



# Effect of Nano-Capsules Containing Risedronate on Calvarial Bone Formation in Rabbit: Radiography and Biochemical Investigation

Hesam Aldin Hoseinzadeh<sup>1</sup>, Ahmad Asghari<sup>1\*</sup>, Gholamreza Abedi<sup>1</sup>, Abolfazl Akbarzadeh<sup>2,3</sup>, Reza Sedaghat<sup>4</sup>

## Abstract

**Objective:** The main purpose of the current study was to determine the effect of nano-capsules containing risedronate on calvarial bone formation in rabbit.

**Materials and Methods:** Fifteen adult rabbits were allocated to the study. Four holes were created in the calvarial bone. Holes 1-4 were filled as described below: hole 1 was right unfilled and kept as control; hole 2 was filled with nano bone; hole 3 was filled using an autogenous bone; and hole 4 was filled with a mixture of nano-capsules containing risedronate. At 4, 8 and 12 weeks after surgery, blood samples were obtained and red blood cell, white blood cell, hemoglobin, hematocrit, mean cell volume, mean cell HGB and platelet counts were determined. Then animals were scarified and bone density was determined using radiography images.

**Results:** Bone formation in nano risedronate + autograft and autograft were  $0.31 \pm 0.03$  and  $0.25 \pm 0.02$ , respectively, while in nano risedronate and control groups were  $0.11 \pm 0.01$  and  $0.08 \pm 0.02$ , respectively ( $P < 0.05$ ). Significant differences were observed in bone density in nano risedronate + autograft as  $0.37 \pm 0.01$ , while the density in the autograft, nano risedronate, and control groups were as  $0.32 \pm 0.01$ ,  $0.14 \pm 0.01$ , and  $0.09 \pm 0.02$ , respectively ( $P < 0.05$ ). Bone density after 12 weeks were  $0.51 \pm 0.01$ ,  $0.36 \pm 0.02$ ,  $0.21 \pm 0.01$ , and  $0.19 \pm 0.02$  in nano risedronate + autograft, nano risedronate, autograft, and control groups, respectively ( $P < 0.05$ ). In all stages of the study, the nano risedronate + autograft group had better bone formation in comparison to the other groups ( $P < 0.05$ ). Different filling materials of defects had no effect on blood hematology indexes ( $P > 0.05$ ).

**Conclusion:** These results suggest nano risedronate + autograft has positive effects on calvarial bone defects healing in rabbit.

**Keywords:** Nano-capsules, Risedronate, Calvarial healing, Radiography, Rabbit

## Introduction

Bisphosphonates are pyrophosphate analogues with affinity for the hydroxyapatite (1). Based on previous literature, bisphosphonates, non-hydrolysable analogues of inorganic pyrophosphate, are the most prominent pharmaceutical drugs for bone turnover (2). Bone turnover or bone remodeling includes a balanced process of bone resorption through osteoclasts, increase in the bone mineral density, and bone formation through osteoblasts (3). The bisphosphonates prevent bone resorption through the osteoclasts to internalize the bisphosphonate via induction of apoptosis (4). Bisphosphonates have other biological effects on other cells such as joint cells, macrophages or chondrocytes (5). Risedronate improved cartilage lesions in the joints in guinea pig models (6). Numerous investigations were done to produce biological materials for bone regeneration and regrowth. Based on

the unavailability of autogenous bone and the problems associated with surgery, non-autogenous, replacement material remains an alternative treatment (7). It is reported the combination of bone grafts with bioactive materials improves the engraftment, bone formation, and bone defect healing (8).

Risedronate acid has been known as an essential bisphosphonate which exerts curative properties in osteoarthritis progression (9). It has the ability to minimize bone mineral loss in the ligament attaches to bone site and increase ligament mechanical properties (10). It has been reported risedronate improved mandibular bone structure, bone density and bone metabolism in glucocorticoid-induced osteoporosis. Fujita et al (11) reported risedronate amplifies the effect of prednisolone on reducing the trabecular bone. Risedronate also increased bone density and bone mineral content in

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<sup>1</sup>Department of Veterinary Surgery, Science and Research Branch, Islamic Azad University, Tehran, Iran. <sup>2</sup>Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran. <sup>3</sup>Universal Scientific Education and Research Network (USERN), Tabriz, Iran.

<sup>4</sup>Department of Anatomy and Pathology, Faculty of Medicine, Shahed University, Tehran, Iran

\*Corresponding Author: Ahmad Asghari, dr.ahmad.asghari@gmail.com



trabecular bone. Co-application of the risedronate and non-steroidal anti-inflammatory drug therapy in the early stages of osteoarthritis protected trabecular bone marrow against lesion and preserved bone mass in rat (4). Despite the fact that curative effect of the risedronate is elicited, there is no previous role for the effect of the risedronate on calvarial bone formation as well as its possible positive and/or negative blood cells in rabbit model. Moreover, there are no reports on the effects of nano-materials on bone formation. Therefore, the main purpose of the current study was to determine the effect of nano-capsules containing risedronate on calvarial bone formation in rabbit.

## Materials and Methods

### Chemicals

$\epsilon$ -Caprolactone, stannous octoate, dimethyl sulfoxide, polyethylene glycol (2000 MW) and risedronate powder were purchased from Sigma Aldrich (St. Louis, MO). The e-Cl was recrystallized by ethyl acetate.

### Preparation of Risedronate Encapsulated With PCL-PEG Copolymer

The double emulsion method (w/o/w) was employed for encapsulation of the risedronate by PCL1000-PEG2000-PCL1000 copolymer. Five mg/mL of risedronate solution was emulsified in dichloromethane (5 mL). A 400 mg of the copolymer was homogenized by a sonicator (Vibromax, USA) at 20 000 rpm for 30 seconds. Then the emulsion was transferred into the 40 mL of polyvinyl alcohol (0.1%) and probe-homogenized at 72 000 rpm for 1 minute. The emulsion combination was quietly stirred at room temperature until the evaporation of the organic phase (Heidolph Instruments). Two cycles of centrifugation at 12 000 rpm for 1 hour (Biofuge 28 RS, Heraeus Centrifuge, UK) were applied for purification of the nanoparticles and reconstituted with deionized and distilled water. Then nanoparticles were filtered by 1.2 mm filter (Millipore, Bedford, MA). During the second emulsification step, the external aqueous phase was used to improve risedronate entrapment in the nanoparticles.

### Animals

In this study, 15 adult male New Zealand white rabbits (mean BW 3-3.5 kg) were purchased from Razi Vaccine and Serum Research Institute (Tehran, Iran). Animals were adapted to the new experimental condition 1 week before the experimentation. Rabbits were kept at constant and optimum environmental and nutritional conditions (temperature, humidity) with a 12-hour light/dark cycle. Commercial chew pellet and tap water were offered to the animals. Animals had free access to pellet and water. Study procedures were done during 10:00 AM-17:00 PM light phase and executed in accordance with the *Guide for the Care and Use of Laboratory Animals to Investigate Experimental Pain in Animals* (12). Animal handling and experimental procedures were done based on the *Guide for the Care and Use of Laboratory Animals* by the

National Institutes of Health (USA) and the current laws of the Iranian government.

### Surgical Protocol

Six hours prior to the initiation of the study, animals were food deprived and water was withheld for 1 hour before surgery. Then 40 mg/kg of ketamine hydrochloride and xylazine (Alafason, Woeden, Holland, 5 mg/kg) was intramuscularly injected. After induction of anesthesia, each animal was placed on a surgical table and shaved on the calvarium, using povidone-iodine (Betadine) as a topical microbicide. An anteroposterior incision (10 cm) was made using No. 15 surgical blade and skin and periosteum were lifted, cranial to caudal, by a fine periosteal elevator. Four holes were created in the calvarial bone using a micromotor (2000 rpm) and milling round surgical trephine (8 mm diameter) until holes reached the meningeal membrane (the soft meningeal membrane was palpable) (13). Sterile saline was used to prevent overheating of the bone in drilling procedure. The cavities created were each filled with a different substance to assay new bone growth. Holes 1-4 were filled as described below: hole 1 was right unfilled and kept as control; hole 2 was filled with nano bone; hole 3 was filled using an autogenous bone; and hole 4 was filled with mixture of nano-capsules containing risedronate. The filling and placement of the material into the pits was done in a counter clockwise direction and without pressure to ensure the particles did not enter the meningeal space. Then periosteum was sutured with 0-4 simple absorbable sutures (13). A number of 0-3 nylon sutures were used for calvarium and the skin was sutured with a single simple suture. As the animals were coming out of anesthesia, they were transferred to a warm place until regaining full consciousness and then transferred into individual cages. Tramadol (20 mg/kg; intramuscular) and cefazolin (20 mg/kg; intramuscular) were injected to relieve pain and prevent infection until one day after operation. In the cases that swelling or inflammation appeared in the region, the sutures were removed and the infection or possible pus was evaluated. Skin sutures were removed 10 days after surgery (14).

### Hematology Analysis

At 4, 8 and 12 weeks after surgery, blood samples (5 mL) were collected using Vacutainer Tubes from the marginal ear vein for hematological measurements (Becton Dickson, Rutherford, NJ). A Coulter S-plus IV (Coulter Electronics Inc., Hialeah, FL) was calibrated with Coulter S-cal. Coulter 4-C plus control material was used. White blood cell ( $10^3/\mu\text{L}$ ), red blood cell ( $10^6/\mu\text{L}$ ), hemoglobin (g/L), percentage of the hematocrit, mean cell volume (fL), mean cell HGB (pg) and platelet ( $10^3/\mu\text{L}$ ) levels were determined.

### Radiologic Investigation

At 4, 8 and 12 weeks after surgery, one animal with the average weight from each group was euthanized by

overdose of intramuscularly injected sodium thiopental. Then the calvarium skin, and the skull containing 4 defects were removed, fixed in formalin (10%), and transferred to radiology section. Radiographs were taken from dorsal-ventral position using an aluminum phantom. The images were analyzed using ImageJ software and the bone formation in each defect was determined per mm aluminum equivalent.

### Statistical Analysis

All data were recorded on and prepared in Microsoft Excel spreadsheets and then analyzed by Student *t* test, Mann-Whitney rank sum test and one-way or two-way variance analysis (ANOVA) using SPSS statistical software. Results were presented as means  $\pm$  SD. Differences between groups were tested by Student-Newman-Keuls tests. Differences between groups were significant at  $P < 0.05$ .

### Results

Based on the radiographic densitometry image, after 4 weeks, bone density in nano risedronate + autograft and autograft groups were  $0.31 \pm 0.03$  and  $0.25 \pm 0.02$ , respectively; while in nano risedronate and control groups were  $0.11 \pm 0.01$  and  $0.08 \pm 0.02$ , respectively. Based on statistical analysis, significant difference was observed between nano drug + autograft and autograft groups ( $P < 0.05$ ) (Figures 1 and 2).

Based on Figures 3 and 4, significant differences were observed on bone density between 4 groups after 8 weeks. As seen, the bone density in nano risedronate + autograft was  $0.37 \pm 0.01$  while in the autograft, nano risedronate, and control groups were  $0.32 \pm 0.01$ ,  $0.14 \pm 0.01$ , and  $0.09 \pm 0.02$ , respectively ( $P < 0.05$ ).

According to Figures 5 and 6, significant differences were observed on bone density after 12 weeks as bone density were  $0.51 \pm 0.01$ ,  $0.36 \pm 0.02$ ,  $0.21 \pm 0.01$ , and  $0.19 \pm 0.02$  in nano risedronate + autograft, nano risedronate, autograft, and control groups, respectively ( $P < 0.05$ ).

The overall bone densitometry measurements is



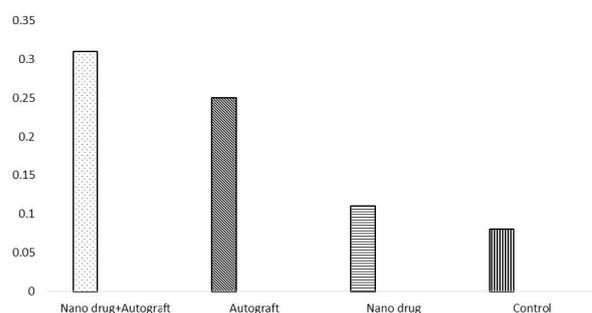
**Figure 1.** The Calvarium Radiographic Image 4 Weeks After Surgery. The first hole: right unfilled and kept as control. The second hole: filled with nano bone. The third hole: filled using an autogenous bone. The fourth hole: filled with a mixture of nano-capsules containing risedronate.

presented in Figure 7. As seen in all stages of the study (4, 8 and 12 weeks), the nano risedronate + autograft had better bone formation in comparison to the other groups ( $P < 0.05$ ).

The effect of calvarial defect filled with nano-capsules containing risedronate on hematology in rabbit is presented in Table 1. No significant effect was observed on white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean cell volume (MCV), mean cell HGB (MCH) and platelets (PLT) levels as hematology indices at 4, 8 and 12 weeks after surgery ( $P > 0.05$ ).

### Discussion

Based on the literature, this is the first report on the role of the nano risedronate + autograft on calvarial healing in rabbit. According to the results, the nano risedronate + autograft had better bone formation compared to the other groups. Tekin et al (15) reported amplified bone density through risedronate and enhanced bone healing and osteogenesis of rabbit. Administration of risedronate (35 mg/wk) plus Ca+D for 12 months significantly increased bone mineral density (10). Limited information exists on the microanatomical distribution of bisphosphonates by localization in animal studies. Bisphosphonates can



**Figure 2.** The Densitometry of Bone 4 Weeks After Surgery.



**Figure 3.** The Calvarium Radiographic Image 8 Weeks After Surgery. The first hole: right unfilled and kept as control. The second hole: filled with nano bone. The third hole: filled using an autogenous bone. The fourth hole: filled with a mixture of nano-capsules containing risedronate.

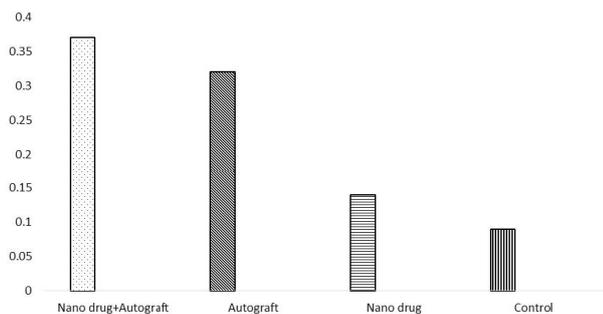


Figure 4. The Densitometry of Bone 8 Weeks After Surgery.

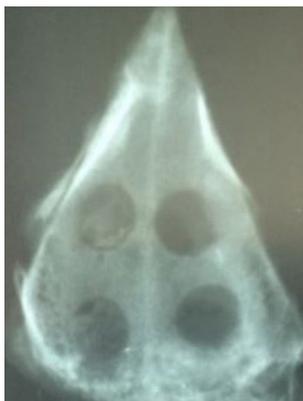


Figure 5. The Calvarium Radiographic Image 12 Weeks After Surgery. The first hole: right unfilled and kept as control. The second hole: filled with nano bone. The third hole: filled using an autogenous bone. The fourth hole: filled with a mixture of nano-capsules containing risedronate.

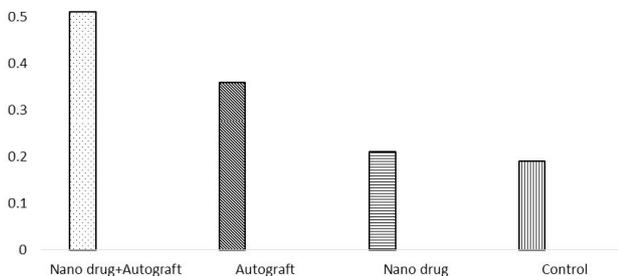


Figure 6. Bone Densitometry 12 Weeks After Surgery.

increase bone formation sites (10). Risedronate decreased cartilage swelling and synovial inflammation (2) and suppressed expression of pro inflammatory cytokines in bone marrow adipocytes (16). In patients suffering from bone loss, risedronate is effective for the recovery of ADT-induced bone loss (17). Bone resorption and the osteoclast number decreased, but no reduction was found in angiogenesis in mice after 2 and 4 weeks (18). It is reported tissue and trabecular bone healing was higher in the osteotomy via zoledronate administration in rabbits (19). A higher bone formation was observed in human periodontitis treated with 1% alendronate gel plus mechanical treatment (scaling and root planning) (20).

The exact mechanism for how risedronate improves

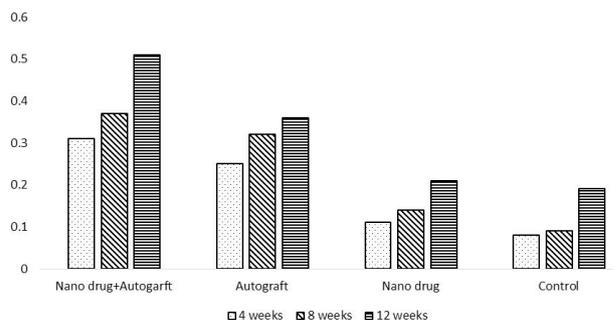


Figure 7. The Overall Bone Densitometry Measurements.

Table 1. Effect of Calvarial Defect Filled With Nano-Capsules Containing Risedronate on Hematology in Rabbit

Hematological Values	Weeks Post-surgery		
	4	8	12
WBC ( $10^3/\mu\text{L}$ )	9.04	8.8	8.5
RBC ( $10^6/\mu\text{L}$ )	5.3	6	5.5
HGB (g/L)	120	130	134
HCT (%)	28	37	39
MCV (fL)	55.6	58.1	61.2
MCH (pg)	22.3	24.3	20.7
PLT ( $10^3/\mu\text{L}$ )	467	564	458

Abbreviations: White blood cell (WBC,  $10^3/\mu\text{L}$ ), red blood cell (RBC,  $10^6/\mu\text{L}$ ), hemoglobin (HGB, g/L), hematocrit (HCT, %), mean cell volume (MCV, fL), mean cell HGB (MCH, pg) and platelets (PLT,  $10^3/\mu\text{L}$ ).

bone density and bone formation is not fully studied; perhaps risedronate acts via parathyroid hormone (PTH) (5). However, there is a report which states risedronate pretreatment might decrease the bone anabolic response to PTH (5). Interestingly, Halasy-Nagy et al (21) reported suppression of bone resorption by risedronate is independent of its role in apoptosis. Risedronate also inhibits pro-osteoclastic cytokine expression in bone marrow (17). Perhaps some activities of Risedronate such as suppression of bone resorption is mediated by inhibition of osteoclast activity and anti-inflammatory effect including substance P production through the tumor necrosis factor alpha (TNF- $\alpha$ ) pathway (22). As observed in this study, nano risedronate as filling material of the defects for 12 weeks had no effect on blood hematology indexes. Therefore, it seems risedronate is safe. However, there was no previous study on the safety of risedronate on blood biochemical parameters, but it is well documented long time exposure to the bisphosphonates causes adverse and toxic effects (23).

To conclude, risedronate is an effective inhibitor of bone resorption. It has been reported risedronate is effective against postmenopausal osteoporosis (24), though the mechanisms through which risedronate prevents bone loss have not been fully elucidated (15). It seems further researches are required to determine direct cellular and molecular mechanisms of action for further application of risedronate in clinical trials.

## Conflict of Interests

Authors declare no conflict of interests.

## Ethical Issues

All protocols of the study were approved by the Ethics Committee of Islamic Azad University, Science and Research Branch, Tehran, Iran.

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None.

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