Diedaqili Tablet Promotes Bony Fusion Accelerates Fracture Healing In Mice

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Abstract

Diedaqli tablet has been found effective for fracture healing in previous clinical studies. In a recent study, we investigated the effects of diedaqli tablet (DDQL) on bone fracture repair in mice. Adult C57BL/6 mice were subjected to transverse femoral fractures and administrated orally with a low dose (40.55mg/ ml), a high dose (162.18mg/ml) DDQL suspension and normal saline daily from day 1 after operation. The femur and blood of mice was analyzed by plain radiography, micro-computed tomography (Micro-CT), histology, biomechanical analysis, and serum Ca, P, and ALP test. The results demonstrated that DDQL can effectively improve the porosis of fracture end and bony union in the course of healing via Micro-CT and hematoxylin-eosin staining (HE staining) analysis. Consistent with morphological findings, biomechanical properties of fracture healing have also been demonstrated. And Diedaqli tablet has a dose-dependent effect. DDQL augmented the release of alkaline phosphatase (ALP) and phosphorus (P) into blood, indicating that it promoted mineralization of hypertrophic cartilage and woven bone growth simultaneously during bone healing. In summary, the preliminary experiment revealed that DDQL can improve bone formation via promoting osteogenic capability, calcium (Ca) and P metabolism, and subsequently accelerates fracture repair and bony fusion.

Introduction

Fracture is a loss of mechanical integrity and continuity of the bone structure, accompanied by local soft tissues injury and vascular trauma[[i]]. It has become a significant global health issue since prevalence of fracture is continuously growing with the development of society and the aging of population. In the United States, there are approximately 15 million fractures per year, among which 1.6 million are hospitalized for traumatic fractures, requiring 1.6 million bone grafts annually at a cost of more than \$60 billion[[ii]]. Fracture healing is a complex dynamic process involving histology, biology, endocrinology and biomechanics[[iii]]. Fracture treatment has always been a key issue in the domain of orthopedics[[iv]]. Surgery including bone grafting, internal fixation and external fixation on fracture is recognized as effective treatment, though may lead to decreased blood supply, postoperative infection and other poor prognosis, resulting in poor fracture healing or even non-union[[v]-[vi]]. In China, Traditional Chinese Medicine (TCM) has received much attention in the treatment of fracture. Recently, further research and clinical studies believe that TCM can promote the reconstruction of blood circulation and the deposition of calcium salt at the fracture site, stimulate the secretion and synthesis of bone growth factor and improve the activity of osteoblasts, which contributes to fracture healing[[vii]-[viii]]. Based on TCM theory, the composition of DDQL originates from the ancient book 'Liang Fang Ji Ye' compiled by a well-known Qing Dynasty doctor Yuanqing Xie, which was used to treat trauma and bleeding by doctors in ancient china. Additionally, several clinical trials have demonstrated that DDQL is safe and effective for the treatment of fracture[[ix]], though the basic research is needed regarding its effect on fracture healing. Thus, this experimental study was designed to explore the effect of DDQL on fracture healing, so as to provide the theoretical basis for clinical effect of DDQL on fracture healing.

Materials And Methods

Experimental Animals

A total of 100 specific pathogen-free (SPF) 8-week-old C57BL/6J male mice with a body weight of 20 ± 2 g were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. The experimental animals were kept in the room which is SPF grade in Animal Experimental Center of Shanghai University of Traditional Chinese Medicine. The laboratory environment was as follows: indoor temperature 20-23°C, relative humidity 40-60%, noise control below 60 dB, light duration of 12 hours of light and 12 hours of night. The feeding room was disinfected once a day, and the pasteurized material was replaced twice a week. The feed was in accordance with the national standard of dry feed for rodents, and was fed in cages with 5 rodents in each cage. The operation of animals in this research strictly conforms to the relevant norms of experimental animal welfare and ethics of Shanghai University of Traditional Chinese Medicine (ethics code: PZSHUTCM191025012).

Animal model

Open osteotomy at femur diaphysis was performed on 8-week-old male C57BL/6 mice to establish a stable femoral open fracture model^{[[1]]}. Fig.1 provides an overview that the animal model of fracture was established, and the process was as follows. The mice abstained from drinking and fasting the night before modeling. Mice were anesthetized with 10% chloral hydrate (Sinopharm Chemical Reagent Co. LTD) at a dose of 0.004 ml/g body weight. After shaving and sterilization of both legs, the shaft of the femur was exposed and cut off with a bone saw machine MDJ-110029 (MYDAJAR Co. LTD). The special 0.5mm intramedullary needle was inserted into the femoral bone marrow cavity through the intercondylar fossa of the femur until sensing loss sensibility which indicated the needle had entered the femoral bone marrow cavity. Finally, the wound was washed with normal saline. The mice after modeling were performed small animal X-ray imaging through Faxitron X-ray MX20 (Faxitron, Inc.) immediately to confirm the fracture line in good position and alignment indicating that the modeling was successful, and injected Gentamicin (Shandong Zhengmu Biological pharmaceutical Co. LTD) continuously for 3 days to prevent infection. In the sham group, only the middle femur region was exposed, and then the muscle, fascia and skin were directly sutured layer by layer with 4-0 line.

Drug Administration and Specimen Harvest

DDQL used in animal administration was purchased from Chongqing Xieran Pharmaceutical Co. LTD. The tablets were crushed into powder by a Chinese medicine crusher. The powder was blended with 0.3% sodium carboxymethyl cellulose solution (CMC-NA) and prepared into low dose (40.55mg/ml) and high dose (162.18mg/ml) DDQL tablet suspension, respectively. DDQL was given to mice by daily oral gavage from the day after modeling until euthanasia, and mice in the control group were fed with normal saline only.

Animals in each group were subject to serological detection, Micro-CT, histology and biomechanics. In week 1(W1), week 2(W2), week 3(W3) and week 4(W4) post-operation, arterial blood of mice was collected from abdominal aorta and stood at room temperature for 30 minutes. Blood was set in a centrifuge at 3000r/min for 20min to extract the yellowish serum from the upper layer and stored in a refrigerator at -80°C. Fractured and contralateral femurs were harvested at the end of experiments in W2 and W4. Soft tissues were removed from the operated and contralateral femurs. The specimens were fixed within 4% paraformaldehyde solution for 24h. Internal fixation needle was removed from the intramedullary cavity and the femur specimens were stored in 75% ethanol for Micro-CT and histologic processing.

Serum content test

The collected serum samples were restored to room temperature and placed in Beckman Coulter AU5800 Automatic biochemical instrument (American Beckman Coulter Co., LTD) to detect the contents of Ca, P and ALP in serum.

Histology

Fixed bone specimens were decalcified with calcium chelating solution (0.5 M EDTA/NaOH, pH 7.5) for 2 weeks. Decalcified bones were then dehydrated and embedded in paraffin wax using Leica EG Embedding Center (Leica Microsystem, Wetzlar, Germany). Paraffin blocks were sectioned into 5µm slices and mounted on glass slides. The sections were de-paraffinized and stained with hematoxylin and eosin (HE) for the bone tissue. The histomorphometric analysis was performed by a blinded observer using Olympus BX43 microscope (Olympus Corporation, Japan).

Biomechanical

The femur specimens of mice were subjected to Instron 5543 type material mechanics tester (Instron Shanghai Material Testing Machine Co., LTD, China) for biomechanical three-point bending test. The program is set as temperature 23°C, humidity 60%, speed 5.00mm/min, span 14mm. The detection index is Maximum Load (N), which refers to the maximum bending load borne by the whole bone before damage, indicating the strength of the healed bone directly reflect the biomechanical properties^{[[ii]]}.

Micro-CT

The femur specimens were scanned in Skyscan1172 type Micro-CT instrument (Belgium Bruker Technology Co., LTD) for 3D imaging analysis. The scanning parameters were as follows: voltage 56KV, current 171µA, scanning row number 666, scanning column number 1000, object to source 59.970mm, camera to source 214.620mm, vertical object position 39.110mm, exposure time 360ms, image pixel size 9.92µm.

After 3d reconstruction by Micro-View software, the region of interest (callus region) was selected on the femoral cross-section. The region of interest was centered on the fracture line and extended 5mm to the

proximal and distal ends of the femoral axis. Bone volume fraction (BVF), that is to say BV/TV, in the region of interest was calculated and analyzed, which referred to the ratio of the volume of mineralized callus to the volume of total callus in the area of interest, reflecting the content of callus tissue at the fracture end[[iii]].

Statistical methods

Statistical analysis was conducted using GraphPad Prism 5.0 (GraphPad Software, CA, USA) software and Excel (Microsoft, CA, USA). All data was obtained from 5 individual mice in each group. Mean and standard deviation values (mean±SD) were calculated for all statistically analyzed parameters. The differences between groups were analyzed using ANOVA with Turkey's post hoc test or unpaired Student's t tests. The p value less than 0.05 was considered statistically significant.

Results

Micro-CT analysis

To study the effect of DDQL on fracture repair in mice, the fracture morphology of mice in W2 and W4 were monitored and analyzed via Micro-CT 3D imaging. As is shown in Fig.2, Micro-CT allows for visual evaluation of callus at the fracture end through 3D reconstruction, and quantitative comparison through the software analysis. In W2, the fracture end was surrounded by newly formed soft callus in each group, among which the callus volume was significantly less in the model group than both DDQL groups. In comparison with the model group, the low dose group had more minor fracture end area and fresh callus than the model group, but the fracture end was still not connected by fibrous tissue. The high-dose group had smaller gap at the fracture end, more newly formed callus, which revealed a current of bone junction. In W4, though the new callus of the model group joined the fracture end, the callus quantity was less and uncalcified. Compared with the model group, low dose group had more calluses and slight calcification, and the fracture ends were completely surrounded by calcified bone and connected surrounding bone cortex in the high dose group. Although the three-dimensional image can only show the size of callus, the bone volume fraction can directly verify the quantification of callus by DDQL. As shown in Fig 3 and Table 1,in W2,the BVF of the low-dose group was higher than that of the model group, but there was no significant difference ($P>0.05$).The BVF of the high-dose group was higher than that of the model group and the low dose group, and the difference between the high-dose group and the model group was statistically significant ($P<0.05$).There was no significant difference between the high dose group and the low dose group ($P>0.05$). In W4,the BVF of the low dose and high dose groups were both higher than that of the model group, and the difference was statistically significant ($P<0.05$). The high dose group was higher than that of the low-dose group, and the difference was statistically significant ($P<0.05$).

HE staining

Since radiographic evaluations were not able to ascertain cartilage and other soft tissues, histological sections of fractured bone were labeled with both cartilage and bone and histomorphometric analysis were performed[[i]]. As shown in Fig 4, the fracture ends of the model group were not connected which was same as Micro-CT analysis, there were gaps surrounded by a large number of necrotic bone fully unabsorbed in W2. A small amount of chondrocyte infiltrated around the fracture ends, and the fibrous bone was sparse. Chondrocyte infiltration and calcified braided bone mass were increased around the fracture end in DDQL groups, and the healing trend was more significant in high dose group. In W4, the fracture ends of the model group were still barely union though a large amount of fibrous bone tissue appeared around the fracture ends. However, the fracture ends of the low-dose and high-dose groups were all connected, and the fibrous bone was transformed into lamellar bone, gradually restoring the basic form of bone.

Biomechanical analysis

As shown in Fig 5 and Table 2, no statistically significant difference was observed between the low-dose group and the model group in W2($P > 0.05$). Statistically significant differences were observed between the high-dose group and the model group, and the femur maximum load of the high-dose group was higher than the model group ($P < 0.05$). Though the femur maximum load of the high-dose group was higher than that of the low-dose group, statistically significant difference was not observed ($P \geq 0.05$). In W4, the femur maximum load of each group was compared, and both the femur maximum load of low-dose and high-dose group were higher than that of model group ($P < 0.05$). Though the high-dose group was higher than the low-dose group, the difference was not statistically significant ($P > 0.05$).

Serum content test

At each point after modeling, the serum Ca,P and ALP content of mice in sham operation group and control group was compared, and no significant difference between the two groups was observed($P > 0.05$), which proved sham operation had no impact on the serum Ca, P and ALP contents in mice.

As shown in Fig 6 and Table 3, the serum Ca content of mice in model group, low-dose group and high-dose group increased in W1, but there was no significant difference ($P > 0.05$). From W2 to W4, the serum Ca content in model group, low-dose group and high-dose group all showed a diminished trend. In W2, the serum Ca content in low-dose group was significantly different from that in sham operation group ($P < 0.05$), while there was no significant difference in other groups ($P > 0.05$). In W3, the serum Ca content in the high-dose group was significantly different from that in the sham group ($P < 0.05$), but there was no significant difference in the other groups ($P > 0.05$). In W4, the serum Ca content of model group was significantly different from that of sham operation group ($P < 0.05$), but there was no significant difference in other groups ($P > 0.05$).

As shown in Fig 7 and Table 4,the serum P content in model group, low-dose group and high-dose group showed a clear trend of increasing within 28 days after modeling, and reached a peak in W3 after modeling.The serum P content of the high dose group was significantly higher than both of the sham

group and the model group in W1($P < 0.05$), but no statistically significant correlation was observed between the other groups ($P > 0.05$). In W2, compared with the sham operation group, the serum P content of model group, low-dose group and high-dose group was significantly different ($P < 0.05$). The serum P content in low dose group and high dose group was higher than the model group, and there was a significant difference ($P < 0.05$). No significant difference in serum P content was found between high dose group and low dose group in W2 ($P > 0.05$). In W3, compared with sham operation group, the serum P content of model group, low-dose group and high-dose group was significantly different ($P < 0.05$). The serum P content in low dose group and high dose group was higher than the model group, there was a significant difference between each group ($P < 0.05$). The serum P content of the high dose group was higher than that of the low-dose group with a significant difference ($P < 0.05$). In W4, the serum P content of model group and high-dose group were higher than the low-dose group, and high dose group was higher than sham group and model group, there was a significant difference between each group ($P < 0.05$).

As shown in Fig 8 and Table 6, The serum ALP content in model group, low-dose group and high-dose group showed an increasing trend within 28 days after modeling, and reached a peak in W3 after modeling. In W1, compared with the sham operation group, the serum ALP content in the high-dose group was higher, and the difference was significant ($P < 0.05$), while the serum ALP content in the other groups had no significant difference ($P > 0.05$). In W2, the serum ALP content of low-dose group and high-dose group was higher than that of sham operation group and model group, and the high-dose group was higher than that of low-dose group, the difference was significant ($P < 0.05$), while the serum ALP content of other groups had no significant difference ($P > 0.05$). In W3, the serum ALP content of low-dose group and high-dose group was higher than that of sham operation group and model group, and the high-dose group was higher than that of low-dose group, the differences were significant ($P < 0.05$), while the serum ALP content of other groups had no significant difference ($P > 0.05$). In W4, the serum ALP content of low dose and high dose was higher than that of sham operation group and model group, with significant difference ($P < 0.05$), while the serum ALP content of other groups had no significant difference ($P > 0.05$).

Discussion

Fracture healing is a complicated dynamic process involving histology, biology, endocrinology and biomechanics, which has always been a key issue in the field of orthopedics[[\[i\]](#)]. About 10% of fractures each year end up as nonunion or delayed union, requiring additional surgery including bone grafting, internal fixation and external fixation[[\[ii\]](#)]. Callus formation and bone resorption depend on the integrity of blood supply and soft tissue, which is general factors for nonunion of fractures[[\[iii\]](#)]. The study has found that the use of rigid plates and reamed nails can affect the vascularization of bone fragments, resulting in high infection rates and delayed union[[\[iv\]](#)]. Although autologous bone transplantation is still the gold standard of bone regeneration, studies found that autologous bone transplantation may give rise to nerve injury, wound infection,

postoperative persistent pain and secondary fracture and other adverse reactions[[v]]. Safer treatment with cost-effective and simple way of administration are thereby still required.

Our HE pathological staining and Micro-CT 3D imaging analyses demonstrated the healing process of femur fracture in mice. Micro-CT, as the gold standard for the evaluation of bone trabecular structure, can measure the structure and mineralization of fracture callus in a noninvasive, quantitative and three-dimensional manner[[vi]-[vii]]. As a simple and low-cost method, HE pathological staining has been widely used in bone morphological assessment. In W2, the callus formation in the high-dose and low-dose groups was significantly better than that in the model group, and the callus content in the high-dose group was significantly higher than that in the low-dose group. In W4, compared with the model group, the callus in the low-dose group was more and showed a slight tendency of calcification, while the fracture end in the high-dose group was completely filled with calcified new bone and associated with the surrounding bone cortex. BVF at the fractured end of mice analyzed by micro-CT detection, refers to the ratio of the volume of mineralized callus to the total callus in the area of interest, reflecting the content of callus tissue at the fractured end, which is relevant to the stiffness and strength of callus[[viii]]. The BVF of low dose group and high dose group were both higher than that of the model group in W2 and W4, which indicated that DDQL tablet could improve bone strength by increasing the content of callus. Moreover, the callus content in the low dose group was significantly lower than that in the high-dose group, indicating that DDQL may have a dose-dependent effect on the degree of callus formation. It verified that diedaqili tablet can effectively promote the formation of callus around the fracture end in mice and shorten the time of fracture healing.

The recovery of bone strength and stiffness is the basic characteristic of fracture healing. Biomechanical testing can be used to evaluate the recovery of bone strength and stiffness. Currently, the main detection methods of biomechanics include three-point bending test, four-point bending test, torsion test and tensile test, etc., and the widely used three-point bending test was selected in this study[[ix]]. According to the Stat theory of mechanics, the bone geometry is suitable to ensure that the maximum tissue strain from the universal load is kept within a certain range, and when this strain capacity is exceeded, the bone tissue will fracture[[x]]. The Maximum Load (Max Load) tested by the three-point bending test refers to the maximum bending load borne by the whole bone before it is destroyed, which indicates the strength of the healed bone and can directly reflect the biomechanical properties. The study observed that the Max Load of femur in both dose group was better than that in the model group in W2 and W4, while the improvement of low dose group in W2 was not obvious. The biomechanical results were consistent with the morphological findings, indicating that DDQL tablet could not only improve the bone morphological

structure of mice femur significantly, but also enhance bone strength and stiffness in fracture healing.

Bone strength can reflect the integrity of bone quality, determined by both bone microstructure and bone mass. Calcium salt deposition is one of the key steps in fracture repair. Bone tissue repair can only be completed by the calcification of bone matrix, and the strength of new bone tissue is closely related to the degree of matrix calcification[[\[xi\]](#)]. The mineralization and mechanical properties of bone depend largely on the deposition levels of Ca and P. Ca and P exist in the blood in the form of ions, which are interactive systems[[\[xii\]](#)]. The normal operation of osteogenesis and bone dissolution is an important link to maintain the stable content of calcium and phosphorus in the blood[[\[xiii\]](#)]. The experimental results of this study showed that in W1 after fracture modeling, serum Ca content of mice showed an increasing trend, which may be caused by the release of a large number of Ca ions into the blood due to the dissolution of bone fragments by osteoclasts. From W1 to W4 after modeling, serum P content increased and peaked in W3, and the serum P content in both high-dose and low-dose groups was higher than that in the model group, indicating that osteogenesis was active and phosphate release increased. From W2 to W4, serum Ca decreased significantly after fracture modeling, suggesting that the deposition of calcium and phosphate in serum increased, which was in the state of osteogenic calcification. These results were consistent with previous studies, however, experimental results showed that DDQL tablets at low and high doses did not significantly change serum Ca in fracture mice.

The activity of ALP synthesized by the osteoblasts is the typical markers of bone turnover, which catalyzes mineralization and bone formation making osteogenesis possible[[\[xiv\]](#)]-[\[xv\]](#)]. As the osteogenic differentiation of cells progresses, its activity gradually increases[[\[xvi\]](#)]. The increase of ALP in each model group within 28 days after the modeling could indicate intensified bone tissue remodeling, which reached the peak in the third week, same as the tendency of serum P content. And the content of ALP in high dose group was obviously higher than that in low dose group and model group, demonstrated DDQL tablet can improve the content of ALP, increase osteogenic activity, promote phosphate release, and increase calcium deposition to promote fracture healing of femur in mice, with a dose-dependent effect[[\[xvii\]](#)].

In conclusion, this study demonstrated that DDQL tablet has an effect of improving bone formation through promoting osteogenic capability, calcium and phosphorus metabolism. We believe that DDQL tablet is a safe, effective and economical oral drug for fracture healing in the long term.

Declarations

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Author contributions

Ye Zhao, Yujie Zhang, Kaoqiang Liu, Yongli Chai were responsible for the implementation of animal experiments, Ye Zhao wrote the report, Kaoqiang Liu was responsible for data statistics, Shen Yao was responsible for the management of research drugs, Ye Zhao and Yujie Zhang as the co-first authors, Hongsheng Zhan was responsible for research quality supervision, Weian Yuan and Zhibi Shen as the co-corresponding authors.

Conflicts of interest

All authors declare that they have no conflicts of interests.

References

1. S.T.Xu,B.F.Ge,Y.K.Xu.Practical OsteopathologyM.Beijing:People's Military Medical Publishing House,2012,in Chinese.
2. Ensrud KE. Epidemiology of fracture risk with advancing age. The journals of gerontology Series A, Biological sciences and medical sciences. 2013;68(10):1236-42.
3. Einhorn TA, Gerstenfeld LC. Fracture healing: mechanisms and interventions. Nat Rev Rheumatol. 2015;11(1):45-54.
4. Odatuwa-Omagbemi DO. Open fractures: epidemiological pattern, initial management and challenges in a sub-urban teaching hospital in Nigeria. Pan Afr Med J. 2019 Jul 19;33:234.
5. Hussain N, Sermer C, Prusick PJ, et al. Intramedullary Nailing Versus Plate Fixation for the Treatment Displaced Midshaft Clavicular Fractures: A Systematic Review and Meta-Analysis. Sci Rep. 2016;6:34912.
6. Metsemakers WJ, Kuehl R, Moriarty TF, Richards RG, Verhofstad MHJ, Borens O, Kates S, Morgenstern M. Infection after fracture fixation: Current surgical and microbiological concepts. Injury. 2018 Mar;49(3):511-522.

7. C.W.Chen.Effect of Diedaqili tablet on fracture healing J. Journal of Clinical Rational drug Use,2013,6(20):63-64,in Chinese.
8. G.C.Han.Treatment of patellar fracture with diedaqili tablet combined with improved Kirschner wire tension band internal fixation J. Henan Journal of Surgery,2020,26(03):142-143,in Chinese.
9. Y.Lin,X.Liu,J.X.Yang,et al.Study on pharmacodynamics of Diedqili Tablet J. Chinese Traditional Medicine emergency,2006,(11):1263-1264,in Chinese.
10. Gunderson ZJ, Campbell ZR, McKinley TO, Natoli RM, Kacena MA. A comprehensive review of mouse diaphyseal femur fracture models. *Injury*. 2020;51(7):1439-1447.
11. Saeid AA, Donaldson SL. Experimental and Finite Element Investigations of Damage Resistance in Biomimetic Composite Sandwich T-Joints. *Materials (Basel)*. 2016 Jun 24;9(7):510.
12. Zhang G, Boyle DL, Zhang Y, et al. Development and mineralization of embryonic avian scleral ossicles. *Mol Vis*. 2012;18:348-361.
13. Lepage SIM, Robson N, Gilmore H, Davis O, Hooper A, St John S, Kamesan V, Gelis P, Carvajal D, Hurtig M, Koch TG. Beyond Cartilage Repair: The Role of the Osteochondral Unit in Joint Health and Disease. *Tissue Eng Part B Rev*. 2019 Apr;25(2):114-125.
14. Giannotti S, Bottai V, Dell'osso G, Pini E, De Paola G, Bugelli G, Guido G. Current medical treatment strategies concerning fracture healing. *Clin Cases Miner Bone Metab*. 2013 May;10(2):116-20.
15. Tzioupis C, Giannoudis PV. Prevalence of long-bone non-unions published correction appears in *Injury*. 2007 Oct;38(10):1224. *Injury*. 2007;38 Suppl 2:S3-S9.
16. Teulières M, Langlais T, de Gauzy JS, Rölfig JD, Accadbled F. Bone Lengthening with a Motorized Intramedullary Nail in 34 Patients with Posttraumatic Limb Length Discrepancies. *J Clin Med*. 2021 May 28;10(11):2393.
17. Zhang X, Zhan X, Zou P, et al. Factors related to infection after fixation in the process of late healed bone fracture. *Exp Ther Med*. 2017;14(2):1126-1130.
18. Day AGE, Francis WR, Fu K, et al. Osteogenic Potential of Human Umbilical Cord Mesenchymal Stem Cells on Coralline Hydroxyapatite/Calcium Carbonate Microparticles. *Stem Cells Int*. 2018 Sep 5;2018:4258613.
19. Ohs N, Collins CJ, Atkins PR. Validation of HR-pQCT against micro-CT for morphometric and biomechanical analyses: A review. *Bone Rep*. 2020 Aug 24;13:100711.
20. Morgan EF, Mason ZD, Chien KB, et al. Micro-computed tomography assessment of fracture healing: relationships among callus structure, composition, and mechanical function. *Bone*. 2009;44(2):335-344.
21. Liu XS, Cohen A, Shane E, et al. Individual trabeculae segmentation (ITS)-based morphological analysis of high-resolution peripheral quantitative computed tomography images detects abnormal trabecular plate and rod microarchitecture in premenopausal women with idiopathic osteoporosis. *J Bone Miner Res*. 2010 Jul;25(7):1496-505.

22. Walker AH, Perkins O, Mehta R, et al. Changes in mechanical properties of rat bones under simulated effects of microgravity and radiation. *Phys. Procedia*. 2015;66:610–616.
23. Chen Z, Beck TJ, Cauley JA, et al. Hormone therapy improves femur geometry among ethnically diverse postmenopausal participants in the Women's Health Initiative hormone intervention trials. *J Bone Miner Res*. 2008;23(12):1935-1945.
24. Claes L, Recknagel S, Ignatius A. Fracture healing under healthy and inflammatory conditions. *Nat Rev Rheumatol*. 2012;8(3):133-43.
25. Jiang Z, Li Z, Zhang W, Yang Y, Han B, Liu W, Peng Y. Dietary Natural N-Acetyl-d-Glucosamine Prevents Bone Loss in Ovariectomized Rat Model of Postmenopausal Osteoporosis. *Molecules*. 2018 Sep 9;23(9):2302.
26. Liang JR, Xiao X, Yang HM, Wang ZY. Assessment of vitamin A requirement of gosling in 0-28 d based on growth performance and bone indexes. *Poult Sci*. 2021 Apr;100(4):101015.
27. Sharma U, Pal D, Prasad R. Alkaline phosphatase: an overview. *Indian J Clin Biochem*. 2014;29(3):269-278.
28. Franceschi R.T., Ge C., Xiao G., Roca H., Jiang D. Transcriptional regulation of osteoblasts. *Cells Tissues Org*. 2009;189:144–152.
29. Chen X, Chen Y, Hou Y, Song P, Zhou M, Nie M, Liu X. Modulation of proliferation and differentiation of gingiva-derived mesenchymal stem cells by concentrated growth factors: Potential implications in tissue engineering for dental regeneration and repair. *Int J Mol Med*. 2019 Jul;44(1):37-46.
30. Sharma U, Pal D, Prasad R. Alkaline phosphatase: an overview. *Indian J Clin Biochem*. 2014 Jul;29(3):269-78.

Tables

Table 1 Comparison of BV/TV among each group at each point. The data of three groups was analyzed by One Way ANOVA.* represents the difference was statistically significant compared with the model group.△represents the difference was statistically significant compared with the low-dose group.

Group	n	Week 2	Week 4
Model	5	43.75±3.33	46.38±2.46△
Low dose	5	44.46±3.58	53.01±4.57*
High dose	5	48.85±2.92*	66.46±2.34*△
F	—	3.533	48.408
P	—	0.062	0.000

Table 2 Comparison of Maximum Load among each group at each point. The data of three groups was analyzed by One Way ANOVA.* represents the difference was statistically significant compared with the

model group. Δ represents the difference was statistically significant compared with the low-dose group.

Group	n	Week 2	Week 4
Model	5	1.06±0.55	4.06±1.08 Δ
Low dose	5	1.58±1.27	6.68±2.49*
High dose	5	2.88±1.79*	7.82±1.63*
F	—	2.579	5.566
P	—	0.117	0.019

Table 3 Comparison of serum Ca content among each group at each point. The data of four groups was analyzed by One Way ANOVA. # represents the difference was statistically significant compared with the sham group.

Group	n	Week 1	Week 2	Week 3	Week 4
Sham	5	2.18±0.02	2.13±0.02	2.10±0.04	2.13±0.06
Model	5	2.18±0.06	2.07±0.06	2.05±0.05	2.06±0.06 [#]
Low dose	5	2.20±0.11	2.00±0.07 [#]	2.02±0.02	2.08±0.04
High dose	5	2.24±0.11	2.03±0.05	1.99±0.13 [#]	2.09±0.02
F	—	0.175	4.761	2.208	2.061
P	—	0.912	0.015	0.127	0.146

Table 4 Comparison of serum P content among each group at each point. The data of four groups was analyzed by One Way ANOVA.* represents the difference was statistically significant compared with the model group. Δ represents the difference was statistically significant compared with the low-dose group. # represents the difference was statistically significant compared with the sham group.

Group	n	Week 1	Week 2	Week 3	Week 4
Sham	5	3.10±0.30	3.09±0.36* Δ	2.70±0.09* Δ	2.76±0.26 Δ
Model	5	3.12±0.10	3.69±0.19 Δ [#]	3.50±0.57 Δ [#]	3.23±0.28
Low dose	5	3.51±0.31	4.08±0.24* [#]	4.30±0.27* [#]	3.40±0.61 [#]
High dose	5	3.83±0.43* [#]	4.13±0.32* [#]	4.82±0.42* Δ [#]	4.27±0.41* Δ [#]
F	—	6.435	13.942	29.485	11.671
P	—	0.005	0.000	0.000	0.000

Table 5 Comparison of serum ALP content among each group at each point. The data of four groups was analyzed by One Way ANOVA.* represents the difference was statistically significant compared with the model group.△ represents the difference was statistically significant compared with the low-dose group.# represents the difference was statistically significant compared with the sham group.

Group	n	Week 1	Week 2	Week 3	Week 4
Sham	5	63.60±3.85	59.60±5.94△	61.60±4.62△	62.40±4.78△
Model	5	66.60±5.60	66.20±4.38△	72.00±10.65△	69.60±8.39△
Low dose	5	68.60±3.36	76.00±8.03*#	97.00±8.09*#	80.80±9.34*#
High dose	5	73.00±8.49#	120.60±5.55*△#	128.6±26.13*△#	87.20±6.69*#
F	—	2.404	81.502	20.151	10.975
P	—	0.105	0.000	0.000	0.000

Figures

Figure 1

Modeling of femur fracture in mice.(a)expose the middle of femur (b)The middle of femur was cut off by a bone sawing machine, and the intramedullary needle was inserted into the femoral bone marrow cavity. (c) Confirm the success of the mice femur fracture model with X-ray (red marked fracture area)

Figure 2

Comparison of mice femur among each group in W2 and W4 after fracture via Micro-CT 3d imaging

Figure 3

Comparison of bone volume fraction among each group in W2 and W4 after fracture

Figure 4

Comparison of mice femur among each group in W2 and W4 after fracture via HE pathological staining

Figure 5

Comparison of maximum load among each group in W2 and W4 after fracture

Figure 6

Comparison of serum Ca content among each group at each point after fracture

Figure 7

Comparison of serum P content among each group at each point

Figure 8

Comparison of serum ALP content among each group at each point