

Effects of dietary supplementation on bone healing in bisphosphonate treated rabbits

Mahmood A. Aljumaily*, Kassim S. Ibrahim**, Hazim Al-allaf***

* Department of Surgery, ** Department of Pathology, *** Department of Imaging and Radiology, College of Medicine, University of Mosul.

(Ann. Coll. Med. Mosul 2010; 36 (1 & 2): 114-120).
Received: 10th May 2010; Accepted: 27th Oct 2010.

ABSTRACT

Objective: The aim of this study was to evaluate the effect of the daily oral administration of vitamin D, calcium, fluoride and vitamin C as dietary supplementation on bone healing in bisphosphonate treated experimental animals (rabbits).

Material and methods: Eight young male rabbits divided into two groups after induction of open ulnar osteotomy, both groups received weekly 1 mg/ kg BW of alendronic acid (alendron) orally starting 6 days before osteotomy for five weeks, the experimental group received daily dose of vitamin D, calcium, fluoride and vitamin C as dietary supplementation from the second post operative day for four weeks. The control group received ordinary diet. At the end of the fifth week the animals' sacrificed and the specimens taken for radiological and computerized tomography (CT) scan densitometry and histomorphometric evaluation carried out for the callus at site of osteotomy.

Results: All ulnar bone osteotomies in both groups united at the end of the fifth week macroscopically and radiologically. The callus density was measured in site of osteotomy by CT scan densitometry, its mean in the experimental group was 681 ± 219 and in the control group was 492 ± 233 . The difference between the experimental and control group was significant, (P value < 0.05). The histological examination of the bone at site of osteotomy showed healing with woven bone predominantly and some lamellar bone and cartilage. The mean of histomorphometric evaluation of healing in site of osteotomy were 9.07 ± 0.80 in experimental group, while in control group were 8.70 ± 0.80 . The difference between two means was not significant

Conclusion: The present study demonstrates that a daily oral administration of vitamin D, calcium, fluoride and vitamin C as dietary supplementation in bisphosphonate treated rabbits enhance bone healing by increase callus density.

Keywords: Dietary supplementation, bisphosphonate, vitamin D, calcium, fluoride, vitamin C, bone healing.

الخلاصة

الأهداف: من أجل دراسة تأثير الكالسيوم وفيتامين دي والفلوريد وفيتامين سي كمضاف غذائي على التئام كسور العظام في الحيوانات المخبرية (الأرانب) المعالجة بالبايسفوسفونيت من دون استخدام وسائل التثبيت الداخلي.

المواد وطريقة العمل: تم استخدام ثمانية من الأرانب الذكور المكثرة محليا في هذه الدراسة. تمت هذه الدراسة في بيت الحيوانات بقسم الجراحة التجريبية في كلية الطب في الشهرين الأخيرين من ٢٠٠٩. تم إحداث كسر جراحي (قص العظم) في عظم الزند بقطع جراحي وبدون التثبيت الداخلي. قسمت الحيوانات إلى مجموعتين كل مجموعة تتكون من أربعة حيوانات. أعطيت المجموعتان مركب الالدرونيت (بايسفوسفونيت) بجرعة ملغرام واحد لكل كيلو غرام من وزن الحيوان واعتبارا من اليوم السادس قبل العملية وجرعة أسبوعية لمدة خمسة أسابيع. أعطيت المجموعة الأولى جرعة يومية من

الكالسيوم وفيتامين دي والفلوريد وفيتامين سي لمدة أربعة أسابيع فيما كانت المجموعة عينة ضابطة. وبعد خمسة أسابيع تم قتل الحيوانات وأخذت عينات العظام للفحص الشعاعي وقياس كثافة العظم الملتئم بواسطة التصوير الطبقي المحوري وتم فحص نسيجي للعظم لملاحظة التئام العظم في مكان قص العظم.

النتائج: التأم كل العظام بعد قصها في نهاية الأسبوع الخامس ظاهريا وإشعاعيا. كانت كثافة العظم الملتئم بواسطة التصوير الطبقي المحوري في محل القطع بعد خمس أسابيع في مجموعة التجربة بمعدل 681 ± 216 وأما المجموعة الضابطة فكانت كثافة العظم في محل القطع 492 ± 233 وهذا فرقا معنويا. أظهر الفحص النسيجي التئام العظم في محل القص بعظم متموج أولي مع بعض الغضاريف وبعض العظام المكتملة. كان معدل مستوى التئام محل قص العظم في حيوانات التجربة $9,07 \pm 0,8$ وفي العينة الضابطة $8,70 \pm 0,8$ وهذا ليس فرقا معنويا.

الخلاصة: إن هذه الدراسة تبين بان إضافة الكالسيوم وفيتامين دي والفلوريد وفيتامين سي كمضاف غذائي يوميا يساعد في التئام كسور العظام في الأرانب المعالجة بالبيسفوسفيت بزيادة كثافتها.

Bone healing is an extremely complex process, and has always been a major medical concern; different pharmacological agents affect bone healing⁽¹⁾. Many systemic and local factors influence fracture healing; nutritional state including vitamins, minerals and trace elements supplementation is one of these factors⁽²⁾. Bisphosphonates increase the callus volume, while delaying the remodeling of the callus woven bone and delay removal of cartilage⁽³⁻⁵⁾. Calcium and Vitamin D3 administration had positive influence on fracture healing^(6,7). Vitamin D3 (cholecalciferol) and its derivatives had been shown to be essential hormones for process of fracture healing⁽⁸⁾. Vitamin C supplementation enhances fracture healing by improving the mechanical resistance of fracture callus and improving the bone mineralization^(9,10). Fluoride dietary supplementation accelerates the fracture healing⁽¹¹⁾.

Bisphosphonates in combination with calcium and vitamin D have become the first line therapy to patients with osteoporosis⁽¹²⁾. Bisphosphonates increases the callus volume and increases mineral density of callus, while delaying remodeling of callus woven bone into lamellar bone⁽¹²⁾. Histomorphometry is the gold standard for assessing bone because it is the only method for direct in situ analysis of bone cells and their activities^(13,14). Histomorphometric (quantitative) evaluation of the callus was shown to be compatible with bone healing achieved in qualitative experimental methods⁽¹⁵⁾. Advent of x-ray computed tomography (CT) has provided the

opportunity to quantitatively and non-destructively assess bone structure and density⁽¹⁶⁾. The mineral density of callus correlated positively with callus strength and stiffness⁽¹⁶⁾.

The aim of this study was to evaluate the effect of the daily oral administration of vitamin D, calcium, fluoride and vitamin C as dietary supplementation on bisphosphonate treated bone healing.

Material and methods

This study was approved by the research ethics committee at the College of Medicine, University of Mosul and follows the council for international organization of medical sciences ethical code for animal experimentation⁽¹⁷⁾. Eight young male aged 4 months locally bred New Zealand rabbits from animal house, College of Medicine, University of Mosul were used in this study at first of November 2009 to thirtieth of April 2010. Their average weight 1470 grams ranged between 1260 grams and 1520grams. The animals were kept in separate metallic cages for one week for adaptation in animals' house. In each cage one animal feed with standard ration and water. The animals received oral dose 1 mg/kg BW of alendronic acid (alendron) weekly starting 6 days before osteotomy and continue for five weeks.

Experimental technique

Food was suspended eight to ten hours prior to administration of anesthesia. To decrease the vagal tonus, each animal received 0.2 mg/kg dose of atropine sulphate by

intramuscular injection. Animals were anesthetized by intramuscular injection of ketamine (50 mg/kg of body weight) and intramuscular injection of diazepam (5.0 mg/kg of body weight). Preoperative antimicrobial prophylaxis consisting of 100 mg/kg of ceftriaxone were injected subcutaneously in proximal part of the same limb. Sample of venous blood aspirated to measure serum calcium, phosphate, and alkaline phosphatase.

The right forelimb was shaved and cleaned by betadine solution. Under an aseptic conditions technique, the right ulna of each animal was accessed by an anterior longitudinal skin incision of approximately 20 mm. After division of the skin and subcutaneous tissue, the fascia, the muscles and tendons were retracted and the periosteum was opened and dissected from the ulna. The ulna shaft was exposed; osteotomy was performed on the exposed portion of the ulna by mean of a one millimeter blade thickness sterile hand saw. No internal fixation used in these osteotomies. The incision was closed, using absorbable 5-0 polyvicryl sutures for the fascia and 4-0 monofilament PDS sutures for the skin, local dressing applied locally using sterile gauze covered with adhesive plaster.

The animals were assigned to two groups; the first group (4 animals) as experimental group received a daily dose of 100 IU vitamin D, 100 mg calcium, 25 µg fluoride and 25 mg vitamin C as dietary supplementation, and continued for 28 consecutive days thereafter. The second group was control group (4 animals) received ordinary diet. After five weeks sample of blood aspirated to measure serum calcium, phosphate, and alkaline phosphatase from animals of both groups. Animals of both groups were anesthetized again as described previously and killed with a 2 ml intracardiac injection of potassium chloride⁽¹⁵⁾. The right ulna of each animal was removed, dissected from the surrounding soft tissue.

The samples were examined radiologically by Siemen- Sirography fluoroscopy equipment 62 K.T.; the KV used in taking x-ray is 30 KV,

50mA, (fig.1). The CT scan examination was carried out to measure the density of callus at the site of osteotomy .The CT scan equipment is light speed, multidetector equipment, General Electric (GE), 32 Yokogawa Medical System, taken TA 0.6 mm slice thickness. The mean of five points was taken at the site of osteotomy to measure the density of callus, the means and standard deviations of these values were calculated (fig. 2).



Figure (1): Radiological examination (X-ray) of rabbit forearm shows healed ulnar osteotomy in stage of union.

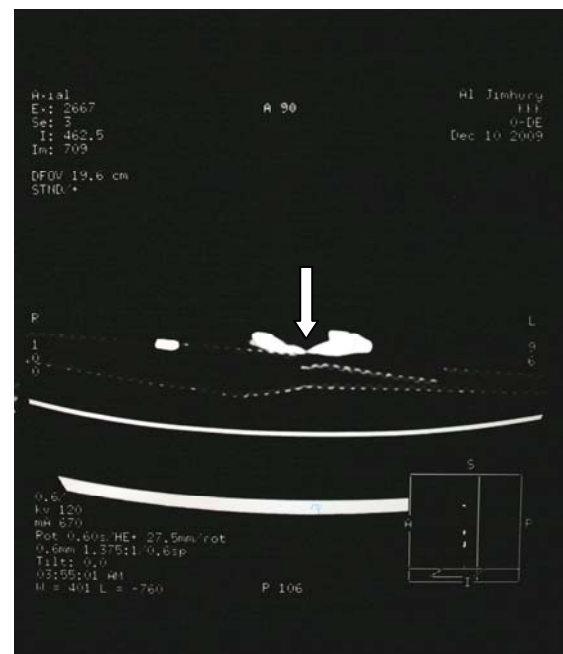


Figure (2): The site of osteotomy identified by CT scan and density measured in five points in site of osteotomy, and its means calculated.

The sites of osteotomy were carefully exposed by removal of all the soft tissue. The ulnar bones were removed, and fixed with 10% formaldehyde solution. After fixation, they were decalcified in 10% formic acid. The decalcification process demineralized the bone, leaving only the soft tissues and bone matrix. This was done to ensure that thin sections could be examined histologically. Thin sections embedded in paraffin wax were cut and stained with haematoxylin and eosin. The site of osteotomy was examined histologically. The progression of fracture-healing in each specimen was quantified with the use of a scale that assigns a grade based on the relative percentages of fibrous tissue, cartilage, woven bone, and mature bone in the callus (histomorphometric evaluation) (fig. 3)⁽⁷⁾. Grade 1 indicates fibrous tissue; grade 2, predominantly fibrous tissue with some cartilage; grade 3, equal amounts of fibrous tissue and cartilage; grade 4, all cartilage; grade 5, predominantly cartilage with some woven bone; grade 6, equal amounts of cartilage and woven bone; grade 7, predominantly woven bone with some cartilage; grade 8, entirely woven bone; grade 9, woven bone and some mature bone; and grade 10, lamellar (mature) bone⁽⁷⁾. The grading was done blindly without knowing which treatment had been given. The mean of fracture healing scores were calculated for each group.

Statistical analysis

Results were reported as mean \pm standard deviation. The unpaired student (t) test was used to calculate the differences between two means. The p value was considered significant if it was less than 0.05.

Results

All animals survived to the end of the study. Neither wound infection nor wound dehiscence were observed in the animals of either group. All animals were (normocalcaemic and normophosphataemic) at time of osteotomy and at end of the study. The normal serum calcium, serum phosphate, serum alkaline phosphatase in normal rabbit was 3.0- 4.2

mmol/l, 1.28- 1.92 mmol/l, and 10-70 IU/L respectively⁽¹⁸⁾.

Macroscopic evaluations demonstrated that all osteotomies were united by the end of the study. Radiological examination showed that all osteotomies were united (fig.1). The mean of CT scan density of callus at the site of osteotomy in experimental group was 681 with a standard deviation 219. The mean of CT scan density of callus at the site of osteotomy in control group was 492 with a standard deviation 233 (fig. 2). There were significant differences in density of callus between the experimental group and control group, (P value is < 0.05), (table 1).

The Histopathological examinations of the osteotomy site showed healed bone with predominantly woven bone with some areas of mature (lamellar) bone with few areas of cartilage, there was no evidence of infection or foreign body reaction (fig. 3).

The mean of histomorphometric evaluation of healing in site of osteotomy was 9.07 with standard deviation 0.80 in experimental group. In control group, the mean of histomorphometric evaluation of healing in the site of osteotomy was 8.70 with standard deviation 0.80. The difference between the two means by t test was not significant, (P value is 0.18) (table 1).

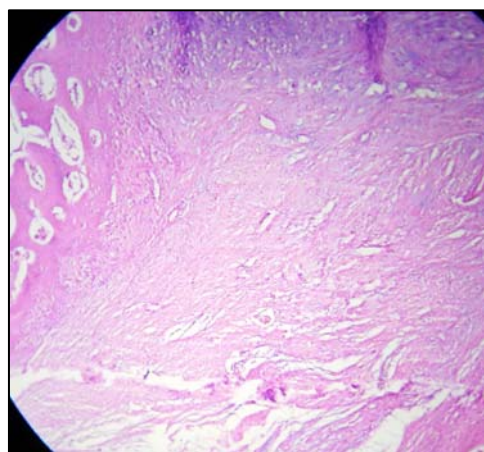


Figure (3): Histological examination showing new bone formation at site of osteotomy in advanced stage of healing (predominantly woven bone with some lamellar bone and small areas of cartilage) without any evidence of chronic inflammation or giant cell reaction at osteotomy site.

Table (1): Histomorphometric evaluation of bone healing in site of osteotomy and callus density evaluation by CT scan in site of osteotomy in experimental group and control group.

	Experimental group		Control group		P value	Significance of difference
	mean	Standard deviation	mean	Standard deviation		
Histomorphometric evaluation of bone healing in site of osteotomy	9.07	0.8	8.70	0.8	0.184	Not significant
Callus density evaluation by CT scan in site of osteotomy	681	219	492	233	0.012	significant

Discussion

The production of a better and stronger healing bone has attracted the interest of many investigators in the past^(2, 12). Numerous substances have been used to increase both the strength and rate of production of fracture callus^(12, 19). Assessment of fracture healing is a common problem in orthopedic practice and research⁽²⁰⁾. Fracture healing can be evaluated through clinical, radiological, mechanical, histological, chemical, or biological study^(1, 2, 15, 20).

Bisphosphonates are used in the clinical treatment of several diseases such as osteoporosis, bone metastases, malignant hypercalcemia, Paget's disease, hyperparathyroidism, and osteogenesis imperfecta. Bisphosphonates inhibit osteoclasts recruitment, and they are anti-catabolic agents. In experimental studies carried out on rats and rabbits, it is believed that bisphosphonates modify the reparative pattern of the callus, it can increase the strength of fracture site by dramatically increasing callus volume; however they delay callus remodeling of woven bone into lamellar bone^(3,5,20,21). Bisphosphonates treatment during fracture does not delay enchondral ossification, but significantly affect bone remodeling⁽⁵⁾.

In this study, the combination of 100 IU vitamin D, 100 mg calcium, 25 µg fluoride and 25 mg vitamin C as a daily dietary supplementation to fractured rabbits which had been treated by 1 mg/kg BW bisphosphonate showed significant increased in the density of

callus in CT densitometry measurement at the site of osteotomy in comparison with control group, (P value < 0.05) (table 1). This finding confirms the idea that this dietary supplementation enhances bone healing when given in combination to animals treated by bisphosphonates. Vitamin D, calcium, fluoride and vitamin C are well known drugs, used widely in the treatment of many orthopedic diseases, and they are safe in therapeutic doses. Their combination in therapeutic doses can be used as dietary supplementation to support bone healing process in patients treated with bisphosphonate.

The histomorphometric evaluation in this study showed no significant difference between experimental and control group (P value is > 0.05), this might be explained by the effect of bisphosphonate through inhibition of osteoclasts and bone remodeling, which delay the maturation of bone and delay its change to lamellar bone. Histopathological examination showed good union without complications (infection or giant cell reaction) in both groups.

In human and experimental animals (rats) it was proved, that calcium ion is an essential structural component of the skeleton and essential for the acceleration of healing of fractured bones^(6,7). Vitamin D is critically important for development, growth and maintenance of a healthy skeleton in human and animals from birth until death^(6,7,8). Vitamin D and its active derivatives could promote fractures healing by improving the histomorphometric parameters, mechanical strength and tendency to increase

transformation of woven bone into lamellar bone in experimental animals (rats and rabbits) ^(22,23,24,25). In rabbits, the amount of ossified tissue was found to be significantly higher in the fluoride treated callus. The bone mechanical properties of healed bones improved also in the fluoride treated callus ⁽²⁶⁾. Vitamin C supplementation to the experimental animals (rats and rabbits) improved the mechanical resistance of fracture callus and made bone healing faster than the control groups ^(9,10). In reviewing the available medical literature there was no report on the combination of vitamin D, calcium, fluoride and vitamin C in bisphosphonate treated experimental animals.

In our study, CT bone mineral density measurement found to be noninvasive, and a reliable tool for quantification of the fracture repair process in experimental animals ⁽²⁰⁾. The mineral density of callus correlated positively with callus strength and stiffness ^(16, 27).

The small number of animals used in this experiment is sufficient to get a conclusion and to stimulate more wide clinical studies when financial and technical support are available, this also fits with animal studies protocol which should be designed to minimize the number of animals used ⁽¹⁷⁾. The rabbits were chosen as the animal model because it is widely used in studies of bone preparations and its bone similar to human bone. The ulna selected because it is easy to access, had good size, its fixation not essential because of close relation to radius, and it is easy to harvest.

In conclusion, our study demonstrated that combination of vitamin D, calcium, fluoride and vitamin C in bisphosphonate treated rabbits improved bone healing process in rabbit ulnar osteotomy. This effect was characterized by increased callus density, without significant effect on histomorphometric criteria.

References

1. Pountos I, Georgouli T, Blokhuis T J, Pape HC, Giannoudis PV. Pharmacological agents and impairment of fracture healing. *Injury* 2008; 39:384-94.
2. Wood II G. General principle of fractures treatment. In: Canale S, Beaty J. Campbell's operative orthopaedics. 2008, 11th ed. Mosby. Philadelphia: 3017-84.
3. Komat S S, Mori S. Bone quality in fracture callus. *Cli calcium* 2005; 15: 977-83.
4. Mashiba T, Mori S. Bone fracture and the healing mechanism. The effect of antiresorptive agents on fracture healing. *Cli calcium* 2009; 19: 673-9.
5. Mcdonald M, Dulai S, Godfrey C, Amanat N, Szynda T, Little J. Bolus or weekly zaldronic acid administration dose not delay endchondral fractures repair. *Bone* 2008; 43: 653-62.
6. Doetsch A, Faber J, Lynnerup N, Watjen J, Bliddal H, Danneskiold B. The effect of calcium and vit D3 supplementation on the healing of the proximal humeral fractures. *Calcif Tissue Int.* 2004; 75: 183- 8.
7. Aslan B, Kalaci A, Bozlar M, Atik E, Yanat A, Tasci A. Effect of vitamin D3 and calcium on fracture healing in rat. *Turkiye Klinileri J Med Sci.* 2006; 26: 296- 302.
8. Schunack W. Vitamin D3 – a prodrug of different D3 hormones. *Med Klin.* 2006; 101 Suppl 1: 20-4.
9. Alcantara T, Delgado A, Vega M, Carrascal M, Munuera L. The effect of vit C on fracture healing in elderly osteogenic disorder shionogi rats. *J Bone Joint Surg-Br.* 2007; 89: 402-7.
10. Yilmaz C, Erdemli E, Selek H, Kinik H, Arikian M, Erdemli B. The contribution of vitamin C to healing of experimental fractures. *Arch Orthop Trauma Surg.* 2001; 121:426- 8.
11. Bialecki D, Evaluation of the repair process in mechanically injured rat bone stimulated by sodium fluoride with non-toxic doses. *Ann Acad Med Stetin.* 1999; 45: 195-209.
12. Seebach C, Kurth A, Marzi I. The influence of bisphosphonates on fracture healing. *Orthopedic* 2007; 36: 136-40.
13. Iwaniec UT, Wronski TJ, Turner RT. Histological analysis of bone. *Methods Mol Biol.* 2008; 448: 325-41.
14. Humadi A, Alhadithi RH, Alkudiyari SI. Validity of the DEXA diagnosis of involuntional osteoporosis in the patients

- with femoral neck fractures. *Indian J Orthop.* 2010; 44: 73-78.
15. Matos MA, Araujo FP, Paixao FB. Histomorphometric evaluation of bone healing in rabbit fibular osteotomy model without fixation. *J Orthop Surg Res.* 2008; 3: 4-8.
 16. Nyman JS, Munoz ZS, Jadhav S, Mansouri A, et al. Quantitative measures of femoral fracture repair in rats derived by micro-computed tomography. *J Biomech.* 2009; 42: 891-7.
 17. Harcourt- Brown F. *Textbook of rabbit medicine* 2002. butterworth Heinemann. Edinburgh: 121- 164.
 18. Rispoli D M, Jepsen K G, Plancher K D. Principle of practice and statistics. In: Miller M D. *Review of orthopaedics.* 2004. 4th ed. Saunders. Philadelphia: 670- 79.
 19. Dzioba RB, Jackson RW. Effects of phosphate supplementation on intact and fractured femora of rats: a biomechanical study. *Can Med Assoc J.* 1977; 19: 1173-5.
 20. Shefelbine SJ, Simon U, Claes L, et al. prediction of fracture callus mechanical properties using micro-CT image and voxel-based finite element analysis. *Bone* 2005; 36: 480-8.
 21. Gertenfeild LC, SacksDJ, Pelis M. et al. comparison of effects of the bisphosphonate aldrionate versus the RANKI inhibitors denosumab on murine fracture healing . *J Bone Miner Res.* 2009; 24: 179-81.
 22. Sheweita SA, Kholshhal KL. Calcium metabolism and oxidative stress in bone fractures: role of antioxidants. *Curr Drug Metab.* 2007; 8: 519-25.
 23. Fu L, Tang T, Miao Y, Dia K. Effects of 1,25 dihydroxy vitamin D3 on fracture healing and bone remodelling in ovariectomized rat femora. *Bone* 2009; 44: 894-8 .
 24. Delgado-Martinez AD, Martinez ME, Carrascal MT, Rodriguez-Avial M, Munuera L. Effect of 25-OH- vitamin D on fracture healing in elderly rats. *J Orthop Res.* 1998; 16: 650-3.
 25. Omeroglu H, AtesY, Akkus O, Korkuzuz F, Bicimoglu A, Akkas N. biomechanical analysis of the effects of single high-dose vitamin D3 on fracture healing in a healthy rabbit model. *Arch Orthop Trauma Surg.* 1997; 116: 271-4.
 26. Shteyer A, Liberman R, Simkin A, Gedalia I. Effect of local application of the fluoride on healing of experimental bone fractures in rabbits. *Calcif Tissue Res.* 1977; 22: 297-302.
 27. Augat P, Merk J, Gemant HK, Claes. Quantitative assessment of experimental fracture repair by peripheral computed tomography. *Calcif Tissue Int* 1997; 60: 194-9.