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Histological evaluation of the role of local angiotensin converting enzyme inhibitors (Lisinopril) on the osseointegration (Experimental Study in Rabbits)

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ABSTRACT

Background: There are very few data about the effect of angiotensin converting enzyme inhibitors (ACEI) locally on the osseointegration around titanium implants. There are wide contraversiy about its effects in relation to other histological tissue inviremens.

Aim of the study: This study was carried out to evaluate the histological behavior of lisinopril as one member of ACEI family on the osseointegration to titanium surface that altered histological responses.

Materials and methods: Twenty four rabbits were used for fixation of titanium secrow of 5mm length and 2mm width in the femor bone of rabbit. 12 rabbits subjected to implant fixation alone as control groups. In the other 12 rabbits the cavities of implant filled with lisinopril befor fixation of implant as experimental groups. Both experimental and control groups are study in intervals of 1, 2, 3 and 4 weeks. Each group examined histomorphometrically under light microscope for the section stained with heamatoxiline and eosin to assist counting of bone cells and bone trabeculea thickness at the end of each four period intervals post operatively supported by examination of rabbit bone specific alkaline phosphatase serum level in each time intervals for all groups by ELISA test.

Results: Hisomorphometric analysis show significant elevation in the number of osteoblast adhered to the implant surface and new bone trabeculea thickness in experimental group mainly at the end of first week with reduction in the deference in osteoblasts number in the 2nd and 3rd week until reach constant levels at the end of 4th week. This results were supported by the level of alkaline phosphatase enzyme serum levels as it highly elevated at first week in experimental group that reduced gradually with progression of osseointegration around titanium implant.

Conclusion: This study was illustrated that lisinopril enhanced osseointigration around titanium implant by facilitate bone cells activation and deffirintiation.

INTRODUCTION

Osseo integration or osteointegration defined as fusion of new bone formation with implanted materials without formation of other tissues between them lead to production of highly differentiated tissue facilitate direct connection and provide preferable functional and structural interaction between implant surface and surrounding living tissues. The osseinduction process produces fusion of bone materials with implant coated layer lead to ankylosis (1). Titanium is the most biocompatible implant materials as it has excellent ability to withstand body fluids and tissues environments. This ability come from titanium oxide layer that formed on the titanium surface naturally after exposure to oxygen that prevent its corrosion in addition to its bio-inertness with high capacity to induce bone cells differentiation and enhance bone remodeling. The physical properties of titanium oxide as it firmly bind to titanium surface and its impermeability in addition to its insoluble provide a strong barrier that inhibits reaction of titanium material with surrounding tissues (2).

The renin-angiotensin system (RAS) is an endocrine regulatory system of circulating system. This system containing major elements includes angiotensin converting enzyme that convert angiotensin I to angiotensin II. The later has significant bone inhibitory functions (3). The RAS exist in local bone remodeling and it play important role in bone remodeling regulation (4). The bone calcium balance affect by the action of Ang II as it reduce calcium uptake by the bone in addition to its ability to inhibit osteoblasts differentiation and activation by binding to specific receptor on the osteoblasts lead to reduction bone regeneration (5). The RAS expressed on the osteoblasts and osteoclasts there for it highly involved in bone remodeling process (6).

AIMS OF STUDY:

A: Histological study of the effects of the ACEI lisinopril on the osseintegration process.
B: serological study of bane forming enzyme alkaline phosphatase to support histomorphometric analysis of bone forming cells.

MATERIALS AND METHODS:

Pure titanium screw made in size of 0.5 mm in length and 0.2 mm width cylindrical in shape with cute end. The material oxidized to produce a micro coat of titanium oxide (TiO2) on the surface of implant. Lisinopril (Prinivil, Zestril) Angiotensin converting enzyme inhibitors (ACE inhibitors) are drugs that block the body's production of angiotensin II. Pure lisinopril powder 99% had been used that examined by using melting point technique. This material sterilized by UV. Waves. Rabbit Bone specific Alkaline Phosphatase (BAP) Elisa kit Used for serum, plasma, cell culture, body fluid and tissue homogenate. Not for therapeutic or diagnostic applications.

Twenty four male rabbits with slandered weight of 1.5 kgm divided into four groups 1,2,3 and 4 weeks. Each group consists from 6 rabbits, three experimental and three control animals. All groups subjected to same surgical procedures. The animal anesthetized generally and the skin incised along the femur bone near the head of its. Without any trauma to the muscles, the two muscles over the femur separated by artery forceps to exposed the bone. Periosteal elevator was used to separate any soft tissue attached to the bone. The periosteum of bone 0.5 cm away from the head of femur removed by hand piece with round carbide bur. The second step make hole for the implant with 1.7mm width taper drill and 5mm length. This hole is stander for all rabbits implant. In the control groups the titanium screw fixed in the socket with screw driver manually until obtain primary stability of implant. No over driven done to prevent bone fracture. In the experimental groups the socket have standard sized fill completely with lisinopril powder before screw driving then the screw was drove over the material. The excess of lisinopril washed out by distillate water. This procedure insures the same amount of lisinopril cover the surface of screw standard for all groups.

Biopsies collection: Immediately after sacrificed the site of operation at the femur of rabbits opened by scalpel very carefully until exposed all length of femur. The femur removed completely and all soft tissue scarified from the bone mainly around the head of screw. The bone kept in buffer formalin 10%. The bone cut in size of 1cm around the screw to get a biopsy of 2cm in size with the screw in the middle of this distance. This biopsy kept in buffer formalin 10 % until examined histologically.

Decalcified section: The biopsies obtain by cutting one cm. around implant. This segment fixed in the 10% buffered formalin immediately after resection for 48 hours. After fixation the specimen was decalcified with decalcified solution that prepared one litter as follows **1:** formic acid 99% concentration 50 ml. **2:** hydrochloric acid 1.18 sp. gr. 50 ml **3:** distilled water 900 ml. Bone decalcification required three weeks to complete removal of calcium from bone then rinse with tap water for 30 minute. Before starting processing the screw removed carefully by screw driver to maintain the serration of new bone around implants. The specimen processed in automatic processor which includes 12 containers. This process take 24 hours, the specimen remain 2 hours in each container as follows:

1: Formalin 10%, 2 containers (4 hours) **2:** Ethanol alcohol, 5 containers (10 hours) the concentration 70%, 80%, 90%, 100% and 100% respectively for dehydration of spacemen. **3:** Xylol, 3 containers (6 hours) to remove the remaining alcohol. **4:** Paraffin wax, 2 containers (4 hours).

This specimen transferred to the histocenter for making block of wax then cut the block by microtome into sections of 4-6 micron and put in water bath to remove the wax and carried by glass slide to starting the staining procedure which includes following steps: **1:** Heamatoxylin 5-15 minutes according to concentration of stain. **2:** Tap water 1-5 minutes to remove the excess stain. **3:** Eosin 2-5 minutes. **4:** Ethanol alcohol 70%, 80%, 90% and 100%. 2 minutes for each concentration. **5:**

Dehydrated by hot plate. **6:** Xylol to remove alcohol. **7:** Fixation of cover slid by DPX. **8:** Examination the slid under light microscope.

Rabbits serum collection: When the groups were time up, the rabbits sacrificed by using very sharp knife to cut the jugular vein and allow heavy bleeding from the neck of rabbit. First few drops discarded to prevent hemolysis then collect the blood to three or four test tubes. The tubes left for two hours at room temperature to allow all the cells to clot. The tubes centrifuged fifteen minutes in 3000 round per minute. After separation, the serum collects by micropipette and collected into eppendorf tubes to be frozen at -20°C. These samples now ready for Elisa test

ELISA tests for rabbit bone specific Alkaline phosphatase (BAP): In this tests used (BAP) from Mybiosource company for antibody and protein ELISA kits (U.S.A.). After serum collected in the eppendorf tubs and kept frozen at -20°C, the Elisa kits opened and the test start as follows:

Equipment required: 1: Precision pipettors and disposable tips to deliver 10-1000µl. **2:**100ml and 1liter graduated cylinder. **3:** Distilled or deionized water. **4:** Tubes to prepare sample solutions. **5:** Absorbent paper. **6:** Micro plate reader capable of measuring absorbance at 450nm.**7:** Washing bottle. **8:** Incubator. **9:** Statically graph.

Reagent preparation: 1: All kit components and samples must bring to the room temperature (25°C) before use. **2:** 10ml of wash solution concentrate (100^x) diluted with 990ml of distilled water to prepare 1000ml of wash solution (1^x). The solution can be kept for two week at (2-8°C). **3:** Other reagents are ready to be used.

Assay procedure: 1: The specific code numbering was secure on the holder to prevent confused. **2:** 100µl of standards or serum samples added to the appropriate well in the antibody pre-coated micro titer plate. **3:** 100µl of 9% physiological saline (PH7 -7.2) added to the blank control well. **4:** 50µl of conjugate added to each well except blank control well. **5:** Cover and incubate the plate for one hour at 37°C. **6:** The micro plate washed using automated washing machine in which the plate washed five times with diluted wash solution (350-400µl/well/wash). The washer was set for a soaking time of 10 seconds and shaking time of 5 seconds between each wash. **7:** The plate were inverted and plot dried by hitting the plat onto absorbent paper until no moisture appeared. **8:** 50µl substrate **A** and 50µl of substrate **B** solution were added to each well including blank control well. **9:** Covered and incubated for 15 minutes at 37°C. **10:** 50µl of stop solution added to each well including blank control well. **11:** mixed well. **12:** Lastly determined the optical density (O.D.) at 450 nm using a micro plate reader immediately. **13:** Each reading duplicated for standard and samples then al O.D. values are subtracted by the mean value of blank control before result interpretation.

Histomorphometric examination under light microscope: The histological examination was performed to the four groups by vertical decalcified section under light microscope in different levels of implant bed. The microscopically finding includes evaluation of cell forming bone as well as bone lamellae. The cell counting and measuring of lamellar thickness was illustrated by using special graduated microscopically lens at power of magnification of 400X.

Criteria of measurements: 1: Four randomly selected location of each section was examined. **2:** Each location divided by graduation of graduated lens into four quarters. **3:** The measurements were applied to each quarter separately and take the mean for these four measurements of the same slide. **4:** The mean of each slides section taken to be considering later in biostatical analysis.

Statically analysis: We have four groups to be examined biostatically. Each group divided into experimental and control equally. Compare means, Paired-Sample T test: were used to determine the correlations between these groups. This test was applied in SPSS 14.00 program under windows 7 operating system. Other statically analyses applied by using Microsoft office excel to obtain relationship among groups and changes during four times intervals of each group.

RESULTS:

A: Alkaline phosphatase (ALP) examination by ELISA test: ELISA test applied to the four groups for each time intervals

1: Analysis of the ELISA test results for alkaline phosphatase at the end of 1st week: There was no any pointers to the presence of ALP in the rabbits of control group in ELISA test while show mild increase in ALP level in experimental group (Diagram 1).

Biostatical analysis: The relation between experimental and control groups was significant at the end of first week (Table 1).

2: Analysis of the ELISA test results for alkaline phosphatase at the end of 2nd week: During the second week after implantation the two groups show appearance of ALP but there is a wide range between them. (Diagram 2).
Biostatical analysis: The relation between experimental and control groups was still significant (Table 2).

3: Analysis of the ELISA test results for alkaline phosphatase at the end of 3rd week.
The relation between two groups still constant after three week from operation but there was mild increase in the level of ALP in control group (Diagram 3) (Table 3).

4: Analysis of the ELISA test results for alkaline phosphatase at the end of 4th week.
The level of ALP markedly increased in control group after four weeks while it stabilized on constant level in experimental groups lead to make the relation between groups not significant(Diagram 4) (Table 4).

5: Analysis of ELISA test results of alkaline phosphatase levels for each group through the month of study.
The appearance of ALP in the experimental groups occurred in first week and show slow rising of its level during the month until reach constant level. The serum analysis for ALP was show negative result in control group in first week. The appearance of ALP noticed after two weeks and increased during third and fourth week to reach standard level (Diagram 5, 6).

B: Histomorphometric analyses by light microscope:
In this examination used decalcified section with hematoxelin and eosin stain for light microscope. In this techniques used graduated lens to measure the bone trabeculea thickness and the number of osteoblast associated with new bone formation.

1: Hestomorphometric analysis at the end of 1st week: The osteoblasts cells in the experimental group appeared in the first week but only granulation tissue are present in control group. Mild new bone formation present in experimental group with thin bone trabeculea formation (Fig. 1). In the control group there was only accumulation of fibers and fibroblast at the site of implantation (Fig. 2).

Biostatical analysis: The statistical analysis of both bone trabeculea and osteoblast show that the relation between experimental and control groups was significant (Table 5, 6) (Diagram 7, 8).

Hestomorhpometric analyses at the end of 2nd week.
In the 2nd week there were very high and much rapped increases in number of osteoblasts in experimental group with appearance of few osteocytes within lacunae in new bone trabeculea. There was first appearance of osteoblast in control group in 2nd week that were few in number and present on very thin new bone trabeculea. The new bone trabeculea increased in thickness and became more prominent and contain lacunae for osteocytes in the experimental groups (Fig. 3, 4).

Biostatical analysis: The biostatical analysis also shows significant relation of experimental groups with control group (Table 7, 8) (Diagram 9, 10).

Hestomorphometric analysis at the end of 3rd week: In the third week the No. of osteoblasts in the experimental group stabilized on nearly constant level with high increasing in the bone thickness. The increasing in the No. of osteoblast was obvious in third week with gradual increase in the bone trabeculea thickness in control group (Fig. 5, 6).

Biostatical analysis: Biostatical analysis gave not significant relation between two groups in the No. of osteoblasts while show significant relation between experimental group and control group in bone trabeculea thickness. (Table 9, 10) (Diagram 11, 12).

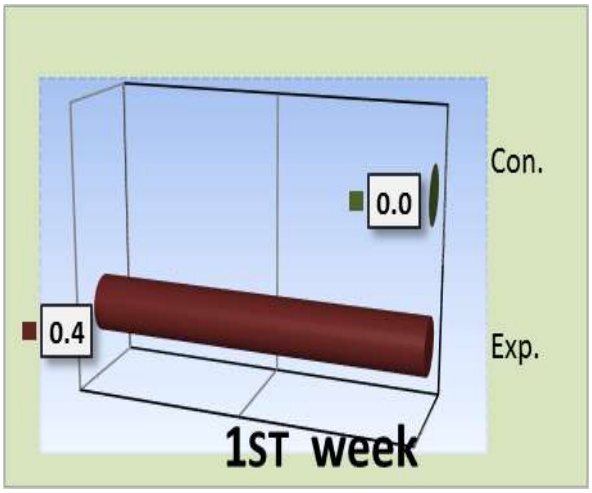
Hestomorphometric analyses at the end of 4th week. In the fourth week the No. of osteoblast reduced in experimental group as large No. of them convert to osteocytes and became equalized with No. of osteoblasts in control group. The bone thickness of two groups became prominent with very low differences between groups (Fig. 7, 8).

Biostatical analysis: Biostatical analysis gave not significant relation among two groups in both osteoblasts No. and bone trabeculea thickness after this period of study (Table 11, 12), (Diagram 13, 14).

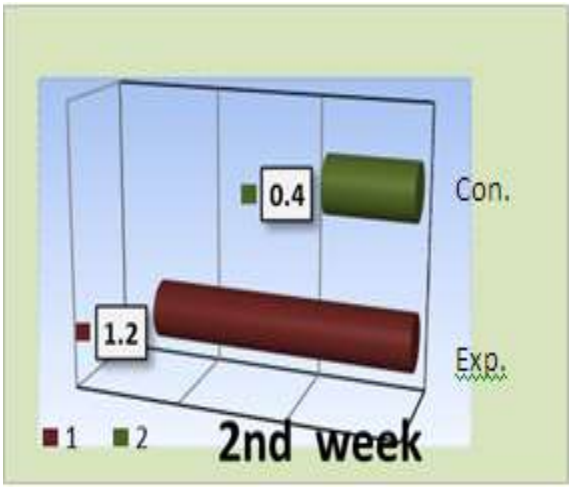
Discussion: The retention of implant to the bone occur in two way, first mechanical retention due to the presence of serration of screw but other retention is bioactive retention that is due to chemical materials cover the implant that induce

fibro-osseous integration that defined as ability of this material to stimulate migration of collagen fibers and osteoblast to the surface of implant and prevent formation of granulation tissue or cartilage (7). Titanium implant is a biocompatible material due to of present titanium oxide on its surface. Titanium dioxide TiO_2 has higher biocompatibility and increase bone adheasion to implant. The use of this surface coat induce osteoblast differentiation that have high level of alkaline phosphatase and high level of osteoclcin and produce osteogenic environments (8). Angiotensin converting enzyme (ACE) controls blood pressure by their constrictive effect. In addition to that control bone resorption that stimulate matrix metalloproteinase throw the osteoblastic receptors but inhibit the alkaline phosphatase activity also inhibit osteoblastic differentiation due to altering the expression of ostegenesis related transcription factor lead to suppression of ostegenesis to osteoblasts (9).

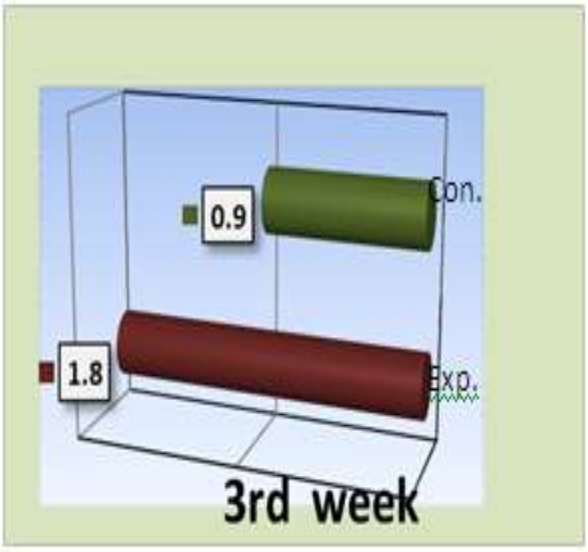
It's an angiotensin converting enzyme inhibiter use mainly as antihypertension drugs. The use of this drug is associated with reduction of osteoporosis and reduces bone fracture in elderly patient (10). The bone mineral density increased in the patient ues ACE inhibitor which has low incidence of bone fracture (11). There are three theories of ACE inhibitors mechanism that influence bone mass. First theory is due to decreased angiotensin II levels which acts on bone cells by a tissue-renin angiotensin system which help in regulation blood flow in capillaries of bone marrow and affects osteoclastic bone resorption. This increase possibility as bone has RAAS system (12). Second theory is due to binding to AT1 receptors on osteoblasts lead to preventing the release of mediators that activate the osteoclasts (13). Lastly by influencing calcium metabolism by increasing parathyroid hormone levels lead to decreasing ionized calcium in fracture site (14).



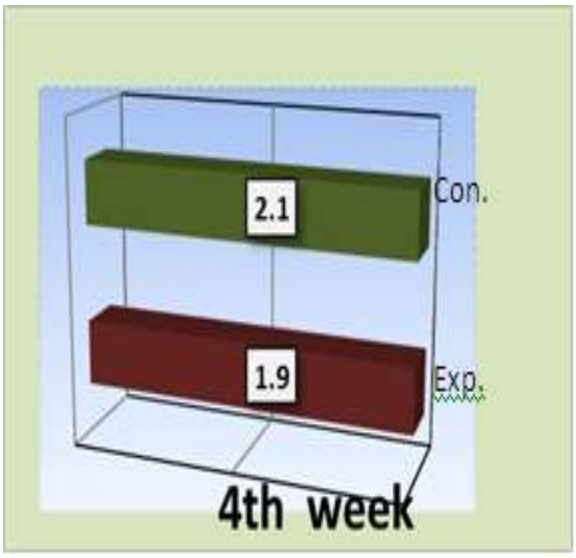
(Diagram 1) APL level at end of 1st week.



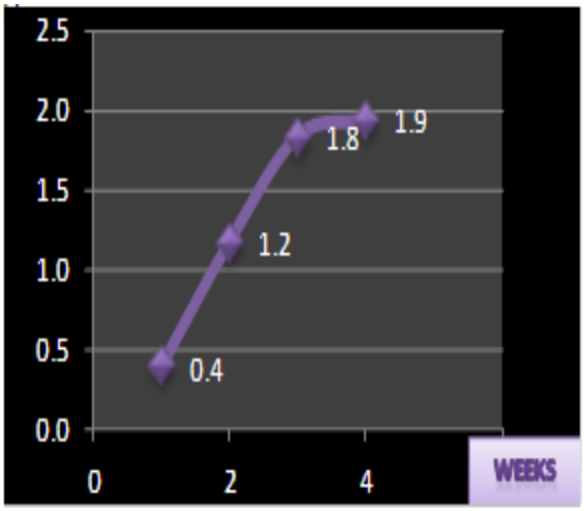
(Diagram 2) ALP level at end of 2nd week

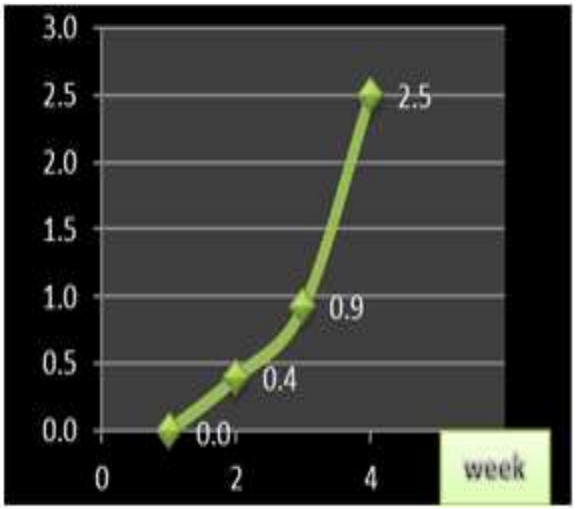


(Diagram 3) ALP level at end of 3rd week.

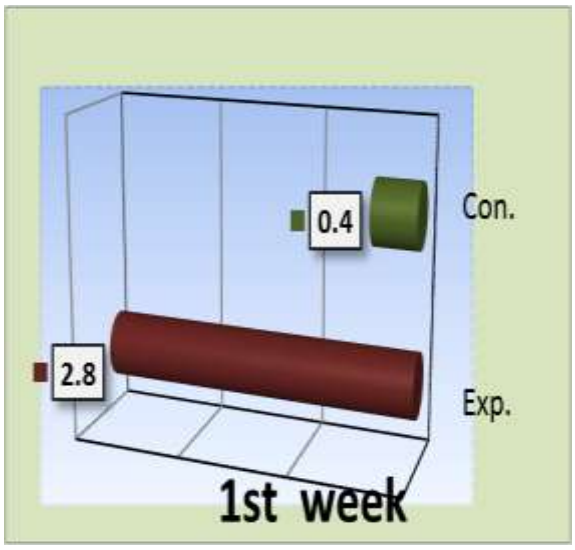


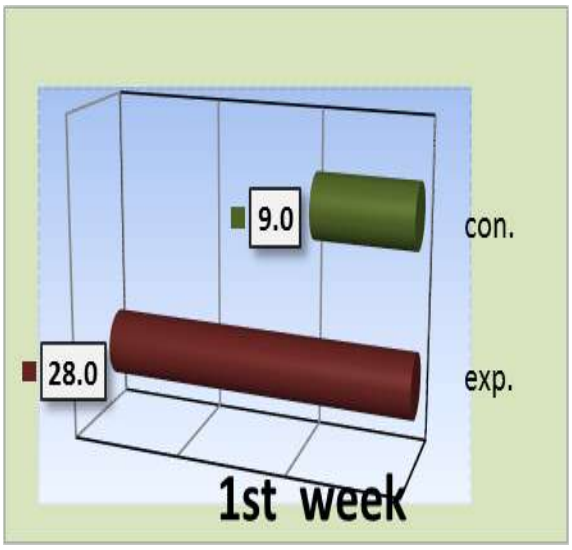
(Diagram 4) ALP level at end of 4th week.



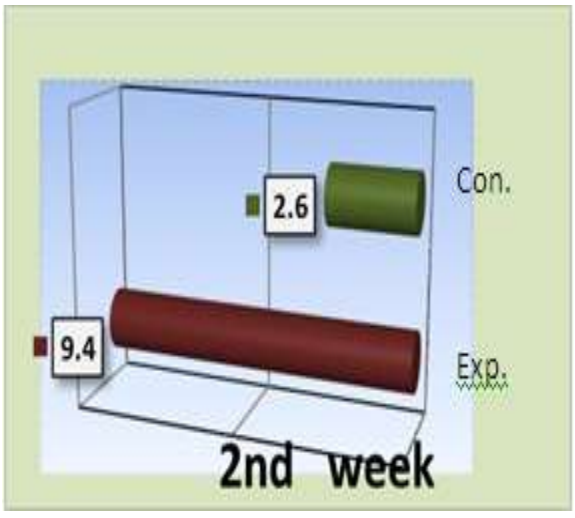


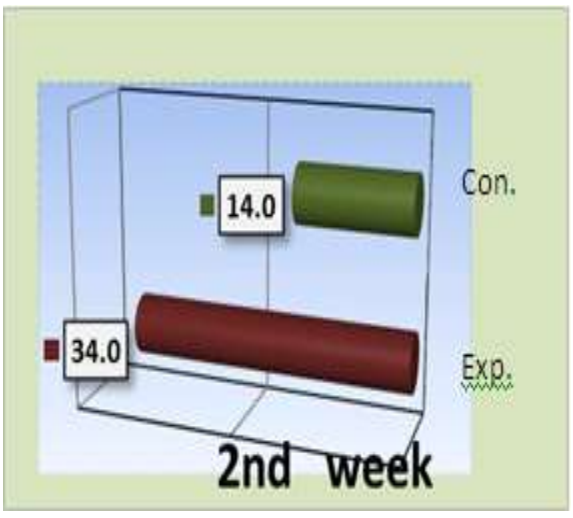
(Diagram 5) ALP level during month in exp. Group (Diagram 6) ALP level during month in con. group



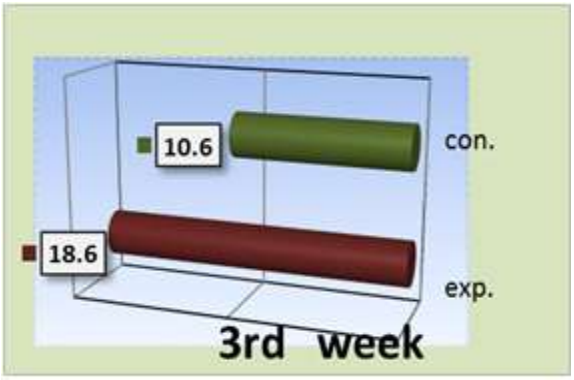


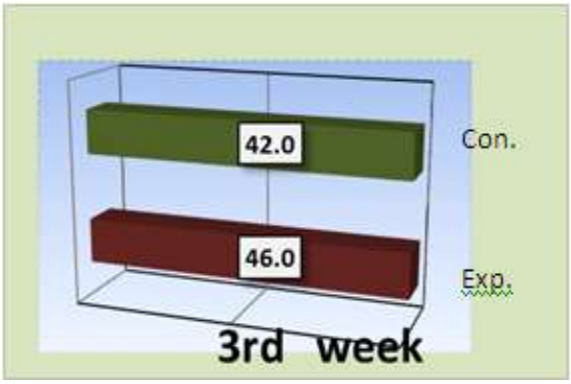
(Diagram 7) bone thickness at end of 1st week (Diagram 8) Osteoblasts No. at end of 1ST week



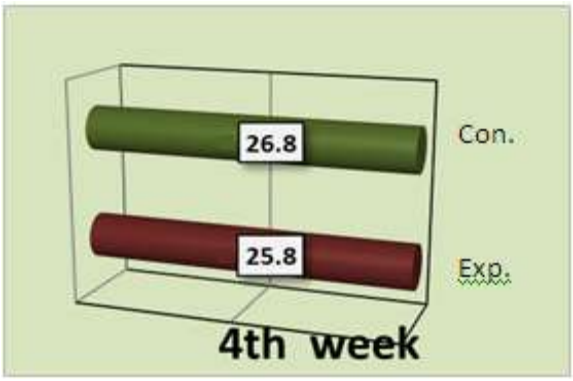


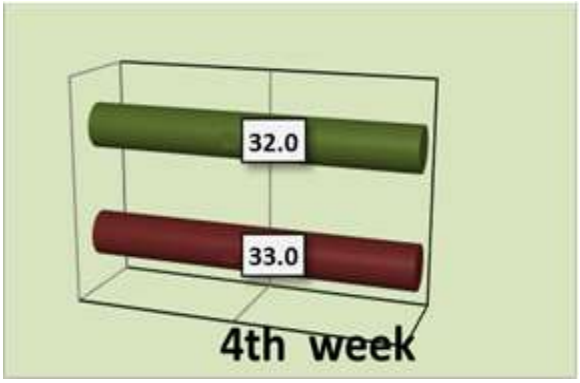
(Diagram 9) bone thickness at end of 2nd week (Diagram 10) Osteoblasts No. at end of 2nd week





(Diagram 11) bone thickness at end of 3rd week **(Diagram 12) Osteoblasts No. at end of 3rd week**





(Diagram 13) bone thickness at end of 4th week **(Diagram 14) Osteoblasts No. at end of 4th week**

(Table 1) Paired sample t. tests for alkaline phosphatase levels at the end of 1st week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).005

(Table 2) Paired sample t. tests for alkaline phosphatase levels at the end of 2nd week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).010

(Table 3) Paired sample t. tests for alkaline phosphatase levels at the end of 3rd week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).001

(Table 4) Paired sample t. tests for alkaline phosphatase levels at the end of 4th week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(not significant).056

(Table 5) Paired sample t. tests for osteoblast number at the end of 1st week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(High significant).000

(Table 6) Paired sample t. tests for bone trabeculea thickness at the end of 1st week

Paired Samples T Test		Sig. ≤ 0.05
Pair	experimental group - control group	(High significant).000

(Table 7) Paired sample t. tests for osteoblast number at the end of 2nd week.

Paired Samples T Test		Sig. ≤ 0.05
Pair	experimental group - control group	(significant).042

(Table 8) Paired sample t. tests for bone trabeculea thickness among at the end of 2nd week.

Paired Samples T Test		Sig. ≤ 0.05
Pair	experimental group - control group	(significant).018

(Table 9) Paired sample t. tests for osteoblast number at the end of 3rd week.

Paired Samples T Test		Sig. ≤ 0.05
Pair	experimental group - control group	(not significant).053

(Table 10) Paired sample t. tests for bone trabeculea thickness at the end of 3rd week.

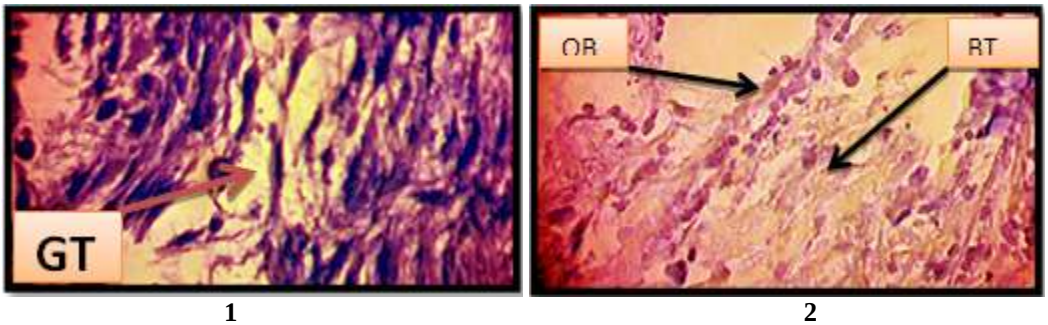
Paired Samples T Test		Sig. ≤ 0.05
pair	experimental group - control group	(significant).000

(Table 11) Paired sample t. tests for bone trabeculea thickness at the end of 3rd week.

Paired Samples Test		Sig. ≤ 0.05
Pair	experimental group - control group	(not significant).063

(Table 12) Paired sample t. tests for osteoblast number at the end of 4th week.

Paired Samples Test		Sig. ≤ 0.05
Pair	experimental group - control group	(not significant).974



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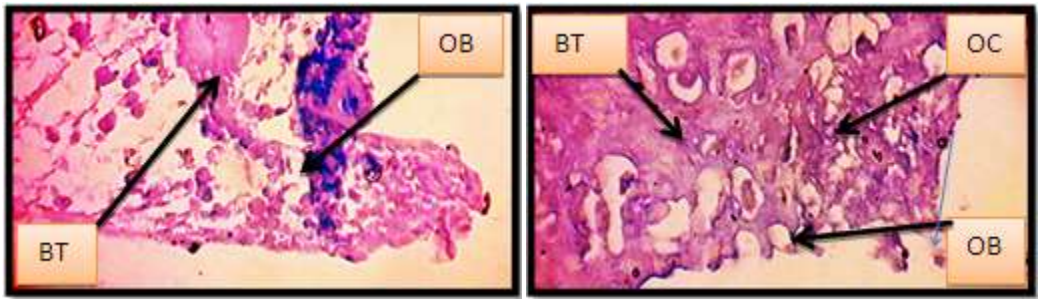
(Fig. 1) Digital micrograph of control group at the end of 1st week. GT.: granulation tissue. (H&E, 400X).
(Fig. 2) Digital micrograph of experimental group at the end of 1st week postoperatively. BT.: Bone trabeculea. OB. Osteoblasts. (H&E, 400X).



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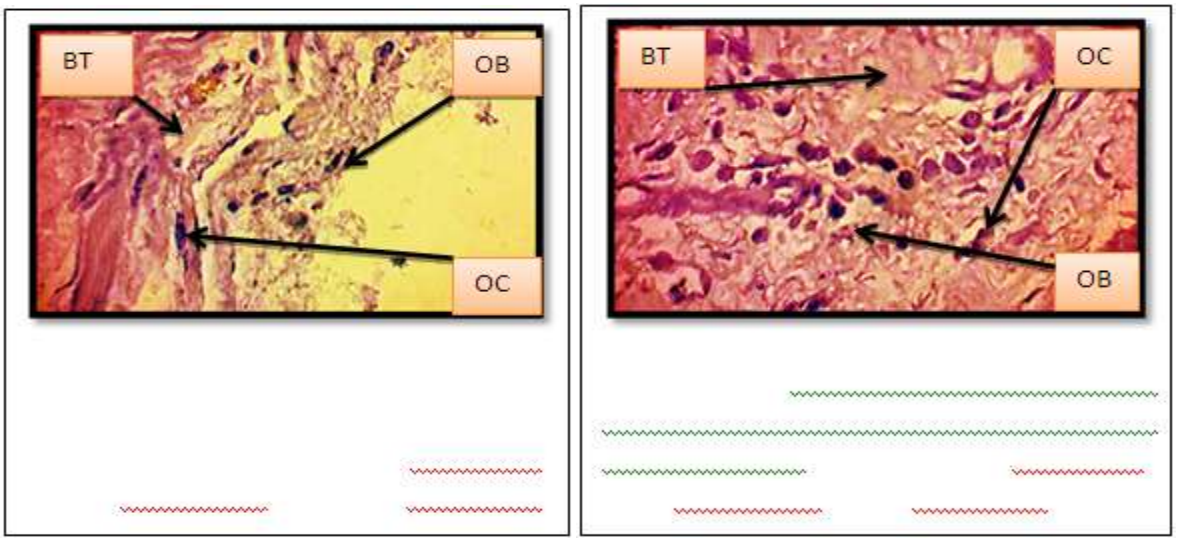
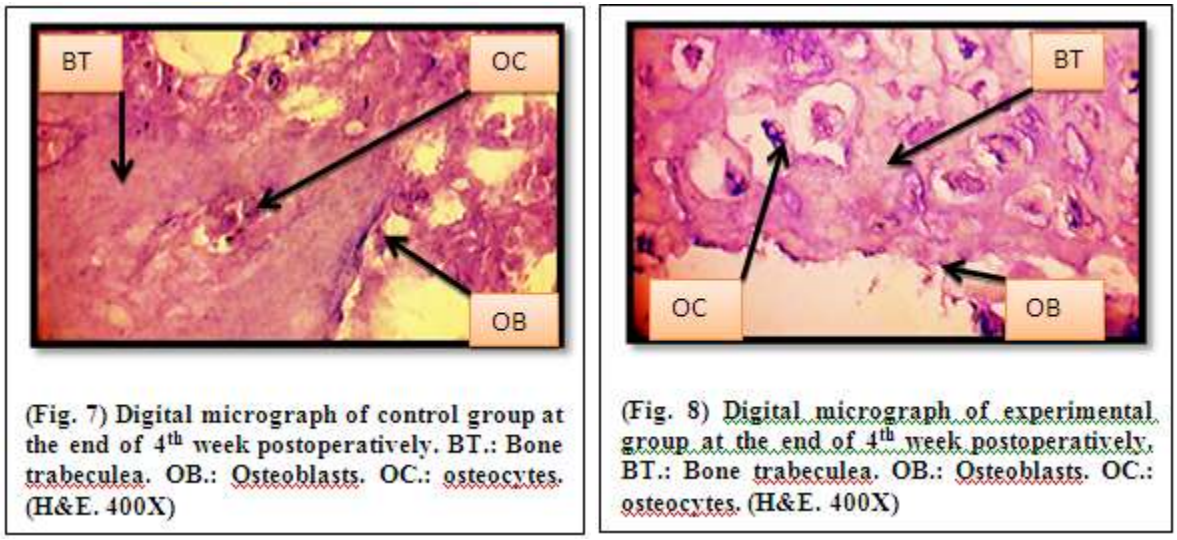
(Fig. 3) Digital micrograph of control at the end of 2nd week postoperatively. BT.: Bone trabeculea. OB.: Osteoblasts. (H&E 400X).

(Fig. 4) Digital micrograph ofexperimental group at the end of 2nd week postoperatively. BT.: Bone trabeculea. OB.: Osteoblasts. OC.: ostecyts (H&E 400X).





(Fig. 7) Digital micrograph of control group at the end of 4th week postoperatively. BT.: Bone trabeculea. OB.: Osteoblasts. OC.: osteocytes. (H&E. 400X)

(Fig. 8) Digital micrograph of experimental group at the end of 4th week postoperatively. BT.: Bone trabeculea. OB.: Osteoblasts. OC.: osteocytes. (H&E. 400X)

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