The impact of curculigoside on osteogenic differentiation and orthodontic tooth movement in osteoporosis under mechanical stretch stress

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Abstract

Curculigoside (CCG), a natural glycoside compound extracted from the traditional Chinese medicinal herb Curculigo orchioides, is known for its osteogenic differentiation-promoting effects. In this study, we investigated the anti-osteoporotic effects of CCG under mechanical stress. Establishing an osteoporotic orthodontic force cell model, we found that CCG treatment could promote osteogenic differentiation in MC3T3-E1 cells and enhance the expression of osteogenic differentiation factors such as COL1, BMP2, ALP, and RUNX2. Furthermore, we applied CCG to osteoporotic rats undergoing orthodontic tooth movement (OTM) to observe its *in vivo* anti-osteoporotic activity. Through the detection of serum ALP levels, X-ray of femur, and Micro-CT of maxillary, we found that CCG could alleviate the reduction in bone density caused by osteoporosis (OP) and decrease the loss of alveolar bone mass during OTM. In summary, CCG can promote osteogenic differentiation of MC3T3-E1 cells under mechanical stress, and it is beneficial for bone formation during OTM in OP.

1. Introduction

Malocclusion, a developmental irregularity affecting the teeth, jaw, and facial structures, is prevalent globally and can impair both facial aesthetics and oral functionality[1]. Severe malocclusions may even have broader implications for systemic health. Osteoporosis (OP), a chronic condition marked by decreased bone density, compromised bone architecture, and heightened bone fragility, raises the risk of fractures[2]. As economies prosper, there is a rising demand for orthodontic care, with a notable increase in patients with OP, complicating the therapeutic process[3]. Research suggests that OP can intensify the deterioration of alveolar bone during active periods of periodontitis, potentially undermining the outcomes of orthodontic treatments that rely on the remodeling of periodontal tissues[4]. Osteoporosis can disrupt the balance of bone reconstruction, affecting the stability of orthodontic tooth movement (OTM) and increasing the risks associated with orthodontic treatment. Studies have demonstrated that in animal models of osteoporosis, there is an increased distance of orthodontic tooth movement coupled with a marked increase in alveolar bone resorption[5, 6]. Moreover, osteoporosis can lead to enhanced root resorption as teeth move orthodontically[3, 7]. Patients with OP are also at a higher risk of losing anchorage[8, 9], which can significantly impair the success of orthodontic interventions.

The process of OTM relies on the jawbone's biological reaction to orthodontic forces[10]. These forces can influence the bone mass and structure of the alveolar bone, a highly plastic tissue that is among the most responsive parts of the skeletal system[11]. Appropriate bite forces can enhance the modeling and mineralization of the alveolar bone, but long-term exposure to imbalanced or excessive mechanical loads may result in bone resorption or structural changes[12–14]. OP can reduce bone density throughout the body, including the alveolar bone[15]. It may increase the risk of tooth loss, which is because the alveolar bone becomes more fragile and more susceptible to the impact of mechanical forces.

Osteoclasts and osteoblasts are the functional cells of jawbone remodeling. During tooth movement, the alveolar bone on the compressed side experiences resorption, while the tension side witnesses new bone formation. These processes adjust dynamically, resulting in qualitative and quantitative changes that establish a new balance[16, 17]. Osteoporosis, characterized by reduced bone formation and increased bone resorption, can affect the efficient and safe movement of teeth. The current pharmacopeia for osteoporosis mainly consists of hormonal agents and bisphosphonates (BPs), which primarily function by inhibiting osteoclastic activity[18]. This is not conducive to OTM, such as BPs that inhibit the movement of orthodontic teeth. Consequently, there is a growing imperative to discover novel osteoporosis treatments that promote bone anabolism. Curculigoside (CCG), extracted from the plant Curculigo, is a type of saponin compound with anti-inflammatory[19], antioxidant[20], and immune-modulating effects[21]. However, there is little research on the regulation of jawbone osteogenesis during tooth movement in OP by CCG. In this study, we investigated the anti-osteoporotic effect of CCG under mechanical stress, and applied CCG to the OTM of osteoporotic rats to observe the *in vivo* anti-osteoporotic activity of CCG.

2. Results

2.1. The effects of H_2O_2 and CCG on MC3T3E1 cells viability.

MC3T3E1 cells were cultured with H_2O_2 at 50, 100, 200, 500, 1000 μM for 1-day, as shown in Fig. 1 (a). It was observed that H_2O_2 exerted a pronounced impact on the cell survival rate, which notably declined by approximately 25% when subjected to 200 μM. This particular concentration was identified as optimal for inducing the cell model of OP($P < 0.001$). Consequently, 200 μM was selected for use in the subsequent experimental procedures. Next, MC3T3E1 cells were treated with CCG for 1-day,. The results, as illustrated in Fig. 1 (b), revealed that CCG at concentrations of 1, 2.5, and 5 μM did not exert a significant influence on the cell survival rate. These findings led to the adoption of the aforementioned concentrations for the continuation of the experiments.

2.2. The effects of CCG on osteogenic differentiation of MC3T3-E1 cells under mechanical stretch stress.

The apparatus used for cell mechanical stress modeling and the schematic diagram are shown in Fig. 2. After a 7-day osteogenic induction period for cells treated with CCG and mechanical stretch stress, both ALP staining and quantitative analysis revealed that the group treated with 5 μM CCG demonstrated a notably enhanced effect on promoting osteogenic differentiation (Fig. 3 (a), (b)). Subsequently, the cells were analyzed for the expression of osteogenesis-related genes by qRT-PCR. The results indicated that the expression levels of genes pivotal to osteogenic differentiation, including COL1, BMP2, ALP, and RUNX2, were significantly elevated in the CCG treatment group. Notably, within this group, the 2.5 μM

concentration of CCG was associated with the most substantial increases in the expression of COL1, BMP2, and RUNX2 (Fig. 3 (c)). In addition, the most pronounced upregulation in ALP expression was observed in the 5 μM group, which is in concordance with the ALP staining and quantification outcomes.

2.3. The effects of CCG on major organs of rats.

The modeling process of animal experiments is shown in Fig. 4. To assess the *in vivo* biological safety of CCG, HE staining was conducted on sections of major organs from rats in the CCG treatment group. The results indicated that there was no significant infiltration of inflammatory cells or substantial tissue damage observed in the primary viscera (Fig. 5).

2.4. The effects of CCG on bone mineral density of OP rats.

The serum ALP of rats levels were measured, and the results revealed that the OP group exhibited significantly lower ALP levels compared to the CTRL group. In contrast, the OP + CCG group showed an increase in serum ALP levels (Fig. 6 (a)). To further investigate the impact of CCG on bone density in rats, To further investigate the impact of CCG on bone density in rats, X-ray was conducted on the femurs of the rats from each group, and Micro-CT scanning was performed on the mandibles. The X-ray indicated a noticeable discontinuity in the cortical bone in the OP group when compared to the CTRL group. In comparison with the OP group, there was a certain degree of restoration in the continuity and bone density of the femoral cortical bone in the OP + CCG group (Fig. 6 (b)).

BV/TV, BS/TV, Tb.Th, and Tb.N of the maxilla were evaluated by Micro-CT and 3D reconstruction (Fig. 6 (c)). The furcation region of the first molar was selected as the region of interest. In comparison with the CTRL group, the OP group showed a reduction in the BS/TV, Tb.Th, and Tb.N. The BV/TV results did not exhibit a statistically significant difference but displayed a downward trend. The administration of CCG was able to partially ameliorate these alterations, as evidenced by a notable increase in BV/TV, BS/TV, Tb.Th, and Tb.N in the OP + CCG group (Fig. 6 (d)).

2.5. The effects of CCG on OTM in rats.

The span from the distal contact point of the maxillary first molar to the mesial contact point of the second molar serves as OTM in rats. OTM was assessed by Micro-CT analysis, the results indicated a marked increase in OTM for the OP group relative to the CTRL group, and the OP + CCG group also exhibited a significant elevation in OTM compared to the CCG group. Nonetheless, no significant differences were observed between the OP and OP + CCG groups (Fig. 6 (e), (f)).

3. Discussion

Alveolar bone is the most metabolically active part of the skeletal system, and OTM occurs through the remodeling of alveolar bone in response to mechanical stimulation[10]. Current researches have demonstrated that a variety of systemic conditions, including OP, can influence the OTM process[22–24]. OP has been shown to potentially cause damage to periodontal tissues such as cementum[4]. As the

population of elderly patients seeking orthodontic treatment grows, the impact of OP on orthodontic procedures is gaining more attention. In this study, we investigated the anti-osteoporotic effect of CCG under certain mechanical stress. And we administered CCG to osteoporotic rats undergoing OTM to assess its *in vivo* activity against OP, providing insights into its potential role in mitigating the adverse effects of OP on orthodontic therapy.

Oxidative stress is one of the pathogenic factors of OP[4, 25, 26], hence we utilized H_2O_2 to treat cells as a condition for creating an OP model. As shown in Fig. 3 (a), a concentration of 200 μM H_2O_2 reduced the cell survival rate to approximately 75%, which is a suitable modeling concentration. In prior research, we observed that specific concentrations of CCG could enhance osteogenic differentiation in MG63 cells. Figure 3 (b) indicates that CCG at concentrations of 1, 2.5, and 5 μM did not adversely affect the vitality of MC3T3-E1 cells. Consequently, we selected these concentrations for further investigation and applied mechanical strain to the cells to mimic the forces experienced during orthodontic treatment. The MC3T3-E1 cell line is a type of pre-osteoblast that has the capacity to differentiate into osteoblasts *in vitro*[27]. In comparison to the MG63 cells, MC3T3-E1 cells demonstrate enhanced osteogenic activity and are more stable in their expression of osteogenic markers[28]. Additionally, these cells are sensitive to mechanical stimuli[29], rendering them an ideal model for investigating the impact of CCG on OP induced by H_2O_2 under mechanical stress conditions. This approach allows us to explore the protective effects of CCG in the context of orthodontic forces and oxidative stress.

The results of ALP staining and ALP activity assay (Fig. 4 (a), (b)) demonstrated that high concentrations of CCG (5 μM) exert an osteogenic effect on the MC3T3-E1 cells. Derived from the traditional Chinese medicinal plant Curculigo orchioides, CCG is a naturally occurring saponin compound that has demonstrated the ability to foster osteogenic differentiation across a spectrum of stem cell populations, including human amniotic fluid stem cells[30], adipose-derived stem cells[31], and mesenchymal stem cells from bone marrow[32]. Consequently, CCG could serve as one of the potential therapeutic agents against osteoporosis.

Appropriate mechanical stress is beneficial for bone remodeling[33]. Osteoblasts play a key role as the primary cellular mediators. Upon experiencing mechanical strain, these cells translate the mechanical signals into biological responses through mechanotransduction pathways. Mechanical stimulation of different strain magnitudes can influence the function of osteoblasts[34]. Studies have indicated that strains exceeding 4000 με can cause cellular damage, while the physiological strain generated in human long bones during movement is approximately 2000 με[35]. Researchers have found that 2000 με can stimulate the differentiation of osteoblasts and also induce the osteogenic differentiation of mesenchymal stem cells[36]. Given that lower magnitudes of mechanical force are insufficient to trigger effective OTM, we opted for a strain level of 2000 με for our subsequent experimental conditions. As depicted in Fig. 4 (c), qRT-PCR results indicate that a concentration of 2.5 μM CCG exhibits the most significant effect, while 5 μM CCG demonstrates the strongest enhancement in ALP gene expression. These findings are in concordance with the outcomes presented in Fig. 4 (a).

The foundation of orthodontic therapy is the adaptive remodeling of periodontal tissues in response to orthodontic forces. Osteoclasts and osteoblasts are the functional cells of periodontal tissue remodeling; osteoclasts are active on the compressed side leading to bone resorption, while osteoblasts are active on the tension side facilitating bone formation[11, 12]. In addition, periodontal ligament stem cells and macrophages also participate in the OTM process[37, 38]. Efficient and safe tooth movement requires appropriate orthodontic forces; insufficient forces may fail to stimulate periodontal tissue responses, while excessive forces could lead to tissue necrosis, impeding direct bone resorption and tooth movement. Research indicates that an orthodontic force of 50 g is more efficient for tooth movement in rats, and higher forces can result in increased root resorption[39, 40]. Thus, in animal experiments, we selected a 50 g orthodontic force for the rat OTM model. HE staining of major organs in rats (Fig. 5) confirmed no significant toxicity from systemic administration of CCG, aligning with the outcomes of cellular experiments. Serum levels of ALP can reflect overall bone metabolic status to some extent. As depicted in Fig. 6 (a), the serum ALP level in the OP group was lower than that of the control group, with the OP + CCG group showing a certain degree of increase. X-ray detection results (Fig. 6 (b)) also mirrored this trend, suggesting that CCG can elevate ALP levels in rats and safeguard overall bone mass in osteoporotic rats. Subsequently, we assessed the OTM distance in rats across different groups (Fig. 6 (e), (f)), noting a significant increase in the OP group compared to the CTRL group, indicating that OP can accelerate the rate of tooth movement in rats, consistent with previous findings[41, 42]. However, in clinical orthodontics, the goal is not merely to accelerate tooth movement but to achieve safe and efficient movement. Therefore, we conducted a three-dimensional reconstruction of the alveolar bone at the M1 furcation area in rats and statistically analyzed the main bone parameters (Fig. 6 (d)). We found that the OP group exhibited varying degrees of reduction in bone parameters such as BS/TV, BV/TV, Tb.N, and Tb.Th. In contrast, the OP + CCG group not only showed a tooth movement distance comparable to the OP group but also a recovery in the corresponding bone parameters to varying extents. This indicates that CCG treatment can protect alveolar bone mass while ensuring the efficiency of tooth movement in osteoporotic rats. Studies have demonstrated that CCG can prevent bone loss by inhibiting osteoclastogenesis[43]. Additionally, CCG has been confirmed to activate the Nrf2 pathway and inhibit the NF-κB pathway in osteoclasts, thereby reducing intracellular levels of reactive oxygen species and mitigating oxidative stress[44]. Since the OTM distance is influenced by both osteoblasts and osteoclasts, CCG may also protect alveolar bone during the OTM process by inhibiting osteoclast activity.

Ensuring the safe and effective movement of teeth in OP orthodontic patients and maintaining the alveolar bone mass is important for orthodontists. Through the establishment of osteoporotic cellular and animal models, this study investigates the influence of CCG on osteoblast differentiation under mechanical stress. It offers potential therapeutic agents for orthodontic treatment conducted in osteoporotic conditions.

In summary, this study demonstrates that under osteoporosis and mechanical stress, CCG can promote osteogenic differentiation in MC3T3-E1 cells. Our findings also indicate that CCG is beneficial for bone

formation during orthodontic tooth movement in states of osteoporosis. These results suggest that CCG could serve as a potential therapeutic agent for orthodontic patients with osteoporosis, facilitating the safe and efficient movement of teeth to the desired position.

4. Methods

4.1. Cell culture

MC3T3-E1 preosteoblasts were purchased from American Type Cell Culture (Manassas, VA, USA). Cells were were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY) and 1% antibiotic/antimycotic. Cells were grown at 37°C and 5% CO_2 in a humidified incubator.

4.2. Cell cytotoxicity assay

Cell cytotoxicity was evaluated using CCK-8 kits. Cells were seeded in a 96-well plate with a cell concentration of 5×10^3/mL. The methods were followed by the manufacturer's protocol. The OD value of each well was measured at 450 nm. The cell viability percentage was calculated by using the formula: (OD of treated cells / OD of control cells) × 100%.

4.3. ALP staining and activity assay

CCG was bought from Shanghai Yuanye. MC3T3-E1 cells were seeded at 10^6 cells per well in a 6-well plate. The experiment was divided into four groups: the blank control group and the low, medium, and high-dose CCG treatment groups (with CCG concentrations of 1.0, 2.5, 5 μmol/L), and the cultures were maintained for 7 d.

ALP staining: Staining solution was prepared according to the kit's instructions (Beyotime, Nanjing) and applied at room temperature in the dark for 30 min. After staining, the cells were observed and photographed under a microscope.

ALP activity assay: Total cellular protein was extracted. The BCA assay kit (Beyotime, Nanjing) was used to calculate the protein concentration of the samples. The working solution was prepared following the kit's instructions (Beyotime, Nanjing). The absorbance of each well was measured at a wavelength of 520 nm using a microplate reader.

4.4. CCG and mechanical stretch stress treatment

Hydrogen peroxide (H_2O_2)and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. A rectangular cell force plate and a four-point bending force system were used for mechanical stretch stress treatment. The MC3T3-E1 cells were seeded to a culture dish with the cell force plate at the bottom. CCG solutions of 0, 1, 2.5, and 5 μM were added after the cells were completely attached, and a H_2O_2 solution at a final concentration of 200 μM was added after 2 h. After 20 h, the force plate was transferred to a tension force dish, and the cells attached to the force plate were loaded to 2000 με mechanical tension for 3 h.

4.5. Quantitative real-time PCR (qRT-PCR)

The total RNA in each group was extracted a with a RNA extraction kit (Foregene, Chengdu, China), and the concentration of RNA in each group was measured. Then the genomic DNA was removed and cDNA was synthesized by reverse transcription reaction using HiFiScript gDNA (CWbio, Chengdu) MasterMix kit. qPCR was performed by MagicSYBR Mixture PCR kits (CWbio, Chengdu). Gene expression levels were determined by normalization to those of GAPDH. The primers used are listed in Table 1.

Table 1
Primers for qRT-PCR

Primer	Sequence
BMP2	F: AGGATTAGCAGGTCTTTG
	R: TCACTGAAGTCCACATAC
RUNX2	F: CCTCTGGTGGCATCTCGTTATCC
	R: CCTCTGGTGGCATCTCGTTATCC
COL1	F: AGTTCTTCTGAGGCACAGACGG
	R: AGTTCTTCTGAGGCACAGACGG
ALP	F: TGGGCATTGTGACTACCACTCGG
	R: CCTCTGGTGGCATCTCGTTATCC
GAPDH	F: TCACTGAAGTCCACATAC
	R: TCCACGACGGACACATTG

4.6. Animal experiments

Animals: The study was approved by Ethics Committee of College of Basic Medical Sciences, Jilin University (SYXK 2023-0010), all methods were carried out in accordance with relevant guidelines and regulations. This study was carried out in compliance with the ARRIVE guidelines.

Groups: Sprague-dawley rats (male, 6–8 weeks, 200–220 g) were all provided by Changchun Yisi Experimental Animal Technology Co., Ltd. (SCXK(Ji) 2023-0002). Rats were kept at SPF status. After 7 days of adaptation, the rats were randomly divided into seven groups (n = 6): CTRL, OP, OP + CCG, OTM, OP + OTM, CCG + OTM, and OP + CCG + OTM.

Establishment of osteoporosis model in rats: Dexamethasone sodium phosphate injection (20 mg/kg) was injected intraperitoneally every other day to sterilize the site to be injected, and the injection could be continued until there was no blood back. At the sixth week after injection, X-ray irradiation was used to irradiate the tibia of the model group. The osteoporosis model was considered to be successfully

established when the tibial cortical bone of the osteoporosis model group became thinner and showed signs of discontinuity compared with the control group

After the successful establishment of the osteoporosis model, the rats were anesthetized by intraperitoneal injection of sodium pentobarbital combined with PBS. A 0.25 mm diameter ligating wire was passed through the bilateral maxillary first and second molars of the rats, and the NiTi spring was stretched. An orthodontic tooth movement model was established. Orthodontic tooth movement model rats were fed with soft food, and the spring shedding was observed every morning and evening. The groups were described above. One week after the establishment of OTM model, CCG solution (15 mg/kg) was given by gavage once every other day. The control group and other no-CCG group were given the same volume of normal saline.

Sample collection: All rats were euthanized 2 weeks after the OTM. Blood serum was collected from each rat's caudal vein. The femur, maxilla, and major organs of the rats were collected and fixed in 4% paraformaldehyde.

4.7. X-ray detection

The fixed femur of each rat was put in X-ray imaging system to observe the status of osteoporosis rats.

4.8. Micro-CT

All fixed maxillas were scanned by Micro-CT (Hiscan XM, Suzhou, China). Three-dimensional reconstruction was performed after scanning. The tooth movement distance of each group was calculated. including bone volume fraction (BV/TV), trabecular thickness (Tb.Th), bone surface fraction (BS/TV), trabecular number (Tb.N), and tooth movement distance.

4.9. ALP analysis of serum

ALP analysis was carried out using a commercial kit (Beyotime, Nanjing) followed by the manufacturer's protocol.

Declarations

Competing Interests Statement:

The authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this study

Author Contribution

Y. T. Li and M. Hu designed the experiments; X. Y. Liu, H. Jiang, H. M. Wang performed the experiments; X. Y. Liu processed the data; X. Y. Liuand Y. T. Li wrote the manuscript; Y. T. Li and M. Hu revised the manuscript.

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Data Availability

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.

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Figures

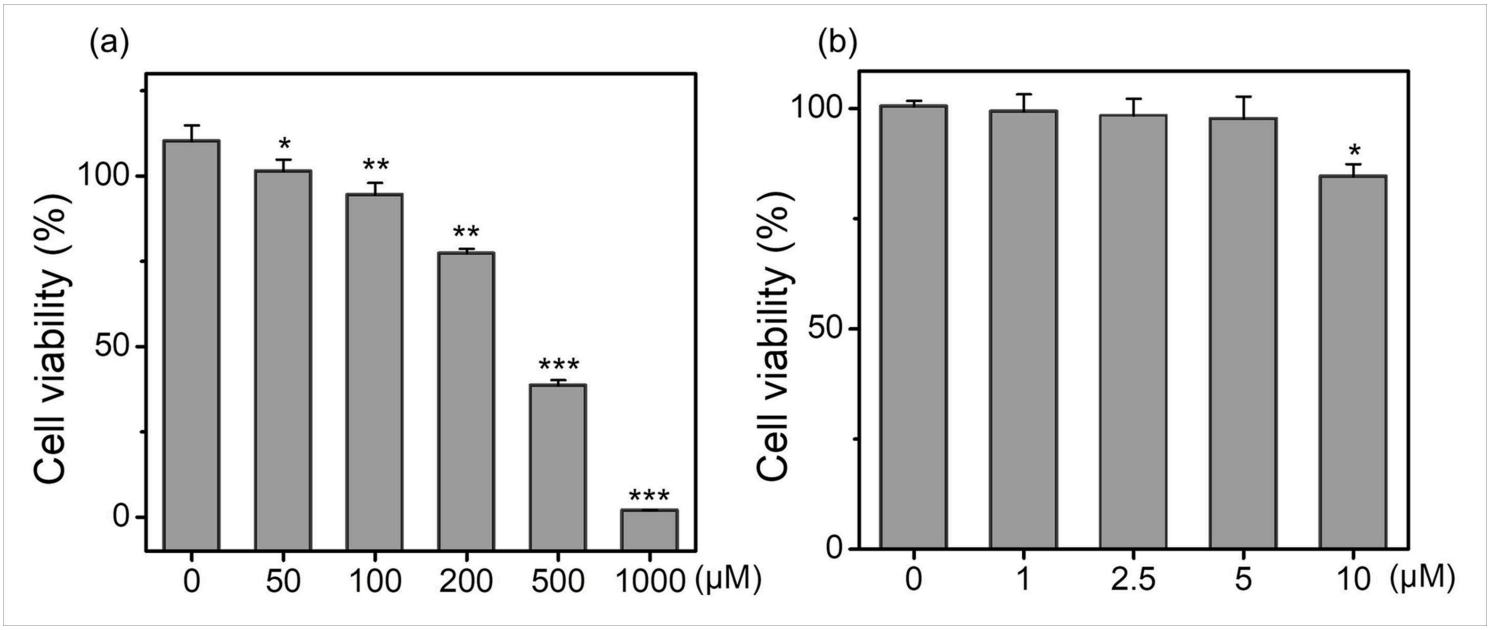

Figure 1

Effect of hydrogen peroxide and CCG and on the proliferation of MC3T3-E1 cells: (a) The survival rate of cells under different concentration of hydrogen peroxide (50, 100, 200, 500, 1000 μM). *P□0.05 vs. 0 μM group, **P□0.01 vs. 0 μM group, ***P□0.001 vs. 0 μM group. (b) Effect of CCG on MC3T3-E1 cells. MC3T3-E1 cells were treated with CCG (0, 1, 2.5, 5 and 10 μM) for 1-day *P□0.05 vs. 0 μM group.

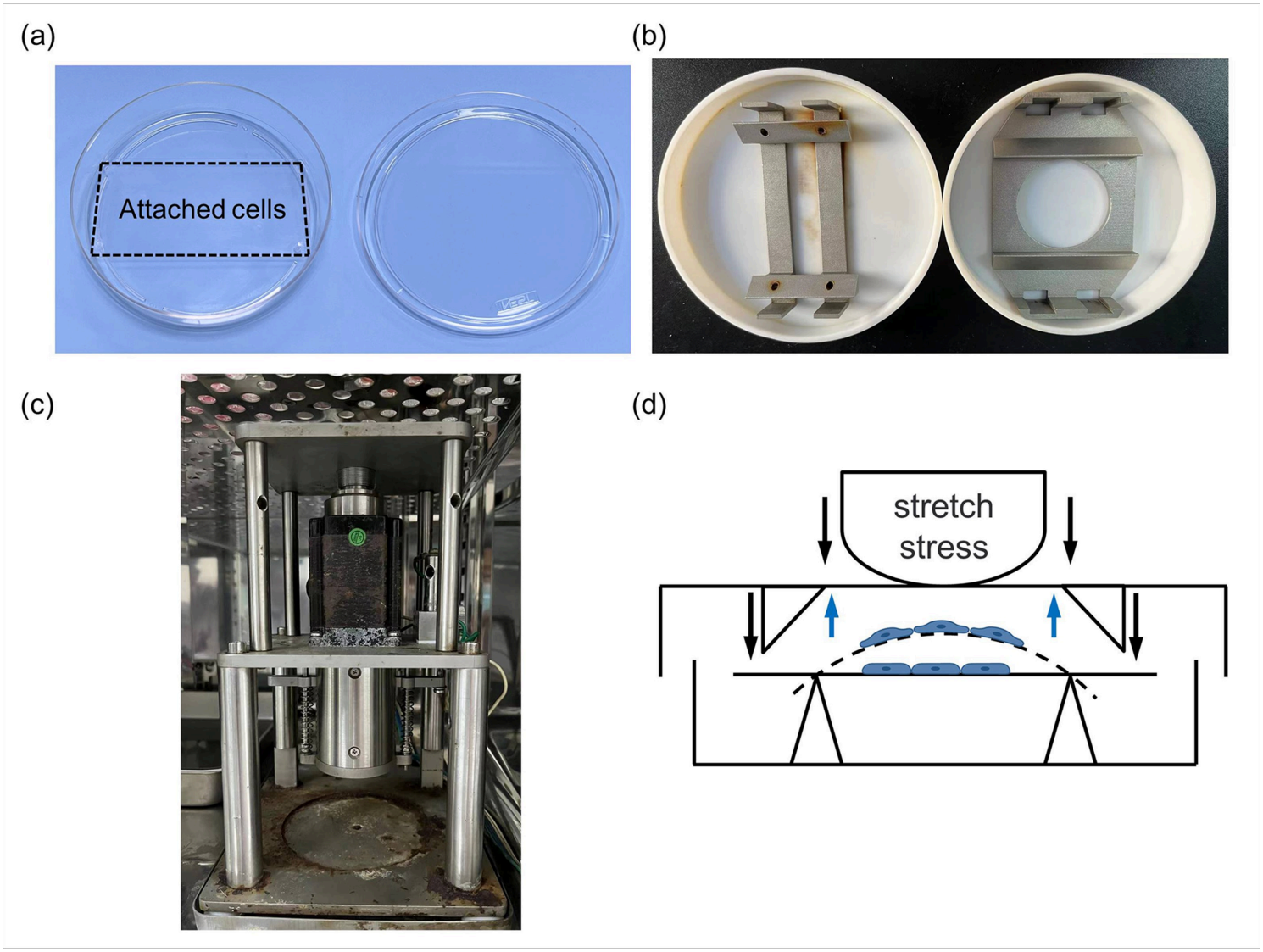

Figure 2

Cell mechanical stress modeling: (a) Cell force plate. (b), (c) Cell force device. (d) Schematic illustration of the mechanical stretch force on the cell.

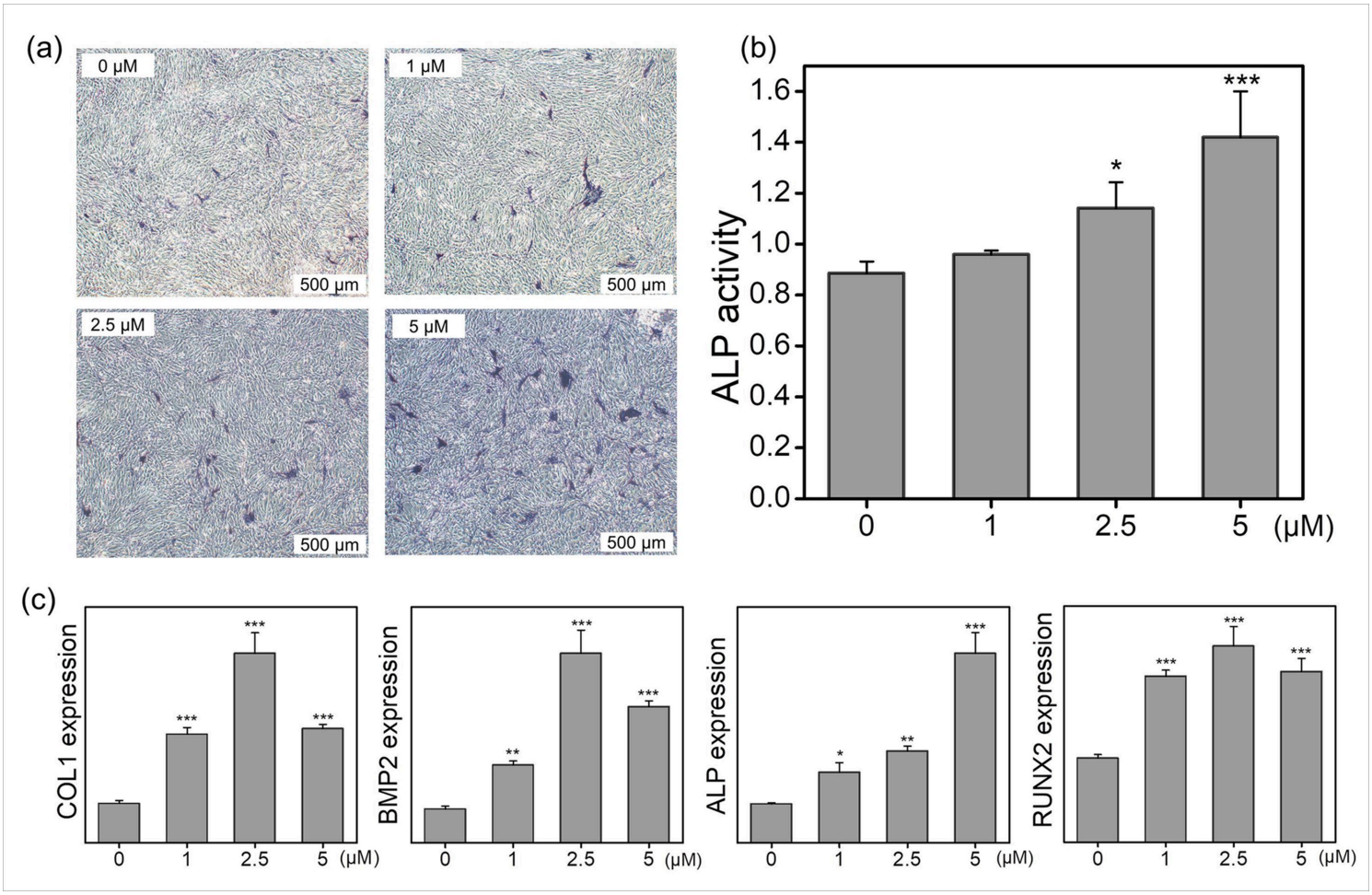

Figure 3

CCG promoted osteogenic differentiation of MC3T3-E1 cells: (a) ALP staining of cells treated with mechanical force and CCG followed by osteogenic induction. (b) Quantitative results of ALP staining. *P 0.05 vs. 0 μM group, ***P 0.001 vs. 0 μM group. (c) CCG treatment increased the mRNA levels of BMP2, ALP, COL-1, RUNX2. *P 0.05 vs. 0 μM group, **P 0.01 vs. 0 μM group, ***P 0.001 vs. 0 μM group.

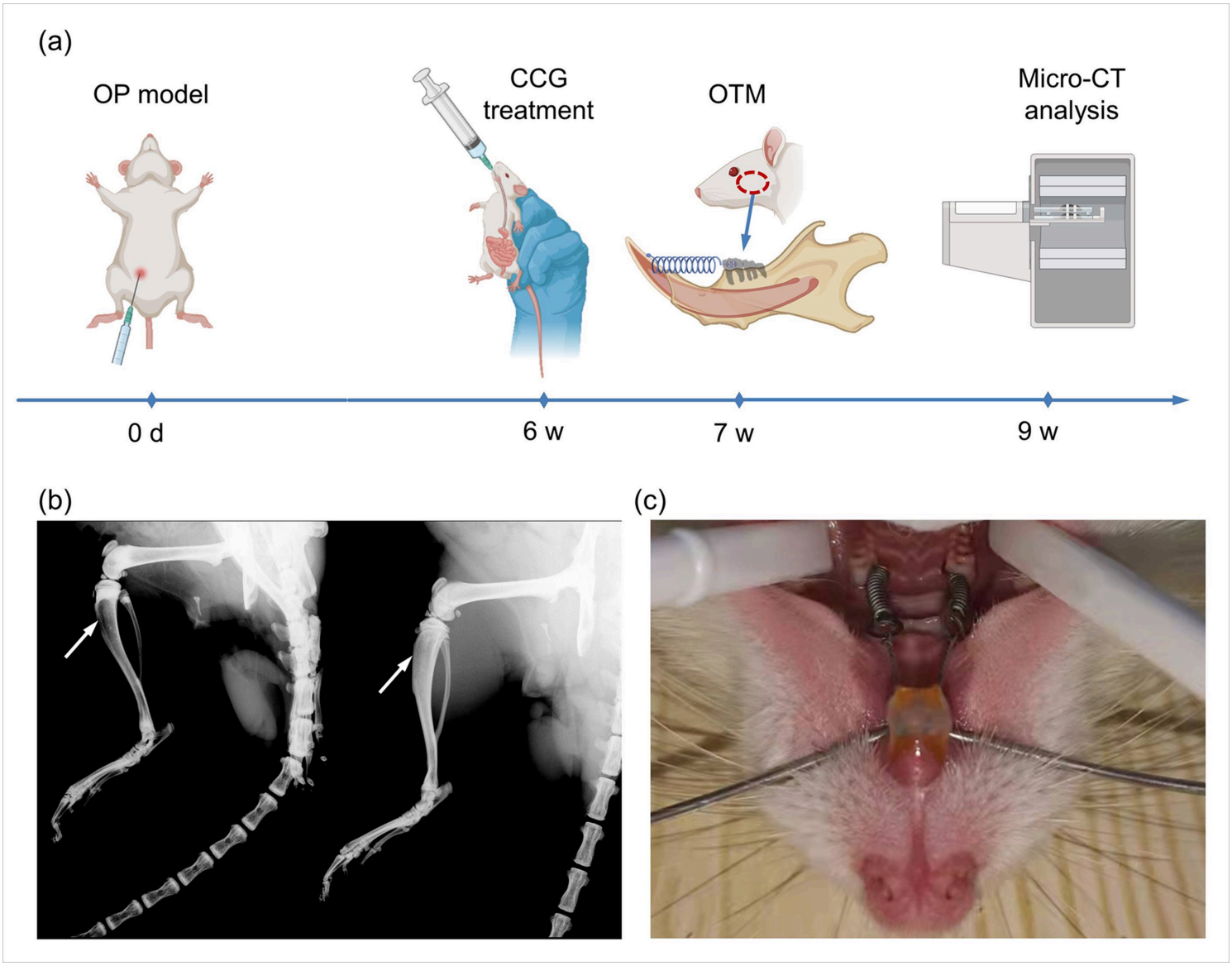

Figure 4

Animal Experimental modeling: (a) Scheme of the animal experiment procedure. (b) X-ray of the leg bone of rats, left: OP group, right: CTRL group. (c) Intraoral photographs of OTM rats.

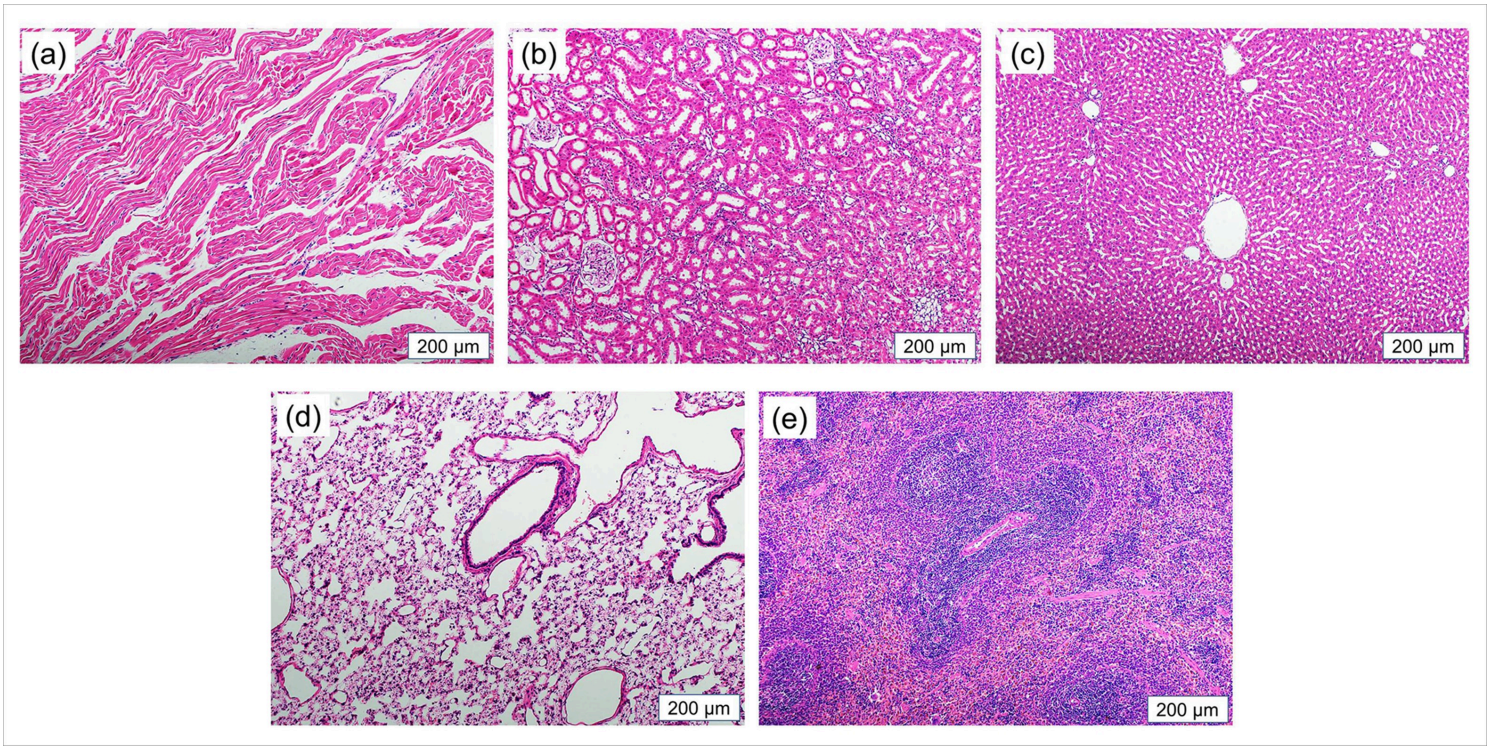

Figure 5

The effects of CCG on major organs in rats: (a) heart, (b) kidney, (c) liver, (d) lung, (e) spleen.

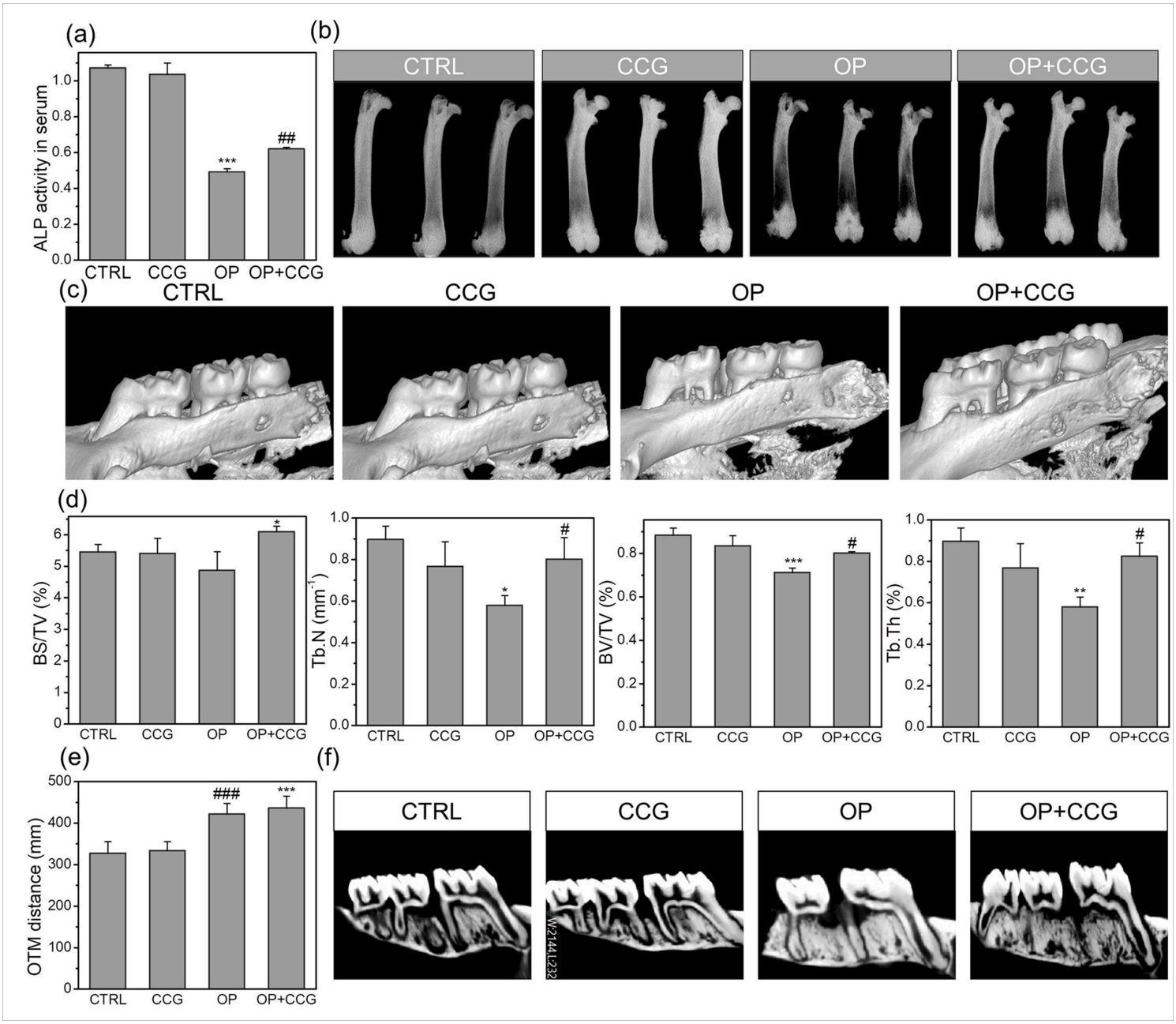

Figure 6

The effects of CCG on bone tissue and alveolar bone tissue in rats: (a) ALP activity of serum samples. (b) Bone mineral density of femurs in rats under X-ray. (c) Three-dimensional reconstruction of the jaw by Micro-CT analysis. (d) Main bone parameters of rat maxillary Micro-CT results: BS/BV, BV/TV, Tb.N, Tb.Th. *P□0.05 vs. CTRL group, **P□0.01 vs. CTRL group, ***P□0.001 vs. CTRL group. #P□0.05 vs. OP group. (e), (f) The effects of CCG on OTM in rats: The effect of CCG on OTM distance in rats. ***P□0.001 vs. CCG group, ###P□0.05 vs. CTRL group.