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Evaluation of Local Calcitonin Therapy on Bone Repair in Sheep Model: A Radiographical Study

(The Effect of Local Delivery of Calcitonin on Bone Formation of Tibial Defect in Sheep: A Radiograhphical Analysis)

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Abstract

Objective: Radio graphical evaluation of healing after local administration of salmon calcitonin of surgically created bone defects in tibia of sheep model.

Study design: Healing of bone defect still represent a challenge to health professional in several areas. In this article, the effect of salmon calcitonin on bone repair was studied.. Differences between drill holes, which filled with new bone were taken and represented as increase radio density. Seven sheep were divided into control (n=3) and experimental group (n=4). Uniform 3−4 surgical defects on the tibia were created. In the experimental group the drill holes was filled with salmon Calcitonin 10 IU/ cavity carried by gelatin sponge, while in control group one hole filled with gelatin sponge to serve as control positive and the other holes left empty to serve as negative control. All animals killed after (2, 4, 8, 12 weeks) postoperatively. The amount of new bone formation was evaluated radio graphically. A radiographic examination was used as a valuable tool for measurement of bone regeneration.

Results: The experimental group, which treated with salmon calcitonin had significantly more bone formation than control group ($P \leq 0.05$) in (4 and 8 weeks) postoperatively.

Conclusions: The topical application of Calcitonin will improve bone formation and this can be evaluated efficiently using digital radiograph. Repair of bone (quantity) after local administration of calcitonin and the amount of regeneration of bone can be estimated radiographically.

Key words: bone regeneration, calcitonin, digital radiograph, animal study model.

INTRODUCTION

Bone loss may be a consequence of various pathologies, trauma or as a result of surgical procedures. An extensive studies on the process of bone repair has been done in worldwide [(1)]. It is well known that, the growing in young individuals, of the trephine defects repair occurs more rapidly than in adults. This is factual for any part of the skeleton and for every species, so, an experimental model in this area should only use skeletally adult animals to obtain patronized and reliable information [(2)]. The sequence of events that take place at fracture healing has been closely studied in different species, where the inflammatory response initiated by impairment to the tissue is present during the first week and followed by the repair and remodeling phases [(3)]. Numerous biological mediators and biomolecules temporally and spatially affect the different stages of bone healing, including inflammation, angiogenesis, osteogenesis, and remodeling. many of these mediators were shown to be influenced systematically [(4)] or locally [(5)]at skeletal bone sites [(6)]. Bone defects are a significant clinical problems [(7)], and the recent advances in medical researches that generated new treatment focused on the acceleration of anabolic process in bone defect [(8)], various approaches were used to accelerate bone regeneration, among these hormones were shown to be effective in bone healing [(9)].

With respect to dentistry, the study of pharmaceutical drugs that favour bone regeneration is of vital importance, mainly in periodontics, maxillofacial surgery, orthodontics, prosthodontics and more recently, in implantology [(10)]. Nowadays, clinically, the most widely used medications are anti-absorption drugs, among these drugs, Salmon

Calcitonin (sCT) [11]. Calcitonin is a calcitropic peptide produced in the parafollicular cells of the thyroid gland. Calcitonin responds to elevated serum ionic calcium levels by decreasing the number and activity of osteoclasts [12, 13]. Its pharmacological action by decreases bone resorption primarily, and causes a transient increase in bone formation secondarily by means of unknown mechanism [14]. Calcitonin can cut down the bone absorption and the rate of bone turnover, which results in relative increase of bone formation, thus improving the bone quantity and quality [15]. Sheep were used as an animal models in this study because it is quite similar in body weight to humans, and sufficiently large to allow serial sampling and multiple experimental procedures. Some similarities between humans and sheep were found in bone structure and seem to have a rate of bone healing that approximates the human rate [16]. In addition, because the effect of sex steroid, i.e., estrogen, on bone resorption and development of osteoblast and osteoclast have been considered, only male sheep were used in the present study to minimize those effects [17].The aim of this study was to assess the effect of locally administration of Salmon Calcitonin following a surgically created bone defects in sheep. The formation and healing of bone defects were determined by radiographic analysis.

MATERIALS AND METHODS

Animal Model And Surgical Procedure

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 seven 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-groups in animals house 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 feed (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. The animals were allocated into two groups, the control group (n=3), and the experimental animals with local application of Salmon Calcitonin (n=4).

The animals were fasted for 24 hours preoperatively. Surgical procedures were performed under general anesthesia under sterile conditions. General anesthesia was achieved using an intramuscular injection of a mixture containing (10mg/kg) Ketamine hydrochloride (Gracure pharmaceutical Ltd, Bhiwadi,(Raj.) India) general anesthetic agent and (2mg/kg) Xylazin (the Egyptian Co. for chemicals & pharmaceuticals (Adwia), Egypt) sedative, analgesic, muscle relaxant solution. The animals were placed in lateral recumbency and rotated over the sternum to the other side during the procedure. The tibia were shaved and cleaned with 10% povidone iodine before manipulation, Skin and periosteal incisions were created separately, and the tibia was exposed. In each tibia, 3− 4 monocortical holes of (5 mm) in width and depth were prepared with a trephine burr until reach the marrow spaces under saline solution irrigation at 1500 rpm. A distance of 5mm was left between each defect. 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. We left one hole filled only with gelatin sponge to serve as a control in same animal.(Fig.1) After obtaining adequate hemostasis, the periosteum was closed with 4/0 resorbable suture 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. The tibia was fastened down to avoid fracture. The animals were given postoperative intramuscular antibiotic were administered with (10mg/kg) of oxytetracycline (Woerden-Holland) twice daily for 3 days postoperatively. The animals were placed in separate cages in a standard environment to allow the animals to live and fed a standard diet. During the study period, the animals were examined for leg fractures, infection, and adverse reactions. 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. Then the animals were killed by an Islamic slaughter. The tibias were carefully dissected free from soft tissues, and hard-tissue samples were transferred into 10% buffered-formalin solution.

Radiographic analysis

Correct positioning of the specimen was confirmed by radiography postmortem. A digital dental x−ray apparatus by Dimax software program (Planmeca , 00880 Helsinki, Finland) and digital sensor (dixi^2, Planmeca, Finland) attached to it calibration stainless steel wire of 10 mm in length. Using the following parameters: 8 mA, 63 Kv, focus object distance of 30 cm, and exposure time of 0.080 sec. the radiographic image was then transferred to the ImageJ 1.47v software (National Institute of Health, USA). Through this software, it is possible to quantify the amount of bone

formation. The quantification of osteoneogenesis was performed by selecting a rectangular area of pre−defined size (≈25mm) to define the " region of interest "(ROI) which represent the original size of bone defect. The region of defect was delineated through drawing a lines of 5mm in length which represent the upper border of defect and these lines are joined with two vertical lines of ≈ 5mm in height which represent the side wall boundaries of the defect Then the opacification area was plotted a polygonal shape along the periphery of radiodense area within the defect cavity using the polygonal shaped and color selector tool of the ImageJ program, representing ossification zone of newly formed bone. The images were calibrated first to get a reading in millimeters by measuring the length of reference wire, so the reference distance was defined before using the function of area measure from analyze tool in the program. (Fig.2). The measurements were performed four times in each defect in different days to minimize intra-observer measurement error and the mean values of all reading for each defect was obtained. The percentage of bone formation was measured by two examiners who were unaware of which group the animals belonged to. The estimation of new bone formation was applied by using an equation as follows:

Area of new bone formation = Total defect area − non formed bone area

$$\text{Percentage of bone formation} = \frac{\text{Area of new bone forma tion}}{\text{Total defect area}} * 100$$

Statistical analysis

After data collection, it processed statistically using the Statistical Package for Social Sciences (SPSS) software (version 19.0, SPSS Inc, USA). The mean of percentage values were submitted to analysis of variance (ANOVA) and Duncan multiple comparisons test. P value ≤ 0.05 were taken to be significant. All the increased area of opacification from the external and internal surface of original defect size was measured.

RESULTS

Radiographic quantification of newly formed bone

A radiographical analysis of radiodense area of the treated and control groups was increased of such period of two to twelve weeks for both groups. Comparing the groups together, it was observed that in four study periods, the averages of the radiodense area of the treated group were always higher than in control group. However, this difference was statistically significant only for the period of 4 and 8 weeks ($p < 0.05$) (Table. 1) .

Radiographic evaluation at second week

When a radiological comparison was made between the control group and Calcitonin treated group at second week postoperatively there was no statistical difference found between them (P= 0.870). (Table.1, Fig.3)

Radiographic evaluation at fourth week

Radiologic bone healing was superior in both groups, and exhibited a more advanced phase of bone formation in the Calcitonin treated defects (P= 0.000). The mean of the percentage value of newly formed bone in the control group (control positive, control negative), and Calcitonin treated group (control of Calcitonin and Calcitonin treated defect) were observed from 102.95 to103.26 and 105.57 to 106.95 respectively. The increased opacity represents periosteal overgrowth at this period of evaluation. (Table.1, Fig.3)

Radiographic evaluation at eighth week

The results of bone formation in locally administered Calcitonin showed a significant superiority versus control group (p= 0.000). The amount of bone formation was increased from both external and internal surface of bone defect and it appears denser in Calcitonin treated group than control group. (Table.1, Fig.3)

Radiographic evaluation at twelfth week

There was no distinguishable difference on the mean of bone formation between the two groups at the end of the twelfth week (P=0.081) with respect to this bone density (Table.1, Fig.3). The cavity of Calcitonin treated group it appeared more denser osteogenic formation compared with control group. However the radiological comparison was made between control group at 2,4,8,and 12 weeks showed a statistically significant increased in amount of new bone formation along the periods of observation (P= 0.000). (Table.1, Fig.4) There were also statistically significant differences in the results of bone formation through the detection periods of evaluation in local Calcitonin administration group along the period of study (P= 0.000). (Table.1, Fig.4).

Discussions

The regeneration of injured or excised bone tissue comprises a complex series of events that initiates with the recruitment, attachment, and proliferation of progenitor cells, followed by cell differentiation into suitable phenotypes that are capable of restoring the damaged tissue [18]. The bone regeneration has been improved by the use of biologic mediators to improve the quantity and quality of the bone being regenerated [19, 20]. Several studies have demonstrated the stimulatory effect of calcitonin on bone growth because of the inhibition of osteoclast activity, emphasizing the importance of investigating the effect of this drug on bone repair [10, 21-22]. Recent developments in digital radiography (DR) have given the dentist the ability to perform radiographic examination with up to 80% reduction of radiation dose when compared with conventional plain film radiography. Digital radiography gives a higher diagnostic yield in following up bone defect, but it do not provide accurate information regarding the course of new bone formation because it is not quantitative but it considered the most suitable radiographical method in assessing the amount of new bone formation than CT scan and DEXA. However, it is more applicable, low cost compare with CT scan and DEXA, and non− invasive imaging techniques, and provides the examiner more information, while at the same time reducing radiation exposure [23].

The images from digital radiograph then transferred to ImageJ program which enable us to measure the three major axis length. This program allow us to measure the differences in dimension of the bone size defect in relation to amount of radio−opacity that fill the defect and its considered an alternative method to achieve the same out puts in measuring the morphological parameters with manual methods but with less time consuming , more reproducible, quantitative, automated image processing, and more practical measures [24,25] . Determining the amount of new bone formation radiographically is important for monitoring the healing and diagnosing and planning changes of bone mass during sickness or treatment [26]. Radiography is a basic method for evaluating bone defect healing, both in clinical use and in animal studies: radiographs are able to visualize callus (new bone) formation after mineralization [27], digital radiograph was taken to examine the location of bone defect and to assess quality of new bone formation. After the sacrifice of animals, high resolution X-ray images are usually taken, which can be used for a variety of measurements, such as bone density or bone dimensions. The formation of a callus (new bone) is one of characteristic used to monitor the healing parameters, but the timing and size of its appearance are variable [28].

There are some existing differences in speed and range of certain healing phases which are dependent on several factors. Therefore, we tried in our research to reduce the differences in some factors to the smallest extent, for instance by using animals of equal age, sex ,weight, and skeletally mature. According to our knowledge this is the only study that has examined the effect of locally administered Salmon Calcitonin on the sheep model. On the account of lacking experimental data, we cannot make a comparison of our result of increase in bone mass resulting from Calcitonin with other studies.

Visual observation of X−ray images over a period of 12 weeks, can trace new bone in both groups with superiority to locally applied Salmon Calcitonin in hastening new bone formation. At 2 weeks defects were plainly evident in most radiograph and there was no statistical difference between two groups in volume and quality of new bone formation when processed in ImageJ program to measure the amount of new bone. In the radiographic images the defects appear blur indicating the beginning of necrotic bone resorption, this stage of healing represent the granulation process which begins from seven days after creation of bone defect or slightly earlier and continues for approximately two weeks [29, 30] . (Fig.5, A,B). Under normal condition we should expect new bone formation to be shown radiographically especially through the initial first fourth weeks of defect healing . Most authorities suggest taking follow−up radiograph to assess the evidence of healing. If there is no bone formation recognize after 4 week, suspicion should be raised about delayed healing [31] . Radiographic examination identified a good healing process within two groups at 4 weeks, with time the bone defects at this stage becomes more radiopaque and the callus (new bone) become mineralizes and the callus begins spanning over the created gap. this initial bridging of the bone defect begins after about a month [30]. it had been noticed that the amount of bone formation was more intensive and of wider range corresponding to treated experimental group. This increase were evident in external surface of original size defect which represent periosteal over growth at this observation period, in fact, this explain the reason of significant difference between the groups (Fig.5,C,D) .

However, the digital radiograph appeared to present a distinct classes of healed defect in both groups from 8 to 12 weeks postoperatively, these finding correlated with ImageJ analysis of corresponding radiographs when applied to measure the amount of bone formation intended to show significant improvement of bony healing in calcitonin treated animals over control group (Fig.5, E,F) . The defects were be difficult to be observed and distinguished from neighboring bone at the end of observation period, suggesting that the defect had completely healed. Eventually, the defects becomes obliterated and this stage of healing attribute a time of three months to the point when the callus is mostly done and begins resorbing to re-establish the continuity of the cortex and the medullary cavity [30] . The defect size had a tendency to increased externally and internally over the original size defect. It is noteworthy that the

density occupied the entire area of defect and its more obvious in treated group at 12 weeks although it is not statistically difference. (Fig.5, G,H). This may indicate that the clinical application of locally administered calcitonin taking advantage as an auxiliary tools to bone healing process and would be as successful as faster of healing induction. The results obtained with the local administration of Calcitonin suggest that, in male sheep, this hormone acts more effectively from the fourth week, with its action being further maintained interim period of eight weeks and being more denser at the end of the study. These findings are in agreement with reports of Pereira et al[32] which showed a smaller radiolucent area of the bone defects of rabbits treated with systemic administration of Calcitonin in the mid time of experimental periods. The observation of the present study, agreeing with the results of authors who demonstrated increase bone density histologically can be compete for Calcitonin action to decrease of resorption cavities and lead to increase bone formation [33, 34]. However , contradicting results of other study were found an immature bone formation and less organized bone trabeculae associated with topical application of Calcitonin in 30 days but it showed more rapid proliferation of bone forming constituents in histological experimental socket in the jaw of dogs in 30 and 60 days [35] .

The main advantage with topical administration is the possibility of administering a higher dose to the region of interest maintaining better localization in bone cavities. Our data imply that calcitonin is efficiently absorbed in the bone at the site of application. The effect of drug was also detected in the control site of the Calcitonin treated animals indicating its absorption of disposited Calcitonin in the surgical site, which was most likely resulted from creeping of Calcitonin near the bone marrow region from treated hole to the neibouring control hole following surgical wounding [36].

Conclusions

The present study showed that single dose of local delivery of Calcitonin improved bone formation in bone defect. This bone formation (quantity) can be evaluated efficiently using digital radiograph and the amount of regeneration of bone was much higher in experimental group in mid periods of the study. Further studies are required to elucidate the effect of local Calcitonin application on humans.

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Fig.1: Application of gelatin sponge socked in salmon calcitonin in surgical bone defect in tibia of the sheep.

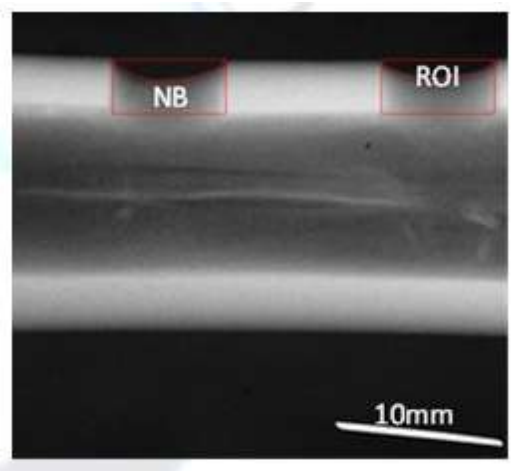


Fig.2: Radiograph demonstrating entire bone defect, (ROI) and amount of new bone formation (NB) . 10mm stainless steel wire for image calibration .

Table.1: Mean And P-Value of Radiographical Analysis Using ANOVA And Duncan Test

Groups \ Periods of healing	Two week	Four week	Eighth week	Twelfth week	P value
Control positive	**A** 82.18 **a**	**A** 102.95 **b**	**A** 107.3 **b**	**A** 106.39 **b**	.001*
Control negative	**A** 81.01 **a**	**A** 103.26 **b**	**A** 108.01 **b**	**A** 106.54 **b**	.000*
Calcitonin	**A** 78.55 **a**	**B** 106.95 **b**	**B** 112.94 **c**	**A** 108.06 **bc**	.000*
Control Calcitonin	**A** 76.96 **a**	**B** 105.57 **b**	**B** 111.10 **b**	**A** 107.56 **b**	.000*
P value	.870	.000*	.000*	.081	

* mean significant at p≤ 0.05
Capital letters refer to comparison among treatments (Vertically),
Small letters refer to comparison among periods (Horizontally)

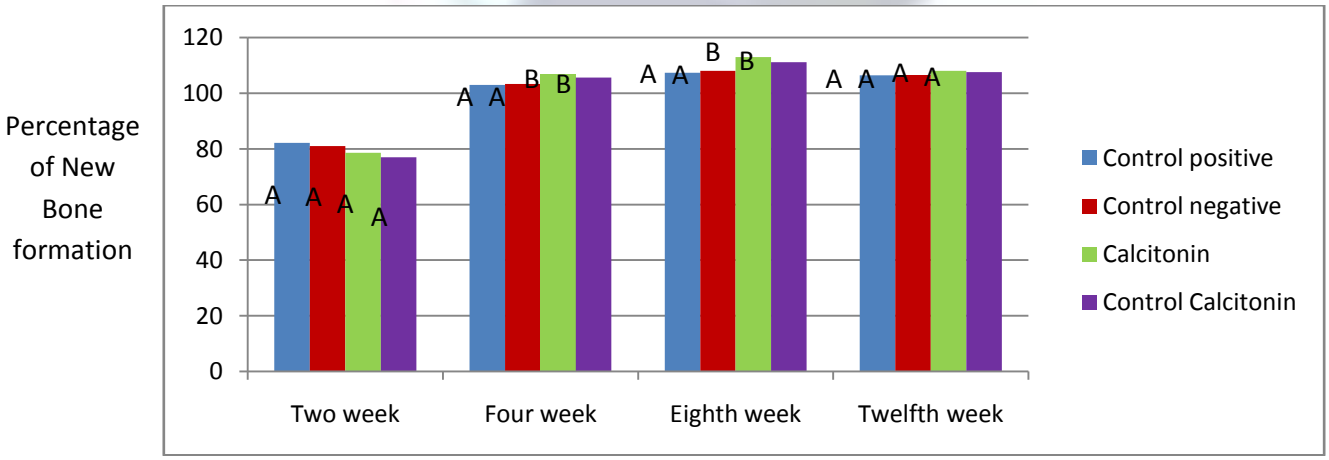


Fig.3. Comparison of Percentages of Amount of Bon Formation Among Different Groups According to Time, Different Letters Indicate Significant Difference At P≤ 0.05.

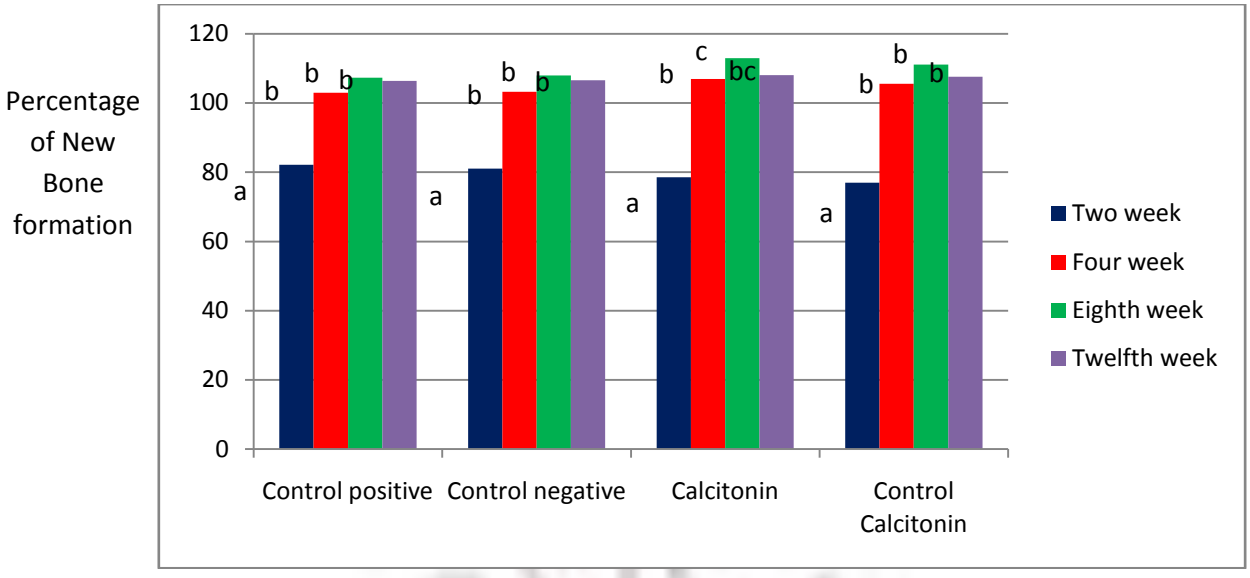
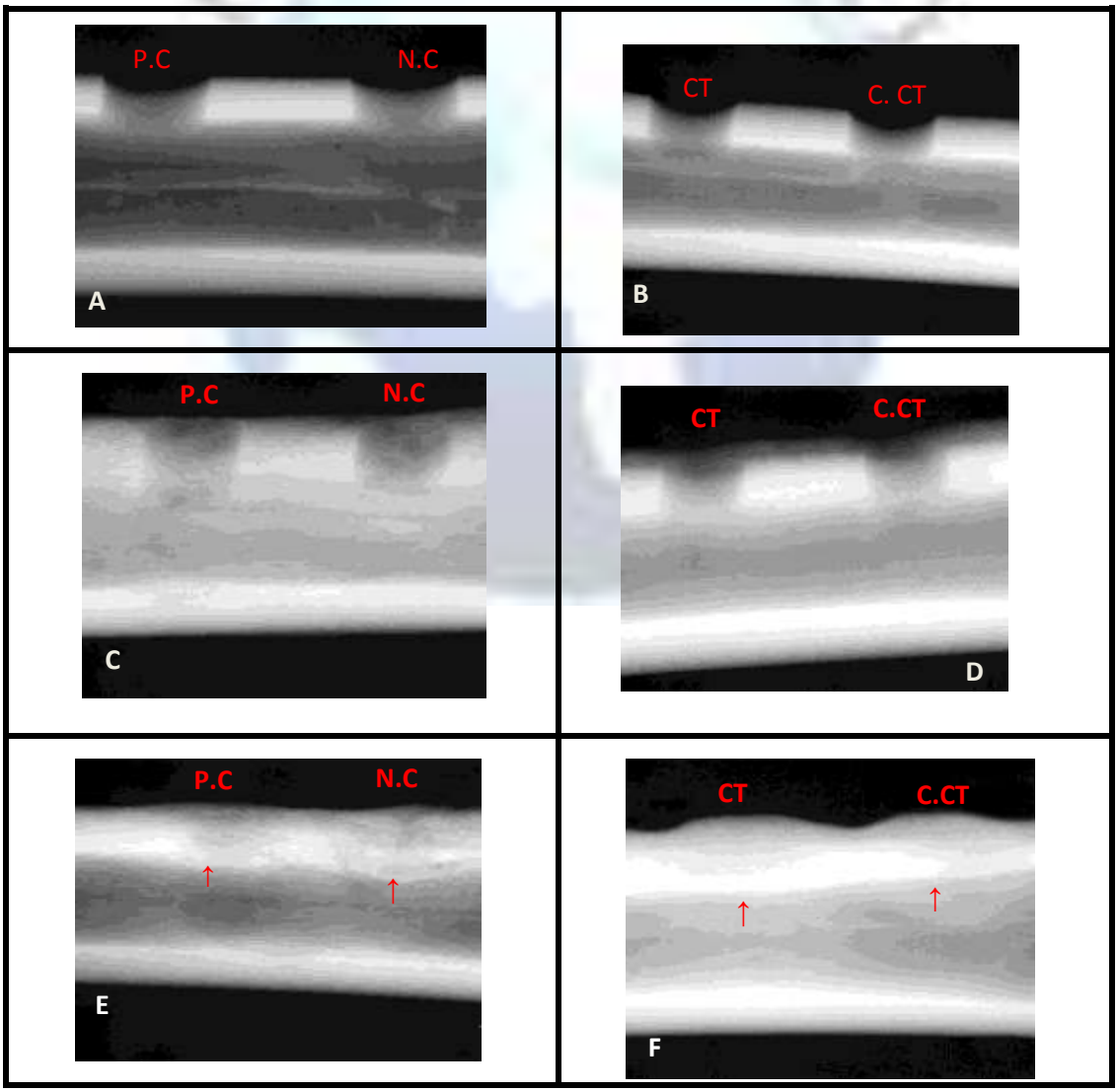


Fig.4. Comparison of Percentages of Amount of Bone Formation in Each Group According to Time, Different Letters Indicate Significant Difference At P≤ 0.05.



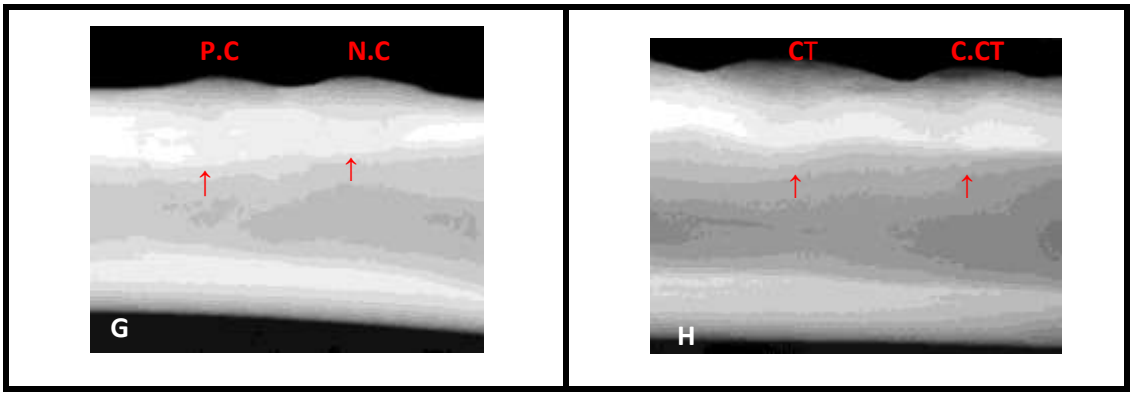


Fig.5: Radiographs show simply detected bone defects at 2 weeks in A, control group - positive control (pc), and negative control (N.C), and B, Calcitonin treated group (C.CT) control calcitonin, and (CT) Calcitonin. In C, D radiograph show slight periosteal overgrowth was detected in 4 weeks and it is clearly noticed in Calcitonin group. E, F radiograph show significant increased in bone formation and more dense in Calcitonin group at 8 weeks. In G, H radiograph the bone formation is more denser in Calcitonin treated group at 12 weeks postoperatively. Arrow represent the location of defect.