Endocrine (2019) 63:376–384
https://doi.org/10.1007/s12020-018-1761-7

ORIGINAL ARTICLE

Effects of combined menaquinone-4 and $PTH_{1–34}$ treatment on osetogenesis and angiogenesis in calvarial defect in osteopenic rats

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Received: 10 June 2018 / Accepted: 11 September 2018 / Published online: 22 September 2018

Abstract

Purpose The aim of this study was to evaluate the effect of combining human parathyroid hormone (1–34) ($PTH_{1–34}$; PTH) and menaquinone-4 (MK-4) on calvarial bone defect repair in osteopenic rats.

Methods Fourteen week olds were subject to craniotomy for the establishment of osteopenic animal models fed through a chronically low-protein diet. After that, critical calvarial defect model was established and all rats were randomly divided into four groups: sham, MK-4, PTH, and PTH + MK-4. The animals received MK-4 (30 mg/kg/day), $PTH_{1–34}$ (60 μg/kg, three times a week), or $PTH_{1–34}$ (60 μg/kg, three times a week) plus MK-4 (30 mg/kg/day) for 8 weeks, respectively. Serum γ-carboxylated osteocalcin (Gla-OC) levels, histological and immunofluorescent labeling were employed to evaluate the bone formation and mineralization in calvarial bone defect. In addition, Microfil perfusion, immunohistochemical, and micro-CT suggested enhanced angiogenesis and bone formation in calvarial bone healing.

Results In this study, treatment with either $PTH_{1–34}$ or MK-4 promoted bone formation and vascular formation in calvarial bone defects compared with the sham group. In addition, combined treatment of $PTH_{1–34}$ plus MK-4 increased serum level of Gla-OC, improved vascular number and vascular density, and enhanced bone formation in calvarial bone defect in osteopenic conditions as compared with monotherapy.

Conclusions In summary, this study indicated that $PTH_{1–34}$ plus MK-4 combination therapy accelerated bone formation and angiogenesis in calvarial bone defects in presence of osteopenia.

Keywords PTH · Menaquinone-4 · Osteogenesis · Angiogenesis · Osteopenic

Introduction

The aging population, along with chronic medical conditions caused by osteoporosis, is a serious problem in today's society. Dietary protein reduction and undernutrition may lead to age-related physiological changes, physical impairment, diseases of the gastrointestinal tract, as well as psychosocial issues [1]. Persistent malnutrition and protein deficiency in the elderly showed deleterious effects on the musculoskeletal system causing decreased bone mineral density [2], leading to increased risk of fractures. The pathologic state of osteopenic is caused by excessive bone resorption as compared with bone formation, diminishing the ability of bone healing from a fracture or osseous defect [3]. Meanwhile, with osteopenic being a global problem, it is urgent for the development of effective methods to improve the ability of bone defect repair in osteopenic conditions.

The human parathyroid hormone (1–34) ($PTH_{1–34)}$ is an anabolic therapy for osteoporosis [4]. Previous study suggested that intermittent administration of PTH stimulated bone formation and increased bone mineral density malnourished rats [5]. Osteoblast surface, osteoid surface, and osteoid volume also increased significantly after intermittent PTH treatment in animals [6, 7]. Moreover, PTH analogs showed positive effect on new bone formation and improved implant fixation in osteopenic or osteoporotic rats

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[8–10]. Several studies suggested that PTH accelerates bone formation by facilitating neovascularization [11–13].

Menaquinones (MKs), which are also known as Vitamin K2, are essential for the γ-carboxylation of osteocalcin (GLA-OC), known to play an important part in bone mineralization [14]. Menaquinone-4 (MK-4) is a specific isoform of menaquinones with four isoprenoid units, previously demonstrated to have a positive effect on human amniotic fluid mesenchymal stem cells (hAFMSCs) on osteogenic differentiation [15]. Previous studies suggested that MK-4 targeted pregnane X receptor (PXR) to stimulate osteoblast differentiation and facilitate bone formation [16–18]. In addition, menaquinones improved angiogenesis and the blood supply in treatment of glucocorticoid-induced osteonecrosis of the femoral head [19].

Elderly patients with osteopenia, often attributable to long-term lack of sufficient dietary protein, are frequently seen in our clinics. Our previous study demonstrated that combination of MK-4 and $PTH_{1–34}$ significantly improved bone strength and increased implant osseointegration around HA-coated implants in osteoporotic rats. However, so far there has been no report on whether combined MK-4 and $PTH_{1–34}$ improved bone formation and angiogenesis in prolonged low protein-induced osteopenic model. In this study, treatment of calvarial bone defects in prolonged low protein-induced osteopenic rat model was used to investigate the effects of combined MK-4 and $PTH_{1–34}$ on bone formation and angiogenesis.

Materials and methods

Experimental animals

Seventy-eight 10-month-old female Sprague–Dawley rats were incorporated in this study. Rats were housed in SPF environment at 12/12 h light/dark cycle. All animals were strictly pair-fed a laboratory diet provided by Provimi Kliba AG (Kaiseraugst, Switzerland) containing 2.5% casein, 0.8% phosphorus, 1% calcium, 70–80% carbohydrates, and 5% fat throughout the experimental period according to previous study [10]. All rats were ensured isocaloric intake through the addition of corn carbohydrate to the low-protein diet, which provided the same energy intake across all groups. All rats had free access to de-mineralized water. All the procedures were carried out according to the Institutional Animal Care and Committee Guide of the Animal Research Committee of the Wenzhou Medical University.

Surgery and treatment

All rats were fed with low protein diet for 14 weeks before craniotomy for the establishment of osteopenic animal model [10]. Following successful establishment of osteopenia, the rats were anesthetized with 50 mg/kg pentobarbital sodium and induced with a unilateral 4 mm calvarial defect. Briefly, following exposed the calvarial bone, 4 mm diameter full-thickness bone defects were created on unilateral parietal bone with trephine under saline perfusion. The incision was closed with sutures after sterilization. After craniotomy, all rats were divided into four groups: sham, MK-4 (menaquinone-4, 30 mg/kg every day; menatetrenone; Eisai Co., Ltd., Tokyo, Japan), PTH (recombinant human $PTH_{1–34}$, 60 μg/kg, three times a week; Forteo®; Eli Lilly, Ltd., Kobe, Japan), PTH + MK-4 group (MK-4, 30 mg/kg every day plus $PTH_{1–34}$, 60 μg/kg three times a week). The doses of PTH and MK-4 were determined according to previous experiments which showed positive effects in osteoporosis models [20].

Serum analysis

Eight weeks after drug administration, the serum of Gla-OC (γ-carboxylated osteocalcin) and Glu-OC (non-γ-carboxylated osteocalcin) was measured with an enzyme-linked immunosorbent assay (Rat Gla-Osteocalcin High Sensitive EIA and Rat Glu-Osteocalcin High Sensitive EIA kits; Takara Bio Inc., Shiga, Japan). The serum of CTX-I (C-terminal telopeptide of type I collagen) was measured using an enzyme-linked immunosorbent assay (Rat Laps EIA; Immunodiagnostic Systems Ltd., Boldon, UK).

Micro-CT analysis

At 8 weeks after drug administration, the calvarium of osteopenic rats was fixed in 4% formaldehyde for 48 h at 4 °C and scanned on micro-CT system (energy 70 kVp, 114 μA, integration time 300 ms, threshold 220, Skyscan 1173; Skyscan, Kontich, Belgium) and reconstructed with three-dimensional images. The volume of newly regenerated bone within the calvarial bone defect and the percentage of bone healing were quantified by Micro-CT analysis.

Histological and immunohistochemical analysis

After Micro-CT scanning, calvarial sample was decalcified in 10% EDTA for 1 month and changed three times per week before being embedded in paraffin. Subsequently, longitudinal sections of 5.0 μm were subjected to hematoxylin and eosin (H & E) staining according to manufacturer's instructions. IHC staining was used to detect the runt-related transcription factor 2 (RUNX2), osteoclacin (OCN), vascular endothelial growth factor (VEGF), Platelet endothelial cell adhesion molecule-1 (CD31) to evaluate osteogenesis and angiogenesis in calvarial defects. The staining was quantified by ImageJ software and the positive

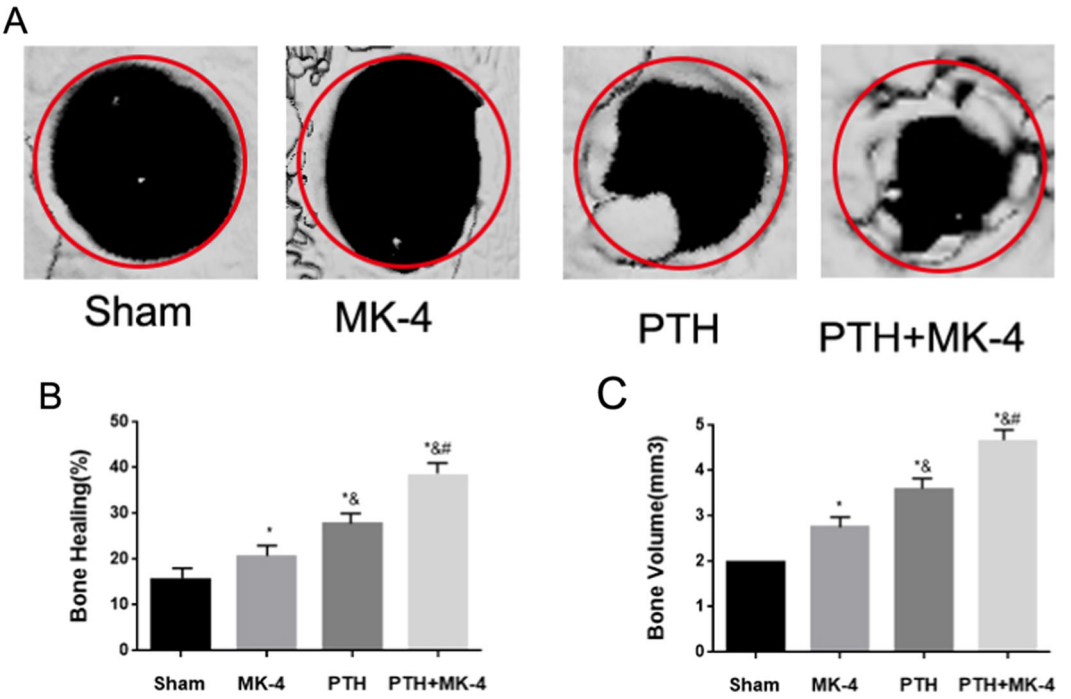

Fig. 1 Three-dimensional reconstructed of calvarial defect calvarial defect at 8 weeks after craniotomy **a**. Quanlity analysis of bone healing **b** and volume of new bone **c** within defect. Data are expressed as mean ± SD, $*P < 0.05$ vs group sham, & $P < 0.05$ vs group MK-4, # $P < 0.05$ vs group PTH, $n = 6$/group

staining was expressed as percentage of the positive area according to previous study [15].

Microfil perfusion

To visualize blood vessels and evaluate angiogenesis, five rats from each group received Microfil perfusion (Flow-tech, Carver, MA, USA) at 8 weeks after craniotomy. Briefly, A 1.5 mm incision was made on the left ventricle and an infusion tube was imbedded into the ascending aorta. Following incision of the inferior vena cava, 200 mL of heparinized saline and 100 ml of 10% formalin were flushed to remove blood and to immobilize blood vessels. Afterwards, 20 ml of Microfil compound were perfused at a rate of 2 ml/min. Finally, the specimens were carefully stored at 4 °C overnight. Tissues were collected and fixed in 4% paraformaldehyde for 48 h and evaluated by Micro-CT.

Sequential fluorescent labeling

Sequential fluorescent labelling of new bone mineralization was performed according to previous reports [21]. At 4 weeks and 6 weeks after drug treatment, the rats were injected with alizarin red (AL; 30 mg/kg, Sigma, USA) and calcein (CA; 15 mg/kg, Sigma, USA) respectively. Mineralized tissues were detected through sequential fluorescent labeling.

Statistical analysis

All measurements were expressed as the mean ± SD and analysis by SPSS22.0 software (SPSS 22.0, Chicago, IL, USA). Significant differences among groups were decided using ANOVA and post-analysis by Tukey's honestly significant difference test. $P < 0.05$ was employed to indicate statistical significance.

Results

Combined treatment of PTH_{1-34} and MK-4 enhanced bone healing in osteopenic calvarial defect

The bone healing was observed by micro-CT at 8 weeks after surgery (Fig. 1). The sham group showed finite bone formation area was 15.43 ± 3.15% at 8 weeks (Fig. 1b). On the contrary, more bone formations were observed in the MK-4 group and the PTH group, and this percentage of bone regeneration were greater in the PTH group (28.75 ± 3.91%) compared with the MK-4 group (20.57 ± 3.64%) at the same time point (Fig. 1b) ($P < 0.05$). The PTH + MK-4 group showed an even higher percentage of new bone formation than the other groups (38.25 ± 4.25%) ($P < 0.05$). The volume of new bone formation showed the same trend as bone formation in each group (Fig. 1c), which suggested that combined therapy increased bone healing.

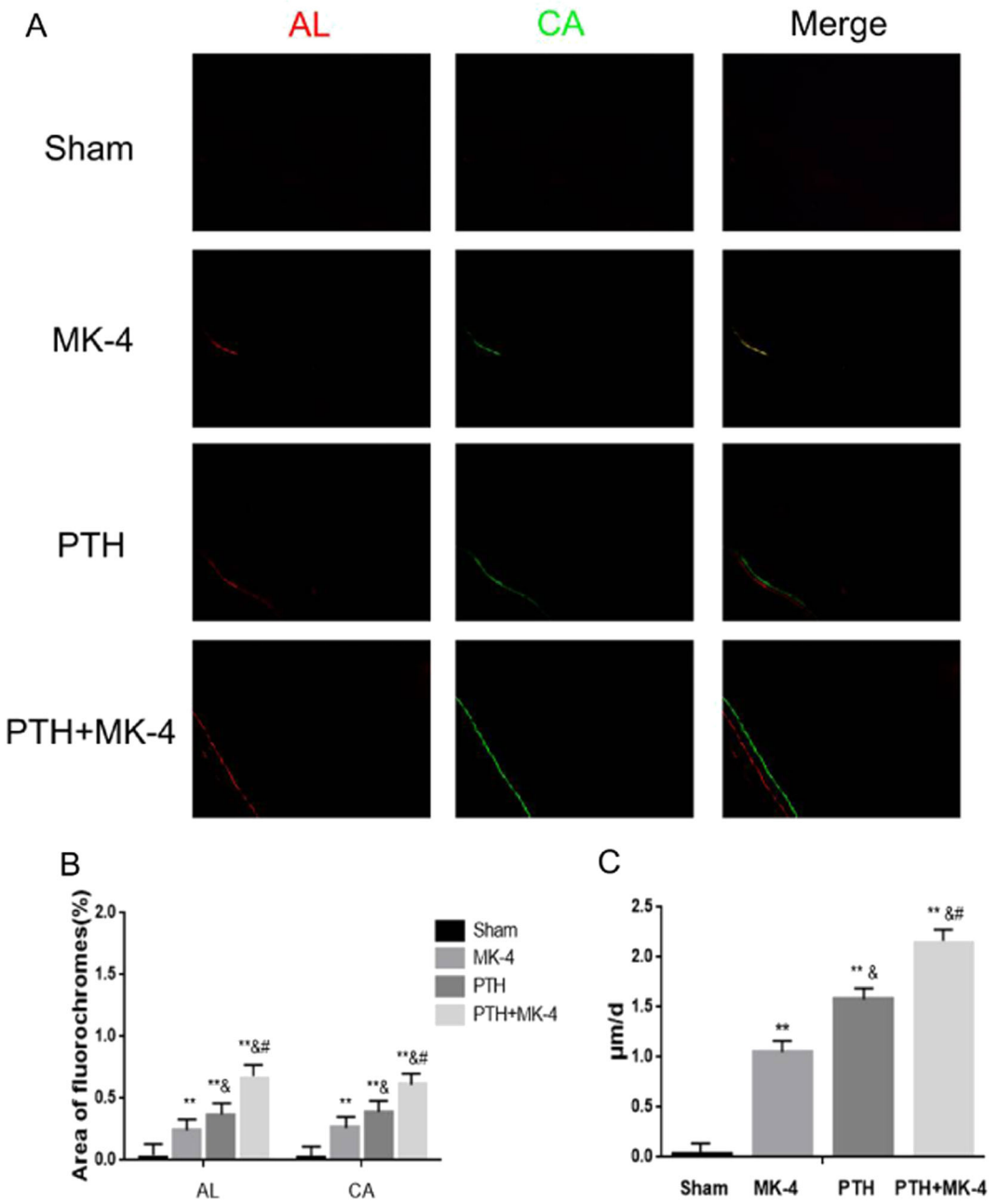

Fig. 2 New bone mineralization were determined histomorphometrically by fluorochrome-labelling analysis. The images in red (AL) represents new bone mineralization labeled by alizarin red injection at weeks 4 and green (CA) represented new mineralization labeled by calcein injection at weeks 6 after drug treatment, respectively. Merged images of the two fluorochromes for the same group **a**. The percentages of AL and CA fluorochrome area for different group **b**. The mineral apposition rate (MAR) for each group **c**. Data are expressed as mean ± SD, & $P < 0.05$ vs group MK-4, # $P < 0.05$ vs group PTH, ** $P < 0.01$ vs group sham, $n = 6$/group

Combined treatment enhanced bone mineralization

The dynamic bone formation and mineralization at the site of calvarial defect were labeled by alizarin red (AL) and calcein (CA) at weeks 4 and weeks 6 after drug treatment (Fig. 2a). At 4 weeks the percentages of AL labeling in the PTH + MK-4 group (0.643 ± 0.206%) was higher than that in the PTH group (0.346 ± 0.125%) or the MK-4 group (0.226 ± 0.115%) ($P < 0.05$) (Fig. 2b). Meanwhile, the mineralization in the PTH group is stronger than the MK-4 group ($P < 0.05$). The same trend was observed in CA labeling, (Fig. 2b) showing that the PTH + MK-4 group (0.598 ± 0.193%) was higher than that in the PTH group (0.367 ± 0.135%) or the MK-4 group (0.258 ± 0.115%) ($P < 0.05$). The maximum distance strip between CA and AL was observed in the PTH + MK-4 group (2.12 ± 0.67 μm/d)

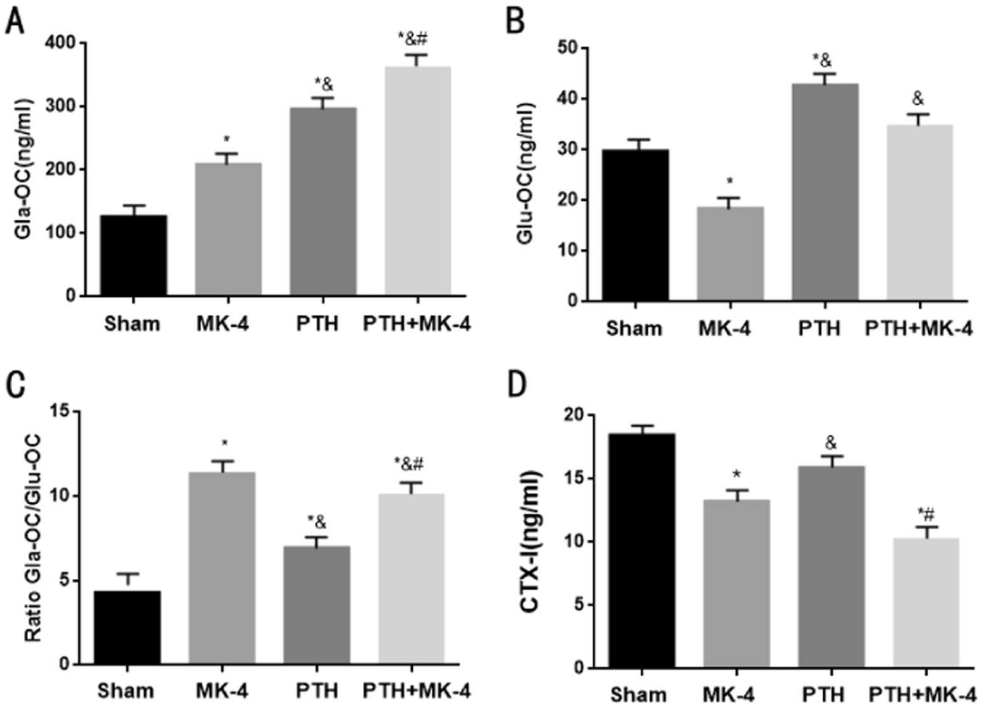

Fig. 3 Serum level of each group at 8 weeks after drug treatment. Serum levels of Gla-OC **a**, Glu-OC **b**, ratio between Gla-OC and Glu-OC **c**, CTX-I **d** in group sham, group MK-4 (menaquinone-4), group PTH (PTH 1–34) and group PTH + MK-4 (menaquinone-4 combined with PTH 1–34). Data are expressed as mean ± SD. *$P < 0.05$ vs group sham, & $P < 0.05$ vs group MK-4, # $P < 0.05$ vs group PTH, $n = 6$/group

than other group ($P < 0.05$) (Fig. 2c), which suggested that the mineralization rate of combined treatment of PTH and MK-4 was higher than other groups.

Biomarker assays

Bone metabolism level was shown in Fig. 3. The level of Gla-OC increased slightly in the MK-4 group (208.95 ± 21.98 ng/ml) while increased significantly in the PTH group (296.75 ± 25.34 ng/ml) compared with the sham group (126.23 ± 16.95 ng/ml) ($P < 0.05$) (Fig. 3a). Meanwhile, the level of Gla-OC was even higher in the PTH + MK-4 group (361.25 ± 30.45 ng/ml) compared with the MK-4 group and the PTH group ($P < 0.05$). Moreover, MK-4 treatment (18.43 ± 3.52 ng/ml) significantly decreased the level of Glu-OC ($P < 0.05$) and PTH treatment (34.66 ± 8.59 ng/ml) increased the level of Glu-OC ($P < 0.05$) as compared with the sham group (29.74 ± 6.75 ng/ml) (Fig. 3b). However, PTH + MK-4 treatment showed no significant difference compare to the sham group ($P > 0.05$). What's more, the ratio between Gla-OC and Glu-OC was increased in the PTH group compared with the sham group, and the highest ratio was observed in the MK-4 group. Meanwhile, this ratio was decreased in the PTH + MK-4 group but still higher compared with the sham group ($P < 0.5$) (Fig. 3c). In addition, levels of CTX-I in the MK-4 group (13.24 ± 3.13 ng/ml) decreased significantly compared to sham group (18.45 ± 3.95 ng/ml) ($P < 0.05$) (Fig. 3d). Meanwhile, CTX I level showed no significant difference in the PTH + MK-4 group (10.23 ± 2.95 ng/ml) compared with the MK-4 group ($P > 0.05$) but decreased significantly compared with sham group ($P < 0.05$).

Evaluation of vascular network within calvarial defect

The vascular network of the calvarial defect was outlined by Microfil perfusion while assessed with micro-CT scanning at 8 weeks (Fig. 4). Three-dimensional images showed infrequent vessels being detected in the sham group. More capillaries were observed in the MK-4 group and the PTH group compared to the sham group. Meanwhile, vascular network showed no significant difference in the PTH group and the MK-4 group. Quantitative analysis reveals highest vessel volume (1.98 ± 0.89 mm) and vessel number (0.184 ± 0.067/mm) but lowest vessel separation (1.21 ± 0.28 mm)in the PTH + MK-4 group compared to other groups ($P < 0.05$) (Fig. 4d). No significant difference were observed between the PTH group and the MK-4 group in vessel volume, vessel number, and vessel separation ($P > 0.05$), but improved significantly compared with the sham group ($P < 0.05$) (Fig. 4d). The analysis of vascular networks indicated that drug administration improved angiogenesis in defects.

HE staining and immunohistochemistry

The changes in bone defect repair are also confirmed by histological analysis at 8 weeks (Fig. 5). No inflammatory cells were detected within each defect region. H&E staining

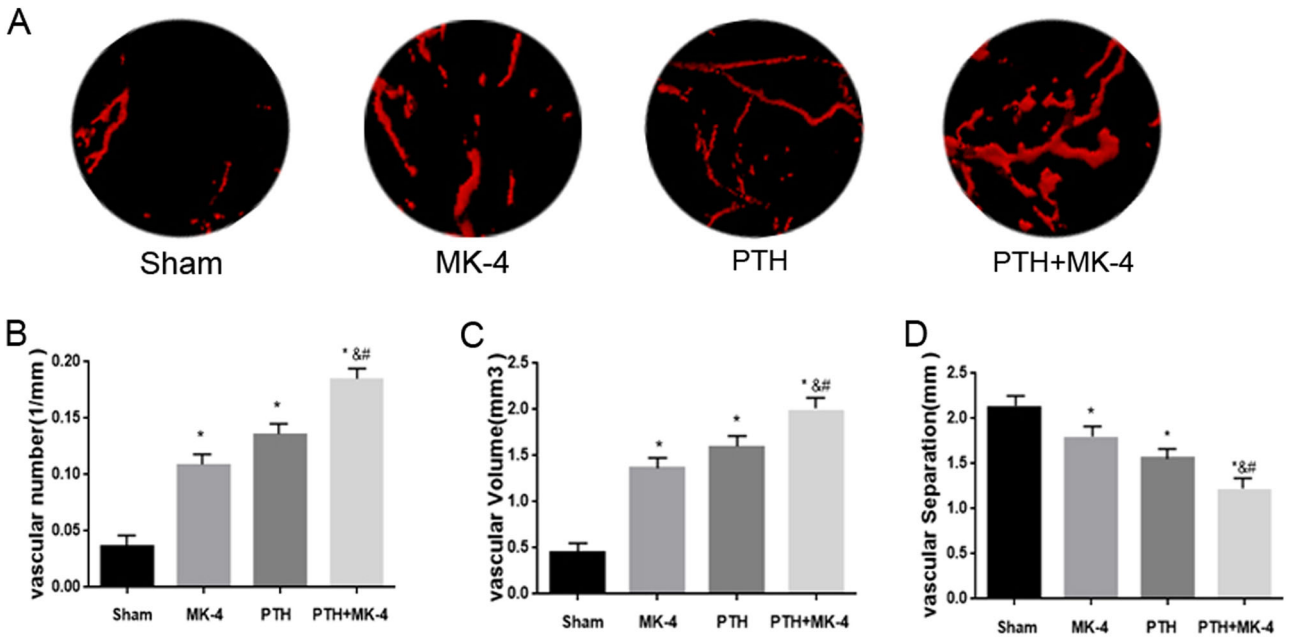

Fig. 4 New blood vessel growth within the calvarial defect visualized with MicroCT imaging at 8 weeks after craniotomy **a**. Analysis of vascular number **b**, vascular volume **c**, and vascular separation **d** in calvarial defect. Data are expressed as mean ± SD, *$P < 0.05$ vs group sham, & $P < 0.05$ vs group MK-4, # $P < 0.05$ vs group PTH, $n = 5$/group

proved that bone regeneration was observed in calvarial defects. At 8 weeks after treatment, little bone formation was observed in the sham group and fibrous-like tissues covered most of the defect areas (Fig. 5). The PTH group and the MK-4 group showed more new bone formation surrounding defect origination than the sham group (Fig. 5). The PTH + MK-4 group revealed the largest bone formation than any other groups (Fig. 5) and extended from the edge of the defect, suggesting combined treatment of MK-4 and PTH1-34 improved bone formation.

The immunohistochemistry staining for angiogenic markers VEGF and CD31 showed that the most obvious positive staining being detected in the PTH + MK-4 group ($P < 0.05$) (Fig. 5). Positive brown staining also observed in the MK-4 group and the PTH group. However, nearly no positive staining for VEGF or CD31 could be detected in the sham group (Fig. 5). Similar tendency was observed in osteogenic maker RUNX2 and OCN. The RUNX2 and OCN were highly expressed in the PTH + MK-4 group compared with the PTH group and the MK-4 group, and barely observed in the sham group ($P < 0.05$) (Fig. 5d, e). This result supported that combination therapy accelerated osteogenesis and angiogenesis in calvarial defects with osteopenic.

Discussion

In this study, we evaluated the effect of combined treatment with MK-4 and PTH on bone formation and angiogenesis in calvarial defects in osteopenic rats chronically fed a low-protein diet. The results of the this study suggested that although MK-4 or PTH alone improved bone healing, combined application of systemic MK-4 and PTH showed stronger effects than monotherapy in osteopenic rats, evidenced by improvements on bone formation and angiogenesis as well as biomechanical parameters.

Osteopenic rats, induced by a chronic low protein diet, were restricted to a 2.5% casein diet [22]. Calvarial defect was selected due to its poor blood supply, leading to high reproducibility in studying the effects of drugs and materials on bone formation and angiogenesis [23].

PTH is an anabolic therapy recommended by the FDA to treat osteoporosis at present [4]. Our results suggested that PTH can increase Gla-OC and Glu-OC in sera, indicating that PTH enhances the synthesis of OC via the stimulation of osteoblasts [24]. In the present study, PTH treatment significantly increased bone formation in the calvarial defect compared to the sham group. This result was in line with previous studies that intermittent administration of parathyroid hormone increased both endochondral and intramembranous bone healing through Wnt signaling and PTHrP/Ihh feedback loop [25]. In addition, PTH reversed the adverse effects of long-term protein undernutrition on bone microarchitecture [1]. This maybe a result of PTH accelerating differentiation of osteoprogenitor cells into osteoblasts as well as inhibition of osteoblast apoptosis [26]. What's more, fracture healing is thought to be closely related to angiogenesis [27, 28]. The relationship between PTHrP and VEGF was found to regulate vascularization

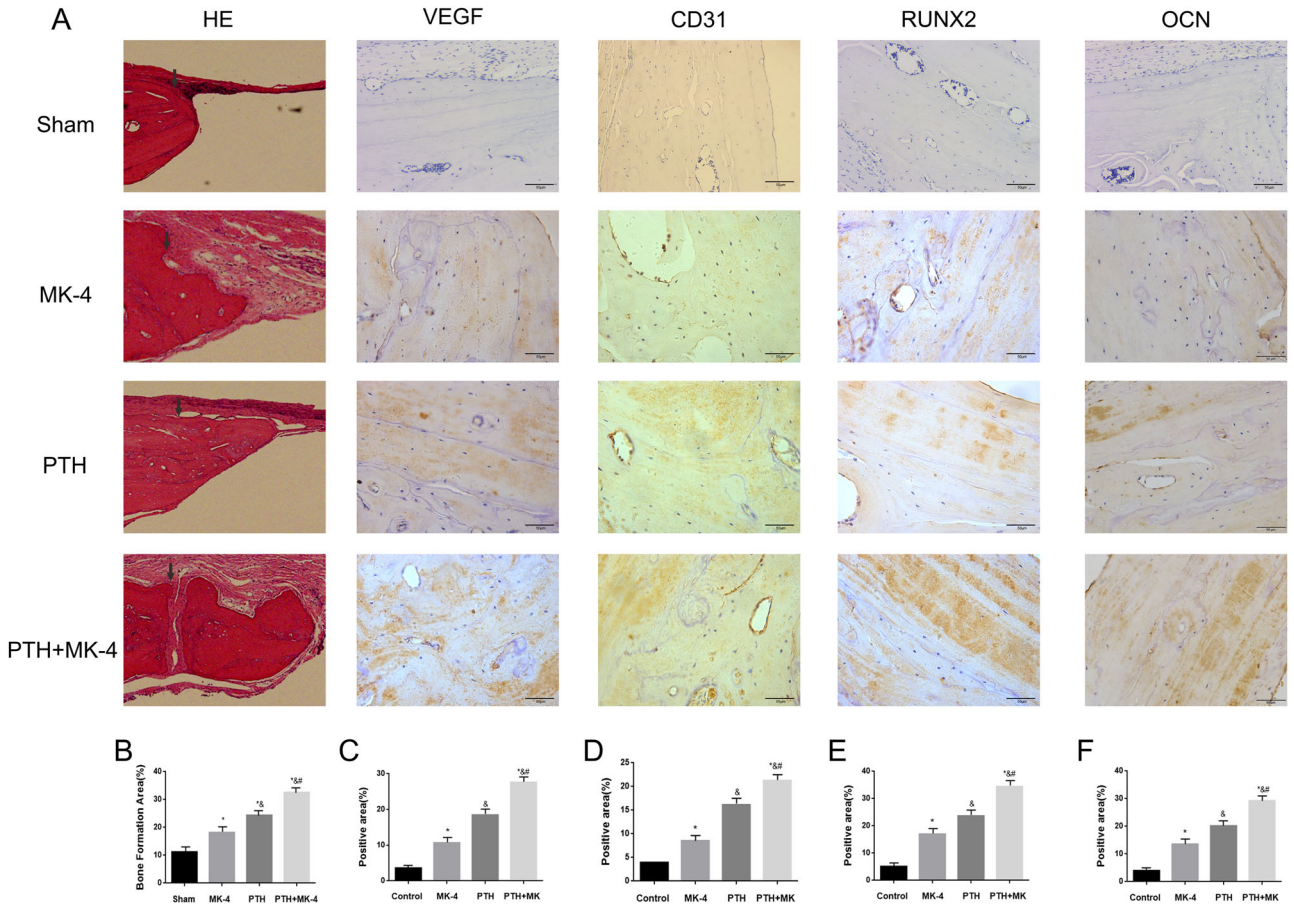

Fig. 5 HE staining and immunohistochemical assay for VEGF, CD31, RUNX2, and OCN expression in each group to observe angiogenesis and osteogenesis in calvarial defect **a**. The brown color represents positive staining. Quantity analysis of bone formation in HE staining **b**, positive area of VEGF **c**, CD31 **d**, RUNX2 **e**, OCN **f**, $*P < 0.05$ vs group sham, & $P < 0.05$ vs group MK-4, # $P < 0.05$ vs group PTH. $n = 6$/group

and bone formation through the C terminal of PTHrP in human osteoblasts and MG-63 cells [11]. Increasing PTH levels could also promote the transition of arterial endothelial cells to chondrocytes [29]. Our results suggested that $PTH_{1–34}$ promoted bone repair in osteopenic condition by accelerating osteogenesis and vascular formation [11].

Menaquinones (MK), an essential compound for γ-carboxylation of OC, converted the glutamic acid (Glu) residue in Gla protein to γ-carboxyglutamic acid (Gla) [30–32]. Previous studies suggested that MKs not only increased bone strength and mineral density but also reduced the risk of fracture in osteoporotic conditions [33, 34]. MK-4 is a short chain isoform of MKs with four isoprenoid units and is the most plentiful form of this vitamin in the human body [35]. Mandatori et al. demonstrated for the first time the positive effects of MK-4 on stem cell osteogenic functions through the GGCX-dependent pathway in 3D dynamic systems. The results of our study indicated that MK-4 significantly decreased Glu-OC but increased Gla-OC level in sera. These results are consistent with previous studies showing that individuals treated with MK-4 consumed Glu-OC and increased Gla-OC in sera [36]. This phenomenon can possibly be attributable to MKs enhancing bone formation as well as suppressing bone resorption through accelerating the production of γ-carboxylate osteocalcin [37, 38]. What's more, our study suggested that MK-4 not only influenced bone metabolism but also improve angiogenesis. However, the effects of MKs on angiogenesis are controversial. Hitoshi suggested that EC proliferation is restrained by MKs in a dose-dependent manner [39]. On the other hand, Zhang demonstrated that MKs showed protective effect on glucocorticoid-induced osteonecrosis through regulating angiogenesis, and MKs promoted endothelial cell migration as well as in vitro tube formation [19]. Vitamin K-dependent protein S has also been shown to be essential for vascular development and homeostasis [40]. The results of these studies suggested that MK-4 accelerated bone formation through promotion of Gla-OC in sera as well as improvements of vascular formation and vascular microenvironment in osteopenic conditions [27].

The serum ratio between Gla-OC and Glu-OC is a marker of bone turnover as well as vitamin K status [41]. In

this study, biomarker assays suggested monotherapy with MK-4 or PTH increased the ratio between Gla-OC and Glu-OC compared with the sham group, with the highest ratio being observed in the MK-4 group. Interestingly, this ratio was not as prominent in the the PTH + MK-4 group, although still higher compared with the sham group. We speculate that combination therapy led to the intermediate ratio of Gla-OC/Glu-OC because of the individual effects of the PTH and MK-4. PTH increased the synthesis of OC via the stimulation of osteoblasts, increasing the level of both Gla-OC and Glu-OC [24]. However, the high ratio seen with MK-4 treatment is likely attributable to action on OC metabolism, which increased Gla-OC while consuming Glu-OC [42, 43].

In addition to effects on osteoclast activity, PTH and MK4 affected other pathways to stimulate bone formation. The c-terminal telopeptide of type I collagen (CTX-I) is decreased in the PTH + MK-4 group as compared with other groups, which suggested combination therapy reversed the bone loss in osteopenic rats. Histomorphometry and micro-CT showed that MK-4 or PTH treatment alone also promoted bone formation and angiogenesis. Meanwhile, the strongest therapeutic effects occurred in the PTH + MK-4 group as compared with monotherapy. Meanwhile, combination therapy promoted the expression of VEGF, CD31, OCN, and RUNX2 in calvarial defect. These results suggested that combination therapy improved both osteogenesis and angiogenesis in calvarial defects in osteopenic rats.

This study contained several limitations. Further studies are needed to assess the dynamic changes in serum markers based on variable dosing combinations following treatment with therapy. Additional studies using qPCR and Western Blot will help confirm osteogenesis and angiogenesis in future experiments. Clinically, combined treatment of $PTH_{1–34}$ and MK-4 may help with reducing the total dose of $PTH_{1–34}$, thereby reducing the treatment cost, drug toxicity, and risk of osteosarcoma.

Conclusion

In summary, the present study confirmed that combination of MK-4 and $PTH_{1–34}$ improved osteogenesis and angiogenesis within calvarial defects in osteopenic conditions compared with monotherapy. Further studies may help to illuminate the molecular mechanisms for the combination therapy of MK-4 and $PTH_{1–34}$ on bone healing.

Acknowledgements The study was supported by the Science Technology Department of Zhejiang Province (Grant No.:2016C37122), National Natural Science Foundation of China (Grant No.:81772348).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Animals were handled with the approval of the Animal Experimentation Ethics Committee of Second Affiliated Hospital of Wenzhou Medical University.

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