Effects of vitamin D, calcium, fluoride and vitamin C as dietary supplementation on bone healing in rabbits

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ABSTRACT

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

Material and methods: Eight young male rabbits divided into two groups after induction of open ulnar osteotomy, the experimental group received daily dose of vitamin D, calcium, fluoride and vitamin C as dietary supplementation from the second post operative day for 28 days. The control group received ordinary diet without any food supplementation. At the end of the fifth week, the animals were sacrificed and the specimens taken for radiological and CT scan densimetry, and histological evaluation carried out for calluses at site of osteotomy.

Results: All ulnar bone osteotomies in both groups united at the end of the fifth week macroscopically and radiologically. There was no significant difference in serum calcium, phosphate, and alkaline phosphatase preoperatively, and at the end of the fifth week. The callus density was measured in site of osteotomy by CT scan densimetry and its mean in experimental group was 331.1 ± 81.3, and control group was 199.7± 32.1. The difference between the experimental and control group was highly significant, (P value is < 0.001). The histological examination of the bone at site of osteotomy showed healing with woven bone predominantly and some lamellar bone and cartilage.

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

Keywords: Vitamin D, calcium, fluoride, vitamin C, dietary supplementation, bone healing.

الخلاصة

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

المواد وطريقة العمل: تم استخدام ثمانية من الأرانب النيوزلندية 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 الأيمن بقطع جراحيا تحت التخدير العمومي وبواسطة المنشار اليدوي وبدون التثبيت الداخلي. قسمت الحيوانات إلى مجموعتين كل مجموعة تتكون من أربعة حيوانات. أعطيت المجموعة الأولى جرعة يومية من الكالسيوم وفيتامين دي والفلوريد وفيتامين سي فيما كانت المجموعة عينة ضابطة. وبعد خمسة أسابيع تم قتل الحيوانات وأخذت عينات العظام للفحص ألشعاعي وقياس كثافة العظم الملتئم بواسطة التصوير الطبقي المحوري وتم فحص نسيجي للعظم لملاحظة التئام العظم في مكان قص العظم.

النتائج: التأمت كل العظام بعد قصها في نهاية الأسبوع الخامس ظاهريا وإشعاعيا. لم يظهر فرق معنوي في مستوى الكالسيوم والفوسفات وإنزيم الفوسفات القاعدي في مصل الدم قبل التجربة وفي نهايتها بعد خمسة أسابيع. كانت كثافة العظم

الملتئم بواسطة التصوير الطبقي المحوري في محل القطع بعد خمسة أسابيع في مجموعة التجربة بمعدل ٣٠٢ ± ١٢٤ وأما المجموعة الضابطة فكانت كثافة العظم في محل القطع ١٩٩,٨ ± ٣٢,١. أظهر الفحص النسيجي التئام العظم في محل القص بعظم متموج أولي مع بعض الغضاريف وبعض العظام المكتملة.

الخلاصة: إن هذه الدراسة تبين بان إضافة الكالسيوم وفيتامين دي والفلوريد وفيتامين سي كمضاف غذائي يساعد في التئام كسور العظام.

Many systemic and local factors influencing fracture healing: nutritional state including vitamins, minerals and trace elements supplementation is one of these factors [1]. Calcium and Vitamin D3 administration had positive influence on fracture healing [2,3]. Vitamin D3 (cholecalciferol) had been shown to be essential hormone for the process of fracture healing [4]. Vitamin C supplementation enhances fracture healing by improving the mechanical resistance of fracture callus and improving the bone mineralization [5,6]. Fluoride dietary supplementation accelerates the fracture healing [7].

Assessment of fracture healing is a common problem in orthopedic practice and research [8]. Fractures healing can be evaluated through clinical, radiological, mechanical, histological, chemical or biological study[1,2,9]. Bone mineral density measurement by computerized tomography (CT) is noninvasive, and a reliable tool for quantification of the fracture repair process in experimental animals [10]. The mineral density of callus correlated positively with callus strength and stiffness [11].

To our knowledge there was no study on effects of the combinations of the vitamin D, calcium, fluoride and vitamin C as dietary supplementation on bone healing. The aim of this study was to evaluate the effect of the daily oral adminstration of vitamin D, calcium, fluoride and vitamin C as dietary supplementation on bone healing in rabbits.

Material and methods

This study was approved by the research ethics committee at the College of Medicine, University of Mosul, and follows the ethical code for animal experimentation of the Council for International Organization of Medical Sciences. Eight young male aged 4 months locally breeded NewZealand rabbits from animal house, College of Medicine, University of Mosul, were used in this study, from the first of November to the 30th of December 2009. Their average weight was 1460 grams (ranged between 1250 grams and 1600 grams). The animals were kept in separate metallic cages for one week, for adaptation in animal's house. In each cage one animal feed with standard ration and water.

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 to 10.0 mg/kg of body weight). Preoperative antimicrobial prophylaxis consisting of 50 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 anteromedial longitudinal skin incision of approximately 20 mm. After division of the skin and subcutaneous tissue, the fascia, the muscles and tendon were retracted and the periosteum was opened and dissected from the ulna. The ulnar shaft was exposed; osteotomy was performed on the exposed portion of the ulna by means of a one mm blade thickness sterile hand saw. The incision was closed by layers, using absorbable 5-0 polyvycril 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 one of the following groups, the first group (4 animals) as experimental group received daily dose of vitamin D, calcium, fluoride and vitamin C as dietary supplementation, the second group (4 animal) as control group.

A total dose of 100 IU vitamin D, 100 mg calcium, 25 µg fluoride, and 25 mg vitamin C dissolved in 10 ml water administrated orally on the second post-operative day and continued for 28 consecutive days thereafter. In the control group, the same volume of bi-distilled water was administered under similar conditions.

After five weeks, samples 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. The right ulna of each animal was removed, dissected from the surrounding soft tissue.

The samples examined radiologically by Siemen- Sirography fluoroscopy equipment 62 K.T.; the KV used in taking x-ray was 30 KV, 50mA, (fig 1). The computerized tomography (CT) scan examination 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 taken at the site of osteotomy to measure the density of callus and five points at the normal bone proximal to osteotomy, the means and standard deviations of these values calculated (fig 2).

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% foramic 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.

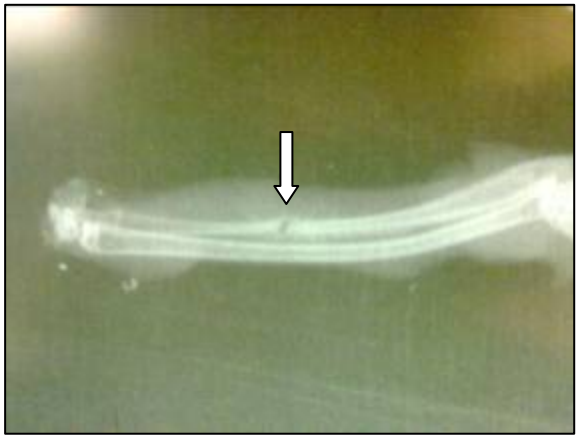

Figure (1): Radiological examination (X-ray) of rabbit forearm shows healed ulnar osteotomy in stage of union.

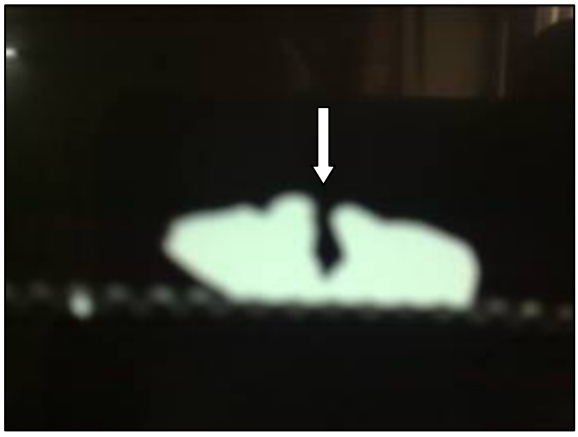

Figure (2): The site of osteotomy identified by CT scan and density measured in five points in site of osteotomy, and its means calculated.

Statistical analysis

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

Results

All animals survived to the end the study. Neither wound infection nor wound dehiscence were observed in the animals of either group. All animal had normal serum calcium (3.2 ± 0.22 mmol/dl), serum phosphate (1.35± 0.18 mmol/dl), and serum alkaline phosphatase (11.6 ± 2.4 IU unit/ dl) at time of osteotomy.

Five week after osteotomy, there was no statistically significant difference (p> 0.05) in the means of serum calcium (3.1 ± 0.14 mmol/dl), serum phosphate (1.4 ± 0.1 mmol/dl), and serum alkaline phosphatase (12. ± 1.6 IU unit/ dl), (table 1).

Macroscopic evaluations demonstrate that all osteotomies were united by the end of the study. The mean of CT scan density of callus at the site of osteotomy in experimental group was 331.1 with a standard deviation 81.3. The mean of CT scan density in normal bone proximal to site of osteotomy in experimental group was 930.7 with a standard deviation 188.3, (table 2). The mean of CT scan density of callus at the site of osteotomy in control group was 199.7 with a standard deviation 32.1. The mean of CT scan density in normal bone proximal to site of osteotomy in control group was 919.3 with a standard deviation 186.8, (table 2). There were highly significant differences in density of callus between the experimental group and control group, (P value is < 0.001). There were no significant differences in density of bone proximal to site of osteotomy between the experimental group and control group, (P value is 0.84, table 2).

The Histopathological examinations of the osteotomy site showed healed bone with predominantly woven bone with some area of mature (lamellar) bone with some area of cartilage, there was no evidence of infection or foreign body reaction, (fig 3). All histological examination of specimens showed approximately same stages of healing in both groups of animals.

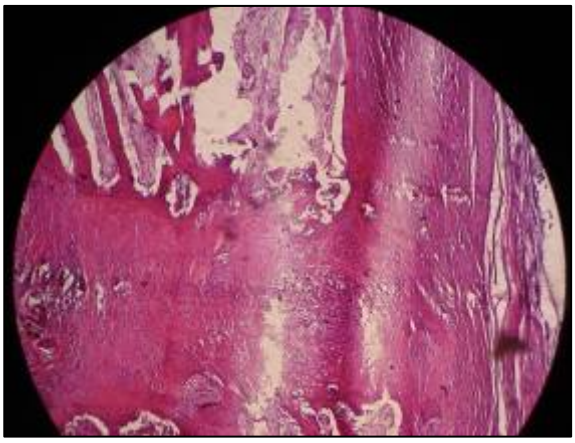

Figure (3): Histological examination showing new bone formation at the site of osteotomy in different stages of healing (woven bone with areas of cartilage and some lamellar bone).

Table (1): The serum calcium, phosphate, alkaline phosphatase of rabbits in preoperative and at the end of the fifth week.

Samples	Preoperative values		Values at end of fifth week		P value	Significance
	Mean	Standard deviation	Mean	Standard deviation		
Serum calcium	3.2	0.22	3.1	0.14	> 0.05	Not significant
Serum phosphate	1.35	0.18	1.4	0.1	> 0.05	Not significant
Serum alkaline phosphatase	11.6	2.4	12	1.6	> 0.05	Not significant

Table (2): CT scan densimetry in site of osteotomy and in normal bone proximal to site of osteotomy, five week after osteotomy of right ulna of rabbits in Experimental group and control groups.

	Experimental group		control group		P value	Significance
	Mean	Standard deviation	Mean	Standard deviation		
CT scan densimetry in site of osteotomy	331.1	81.3	199.7	32.1	< 0.001	Highly significant
CT scan densimetry in normal bone proximal to site of osteotomy	930.7	188.3	919	186.8	0.84	Not significant

Discussion

The production of a better and stronger healing bone has attracted the interest of many investigators in the past. Numerous substances have been used to increase both the strength and rate of production of fracture callus [12].

Calcium ion is an essential structural component of the skeleton and essential for the acceleration of healing of fractured bones [13]. Vitamin D is critically important for development, growth and maintenance of a healthy skeleton from birth until death [4]. 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 [14,15,16]. 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[17]. Vitamin C supplementation improved the mechanical resistance of fracture callus and made bone healing faster [18,19, 20]. The vitamin D, calcium, fluoride and vitamin C well known drugs, used widely in treatment of many orthopedic diseases, and safe drugs in therapeutic doses. Their combination in therapeutic doses can be used as dietary supplementation to support bone healing process in humans.

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 rabbits with fractured ulna, highly significantly increased the density of callus in CT densimetry measurement at the site of osteotomy in comparison with control group, (P value is < 0.001), (table 2). There was no significant difference in density of normal bone proximal to the site of osteotomy between experimental and control groups (P value is 0.84), (table 2). Macroscopically all osteotomies were united by the end of the study in both groups. Histopathological examination showed good union without complications in both groups. It is well known that the mineral density of callus correlated positively with callus mechanical properties[11].

There was no significant difference in serum calcium, serum phosphate, and serum alkaline phosphatase preoperatively, and at the end of the fifth week. Our results preoperatively and at the end of the fifth week fall within the normal range. The normal serum calcium, serum phosphate, serum alkaline phosphatase in normal rabbits were 3.0- 4.2 mmol/l, 1.28-1.92 mmol/l, and 10-70 IU/L respectively [21]. These findings indicate that animals had normal serum calcium and phosphate through all time of study.

The rabbits were chosen as the animal model because they are widely used in studies of bone preparations, and its bone is similar to human bone. The ulna was selected because it is easy to access, had good size, its fixation not essential, and it is easy to harvest. 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 [22].

In conclusion, our study demonstrates that combination of vitamin D, calcium, fluoride and vitamin C improve bone healing process in rabbits model osteotomy; this effect is characterized by increase callus density.

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