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The influence of local administration of calcitonin and alendronate on bone formation marker (osteocalcin) in critical size defect: An experimental study in sheep

(The Effect of Local Delivery of Calcitonin and Alendronate on Osteocalcin in Critical Size Bone Defect of Sheep)

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ABSTRACT

Aims: This study was undertaken to investigate the influence of local administration of Calcitonin and Alendronate on bone formation marker (Osteocalcin).

Materials and Methods: Eleven male sheep were divided into 3 groups: control (n=3), Calcitonin treated (n=4), and Alendronate treated animals (n=4), and followed up at 2, 4, 8, and 12weeks. Bone defects 5mm in diameter and 5mm in depth were created with a standard trephine bur in tibia of the sheep. The tested materials were placed in the defect. Serum Osteocalcin was measured in four period of times (pre-operative time, 24 h, 72 h, and 7 days post-operatively) using ELISA.

Results: Alendronate and Calcitonin revealed a tendency to increase in Osteocalcin level but not reach to statistically significance when comparing to control group.

Conclusions: Locally applied Alendronate and Calcitonin may be considered among therapeutic option to improve bone formation process in different bone remodeling cases. Further studies are required to elucidate the effect of local delivery of Alendronate and Calcitonin on bone formation markers.

Keywords: Bone formation, Bisphosphonates, Calcitonin, Osteocalcin.

INTRODUCTION

Bone turnover is the total volume of bone that is both resorbed and formed over a period of time [(1)]. The bone turnover in adults, mainly occurs through bone remodeling, a focal process that involves the coupled activity of osteoclasts and osteoblasts [(2)]. Changes of the amount of bone turnover cause local changes in bone volume and affect the average age of tissue in a bone, resulting in alterations in tissue of mineralization gradation and trabecular microarchitecture [(3–5)].

In fracture healing, damaged bone is removed and callus in the form of woven bone is laid down. Dead bone can persist for thousands of years, but living bone must be replaced by new bone periodically as it loses its mechanical capability [(6)]. The goal of prevention is to reestablish the normal bone turnover rate and preserve bone mass. High bone turnover, with bone resorption exceeding bone formation, is a major mechanism of osteoporosis in postmenopausal. The activity of remodeling can be detrimental mainly because it removes the damaged bone before it replaces, producing loci of provisionally exaggerated weakness. However, when bone remodeling is balanced, the improved strength flowing from repair has been found to be more than abundant to offset any focal weakness happening in the process [(7, 8)]. Biochemical bone turnover markers (BTMs) are divided into two groups: markers of bone formation and markers of bone resorption, among these biochemical markers of bone formation are Osteocalcin (Bone Gla Protein) and Specific Bone Alkaline Phosphatase (ALP) [(9)].

Bone defect healing is considered challenge for health professionals [10, 11]. Dental surgeons have been looking for means of stimulating osteogenesis in defects of the jaws to restore function and esthetic [12].

Considering the healing process, substances inserted into a recipient bone bed may have osteoconductive, osteopromotive, and osteoinductive properties [13]. Bisphosphonates (BPs) are anti−catabolic drugs revealed to be effective in hastening bone regeneration through inhibition of osteoclast cell function, and promotion of osteoblastic activity, which increase speed of bone formation [14]. Several investigators reported that these medications improved bone mineral amount and density, and increased the strength of regenerated bone and decreased rate of bone resorption. Administration of BPs locally is predictable to increase the bone formation. This could happen by achieving a high local concentration, which inhibits bone absorption by osteoclasts more effectively than that gained by systemic administration, with an advantage of absence of adverse effects of systemic administration [15-17].

Calcitonin (CT) is reported to be effective medication for treatment of a large surgical bone defect that need a long standing process to heal of such cavities normally depends upon restorative physiological processes, in which several factors take place [18]. Calcitonin (CT), a Ca^{+2}-regulator hormone, produced by the C-cells of the thyroid tissue has been reported to stimulate hard tissue formation [19, 20] .The primary action of CT on bone tissue is suppression of osteolysis and induction of Ca^{+2} release. It has been reported that CT inhibits osteoclastic bone resorption by binding to specific cell surface receptors [21]. In addition to its inhibitory effect on osteoclasts, it was also shown that CT had a direct stimulatory effect on acceleration bone formation and mineralization and it is a biocompatible to bone tissue [22].

Thus, the purpose of the present investigation was to evaluate the Osteocalcin as a bone formation marker to demonstrate the local effect of Calcitonin and Alendronate on bone turnover. Unfortunately, and within the limit of this study we did not find any published previous studies that involves local effect of antiresorpative drug on bone turnover markers.

MATERIALS AND METHODS

The protocol and guidelines of this study were approved by Scientific Committe/ Department of Oral and Maxillofacial Surgery/ College of Dentistry, University of Mosul. This study carried out on eleven adult male sheep of 7-8 months old, each weighing 25-30kg from local market were included in the study. During the entire period of the study, the animals were permanently housed indoor in animal house of College of Dentistry/ University of Mosul. They were kept in−group housing under photoperiod cycle of light 6:00 to 18:00 h and dark from18:00 to 6:00 h, temperature 20±2 C°. Animals were supplied with food twice daily with standardized diet (Ipek Yem, Turkey) with tap water. Animals were quarantined for 7 days prior to surgical procedure to check the general statement and ensure the absence of general or infectious disease. All animals were operated on at the experimental unit in the Oral and Maxillofacial department, Faculty of Dentistry.

Preparation and Premedication

The operated animals were kept fasting for 12 hours before surgery, the food was withheld but water was available. The animals were allocated to three groups, each experimental group consist of four sheep, the first group were used for local application of salmon calcitonin; the second group were used for local alendronate administration; the last three sheep were selected for control group.

The experimental animals were sacrificed by an Islamic slaughter at 2, 4, 8, 12 weeks after surgical procedure for each group. Each animal was scheduled to four surgeries working on each limb at different time interval according to planned period of sacrificed beginning from 12 weeks in the first limb to be finished at the end of 2 week in the last limb.

Induction of anesthesia

At the day of operation, the animal received general anesthesia inducted by intramuscular injection of a mixture containing (10mg/kg) ketamine hydrochloride (Gracure pharmaceutical Ltd, Bhiwadi,(Raj.) India) general anesthetic agent and (2mg/kg) Xylazine (the Egyptian Co. for chemicals & pharmaceuticals (Adwia), Egypt) sedative and analgesic solution.

Surgical procedure

The animals were placed in lateral recumbency and rotated over the sternum to the other side during the procedure. The operation side was shaved, and disinfected with 10% povidone iodine (Iraq). Local anesthesia of 2% Lidocaine HCL with epinephrine 1:80,000 (New Stetic/ Colombia) . Local anesthetic agent was administered by infiltration at surgical

site prior the incision for hemostasis. A skin incision was made along the longitudinal axis at lateral aspect of tibia a bout 5cm in length, fascia was dissected, and a full-thickness flap was reflected to expose the bone under a septic condition. After exposure and reflection of soft tissue, 5 mm diameter trephine bur on a slow speed surgical handpiece under constant saline solution irrigation was used to create bony defects. This dimension was chosen because a critical size defect implies that the defect does not heal spontaneously during the life time of the animal, as defined by Schmitz and Hollingers [23]. Three to four drill holes of 5mm diameter in width and depth were created on one side at the exact location of tibia leaving a distant at about 5mm between each hole, and then the bone cavities were carefully washed to eliminate bone debris and dried using gauze before being filled with the material.

Grouping and preparation of injected material

In the control group the proximal and distal hole left empty to serve as a negative control and the middle hole filled with gelatin sponge (Gela tamp, Colte´ne/ Whaledent, Germany) to serve as a positive control.The group treated with Salmon Calcitonin, each cavity filled with 10 IU (international unit) of injectable synthetic salmon calcitonin (Miacalcic, 100 IU, Novartis, Basel, Switzerland) carried by gelatin sponge. One international unit (IU) corresponds to about 0.2µg of drug. In the locally applied Alendronate group the defects filled with gelatin sponge socked in 2mg of Alendronate Sodium rehydrate (Merck, USA) dissolved in 0.1 distal water.

On each limb, one hole filled with gelatin sponge only to serve as a control hole for that tested group. At completion of material placement, the flap was gently approximated and the primary wound closure was performed in layers. Closure of subcutaneous tissue was achieved using 4/0 synthetic, braided, absorbable polyglycolic acid surgical suture (Scotland), and the skin was closed with 3/0 non resorbable black silk suture (Huaian Angel medical instruments Co., LTD, China) which was removed 10 days postoperatively. Finally topical aerosol of chlortetracycline antibiotic (Iran) was applied to surgical wound then the wound were dressed by gauze to protect it from straw entering the wound during recovery and for the first couple of hours after surgery.

Postoperative care

Immediately postoperative intramuscular antibiotic were administered with (10mg/kg) of oxytetracycline (Woerden-Holland) , the same dose of I.M. antibiotic was repeated every 12 hours for 3 days. Along the period of study, the animals received a balanced diet and periodical veterinary care with no evidence of serious adverse local or systemic complication observed throughout the period of study.

Blood collection

The blood samples were collected from the jugular vein of overnight fasting animals. The samples were 18 selected randomly from the control and tested groups. A 5ml of blood were drawn from each sheep then put it in vacuum plain tube. The blood samples were drawn from each operated sheep four times, one immediately before the operation, the second at 24 h postoperatively, the third at 72 h, and the last aspiration was in the seventh day after surgery. Within 30 minutes of collection, sera were separated as soon as possible from the clot of red blood cell after centrifuge at 3000 rmp for 10 minutes and all serum were collected and stored at -20 Cº until assayed.

Serum biochemistry

Serum Osteocalcin was measured by standard sandwich enzyme-linked immune sorbent assay technology (Elisa) using a kit specific fore sheep Osteocalcin (My Bio Source. Com., USA) following the manufacturer's instructions provided. The optical density (OD) for serum samples were read in micro plate reader in standard size (Biotek, USA).

Statistical analysis

The results are expressed as the mean ± SD. Comparisons between groups were processed using the Statistical Package for Social Sciences (SPSS) software (Version 19.0,SPSS Inc., USA) descriptive analysis for each variable under study were made and using one-way analysis of variance (ANOVA) with post hoc Duncan test for comparing two factors (treatment and time) and its interaction. Every decision in statistical analysis was taken at 5% level of significance.

RESULTS

The ANOVA showed significant increase in the level of Osteocalcin in all groups at last days of observation periods comparing to it is base line (pre-operative time). Table 1, 2, Fig. 1.

The effect of interaction between studied groups and time interval showed slightly higher differences but statistically not significant in Osteocalcin level of Alendronate and Calcitonin treated group when compare with control group. Such behavior may be visualized through Table 3, 4, Fig 2.

Estimation of Osteocalcin levels in serum

In control group, the levels of serum Osteocalcin in blood samples showed mild but gradual increase to reach a significant level at the end of 7 days when compare with pre-operative time (base line), where mean value were 1.391±0.183 ng/ml at base line to 2.048±0.535 ng/ml, $P<0.05$, Table 1, 2, Fig. 1.

In Salmon Calcitonin treated animals there is an increase in Osteocalcin ($P< 0.0001$) with maximal increase occurring at day 7 post-operatively, observed values were 1.479±0.224 ng/ml at base line and 2.306±0.471 ng/ml respectively, Table 1, 2, Fig. 1.

The changes of serum Osteocalcin for Alendronate treated animals is shown in Tab.1, Fig 1. It showed higher significance level at seventh day after surgery ($P<0.0001$), the mean values was 2.333±0.044 ng/ml comparing to 1.459±0.126 at base line, Table 1, 2, Fig.1

There were differences in Osteocalcin level and tendency to increase in treated groups among periods of time when comparing to control group, but this differences were not statistically significant, Table 3, 4, Fig 2.

DISCUSSION

Previous studies have mainly focused on systemic effect of calcitonin and alendronate on bone formation and resorption markers, we didn't find any research articles dealing with this local application of antiresorpative agents on bone formation marker (Osteocalcin). Serum biochemical data showed that the treated sheep (Alendronate or Calcitonin treated) subtle changes in Osteocalcin levels were increased slightly but not statistically significant comparing to control group in all detection periods of time that measured.

Serum Osteocalcin (OC), the vitamin K dependent, non− collagenous bone protein, is regarded as an indicator of osteoblast function and is increased in high turnover bone disease [(24)]. Serum Osteocalcin is a valid bone marker of bone formation, this is particularly evident when bone formation is decreased and resorption increased. Novaes et al, who reported that Osteocalcin, as one of the important indicators of osteogenic differentiation and bone tissue formation[(25)]. Osteocalcin is not expressed during early stage of osteoblastic differentiation but it is prominently a marker of late, mature osteoblast. The expression of Osteocalcin is induced in vitro at the onset of mineralization, clearly after the expression of other osteoblastic markers such as alkaline phosphatase and type I collagen. Thus, the expression of Osteocalcin is considered a specific feature of the late phase of bone formation [(26)]. OC is chemotactic for osteoclast and regulate osteoblast activity too [(27)].

Serum Osteocalcin is increased following fracture, remaining elevated for at least 3 months post fracture [(28)].

Bisphosphonates have osteostimulative properties both in vivo and in vitro studies as shown by increase in matrix formation [(29, 30).] Bisphosphonate treatment promotes osteoblastic differentiation in calvarial wounds in vivo [(31)], induce the osteoblasts to secrete inhibitors of osteoclast-mediated resorption and also stimulate the formation of osteoblast precursors and mineralized nodules, thereby promoting early osteoblastogenesis [(32, 33)] . The Alendronate stimulated the bone formation in the autogenous free bone grafts [(34)]. Alendronate may be considered among the therapeutic options available to improve bone formation process in different bone remodeling cases [(35)]. These studies support our results that Alendronate and Calcitonin activate OC which act as an indicator of increased of osteoblastic activity because of indirect inhibition of resorptive mechanism.

Alendronate treatment has conflicting results besides of bone formation caused excessive osteoclastic bone resorption followed by a secondary increase in osteoblastic activity leading to increased bone turnover [(36, 37)]. A risk of avascular necrosis in jaws with high doses of bisphosphonate therapy was reported, in such cases, osseointegration of dental implants might be jeopardized. There is no such risk with locally active bone remodelling cases. This is because the required dosage and duration of the bisphosphonate therapy should be less than in systemic disease [(38)].

The effect of calcitonin now thought to be dependent on calcitonin binding to specific receptors on osteoclasts cause inhibition of bone resorption and decreasing the activity of those resorptive cells [(39).] Calcitonin also binds to specific receptors on cells of the monocyte− macrophage series, proposing that it also acts to decline in bone resorption by retarding recruitment of osteoclasts [(40)].

The effects of calcitonin on the bone formation process are less clearly understood. The reports from previous studies have shown both increases and decreases in bone formation indices. Confirmation of previous observed vivo studies have recognized that prolonged (daily) calcitonin treatment can decrease the rate of bone formation [(41)]. This is usually supposed to be an indirect effect cause by calcitonin's action to inhibit bone resorption, so that a calcitonin-dependent decrease in resorption leads to a proportional decrease in compensatory bone formation. In contrast to these observations, previous in vitro studies have shown that calcitonin can increase the growth of bone and cartilage [(42)], suggesting direct stimulation; some in vivo data are reliable with this premise that support anabolic effect of calcitonin treatment on osteoblast cell line[(43)] .

Osteocalcin level correlated to topical application of both calcitonin and alendronate on critical size bone defect revealed slight increase in peak level of OC in treated experimental groups over control group in relation to the examination periods, but this did not reach statistical significance. While all groups showed increase of the OC levels at the end of 7th day comparing to its base line values. The increase in OC levels due to trauma from the surgical wound and it was higher in the treated groups due to synergistic effect of drugs and trauma. Each of Alendronate sodium and Calcitonin used in the research, even with small dose is effective in accelerating bone formation and give clue to increased mineral density of regenerated bone and accelerate bone formation and providing a chance to decrease the time of bone healing. The explanation might be that the locally applied Alendronate and Calcitonin was received directly on local bone surface where the osteoclast stimulated by the drug and have a high affinity for bone mineral and local application is feasible. Low dose of Bisphosphonate and Calcitonin administered locally is expected to achieve a high local concentration that could inhibit bone absorption by osteoclasts more effectively, and Serum OC appears to be a specific marker for bone formation and can predict the histological profile in the treated cases.

CONCLUSION

Local administration of Calcitonin and Alendronate are an effective therapeutic strategy that could be safe. The Osteocalcin that reflect the overall osteoblastic and osteoclastic activity in the skeleton and in some situations may serve as surrogates for histologic examination of bone, and possibly reduce the need for frequent bone density testing.

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Table 1: Descriptive Statistic of Serum Osteocalcin For Each Group In Relation To Time

Group		N	Mean	Std.Deviation
Control	Pre-op.	4	1.3910	.18336
	24 Hrs	4	1.5937	.16347
	72 Hrs	4	2.0155	.31182
	7 Days	4	2.0477	.53473
Calcitonin	Pre-op.	7	1.4788	.22393
	24 Hrs	7	1.6035	.15004
	72 Hrs	7	2.1234	.36054
	7 Days	7	2.3057	.47145
Alendronate	Pre-op.	7	1.4593	.12594
	24 Hrs	7	1.6114	.23791
	72 Hrs	7	2.2088	.17064
	7 Days	7	2.3332	.43901

Table 2. Comparison Among Osteocalcin Values Of 3 Groups At Different Times Using ANOVA Test.

Group		Sum of squares	Df	Mean Squares	F	P-value
Control	Between Groups	1.247	3	.416	3.750	041
	Within Groups	1.331	12	.111		
	Total	2.578	15			
Calcitonin	Between Groups	3.345	3	1.115	10.496	.000
	Within Groups	2.549	24	.106		
	Total	5.894	27			
Alendronate	Between Groups	3.923	3	1.308	17.773	.000
	Within Groups	1.766	24	.074		
	Total	5.689	27			

Table 3. Descriptive Statistic Of Osteocalcin Levels Of Different Group According To Times.

Time		N	Mean	Std.Deviation
Pre-op.	Control	4	1.3910	.18336
	Calcitonin	7	1.4788	.22393
	Alendronate	7	1.4593	.12594
24 Hrs	Control	4	1.5937	.16347
	Calcitonin	7	1.6035	.15004
	Alendronate	7	1.6114	.23791
72 Hrs	Control	4	2.0155	.31182
	Calcitonin	7	2.1234	.36054
	Alendronate	7	2.2088	.17064
7 Days	Control	4	2.0477	.53473
	Calcitonin	7	2.3057	.47145
	Alendronate	7	2.3332	.43901

Table 4. Comparison Of Serum Osteocalcin Values Of 3 Groups According To Each Periods Of Time Using ANOVA Test.

Time		Sum of Squares	df	Mean squares	F	P-value
Pre-op.	Between Groups	.020	2	.010	.306	.741
	Within Groups	.497	15	.033		
	Total	.517	17			
24 Hrs	Between Groups	.001	2	.000	.011	.989
	Within Groups	.555	15	.037		
	Total	.556	17			
72 Hrs	Between Groups	.096	2	.048	.578	.573
	Within Groups	1.246	15	.083		
	Total	1.342	17			
7 Days	Between Groups	.232	2	.116	.521	.604
	Within Groups	3.348	15	.223		
	Total	3.580	17			

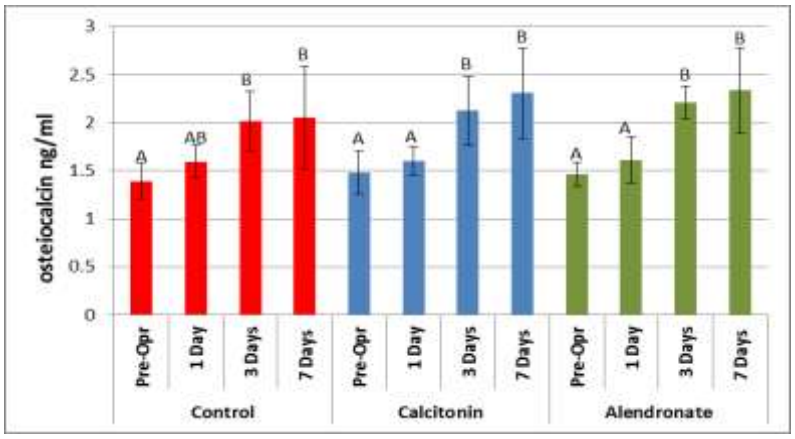

Figure 1: Comparison of Osteocalcin Among Different Times For Each Group, Different Letters Indicates Significant Different At $P \leq 0.05$.

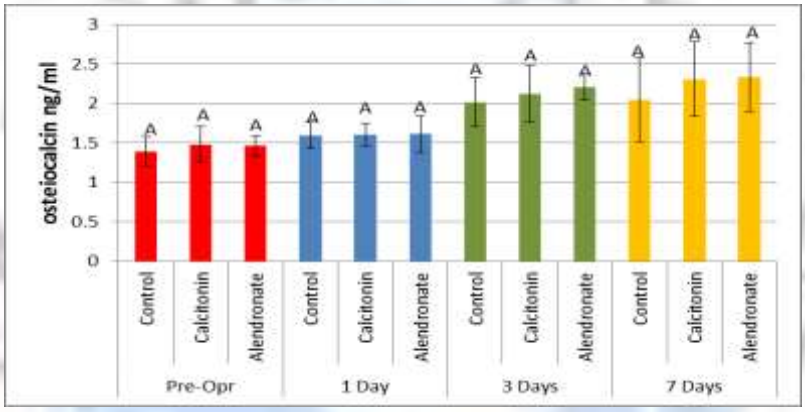

Figure 2: Comparison of Osteocalcin Among Different Groups According To Time, Different Letters Indicates Significant Different At $P \leq 0.05$