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Effect of calcium citrate on bone integration in a rabbit femur defect model

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ABSTRACT

Objective: To explore effect of calcium citrate on bone integration in a rabbit femur defect model, and to compare the bone formation with different sizes by radiological and histological study. **Methods:** Twenty-four male Japanese white rabbits were randomly divided into three groups (Group A, B, C) in this study. Under anesthesia, defects of four sizes (1.2, 1.5, 2.0 and 2.5 mm) were created in each of the rabbits. Commercially pure calcium citrate powder was placed inside the medullary compartment of the femur (Experimental), while in the contralateral femur (Control) nothing was implanted. The defects were analyzed using radiography and histological analysis by using Imagepro-Plus 6.0 software after animal was sacrificed at 4th(Group A), 6th(Group B) and 8th(Group C) weeks postoperatively. Four samples were analyzed for each size of defect and each healing period. **Results:** The histological and the radiologic evaluation were performed after sacrification of all rabbits on postoperative 4th and 6th weeks, It showed significant difference between the experimental group and the control group when these defects were less than or equal to 2.0 mm. No statistical difference was observed when these defects were larger than 2.0 mm at all healing periods except at the 4th week. **Conclusions:** Calcium citrate affects the early periods of bone defects healing mechanism in Japanese white rabbits positively, especially when the defect is not too large. We suggest further studies on calcium citrate to determine the effects of various dosages, administration ways and the experimental time on the bone defects.

1. Introduction

The treatment of bone nonunion, bone defects caused by trauma and the limb salvage treatment of bone tumors, even some of the bone reconstruction surgery is often related to bone graft. Extensive bone injuries that require grafting typically involve one of two kinds of bone material: autograft or allograft.

Autograft and allograft are traditional options for treatment of larger bone defects[1,2], however, their use is associated with several disadvantages. Autograft is considered to be more effective but it requires another operation with additional loss of blood and a prolonged anesthetic time. It

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may also lead to donor site complications like neurovascular injury, infection, hematoma formation and chronic pain[3-5], which is of limited availability and carry the possibility of donor site morbidity[1,2]. Allograft and xenograft bone substitutes have been studied to overcome the risks associated to autogenous bone, but their use is complicated by issues such as immunogenicity and consequent rejection, graft sequestration, infection, and the potential for disease transmission[1,2]. Considering all these limitations, alloplastic grafts appears to be safe and convenient[6-11], and synthetic grafts are preferred in place of autologous grafts. Various studies have proved the efficacy of calcium citrate as a substitute for an autologous graft. Calcium citrate appears to be the most appropriate when a bone substitute with some mechanical strength is needed, as calcium citrate is composed of the natural minerals found in human bone and it is similar with the natural structure of cancellous bone. Calcium citrate is a biocompatible material with osteoconductive properties. It is available in various forms and the bone formation and graft incorporation varies according to the type.

The aim of the present study was to prepare calcium citrate and explore its effect on bone integration in a femur defect rabbit model. We investigated the bone formation with different sizes of holes and compared them by radiological and histological study.

2. Materials and methods

2.1. Animals and reagent

Twenty-four healthy male Japanese white rabbits weighing between 2.0-3.5 kg were used as experimental animals for the study. All the rabbits were divided into three groups (Group A, B, C) randomly. Before the study, general health of the rabbits was monitored for 7 days. The rabbits were kept in standard cages in an experimental animal room and were fed with a standard laboratory diet and water. This study protocol was approved by the ethical committee for animal experiments of Wenzhou Medical College, Zhejiang, China.

The calcium citrate was purchased from Sigma.

2.2. Surgical procedure

All the animals were anesthetized by intraperitoneal administration with 10% trichloroacetaldehyde monohydrate, at a dose of 3 mL per 1 kg body weight. The skin was disinfected and shaved; the shaved and disinfected incision site was treated topically with 1% lingocaine solution. After administering local anesthetic, a longitudinal incision of 4 cm was made along the frontal aspect of both femurs. Subcutaneous tissue, muscles and ligaments were dissected to expose the external surface of the femur in the area of the diaphyseal bone. Kirschner wire with different sizes (1.2 mm, 1.5 mm, 2.0 mm, 2.5 mm in diameter) were used to drill holes reaching the bone marrow. Overheating and additional bone damage was prevented by using cooling saline.

Calcium citrate powder (15 mg) was placed inside the medullary compartment of the femur (Experimental), while in the contralateral femur (Control) nothing was implanted. The wounds were carefully sutured. The animals were given 800 000 u penicillin intramuscular injections every 12 hours for the first 48 hours after the operation. Within 2-3 days, the animals resumed normal ambulation and did not show signs of pain or distress.

The animals were killed by ether overdose, at 4th(Group A), 6th(Group B) and 8th(Group C) weeks postoperatively. The femur were resected, fxed in 10% formalin solution and radiographed.

2.3. Evaluation

2.3.1. X-ray examination

X-ray photographys were taken at 4th, 6th and 8th weeks postoperatively.

2.3.2. Histological analysis

All histological procedures and evaluations were carried out in the Laboratory of Orthopaedic Research Institute and Scientific Research Center of Second Affiliated Hospital of Wenzhou Medical College. Hard tissue samples were fixed with 10% buffered formalin for 24-72 hours and decalcified with 10% formic acid for 3 weeks. After washing under tap water overnight, samples were embedded in paraffin. Three sections with 4 μm thickness were cut at the central region of each specimen to obtain maximum standardization of the cutting surface. All sections were xyelene deparaffinized at 56 ℃, then incubated in absolute 96% ethanol. For histomorphological evaluation, sections were stained with routine hematoxylin-eosine. Routine histology and histomorphometric analyses were performed using transmission light microscopy (Axioskop Carl Zeiss GmbH, Jena, Germany) and image-analysis software (KS 300; Kontron Electronic GmbH, Munchen, Germany).

The bone area ratio was estimated by analyzing the newly formed bone in compact bone area and counting total dot numbers with an image analyzing software (Image J, National Institute of Health, Bethesda, USA). The percentage ratio of the newly formed bone area against the total compact bone area was analyzed statistically by ANOVA.

2.4. Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences (SPSS) 16.0 software (SPSS Inc., Chicago, IL, United States). In statistical analysis Mann-Whitney U-test, Wilcoxon signed ranks test, and Kolmogorov-Smirnov test were used for evaluation. Statistical significance was assigned to $P<0.05$.

3. Results

3.1.General observation

All animals tolerated the surgical procedures well, and the clinical healing process was generally uneventful, with no dehiscence of the surgical wound, signs of inflammation, or other complications.

3.2. Radiographic evaluation

At 4 weeks, a definite defect margin was observed with a small amount of radiopacity visible along the lateral margin in all the control group specimens; but in the experimental group, the defect margin was partially obscured and there were irregular and asymmetric radiopaque areas at 0.2-0.4 mm from the defect margin.

At 6 weeks, the specimens in the control group seems just like the experimental group specimens at 4 weeks; but in the experimental group, the defect margins could not be distinguished and the radiopaque area extended moderately inwards for a uniform distance, even some bony islets that were separated from the marginal bone were evident in the central area of the defect.

At 8 weeks, the 1.2 mm and 1.5 mm defects were filled with radiopaque substance both in the experimental group and in the control group. The 2.0 mm defects in the experimental group were almost filled with radiopaque substance except in the central portion; but in the control group, only some evident bony islets that were separated from the marginal bone could be oberved. A bony bridge had formed from the marginal bone to the center, and the defect margin was no longer defined on all of the 2.5 mm defects (Figure 1).

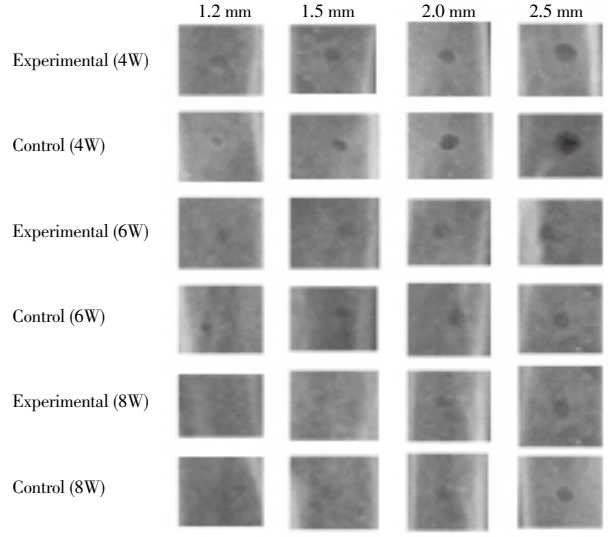

Figure 1. Radiographic views of the surgical defects at different healing periods.

3.3. Histologic evaluation

In all groups, different percentages of connective tissue, new bone formation were seen in defected areas.

At 4 weeks, in the control group, a small amount of wedge-shaped bone had regenerated. Most of the newly formed bone was woven bone, which showed limited bone marrow and a large number of osteoclasts. Bone regeneration in the experimental group was slightly greater than the control group, and moderate amounts of bone regeneration and bone marrow began to appear. Active new bone formation was also present, with osteoid seams lined by plump osteoblasts.

At 6 weeks, in the control group, bone regeneration was significantly greater than that at 4 weeks, and moderate amounts of bone regeneration and bone marrow began to appear. While in the experimental group the newly formed bone had a moderate amount of bone marrow and had matured. Bone regeneration from the margin was markedly increased in both width and length. Bony islands that appeared in some specimens on the center of the defects contained moderate amounts of bone marrow.

At 8 weeks, in the 1.2 mm and 1.5 mm defects group, lamellar bone was formed in the newly formed bone, even in the 2.0 mm defects, bony islets. Newly formed woven bone had regenerated around the defect margin in the 2.5 mm group (Figure 2).

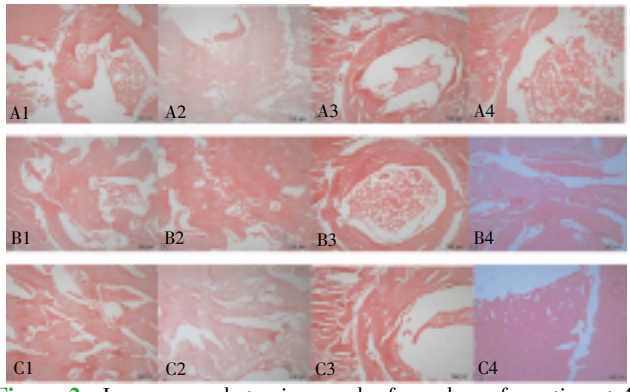

Figure 2. Low power photomicrograph of new bone formation at 4, 6, 8 weeks from the calcium citrate group and control group (Original magnification10×, HE staining).
*A(4 week time-point), B(6 week time-point), C(8 week time-point).
**The diameter of drilled hole: 1(1.2 mm in experimental group), 2(2.5 mm in experimental group), 3(1.2 mm in control group), 4(2.5 mm in control group).
*** Low power photomicrograph of new bone formation in the diameter of drilled hole 1.5 mm and 2.0 mm was not showed here.

3.4. Estimated bone area ratio

The new bone area ratio at postoperative weeks 4, 6, and 8 are listed in Figure 3. The mean new bone area ratios of the experimental group at 4 weeks were 25.0%, 21.1%, 17.1%, and 9.5% in the 1.2, 1.5, 2.0 and 2.5-mm-defect groups, at 6 weeks were 56.0%, 48.9%, 38.1% and 20.0%, and at 8 weeks were 72.6%, 62.1%, 50.3% and 28.0%, respectively. The mean new bone area ratios of the control group at 4 weeks were 21.5%, 16.9%, 14.1% and 7.1% in the 1.2, 1.5, 2.0 and 2.5-mm-defect groups, at 6 weeks were 51.1%, 46.4%, 34.0% and 18.1%, and at 8 weeks were 67.8%, 58.1%, 47.5% and 25.4%, respectively. The new bone area ratio differed significantly between the experimental group and the control group when the defects were 1.2 mm and 1.5 mm at 4 and 6 weeks after the surgery ($P<0.05$). But there was no significant difference among these groups at the 8th week ($P>0.05$). The estimated bone area ratio of (72.60±3.38)% of calcium citrate Group (1.2 mm-defect; 8 weeks) was the highest average value of all of the samples. In the 2.5 mm-defect groups, there was no significant difference between the experimental group and the control group at all healing periods except at the 4th week ($P<0.05$).

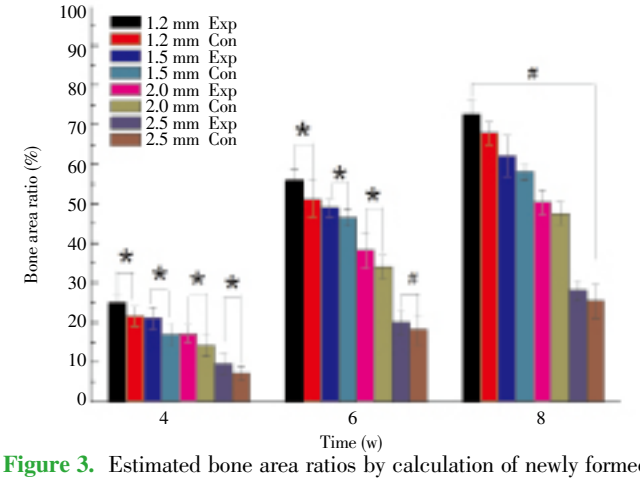

Figure 3. Estimated bone area ratios by calculation of newly formed bone area against total compact bone area after 4-week, 6-week and 8-week implantation.
Exp: experimental group; Con: control group.
*: experimental group vs. control group revealed $P<0.05$.
#: experimental group vs. control group revealed $P>0.05$.

4. Discussion

For bone regeneration and bone tissue engineering applications, an ideal biomaterial should have the properties of favorable biocompatibility, bioconductivity, and biodegradability. An optimal biomaterial used as a bone substitute should not only be a temporary scaffold for supporting the adhesion, growth, proliferation, and differentiation of the ´seed´ cells (such as hBMSC), but also be able to degrade into non-toxic products, which can be metabolized via the physiological mechanisms[12,13].

Calcium citrate has the potential to be a well-absorbed material that supplies calcium ions to bones, it has been used to supply calcium to deficient individuals, especially postmenopausal women[14-16], and it was also commonly used to prevent or treatment of osteoporosis as calcium supplements and could decrease bone loss and increase bone mineral density[17].

The present work describes the *in vivo* behavior of calcium citrate for the first time. Radiography, histological resultsshowed that the implantation of calcium citrate induces a rise in bone formation in the marrow of rabbit femur at early phases of fracture healing. The evaluation at 4th and 6th week showed significant change between the experimental group and the control group. Bone marrow began to appear and bony islets were observed after 4 weeks of healing in the experimental group. The defect closure and the new bone area ratio gradually increased with the healing time, these parameters differed significantly between weeks 4 and 8. An observation period of at least 8 weeks was recommended by Bodde *et al*. A slight increase was observed at 8th week but was not statistically significant.

Bone marrow stromal stem cells (BMSCs) differentiate into osteoblasts is the main way for bone formation, it follows an orderly cascade of events including proliferation, mesenchymal commitment, matrix deposition and matrix mineralization. Each of these stages was characterized by the expression of lineage-specific genes[18]. As reported in many papers, Ca^{2+} was an extracellular factor that regulated viability and osteogenic differentiation of BMSCs. Calcium is essential for cells mineralization and elevating concentration of Ca^{2+} slightly would stimulate genes expression associated with osteogenesis differentiation including OCN and OPN[19,20]. In the previous *in vitro* studies, it was observed that the proliferation and differentiation of BMSCs cultured on calcium citrate were significantly enhanced than the control. Adluri[21] showed that calcium citrate could stimulate the proliferation and mineralization of osteoblast cells and increase the activity of ALP significantly, and results in this study also confirmed that calcium citrate increased the differentiation and proliferation of osteoblasts in situ and increased bone formation.

Many engineering biomaterials are able to release calcium ions, including calcium phosphate and hydroxyapatite, which stimulate osteoblast regeneration and proliferation[22-24]; but these materials resorb more slowly, and the calcium ions released is in lower level. This feature may affect new bone formed in the early time point. Results in this study showed that calcium citrate can stimulate fracture healing in Japanese white rabbits in the early time point. The reason may be that calcium citrate resorb more faster than calcium phosphate, hydroxyapatite and some other biomaterials, so the calcium ions released is in higher level. Further study is needed to verify this.

This model has several limitations, and need further improved: firstly, the experiment time span is not long enough, we should evaluate the experiment result at the 2th,4th, 8th,12th and 24th weeks postoperatively in order to better observe the effect of calcium citrate on bone integration in a rabbit femur defect. Secondly, "critical size defects (CSD)" is mentioned in many animal experiment. The CSD has been defined as the smallest intraosseous wound in an animal that will not heal spontaneously when left untreated for a certain time period[25] or which shows less than 10% bone regeneration during the lifetime of the animal[26]. The CSD has been used as an experimental model for evaluating the effectiveness of newly developed biomaterials[27]. The experiment is not designed according to the CSD, so it increases the difficulty of dealing with our post-experiment result. Furthermore, further studies should be performed to compare calcium citrate with other bone substitutes, and possibly in combination with other osteogenic proteins or growth factors.

Citric acid reacts with many ceramic materials, such as HA, which have been designed and surface-treated to promote cell adhesion[28,29]. Calcium citrate had not been used as a bone graft material, simply because it is difficult to shape in order to provide long-term three-dimensional framework, although calcium citrate is rapidly resorbed *in vivo* releasing calcium ions for the new bone formation.

Future studies examining how to make calcium citrate shaped and what,s the biologic response of this material in other bony sites may reveal further insight into their future clinical use, Of course, there is still a long way to go.

In summary, the present study reveals that calcium citrate affected positively the early periods of fracture healing mechanism in Japanese white rabbits, especially when

the defect is not too large. But the specific mechanism for calcium citrate to stimulate bone formation is unclear. We think that more evidences are required for the better understanding of potential of calcium citrate at the treatment of intrabony defects.

Conflict of interest statement

We declare that we have no conflict of interest.

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