



# Local administration of aspirin with $\beta$ -tricalcium phosphate/poly-lactic-co-glycolic acid ( $\beta$ -TCP/PLGA) could enhance osteoporotic bone regeneration

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## Abstract

Composite materials  $\beta$ -tricalcium phosphate ( $\beta$ -TCP) and poly-lactic-co-glycolic acid (PLGA) have achieved stable bone regeneration without cell transplantation in previous studies. Recent research shows that aspirin (ASP) has great potential in promoting bone regeneration. The objective of the present study was to incorporate PLGA into  $\beta$ -TCP combined with a lower single-dose local administration of ASP to enhance its *in vivo* biodegradation and bone tissue growth. After the creation of a rodent critical-sized femoral metaphyseal bone defect, PLGA -modified  $\beta$ -TCP (TP) was prepared by mixing sieved granules of  $\beta$ -TCP and PLGA (50:50, v/v) for medical use, then TP with dripped 50  $\mu$ g/0.1 ml and 100  $\mu$ g/0.1 ml aspirin solution was implanted into the defect of OVX rats until death at 8 weeks. The defected area in distal femurs of rats was harvested for evaluation by histology, micro-CT, biomechanics and real time RT-PCR. The results of our study show that a single-dose local administration of ASP combined with the local usage of TP can increase the healing of defects in OVX rats. Single-dose local administration of aspirin can improve the transcription of genes involved in the regulation of bone formation and vascularization in the defect area, and inhibits osteoclast activity. Furthermore, treatments with a higher single-dose local administration of ASP and TP showed a stronger effect on accelerating the local bone formation than while using a lower dose of ASP. The results from our study demonstrate that the combination of a single-dose local administration of ASP and  $\beta$ -TCP/PLGA had an additive effect on local bone formation in osteoporosis rats, and bone regeneration by PLGA/ $\beta$ -TCP/ASP occurred in a dose-dependent manner.

**Keywords** Osteoporotic bone defect ·  $\beta$ -Tricalcium phosphate · Poly-lactic-co-glycolic acid · Aspirin · Regeneration

## Introduction

Osteoporosis is a common chronic disease that has become an epidemic, and by far the most common cause of age-related bone loss. Estrogen deficiency is associated with an increased osteoclast activity and an increased bone

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remodelling with the predominance of bone resorption over bone formation, resulting in decreased bone mass and increased bone fragility [1]. Once bone fracture or defect occurs, plastic surgery is needed for bone repair. One of the most common programs which has been widely accepted in treating bone fractures and regenerate bone loss is the autograft [2, 3]. Autografts are a solution for such problems but are limited in size, come with a painful surgical procedure and the dissection of autologous bone from an intact site, hence creating an additional lesion. The aims of using tissue engineering are to achieve the morphology and structure of the scaffold to increase adhesion of osteoblasts and osteoprogenitor cells, promote differentiation and migration, and allow the synthesis of homogenous bone matrix without necrosis [4]. Calcium phosphate-based ceramic scaffolds for bone graft substitutes have long been the focus of extensive research work [5, 6]. There have been numerous reports on bone tissue engineering using  $\beta$ -tricalcium phosphate ( $\beta$ -TCP), a bioactive ceramic material with good properties of resorption, osteoconductivity, cellular adhesion, mechanical strength, and compatibility with host bone tissue [7]. With regard to biomaterial scaffolds, an ideal one should meet numerous requirements, including excellent biocompatibility and superior osteoconductivity and osteoinductive properties. However, most of the scaffolds currently used (i.e., those made from ceramics, polymers, and metals) only have osteoconductive abilities and thus lack powerful osteoinductive properties [8]. Therefore, to overcome this issue, bioactive molecules, such as growth factors or cytokines, have been introduced into scaffolds [9].

Aspirin (ASP) is a common and safe compound that has been used as an effective antipyretic, analgesic and anti-inflammatory drug. However, additional effects have been identified. Based on preliminary study, aspirin is suggested to possess anti-postmenopausal osteoporosis effects in the ovariectomized rat model [10], which indicates a possible clinical application for aspirin in the prevention of bone loss. It inhibits osteoclastogenesis, and improves osteogenesis by affecting multiple biological pathways, such as inhibition of COX2, COX1, and PGE2 activity [11]. Through acting on the Fas/FasL signaling pathway, ASP intervenes in the development of osteoporosis by inhibiting the apoptotic effect of T lymphocytes on bone marrow-derived mesenchymal cells, promoting osteogenic differentiation and inhibiting osteoclast differentiation [12]. Thus, we speculated that aspirin could favor bone regeneration by affecting multiple biological pathways.

All the evidences above consolidate the notion that  $\beta$ -TCP and aspirin represents a promising therapeutic approach for the treatment of osteoporotic defect induced by postmenopause. However, these is little known about the effect of combined treatment with local administration of aspirin and  $\beta$ -TCP/poly-lactic-co-glycolic acid (PLGA)

composite on osteoporotic defect in bone loss induced by ovariectomized (OVX). Thus, we hypothesized that combined treatment with local administration of aspirin and  $\beta$ -TCP/PLGA composite treatment would enhance healing of metaphyseal defects in OVX rats via enhanced stimulation of bone formation and that this is significantly better than the effect of using the  $\beta$ -TCP/PLGA composite alone. The purpose of this study is to observe the effects of combined treatment with local administration of aspirin and  $\beta$ -TCP/PLGA composite on defect healing in OVX rats.

## Materials and methods

### Animals

A total of 50 virgin adult (3 months old) female SD rats weighing 220–250 g were used, and the present study was approved by the animal service of First Affiliated Hospital of Wannan Medical College, Yanjishan Hospital. The rats were bred under conditions of controlled temperature ( $22 \pm 0.5$  °C), humidity ( $50 \pm 10\%$ ) and lighting (12 h light–12 h dark cycle, light period began at 07.00 h). All the rats were given free access to distilled water and fed standard rat chow. The animal care and experimental procedures were carried out in accordance with the Guidelines for Animal Experimentation of Wannan Medical College, with the approval of the Institutional Animal Care and Use Committee.

### Preparation of $\beta$ -TCP/PLGA composite

$\beta$ -TCP/PLGA composite was prepared based on the procedures described by Zhang et al. [13]. PLGA (St. Louis, MO, USA) was used for the microsphere preparation. Briefly, 1 g of PLGA was dissolved in 6 ml of dichloromethane and mixed by vortex agitation for 30 s to create a homogenous solution. The mixture was transferred into a stirring beaker containing 100 ml of 0.3% aqueous PVA (St. Louis, MO, USA) solution (w/v). Subsequently, the solution was stirred (800–1000 rpm) for 5 h to evaporate the solvent and allow for stabilization of the microspheres. Next, the prepared  $\beta$ -TCP (Olympus Terumo Biomaterials, Tokyo, Japan) powder microspheres were added to PLGA solution (50:50, v/v). Finally, the obtained  $\beta$ -TCP/PLGA composite was then separated and washed three times with distilled water. Porous composites were obtained after further lyophilization. Scaffolds were lyophilized and stored at  $-20$  °C until further use.

## Surgery and treatment

After bilateral ovariectomy ( $N = 45$ ) or sham operation ( $N = 5$ ) according to previous reports [14, 15], 12 weeks were allowed to pass before defect surgery for the establishment of standard osteoporotic animal models. Each five randomly selected OVX rats and the five sham-operated ones were euthanized. The distal femurs were harvested for bone mineral density (BMD) evaluation by micro-CT to confirm the establishment of osteoporosis. Afterwards, femoral cylindrical defects were created from the anterior to posterior direction in the distal femur, which were standardized at 3 mm in diameter, penetrated internal and external at about 5 mm in length under generous irrigation with NaCl 0.9%, and lay above the distal epiphyseal growth plate as previously described [16, 17], then all the animals were randomly divided into four groups, control group (CON),  $\beta$ -TCP/PLGA composite group (TP) and local administration of low and high density aspirin and  $\beta$ -TCP/PLGA composite group (LATP and HATP); The defects from the group TP, LATP and HATP were filled with  $\beta$ -TCP/PLGA composite material, and the defects from the group LATP and HATP were dripped with 50  $\mu$ g/0.1 ml and 100  $\mu$ g/0.1 ml aspirin solution. The doses and patterns of ASP were determined

according to the previous experiments [11, 18]. Lastly, surgical sites were sutured and the animals received antibiotics (penicillin, 400,000 U/day) for 3 days. Analgesic was given pre-emptively and postoperatively for 3 days by subcutaneous injection at 0.03 mg/kg body weight. Before the animals were sacrificed, calcein green (Sigma-Aldrich Inc., 20 mg/kg) was injected subcutaneously at 10 and 3 days before the end of the experiment, respectively. The retrieval procedure was performed at 8 weeks (10 rats at each time point), when the animals were sacrificed using an overdose of barbiturate (Mebumal, ACO L € akemedel AB, Solna, Sweden). For the subsequent analytical technique at 8 weeks, the skin was carefully reopened and the bone defect site with the overlying membrane and soft tissue were harvested en bloc and preserved in formalin (Fig. 1).

## Bone micro-CT scanning

We dissected the femurs, cleaned the soft tissues, fixed them in 10% neutral formalin for 48 h, and then immersed them into 70% ethanol. Bone microarchitecture in the middle and distal femur was scanned using micro-computed tomography ( $\mu$ -CT; VivaCT 40; Scanco, Brüttisellen, Switzerland) with 15  $\mu$ m resolution, tube voltage of 70 kV and tube current of

**Fig. 1** Filling of the distal femoral defect (a). The distal femoral defect was always left unfilled, serving as a control group. The defects of other groups were filled in such a way that in total there were three samples for each material at each time point. Composite materials include TP (b), LATP (c) and HATP (d) scaffolds



114  $\mu\text{A}$ . The reconstruction and 3D quantitative analyses were performed using the software provided by a desktop micro-CT system (VivaCT 40; Scanco). For evaluation of bone regeneration and microarchitecture within the defect, volume of interest (VOI, diameter = 3 mm) was drawn within the femoral metaphysis defect and analyzed using the CTAn software (Bruker, Belgium) to calculate the new bone formation (BV/TV), bone mineral density (BMD), trabecular thickness (Tb.Th), trabecular number (Tb.N) and the distance between trabeculae (Tb.Sp) according to previous reports [19, 20].

### Histological analysis

After micro-CT scanning, the femurs were fixed in formalin and dehydrated in ascending ethanol series. Subsequently, bones were embedded in methylmethacrylate and cut into 4-mm sections for staining and 7-mm sections to assess fluorescence labels. The sections were stained with hematoxylin and eosin (H&E) to assess the microarchitecture. Unstained sections were analyzed using fluorescence microscopy to determine the mineral apposition rate (MAR) using the two fluorescent labels. Histomorphometric analysis was performed with the Osteomeasure software (OsteoMetrics, Decatur, GA, USA) as described previously [21, 22].

### Biomechanical testing

Compression testing of the distal femoral metaphysis was conducted as previously described [23]. The end of the dorsal distal femur (both condyles) was placed in a 5 mm wide and 2 mm-deep notch of an aluminum alloy base, which was fixed to the mechanical testing system (MTS Landmark Systems, USA). This position resulted in a stable three-point contact with the base so that the femur could not slip during the breaking test. A 1 N preload was applied to the ventral aspect of the condyles and then compressed until failure at a rate of 2 mm/min. The ultimate load at failure was determined as the strength of the condyles.

### Real time RT-PCR

CON, TP, LATP and HATP rats ( $n = 5$  per each group) were sacrificed 8 weeks after implanting the material. Total RNA was prepared from the entire mass of newly formed bones in the implanted sponges of each animal using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA). Total RNA (1  $\mu\text{g}$ ) was used for cDNA synthesis with Super Script Reverse Transcriptase II and oligo dT<sub>12–18</sub> primers (Invitrogen). Oligonucleotide primers for real time RT-PCR were designed with product sizes < 200 bp using primer express 3.0 (Applied Biosystems) as shown in Table 1. Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used to detect the accumulation of PCR product during cycling with the ABI Step One Plus sequence detection system (Applied Biosystems). The RMO cycling conditions were as follows: predenaturation at 95 °C for 10 min, and amplification using three-step cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s, for 40 cycles as previously described [24].

### Statistical analysis

Data were expressed as mean  $\pm$  standard deviation (SD). Statistical analyses were performed using the statistics package SPSS 19.0 (SPSS, Chicago, IL, USA). Multiple comparisons between groups were carried out using one-way ANOVA and Tukey's post hoc test. The significance level of 0.05 was applied for all analyses.

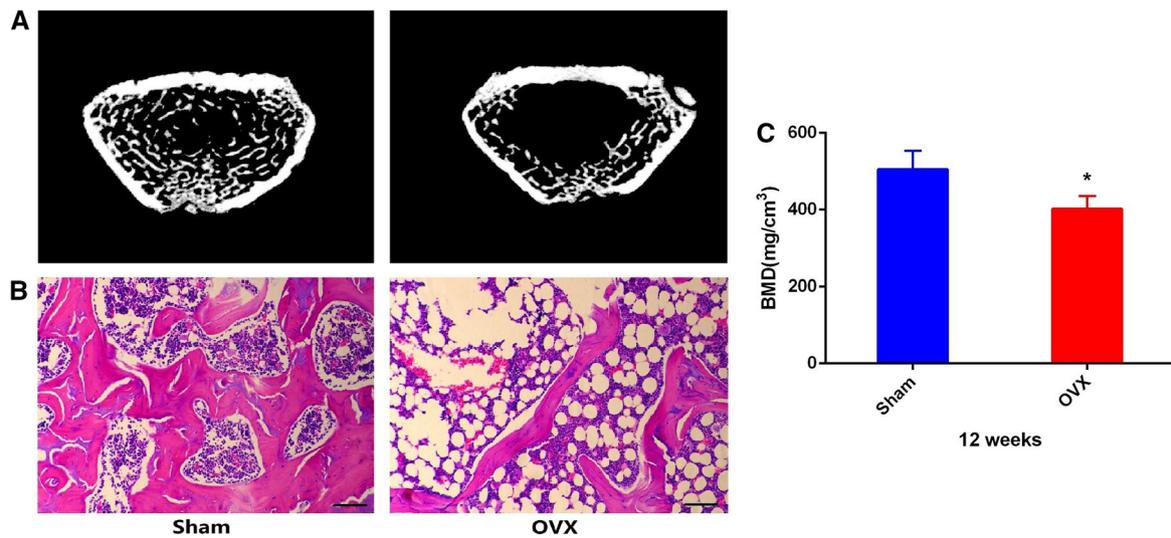
## Results

### Confirming the rat osteoporotic model

HE stain images and micro-CT data showed that, relative to the sham group, OVX rats had significantly lower subchondral trabecular bone volume and thickness, and dramatically higher trabecular separation. Quantitative analysis by micro-CT showed that the BMD of trabecular bone in OVX rats was  $402.32 \pm 33.14 \text{ mg/cm}^3$ , significantly lower than that of sham rats ( $505.43 \pm 47.87 \text{ mg/cm}^3$ ;  $P < 0.05$ ; Fig. 2a–c).

**Table 1** Primers used for real time RT-PCR

Genes	Forward (5'–3')	Reverse (5'–3')
GAPDH	GGTGGACCTCATGGCCTACA	GGGCCTCTCTTGTCTCTCA
Runx2	TTGCCTCTGACACGGCTTTAC	AGCAAAGAGGCCGCATTAAG
OCN	TCACTCTGCTGGCCCTGACT	CCCTCCTGCTTGGACATGAA
OPN	AGCCAGCCAAGGACCAACTA	GCCAAACTCAGCCACTTTCAC
BMP-2	GTCCTTTTCCCCTGGCTGAT	AACATGGAGATTGCGCTAAGC
TRAP	GTGCTGGCTGGAAACCATGA	GTCCAGCATAAAGATGGCCACA
VEGF	TGCCACCCGAGGAGTTCA	GGCCCTGGTGAGGTTTGAT



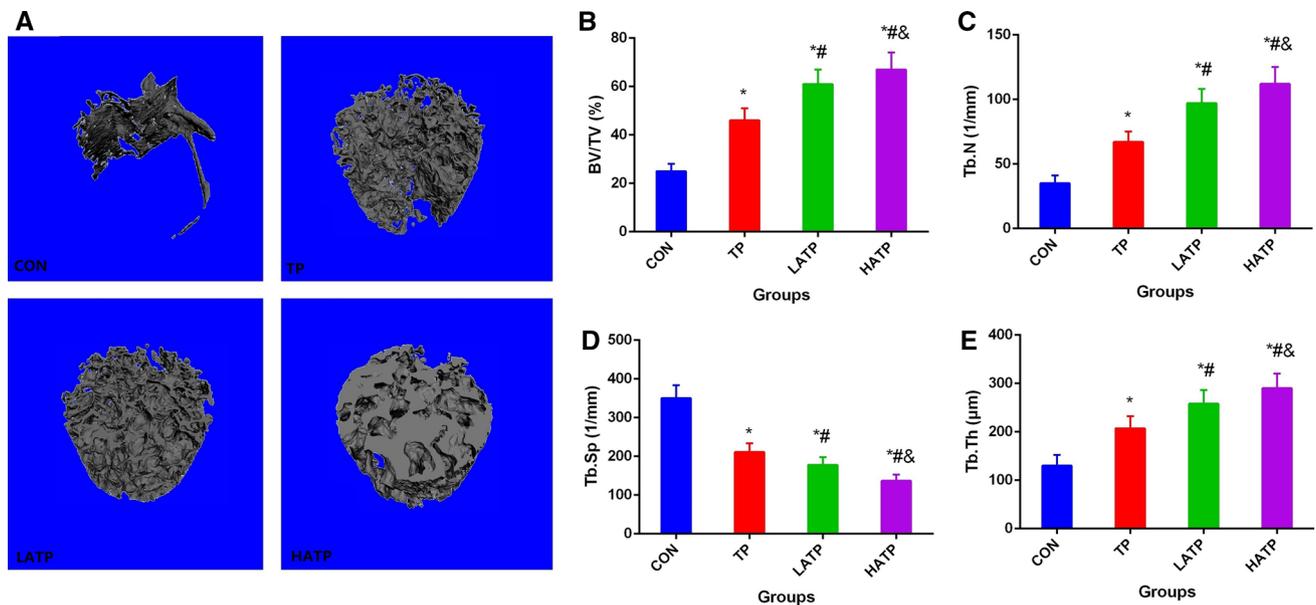
**Fig. 2** **a** Two-dimensional micro-CT images in sham and OVX groups. **b** The BMD of trabecular bone in the OVX group was significantly lower than that of the sham group. **c** Hematoxylin–eosin

staining results for the distal femurs of sham and OVX groups ( $\times 20$ ). \* $P < 0.05$  versus Sham group (by Student's  $t$  test)

### Microstructure parameters

The images of the three-dimensional reconstructions of the trabecular in the defects are clearly shown in Fig. 3a and the quantitative results were expressed as BV/TV, Tb.Th, Tb.N and Tb.Sp (Fig. 3b–e). After 8 weeks of treatment, the microarchitecture parameters BV/TV, Tb.Th, Tb.N

were significantly lower, and the microarchitecture parameters Tb.Sp were significantly higher than the CON group ( $P < 0.05$ ), and notable difference in BV/TV, Tb.Th, Tb.N and Tb.Sp was observed among the group TP, LATP and HATP ( $P < 0.05$ ). Moreover, the rats in the group HATP presented the strongest effect on BV/TV, Tb.Th, Tb.N and Tb.Sp regardless of treatment groups.



**Fig. 3** **a** Conditions of repair in defect was exhibited by 3D reconstruction after euthanasia of each rat in four groups for 8 weeks. **b–d** Quantitative results of micro-CT analysis expressed as BV/TV, Tb.N, Tb.Sp, and Tb.Th. Data were expressed as mean  $\pm$  SD; error bars in

the figure are presented as SD,  $N = 5$  specimens/group. \* $P < 0.05$  versus CON group, # $P < 0.05$  versus TP group, & $P < 0.05$  versus LATP group (by one-way ANOVA and Tukey's post hoc test)

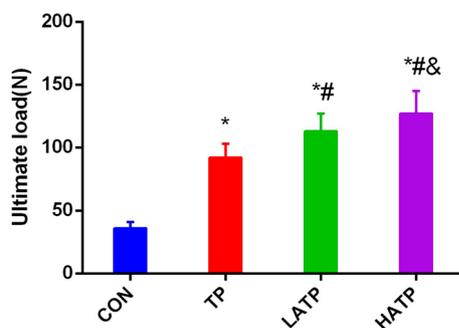
## Biomechanical test

Results of biomechanical test of femoral condyles were expressed as the ultimate load (Fig. 4). After treatment for 8 weeks, the strongest effects on strength of femoral condyles were observed in the HATP group. After treatment with ASP and  $\beta$ -TCP/PLGA composite for 8 weeks, the ultimate load of TP, LATP and HATP groups were significantly increased by 2.12-, 2.65- and 3.12-fold, respectively, compared with the group CON and a significant difference was observed among the four groups ( $P < 0.05$ ).

## Histology analysis

At 8 weeks post surgery, defects were filled with a thin, loose connective tissue with minimal new bone formation from CON group (Fig. 5A), while the quantity of the newly formed bone was observed in group TP, LATP and HATP. Compared with the CON group, the defect sites were filled with much new bone formation and a greater number of defect zone disappeared from the group TP (Fig. 5B), and more new bone formation and less residual biomaterials were seen in the defect area from group LATP and HATP (Fig. 5C, D). Compared with treatment by TP or LATP, defect sites exhibited more bone formation from the defect margin to the center and the specimens showed more advanced stages of remodeling and consolidation in the group HATP (Fig. 5D).

The quantitative results were shown as the percentage of remaining biomaterials and bone fill in the defects (Fig. 5E, F). At 8 weeks, the percentages of bone fill from groups TP, LATP and HATP significantly increased by 2.13-, 2.67- and 3.34-fold at 8 weeks ( $P < 0.05$ ), respectively, compared with group CON (Fig. 5E); compared with group TP, combination therapy with lower and higher ASP and  $\beta$ -TCP/PLGA decreased the percentage of remaining biomaterials from



**Fig. 4** Biomechanical results expressed as ultimate load force. Data were expressed as mean  $\pm$  SD; error bars in the figure are presented as SD,  $N=5$  specimens/group. \* $P < 0.05$  versus CON group, # $P < 0.05$  versus TP group, & $P < 0.05$  versus LATP group (by one-way ANOVA and Tukey's post hoc test)

groups LATP and HATP by 2.04- and 2.82-fold, respectively ( $P < 0.05$ ) (Fig. 5F).

Fluorochrome double labelling was performed to evaluate the effects of ASP on the mineral apposition rate (MAR; Fig. 5a, b) of the defect area. As shown in Fig. 5G, HATP-treated rats had a significantly higher MAR in contrast to the other treatment groups ( $P < 0.05$ ).

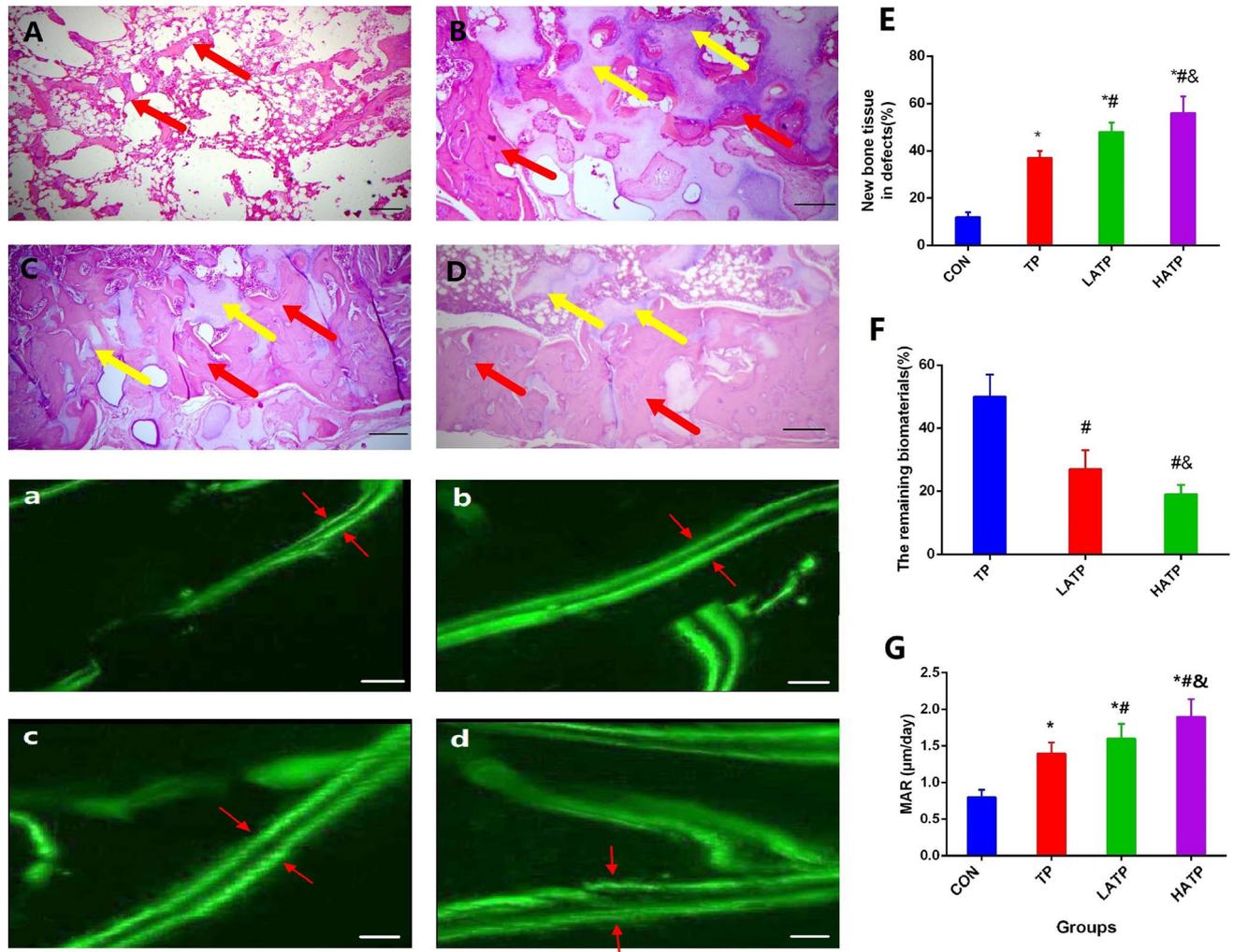
## Expression of osteogenic and angiogenic markers by $\beta$ -TCP/PLGA and ASP

We isolated total RNA from the scaffolds inserted in defects 8 weeks after surgery, and measured the mRNA levels of Runx2, OCN, OPN, BMP-2, VEGF and TRAP (Fig. 6a–f). The levels of Runx2, OCN, OPN, BMP-2 and VEGF were significantly upregulated in the TP, LATP and HATP group compared with the CON group ( $P < 0.05$ ). Of these genes, BMP-2 mRNA was upregulated the most, showing an approximate 2.4 fold increase as a result of the delivery of  $\beta$ -TCP/PLGA and ASP ( $P < 0.05$ , Fig. 6d). We also examined the expression levels of angiogenesis and degradation-related genes, such as VEGF using the same RNA samples. The levels of VEGF were significantly higher in the HATP group than in the CON group (Fig. 6e); and the levels of TRAP were significantly lower in the TP, LATP and HATP groups than in the CON group ( $P < 0.05$ , Fig. 6f).

## Discussion

The findings of this study showed that the combination therapy with local single-dose ASP and PLGA/ $\beta$ -TCP in an ovariectomized (OVX) rat model of bone loss following establishment of a bone defect model at the femoral metaphysis surgery increased bone healing. Additionally, the lower and higher dose of ASP regimens increased the bone regeneration in the defect area.

In the clinic, primary osteoporosis can be the result of hormone deficiencies in postmenopausal women, caused by many factors or it could be classified as idiopathic in nature [25]. A marked increase in stochastic remodeling at menopause causes bone loss, severe deterioration of bone microarchitecture, and reduction in bone quality. There is a wealth of evidence from basic science studies showing that these problems resulted largely from the deficiency of ovarian hormones, especially estradiol ( $E_2$ ) [26]. Varieties of OVX animals have been employed to investigate OP. In rats, bone loss occurs some days after OVX at different bone sites in both cancellous and cortical bone [27–29]. OVX causes enlarged bone marrow cavity, showing a similar pathological regression of bone in postmenopausal women. Therefore, the OVX rat has been regarded as a canonical protocol to create the OP model in animals. Femoral metaphysis was chosen as



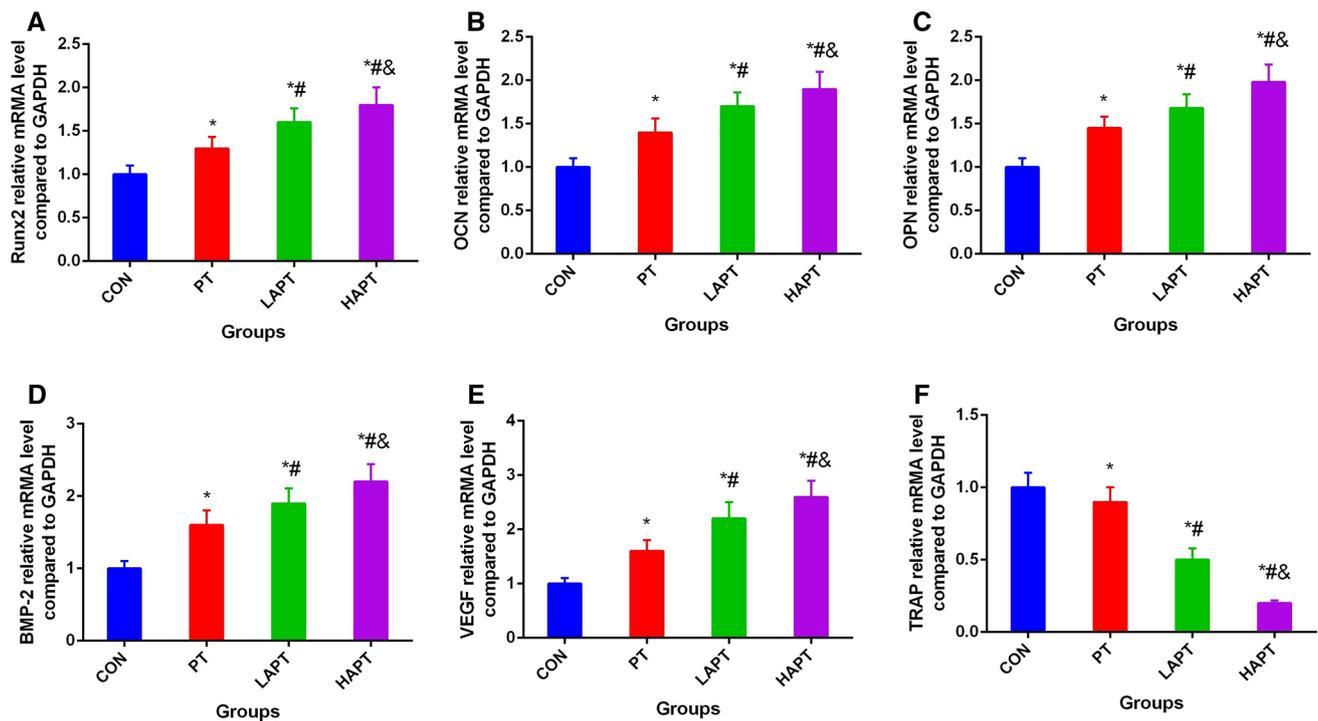
**Fig. 5** A–D HE staining revealed new bone matrix deposition within defects in the above mentioned groups; a–d double calcein green labelling was performed in the femurs bone section of the treatment groups (magnification,  $\times 200$ ); **E** result of the remaining percentage of biomaterials by HE examination; **F** result of bone fill in the defects by HE examination; **G** quantitative analysis of mineral apposition

rate (MAR). **A, a** Group CON, **B, b** group TP, **C, c** group LATP and **D, d** group HATP; red arrow: new bone or mineralized zone, yellow arrow: biomaterials, bar 200  $\mu\text{m}$ ; data were expressed as mean  $\pm$  SD; error bars in the figure are presented as SD,  $N=5$  specimens/group. \* $P<0.05$  versus CON group, # $P<0.05$  versus TP group, & $P<0.05$  versus HATP group (by one-way ANOVA and Tukey's post hoc test)

the implant site because in OVX rats, cancellous bone mass in the metaphyseal region of long bones is rapidly reduced and it is large enough in rats to allow implantation, which indicated that this was a suitable condition as a model of osteoporosis [30].

The treatment of the OVX rats with PLGA/ $\beta$ -TCP with ASP increased the bone mass in the defected site when compared with the untreated group and PT group and significantly increased the bone strength. In the present work, we endeavored to develop a sound  $\beta$ -TCP bone substitute system by incorporating ASP and PLGA into CPC to enhance bone tissue growth. Bone regeneration has been achieved via osteoinductive growth factors, osteogenic cells, and scaffolds or their combination. Currently, the

field is trending toward attempts to create a biomaterial with conductive or inductive properties for bone formation to approach or even exceed the growth rate of autogenous bone [31]. Recently, some inductive factors were added into the grafts to reconstruct lost bone [32, 33]. Although growth factors, such as bone morphogenic protein-2 (BMP-2) and bone morphogenic protein-7 (BMP-7), have been the primary choices as osteoinductive agents for bone repair [17, 34], the high cost and limited active period largely restrict a wider clinical application of growth factors. In this study we chose ASP as a bone-inducing drug because these drugs are inexpensive and safe, as well as shows osteoinductive functions. PLGA/ $\beta$ -TCP with ASP 50  $\mu\text{g}$  or PLGA/ $\beta$ -TCP with ASP 100  $\mu\text{g}$  obtained more



**Fig. 6** mRNA expression of Runx2 (a), OCN (b), OPN (c), BMP-2 (d), VEGF (e) and TRAP (f) within the defect margin. Rats were sacrificed 8 weeks after surgery and total RNA extracted from the defect sites of each animal was subjected to real time RT-PCR. Data were

expressed as mean  $\pm$  SD; error bars in the figure are presented as SD,  $N=5$  specimens/group. \* $P<0.05$  versus CON group, # $P<0.05$  versus TP group, & $P<0.05$  versus HATP group (by one-way ANOVA and Tukey's post hoc test)

than in those treated with PLGA/ $\beta$ -TCP at an experimental period. Bone regeneration by implanted PLGA/ $\beta$ -TCP has been shown to originate from the defect margin and around implanted PLGA/ $\beta$ -TCP if it has been implanted into a critical-sized distal femoral defect.

Local use of ASP had an additive effect on bone formation at the defected area in an OVX rat model. Why did the local ASP treatment show a stronger effect on bone strength preservation and bone formation on the implant PLGA/ $\beta$ -TCP? The most plausible explanation may be that ASP could alter local bone formation in osteoporosis subjects. Currently, several lines of evidence have suggested that aspirin could favor bone regeneration through inhibition of osteoclastogenesis and improvement in angiogenesis and osteogenesis by suppression of expression of pro-inflammatory cytokines, inhibition of activity of cyclooxygenase 1 and 2, and prostaglandin-E<sub>2</sub>, and up-regulation of telomerase activity in bone marrow mesenchymal stem cells [35, 36]. Moreover, ASP can intervene the development of osteoporosis by inhibiting the apoptotic effect of T lymphocytes on bone marrow-derived mesenchymal cells, promoting osteogenic differentiation and inhibiting osteoclast differentiation acting on the Fas/FasL signaling pathway [37], as well as altering the expression of matrix metalloproteinase-1 (MMP-1) and interleukin (IL)-6 [38].

In the present study, we also found that PLGA/ $\beta$ -TCP with ASP promote bone formation by regulating the transcription of genes involved in bone remodeling, and had a greater effect on defected area restoration. Osteogenesis during bone healing and remodeling is coupled with angiogenesis. Conversely, blood vessels are thought to influence the osteogenic generation of new bone [39]. In the mammalian skeletal system, growth of the vascular network is regulated by signals provided by chondrocytes and other bone cells, among which the vascular endothelial growth factor (VEGF) is best understood [40]. Osteoblast differentiation and mineralized bone formation are tightly controlled by the expression of osteogenic transcription factors. Runx2 binds to the promoter regions of all osteoblast-specific genes and controls their expressions as a master regulator for osteoblast differentiation [25]. ALP is the abundant matrix protein expressed in the early stages of osteoblastic differentiation and their expressions are followed by OCN and OPN [25]. TRAP as a bone resorption marker enzyme, released by osteoclasts, well reflects the osteoclast activity directly along with bone resorption status in vivo. In this study, the levels of Runx2, OCN, OPN, BMP-2 and VEGF were significantly upregulated, but the levels of TRAP were significantly downregulated, which suggested that the local environment is more conducive to bone formation. Our

current data support the conclusion that the mRNA expression of these markers was coupled with new bone formation in the defect region. Our results also indicate that PLGA/ $\beta$ -TCP with ASP-mediated acceleration of new bone formation in rat distal femoral defects correlated with osteogenic gene induction. In other words, PLGA/ $\beta$ -TCP with ASP may synergistically promote osteoblast activity, inhibits osteoclastic activity, and increases blood vessel formation.

First of all, the number of animals is limited due to the pre-clinical pilot study design. In addition, we only studied for a short period of time and did not observe the situation after 8 weeks. Secondly, we only referred and applied two doses of ASP to observe the effect of improving bone integration. We did not observe a gradient dose, and did not know the most appropriate dose. Therefore, the effect of different doses and the best dose will be further observed at a later stage. The most important thing is that we have not designed ASP and PLGA/ $\beta$ -TCP in vitro cell research and an older animal model which may be suitable for representing elderly osteoporotic bone defects, and thus it is necessary to further study it.

In summary, our findings regarding the effect on new bone formation in the critical-sized osseous defects, indicate that using a single-dose local administration of aspirin together with  $\beta$ -TCP/PLGA composite would be promising as a method of achieving rapid and bone regeneration of osseous defects. It is also revealed that bone regeneration by PLGA/ $\beta$ -TCP/ASP occurs in a dose-dependent manner because notable significant differences were observed in bone regenerative properties between PLGA/ $\beta$ -TCP with 100  $\mu$ g ASP and PLGA/ $\beta$ -TCP with 50  $\mu$ g ASP.

## Compliance with Ethical Standards

**Conflict of interest** All authors have no conflicts of interest.

**Ethical approval** This study did not involve human participants.

**Informed consent** This study does not involve human participants and therefore does not require informed consent.

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