Biocompatibility and Bioactivity of BCP/Dox Scaffolds Containing Doxycycline Microspheres for Rat Silencing Pparγ Gene Transfected Bone Mesenchymal Stem Cells

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Research

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Abstract

Background: In our previous study, we have found that PPARγ-silenced BMSCs decreased adipogenic differentiation, but increased osteogenic differentiation after being induced by doxycycline. We demonstrated biphasic calcium phosphate (BCP) scaffold coated with multilayer of hydroxyapatite/poly-L-Lactide (HA/PLLA) nanocomposites is an excellent substitute for damaged and defect bone in bone tissue engineering. Combination of biomaterial scaffolds and therapeutic agents could contribute to a more predictable outcome with the potential of inducing bone formation while preventing bacterial infection. The delivery of BMSCs into Dox implant scaffolds aiming at enhancing the influence of BMSCs on the biocompatibility of the Dox implant has not been reported yet.

Methods: The Poly-lactic-co-glycolic acid-Methoxypolyethylene glycols (PLGA-mPEG) microspheres were prepared by encapsulating the doxycycline, and they were incorporated into three dimensional BCP scaffold to build a doxycycline sustained release system of BCP scaffold. The preprocessed BCP scaffold is present to tBMSCs, then tBMSCs viability, tBMSCs proliferation and differentiation capacities are detected in vitro.

Results: The microspheres were uniformly loaded on the BCP scaffolds and the pore structure was not affected, the BCP/Dox scaffolds were a good porous scaffold for the sustained release Dox for 2 months. The BCP/Dox scaffolds could promote transfected tBMSCs adhesion, proliferation and osteogenic differentiation *in vitro*.

Conclusions: The BCP/Dox scaffold is a suitable carrier for localized delivery of the Dox, and he BCP/Dox scaffolds could promote adhesion, proliferation and osteogenic differentiation of undifferentiated tBMSCs in vitro, but more work is needed to research to meet the demands of tissue engineering.

1. Background

Large segmental bone defect repair is a clinical and scientific challenge with increasing interest focused on combining gene transfer with tissue engineering techniques. In terms of bone tissue engineering, bone marrow stroma stem cells (BMSCs) loaded onto porous scaffolds, providing a prospective approach for the reconstruction of even large bone defects, have recently received special interest [1–4]. However, their guided differentiations are mainly realized by the modulation of adequate growth factors in previous studies [5]. However, this strategy is certainly limited to load many factors on scaffold to increase BMSCs differentiation. Also, inevitable initial burst release would significantly short the whole drug release period. Therefore, future advances in bone regeneration will likely incorporate therapies that mimic critical aspects of natural biological processes, using the tools of gene therapy and tissue engineering [2, 6].

In our previous study, we have examined the effect of the silencing PPARγ gene on the BMSCs differentiation, and the transfected BMSCs (tBMSCs) decreased adipogenic differentiation and increased osteogenic differentiation when they induced by doxycycline (Dox, tetracyclines) [7]. However, the effects of tBMSCs by silencing PPARγ gene on repair of segmental bone defects have not been reported, but it is

the necessary in way which developed gene and tissue engineering technology to deal with bone defect. Currently, It is well demonstrated that tetracyclines have the therapeutic potential to improve bone mass by inhibiting bone resorption[8–10] and enhancing bone formation[9–11], and they have been used since 2012 in the clinic to treat osteoporotic/osteopenic bone loss[9]. Additionally, tetracyclines are also employed as genetic switching system for the expression of target proteins in the gene and biochemical research [12–15]; in the Tet-On system, the doxycycline-Tet repressor interaction is used to affect a genetic construct controlling its expression [16].

In the last decade, synthesis of tissue engineering material has made a great progress with the rapid development of biomaterial-based bone substitutes. In our previous study, we demonstrated biphasic calcium phosphate (BCP) scaffold coated with multilayer of hydroxyapatite/poly-L-Lactide (HA/PLLA) nanocomposites is an excellent substitute for damaged and defect bone in bone tissue engineering [17].

Regarding bone regeneration strategies, the association of a biomaterial scaffold and a therapeutic agent that might induce bone formation while preventing bacterial infection could contribute to a more predictable outcome. The introduction of BMSCs into Dox implant scaffolds aiming at enhancing the influence of BMSCs on the biocompatibility of the Dox implant has not been reported yet.

In this study, PLGA-mPEG microspheres were prepared by encapsulating the doxycycline that stimulates bone formation and remodeling, and they were incorporated into three dimensional BCP scaffold. Then a doxycycline sustained release system was preprocessed in the BCP scaffold. The preprocessed BCP scaffold is present to tBMSCs, then tBMSCs viability, tBMSCs proliferation and differentiation capacities are detected in vitro. (Fig. 1)

2. Methods

2.1. Reagents and Materials

Sprague-Dawley (SD) was obtained from the Animal Center of Tongji Medical College of Huazhong University of Science and Technology (Wuhan, China); Low-glucose DMEM and Fetal bovine serum (FBS) (Hyclone, USA); Doxycycline Hydrochloride (Sigma, USA); Oil Red O, Alizarin red, OriCellTMSprague-Dawley (SD) Rat BMSC Adipogenic Differentiation Medium(RBADM), OriCellPTMP Sprague-Dawley (SD) Rat BMSC Osteogenic Differentiation Medium (RBODM) (Cyngen, USA); Alkaline phosphates (ALP) kit (Nanjing Jiancheng, China);Methyl thiazolyl tetrazolium MTT (Beyotime, China)Methoxy polyethylene glycol (Aladdin, USA)

2.2 .Preparation of BCP/Dox scaffolds

Fabrication of BCP scaffold coated with HA/PLLA: the BCP scaffolds were fabricated into round slices with a diameter of 5 mm and a thickness of 2 mm. The biodegradable BCP scaffolds (synthesized by State Key Laboratory of Material Processing and Die and Mould Technology, College of Materials Science and Engineering, Huazhong University of Science and Technology) consisted of about 40% of HA and

60% of β-TCP, coated with three-layer HA/PLLA using a polymeric reticulate method[17]. The porosity of the scaffolds were from 90.8–92.3%, and the pore sizes of open macropores were about 300–600 μm. The average pore size of the scaffolds macropores was approximately 450 μm. The compressive strength was at the range of 4.81–5.73 MPa by a 10 × 10 × 10 mm^3 scaffold.

Fabrication of Dox containing PLGA-mPEG microspheres: Dox-containing PLGA-mPEG microspheres (synthesized by State Key Laboratory of Material Processing and Die and Mould Technology, College of Materials Science and Engineering, Huazhong University of Science and Technology) were fabricated using the double emulsion-evaporation method [18].

The round slices BCP scaffolds coated with HA/PLLA was immersed in PLGA-mPEG/Dox microspheres solution for 30 min, then was frozeen-drying overnight at -70 °C. The BCP scaffolds properties were investigated by Fourier-transform Infrared spectroscopy (FTIR) and SEM analysis in our study. For presence of specific chemical groups in BCP/Dox scaffolds analysis, FTIR spectra were obtained within the range between 4000 and 500 cm^{-1} on VERTEX 70 FTIR spectrometer (VERTEX, Bruker) with a resolution of 1 cm^{-1}, using attenuated total reflectance (ATR) technique[17]. The morphologies were examined by FEI-scanning electron microscopy (SEM, Quanta 200, Holland FEI).

2.3. Dox release in vitro

The BCP scaffolds loaded with Dox were incubated in 5 mL PBS at 37 °C.The elute of Dox was examined at 1 h, 4, 8 h, 16 h, 24 h, 7 d, 14 d, 28 d, 42 d and 63 d, respectively. At each time interval, 3 mL supernatant was removed and replaced with fresh PBS. Then the Dox concentration was measured using a UV spectrophotometer (Hitachi U2910) at the λ_{max} value of 274 nm, and the amounts of the released Dox in the collected supernatants were calculated.

2.4. BMSCs isolation and cell transfection

Rats were sacrificed by cervical dislocation, the femora and tibiae were removed, the marrow cavity was flushed with low-glucose DMEM supplemented with 10% FBS and antibiotics. The bone marrow suspension was incubated in 75cm² flasks in an atmosphere of 5% CO_2 at 37°C. After 48h of incubation, nonadherent cells were removed.Culture medium was replaced every 3 days. When adherent cells reached 90% confluence, they were detached by mild treatment with trypsin-EDTA, 5 min, and 37°C and replated at one-third of the confluent density for continued passage. Cells of passage 4 were used for the following experiment [7]. The protocol of BMSCs transfection of PPARγ knockdown was described in detail in our previous research [7]. We constructed rat PPARγ gene shRNA Tet-on lentiviral vector (pTRIPZ/PPARγ-shRNA), and the lentiviral vector facilitated tetracycline (which has the characteristics of bone targeting)-inducible knockdown specific to PPARγ gene. After being transfection for 48 h, total RNA or proteins were extracted to examine the efficiency of PPARγ knockdown. Then transfected it into BMSCs, the silencing effects induced by tetracycline is examined. After transient transfection of BMSCs, the successfully transfected BMSCs (tBMSCs) were incubated in 75 cm² flasks in an atmosphere of 5% CO_2 at 37°C. At

the cell density of 90%, cells were digested with 0.25% trypsin and passaged. The fourth passage untransfected rat BMSCs were used as control for the following experiments.

2.5. Evaluation of adhesion and proliferation

The round slices BCP/Dox scaffolds were sterilized by ethylene oxide gas. BCP/Dox scaffolds were soaked with DMEM-LG supplemented with 15% FBS for 15 min, and prepared for further experiment after removal of medium. Round slice BCP/Dox scaffolds were added to 24-well plate, and no round slices were added in the control group. Five parallel replicates were used for each group. About 40 μL of BMSCs or tBMSCs (1.0×10^7 cells ml^{-1}) were added into the scaffolds until scaffolds saturation. Then the 24-well plate was incubated at 37°C for 4 h. After being washed with 1 mL PBS to remove the non adherent cells, cells were further incubated with 1 mL medium. The culture medium was changed by fresh medium every 2 d.

Adhesion rate: after 6- and 24 h of culture respectively, the scaffolds were removed from the wells and the media were collected and non-attached cells were counted for 6 or 24 h, respecitively. The adhesion rates were calculated as percentage of attached BMSCs.

Proliferation rate: The viable cell numbers in each 24-well plate were then determined from the standard curve based on their MTT absorbency. After 1, 3 and 5 d, five wells of each group were chosen for the MTT assay. The medium was removed and 100 μL of the MTT solution (5 mg ml^{-1}) was added to each well. Following incubation at 37°C for 4 h in a fully humidified atmosphere at 5% CO_2. Subsequently, the medium was removed, and 650 μl/well dimethyl sulfoxide (DMSO) was added to each well, then the solution was transferred to 96-well plates. The absorbance of each well was measured at 570 nm using an ELISA microplate reader (Bio-Rad). The results were statistically analyzed to express the mean and standard deviation (SD) [19].

2.6. Evaluation of adipogenic differentiation

As mentioned above to inoculate BMSCs and tBMSCs cells on scaffolds in 24-well, one round slice BCP scaffolds were added to each 24-well, for the control group, there was no round slices. Five wells of each group were osteogenic induction cultured with RBADM. The culture medium was changed by induction medium every 2 d. Cells were cultured in adipogenic medium for about 14 d until the density of the cells reached 80%. After being washed three times in PBS, cells were fixed in 3.7% freshly cold paraformaldehyde for 10 min at 4°C. Then cells were washed three times with 60% isopropanol and stained with filtered Oil red O solution (0.3% w/v) at room temperature. After 10 min, cells were washed and observed under an inverted phase contrast microscope. Finally, cells were destained in 1 ml 100% isopropanol for 15 min, and the OD of the destained solution was measured at 540 nm [20] .

2.7 .Evaluation of osteogenic differentiation

The efficiency of the transfected cells induced into osteoblasts was investigated by measuring the ALP. BMSCs or tBMSCs were resuspended with the culture medium (1.0×10^5 cells ml^{-1}) and then seeded into

24-well plate at a magnitude of 3×10^4 cells/well, and the culture medium was changed by fresh common medium every 2 d.

As mentioned above to inoculate BMSCs and tBMSCs cells on scaffolds in 24-well, five wells of each group were osteogenic induction cultured with RBODM. The culture medium was changed by induction medium every 2 d. ALP activity was measured using Nanjing Jiancheng Kit (Nanjing, China) after 6, 9 and 12 d. A colorimetric endpoint assay that measures the conversion of *p*-nitrophenol phosphate to *p*-nitrophenol by the enzyme ALP [7, 11].

At the same time, five wells of each group were chosen for the ALP assay after 9 d, the culture medium and scaffolds were removed, and cells were washed three times in PBS and fixed in 3.7% paraformaldehyde for 10 min at 4°C. Cells were then stained with Nanjing Jiancheng ALP Kit (Nanjing, China).

Other cells were cultured in osteogenic medium for 12 d until the cells reached 80% confluency. The culture medium and scaffolds were removed at the end of incubation; cells were washed three times in PBS and fixed in 3.7% paraformaldehyde for 10 min at 4°C. Cells were then stained with 0.5% alizarin red (pH 4.2) for 30 min and rinsed three times with water followed by a 15 min wash with PBS. Alizarin red was eluted in 10% cetylpyridinium chloride in PBS [21], and the number of calcium nodule was measured by spectrophotometry. [22]

2.8. Statistical Analysis.

The results were presented as mean ± standard deviation (SD). Statistical comparisons were performed using Student's *t*-test or non-parametric equivalents of analysis of variance (ANOVA). $P < 0.05$ were considered statistically significant.

3. Results

3.1. Microspheres morphology

The PLGA-mPEG microsphere contained 0.5wt% Dox, and the encapsulation efficiency of microspheres was 24.7%. Based on SEM images, the average diameter of the microspheres was between15 μm and 20 μm. And this size was suitable be incorporated into the coated BCP scaffold, of which the average pore size of the macrospores was approximately 450 μm (between 300 μm and 600 μm) [17]. The microspheres were uniformly loaded on the BCP scaffolds and the pore structure was not affected (Fig. 2A).

3.2. Physicochemical characterization of the scaffolds scaffold

The black line shows the characteristics absorption peaks of Dox were identified mainly the band in the 1000–550 cm^{-1} region.

The red line exhibit the characteristic absorption bands of BCP scaffolds coated with HA/PLLA. The PO_4^{3-} were the principal molecular components of BCP contributing to the IR absorbance in the 1200–550 cm^{-1} regions. The characteristic peak at 1040 cm^{-1} correspons to the stretching vibration of PO_4^{3-}, whereas that at 599 cm^{-1} denotes to the deformation vibrations of PO_4^{3-}. The characteristics of the –OH bands in HA were identified by observing the broad band from about 3700 to 2500 cm^{-1}. The peak of this band was centered at about 3131 cm^{-1}, which was typical stretching vibration of OH – ions. The characteristics of carbonyl group and methyl in PLLA were identified by observing the band at 1452 and 1360 cm^{-1}, respectively.

The blue line exhibit the BCP scaffold encapsulated with Dox. The predominant broad absorption band associated with PLLA and Dox, as shown in Fig. 2.

3.3. Dox release kinetics from microspheres

There was critical interpretation of the phenomenon, especially the first 16h; microspheres containing 0.5wt% Dox released 21.4% of the drug in the first 4 h, 32.6% by 8 h and 65.5% by 7 d and slow release started from 7 d continued for 2 months. (Fig. 2C)

The osteogenic differentiation ability of tBMSCs was low without Dox, and increased with the increase of Dox concentration from 2 μg/ml to 8 μg/ml, and the osteogenic differentiation ability was the highest at 8 μg/ml, then decreased gradually the concentration increasing. (Fig. 3A)

3.4. tBMSCs grown on the BCP/Dox scaffolds

BMSCs were successfully seeded onto the BCP/Dox scaffolds for 6 h and 24 h. Adhesion rates of BMSCs were faster than those of tBMSCs on BCP/Dox scaffolds 6 h after the incubation, but there was no significant difference at each time point ($p > 0.05$). (Fig. 3B)

The cells rised to adhere virtually 24 h after the incubation and the activity of proliferation in both cells was increas significantly after 3md, and had the potential to grow dynamically after 5 d, but there were no significant differences between the two groups ($p > 0.05$). (Fig. 3C)

A few cells were found adhere to the surface of BCP/Dox scaffolds. Figure 3D displays the distinct morphologies of tBMSCs on scaffolds after 5 d culture. The tBMSCs were mostly polygonal, shuttle type and unevenly dispersed on the surface and numerous filopodia like structures anchored the cell bodies and entered the pores. The tBMSCs on the BCP/Dox scaffold could largely spread out, suggesting good cell adhesion capability. These biological results in vitro confirm that BCP/Dox scaffolds had good cytocompatibility and were useful for bone regeneration. And there were unmodified Dox microspheres in the space surrounding the cells. (Fig. 3D)

3.5. Adipogenic differentiation

BMSCs induced to differentiate into adipocytes for 14 d were harvested 14 d after differentiation, and the synthetic levels of lipid droplet were evaluated. The Oil red O staining image analysis is shown in Fig. 4A

and 4B, the adipogenic differentiation rate in transfected BMSCs were decrease when compared with BMSC and BMSC combined with BCP/Dox scaffolds at two weeks post-differentiation. Furthermore, the quantitative analysis of oil red also revealed a decrease in the fat content levels of cell oil (Fig. 4B). The transfection of BMSCs combined with BCP/Dox scaffolds almost completely inhibited lipid droplet formation after 14 d in culture (Fig. 4B). These results indicate that BCP/Dox scaffolds inhibits the adipogenesis of BMSCs, and the transfection of BMSCs combined with BCP/Dox scaffolds have the best results of inhibits adipogenesis.

3.6. Osteogenic differentiation

BMSCs induced to differentiate into osteoblasts were harvested at 6, 9 and 12 d after differentiation, and the expression levels of ALP, which involved in osteogenic differentiation, were evaluated. A significant differences was found in ALP activity in the presence of BCP/Dox scaffolds in the tBMSCs group, and the maximum levels of ALP, achieved on 12 d, and the differences are statistically significant ($P < 0.01$), indicating BCP/Dox scaffolds increased the ALP levels and transfection BMSCs also increased ALP activity when compared with BMSCs (Fig. 4C). Furthermore, the ALP staining image analysis is shown in Fig. 5A, the osteogenic differentiation rate in transfected BMSCs were increas when compared with BMSC and BMSC combined with BCP/Dox scaffolds at 9 d post-differentiation.

Additionally, transfection BMSCs promoted calcium deposition after 12 d of osteogenic induction. Figure 5B shows that there were yellow granules within three groups cells, and the difference was significant, tBMSC + BCP/Dox group > BMSC + BCP/Dox group > BMSC group, both transfection BMSCs and BCP/Dox scaffolds promoted the formation of bone nodules as demonstrated by alizarin red staining. These data suggest that both transfection BMSCs and BCP/Dox scaffolds exert osteogenic effects.

4. Discussion

In the bone tissue engineering field, there has been special interest in BMSCs loaded onto porous scaffolds, providing a prospective approach for the reconstruction of even large bone defects [1, 4]. However, their guided differentiations are mainly realized by the modulation of adequate growth factors in previous reports in previous studies [5]. However, this strategy is certainly limited to load many factors on scaffold to increase BMSCs differentiation; Also, inevitable initial burst release would significantly short the whole drug release period. In this study, we synthesized a biomimetic composite biomaterial; BCP scaffold loaded with Dox microsphere and examined its ability to promote attachment, proliferation and differentiation of BMSCs by silencing PPARγ gene in vitro. The results showed that BCP/Dox scaffolds was a good porous scaffold for the sustained release Dox and could promote adhesion and proliferation and differentiation of tBMSCs.

BMSCs have multiple potentials differentiation and have been suggested in tissue engineering and bone regeneration studies, and BMSCs being the first choice in most cases [4, 23–25]. Gene transfer technology has opened novel treatment avenues towards the treatment of damaged musculoskeletal tissues[4, 26], and this technology are successfully combined with tissue engineering, and there are many

studies suggest that BMSCs transfected with BMP gene[12, 27], VEGF gene[27, 28] or TGF-β gene[29] enhanced bone regeneration. But the effects of BMSCs by silencing PPARγ gene on repair of segmental bone defects have not been reported and remain poorly understood, and then the first step is evaluation of the ability of the tBMSCs, such as adhesion, proliferation, adipogenic differentiation and osteogenic differentiation.

It has been recently emphasized that PPARγ is commonly termed the master regulator of adipogenesis[30, 31]. However, osteogenesis and adipogenesis of BMSCs maintain a homeostasis under physiological conditions. Once the homeostasis of osteogenic and adipogenic differentiation of BMSCs are disrupted, disorders such as osteoporosis may occur[32]. In our previous study, tBMSCs silencing PPARγ gene decreased adipogenic differentiation and increased osteogenic differentiation when induced by Dox[7].

The BCP/Dox could promote adhesion and proliferation and differentiation of tBMSCs in vitro. The first step in assessing the feasibility of gene therapy for bone defects was to determine whether gene transfection could enhance or destroy the bioactivity of seeded cells. In vitro, the proliferation and differentiation of tBMSCs were comparative with BMSCs, and in vitro SEM studies demonstrated that transfected tBMSCs can adhere, proliferate in the BCP/Dox scaffolds. In our study, Dox at 2–10 μg/ml could increase the proliferation and differentiation of tBMSCs, and the main reason was that Dox was used genetic switching system, and Dox induced tBMSCs to decreased adipogenic and increased osteogenic differentiation [7]. The tBMSCs in BCP/Doxloaded with tBMSCs group expressed of higher levels of ALP in vitro, which could be associated with availability of high levels of phosphate ions, essential to the onset of the mineralization process. There is also research found that biological materials with Dox or MIN could increase cell number of functional active cells and promote mineral deposition *in vitro*[11].

However, Dox would additionally significantly increase the proliferation of BMSCs and osteogenic differentiation in other ways with dose-related. Currently, Dox or MIN (1 mg/ml) has been found to induce BMSCs proliferation and to increase the extent of matrix mineralization [11, 33]. MIN (up to 3 μg/ml) was reported to increase the efficiency of rat BMSCs regarding colony-forming capacity[34]. Minocycline as a same kind of Dox could increase the proliferation and differentiation of BMSCs and osteoblasts in vitro, enhance bone formation, decrease connective tissue breakdown and diminish bone resorption[11, 35]. Dou et al. [36]suggested that the composite containing minocycline had good bioactivity. Therefore, it seems that tetracyclines at low level (less than 50 mg/ml) may induce osteogenesis[8].

The BCP/Dox scaffolds were a good porous scaffold for the sustained release Dox. In previous research, BCP scaffold coated with three layers HA/PLLA nanocomposites was appropriate in the BCP matrix when comprehensively considering the requirement of porosity and mechanical strength for tissue engineering scaffold[17]. In our study, BCP scaffold is a suitable carrier for localized delivery of the Dox in a clinically relevant fracture healing time to expedite skeletal repair. The Dox was sustained release for two months by microsphere, whilst shorter than fracture healing mean time 3 months. According to Feng et al [37] and

Deitchman et al [38] that the release of tetracycline from biomaterials is due to the strong chelating interaction between tetracycline and the calcium mineral component of biomaterial, the tetracycline release time would be longer in the interface of calcium mineral component of BCP than Dox from microsphere alone. More recently, local delivery of tetracyclines showed similar effects in periodontal disease [9, 37]. In addition to achieve excellent antimicrobial activity, there are also researches that the local delivery c and maintenance of the characteristic biological activity of these cells [11, 39].

The tBMSCs response to the BCP/Dox scaffolds was dose- and time-dependent to the proliferation and differentiation; therefore, further research should be done on optimizing the concentration of Dox and on tBMSCs. Moreover, considerable work in vitro and in vivo should be performed to further test the best time for tBMSCs adherion and proliferation on BCP scaffold.

5. Conclusion

In summary, the results showed that BCP/Dox scaffolds was a good porous scaffold for the sustained release Dox and slow release keeping for 2 months to meet the demands of sustained release system. The BCP/Dox scaffolds could promote adhesion and proliferation and differentiation of undifferentiated tBMSCs, and the BCP/Dox/tBMSCs could effectively induce osteogenesis in vitro, more work is needed to research to meet the demands of tissue engineering.

Abbreviations

biphasic calcium phosphate: BCP

hydroxyapatite/poly-L-Lactide: HA/PLLA

Poly-lactic-co-glycolic acid-Methoxypolyethylene glycols: PLGA-mPEG

bone marrow stroma stem cells: BMSCs

transfected BMSCs: tBMSCs

hydroxyapatite/poly-L-Lactide: HA/PLLA

Fetal bovine serum: FBS

Fourier-transform Infrared spectroscopy: FTIR

Declarations

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Not applicable

Consent for publication

Not applicable

Availability of data and materials

Not applicable

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

JK designed and initiated the study. TL performed all assays and analyzed the data. TL and ZL drafted and revised the manuscript. All authors read and approved the final manuscript.

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Figures

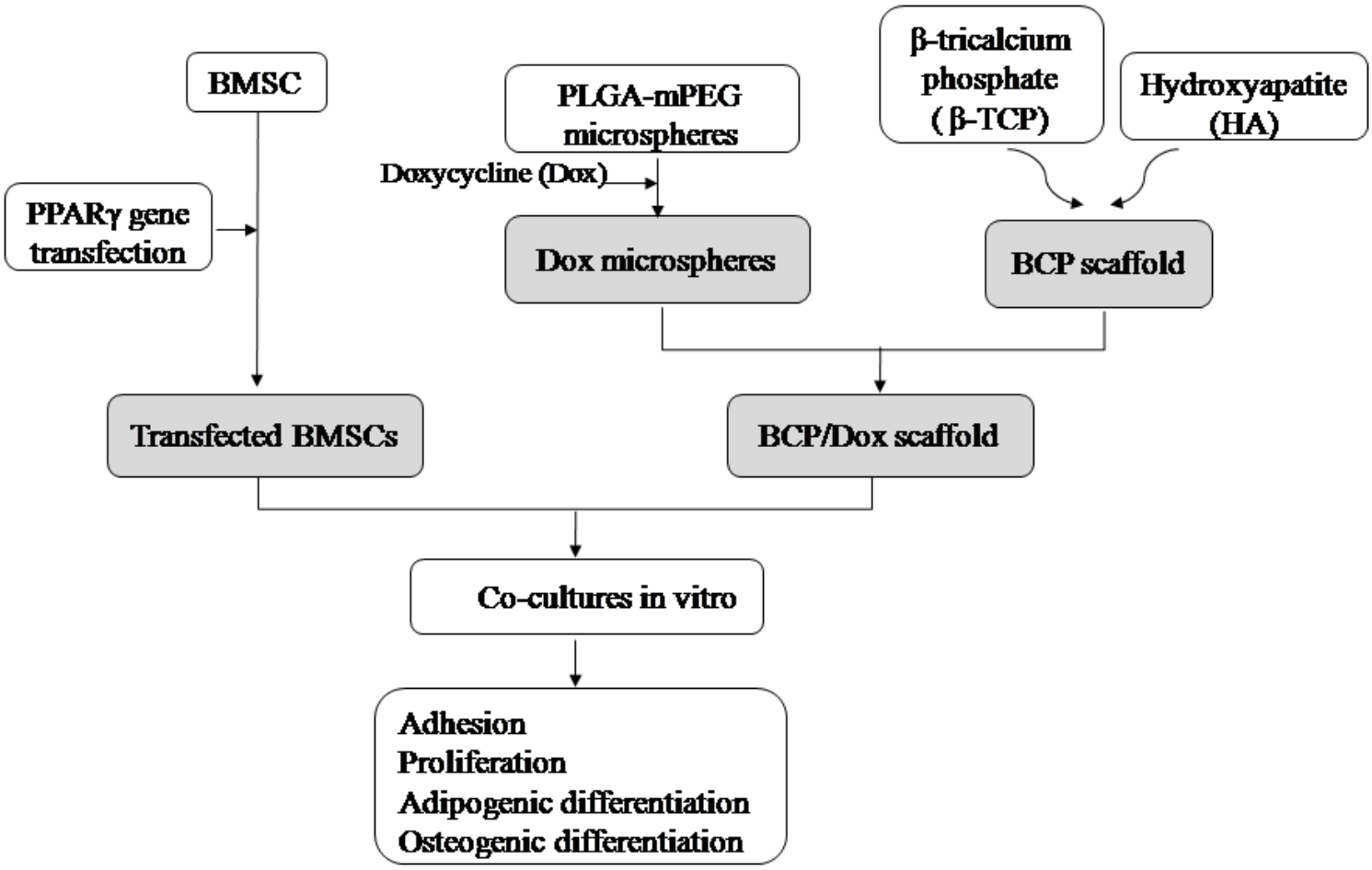

Figure 1

The flow chart. The BCP scaffold and Dox microspheres were synthesized, then their composite scaffold were determined as sustained release system, to explore the biocompatibility of the complex with the cultured transfected BMSCs in vitro.

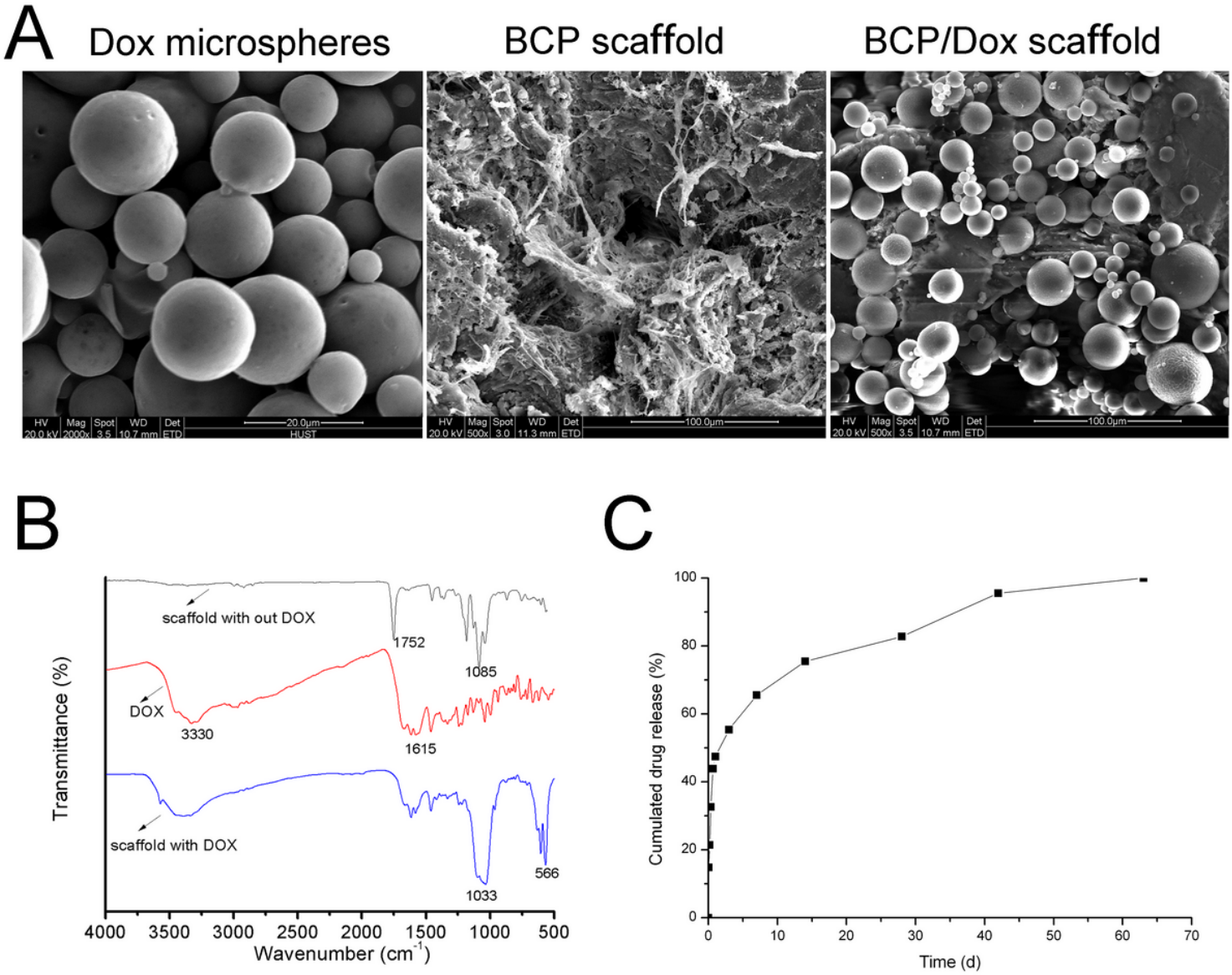

Figure 2

(A) SEM images of Dox microspheres. The average diameter of the microspheres was between15μm and 20μm; the average pore size of the macrospores was approximately 450 μm (between 300 μm and 600 μm); and BCP scaffolds loaded with Dox microspheres, the size was suitable be incorporated into the coated BCP scaffold.Fig. 2 (B) FTIR spectra of pure Dox, BCP/Dox and BCP scaffold. The absorption band in the region of 1000□500 cm-1 is characteristic of Dox loaded on the surface of the scaffolds.Fig. 2 (C).In vitro release kinetics of the Dox from BCP/Dox scaffold. BCP/Dox scaffold released 21.4% of the drug in the first 4h, 32.6% by 8 h and 65.5% by 7 d and slow release started from 7 d keeping for 2 months.

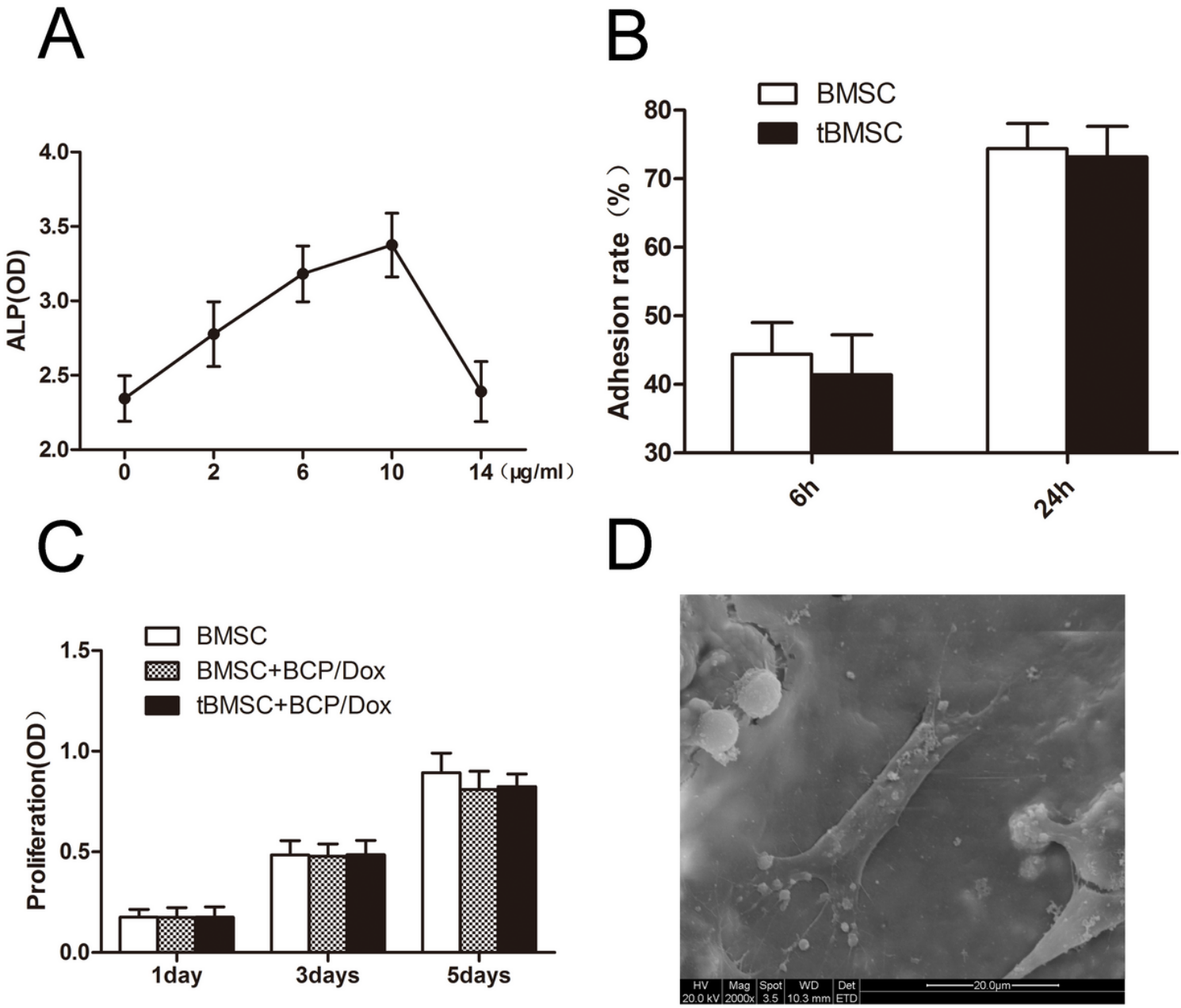

Figure 3

(A). Effection of different levels of Dox on ALP express of BMSCs. The osteogenic differentiation ability of tBMSCs increased of concentration of Dox from 2 μg/ml to 8 μg/ml, and the osteogenic differentiation ability was the highest at 8 μg/ml, and then decreased gradually the concentration increasing.Fig.3 (B). Adhesion rates of BMSCs and tBMSCs on the BCP/Dox scaffolds at 6 and 24h. Adhesion rates of BMSCs were greater than those of tBMSCs on BCP/Dox scaffolds 6 h and 24 h after the incubation, but there was no significant difference at each time point ($p>0.05$)Fig.3 (C). Proliferate rate of BMSCs and tBMSCs on the BCP/Dox scaffolds at 1, 3 and 5 d. the activity of proliferation in both cells was increased significantly after 3 d, and had the potential to grow dynamically after 5 d, but there were no significant differences between the two groups ($p>0.05$). Fig.3 (D). SEM morphologies of the tBMSCs on scaffolds at

5 d. The tBMSCs were mostly polygonal, shuttle type and unevenly dispersed on the surface and numerous filopodia like structures anchored the cell bodies and entered the pores.

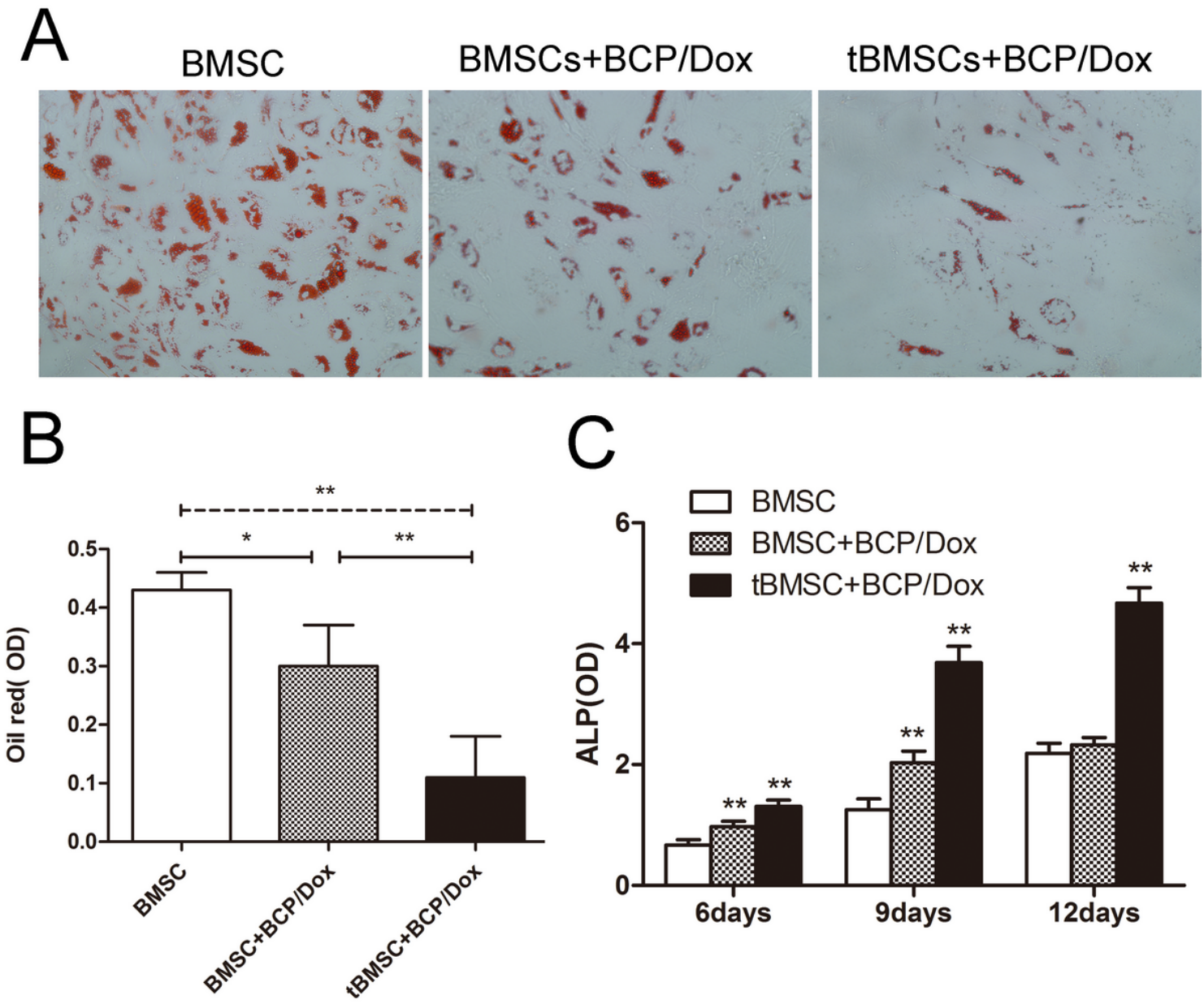

Figure 4

(A) Oil red O staining, lipid droplets were stained with Oil Red O on day 14 of BMSC adipocyte differentiation and examined using light microscopy, each group has Oil red cell, tBMSC+BCP/Dox group BMSC+BCP/Dox group BMSC group. tBMSC+BCP/Dox group almost completely inhibited.Fig.4 (B) The OD values of the destained solution of Oil red O from cells in adipogenic medium. Each group has Oil red cell after 14d, tBMSC+BCP/Dox group reduced more than the other groups, and BMSC+BCP/Dox group reduced more than BMSC group, the difference was significant (*p<0.05,**p<0.01).Fig.4 (C) ALP activity of two cells cultured with or without BCP/Dox scaffolds after 6, 9 and 12d. The largest ALP activity was tBMSC+BCP/Dox group, then t BCP/Dox scaffolds group and BMSC group, and the differences are statistically significant (**p<0.01).

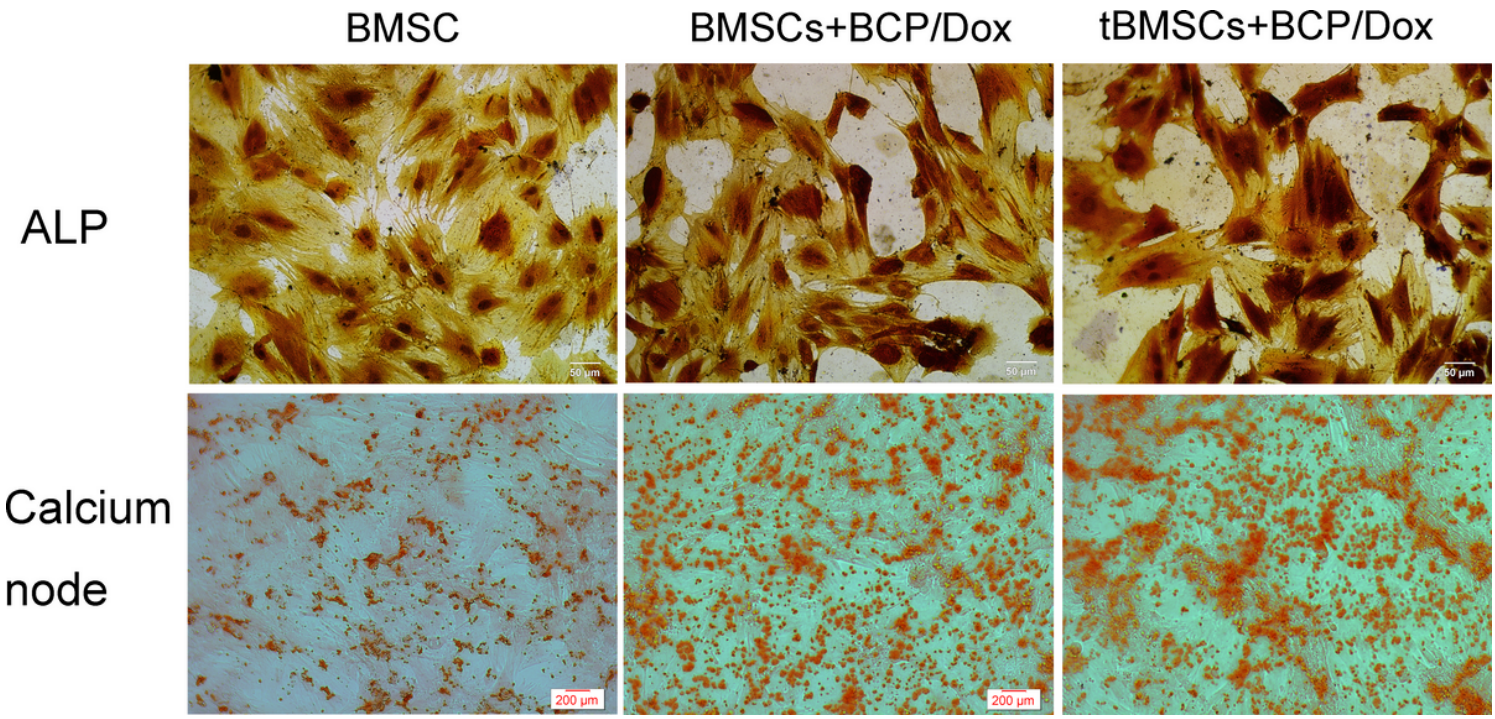

Figure 5

(A) ALP Staining was used to detect osteogenic differentiation in cells cultured with or without BCP/Dox scaffolds after 9 d. There were yellow granules were in three types cells cytoplasm, tBMSC+BCP/Dox group tBMSC+BCP/Dox group greater than the other groups.Fig.5 (B) Alizarin red staining was used to detect mineralization of nodules in cells cultured with or without BCP/Dox scaffolds after 12d. There were calcium nodes in three groups, tBMSC+BCP/Dox groupgreater than the other groups.