Introduction

Osteoporosis is a bone dysfunction, causing the underlying bone microstructure destruction, progressively increasing the bone fragility, till to cause a fracture. After the occurrence of osteoporotic fractures, bone cement is often required to restore the mechanical strength of the fractured bone and accelerate the process of bone repair. At present, the commonly used filling bone cements mainly include polymethyl methacrylate (PMMA), calcium phosphate cement (CPC), and calcium sulfate cement (CSC). CPC is composed of calcium (Ca) ions and phosphate (PO$_4^{3-}$) radical, which are also the main inorganic components of natural bone. In human body, CPC degrades slowly up to be totally replaced by new bone. Furthermore, the injectable, self-curing, and low curing heat properties of CPC make it suitable for minimally invasive surgery and complex geometric bone defects. With these advantages, CPC has received increasing attention in clinical applications. CPC is applied to fix osteoporotic vertebrae and burst vertebral fractures, as well as be used as pedicle screw to strengthen fixation. However, CPC itself has poor osteoinductivity and cannot improve postoperative bone regeneration ability.
strength, and sometimes even worsens bone deficiency. Therefore, it has become a hotspot to improve the biological activity and osteoinductivity of CPC.

Many bioactive agents (such as gelatin, growth factor, and Mg ions) have been incorporated into CPC to improve its bone regeneration ability. Among these agents, bioactive metal ions are widely used as osteointegration improvement agent in researches. In addition, Li is favorable for many bone-related events which are verified in many recent studies. For examples, literatures reported that Wnt signal-RANKL/OPG and Wnt/β-catenin pathways were involved in the superior osteogenesis of Li-incorporated Ti surface. Furthermore, Li-incorporated bioactive scaffold support the regeneration of osteochondral defects and can stimulate bone marrow stromal cell-derived exosomal miR-130a secretion, promoting angiogenesis. Bain et al. verified that Li ions could activate endogenous β-catenin signaling, and thus inducing the expression of alkaline phosphatase (ALP) mRNA and protein, similar to the treatment with BMP-2 protein. More importantly, LiCl increases bone formation by suppressing formation of APC-GSK-3β complex and preventing β-catenin phosphorylation. All these studies have confirmed that Li is a promising choice for the improvement of bone regeneration of bone implants. However, there is little knowledge about the osteogenic potential of Li in CPC and of great importance to explore this issue.

In order to improve the osteointergation of CPC, this study intends to incorporate Li into CPC to maintain the local slow release of Li. In vitro and in vivo performances of Li-doped CPC were studied.

Methods

Preparation of Li-doped CPC

CPC (Hydroxyapatite, relative molecular weight 502.31; Aladdin reagent, China) was mixed with deionized water (liquid-solid mass ratio is 0.5), and was transferred into stainless steel molds (φ=10 mm, h=2 mm for in vitro cell experiments; φ=2 mm, L=4 mm for use for animal experiments). The samples were taken out of the mold after solidify for 4 h. Lithium chloride (LiCl) was dissolved into deionized water (500 μg/mL) and Li-doped CPC was prepared according to the aforementioned steps. The obtained composite were denoted as CPC@Li. The surface morphology of the composites was observed using scanning electron microscopy (S-3400N, Hitachi, Japan).

Li ions release detection

CPC@Li composite was immersed in 5 mL of phosphate buffered saline (PBS, pH=7.4) at 37°C for 1, 2, 4, 7, 10, and 14 days. At each time point, the supernatant was collected filled with equal volume (5 mL) of new PBS. The Li ions in the collected extracts were detected by an inductively coupled plasma atomic emission spectroscopy (ICP-MS, iCAP RQ, ThermoFisher, USA).

Cell culture

α-MEM medium containing 10% v/v fetal bovine serum and 1% v/v penicillin was used to culture rat bone marrow stem cells (rBMSCs). The culture condition is 5% CO2, 37°C. rBMSCs at passage 3–5 were used for osteogenic differentiation experiments. CPC and CPC@Li composites were sterilized in 75% ethanol and rinsed with PBS prior to use.

Cell morphology

CPC and CPC@Li composites were placed in a 24-well plate, and 1 mL of cell suspension with a density of 5×10^4 cells/mL was added. After incubation for 4 and 24 h, the composites were washed twice with PBS. Afterward, the cells were rinsed, fixed, permeabilized, and blocked. Subsequently, 4’,6-diamidino-2-phenylindole (DAPI) and FITC-phalloidin (Sigma, USA) were used to stain the nucleus and cytoskeleton, and the cell morphology was observed under a fluorescence microscope (Olympus IX 71, Olympus, Japan).

Cell proliferation

rBSMC were grown on CPC and CPC@Li composites for 1, 4, and 7 days. At each time point, the culture medium was replaced with the fresh culture medium containing CCK-8 working solution. After another 40 min culture, 110 μL of the liquid was transferred into a 96-well plate, and the absorbance value at 450 nm was detected using a microplate reader (Bio-Tek, USA).

ALP activity assay

CPC and CPC@Li composites were placed in a 24-well plate, and 1 mL of cell suspension with a density of 2×10^4 cells/mL was added. After incubation for 3 and 7 days, the cells were fixed with paraformaldehyde. Alkaline phosphatase (ALP) activity was qualitatively assessed by BCIP/NBT staining working solution (Beyotime Biotechnology, China). ALP stained composites were observed using the bright field of a fluorescence microscope. The ALP activity detection kit is used to quantitatively detect ALP activity. Under alkaline condition, the substrate Para-nitrophenyl phosphate (pNPP) reacted with alkaline phosphatase to generate yellow product para-nitrophenol, which can be detected at the absorbance of 400–415 nm. BSA kit was used to detect cell protein expression. ALP activity is the ratio of ALP quantification to protein quantification.
Quantitative real time-polymerase chain reaction (qRT-PCR) assay

qRT-PCR was used to evaluate the osteogenesis-related genes expression in rBSMCs after cultured on various surfaces for 7 and 14 days. Trizol was used to lyse cells and then RNA concentration was detected using Naodrop spectrophotometer. RNA was reversely transcribed into cDNA and then target gene primers were added to amplify the cDNA. Finally, a PCR fluorescence quantifier was used to detect the Ct value. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression. Target genes include: osteopontin (OPN), osteocalcin (OCN), type I collagen (Collagen-I, COL-I), and ALP. GAPDH was set as a housekeeping gene, and the sequences of gene primer are shown in Table 1.

Animal experiment

All the animal experiments were approved by the Institutional Animal Care and Use Committee of Guangdong Provincial People’s Hospital. SD rats (5 weeks, ~200 g) were used for bone implantation experiments (n = 3 for each group). Rats were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg). The bilateral ovarian tissues of rat were excised and raised for 8 weeks to construct an osteoporosis model. After the model is constructed, the hind limbs of the rats were shaved and disinfected with 2% iodophor. A manual electric drill was used to pre-drill a 2 mm diameter hole along the direction of the medullary cavity, and the composites were then implanted. Antibiotics were given continuously for 3 days after the operation to prevent infection at the surgical site. Each hind limb was implanted with one sample. The rats were sacrificed 8 weeks after the operation and bilateral femur samples were collected. The bone samples were fixed with 4% paraformaldehyde, followed by dehydration, paraffin embedding, and sectioning. The obtained slices were stained with VG (Van’s Gieson), and the slices were observed under the bright field of a fluorescence microscope.

Data analysis

Data are expressed as mean ± standard deviation and analyzed using SPSS19.0 software. $p < 0.05$, $p < 0.01$, or $p < 0.001$ indicates that the difference is statistically significant.

Results and discussion

The morphology of CPC and CPC@Li composites after self-curing is shown in Figure 1(a) and (b), respectively. A granular structure with voids was observed in CPC and CPC@Li composites, suggesting that the addition of Li ions to the curing solution did not affect the curing morphology of CPC bone cement. The cumulative release curve of Li ions from CPC@Li is shown in Figure 1(c). It can be seen that Li was sharply released within 1 day (about 600 ppb), and slowly released in the next 13 days, with an amount of 800 ppb.

Figure 2 shows the morphology of rBMSCs cultured on CPC and CPC@Li composites for 4 and 24 h. After 4 h of adhesion, for CPC group, the cells showed a few lamellipodia. The cells cultured on CPC@Li sample not only exhibited lamellipodia, but also displayed a small amount of filopodia. The spreading area of cells cultured on CPC@Li was larger than that on neat CPC. After 24 h of cell culture, no significant changes in the cell morphology and spreading area were observed for neat CPC group, but more pseudopodia stretched out. Whereas rBMSCs cells grown on CPC@Li composites for 24 h, the cells showed a significantly higher spreading area comparing to 4 h, creating connections among them through the pseudopodia. The results indicate that the introduction of Li into CPC can promote the early adhesion of rBMSCs cells.

Cell proliferation results are shown in Figure 3. An increased proliferation over time was measured for both CPC composites. At short-time point (1 day), no significant difference in the cell proliferation was observed between neat CPC and CPC@Li composites. At day 4, cells grown on the surface of CPC@Li composite exhibited significantly higher proliferation than neat CPC (p < 0.01). At long-time point (7 days), this difference was more significant (p < 0.001), indicating that CPC@Li composite was more conducive to the proliferation of rBSMCs. Generally, cells first adhere and spread on implant surface and then grow and proliferate. Once the adhesion and spreading processes are accelerated, cells exhibited better growth and proliferation. Owing to a better adhesion and spreading abilities of rBMSCs on CPC@Li composite, the cells showed higher levels of proliferation rate at longer-time (4 and 7 days).

The process of new bone formation includes the synthesis, mineralization, and maturation of bone matrix.

### Table 1. Primer sequence of osteogenesis-related genes of rBSMCs.

| Genes   | Forward sequence | Reverse sequence |
|---------|------------------|------------------|
| OPN     | TGCAAAACACCGTTGTAACAAAGGC | TGCAGTGGCGGTTTGCATTCTT |
| COL-I   | CTCGCCAGAAGAAATATGTATCACCC | GAAAGGAAGTTTCTCAAGACC |
| OCN     | GCCCTGACTGCATTCTGCCCTCT | TCACCAACTTACGCCTCCTCCTG |
| ALP     | CGTCTCCTGTTTGGATTATGCT | CCCAGGCAACGTGGTCAAG |
| GAPDH   | ACAGCAACAGGGTGGTGAC | TTTGAGGGTGCAGCGAACTT |
Figure 1. Surface morphology of top section of CPC (a) and CPC@Li (b) composites (magnification 1000×, scale bar 20 μm). Cumulative release of Li ions from CPC@Li composite immersed in PBS (c).

Figure 2. Adhesion and spreading of rBSMCs after being cultured on CPC and CPC@Li composites for 4 and 24 h (magnification 400×, scale bar 30 μm). Fluorescence images were obtained by staining nuclei with DAPI (blue fluorescence) and F-actine of cytoskeleton with FITC-phalloidin (green fluorescence).
Figure 3. Cell viability of rBSMCs cells grown on CPC and CPC@Li composites for 1, 4, and 7 days. Data are mean values from n=4. **p<0.01. ***p<0.001.

Figure 4. Qualitative ALP staining (magnification 100×, scale bar 200 μm) (a) and quantitative analysis of ALP activity (b) of rBSMCs cultured on CPC and CPC@Li composites for 3 and 7 days. ***p < 0.001.
Figure 5. Expression of osteogenesis-related genes in rBSMCs grown on CPC and CPC@Li composites for 7 and 14 days. Data are expressed as relative gene expression with respect to GAPDH.

*p < 0.05. **p < 0.01. ***p < 0.0001.

Figure 6. VG staining of the obtained bone femur after implantation for 8 weeks (magnification 100×, scale bar 100 μm). Red triangles indicate the implants and yellow triangles indicate the newly formed bone.

Osteoblasts first synthesize extracellular matrix (such as COL-I and OCN) and then release enzymes (such as Ca^{2+} and ALP) through matrix vesicles. Calcium ions are deposited on collagen fibers under the action of ALP to complete the matrix mineralization process and finally form bone tissue. Therefore, ALP is an early signal of osteoblast differentiation, and the expression of related genes also determines the differentiation trend of osteoblasts. Figure 4
The expression levels of four osteogenesis-related genes (ALP, COL-I, OCN, OPN) were also detected at days 7 and 14, and the results are shown in Figure 5. After 7 days of culture, the expression levels of ALP and OCN genes in rBMSCs cultured on CPC@Li composite was significantly higher than neat CPC (p < 0.01); while the expression levels of the other two genes (COL-I and OPN) were in a similar level for both CPC composites. At long-time (14 days), the expression levels of all the four genes in rBMSCs for CPC@Li composite were significantly higher than neat CPC (p < 0.01 for ALP, p < 0.001 for COL-I, p < 0.01 for OCN, p < 0.05 for OPN). The above results indicate that the introduction of Li into CPC is conducive to the expression of osteogenic genes in rBSMCs.

A rat osteoporosis model was used to evaluate the osseointegration effect of CPC@Li. The bilateral ovariectomy was applied in female rats to construct a classic osteoporotic rat model. The composites were implanted for 8 weeks and then the femur was taken out. The femurs were harvested 16 days after implantation to construct CPC@Li composite osteoporosis rat model. The composites were implanted to the left tibia of female rats to construct a classic osseointegration effect of CPC@Li. The bilateral ovariectomy was applied in female rats to construct a classic osteoporosis rat model. The composites were implanted for 8 weeks and then the femur was taken out. The femurs were harvested 16 days after implantation to construct CPC@Li composite osteoporosis rat model.

The expression of osteogenic genes in rBSMCs. The bilateral ovariectomy was applied in female rats to construct a classic osteoporosis model. The novel Li-doped CPC is expected to be applied to patients suffered osteoporotic fractures. The bilateral ovariectomy was applied in female rats to construct a classic osteoporosis model. The novel Li-doped CPC is expected to be applied to patients suffered osteoporotic fractures.

The expression of osteogenic genes in rBSMCs cultured on CPC@Li composite was significantly higher than neat CPC (p < 0.001). The expression of osteogenic genes in rBSMCs cultured on CPC@Li composite was significantly higher than neat CPC (p < 0.001). The expression of osteogenic genes in rBSMCs cultured on CPC@Li composite was significantly higher than neat CPC (p < 0.001).

The above results indicated that CPC@Li is more benefit for the new bone formation and exhibited desirable osseointegration.

**Conclusion**

A Li-incorporated CPC composite was fabricated and provided a sustained release of Li ions over time. This study confirmed that Li-doped CPC not only had a significant effect in promoting osteogenic differentiation in vitro, but also showed good new bone formation and osseointegration in an osteoporotic model. The novel Li-doped CPC is expected to be applied to patients suffered osteoporotic fractures.

**Declaration of conflicting interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.
14. Huang TB, Li YZ, Yu K, et al. Effect of the Wnt signal-RANKL/OPG axis on the enhanced osteogenic integration of a lithium incorporated surface. *Biomater Sci* 2019; 7(3): 1101–1116.

15. Liu W, Chen D, Jiang G, et al. A lithium-containing nanoporous coating on entangled titanium scaffold can enhance osseointegration through Wnt/β-catenin pathway. *Nanomed Nanotechnol Biol Med* 2018; 14(1): 153–164.

16. Deng C, Yang Q, Sun X, et al. Bioactive scaffolds with Li and Si ions-synergistic effects for osteochondral defects regeneration. *Appl Mater Today* 2018; 10: 203–216.

17. Liu L, Liu Y, Feng C, et al. Lithium-containing biomaterials stimulate bone marrow stromal cell-derived exosomal miR-130a secretion to promote angiogenesis. *Biomaterials* 2019; 192: 523–536.

18. Bain G, Müller T, Wang X and Papkoff J. Activated β-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction. *Biochem Biophys Res Commun* 2003; 301(1): 84–91.

19. Silva AK, Yi H, Hayes SH, Seigel GM and Hackam AS. Lithium chloride regulates the proliferation of stem-like cells in retinoblastoma cell lines: a potential role for the canonical Wnt signaling pathway. *Mol Vis* 2010; 16(5): 36–45.

20. Zhu Z, Yin J, Guan J, et al. Lithium stimulates human bone marrow derived mesenchymal stem cell proliferation through GSK-3β-dependent β-catenin/Wnt pathway activation. *FEBS J* 2014; 281(23): 5371–5389.

21. Urist MR. Bone: formation by autoinduction. *Science* 1965; 150(3698): 893–899.