL RANKL (RL) without or with BMP2/7 heterodimer, BMP2 homodimer, BMP7 homodimer, or 1:1 mixture of BMP2 and BMP7 homodimers of different concentrations. Values shown are means ± SD, $n=4$, $^{**}p<0.01$, $^{*}p<0.05$ indicated the significant difference versus RL control group. TRACP, tartrate-resistant acid phosphatise.

BMP homodimers, their effects on the osteoclastogenesis remains unclear. To our knowledge, this is the first study that delineates the effects of BMP heterodimer on the sequential cellular events of *in vitro* osteoclastogenesis.

RANKL alone can directly stimulate the osteoclastogenesis of preosteoclasts (RAW264.7 cells).[32,33,37,38] In contrast, although BMP-specific receptors were detected on the surface of mature osteoclasts,[28–30] BMPs alone failed to induce the fusion of preosteoclast into osteoclasts without RANKL.[30,37] We also found that BMP2/7 heterodimer or BMP2 or BMP7 homodimer alone could not induce *in vitro* osteoclastogenesis (Data now shown). The results indicated that BMP, irrespective of heterodimer or homodimer, could influence the process of osteoclastogenesis only by intervening in the RANKL signaling pathway. Interestingly, soluble BMP receptor-IA could significantly inhibit the promoting effect not only of BMP2, but also of RANKL on osteoclast formation,[30] suggesting that RANKL could also react with BMP receptors. Hitherto, the interaction between the RANKL-mediated and BMP-mediated pathways remains unclear.

The maximum effects on promoting the proliferation of RAW264.7 cells were obtained at 5 ng/mL for BMP2 and BMP7 homodimers, but at 150 ng/mL for BMP2/7 heterodimer (Fig. 1). BMP2 or BMP7 homodimers of high concentrations (150–200 ng/mL) did not drastically promote the proliferation of RAW264.7 cells, suggesting that the overstimulation of osteoclastic activity caused by BMP homodimers of high doses might not be attributed to the over-proliferation of preosteoclasts. In contrast, the BMP2/7 heterodimer of such high concentrations (150–200 ng/mL) significantly stimulates the proliferation of RAW264.7 cells. However, this can be avoided in the real application of the BMP2/7 heterodimer, because the BMP2/7 heterodimer of a low concentration range (5–50 ng/mL) was already adequate to drastically promote all the cellular events of osteoblastogenesis to the maximum levels.[23]

The maximum promoting effect of BMP2 occurred at 5 ng/mL for both preosteoblasts[23] and preosteoclasts (Fig. 1). In contrast, the maximum effect of BMP2/7 occurred at 5 ng/mL for preosteoblasts, whereas at 150 ng/mL for preosteoclasts. The maximum effect of BMP7 occurred at 100 ng/mL for preosteoblasts, whereas it occurred at 10 ng/mL for preosteoclasts. These functional specificities of the respective BMPs might be associated with their functional specificities in modulating the balance of osteoblastic and osteoclastic activities.

Interestingly, under the stimulation of the BMP homodimers, the total surface area of the subsequently formed osteoclasts did not correlate well to the total numbers of preosteoclasts. For example, BMP2 homodimer of 150–200 ng/mL did not significantly enhance proliferation of preosteoclasts (Fig. 1), but significantly induced the highest levels of the total surface area of osteoclasts (Fig. 2). On the other hand, BMP7 homodimer of 5 ng/mL did significantly enhance the proliferation of preosteoclasts (Fig. 1), but failed to do so in the fusion of preosteoclasts (Fig. 2). These findings suggested that BMP homodimers could also significantly modulate the fusion of preosteoclasts into osteoclasts in addition to their effects on their proliferation. It seems consistent with a recent study that preosteoclasts that were committed to proliferate might not be prone to arrest the cell cycle and, thus, not to fuse.[39] However, this seemed not to be the case for BMP2/7 heterodimer. The dose pattern of the BMP2/7's effects on cell fusion correlated well to that on cell proliferation. This finding exhibited the specificities of BMP2/7 heterodimer in stimulating osteoclastic fusion compared with BMP2 and BMP7 homodimers. Hitherto, how BMPs act on the fusion of preosteoclasts remains unclear. Although the presence of RANKL is indispensible for the stimulating effect of BMPs on osteoclast formation, BMPs' effects were not attributed to the changes in RANK, c-Fms, or GM-CSF expression.[30]

Stereological analysis showed that BMP2 homodimer and BMP2/7 heterodimer could both increase the number of osteoclasts and enlarge the average surface area of an individual osteoclast. These two BMPs of the concentrations that significantly promoted the total surface area of osteoclasts could also significantly enhance the total number of osteoclasts. However, no significant effect of the BMP7 homodimer could be found on promoting the average surface area of the osteoclast at all the selected concentrations. This result indicated that, under the stimulation of BMP7 homodimer, it was the significant enhancement in the number but not the

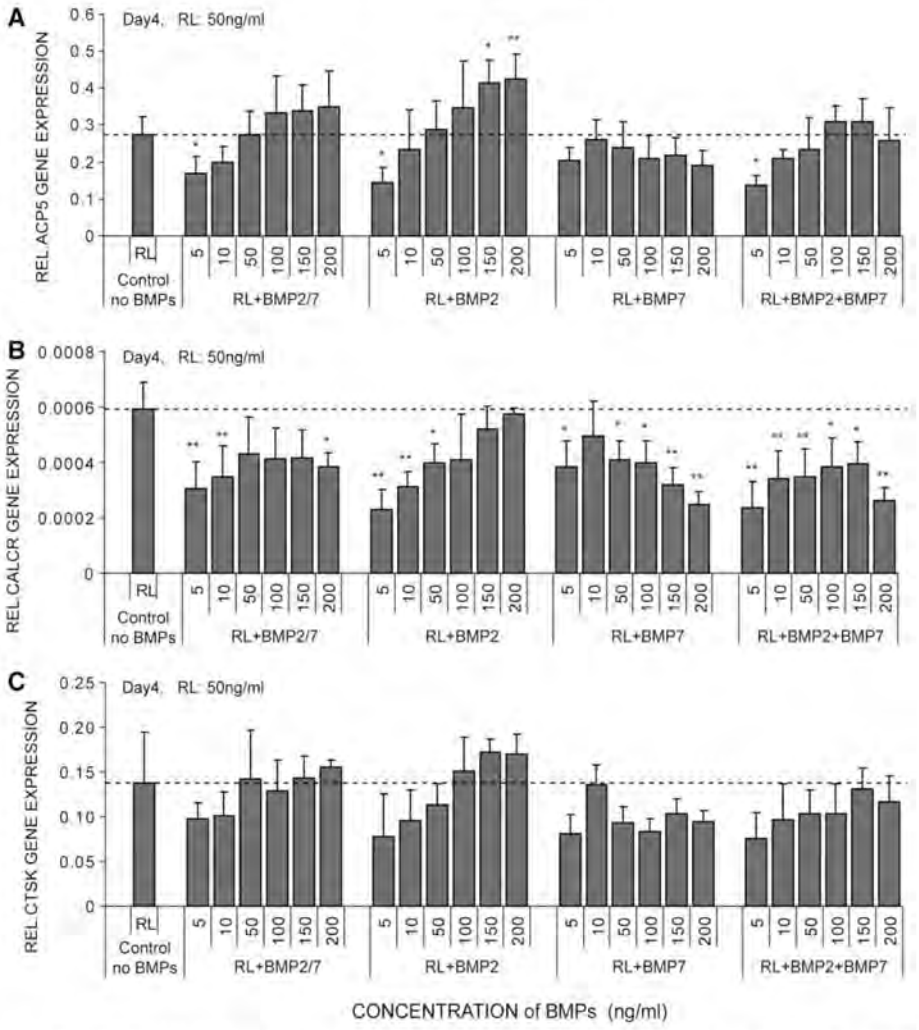

FIG. 4. Graph depicting the relative mRNA expression of *Acp5* **(A)**, *Calcr* **(B),** and *Ctsk* **(C)** under a 4-day stimulation of 50 ng/mL RANKL (RL) without or with BMP2/7 heterodimer, BMP2, BMP7, or 1:1 mixture of BMP2 and BMP7 homodimers. RL: RANKL control group (50 ng/mL RANKL without BMPs); BMP experiment groups (50 ng/mL RANKL with various BMPs of different concentrations). mRNA levels were normalized by corresponding *β-actin* mRNA expression and were shown as means ± SD, $n = 3$ per group, two experiments, $^{**}p < 0.01$, $^{*}p < 0.05$ indicated significant difference versus RL control group.

average surface area of osteoclasts that contributed to the significant enhancement in the total surface area of osteoclasts. This finding indicated that BMPs could dose dependently modulate the fusion of osteoclasts by influencing either the total number of osteoclasts or the average surface area of an osteoclast or the combination of these two aspects. The mechanism that accounted for such effects of BMPs needs further clarification. The motility of the multinucleated "founder" cells to catch monocytes for further fusion[40] can be a target of BMPs to control the numbers and the size of osteoclasts, since BMPs can modulate the chemotactic migration of many cell types.[23,41–43]

Apart from the formation of osteoclasts, BMPs can also significantly influence the osteoclastic differentiation such as the mRNA expression of *Acp5* (Fig. 4) and TRACP activity (Fig. 3). Interestingly, BMP of certain concentrations may enhance the resorption activity of osteoclasts by lowering the expression of calcitonin receptor and, thus, decreasing the sensitivity of osteoclasts to calcitonin. In contrast, no significant changes could be found on the relative mRNA levels of *Ctsk*.

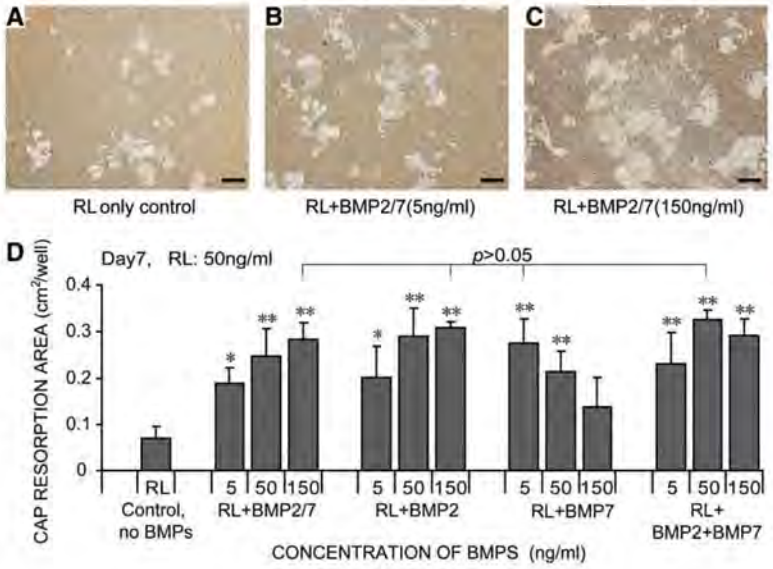

FIG. 5. Graph depicting the resorption area of RAW264.7 cells under a 7-day stimulation of 50 ng/mL RANKL (RL) without or with BMP2/7 heterodimer, BMP2, BMP7, or 1:1 mixture of BMP2 and BMP7 homodimers on Osteoclast Activity Assay Substrate (OAAS) plates. **(A)** RANKL control group (50 ng/mL RANKL without BMPs), RL; **(B–C)** BMP2/7 at 5 ng/mL, or 150 ng/mL significantly increased the CaP resorption area on the basis of 50 ng/mL RANKL stimulation. **(D)** CaP resorption area per well was stereologically analyzed. Values shown are means ± SD, $n = 4$, $^{**}p < 0.01$, $^{*}p < 0.05$ indicated significant difference versus RL control group. Color images available online at www.liebertonline.com/tea

Except for 150 ng/mL BMP7 homodimer, all the BMPs of the selected concentrations could significantly stimulate the osteoclastic function—CaP resorption (Fig. 5). Unexpectedly, 5 ng/mL BMPs, irrespective of homodimer or heterodimer, could not significantly enhance either proliferation of preosteoclast or the total surface area of osteoclasts, or osteoclast-related genes, but significantly enhance resorption activity. This finding indicated that BMP could also influence the function of osteoclasts. It is consistent with the previous study that the resorption activity of mature osteoclasts could be significantly enhanced by BMP.[37] This effect of BMPs may be partially mediated by sustaining the survival of osteoclasts.[30] Although cell fusion of preosteoclasts is not essential for bone resorption, it significantly enhances the efficiency of the action.[40] However, our finding indicated that the resorption activity did not correlate well to the total surface area of osteoclasts. The functional status of osteoclasts that are modulated by BMPs was another contributing factor.

Although the co-expression of two different BMP genes have been detected in developing limbs and during fracture healing, and co-purification of BMP2 and BMP7 proteins have also been reported in previous studies,[44–47] the modulating effect of BMP heterodimers on osteoblastic and osteoclastic activities during skeletal development and bone healing remains unclear.[14] Although both BMP2/7 heterodimer and the two homodimers were already capable of significantly enhancing resorptive ability of osteoclasts at a concentration of as low as 5 ng/mL, only the BMP2/7 heterodimer could spontaneously significantly induce the proliferation, differentiation of osteoblasts in such a low concentration.[23] Consequently, the BMP2/7 heterodimer may be a factor that balances osteoblastic and osteoclastic activities during osteogenesis, especially on the site of less BMP producers where they could only provide a lower concentration of BMP homodimers, such as in the beginning phase of fracture healing and embryonic development.[47]

Low-concentration (5–50 ng/mL) BMP2/7 heterodimer can be a promising strategy to repair large-volume bone defects. The BMP2/7 heterodimer induced a significantly higher level of osteoblastic activity than the two homodimers in this concentration range.[23] On the other hand, it induced a similar level of osteoclastic resorption as BMP homodimers. An appropriate osteoclastic activity is also important for the stimulation of osteoblastic activities,[48] and facilitates the rapid maturation of new bone. This finding suggested that low-concentration BMP heterodimers could facilitate more osteogenesis than BMP homodimers. This deductive conclusion was recently corroborated by our microCT evaluation of bone regeneration that was induced by the low-concentration BMP2/7 heterodimer in a peri-implant defect. It showed that 30 ng/mL BMP2/7 heterodimer could more rapidly induce bone regeneration in a significantly higher amount and maturation than BMP2 or BMP7 homodimers.[49]

It remains unclear as to which signaling mechanisms contribute to these functional advantages of BMP heterodimers over homodimers. However, we may find some clues in recent studies. The BMP2 homodimer has a high affinity to type I receptor BMPIa but a low affinity to type II receptor ActRIIb, while the BMP6 homodimer has a low affinity to type I receptor BMPIa but a high affinity to type II receptor ActRIIb.[50] In contrast, BMP2/6 heterodimer simultaneously has a high affinity to both type I and type II receptors, which results in significantly higher signaling.[51] Another mechanism can be the lower affinity of antagonists to BMP heterodimers.[14,52]

One limitation in this study was that the limited concentration range and also selected cell type could not directly extrapolate to the *in vivo* situations. Further studies need to be performed to obtain a deeper understanding of the BMP2/7 heterodimer's effect on osteoclastogenesis.

Conclusions

The BMP2/7 heterodimer could dose-dependently modulate every cellular event of *in vitro* RANKL-mediated osteoclastogenesis, such as the proliferation and fusion of preosteoclasts, gene expression, and function of osteoclasts. The optimal concentrations of BMP2/7 appeared to be different among the monitored cellular events. Low-dose (5–50 ng/mL) BMP2/7 heterodimer could significantly enhance the osteoclastic function (e.g., CaP resorption), which might facilitate a more rapid and earlier remodeling of its induced new bone.

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Disclosure Statement

All authors have no conflicts of interest.

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Chapter 6

A Traditional Chinese Medicine – Icariin- Enhances the Effectiveness of Bone Morphogenetic Protein 2

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Chinese Medicine（submitted）

Running title: Icariin enhances the effect of BMP-2

Abstract

Background: Bone morphogentic protein-2 (BMP-2) can induce new bone regeneration and enhance the osteogenic activity. A traditional Chinese Medicine Icariin has an osteogenic potency. We hereby hypothesize that administrating BMP-2 with Icariin could promote osteogenesis. The aim of this study was to compare *in vitro* the osteogenic effectiveness of BMP-2 with and without the presence of the Traditional Chinese Medicine Icariin.

Material and methods: Icariin and BMP-2 were administered together at different concentrations into the culturing medium of the pre-osteoblasts cell line MC3T3-E1. We applied BMP-2 (0ng/ml, 50ng/ml, 100ng/ml and 200ng/ml) with or without 6.8×10^3ng/ml Icariin to promote the osteoblastogenesis of the MC3T3-E1 cell line. We evaluated the efficacy by assessing: The proliferation of cell numbers after 1, 4 and 7 days; The alkaline phosphatase activity, which is a marker for early differentiation; and the osteocalcin which is a marker for late differentiation after 4, 7 and 10 days; Calcium deposition, which is a marker for final mineralization, after 27 days; The expression of osteoblastogenic genes such asalkaline phosphatase, Collagen I, osteocalcin, and Runx2 and endogenous BMP-2 after 1, 4 and 7 days was evaluated.

Results: Adding Icariin resulted mostly in significantly higher osteoblastogenic genes and proteins in a trend increasing in timeIrrespective of the concentration of BMP-2, the addition of Icariin resulted in a significantly higher cell proliferation and mineralization. The administration of these two agents accelerated further the osteogenic-related gene expression, especially OCN and Runx2.

Conclusions:

The results indicated that administering Icariin and BMP-2 working together can induce osteogenic activities in MC3T3-E1 cell line and promote osteoblastogeness.

Key words: Traditional Chinese Medicine,Icariin, Bone Morphogenetic Protein-2, Bone Tissue Engineering, Osteoblastogenesis. MC3T3 cell line, Osteogeness

Background

Various types of bioactive factors such as growth factors or biomolecules have been reported to possess osteogenic properties [1-4]. Bone morphogenetic protein 2 (BMP-2) is an osteoinductive growth factor, which is commercially available in its recombinant human form (rhBMP2) [5, 6]. However, the effective dose of milligrams of BMP-2 clinically was always very high [7]. Since BMP-2 is quite expensive, the costs are high. The high dose could also lead to a series of potential side effects, such as abnormal bone resorption [8] and unexpected bone formation in ectopic area [9]. Reducing the dose of BMP-2 could be one way to solve the problem but there is a great need to find a substitute for the expensive BMP-2. Researchers have found that the main component of a traditional Chinese medicine, icariin ($C_{33}H_{40}O_{15}$, molecular weight: 676.67), has a potential osteogenic property for bone tissue engineering [10]. Icariin is one of the primary active ingredients of Herba Epimedii, which is “yingyanghuo” in Chinese. Using modern separation techniques, Icariin has been successfully extracted from Herba Epimedii [10]. It was recorded in the Chinese pharmacopoeia for the purpose of anti-rheumatics (anti-inflammation), tonics (health promotion), and aphrodisiacs [11]. The osteogenic potential and the low price of Icariin make it a very attractive candidate as a substitute of BMP-2, or for enhancing the therapeutic effects of BMP-2. Previous studies have demonstrated that Icariin promoted osteoblast differentiation and mineralization *in vitro*[12, 13].However, the osteogenic effects of the administering Icariin with BMP-2 have not been investigated and the detailed molecular mechanisms underlying the effects are also unclear.

In this study, we investigated the osteogenic effects of administering the BMP-2 with Icariin on the proliferation and differentiation of MC3T3-E1 cells, since MC3T3-E1 cells, which are a precursor of functionalized osteoblast, can show the potency of osteogenic agents [14, 15]. We hypothesize that Icariin could enhance the osteogenic potency of BMP-2 and consequently reduce the dose of BMP-2.

Materials and methods

Materials

MC3T3-E1 came from the Chinese Academy of Science (Shanghai, China). Icariin, with a purity of more than 99%, was purchased from Tauto Biotech (Shanghai, China). BMP-2 was from R&D system (Minneapolis, MN, USA). α-Minimal essential medium (α-MEM) was obtained from Genom Bio-medicine Co. (Hangzhou, China). Fetal bovine serum (FBS)

was the product of Hangzhou Tianhang Bio-technology Co (Hangzhou, China). Cell Counting Kit (CCK-8) was purchased from Donjindo (Kumamoto, Japan). LabAssayTM alkaline phosphatase (ALP) was from Wako (Osaka, Japan), osteocalcin (OCN) EIA kit was from Biomedical Technologies, (Stoughton, MA). bicinchoninic acid (BCA) Protein Assay kit (Beyotime, Shanghai, China) cell lysates (Sigma-Aldrich, St. Louis, MO) Alizarin red, L-ascorbic acid and β-glycerophosphate were all purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Testing Groups

Eight groups of different concentrations of BMP-2 with or without Icariin were established. Each group had 3 samples, which was the number used in a previous study. See Table 1.

Cell culture

MC3T3-E1 cellswere cultured in α-Minimum essential medium (α-MEM) containing 10% fetal bovine serum (FBS). The medium was refreshed every 3 days. Exponentially growing cells were plated at 5×10^3 cells/well in 96-well plates for cell proliferation, at 2×10^5 cells/well in 6-well plates for ALP activity assay and OCN detection, or 2×10^4 in 48-well plates for alizarin red staining. After 24-h incubation, cells were subjected to a low-serum medium (2% FBS) for another 24 h and then followed by BMP-2 treatment.

Cell proliferation assay

To investigate the proliferation of MC3T3-E1 cells in response to different concentrations of BMP-2 with and without Icariin, 5000cells per well were used in 96-well plates. The numbers of newly proliferated cells were determined after 1 day, 4 days, and 7 days using Cell Counting Kit-8. The MC3T3-E1 cells were placed on 96well plates with 5000 cells per well in a humidified incubator for 24 hours. The cells were subjected to a low serum medium of 2% FBS for another 24 hours and then fresh media containing different concentrations of BMP-2 with and without Icariin were added (see table 1). The cell numbers were determined using a CCK-8 assay after 1, 4, and 7 days. At predetermined times, the cells were washed three times with PBS to eliminate non-viable cells, and then incubated with 10 μl of CCK-8 solution for 1 hour in the incubator. The optical density was measured using a microplate reader at a wavelength of 450 nm. Three wells were tested in each group for each incubation time and the resulting absorbance for each of the wells was

averaged.

Alkaline Phosphatase (ALP) activity assay

To determine the early difference between pre-osteoblasts MC3T3-E1 cell stimulated with different concentrations of BMP-2 with and without Icariin, the ALP activity and total protein content were measured after 4, 7 and 10 days. The ALP activity was determined using a commercially available LabAssay ALP colorimetric assay kit. The total protein content was measured at 570nm using a commercial BCA Protein Assay kit. The values that represented ALP activity were expressed as mmol p-NP/mg total protein.

Osteocalcin (OCN) expression assay

To assess the differentiation difference between MC3T3-E1 cellsstimulated with different concentrations of BMP-2 with and without Icariin, the amount of OCN secreted into the cell culture medium was determined. The cell supernatants were collected after 4, 7 and 10 days and were centrifuged at 10,000 rpm for 5 minutes at 4℃. The OCN concentrations of the supernatants were determined by ELISA using a mouse OCN EIA kit.

Mineralization

We compared the mineralization of MC3T3-E1 cells stimulated with different concentrations of BMP-2 with and without Icariin. The 2×10^4 cells in 48-well plates were treated with a mineralizing medium of 10% FBS, 50mg/mL Lascorbic acid, and 5mM b-glycerophosphate containing different concentrations of BMP-2 with and without Icariin. The medium was replaced every 3 days. After 27 days, the mineralized nodules were determined using alizarin red staining. The cultured plates were photographed with NIS-Elements F2.20 (Nikon Eclipse 80i, Tokyo, Japan), and the calcified area was measured using an Image-Pro Plus 6.0 analysis. All the experiments were repeated three times.

Real time PCR measurement of gene expression

The effects of different concentrations of BMP-2 with and without Icariin in stimulating osteogenic gene expression were also examined by quantitative RT-PCR. The messenger ribonucleic acid (mRNA) expression of bone protein alkaline phosphatase (ALP), BMP-2,

osteocalcin (OCN), collagen-I (Col Ⅰ), and runt related transcription factor 2 (Runx-2) was determined after 1, 4 and 7days of osteogenic induction. The total RNA was extracted from the cells by using a RNeasy Mini Kit and RNase-Free DNase Set. Single stranded complementary deoxyribonucleic acid (cDNA) was synthesized from total ribonucleic acid (RNA) with a Primescrip^{TM} RT Reagent Kit. A real-time polymerase chain reaction (PCR) was performed using 1μl of cDNA product in a 25μl reaction volume with Mastercycler ep realplex Real Time PCR System (Eppendorf, Germany). SYBR® Premix Ex TaqTM II, specific primers (see below), and 1μl of cDNA were used in each PCR reaction (95℃ for 30 s, 40 cycles of denaturation at 95℃ for 5 s, and annealing and extension at 60℃ for 30 s). The sense and antisense primers were designed with the Primer Express 3.0 based on published mouse cDNA sequences. SYBR was used as an internal control gene. Primer sequences were as given in Table 2. All real time PCR reactions were performed in triplicate and the results after calibration with β-actin expression were calculated using the comparative threshold cycle (DDCT) method and are presented as fold increase relative to the non-stimulated control.

Western blotting

For the western blotting examination, MC3T3-E1 were cultured in medium of different concentrations of BMP-2 with and without Icariin for 1, 4 and 7 days. The cells were washed three times with cold PBS and collected by centrifugation and washed once with PBS. The washed cells were lysed on ice for 30 min in RIPA lysis buffer (Thermo, DE) supplemented with protease inhibitor cocktail, phosphatase inhibitor cocktail and phenylmethanesulphonyl fluoride (PMSF) (Kangchen,Shanghai, China). Protein concentration was measured using a BSA protein assay kit. 20 μg of the sample was resolved on 10% SDS-PAGE gel and electro-transferred to polyvinylidene difluoride membrane. Membranes were blocked and incubated with appropriate primary antibodies including ALP (ablcam, USA)and Runx2 (CST, USA) at dilution of 1:1000. For normalization of protein loading, mouse anti-rat b-actin (Sigma) antibody was used at 1:10000 dilution. Finally, membrane reactions were visualized using horseradish peroxidase (HRP)-conjugated secondary antibodies (Beyotime, dilution, 1:1000) with ECL plus reagents (Amersham Pharmacia Biotech, Buckinghamshire, England) by UVItec ALLIANCE 4.7 gel imaging system. Protein band intensities on scanned films were

compared to their respective controls using Quantity One Image software. Bands were first rounded up using a volume rect tool, then target area intensity was calculated. Density of β-actin was used as control for protein expression of ALP and Runx2.

Statistical analysis

Statistical comparisons of the results obtained with different concentrations of BMP-2 with and without Icariin were made using a one-way variance analysis. Then further comparisons were made using Bonferroni corrections. The level of significance was set at $p<0.05$. The statistical package for social science (SPSS) software, version 17 from SPSS Inc., Chicago, IL for a Windows computer system was used for the statistical analysis.

Results

Cell proliferation

Icariin significantly enhanced the cell proliferation at all the three times compared with the control group without bioactive agents (Fig. 1). The administration of BMP-2 at 0, 50, 100, and 200mg/ml with Icariin resulted in a significantly enhanced cell proliferation compared with BMP-2 used alone at all the three times (Fig.1).

ALP activity

Icariin significantly enhanced the ALP expression with BMP-2 compared with the control group without bioactive agents at all the three times (Fig. 2). When BMP-2 was present at 50 or 100 ng/ml, Icariin significantly increased the ALP activity after 4 and 10 days culturing but not after 7 days (Fig. 2). When the BMP-2 concentration was 200ng/ml, Icariin significantly increased the ALP activity at all the three times.

OCN expression

Icariin significantly enhanced the OCN expression compared with control group without bioactive agents at all the three times (Fig. 3). The OCN expression increased significantly with Icariin in most Icariin groups compared with the groups without Icariin (Fig. 3). Only when BMP-2 was 200ng/ml on day 7, was no significant difference observed (Fig. 3).

Cell matrix mineralization

After a 27-day culture, Icariin significantly increased the mineralization compared with

control group without bioactive agents (Fig. 4). The administration of Icariin with BMP-2 significantly increased the mineralization compared with BMP-2 only (Fig. 4).

Real-time PCR quantification measurement of gene expression

Icariin resulted in a significant increase of ALP expression compared with the control group without bioactive agents after 4 and 7 days, but not after 1 day (Fig. 5A). The results of the administration of icariin with BMP-2 are shown in Fig.5A. The administration of icariin with BMP-2 at 200ng/ml significantly increased ALP expression in all times. However, the administration of Icariin with BMP-2 at 100ng/ml did not show any significant effects. When BMP-2 was at 50ng/ml, the administration of BMP-2 with icariin only significantly increased the ALP expression after 4 days.

BMP-2 gene expression was dramatically improved with Icariin on day 7 ($P<0.01$) (Fig.5B). However, the administration of Icariin with BMP-2 at 50ng/ml only slightly increased the BMP-2 expression after 7 days ($P<0.05$) and Icariin and BMP-2 at 100ng/ml showed improvement after 1 day ($P<0.05$). The BMP-2 gene expression was not increased by the administration of icariin with BMP-2 at 200ng/ml on 1 day and 4 days, but was reduced after 7 days($P<0.01$) (Fig. 5B).

The addition of Icariin increased Col Ⅰ gene expression after 1 and 4 days (Fig. 5C). The results of the administration of icariin with BMP-2 were shown in Fig. 5C. The administration of Icariin with BMP-2 at 50ng/ml increased Col Ⅰ gene expression only after 4 days, and BMP-2 at 100ng/ml with the addition of Icariin did not improve the Col Ⅰ gene expression significantly. When BMP-2 was at 200ng/ml, the administration of Icariin increased the Col Ⅰ expression after 4 and 7 days. .

The presence of Icariin increased more OCN gene expression after 4 and 7 days (Fig. 5D). Cells treated with Icariin and BMP-2 at 50ng/ml, 100ng/ml and 200ng/ml expressed dramatically more OCN gene than with BMP-2 alone after 1 day, 4 and 7 days except for BMP-2 at 50n/ml on day 1 ($P<0.01$)(Fig. 5D).

A statistical difference in the gene expression of Runx2 was observed when Icariin was added to the cells only after 1 day (Fig. 5E). The Runx2 gene expression was increased by the administration of Icariin with BMP-2 at 50ng/ml, 100ng/ml and 200ng/ml after 1, 4 and 7 days compared to the BMP-2 alone, except for BMP-2 at 50ng/ml after 1 day.

Western blotting

Results of western blotting showed that Runx2 expression seemed to be upregulated by BioCaP combined with Icariin and BMP-2 compared with BMP-2 incorporated into BioCaP granules. Though ALP expression had no significant changes when the addition of Icariin into the BioCaP granules incorporated with BMP-2 (Fig. 6).

Discussion

BMPs' signaling is crucial and indispensable for osteogenesis. The cellular and therapeutic effects of BMPs are mediated by their downstream signaling pathways that are initiated by BMPs binding with transmembrane serine/threonine kinase receptors [16]. BMPs can trigger two main downstream signaling pathways: Smad-dependent and Smad-independent [17]. The BMP signaling cascade is comprised of two types of receptor serine/threonine protein kinases, type Ⅰ and Ⅱ receptors, and transcription factors belonging to the SMAD family that transduce the BMP signal to the nucleus[18]. Many agents, such as Icariin [19], simvastatin and Vitamine D3, promote osteogenesis through stimulating the endogenous BMPs. , [20]. However, up till now, there is no evidence that these drugs are capable of inducing new bone formation in an ectopic site (subcutaneously) as BMPs does. It suggests that exogenous BMPs such as BMP-2 may be still indispensable for repairing large bone defects, since these defects have a similar micro-environment as an ectopic site [21].

In this study, we first focused on the effect BMP-2 administered with Icariin on *in-vitro* osteoblastogenesis. Firstly, it was shown that Icariin had significant positive effects on the proliferation and the osteogenic effects of MC3T3-E1 cells. Secondly, Icariin administered with BMP-2 of 50, 100, or 200ng/ml showed that Icariin significantly increased the osteogenic effectiveness of BMP-2. Compared with using BMP-2 alone, using the two together achieved a better osteogenic effect on the level of proliferation, protein, mRNA expression and mineralization.

Icariin used with BMP-2 significantly increased the effects compared with BMP-2 alone. A previous study demonstrated that the Runx2, BMP-2 and BMP-4 mRNA expression level were significantly increased with Icariin [22]. Icariin exerts its osteogenic potential by inducing Runx2 expression, by the production of BMP-2, and by the BMP activation of signaling [23]. A recent study indicated that Icariin can modulate bone

formation via the BMP-2/Smad 4 signal transduction pathway in human osteoblastic cell line [19]. Meanwhile, it inhibits osteoclastogenesis induced by lipopolysaccharide (LPS) by suppressing the activation of the p38 and Jun N-terminal kinase (JNK) pathway [24].

Although the signaling of Icariin in promoting osteoblastogenesis and osteogenesis was already partially unveiled, the mechanism of the synergistic effect of exogenous Icariin and BMP-2 has been poorly investigated. In the present study, when we focused on the mRNA expression levels, the administration of these two agents together accelerated further the gene expression related to osteogenesis, especially the OCN and Runx2 expression. Treatments with Icariin and BMP-2 for 1, 4 and 7 days strongly increased the mRNA expression of Runx2, which is the master transcription factor in osteogensis [1]. Other markers which represented the different stages of osteogenic potential were mostly increased. Ohba et al [25] suggested two possible mechanisms for the involvement of BMP signaling in the effects of Icariin. Firstly, Icariin may activate BMP signaling indirectly through extracellular BMPs. Secondly, Icariin may activate BMP signaling directly by interacting with Smads via unknown mechanisms. BMP-2 promotes osteogensis mainly through increased intracellular alkaline phosphatase activity, osteocalcin and collagen protein synthesis [26].

In the present study, Icariin alone significantly increased the BMP2 gene expression after 7 days. However, the administration of Icariin with BMP-2 at 50ng/ml on day 7 and at 100ng/ml on day 1 compared with BMP-2 alone gave only a little improvement. What is more, the administration of Icariin with BMP-2 at 200ng/ml failed to increase the BMP-2 gene expression on day 1 and 4, even reduced it on day 7. This reminded us that osteogenic activity induced by BMP-2 would reach a plateau at 200-400g/ml [27]. Thus, we hypothesized that Icariin may directly activate BMP signaling, since when administrating both two agents to the cells may produce high concentration of BMP-2 mRNA, this situation may contribute to the failure increasing of BMP-2 gene expression. What's more, the administration of both Icariin and BMP-2 to MC3T3-E1 cells after 7 days may further increase the expression of noggin, which is a BMP inhibitor [28], since the effect of Icariin and BMP-2 at 200ng/ml decreased after 7 days culture.

The toxicity of an agent which is going to be used clinically is always the first concern. Consistent with the previous findings [23, 29], we have chosen the optimal concentration of Icariin in stimulating proliferation of MC3T3-E1, which is 6.8×10^3 ng/ml in our previous study [30]. Therefore, this concentration was chosen for the current study.

In this study, we only applied one kind of osteogenic cell line, which might behave differently from the others. The viability of the primary osteoblasts which were harvested from a 8-month-old female Imprinting Control Region mice reached a maximum at 10^{-8}M of Icariin [7]. The cell viability with 10^{-9}M of Icariin was significantly increased by 136.50±6.47% in hFOB 1.19 human osteoblastic cell line cells compared to the control cells [19]. We adopted MC3T3-E1 because it was a well-established model for testing osteoblastogenesis *in vitro*[27]. Additionally this cell is very similar to a primary calvarial osteoblast [31]. Since the results from this study are very promising for the administration of these two agents, we may further adopt their effect on human primary bone marrow stem cells in order to give a stronger indication for clinical use. The mechanism of the osteogenic effect should also be investigated further.

In our previous study, we have developed for the first time a novel biomimetic calcium phosphate bone substitute (BioCaP) as a dual release system [32]. In the future, we would like to apply the model of the administration of Icariin with BMP-2 when it is internally incorporated into BioCaP based delivery system to evaluate this model on bone regeneration in animal models.

Conclusions

In conclusion, Icariin can stimulate osteogenic activity; the administration of Icariin with BMP-2 can greatly enhance the osteogenic effect of BMP-2 by stimulating the osteogenic activity of M3T3-E1 cell. This suggests that traditional Chinese medicine with bone growth factors such as Icariin might be applied to healing bones in bone tissue engineering.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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Table 1.Testing groups. The MC3T3-E1 cells cultured at different concentration (0ng/ml, 50ng/ml, 100ng/ml, 200ng/ml) of BMP-2 co-administration with icariin (6.8×10^3ng/ml) or not.

Groups	1	2	3	4	5	6	7	8
Icariin	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(+)
BMP-2	(-) 0ng/ml	(-) 0ng/ml	(+) 50ng/ml	(+) 50ng/ml	(+) 100ng/ml	(+) 100g/ml	(+) 200ng/ml	(+) 200ng/ml

Table 2. Primer sequences for real-time quantitative polymerase chain reaction analysis of the gene expression. The specific informations of alkaline phosphatase (ALP), BMP-2, collagen I, osteocalcin (OCN), and Runx2 genes.

Gene	**Accession No.**	**Primers (F=forward; R=reverse)**
ALP	NM_007431	F: 5'- TGCCTACTTGTGTGGCGTGAA -3'; R: 5'- TCACCCGAGTGGTAGTCACAATG -3'
BMP-2	NM_007553	5'-AAGAGACATGTGAGGATTAGCAGGT-3'and 5'-GCTTCCGCTGTTTGTGTTTG-3'
Collagen I	NM_007742	F: 5'- ATGCCGCGACCTCAAGATG -3'; R: 5'- TGAGGCACAGACGGCTGAGTA -3'
Osteocalcin (OCN)	NM_007541	F: 5'- AGCAGCTTGGCCCAGACCTA -3'; R: 5'- TAGCGCCGGAGTCTGTTCACTAC -3'
Runx2	NM_009820	F: 5'- CACTGGCGGTGCAACAAGA -3'; R: 5'- TTTCATAACAGCGGAGGCATTTC -3'
β-actin	NM_007393	F: 5'- AGGAGCAATGATCTTGATCTT -3'; R: 5'- TGCCAACACAGTGCTGTCT -3'

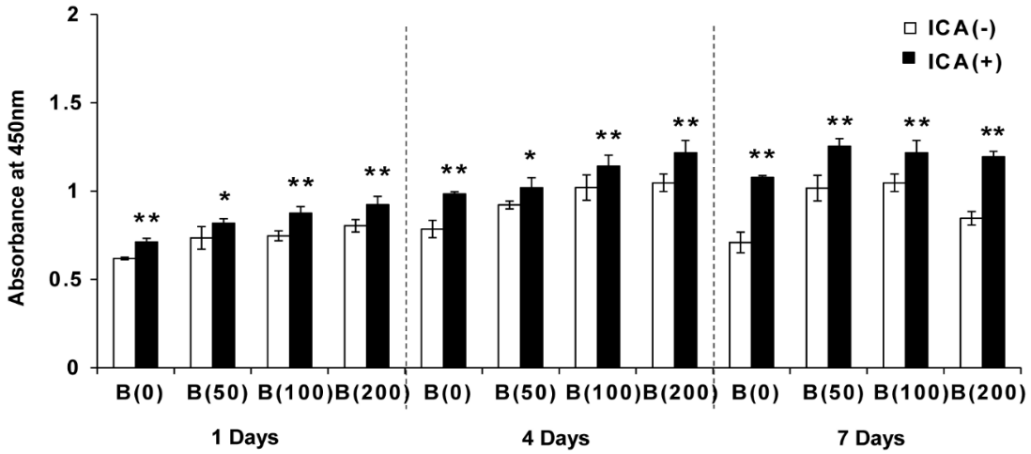

Fig.1. The proliferation of MC3T3-E1 under different concentrations of BMP-2 with or without icariin. The absorbance was measured on day 1, 4 and 7. Concentrations of BMP-2 were 0 ng/ml (A), and 50ng/ml, 100ng/ml, 200ng/ml (B). Mean values (n=3 samples per group) were represented together with the standard deviation. Icariin (ICA) was supplemented at 6.8×10^3ng/ml. *P<0.05 **P <0.01 ICA positives versus ICA negatives separately.

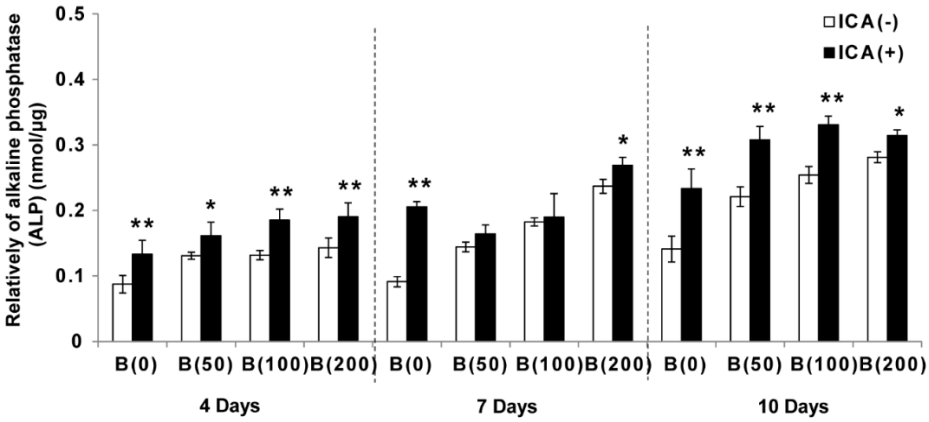

Fig. 2. The ALP activities of MC3T3-E1 under different concentration of BMP-2 with or without icariin treatments. The ALP activities were determined by colorimetric assay on day 4, 7 and 10. Concentrations of BMP-2 were 0 ng/ml (A), and 50ng/ml, 100ng/ml, 200ng/ml (B). Mean values (n=3 samples per group) were represented together with the standard deviation normalized by total cellular protein. Icariin (ICA) was supplemented at 6.8×10^3ng/ml. *P<0.05, **P<0.01 ICA positive versus ICA negative separately.

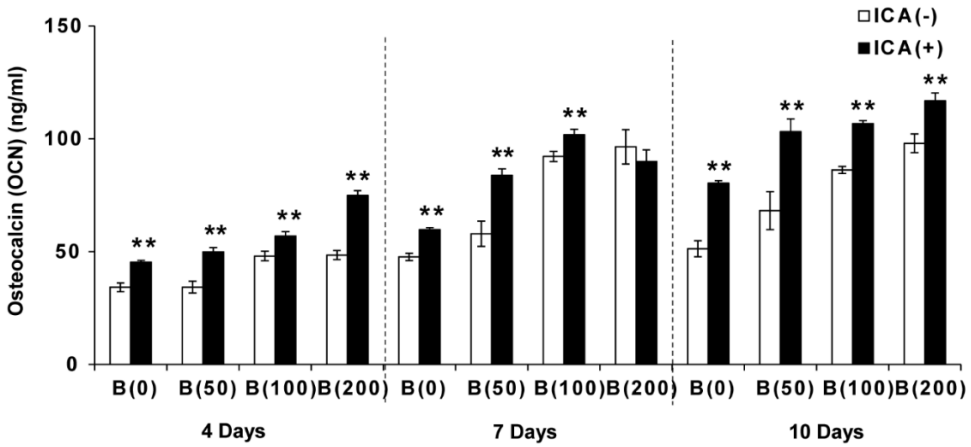

Fig. 3. The OCN expressions of MC3T3-E1 under different concentration of BMP-2 with or without icariin treatments. The OCN expressions were determined by ELISA on day 4, 7 and 10. Concentrations of BMP-2 were 0 ng/ml, and 50ng/ml, 100ng/ml, 200ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Icariin (ICA) was supplemented at 6.8×10^3ng/ml. *P<0.05, **P<0.01 ICA positive versus ICA negative separately.

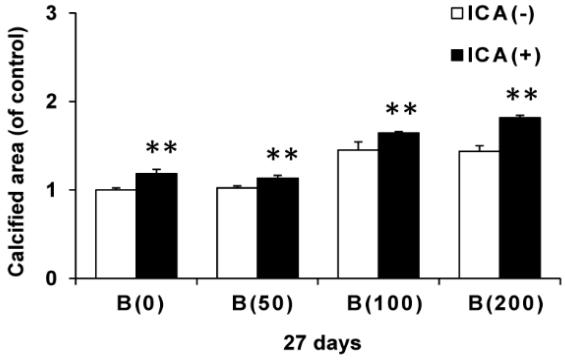

Fig. 4. The Quantitative calculation of calcified area in different groups on day 27. (A) Concentrations of BMP-2 were 0 ng/ml, and 50ng/ml, 100ng/ml, 200ng/ml. (B) Microscopic images of alizarin red stained 27 days after eight groups treatment. Mean values (n=3 samples per group) were represented together with the standard deviation. Icariin (ICA) was supplemented at 6.8×10^3ng/ml. *P<0.05, **P<0.01 ICA positive versus ICA negative separately.

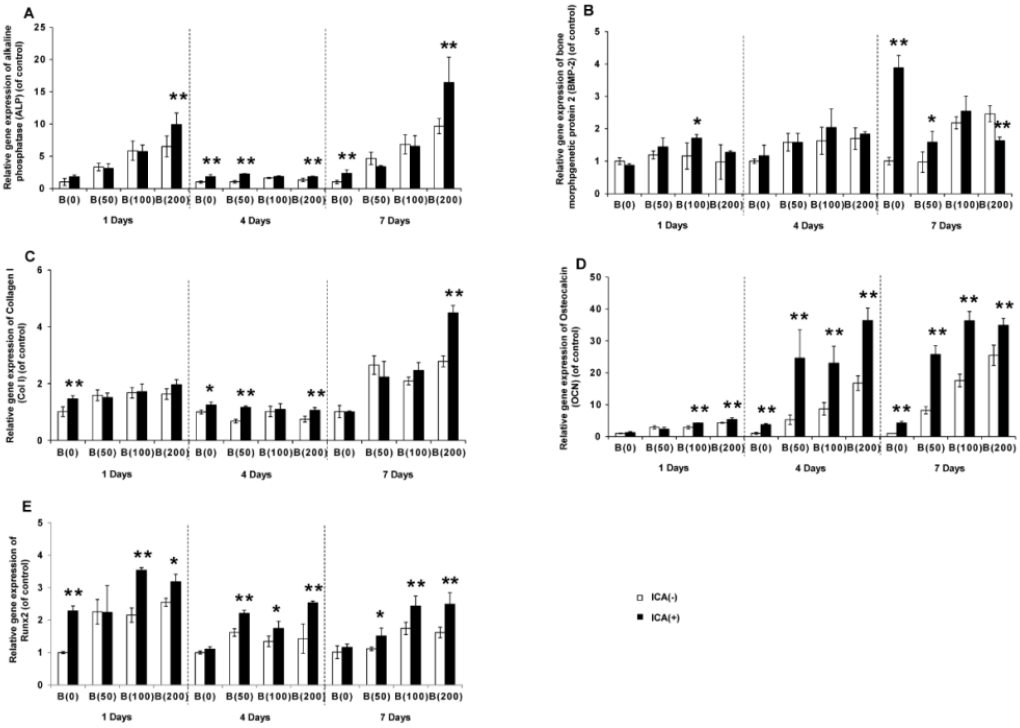

Fig. 5. The time course changes in mRNA expression. The mRNA expression of (A) ALP, (B) BMP-2 (C) Col Ⅰ, (D) OCN, (E) Runx2 in MC3T3-E1 on day 1, 4 and 7 days under different concentration of BMP-2 with or without icariin treatments. Concentrations of BMP-2 were 0 ng/ml, and 50ng/ml, 100ng/ml, 200ng/ml. Mean values (n=3 samples per group) were represented together with the standard deviation. Icariin (ICA) was supplemented at 6.8×10^{3}ng/ml. *P<0.05, **P<0.01, ICA positive versus ICA negative separately.

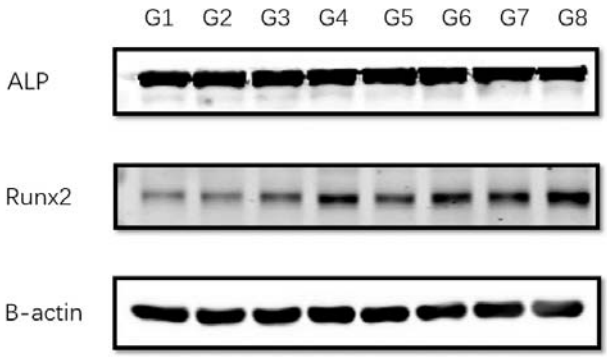

Figure 6. The Protein expression of osteoblastic markers in MC3T3-E1 treated with different concentration of BMP-2 with or without icariin. **The protein expression was detected by western blot.**The details of each group was explained in table 1.

Chapter 7

Combination of Icariin and bone morphogenetic protein 2: A potential osteogeniccompound for bone tissue engineering

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In preparation

ABSTRACT:

Introduction: Icaiin, a typical flaonol glycoside, is the main active component of Herba Epimedii, which was used to cure bone-related diseases in China for centruies. It has been reported that Icariin can be delivered locally by biomaterials and it has an osteogenic potential for bone tissue engineering. In this study, the osteogenic potential of biomimetic calcium phosphate bone substitute (BioCaP), a novel drug delivery system, internally-incorporated Icariin was evaluated. The BioCaP with combined regimens of Icariin and bone morphogenetic protein 2 was investigated *in vitro* using MC3T3-E1 cell line as well as to repair the critical-size bone defects (8mm in diameter) in SD male rats skull.

Materials and Methods: BioCaP was fabricated according to a well-established biomimetic mineralization approach. *In vitro*, the effects of BioCaP alone or BioCaP with Icariin or/and BMP-2 on cell proliferation and osteogenic differentiation of MC3T3-E1 cells were systematically evaluated. *In vivo*, five groups were established: (1) BioCaP with neither Icariin nor BMP-2; (2) BioCaP with BMP-2; (3) BioCaP with Icariin; (4) BioCaP Icariin and BMP-2; (5) Autogenous bone in CSBD. Twelve weeks after implantation in the critical-size defects in rat skull, samples were withdrawn for Micro-CT and histological analysis.

Results: *In vitro*,BioCaP incorporated with or without Icariin had positive effects on osteogenic differentiation of MC3T3-E1. And especially in the presence of Icariin, BioCaP could have better osteogenic efficiency. However, they didn't have influence on cell proliferation. Moreover, coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone, especially on the protein and gene expression of ALP and OCN, and mineralization as well. *In vivo*, the volume of newly formed bone associated with BioCaPinternally-incorporated with both Icariin and BMP-2 was significantly higher than the control and BioCaP with Icariin or BMP-2. BioCaP with Icariin induced more new bone formation than the BioCaP only. Micro-CT analysis revealed that bone mineral density of newly formed bone for BioCaP with Icariin and/or BMP-2 have no significant differences.

Conclusions: BioCaP incorporated with Icariin could enhance *in vitro* osteogenic differentiation, but had no proliferation increasement. Coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone Icariin combined with BMP-2 internally-incorporated into BioCaP have a synergistic stimulatory effect for the repair critical-size bone defects in rat skull.

Key words: Biomimetic calcium phosphate; MC3T3-E1; Critical-sized bone defect; Icariin; BMP-2

1. Introduction:

Bone defects are quite common and cause a quite large clinical and biomedical burden. As a result of inflammatory disease, tumor, trauma, or anatomical and congenital disorders, When the defects can't be self-healed, it requires bone grafting or bone substitutes, which are of paramount importance to achieve a suitable functional and great aesthetic restoration [1, 2]. The golden standard for bone reconstruction is still the autologous bone graft [3]. There are many advantages using autologous bone due to its natural osteoinductivity, but there are also disadvantages associated with harvesting of the bone transplant, such as limited availability of bone volume, donor-site morbidity and risk of infection. The application of allografts, xenografts or biosynthetic substitutes eliminates these disadvantages associated with autologous bone [4].

Biosynthetic substitutes, such as calcium phosphate (CaP) based biomaterials have been successfully used as a graft material, because of their good biocompatibility, osteoconductivity, and chemical composition, which resembles the composition of the natural bone matrix [5-9].Recently, biomimetic calcium-phosphate materials have been received an increased interest, because of their capacity to carry (delivery) bioactive agents without compromising their bioactivity [10-14]. Biomimetic materials are capable of eliciting specific cellular responses and directing new tissue formation [15]. Based on the mature technic, Liu et al. recently developed a biomimetic calcium-phosphate bone substitute (BioCaP) using a refined approach. The BioCaP granule is used as a dual protein-delivery system which poses an internally-incorporated mode. Protein and calcium phosphate were precipitated together to form BioCaP granules in this system in which a depot of protein was incorporated in the center of the granules as an internal depot. Previous data showed that the volume densities of bone, bone marrow, and blood vessels were significantly higher in granules where BMP-2 was incorporated internally [16].

Recently, we have found that Icariin is a strong bone anabolic agent, which is comparable with BMP2 in terms of enhancing MC3T3-E1 cells proliferation and osteogenic differentiation [17]. Icariin is a small molecule compound, which is the main pharmacological component of Herba Epimedii, a centuries old traditional medicine herb [18]. Previous studies have shown that Icariin can increase osteogenic differentiation [19-21] and inhibit osteoclastic formation [22, 23]. When loaded into calcium phosphate cement scaffold [24], β-tricalcium phosphate ceramic [25] or chitosan/hydroxyapatite [26] it could promote bone regeneration. In addition, a 24-month randomized double-blind

placebo-controlled clinical trial indicated that a daily dose of 60mg Icariin and 15mg daidzein, and 3mg genistein has beneficial effects in preventing bone loss in postmenopausal women, without resulting in a detectable hyperplasia effect on the endometrium [27].

Strategies to reduce the high dose of BMP-2 used clinically are needed due to safety and economic reasons---BMP-2 can intrigue unwanted calcification [28] abnormal bone resorption [29] unexpected bone formation in ectopic area[30], and may stimulate cancerous cell growth[31]. The easiest way is to reduce the dose level of BMP-2. Therefore we applied combination of Icariin and BMP-2 in our previous study *in vitro* to test the possibility.

Our previous study has demonstrated that Icariin can promote the osteogenic efficience of BMP-2 in MC3T3-E1 cells, which is a precursor of functionalized osteoblast. Based on the above knowledge, applying a combination of Icariin and BMP-2 internally-incorporated into the interior of BioCaP granule appears to be a promising approach to repair bone defects.

In this article, we were about to investigate whether the BioCaP internally -incorporated with Icariin induces osteogenic proliferation and differentiation of MC3T3-E1 *in vitro*. In the meanwhile, we tested the hypothesis that above modified bone substitute carry Icariin and/or BMP-2 could be used to promote the repair of critical sized bone defect in a rat skull model.

2. Material and Methods

2.1 Fabrication of biomimetic calcium phosphate (BioCaP) bone substitute

According to the biomimetic coating principle[32, 33], BioCaP was fabricated by refining [16]. Briefly, a supersaturated CaP solution (200 mM HCl, 20 mM $CaCl_2 \cdot 2H_20$, 680 mM NaCl, and 10 mM Na_2HPO_4) with or without agents (Icariin (≥99%, Tauto Biotech, Shanghai, China) and/or BMP-2(R&D system, Minneapolis, MN, USA)) (the concentration of the two agents *in vitro* and *in vivo* respectively, see table 1) buffered by TRIS (250 mM) to a PH of 7.4. This solution was incubated in a shaking water bath (50 agitations/minute) at 37°C. Rapid precipitation appeared at PH of 6.25. Protein was added to this CaP solution and co-precipitated (incorporated) into the interior of BioCaP (viz., internal depot of protein). After 24 h of incubation, the precipitation was retrieved, gently washed by Milli-Q water, filtered, and compressed to form a tablet (diameter: 5 mm; thickness: 0.4 mm) using

a vacuum exhaust filtering method with a vacuum filter (0.22-lm pore, Corning, NY, USA) and an air pump. After drying at room temperature, BioCaP tablets were ground and filtered to obtain granules with a size of 0.3-0.6mm using metallic mesh filters. For sterilization, all the solutions were filtered with the vacuum filter (0.22-μm pore) before co-precipitation. All the procedures were performed under aseptic conditions.

2.2 *In vitro* experiment

2.2.1 Experiment groups

In order to test the osteogenic efficency of the produced BioCaP incorporated with Icariin and BMP-2, MC3T3-E1 cell line was treated by the following experimental groups:

(1) Cultural medium (C);

(2) BioCaP granules alone (Bio);

(3) BioCaP granules with an internal depot of Icariin (Bio+I, experimental);

(4) BioCaP granules with an internal depot of BMP-2 (Bio+B, experimental);

(5) BioCaP granules with an internal depot of Icariin and BMP-2 (Bio+I+B, experimental).

2.2.2 Culture of MC3T3-E1 with BioCaP

MC3T3-E1 Cell (Chinese Academy of Science, Shanghai, China) suspensions were plated at 4×10^5 cells/well in 6 well plated for cell proliferation, ALP activity assay, OCN expression, alizarin red staining and osteogenic gene expression. After 24 hours incubation, 12-15mg BioCaP alone or BioCaP with Icariin or/and BMP-2 was seeded at each well.

2.2.3 Cell proliferation assay

To investigate cell proliferation of MC3T3-E1 cells in response to BioCaP alone or BioCaP with Icariin or/and BMP-2, the number of cells was determined after stimulation for 1, 4 and 7 days using Cell Counting Kit-8 (Donjindo, Kumamto, Japan) as previously described.

2.2.4 ALP activity assay

To determine the early differentiation of preosteoblasts stimulated by BioCaP alone or BioCaP with Icariin or/and BMP-2, ALP activity and protein content were measured after treatment on day 1, 4 and 7. ALP activity in the cell lysates was determined using the

LabAssay ALP colorimetric assay kit (Wako, Osaka, Japan). The cell number was estimated by determining total protein content measured at 570nm using a commercial bicinchoninic acid (BCA) Protein Assay kit (Beyotime, Shanghai, China). The values representing ALP activity were expressed as mmol p-NP/mg total protein.

2.2.5 OCN expression assay

To assess the terminal differentiation of MC3T3-E1 cellsstimulated by BioCaP alone or BioCaP with Icariin or/and BMP-2, OCN secreted into the cell culture medium was determined. The cell supernatants were collected on days 4, 7 and 10 and were centrifuged (10,000 rpm, 4℃, 5 min) before detection. The supernatant OCN concentration was determined using the ELISA mouse OCN EIA kit (Biomedical Technologies, Stoughton, MA).

2.2.6 Cell matrix mineralization

We compared the mineralization of MC3T3-E1 cells stimulated by BioCaP alone or BioCaP with Icariin or/and BMP-2. Triplicate cell cultures were prepared in the same way as described previously and then treated with mineralizing medium (10% fetal bovine serum (FBS) (Hangzhou Tianhang Bio-technology Co., Hangzhou, China), 50mg/mL L-ascorbic acid-2-phosphate (AsAP), and 10mM β-glycerophosphate (β-GP) (Sigma-Aldrich, St. Louis, MO, USA)) containing. The medium was replaced every 3 days. After 3, 4 and 5 weeks, mineralized nodules were determined by alizarin red (Sigma-Aldrich, St. Louis, MO, USA) staining. Culture plates were photographed by NIS-Elements F2.20 (Nikon Eclipse 80i, Tokyo, Japan), and the calcified area was quantified using Image-Pro Plus 6.0 analysis. After being photographed, 10% cetylpyridinium chloride (CPC, Sigma-Aldrich LLC, GER) was used to dissolve the mineralized nodules and release calcium-bound alizarin red S into solution. Then we detected the colorimetric absorbance at 560 nm.

2.2.7 Real-time PCR Quantification of gene expression

Effects of BioCaP alone or BioCaP with Icariin or/and BMP-2 in stimulating osteogenic gene expression were also examined by quantitative RT-PCR. The messenger ribonucleic acid (mRNA) expression of ALP, BMP2, osteocalcin (OCN), collagen-I (Col I), and runt-related transcription factor 2 (Runx-2) was quantitatively determined after 1d, 4d and

7d of osteogenic induction. Total RNA was extracted from the cells using RNeasy Mini Kit and RNase-Free DNase Set (Giagen sample & assay technologies, Germany). Single stranded complementary deoxyribonucleic acid (cDNA) was synthesized from total ribonucleic acid (RNA) with a Primescrip™ RT Reagent Kit (Takara Biotechnology, Dalian, China). Real time polymerase chain reaction (PCR) was performed using 1μl of cDNA product in a 25μl reaction volume with Mastercycler ep realplex Real Time PCR System (Eppendorf, Germany). SYBR® Premix Ex TaqTM II (Takara Biotechnology, Dalian, China), specific primers (see table 2), and 1μl of cDNA were used in each PCR reaction (95℃ for 30 s, 40 cycles of denaturation at 95℃ for 5 s, and annealing and extension at 60℃ for 30 s). The sense and antisense primers (see Table 2) were designed with the Primer Express 3.0 based on published mouse cDNA sequences. β-actin was used as an internal control gene. All real - time PCR reactions were performed in triplicate. The results were calculated using the comparative threshold cycle (DDCT) method - after calibration with β-actin expression - and are presented as fold increase relative to the non-stimulated control.

2.2.8 Statistical analysis

Statistical comparisons among results obtained with BioCaP alone or BioCaP with Icariin or/and BMP-2 were made by a one way analysis of variance. Post hoc comparisons were made using Bonferroni corrections. The level of significance was set at $p<0.05$. Statistics package for social science (SPSS) software (version 17, SPSS Inc., Chicago, IL) for a Windows computer system was employed for the statistical analysis.

2.3 *In vivo* experiment

2.3.1 Experiment groups

Surgical procedures were performed on 8-week-old male SD rats. The animal experiments were approved by the Ethics Committee of Zhejiang Chinese Medical University, China. Five groups were established to treat CSBD (n=6 animals per group):

(1) BioCaP granules with neither Icariin nor BMP-2 (BioCaP, negative control) in CSBD;

(2) BioCaP granules with an internal depot of BMP-2 (BioCaP int. BMP-2, experimental) in CSBD;

(3) BioCaP granules with an internal depot of Icariin (BioCaP int. Icariin, experimental) in

CSBD;

(4) BioCaP granules with an internal depot of icariin and BMP-2 (BioCaP int. Icariin and BMP-2, experimental) in CSBD;

(5) Autogenous bone in CSBD (Autogenous, positive control).

2.3.2 Surgical procedure

The animals were anaesthetized by intraperitoneal injection of Sumianxin Ⅱ (0.5ml/kg) (Military Veterinary Institute, Quartermaster University of PLA, Changchun, China). A 2cm sagittal incision was made on the scalp, and the calvarium was exposed by blunt dissection. One critical-sized bone defect was created by means of a 8-mm diameter trephine bur (Hu-Friedy, USA) with special care to avoid perforating the dura mater [34] and superior sagittal sinus. The granules of every experiental group was randomly implanted into the critical-sized calvarial defects. Then the defects were covered by Bio-gide membranes. The incision was closed in layers using 4-0 sutures. The rats were able to function normally after this procedure.

2.3.3 Micro-CT evaluation

Three-dimensional (3D) reconstructions of the embedded specimens were scanned using a high resolution micro-CT system (μCT 40, Scanco Medical AG, Bassersdorf, Switzerland). Specimens were placed vertically in a polyetherimide cylinder-shaped holder and scanned at 70kV source voltage, 113μA current and a 18μm isotropic voxel size. Grey values of each specimens which was depending on radiopacity of the scanned material, was converted into corresponding value of degree of mineralization by the analysis software (Scanco Medical AG). Newly formed bone and BioCaP can easily be discriminated, since the mineralization degree of BioCaP was significantly whiter than the bone's. According to the previous study, a novel approach so called "onion-peeling" algorithm (Scanco Medical AG) was used to distinguish between the newly formed bone deposited on the BioCaP and BioCaP itself and specific threshold settings. By using this approach, the micro-CT results were comparable with histomorphometrical ones [35]. Briefly, a low threshold of 560 mg hydroxyapatite (HA)/cm^3 to discriminated bone tissue from connective tissue and (bone marrow), and the grey values were scaled from 1 to 1000 and the threshold was set at 200 to distinguish BioCaP from bone tissue. These two thresholds were calculated by averaging

the thresholds resolved in three slices of three samples by two individual researchers. Micro-CT measurements included bone volume (BV), bone density (BV/TV), material volume (MV), and bone mineral density (BMD).

2.3.4 Histological procedures

Specimens with 2-3mm surrounding the CSBD were fixed in a 10% neutral buffered formalin solution. The undecalcified specimens of 30 rats were dehydrated in ascending concentration of ethanol from 70% to 100% and finally embedded in methl methacrylate (MMA) [36]. The specimens were sawed vertical to the surface of the defects, into 10-12 slices of 1mm thick and were subsequently polished to a final thickness of about 600μm thickness. The slices were surface-stained with McNeal's Tetrachrome, basic Fuchsine and Toluidine Blue [37] and examined with a light microscope with a digital camera (Leica, Wetzlar, Germany).

2.3.5 Histomorphometric analysis

In addition to a subjective histological description, three sections, randomly choosing from each defect, were used for the quantitative histomorphometric analysis. The measurements on volume density of newly formed bone were quantified using a personal computer-based image analysis system (image Pro 5.0, Media Cybernetic, Sliver Springs, MD, USA) and reported as a percentage of the whole calvarium defect area[36].

2.3.6 Statistical characterization

All data are presented as the mean value together with the standard deviation (SD). Using the software SPSS 16.0 (SPSS Science), statistical significance was assessed by a Turkey's *post-hoc* test of a one way analysis of variance (ANOVA). The significance level was set at $p<0.05$.

3. Results

3.1 *In vitro* experiment

3.1.1 Cell proliferation assay

BioCaP with or without Icariin didn't affect cell proliferation of MC3T3-E1 on all three time points compared with control, and BioCaP with Icariin didn't improve the cell proliferation compared with BioCaP ganules alone. However, BioCaP with BMP-2 or

Icariin and BMP-2 significantly increase the cell proliferation on later days (4 and 7 days) compared with the control but not on the first day. BioCaP incorporated with both of Icariin and BMP-2 had higher cell proliferation than BioCaP with BMP-2 alone on day 4.

3.1.2 ALP activity assay

After 4 and 7 days treatment, BioCaP with both of Icariin and BMP-2 had higher ALP activity than BioCaP granules alone(1.6 times and 2.3 times) and the control group (1.9 times and 2 times). Moreover, with the addition of Icariin, BioCaP with both of two agent produced more ALP than BioCaP with BMP-2 alone on day 1 (1.3 times) and day 7 (1.1 times). Also, higher ALP activity was produced by BioCaP with Icariin than nontreated control (2 times) and the BioCaP alone (1.7 times) group on day 4 (Fig. 2).

3.1.3 OCN expression assay

BioCaP alone produced less OCN expression than control on day 7 and 10. Interestingly, when BioCaP incorporated with Icariin, it has more OCN secretion than the control after 7 (1.1 times) and 10 days (1.2 times) stimulation. BioCaP incorporated with both agents had higher OCN expression than the control on the three time points (1.4 times, 1.4 times and 1.3 times) (Fig. 3). After 7 days treatment, BioCaP incoporated with Icariin and BMP-2 produced higher OCN than BioCaP with BMP-2 (1.4 times), but had no statistical differences on day 4 and 10.

3.1.4 Cell matrix mineralization

On all three time points, all other 4 groups had significant higher minerlization compared with the cultural medium one. BioCaP with Icariin had far more areas of mineralized nodules compared with the BioCaP granules alone group on the later days (1.6 times on 4 weeks point and 2 times on 5 weeks point). Incorporating with Icariin, BioCaP with both of two agents could have more mineralization on 3 weeks (1.2 times) and 4 weeks (1.3 times). After 5 weeks treatment, there were no significant differences between BioCaP with two agents and BioCaP with BMP-2 alone in terms of mineralization (Fig. 4).

3.1.5 Real-time PCR Quantification of gene expression

From day 1 to day 7, expression of ALP genes was significantly upregulated with the stimulation of BioCaP with Icariin (6.8, 2.7 and 1.8 fold), but was not by BioCaP alone on

day 4(Fig. 5A). ALP gene expression was dramatically promoted by BioCaP with BMP-2(6.8, 5.6 and 4.9 fold) and BioCaP with Icariin and BMP-2 (7.1, 7.7 and 10.3 fold) on all three time points. BioCaP with both of two agents increased the ALP expression than the BioCaP with BMP-2 alone on day 4 and 7 (1.4 and 2.1 fold).

A significant reduction in the expression of BMP-2 gene could be detected from day 1 to day 4 when treated with BioCaP alone. Without the absence of Icariin, BioCaP significantly enhance the expression of BMP-2 gene on day 4 and 7 compared to control (2.5 and 4.4 fold). Furthermore, BioCaP with Icariin dramatically increase the BMP-2 gene expression in comparison with BioCaP alone on all three time points especially on day 4 (14, 40.3 and 5.8 fold) (Fig. 5B). Noticeably, the present of Icariin signficantly reduce the expression of BMP-2 genes on BioCaP with BMP-2 (-3.5, -2.2 and -9.9 folds) after 1 day, and 4,7 days' stimulation.

Different from the other osteogenic gene expression, BioCaP with or without Icariin couldn't promote the Col Ⅰ expression after 4 and 7 day treatment even reduced on 1 days. However, with or without Icariin, BioCaP incorporated with BMP-2 upregulated Col 1 gene expression on all three days (Fig. 5C). Addition of Icariin could promted the expression of Col1 gene of BioCaP with BMP-2 after 7 days' treatment (1.3 fold).

BioCaP incorporated with Icariin had significantly higher OCN gene expression compared to BioCaP alone on day 7 (2.4 fold). With the presence of Icariin, BioCaP with BMP-2 upregulated OCN gene expression in comparison with BioCaP with BMP-2 alone on 1,4 and 7 days (2.0, 1.5, 1.1 fold) (Fig. 5D).

BioCaP with or without Icariin couldn't enhance the expression of Runx2 on day 1 in comparison with the control. After 4 and 7 days' stimulation, BioCaP with Icariin significantly increase the Runx2 gene expression (2.2 and 1.8 fold) when compared with the control. Interestingly, presence of Icariin greatly promoted the ability to upregulate the Runx2 expression when compared to BioCaP alone on day 4 and 7(2.6 and 2.5 fold) (Fig. 5E). Addition of Icariin increased the expression of Runx2 gene after 4 days (1.5 fold) but had no statistical difference after 1 day and 7 days treatment.

3.2 *In vivo* experiment

3.2.1 Clinical observations

After 12 weeks implantation, a total of 30 specimens were harvested. The healing

period was uneventful and all the surgical sites healed well without any obvious wound complication. No visual signs of inflammatory activity or other adverse tissue reactions were observed.

3.2.2 Micro-CT analysis

Newly formed bone and BioCaP were discriminated by the analysis software. The BioCaP group didn't have any new bone formation. Micro-CT analysis revealed that the volume density of newly formed bone (mm^3/mm^3) of BioCaP internally-incorporated with BMP-2 and the one with both Icariin and BMP-2 was significantly higher than the other groups. BioCaP with Icariin has more volume density of new bone than the BioCaP only group. Interestingly, the BioCaP with both Icariin and BMP-2 obtained much higher volume density of new bone than any other groups (Fig. 6). However, no differences were found between the BioCaP internally-incorporated with Icariin, with BMP-2, and with both of them concerning to bone mineral density (Fig. 7).

3.2.3 Descriptive light microscopy

The control group (CSBD with no bone substitute) confirmed that the defect can't heal 12 weeks post-operation. After 12 weeks implantation, nearly no newly formed bone was observed in the groups of BioCaP only. The new bone formation can be seen more or less in the other three graft groups. New bone only appeared in close contact with the BioCaP granules surroundings, in other words, thin layer of new bone capsulated the BioCaP internally-incorporated with Icariin. Interconnected bone network could be observed in the BioCaP with BMP-2 and with both of Icariin and BMP-2 groups clearly, and the BioCaP granules embedded in quantity of new bone. Interestingly, the group, BioCaP with both of Icariin and BMP-2 seems to have more bone formation since the new bone covered almost whole areas of the defects, while the BioCaP with BMP-2 only group covered part of the defect (Fig. 8).

3.2.4 Histomorphometric results

Quantitative evaluations of the amount of bone formation after 12 weeks implantation are in fig.4. There was no bone formation capsulated the BioCaP granules in the BioCaP only groups. BioCaP with both of Icariin and BMP-2 produced significantly higher volume density of new bone than BioCaP with Icariin or BMP-2. More volume density of bone was

observed in the BioCaP with BMP-2 than with Icariin (Fig. 9).

4. Discussion

Previous study showed that the Runx2, BMP-2 and BMP-4 mRNA expression level were significantly up-regulated by Icariin treating. Icariin exerted its osteogenic effects through the induction of Runx2 expression, the production of BMP-2, and the BMP signaling activation. In addition, recent studies indicated that Icariin can modulate the process of bone formation via BMP-2/Smad 4 signal pathway in human osteoblastic cell line [38-40]. However, until now oral administration was the common mode of Icariin delivery. Due to the half-life of the agents and the way of blood circuation reaching the local tissue in systemic drug delivery, Icariin could not maintain an appropriate concentration in bone defects continuously.(wuyuxiong) Moreover, the previous findings strongly suggest that BioCaP granules can be used as an attractive protein delivery vehicle [16]. Therefore, a local sustained release system of Icariin in bone defect areas desperately needs to be developed and evaluated. We demonstrated the addition of Icariin significantly increased the osteogenic effects of BMP-2 *in vitro* in the previous study. In this study, we were eager to estimate the osteogenic potential of BioCaP alone or BioCaP incorporated with Icariin or/and BMP-2*in vitro and in vivo* here.

We for the first time evaluated the BioCaP internally-incorporated with bioactive agents *in vitro*. BioCaP incorporated with or without Icariin didn't increase the proliferation of MC3T3-E1 cells. Runx2 controls osteoblast proliferation and promotes a transition from a proliferative to a post-proliferative stage prior to osteoblast differentiation [41, 42]. It remains to be elucidated whether the significant up-regulation of Runx2 could partially account for the significant down-regulation of MC3T3-E1 cell proliferation. Phimphilai et al. [43] reported that the transcriptional activity of Runx2 requires BMP signaling and that the sensitivity of cells to BMPs is enhanced by Runx2. Consistent with Phimphilai, in this study, BMP-2 and Runx2 gene expression was upregulated by BioCaP incorporated with Icariin on later time (day 4 and 7). The upregulation of BMP-2 gene expression stimulated by BioCaP with Icariin in line with the speculation in our previous study that Icariin may activate BMP signaling indirectly through extracellular BMP-2 after BMP-2 are produce [17]. However, BioCaP with Icariin often couldn't increase the osteogenic gene expression on the early time (day 1). The reason for the present findings may be that the release rate of Icariin was too slow on the first day, leading very low concentration of Icariin around

MC3T3-E1 that consequently became less effective.

As the early marker of osteogenic differentiation, in addition of Icariin, the ALP protein expression of BioCaP incorporated with BMP-2 could be promoted 1.3 times and 1.1 times on day 1 and 7 respectively. In the meantime, the ALP gene expression could also upregulated on day 4 and 7 (1.4 and 2.1 fold respectively). OCN, the late marker of differentiation related to the matrix deposition and mineralization, could be increased by the presence of Icariin in terms of BioCaP incorporated with BMP-2 (1.4 times on day 7 of protein and 2.0, 1.5, 1.1 fold on all three time points of gene expression). Runx2 gene expression had similar pattern as ALP and OCN gene expression. Different from the other osteogenic related gene expression, the addition of Icariin failed to promote the BMP-2 gene expression of BioCaP with BMP-2 instead downregulated the BMP-2 mRNA. Involvement of BMP signaling in the effects of Icariin is for sure [44]. Therefore, it's possibly because the transiently high dosage of BMP-2 may lead to a series of potential side effects, such as the overstimulation of osteoclastic bone resorption [45]. On the other hand, Icariin has been shown to inhibit lipopolysaccharide (LPS)-induced osteoclastogenesis by suppressing the activation of the p38 and Jun N-terminal kinase (JNK) pathway [23]. The anti-osteoclastogenesis effects of Icariin may compromise the overstimulated osteoclastic bone resorption impact of BMP-2.

Mineralization is important for bone regeneration. Some hydroxyapatite-based scaffolds are used in bone tissue engineering to provide artificial highly mineralized environments for enhancing osteogenic differentiation and bone regeneration [24]. BioCaP granules alone was found to be effective for promoting mineralization on MC3T3-E1 *in vitro*. Icariin agent alone increased the *in vitro* mineralization [17, 19], while addition of Icariin synergistically enhanced the mineralization ability of BioCaP in a time-dependent manner as expected. Meanwhile, the presence of Icariin stimulated the mineralization of BioCaP with BMP-2 as expect. These data for the combined effect indicated that Icariin is useful for enhancing mineralization.

The release kinetic is a crucial factor for the osteoinductive efficiency of bioactive agents. BMP-2 is simply adsorbed onto the bone-defect-implanting materials, which is associated with a high dose burst release and thus low osteoinductive efficiency [46]. In contrast, the internally incorporated BMP-2 showed a gradual, sustained and cell-mediated release of bioacitve agents and thus a siginificanly higher osteoinductive efficiency than the adsorbed BMP-2 [16]. Therefore, the fabricated methods of agent loaded into the BioCaP in

this study were internally incorporation.

Skull defects are favored models for animal and clinical research on bone induction because bone regeneration in this area is either slow or incomplete. Skull bone healing is so slow that cranioplasty operations, especially in the adult human beings, often fail[34]. The creation of nonunions in animals within the skull was size dependent. Defects of a size that will not heal during the lifetime of the animal may be termed critical size defects[47]. Trephine defects in the vault of the adult cranium measure 8 mm in the rat [34, 48]. The key to establish the rat calvarium critical-sized bone defects is with extraordinarily special care to avoid perforating the dura mater which may cause excessive bleeding that may contribute to the death of the rats [34]. Critical-sized skull defects in adults fail to heal spontaneously, whereas juveniles retain the ability to reossify skull defects of almost any magnitude [49]. Thus we selected the adult (8-weeks old) SD rats as the experiment animal for this study.

The degradability of CaP-based materials is very crucial for the *in vivo* longevity and efficacy of its biological effects [50]. The material's dissolubility and the cell-mediated resorption regulated its degradations [51]. In the current study, all BioCaP granules which delivered agents were observed to be in close contact with bone or completely encapsulated in the newly formed bone. This finding in consistent with the previous studies [16] that BioCaP is highly biocompatible. The slow release of BMP-2 plays a superior role in bone formation [37, 52]. In the previous studies, the agents incorporated into the BioCaP granules resulted in a sustained release of agents *in vitro* and BMP-2 delivered by BioCaP granules led to a highly osteoinductive efficiency *in vivo*[53]. Only BMP-2 have been experimented to be internally-incorporated into the BioCaP granules, here we for the first time verified that besides BMP-2, Icariin also can be delivered and released properly and still have good biocompatibility as well. The BioCaP granules which were internally –incorporate by Icariin surrounded by newly formed bone were the evidence that Icariin also can be sustained release by BioCaP. In our previous *in vitro* experiment, we demonstrated that Icariin alone is a strong osteogenic agent and mixture of Icariin and BMP-2 can significantly enhance the osteogenic activity of BMP-2 [54], here we confirm it *in vivo* also.

Although histological analyses provide unique information on cellularity and dynamic indices of bone remodelling, they have limitations in assessing the micro-architectures ofbone. Histological analyses are derived from stereological analysis of a few 2D sections,

usually assuming that the underlying structure is plate like [55, 56]. On the other hands, micro-CT can directly measure the micro-architectures of bone independent of stereological models [57]. These landmarks, however, are not always identifiable, raising questions regarding their reliability, variability and even subjectivity. The lack of agreement between clinicians in the qualitative study suggests that assessment of osseous landmarks is somewhat subjective[58]. In this study, these two approaches were used together to comprise their limitations.

In this *in vivo* study, we acquired positive results in rats, which may have to be repeated and confirmed in larger species, as dogs or sheep before human clinical trials can be initiated. Besides, more time points should be tested also.

Benefiting from our results, we suggested that (1) in the presence of Icariin, BioCaP could have better osteogenic potential when stimulated MC3T3-E1; (2) addition of Icariin promoted the osteogenic differentiation of MC3T3-E1 treated with BioCaP incorporated with BMP-2 ; (3) BioCaP incorporated with Icariin increased the bone formation; (4) addition of Icariin significantly enhanced the new bone formation of BMP-2 incorporated into the BioCaP granules in the critical-sized bone defects in SD rats.

5. Conclusion

In conclusion, BioCaP incorporated with Icariin could enhance *in vitro* osteogenic differentiation. Coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone. Our histological and histomorphometrical findings confirmed our postulation: addition of Icariin increased the new bone formation of BMP-2 incorporated into the BioCaP granules in the critical-sized bone defects in SD rats. The BioCaP granules, which have good biocompatibility, with the help of mixture of Icariin and BMP-2 may be a promising bone substitute for clinical applications.

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Table1. Concentration of agents introduced into the CaP solutions before buffering

	concentration of agent	
	In vitro	*In vivo*
BioCaP+Icariin	5mg/L	10mg/L
BioCaP+BMP-2	0.5mg/L	0.5mg/L
BioCaP+Icariin +BMP-2	5mg/L+ 0.5mg/L	10mg/L+0.5mg/L

Table 2. Primer sequences for real-time quantitative polymerase chain reaction analysis of the gene expression.

Gene	**Accession No.**	**Primers (F=forward; R=reverse)**
ALP	NM_007431	F: 5'- TGCCTACTTGTGTGGCGTGAA -3'; R: 5'- TCACCCGAGTGGTAGTCACAATG -3'
BMP-2	NM_007553	5'-AAGAGACATGTGAGGATTAGCAGGT-3' and 5'-GCTTCCGCTGTTTGTGTTTG-3'
Collagen I	NM_007742	F: 5'- ATGCCGCGACCTCAAGATG -3'; R: 5'- TGAGGCACAGACGGCTGAGTA -3'
Osteocalcin (OCN)	NM_007541	F: 5'- AGCAGCTTGGCCCAGACCTA -3'; R: 5'- TAGCGCCGGAGTCTGTTCACTAC -3'
Runx2	NM_009820	F: 5'- CACTGGCGGTGCAACAAGA -3'; R: 5'- TTTCATAACAGCGGAGGCATTTC -3'
β-actin	NM_007393	F: 5'- AGGAGCAATGATCTTGATCTT -3'; R: 5'- TGCCAACACAGTGCTGTCT -3'

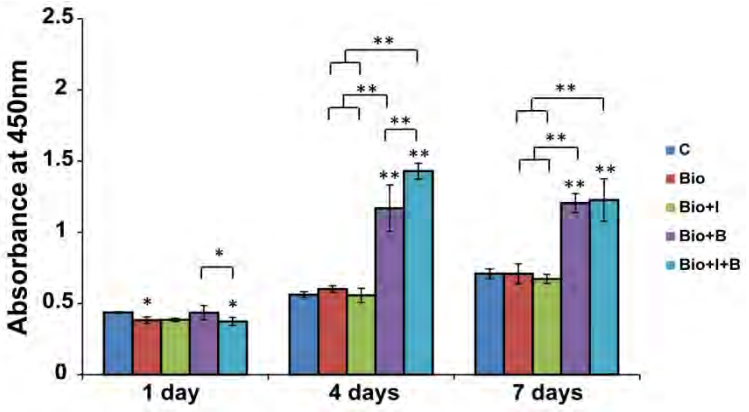

Fig 1. The proliferation of MC3T3-E1 cells stimulated by BioCaP alone or BioCaP with Icariin or/and BMP-2. The absorbance was measured on days 1, 4 and 7. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01, ** without lines means versus control (C). Control (C); biomimetic calcium phosphate (BioCaP); Icariin (I); BMP-2 (B).

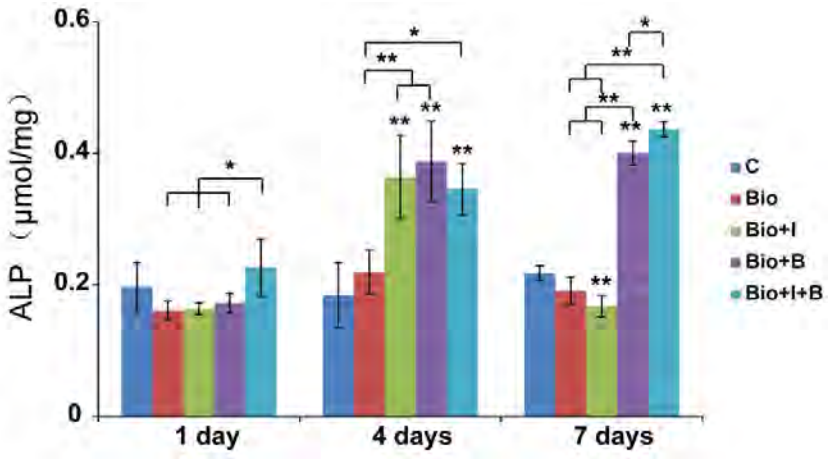

Fig 2. The ALP activities of MC3T3-E1 cells after treatment with BioCaP alone or BioCaP with Icariin or/and BMP-2. The ALP activities were determined by colorimetric assay on day 1, 4 and 7. Mean values (n=3 samples per group) were represented together with the standard deviation normalized by total cellular protein. Error bars denote the standard deviation. *P<0.05, **P<0.01, ** without lines means versus control (C). Control (C); biomimetic calcium phosphate (BioCaP); Icariin (I); BMP-2 (B).

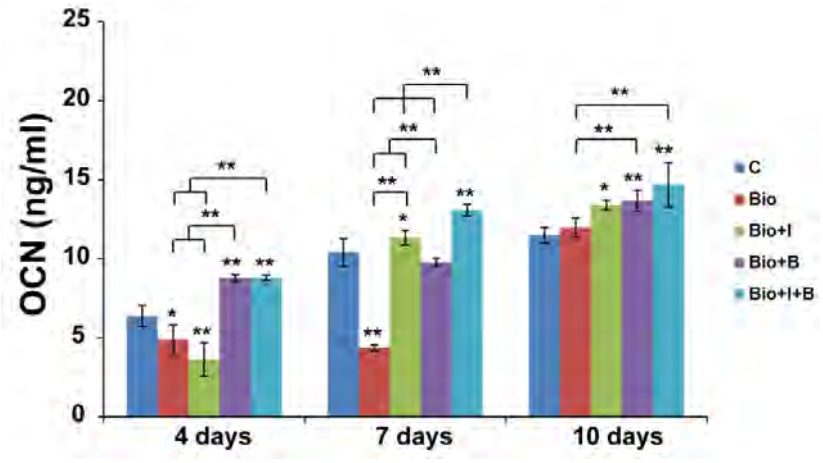

Fig 3. The OCN expressions of MC3T3-E1 cells after treatment with BioCaP alone or BioCaP with Icariin or/and BMP-2. ELISA was used to determine OCN expression on day 4, 7 and 10. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01, ** without lines means versus control (C). Control (C); biomimetic calcium phosphate (BioCaP); Icariin (I); BMP-2 (B).

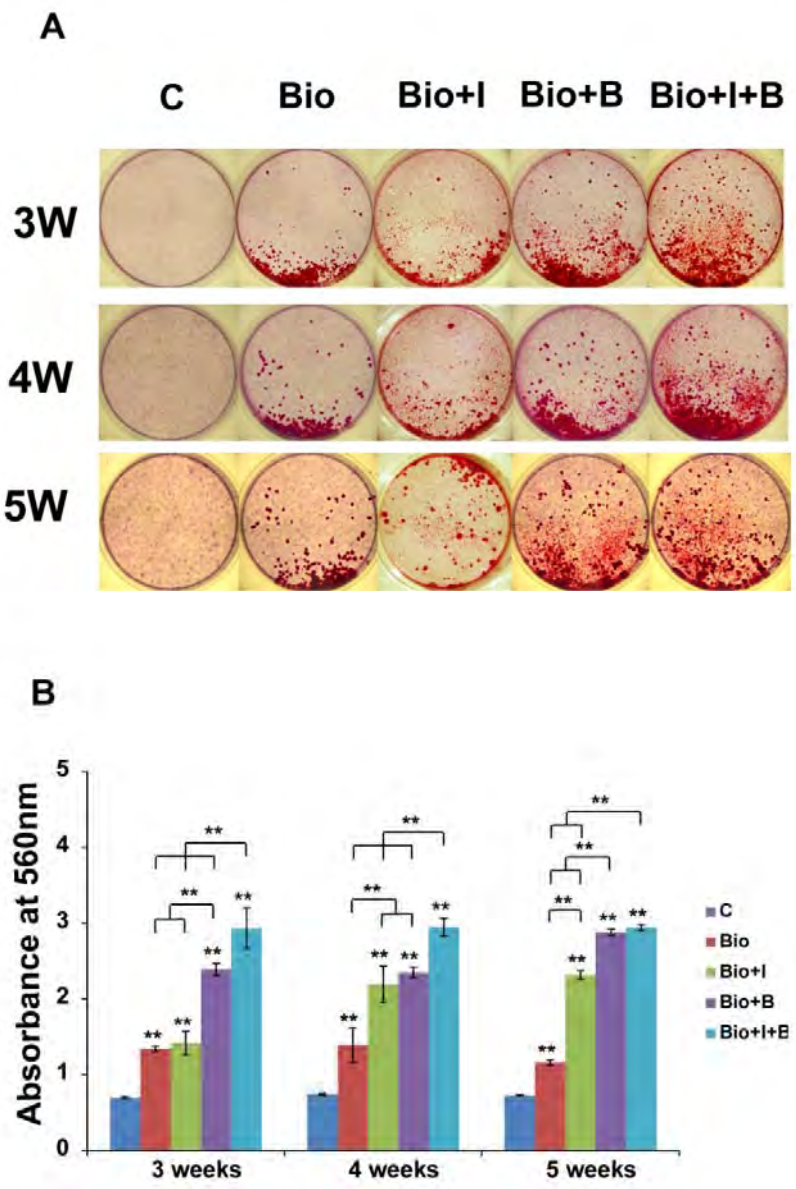

Fig 4. (A) Marocopic images of alzarin red stained 3,4 and 5 weeks after five groups treatmet. (B) The mineralization of MC3T3-E1 cells under the BioCaP alone or BioCaP with Icariin or/and BMP-2 treatment on 3, 4 and 5 weeks and Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01, ** without lines means versus control (C). Control (C); biomimetic calcium phosphate (BioCaP); Icariin (I); BMP-2 (B).

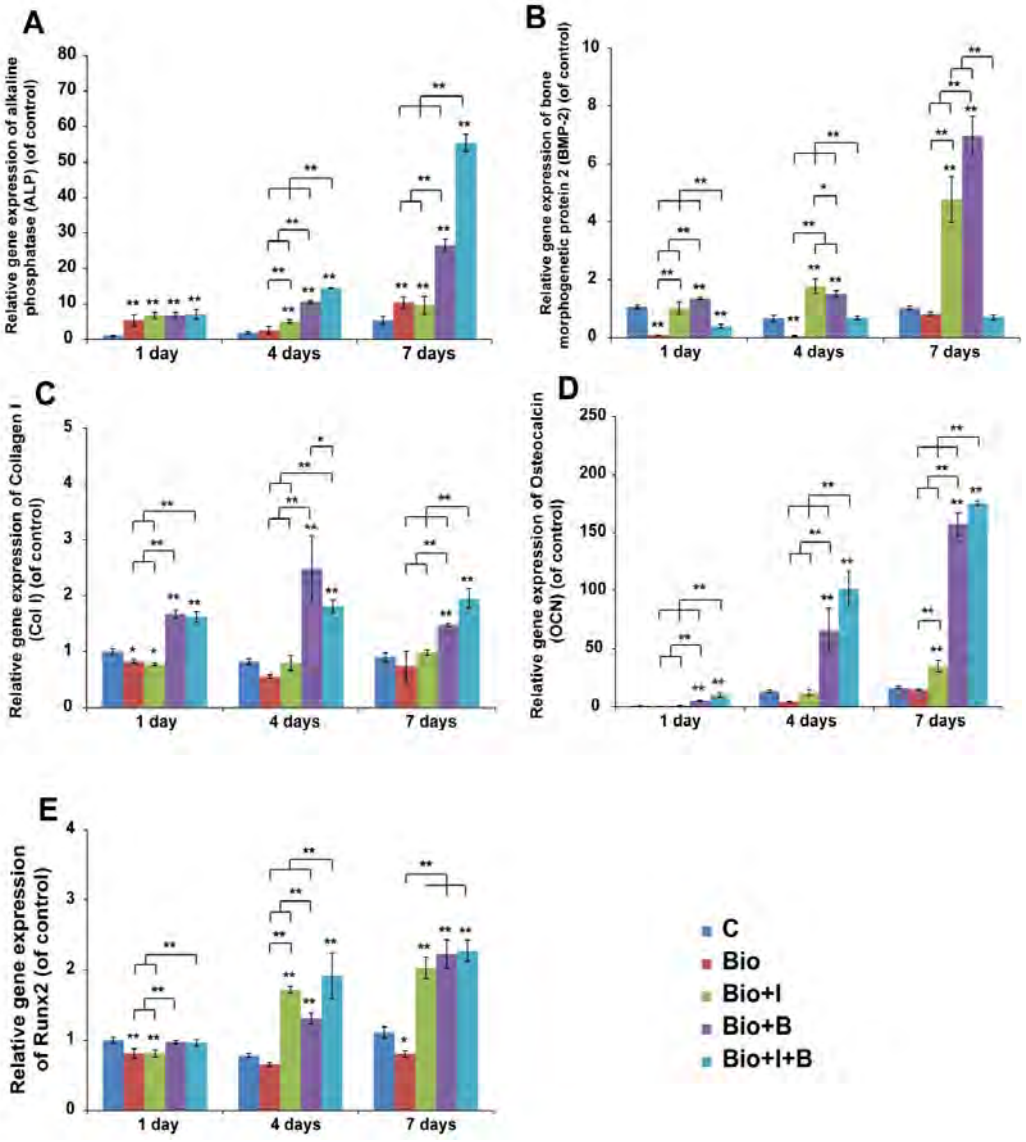

Fig 5. The time course changes in mRNA expression of (A) ALP, (B) BMP-2 (C) Col1, (D) OCN, (E) Runx2 in MC3T3-E1 on day 1, 4 and 7. Mean values (n=3 samples per group) were represented together with the standard deviation. Error bars denote the standard deviation. *P<0.05, **P<0.01, ** without lines means versus control (C). Control (C); biomimetic calcium phosphate (BioCaP); Icariin (I); BMP-2 (B).

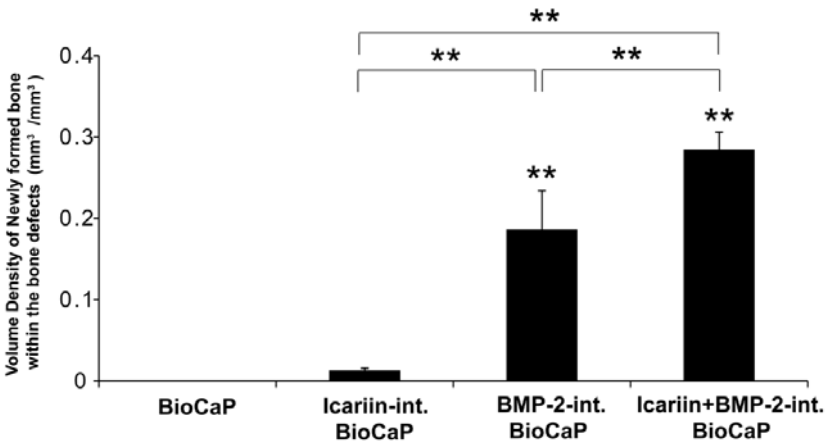

Fig 6. The volume of newly formed bone within the bone defect after 12 weeks postoperation for each group which is analysed by Micro-CT. Mean value (n=6 specimens per group) are represented together with the standard deviation. **P<0.01

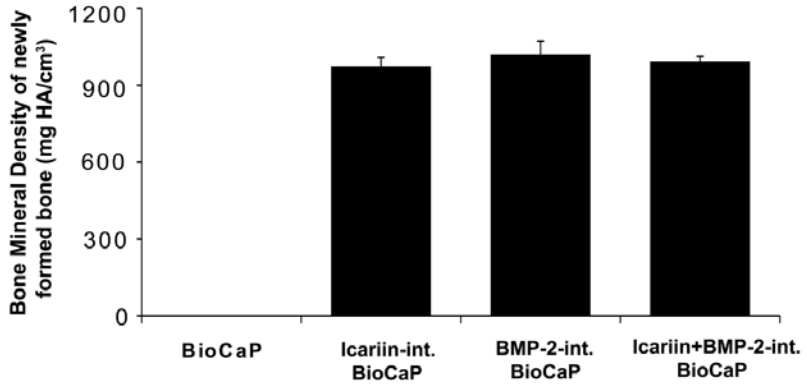

Fig 7. The bone mineral density (mg HA/cm3) of the newly formed bone within the bone defect after 12 weeks postoperation for each group which is analysed by Micro-CT. Mean value (n=6 specimens per group) are represented together with the standard deviation.

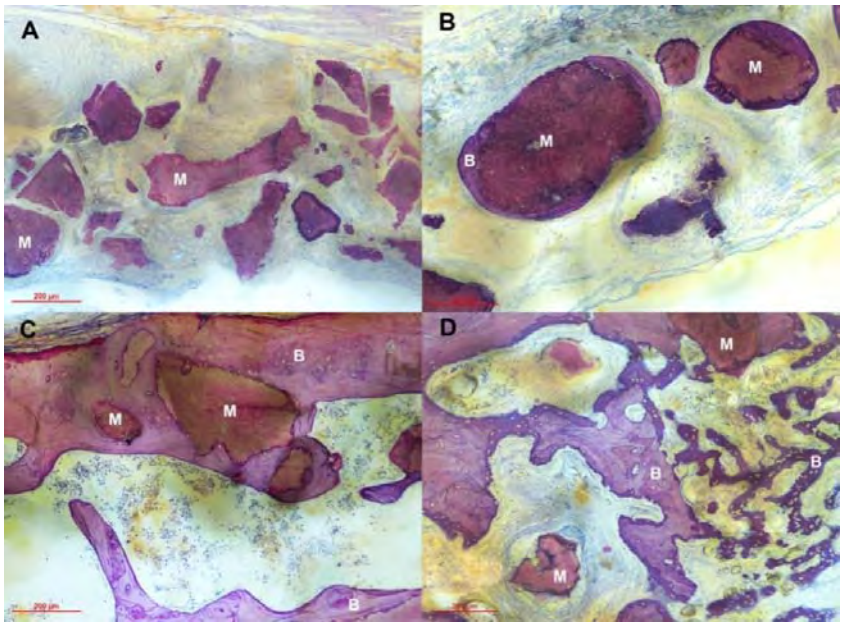

Fig 8. Representative histological micrographs of bone defect of each group at 12 weeks after implantation. The BioCaP granules (M) were surround by newly formed bone(B) in B,C, D. Groups: (A) BioCaP bearing neither Icariin or BMP-2; (B) BioCaP bearing an internally-incorporated Icariin; (C) BioCaP bearing an internally-incorporated BMP-2; (D) BioCaP bearing an internally-incorporated Icariin and BMP-2. The slices were surface-stained with McNeal's Tetrachrome, basic Fuchsine and Toluidine Blue. Scale bar= 200 μm.

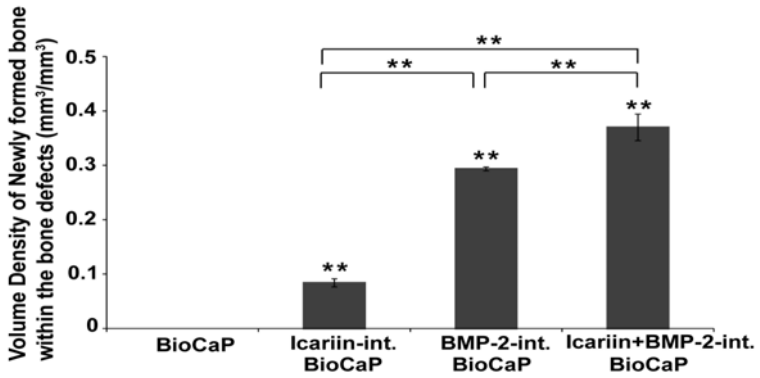

Fig 9.The volume of newly formed bone within the bone defect after 12 weeks postoperation for each group which is analysed by histology. Mean value (n=6 specimens per group) are represented together with the standard deviation. ***P*<0.01

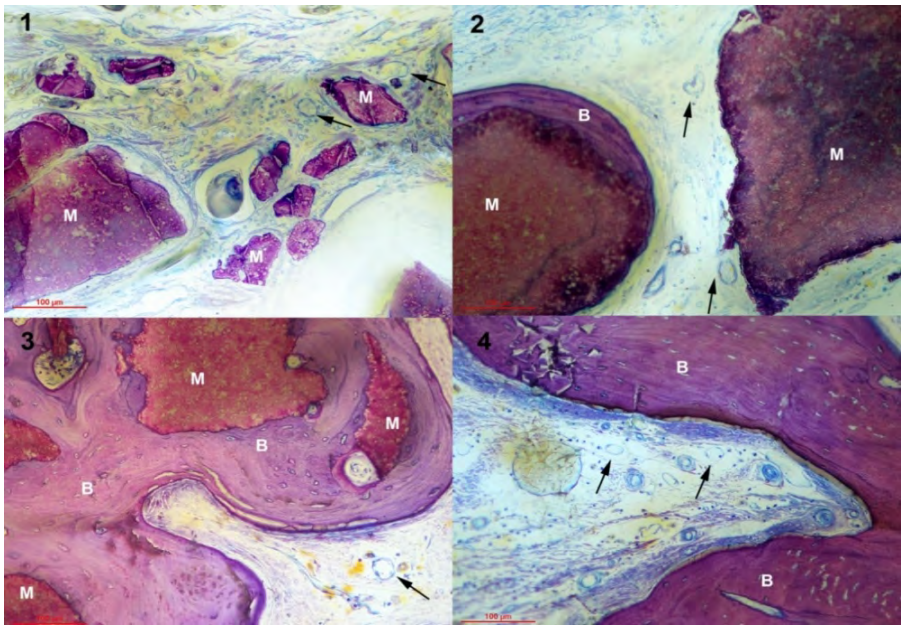

Fig. 10. Representative histological micrographs of bone defect of each group at 12 weeks after implantation. The BioCaP granules (M) were surround by newly formed bone(B) in 2,3,4. The vascular was observed in 1,2,3,4 Groups: (A) BioCaP bearing neither Icariin or BMP-2; (B) BioCaP bearing an internally-incorporated Icariin; (C) BioCaP bearing an internally-incorporated BMP-2; (D) BioCaP bearing an internally-incorporated Icariin and BMP-2. The slices were surface-stained with McNeal's Tetrachrome, basic Fuchsine and Toluidine Blue. Scale bar= 100 μm.

Chapter 8

GENERAL DISCUSSION

Icariin

More and more researches show that icariin has an osteoinductive potential, due to its properties of inducing osteogenesis, chondrogenesis, and angiogenesis. The multiple function of icariin, especially the induction of osteogenesis, is remarkable. The loading of icariin in calcium phosphate biomaterials provides a good alternative to for delivering icariin locally for bone repair, since the calcium phosphate materials have been used as osteoconductive scaffolds. It has been known that the local use of icariin demonstrated positive effects in bone formation at an early stage [1]. Several studies have tried to clarify the molecular mechanisms underlying the osteogenic effects. In summary, icariin may exert its osteogenic effects through the induction of BMP-2 and NO synthesis and the BMP-2/Smad4 signal transduction pathway, by up-regulating the expression of BMP-2, BMP-4, Smad4, Cbfa1/Runx2, OPG, RANKL, and the OPG/RANKL ratio[2, 3]. Icariin can inhibit LPS-induced osteoclastogenesis by suppressing the activation of the p38 and JNK pathway, which in turn contributes to strengthening the bone[4]. The positive effects of icariin on a potent chondrogenic effect might be the up-regulation of the expression of aggrecan, collagen II, and Sox9 genes and down-regulation of the expression of the collagen I gene of chondrocytes [5] (Chapter 2).

Icariin and BMP-2

Many agents exert their promoting effect on osteogenesis through stimulating the endogenous BMPs, such as Icariin[3], simvastatin and Vitamine D3[6] (Chapter 3). The administration of exogenous bioactive agents, such as FGF-2 [7] and VEGF [8] with BMP-2 to increase the osteogenic effects has become a hot focus in the field of bone regeneration (Chapter 6). Among them, Icariin has become a hot focus in the field of bone regeneration because of its low cost and osteogenic potential(Chapter 3).

Ohba et al [9] suggested two possible mechanisms for the involvement of BMP signaling in the effects of Icariin. Firstly, Icariin may activate BMP signaling indirectly through extracellular BMPs. Secondly, Icariin may activate BMP signaling directly by interacting with Smads via unknown mechanisms (Chapter 6). Our study in Chapter 3 suggested that Icariin did up-regulate the gene expression of BMP-2 and produced more BMP-2 mRNA than the BMP-2 groups at Day 7. This indicated that Icariin may activate BMP signaling indirectly through extracelluar BMP-2 after BMP-2 are produced. However, the administration of Icariin with BMP-2 at 50ng/ml on day 7 and at 100ng/ml on day 1

compared with BMP-2 alone gave only a little improvement in Chapter 6. What is more, the administration of Icariin with BMP-2 at 200ng/ml failed to increase the BMP-2 gene expression on day 1 and 4, even reduced it on day 7. This reminded us that osteogenic activity induced by BMP-2 would reach a plateau at 200-400g/ml [10]. Thus, we hypothesized that Icariin may directly activate BMP signaling, since when administrating both two agents to the cells may produce high concentration of BMP-2 mRNA, this situation may contribute to the failure increasing of BMP-2 gene expression. What's more, the administration of both Icariin and BMP-2 to MC3T3-E1 cells after 7 days may further increase the expression of noggin, which is a BMP inhibitor [11], since the effect of Icariin and BMP-2 at 200ng/ml decreased after 7 days culture (Chapter 6). Coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone, especially on the protein and gene expression of ALP and OCN, and mineralization as well (Chapter 7).

BioCaP

BioCaP is prepared in a biomimetic envionment which can retain the bioactivity of growth factor [12, 13]. This biomimetic environment [(200mM HCl, 20mM $CaCl_2 \cdot 2H_2O$, 680 mM NaCl, and 10mM Na_2HPO_4) buffered by TRIS (250mM) to a PH of 7.4] is the key factor in the fabrication of BioCaP[14]. Besides, during the preparation, the sterility of biomedical materials is very important for clinical trials. The whole processing of this materials, including vacuum filtering, was carried in a biological safety cabinet. In our *in vitro* experiment the BioCaP with Icariin can significantly promote the differentiation of MC3T3-E1 as well as *in vivo* results in Chapter 7 indicated the perfect sterility of BioCaP materials. In BioCaP, drugs and calcium phosphate were precipitated together to form BioCaP granules in which a depot of drugs was incorporated in the center of the granules as an internal depot. The *in vivo* experiments in Chapter 7, all BioCaP granules which delivered agents were observed to be in close contact with bone or completely encapsulated in the newly formed bone also indicated the highly biocompatibility of BioCaP.

Animal model

Skull defects are favored models for animal and clinical research on bone induction because bone regeneration in this area is either slow or incomplete. Skull bone healing is so slow that cranioplasty operations, especially in the adult human beings, often fail[15]. The

creation of nonunions in animals within the skull was size dependent. Defects of a size that will not heal during the lifetime of the animal may be termed critical size defects[16]. Trephine defects in the vault of the adult cranium measure 8 mm in the rat [15, 17]. The key to establish the rat calvarium critical-sized bone defects is with extraordinarily special care to avoid perforating the dura mater which may cause excessive bleeding that may contribute to the death of the rats [15]. Critical-sized skull defects in adults fail to heal spontaneously, whereas juveniles retain the ability to reossify skull defects of almost any magnitude [12]. Therefore, we selected the adult (8-weeks old) SD rats as the experiment animal for this study (Chapter 4, 7 and 8).

Micro-CT and histological analysis

Although histological analyses provide unique information on cellularity and dynamic indices of bone remodeling, they have limitations in assessing the micro-architectures of bone. Histological analyses are derived from stereological analysis of a few 2D sections, usually assuming that the underlying structure is plate like [18, 19]. On the other hands, micro-CT can directly measure the micro-architectures of bone independent of stereological models [20]. These landmarks, however, are not always identifiable, raising questions regarding their reliability, variability and even subjectivity. The lack of agreement between clinicians in the qualitative study suggests that assessment of osseous landmarks is somewhat subjective [21]. In Chapter 4, 7 and 8, these two approaches were used together to comprise their limitations.

CONCLUSION

The main scope of the work conducted in this dissertation was to

1. The extremely low cost and the high abundance of icariin and its excellent function for bone regeneration make it very appealing for clinical applications. Therefore, it could be candidate for an assistant of BMPs or as a substitution. On the whole, the developing techniques give us the confidence to believe that icariin might have a very bright future in bone tissue engineering.

2. The findings indicate that icariin is a strong bone anabolic agent, which is comparable with BMP-2 in terms of enhancing MC3T3-E1 cells proliferation and osteogenic differentiation. The low cost of icariin and its osteogenic potential makes it very appealing for bone tissue engineering.

3. Icariin can stimulate osteogenic activity; the administration of Icariin with BMP-2 can greatly enhance the osteogenic effect of BMP-2 by stimulating the osteogenic activity of M3T3-E1 cell. Coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone. This suggests that traditional Chinese medicine with bone growth factors such as Icariin might be applied to healing bones in bone tissue engineering.

4. Our findings show that BioCaP incorporated with icariin could enhance *in vitro* osteogenic differentiation. Coadministration of icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone. Our histological and histomorphometrical findings confirmed our postulation: addition of icariin increased the new bone formation of BMP-2 incorporated into the BioCaP granules in the critical-sized bone defects in SD rats. The BioCaP granules, which have good biocompatibility, with the help of mixture of icariin and BMP-2 may be a promising bone substitute for clinical applications.

FUTRUE SCOPE

We are on the way to develop an ectopic animal model to evaluate the osteoinductive efficiency of BioCaP internally-incorporated with Icariin or/and BMP-2. The BioCaP granules which are internally-incorporated with Icariin or/and BMP-2 might be considered as a promising tissue engineering system in clinical trials in the near future.

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21. Norman, D.G., et al., Quantitative topographic anatomy of the femoral ACL footprint: a micro-CT analysis. Medical and Biological Engineering and Computing, 2014. 52(11): p. 985-95.

Chapter 9

GENERAL SUMMARY

General summary

Traditional Chinese medicines have been recommended for bone regeneration and repair for thousands of years. Currently, the Herba Epimedii and its multi-component formulation are the attractive native herbs for the treatment of osteoporosis. Icariin, a typical flavonol glycoside, is considered to be the main active ingredient of the Herba Epimedii from which icariin has been successfully extracted. Most interestingly, it has been reported that icariin can be delivered locally by biomaterials and that it has an osteogenic potential for bone tissue engineering. The osteogenic potential of icariin could be attributed to its multiple functions in the musculoskeletal system which is involved in the regulation of multiple signaling pathways in anti-osteoporosis, osteogenesis, anti-osteoclastogenesis, chondrogenesis, angiogenesis, and anti-inflammation. The osteogenic potential and the low price of icariin make it a very attractive candidate as a substitute of osteogenic protein–bone morphogenetic proteins (BMPs), or as a promoter for enhancing the therapeutic effects of BMPs.

This dissertation discusses the research on the osteogenic potential of coadministration of Icariin and BMP-2 *in vitro* and *in vivo*. For the first time we have combined these two agents together and internally-incorporated them into the center of a biomimetic calcium phosphate bone substitute (BioCaP). We evaluate this system in MC3T3-E1 cell line and critical sized bone defect in calvarial of SD rats. Meanwhile, we developed a 3-dimensional construct by combing Icariin, allogeneic bone marrow-derived mesenchymal stem cells (BMSCs), and a siliceous mesostructured cellular foams-poly (3-hydroxybutyrate-co3 -hydroxyhexanoate) (SMC-PHBHHx) composite scaffold.

The general aim of this dissertation includes 6 aspects:

1. To determine the optimal concentration of icariin. And to evaluate the osteogenic differentiation of MC3T3-E1 under treatment of Icariin.
2. To compare the osteogenic differentiation of MC3T3-E1 under stimulation of BMP-2 alone and coadministration of Icariin and BMP-2.
3. To explore the osteogenic effect of BioCaP internally-incorporated with Icariin or/and BMP-2 *in vitro.*
4. To discuss whether Icariin can promote the efficacy of allogeneic BMSCs-based tissue engineering technique in repairing large-volume bone defects.
5. To delineate the functional characteristics of BMP2/7 heterodimer in inducing the *in vitro*

osteoclastogenesis.

6. To evaluate the therapeutic effect of BioCaP internally-incorporated with Icariin or/and BMP-2 in the repair of calvarial critical-sized bone defect of SD rats.

In this dissertation, the osteogenic potential for bone tissue engineering of Icariin had been reviewed (Chapter 2). More and more researches show that icariin has an osteogenic potential, due to its properties of inducing osteogenesis, chondrogenesis, and angiogenesis. The multiple function of icariin, especially the induction of osteogenesis, is remarkable. The loading of icariin in calcium phosphate biomaterials provides a good alternative to for delivering icariin locally for bone repair, since the calcium phosphate materials have been used as osteoconductive scaffolds. Because of its osteogenic potential and the low price, it can be a very attractive candidate as a substitute of BMP-2 (Chapter 3).

In Chapter 3, we investigated the potential of Icariin to stimulate osteogenesis using an *in vitro* studies to compare icariin's ability as a function of time and dose to induce osteoblastogenesis in MC3T3-E1 cells with that of BMP-2. The optimal concentration of icariin in stimulating the proliferation of MC3T3-E1 was 6.8×10^3ng/ml which is consistent with previous researches. There is no significant difference between icariin and BMP-2 relative to cell proliferation, alkaline phosphatase (ALP) activity, and osteocalcin (OCN) expression. Icariin had better ALP activity after a 7-day culture and OCN expression after 4 and 10 day cultures compared with BMP-2. Moreover, icariin produced more mineralized nodules of a larger area than BMP-2. Compared with BMP-2, the results indicate that icariin could be a good option for bone regeneration clinically, since it is an inexpensive and safe drug with an excellent osteogenesis potential.

The concentration of 6.8×10^3 ng/ml of Icariin was used in Chapter 3 as Chapter 2 did. We applied BMP-2 (0ng/ml, 50ng/ml, 100ng/ml and 200ng/ml) with or without 6.8×10^3ng/ml Icariin to promote the osteoblastogenesis of the MC3T3-E1 cell line. Adding Icariin resulted mostly in significantly higher osteoblastogenic genes and proteins in a trend increasing in time. Irrespective of the concentration of BMP-2, the addition of Icariin resulted in a significantly higher cell proliferation and mineralization. The administration of these two agents accelerated further the osteogenic-related gene expression, especially OCN and Runx2 in Chapter 6. Thus, we hypothesized that Icariin may directly activate BMP signaling, since when administrating both two agents to the cells may produce high

concentration of BMP-2 mRNA, this situation may contribute to the failure increasing of BMP-2 gene expression. What's more, the administration of both Icariin and BMP-2 to MC3T3-E1 cells after 7 days may further increase the expression of noggin, which is a BMP inhibitor, since the effect of Icariin and BMP-2 at 200ng/ml decreased after 7-days culture.

Icariin at optimal concentration (6.8×10^{2}ng/ml) could significantly upregulate the osteogenesis- and angiogenesis-related genes and proteins, such as Runx2, ALP, osteocalcin, vascular endothelial growth factors, and fibroblast growth factors, as well as the mineralization of BMSCs in Chapter 4. The different cell lines could attribute to the different optimal osteogenic concentration of Icariin. In the *in vivo* part of the Chapter 4, eight weeks after implantation in calvarial critical-size bone defects, the constructs with icariin were associated with significantly higher bone volume density, trabecular thickness, trabecular number, and significantly lower trabecular separation than the constructs without icariin.

In Chapter 5, BMP2/7 heterodimer could dose dependently modulate each osteoclastogenic event with different concentration patterns from the BMP homodimers. Low-concentration BMP2/7 heterodimer may favor a rapid and spontaneous remodeling of its induced bone and, thus, bear a promising potential in cytokine-based tissue engineering.

It has been reported that icariin can be delivered locally by biomaterials and it has an osteogenic potential for bone tissue engineering. In Chapter 7, the osteogenic potential of biomimetic calcium phosphate bone substitute (BioCaP), a novel drug delivery system, internally-incorporated icariin was evaluated *in vitro*(MC3T3-E1). The BioCaP with combined regimens of icariin and bone morphogenetic protein 2 was investigated to repair the critical-size bone defects (8mm in diameter) in SD male rats' skull. *In vivo*, five groups were established: (1) BioCaP with neither Icariin nor BMP-2; (2) BioCaP with Icariin; (4) BioCaP with BMP-2; (5) BioCaP Icariin and BMP-2; (5) Autogenous bone. 12 weeks after implantation in the critical-size defects in rat skull, samples were withdrawn for Micro-CT and histological analysis. BioCaP incorporated with or without icariin had positive effects on osteogenic differentiation of MC3T3-E1. And especially in the presence of icariin, BioCaP could have better osteogenic efficiency. However, they didn't have influence on cell proliferation. Coadministration of Icariin and BMP-2 into the BioCaP had better osteogenic potential than BioCaP with BMP-2 alone, especially on the protein and gene

expression of ALP and OCN, and mineralization as well. The volume of newly formed bone associated with BioCaP internally-incorporated with both icariin and BMP-2 was significantly higher than the control and BioCaP with icariin or BMP-2. BioCaP with icariin induced more new bone formation than the BioCaP only. Micro-CT analysis revealed that bone mineral density of newly formed bone for BioCaP with icariin and/or BMP-2 have no significant differences. These results confirmed that BioCaP incorporated with icariin could enhance *in vitro* osteogenic differentiation, but had no proliferation increase. Icariin combined with BMP-2 internally-incorporated into BioCaP have a synergistic stimulatory effect for the repair critical-size bone defects in rat skull.

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Publications and presentations

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1. Xin Zhang, Tie Liu, Yuanliang Huang, Daniel Wismeijer, Yuelian Liu. Icariin: does it have an osteoinductive potential for bone tissue engineering? *Phytotherapy Research*.2014,28(4):498-509

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Presentation

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2. Icariin: a traditional Chinese medicine enhances the osteoinductive efficiency of BMP-2. Annual Meeting of the Dutch Society for Calcium and Bone Metabolism, NVCB 23nd Annual Meeting. November, 2013. (Oral presentation).

National meetings (The Netherlands)

1. 22nd Annual Meeting of Netherlands society for Biomaterials and Tissue Engineering (NBTE), 2013 (oral presentation).

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