The osteogenesis of Ginsenoside Rb1 incorporated silk/micro-nano hydroxyapatite/sodium alginate composite scaffolds for calvarial defect

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Ginsenoside Rb1, the effective constituent of ginseng, has been demonstrated to play favorable roles in improving the immunity system. However, there is little study on the osteogenesis and angiogenesis effect of Ginsenoside Rb1. Moreover, how to establish a delivery system of Ginsenoside Rb1 and its repairment ability in bone defect remains elusive. In this study, the role of Ginsenoside Rb1 in cell viability, proliferation, apoptosis, osteogenic genes expression, ALP activity of rat BMSCs were evaluated firstly. Then, micro-nano HAp granules combined with silk were prepared to establish a delivery system of Ginsenoside Rb1, and the osteogenic and angiogenic effect of Ginsenoside Rb1 loaded on micro-nano HAp/silk in rat calvarial defect models were assessed by sequential fluorescence labeling, and histology analysis, respectively. It revealed that Ginsenoside Rb1 could maintain cell viability, significantly increased ALP activity, osteogenic and angiogenic genes expression. Meanwhile, micro-nano HAp granules combined with silk were fabricated smoothly and were a delivery carrier for Ginsenoside Rb1. Significantly, Ginsenoside Rb1 loaded on micro-nano HAp/silk could facilitate osteogenesis and angiogenesis. All the outcomes hint that Ginsenoside Rb1 could reinforce the osteogenesis differentiation and angiogenesis factor’s expression of BMSCs. Moreover, micro-nano HAp combined with silk could act as a carrier for Ginsenoside Rb1 to repair bone defect.

International Journal of Oral Science (2022) 14:10 ; https://doi.org/10.1038/s41368-022-00157-5

INTRODUCTION
Bone defect caused by injuries, tumor resection or osteoporosis resulted in severe dysfunction and interfered with people’s work and life remarkably. Bone mesenchymal stem cells (BMSCs), precursors of osteogenic cells, exhibit the potential capability of differentiating into osteoblastic cells and could be employed for the therapy of bone defect.1 To improve the efficiency of osteoanagenesis in patients, it’s crucial to stimulate the differentiation of BMSCs into osteoblasts. Recently, some traditional Chinese medicine have demonstrated their osteoinductive ability for BMSCs and dedicated the therapeutic properties for bone defects.2

Ginseng, as one of the herbal medicine, is widely used in a range of therapeutic and healthcare applications in China and other Asian countries.3 Ginsenoside is dammarane-type triterpene saponins acquired from ginseng and display a variety of pharmacology features like anti-cancer, anti-inflammatory, anti-oxidative and anti-apoptosis effect.4,9 These different pharmacology features are predominantly due to the steroid structure, which allows them to engage in interaction with cellular membranes, membrane-bound ionic channels, and exocellular and endocellular acceptors to generate changes at the transcriptomic level.10 Ginsenoside Rb1, the affluent ginseng saponin exists in ginseng roots, confers the pharmacology features, particularly in the heart and vessel system, endocrine system, and immunosystem.10 Some researches had reported that Ginsenoside Rb1 could inhibit the programmed cell death in isoproterenol-triggered cardiomyocytes, as well as the doxorubicin-triggered H9C2 cells.11,12 However, there was little study highlight the roles of Ginsenoside Rb1 in osteoblastic differentiation of BMSCs and bone defect repairing.

Herein, our team detected the osteoblastic differentiation of BMSCs induced by Ginsenoside Rb1. Furthermore, a local persistent releasing system of Ginsenoside Rb1 in the bone defect area was proposed. Hydroxyapatite (Ca10(PO4)6(OH)2, HAp) biological ceramic, as a naturally formed constituent of bony tissues, was well known to be bioactive and biocompatible in organisms, without antigenic characteristics and cytotoxicity.11,12 Our research has displayed that because of the outstanding specific surface area, micro-nano hybrid structured HAp (micronano HAp) particulates could be utilized as the carrier of drug delivery system to reinforce osteoinduction ability.2 Silk fibroin is a representative naturally formed biological polymer stemmed from Bombyx mori cocoons, and it possesses high versatility and minimal inflammatory reaction on account of its good biocompatibility and bio-degradability.13 However, the pure silk hydrogel was lack of osteogenic activity in vivo.14 In a previous study, with the addition of sodium alginate (SA), a polyanion co-polymer stemmed from brown sea algae, silk and Ca2+ could together generate an uniformed interpenetration aquagel to acquire a
steady scaffold. Currently, the present research on using Ca$^{2+}$ HAp, encapsulated with Ginsenoside Rb1, to cross-link both silk and SA to acquire steady dual net scaffolds utilized in bone defect was carried out to explore its osteogenesis effect. Based on the findings above, we aim to explore the therapeutic potential of Ginsenoside Rb1 as bone anabolic agents, as well as to establish a drug delivery system by taking advantage of micro-nano HAp and silk to load Ginsenoside Rb1, seeking to find a new guideline of biomaterials-drug-based healing strategies.

RESULTS

Ginsenoside Rb1 maintained cell viability and inhibited apoptosis of BMSCs

The cytotoxicity experiment was conducted to investigate the suitable level of Ginsenoside Rb1 for BMSCs and it turned out to be that the concentration of 80 μmol·L$^{-1}$ were apparently excessive, leading to more than half BMSCs death (50.17% ± 0.47%) ($P < 0.05$) (Fig. 1a). Cell Counting Kit-8 (CCK8) assay showed that Ginsenoside Rb1 at 10–40 μmol·L$^{-1}$ may have no superiority to enhance cell viability compared with 0 μmol·L$^{-1}$ group (Fig. 1b).

Meanwhile, Ginsenoside Rb1 exerted its advantages in reducing the apoptotic level in contrast to the controls. BMSCs apoptosis level treated with Ginsenoside Rb1 at 10 μmol·L$^{-1}$ for 1 day were 9.74% ± 0.24%, while the control group was 13.83% ± 1.10% ($P < 0.05$, Fig. 1c, d), which exerted the advantage of Ginsenoside Rb1 on reducing the apoptotic level of BMSCs. Those outcomes revealed that the Ginsenoside Rb1 play an essential role in cell viability maintenance and apoptosis inhibition effect on BMSCs.

Detection of Ginsenoside Rb1 in enhancing osteogenesis differentiation and angiogenesis factor expression of BMSCs

In this study, the mRNA expressing of runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP), osteocalcin (OCN), vascular endothelial growth factor (VEGF), Angiopoietin-1 (ANG-1) in BMSCs was detected by the treatment of Ginsenoside Rb1 at the concentrations of 10, 20, and 40 μmol·L$^{-1}$ (Fig. 2a–g).

Ginsenoside Rb1 enhanced the mRNA expression of Runx2, ALP, OCN, and OPN of BMSCs at day 7 (Fig. 2h).

Figure 1: Proliferative ability and programmed cell death of BMSCs exposed to ginsenoside Rb1. a Cell toxicity assessment with diverse levels of ginsenoside Rb1. b Cell viability of BMSCs posterior to exposure to ginsenoside Rb1 by CCK8 analysis. c, d Apoptosis experiment of BMSCs. (*in contrast to 0 μmol·L$^{-1}$ group at every temporal point, *$P < 0.05$; n = 3)
Taken together, the results proved Ginsenoside Rb1 was of great potential in promoting osteogenesis differentiation, as well as angiogenesis factor expressing of BMSCs.

ERK and AKT signal paths involved in the Ginsenoside Rb1-stimulated osteogenic differentiation

To reveal the role of the ERK and AKT signal paths in the functioning of Ginsenoside Rb1, we investigated the total and phosphorylation levels of ERK and AKT under 20 μmol·L⁻¹ concentration at the time of 0, 15, 30, 60, and 120 min. Results of western blotting showed that AKT and ERK were both phosphorylated posterior to Ginsenoside Rb1 (20 μmol·L⁻¹) induction at the first 15 min, and peaked at 30 min. Then, they gradually fall back. As for ERK signaling, it had an extra high wave at the time point of 120 min (Fig. 3a, b). Meanwhile, it showed that Ginsenoside Rb1 inhibited after the treatment of ERK signal path suppressor PD98059 or AKT signal path suppressor LY294002, respectively (Fig. 3e).

Ginsenoside Rb1 promotes the homing for HUVECs and BMSCs and promotes capillary tube forming of HUVECs

To explore the potential of Ginsenoside Rb1 in the recruitment of human umbilical vein endothelial cells (HUVECs in short) and BMSCs, the homing capability of Ginsenoside Rb1 for HUVECs and BMSCs was tested. The transwell migration test result showed that Ginsenoside Rb1 at the levels of 10, 20, and 40 μmol·L⁻¹ could facilitate the motility of HUVECs and BMSCs significantly in vitro, as shown in Fig. 4. Since the capillary tube net forming is vital for angiogenetic activities, capillary tube formation ability of HUVECs with the treatment of Ginsenoside Rb1 at concentrations of 10, 20, and 40 μmol·L⁻¹ were investigated and overall capillary tube length and the quantity of branching points per field were quantified as presented by
Vessels were generated within a capillary net under Ginsenoside Rb1 at concentrations of 10, 20, and 40 μmol·L⁻¹ obviously. In contrast, HUVECs in control group without Ginsenoside Rb1 failed to generate vessels posterior to 5 h on growth factor-decreased matrigel. Notably, number of branch nodes, length, junctions, and meshes per field were increased, in contrast to the controls, demonstrating the underlying angiogenesis of Ginsenoside Rb1.

Detection of micro-nano HAp granules

The acquired micro-nano HAp particulates displayed irregularity morphologically in shape under the micrographs with low magnification (Fig. 6a, b). The energy-dispersive X-ray spectroscopy (EDS-XRS; QUANTAX 400-30, BRUKER, Karlsruhe, Germany) was adopted to analyze the elements on the scaffold surface. It showed that granules detected contained calcium (37.88% in wt), phosphorus (16.82%), carbon (19.98%), and oxygen (25.32%).
Ginsenoside Rb1 promotes bone regeneration in the histological analysis. Meanwhile, the non-decalcified samples dyed in van Gieson’s picro fuchsin (Fig. 8) displayed that the new bone formation was commencing after the operation. More new bone formation was identified within the groups of silk/HAp/Rb1 (8.52% ± 0.41%) and silk/HAp/Rb1 + BMSCs (11.31% ± 0.97%), especially in the silk/HAp/Rb1 + BMSCs group. Moreover, the new bone in the silk/HAp/Rb1 + BMSCs group was more than the other three groups.

DISCUSSION

Traditional Chinese medicine has been inherited for thousands of years in China. As a reinforcing qi medicine, ginseng has been considered as the tonic of herbs. Ginsenosides, the active ingredients naturally present in ginseng, have a variety of beneficial effects on some disease, such as metabolic, vascular, and central nervous system disease. Particularly, Ginsenoside Rb1 contains a highly concentrated form of ginsenosides and exerts various pharmacological effects on metabolic disorders, including the modulation of its antioxidant, anti-inflammatory, anti-apoptosis effects, and promoting osteogenesis. Therefore, it was reasonable to speculate that Ginsenoside Rb1 might be a substitute for exogenous cytokines or growth factors to improve the therapeutic efficiency of bone defect.

Ginsenoside Rb1 has been shown to promote the proliferation of endogenous neural stem cells. This study found that Ginsenoside Rb1 at the concentrations of 10, 20, and 40 μmol·L⁻¹ could maintain the viability of BMSCs, and reduced apoptosis cell ratio after 1 day’s treatment. However, some investigations demonstrated that Ginsenoside Rb1 inhibit cell activity of rat hepatic stellate cells, and this paradox attributed potentially to the inconsistent cellular response of different cell types and the different concentration of Ginsenoside Rb1. Moreover, it was notable to find out that Ginsenoside Rb1 had an excellent ability in improving osteogenesis differentiation of BMSCs. The osteogenesis function was demonstrated by the mRNA expressing of Runx2, ALP, OPN, OCN, and ALP activity. Runx2 showed an essential effect on modulating the expressing of osteoblastic genes at the early stage. The mRNA expression of Runx2 in 10, 20, and 40 μmol·L⁻¹ Ginsenoside Rb1-treated groups was induced at 12 h and 24 h, except 40 μmol·L⁻¹. OPN was related to the mature phase of osteoblastic cells in the period of attachment and substrate syntheses prior to mineralisation, and OCN was related to the matrix deposition and mineralisation. Their expressions were both enhanced after 12 h treatment, and fall back at 24 h, but still higher than the control group except 40 μmol·L⁻¹. Some investigations demonstrated that the osteoblast differentiation has been promoted by Ginsenoside after 72 h of culture, while others reported that the osteoblasts differentiation have been enhanced by diverse level of Ginsenoside (10⁻⁵, 10⁻⁴, and 10⁻³ μmol·L⁻¹). This phenomenon may be caused by the different experimental conditions and cell types tested in individual studies.

Although the data manifested the capability of Ginsenoside Rb1 in stimulating osteogenesis, we also wanted to determine the underlying mechanisms by which this phenomenon took up. The ERK signaling pathway had been demonstrated to modulate osteoblastic growth, programmed cell death and differentiative activities via modulating the expressing of cellular cycle regulators. Meanwhile, AKT signaling pathway was...
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The osteogenesis of Ginsenoside Rb1 incorporated silk/micro-nano HAp particulates were capable of reticulating into a net, Ginsenoside Rb1 was capable of penetrating into the HAp particulates, which was capable of validly slowing down the releasing velocity. For that reason, it revealed that micro-nano HAp particulates exhibited a brilliant capability of controlling the Ginsenoside Rb1 delivery in vitro.

The early forming of novel vessel nets by HUVECs and the rapid bone regeneration by BMSCs of engineered constructs is pivotal for the successful result of bone defect restoration. Vessels produce a different metabolism and molecule micro environment, regulating the bone vasculature growth, sustaining perivascular osteoprogenitors, and they couple angiogenetic activities with osteogenesis. The homing experiment demonstrated that Ginsenoside Rb1 promoted the migration of HUVECs and BMSCs significantly. Moreover, a remarkable elevation in the quantity of tube formation was found in the Ginsenoside Rb1-exposed groups, revealing that Ginsenoside Rb1 could promote the proliferation of HUVECs, and facilitate subsequent angiogenesis.

Even although preliminary investigation indicated the potential ability of Ginsenoside Rb1 to promote osteogenesis and angiogenesis, the ultimate objective is to employ them in clinical application. Restoration of bone defect always needs fillers ranging from autologous bone to heterogeneous bone, which including a series of shortcomings. In the present study, coinciding with the outcomes in vitro, Ginsenoside Rb1 encapsulation in the micro-nano HAp particulates elevated the capability of HAp in regulating osteogenesis, the ultimate objective is to employ them in clinical application.

CONCLUSION
In conclusion, the present study had provided more valuable evidence for traditional Chinese medicine. Ginsenoside Rb1 is of significant effectiveness in promoting cell proliferation and...
were cultivated when there was approximately 80% confluence. The cultivation intermediary was added every 2 days, and the cells were fed with fresh media every 3 days and cells–intermediary was substituted by 200 μl of fresh media.

HUVECs were chosen as the typical lineage cells herein to explore the bioactivity of GD on angiogenesis. HUVECs were cultivated in Endothelial Cell Medium (Sciencell, Carlsbad, CA, USA). The long-term effects of Ginsenoside Rb1 on the cellular viability and proliferation of BMSCs was estimated by Cell Counting Kit-8 (CCK-8) analysis. BMSCs were inoculated into 96-well dishes at 2 × 10^4 cells per well. Posterior to a 24-h cultivation, they were exposed to 0, 5, 10, 20, 40, and 80 μmol·L^{-1} Ginsenoside Rb1, separately. At 24 h, the cell toxicity assessment was completed via MTT analysis. As per the supplier’s specification, 20 μL 5 mg·mL^{-1} MTT (Amresco, America) liquor was supplemented and cultivated under 37°C for 240 min to generate MTT formazan. After that, the intermediary was substituted by 200 μL DMSO (Sigma, America) for the purpose of dissolving the formazan, and the absorption was identified at 590 nm via the ELX Ultra micro plate analyzer (Bio-tek, America). The proportion of viable cells was determined via contrasting the specimen absorption in the presence or absence of Ginsenoside Rb1.

Apoptotic experiment
An annexin V-FITC tool (Becton Dickinson) was employed to realize the quantification of programmed cell death. Posterior to the exposure to Ginsenoside Rb1 at the concentrations of 0, 10, 20, and 40 μmol·L^{-1} for 1, 4, and 7 days separately. The same volume of vehicle (1 μL DMSO/per mL intermediary) was supplemented to generate the controls (0 μmol·L^{-1}). As per the supplier’s specification, cellular proliferative ability was evaluated via CCK-8 analysis. Every assay was completed at least for three times.

Fig. 6 Features of micro-nano HAp particulates. a The SEM images of micro-nano HAp particulates (scale bar = 10 μmol·L^{-1}). b The elements on the scaffold surface were analyzed. c, d The cumulative releasing proportion (%) of ginsenoside Rb1 loaded in the micro-nano HAp particulates at 2000 μmol·L^{-1}.
Osteoblastic, angioblastic gene expression, and ERK, AKT inhibitor treatment analysis by qRT-PCR

BMSCs were placed onto 6-well dishes at 2 × 10⁵ cells per well, and cultivated for 24 h, before cultivation with Ginsenoside Rb1 at the final contents of 0, 10, 20, and 40 μmol·L⁻¹, separately. Overall RNA was separated from the cells posterior to 12 h and 24 h Ginsenoside Rb1 exposure via the Trizol reagent (Invitrogen, America), as per the supplier’s specification. cDNA was prepared via a cDNA Preparation Reverse Transcriptional Tool (Fermentas, America). Realtime PCR analysis for Runx2, OPN, OCN, VEGF, and ANG-1 was completed via a Light-Cycler system through SYBR Pre-mix Ex Taq™ (Takara, Japan) as per the supplier’s specification.

The parameters for realtime PCR were stated below: denaturating under 95 °C for 10 s; 50 cycles under 95 °C for 10 s and 60 °C for 30 s; and an eventual dissociating phase (95 °C for 300 s) supplemented at the end of the magnification process. β-Actin was utilized as the inner control. The data were studied via the comparative Ct (2⁻ΔΔCt) approach and were described as a fold change in contrast to the controls. Every assay was completed at least for three times. The primer sequences herein were presented by Table 1.

To investigate the ERK and AKT signaling pathway, ERK and AKT inhibitor treatment analysis had been conducted. BMSCs exposed to Ginsenoside Rb1 at 0 and 20 μmol·L⁻¹ were cultivated in the intermediary added with ERK signal path suppressor PD98059 (Beyotime), or AKT signal path suppressor LY294002 (Beyotime) for 7 days, at final concentration 20 μmol·L⁻¹ and 20 μmol·L⁻¹, separately. Overall RNA was separated and synthesized cDNA, and realtime PCR was completed on Runx2, ALP, OPN, and OCN as aforementioned.

Fig. 7 Sequence fluorescence labeling of TE, AL, and CA for groups silk/HAp, silk/HAp/Rb1, silk/HAp+BMSCs, and silk/HAp/Rb1+BMSCs. a The pictures in yellow (TE; a1, b1, c1, d1), red (AL; a2, b2, c2, d2) and green (CA; a3, b3, c3, d3) indicated the rate of calvariae forming and mineralisation at 2, 4, and 6 weeks posterior to operation, separately. a4, b4, c4, d4 Meraged pictures of the 3 fluorescent dyes for the identical group. Scale bar = 100 μm. e The proportion (%) of TE, AL and CA dyeing via histomorphometry assay (*P < 0.05)
Alkaline phosphatase Staining and activity
BMSCs at $5 \times 10^4$ cells per well were cultivated nightlong in 24-well dishes. ALP dyeing and activity quantitation were completed at the seventh day posterior to the exposure to Ginsenoside Rb1 at 0, 10, 20, and 40 $\mu$mol·L$^{-1}$, separately. For the AKT and ERK suppressor exposure assay, BMSCs exposed to PD98059 (Beyotime) or LY294002 (Beyotime) for 7 days at final concentration $20 \mu$mol·L$^{-1}$ and $20 \mu$mol·L$^{-1}$, together with $20 \mu$mol·L$^{-1}$ Rb1. For ALP staining, all specimens were cleaned with PBS for 3 times and subjected to fixation with 4% paraformaldehyde for 600 s, and afterward cultivated in a matrix solution from an ALP dyeing tool (Beyotime), as per the supplier’s specification. After staining, the results were observed via a digital camera (ECLIPSES 100, NIKON, Tokyo, Japan). For ALP activity quantitation, the cells were cleaned 3 times in PBS, and 200 $\mu$L of lysis buffering solution was supplemented into the cellular layer and maintained on ice for 20 min. The cellular lysate was subjected to sonication for 60 s and treated with centrifugation at $12,000 \times g$ and subjected to centrifugation for $4 \mu$L under $4 \degree C$ for 600 s. ALP activities were analyzed using Alkaline Phosphatase Assay Tool (Beyotime, Suzhou, PRC) as per the supplier’s specification. The OD at 405 nm were measured to determine ALP activities. Overall protein levels were evaluated via a Pierce™ BCA Protein Analysis Tool (Thermo Fisher Scientific, America). OD results were standardized to bovine serum albumin standard curve, at 562 nm. ALP activities were evaluated as OD results at 405 nm per mg of overall protein.

Homing assay for HUVECs and BMSCs
In the homing assay for HUVECs and BMSCs, transwell migration tests (Corning Costar, America) were completed. In short, $3 \times 10^5$ HUVECs or BMSCs were inoculated onto the upper insert with $8 \mu$m apertures, and the lower chamber containing different concentrations of Ginsenoside Rb1. Posterior to a 24 h co-culture, the upper cells were removed, and the cells beneath the transwell were dyed with crystal violet and quantified analysis after dissolved by acetic acid.

In vitro sprouting analysis
The in vitro sprouting assay was finished as described in the past, and GF reduced Matrigel (BD Biosciences, America) was subjected to thaw under $4 \degree C$ and was added into 24-well plates on the ice. The dishes were afterward moved into an incubating device under $37 \degree C$ for 0.5 h to realize the gelation. Meanwhile, HUVECs were detached by trypsin and counted before resuspended in ECM with diverse levels of Ginsenoside Rb1. Then HUVECs were placed onto the gel at $10^5$ cells/well. Posterior to cultivation for 300 min under $37 \degree C$ in an incubating device, the plates were studied via a microscopic device (Nikon, Japan). We obtained $\geq 5$ fields for every matrix and the overall length of capillary tubes and quantity of branching points per field were calculated via a researcher blinded to our assay by virtue of NIH Image J 1.45 program (Bethesda, America).

The release kinetics of Ginsenoside Rb1 from HAp
According to our previous study, the levels of drugs utilized in the bony defect model at the multiple of 100 folds of that in vitro could acquire the best osteogenesis effect. Ginsenoside Rb1 at the concentrations of $2000 \mu$mol·L$^{-1}$ were frozen in $−80 \degree C$ overnight, and distillation was executed to realize the evaporation of the solvent DMSO (Sigma, America).

Then 1 mL SBF was supplemented to every compound and cultivated under $37 \degree C$, subsequently, the supernate was harvested and preserved under $4 \degree C$. At every chosen temporal point (1, 3, 6, 12, 24 h, 4, and 7 days). Afterward, the sample was subjected to resuspension in new SBF and cultivated till the following temporal point. The releasing of Ginsenoside Rb1 was subjected to quantification via the HPLC instrument (Shimadzu 2010C, America), and the data are described via the accumulative releasing as a function of the releasing time.

Accumulative quantity of release (%) $= 100 \times M_t/M_{\infty}$ in which $M_t$ denotes the quantity of Ginsenoside Rb1 generated from a specimen at temporal point t. The sum of Ginsenoside Rb1 in a specimen was computed and considered $M_{\infty}$ herein. We examined 3 specimens for every group and the outcomes were presented as mean values.
Preparation of composite silk fibrin hydrogel with Ginsenoside Rb1 loaded HAp
Silk fibrin hydrogel gel was prepared as follow: The silkworm cocoons, purchased from Sigma-Aldrich (St. Louis, America), were cut and boiled for 30 min. The silk was washed thoroughly with deionized water. Posterior to deagglomeration, the silk was subjected to dehydration in a drying device under 60 °C for 6 h and afterward subjected to a homogenizer in a CaCl2 solution. The mixture was subjected to dialysis in deionization water for 3 d. The obtained aqueous silk liquor was afterward subjected to lyophilization under −80 °C to acquire the purified regenerated B. mori SF. Then the SF was dissolved by deionized water, and the silk fibrin hydrogel gel in this study was 6%w/v.15

The micro-nano HAp particulates were prepared via the hydrothermal transform of the α-tricalcium phosphate (α-TCP, [α-Ca3(PO4)2]) particulates in CaCl2 water solution as per our research in the past. 0.1 g of the acquired micro-nano HAp particulates was soaked in 75 μL Ginsenoside Rb1 at 2000 μmOL−1 nightlong. Before lyophilization to realize the evaporation of the solvent DMSO (Sigma, America). Then the silk, and sodium alginate (SA) were produced as 6% (w/v), and 2% (w/v) stock solutions, respectively. Then, 100 μL silk liquid and 100 μL 5A liquor were mixed to acquire a silk/SA mix. Moreover, 100 mg HAp particles were supplemented to the silk/SA mix and agitated for a uniformed distribution. The mixture was allocated into a cylindrical Teflon mold of 5 mm diameter. For the animal experiment, 20 μL BMSCs were plated at 1 × 10⁵ cells per cm² on the composite gelation. Then the mixture was preserved under 37 °C till gelatinization.

Critical-size cranial defect study in vivo
A 5 mm diameter of full-thickness rat cranial bone defect is a commonly used model for evaluating the in vivo bone formation ability of the designed complexes.16 Then, 12 female 12-week-old Sprague-Dawley rats were acquired from our hospital’s Animal Center (PRC) for a cranial defect repair experiment, which was approved by Animal Experiment Ethics Board of our hospital affiliated to SJTU (HKDL2016321). Thereafter, the animals were separated into 4 groups, including silk fibrin hydrogel gel containing no Ginsenoside Rb1-loaded HAp granules (group A, silk/HAp, n = 3), silk fibrin hydrogel gel containing Ginsenoside Rb1-loaded HAp granules (group B, silk/HAp/Rb1, n = 3), BMSCs loaded silk fibrin hydrogel gel containing no Ginsenoside Rb1-loaded HAp granules (group C, BMSCs/silk/HAp, n = 3), and BMSCs loaded silk fibrin hydrogel gel containing Ginsenoside Rb1-loaded HAp granules (group D, BMSCs/silk/HAp/Rb1, n = 3). Sequential fluorescent labeling In terms of the 8-week observation, a multicolor sequence fluorescence labeling for newly formed bones and mineralisation was completed. In short, via intraperitoneal injection, the rats were exposed to 25 mg/kg tetracycline hydrochloride (TE, Sigma, America), 30 mg/g−1 alizarin red (AL, Sigma, America), and 20 mg/kg−1 Ca (Sigma), at 2, 4, and 6 weeks posterior to the treatment, separately.

Histology and histomorphometry analysis
The cranial bone specimens were subjected to dehydration in elevating levels of alcohol from 70% to 100%, and afterward subjected to polymethylmethacrylate (PMMA) embedment. We fabricated 3 longitudinal slices for every sample as depicted in our past researches.17 Initially, the specimens were studied for fluorescence labeling via CLSM (Leica TCS, Germany), and the fluorochrome dyeing for newly formed bones and the quantification of mineralisation was achieved. The data on yellow (TE), red (AL), and green (CA) denote the osteogenesis and mineralisation at 2, 4, and 6 weeks posterior to operation, separately. Eventually, the specimens were dyed in Van Gieson’s picro fuchsin for histology analysis. The region of new bone formation was subjected to quantification from the serial slice harvested from every specimen, via Image Pro Plus 6.0, and presented as a proportion (%) of the entire bone defect region, separately.

Statistics
Herein, the outcomes of repeatedly performed assays were described as the average ± SD. The remarkable diversity between datasets (*P < 0.05) was studied via one-way ANOVA.

ACKNOWLEDGEMENTS
This project was supported by National Natural Science Foundation of China (81600828), Shanghai Sailing Program (16YF1406600).

ADDITIONAL INFORMATION
Competing interests: The authors declare no competing interests.

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Table 1. List of primers used and respective forward and reverse sequences

| Gene   | Forward sequence                     | Reverse sequence                |
|--------|--------------------------------------|----------------------------------|
| β-actin| 5′-GTAAGAGACTCTATGCGCAACA-3′         | 5′-GGACTCTATCGACTCTGCTG-3′       |
| Runx2  | 5′-ATCCAGCCACCTCCTAACC-3′            | 5′-GGGACATTGGAGAATCATTG-3′       |
| ALP    | 5′-TTTCTACCTTGCTCATTCCG-3′           | 5′-GGCTGTGACTTGGAGACCAG-3′       |
| COL I  | 5′-CTGCCCAAGAGAAATGTGATCAC-3′        | 5′-GAAGCAAAGTCTCCTCCAAGACC-3′    |
| OCN    | 5′-GGCCTGATGCTATGTGGTCCT-3′          | 5′-TCACCATTTAATGGCCCTCTG-3′      |
| OPN    | 5′-CCAGGGCGTGGAAAACACAGACC-3′        | 5′-GGCTGTGAGTGGACCTGACTG-3′      |
| VEGF   | 5′-GGCTCTGAGAAACATTGAATTTTCT-3′      | 5′-GCAATAGCTGGCTTGAGAAC-3′       |
| ANG1   | 5′-GGACAGCGCAGGCAACACAGAGCAGC-3′     | 5′-CCACAGGCGTCAAACACACCAACC-3′   |
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