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Evaluation of the Role of local Atorvastatin on the Osseointegration (Experimental Study in Rabbits)

Asmaa Ali[1], Asst. Prof. Dr. Nada M. Hassan Al-Ghaban[2]

[1]B.D.S, M.Sc. in Periodontology, College of Dentistry, Baghdad University, Iraq
[2]B.D.S, M.Sc., PhD. Oral histology and biology, Oral diagnosis department, College of Dentistry, Baghdad University, Iraq

ABSTRACT

BACKGROUND: Statins are a commonly prescribed cholesterol-lowering drug; however, it has been shown at the recent time that they also have the beneficial side effect of enhancing bone matrix formation. There are very few data about the effect of Atorvastatin locally on the osseointegration around titanium implants.

AIM OF THE STUDY: This study was accomplished to evaluate the histological action of Atorvastatin as one member of the Statins family on the Osseo-integration to the titanium surface that modify histological responses.

MATERIALS AND METHODS: Titanium screw of 5mm length and 2mm width was used for fixation in the femur bone of twenty four rabbits. Control groups consist of 12 rabbits subjected to implant fixation alone. In the other 12 rabbits the cavities of implant filled with Atorvastatin powder before fixation of the implant as the experimental groups. We study both experimental and control groups 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 interval post operatively. Also, we examined the rabbit bone specific alkaline phosphatase serum level in each time interval for all groups by ELISA test.

RESULTS: Histomorphometric analysis shows 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 1st week with the reduction in the deference inosteoblasts number in the 2nd and3rd week until reach constant levels at the end of 4thweek. These results were supported by the level of alkaline phosphatase enzyme serum levels as it highly elevated at the first week in the experimental group that reduced gradually with progression of osseointegration around the titanium implant.

CONCLUSION: This study was illustrated that Atorvastatin enhanced osseointegration around the titanium implant by facilitating bone cells activation and differentiation.

INTRODUCTION

Fusion of new bone formation with implanted materials without formation of other tissues between them leads to production of highly differentiated tissue facilitate direct connection and provides preferable functional and structural interaction between implant surface and surrounding living tissues, which known as osseointegration. The Osseo induction 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 tissue environments, this ability come from the 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 provides a strong barrier that inhibits the reaction of titanium material with surrounding tissues[(2)].There is increased interest in cellular, biomaterials-based delivery of pro-osteogenic drugs for bone regeneration. Controlled delivery of shelf-stable small molecules, such as statins, that can stimulate endogenous cells to produce functional bone is particularly attractive because it circumvents important regulatory issues such as cell implantation and practical issues of implementation and clinical use, resulting in the development of a shelf-stable and relatively low cost therapeutic for bone regeneration[(3)]. Statins gather a class of agents, which inhibit hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase enzyme, leading to management of cholesterol production.

Atorvastatin (ATV), an inhibitor of HMG-CoA reductase enzyme, has been widely used in clinical practice in order to prevent cardiovascular accidents [4]. Apart from their hypolipemiant function, statins have stood out by their additional secondary effects, including anti-inflammatory, antioxidant, antithrombotic and endothelium stabilization actions [5], as well as angiogenesis promotion and increase of osteoblastic differentiation, inducing bone formation [6].

AIMS OF STUDY:

A: Histological study of the effects of the Atorvastatin on the Osseo-integration process.

B: serological study of alkaline phosphatase to support histomorphometric analysis of bone forming cells.

MATERIALS AND METHODS:

Pure titanium screw made cylindrical in shape with cute end with size of 0.5 mm in length and 0.2 mm width. They were oxidized to produce a micro coat of titanium oxide (TiO_2) on the surface of implant. Pure Atorvastatin powder 99% had been using 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.Twenty four male rabbit's weight 1.5 kg as a standard weight, divided into four groups 1, 2, 3 and 4 weeks. Each group contains 6 rabbits, 3 experimental and 3 controls. All groups subjected to standard surgical procedures. The animal anesthetized generally and the skin incised along the femur bone near the head. The two muscles over the femur separated by artery forceps to expose the bone without any trauma. Periosteal elevator was used toseparate any soft tissue attached to the bone. Then 0.5cm away from the head of femur we remove periosteum of the bone by handpiece with a round carbide bur, and make hole with 1.7mm width taper drill and 5mm length for the implant. This procedure and size of the hole is stander for all rabbits implant. In the control groups the titanium screw fixed in the socket with screw drivermanually until obtaining primary stability of the implant. No over driven done to prevent bone fracture, while in the experimental groups the hole had filled completely with Atorvastatin powder,and then the screw was tightened over the material. The excess of Atorvastatin washed out by distill water.

BIOPSIES COLLECTION: The site of operation at the femur of rabbits immediately opened after sacrificed by scalpel very carefully until exposed all length of femur. The femur removed completely and all soft tissues scarified from the bone, mainly around the screw head. The bone was cut 1cm around the screw toget a biopsy of 2cm in size with the screw in the middle of this distance. This biopsy kept in buffer formalin 10 % until histological examination.

DECALCIFIED SECTION:. After fixation the specimen was decalcified with a solution that prepared (one litter) as follows **1:** formic acid 99% concentration 50 ml. **2:** hydrochloric acid 1.18 sp. gr. 50 ml **3:**distill water 900 ml. Bone decalcification require three weeks to complete removal of calcium from bone, then rinse with tapwater for 30 minutes. Before starting processing, we remove the screw cautiously by screw driver to preserve the serration of new bone around implants. The specimen processed in automatic processor, which includes 12 containers. This process takes24 hours, the specimen remains 2 hours in each container as follows:**1:** 10% Formalin, 2 containers (4 hours) **2:** Ethanol alcohol, 5 containers (10 hours) the concentration 70%, 80%, 90%,100% and 100% respectively to dehydrate the spacemen. **3:** Xylol, 3 containers (6 hours) to eliminate the remnants of alcohol. **4:** Paraffin wax, 2 containers (4 hours).This specimen transported to the histocenter for making a block of wax, then cut the block by microtome into sections of 4-6micron and put in water bath to remove the wax and carried by a glass slide to starting the staining procedure which includes following steps: **1:** Heamatoxylin 5-15 minutes depending on the concentration of stain. **2:** Tap water 1-5 minutes to remove theexcess 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 lightmicroscope.

RABBITS SERUM COLLECTION: When the time is up, the rabbits sacrificed by using a very sharp knife to cut thejugular vein and allow heavy bleeding from the neck of a 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 rounds 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 test used (BAP) from the Mybiosource Company for antibody and protein ELISA kits (U.S.A.). After serum collected in the Eppendorf tubes and kept frozen at -20C, 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:** Microplate 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:** 10mlof 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 weeks 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 orserum samples added to the appropriate well in the antibody pre-coated micro titer plate. **3:** 100µl of 9% physiologicalsaline (PH7 -7.2) added to the blank control well. **4:** 50µl of conjugate added to each well, except the blank control well. **5:** Cover and incubate the plate for one hour at 37°C. **6:** The microplate washed using an automated washing machine in which the plate washed five times with a diluted wash solution (350-400µl/well/wash). The washer was set for a soaking time of 10seconds and shaking time of 5 seconds between each wash. **7:** The plate was inverted and plot dried by hitting the plate 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 1 minute 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 is 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 at different levels of implant bed. The microscopically finding includes evaluation of cell forming bone as well as bone lamellae. The cell count and measurement of lamellar thickness was illustrated by using special graduated microscopically lens at the power of magnification of 400X.

CRITERIA OF MEASUREMENTS: 1: Four randomly selected location of each section was examined. **2:** Each location divided by graduating 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 considered later in biostatical analysis.

STATISTICAL ANALYSIS: We have four groups to be examined biostatistically. Each group divided equally into experimental and control. 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 statistically analyses applied by using Microsoft office excel to obtain a relationship between groups and changes during four time intervals of each group.

RESULTS

A: Alkaline Phosphatase (ALP) Examined By Elisa Test: ELISA test applied to the 4 groups for each timeinterval

1: Analysis Of The Elisa Test Results For Alkaline Phosphatase At The End Of 1st Week: ALP was absent in the rabbits of control group in an ELISA test while show mild increase in ALP level in the experimental group(Diagram 1).

Biostatical Analysis: At the end of 1st week the relation between experimental and control groups was significant (Table1).

2: Analysis Of The Elisa Test Results For Alkaline Phosphatase At The End Of 2nd Week: During the second week after implantation both 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 3rdweek: The relation between two groups still constant after three weeks of operation, but there was a mild increase in the level of ALP in the 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 the control group after four weeks while it stabilized on constant level in experimental groups lead to make the relation between groups not significantly(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 the 1st week and show slow rising of its level during the month until reach constant level. The serum analysis for the ALP was showing negative results in the control group in the first week. The appearance of ALP noticed after two weeks and increased during the third and fourth week to reach a 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 technique used graduated lens to measure the bone trabeculea thickness and the number of osteoblast associated with new bone formation.

1: Histomorphometric Analysis At The End Of 1st week: The osteoblasts cells in the experimental group appeared in the1st week, but the only granulation tissue is present in the control group. In the control group there was only an accumulation of fibers and fibroblast at the site of implantation (Fig. 1).While in experimental group there were thin bone trabeculea formation (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, 9) (Diagram 7, 13).

Histomorhpometric Analyses At The End Of 2nd Week
In the 2nd week there was very high and much faster increase in the number of osteoblasts in the experimental group with the appearance of few osteocytes within lacunae in new bone trabeculea. There was the first appearance of osteoblast in the 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).

Biostatistical Analysis: Also shows significant relation of experimental groups with control group (Table 6, 10) (Diagram 8, 14).

Histomorphometric Analysis At The End Of 3rd week: There is stabilization in the number of osteoblasts in the experimental groupon a nearly constant level with high increasing in the bone thickness. The increasing in the No. of osteoblast was obvious in 3rd week with gradual increase in the bone trabeculea thickness in control group (Fig. 5, 6).

Biostatical Analysis: There is not significant relation between the two groups in the number of osteoblasts while show significant relation between experimental group and control group in bone trabeculea thickness. (Table 7, 11) (Diagram 9, 15).

Histomorphometric Analyses At The End Of 4th week. During the fourth week the number of osteoblast reduced in the experimental group as a large number of them convert to osteocytes and became equalized with a number of osteoblasts in the control group. The bone thickness of two groups became prominent with very low differences between groups (Fig. 7, 8).

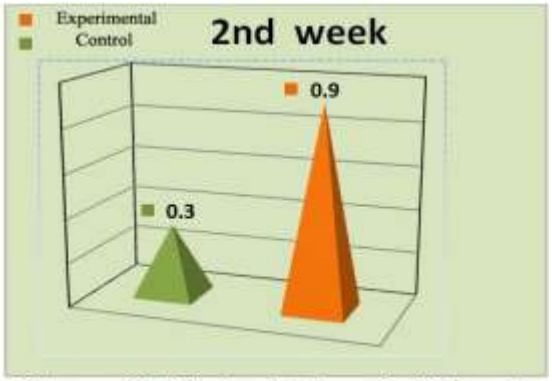
Biostatical Analysis: Biostatical analysis gave not a significant relation between the two groups in both osteoblasts number and bone trabeculea thickness after this period of study (Table 8, 12), (Diagram 10, 16).

DISCUSSION:

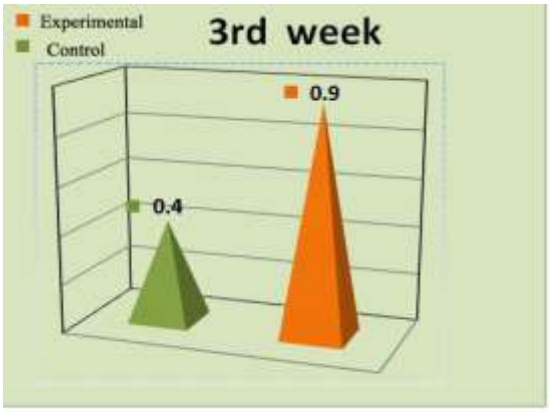
The retention of implant to the bone occurs in two way, first mechanical retention due to the presence of serration of the screw, but other retention is a bioactive retention that is due to chemical materials cover the implant that induce fibro-osseous integration that defined as the ability of this 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 the presence of titanium oxide on its surface. Titanium dioxide TiO_2 has higher biocompatibility and increase bone adhesion to implant. The use of this surface coat induces osteoblast differentiation which has increased levels of alkaline phosphatase and high level of osteocalcin and produce osteogenic environments [8].

The demonstrated potential of statins to promote bone regeneration has spurred an interest in the development of local delivery strategies for statins. Local delivery is of interest for a number of reasons. First, systemic administration of statins can result in rare but serious side effects, most notably liver toxicity, myositis (inflammation of the muscle), and rhabdomyolysis (severe muscle inflammation and damage)[9] .Second, local delivery allows an adequate dosage to be delivered to the desired area without relying on systemic administration, which depending on the situation may be hindered by impaired vascularity. Local administration may lessen the possibility of widespread muscle and liver damage and obviate any unintended effects of statin usage given its pleiotropic effects while providing therapeutic benefit to the delivery area. Additionally, the concentrations needed to take advantage of the antimicrobial nature of the statins would be difficult to achieve with systemic delivery, but are feasible with local delivery[10].

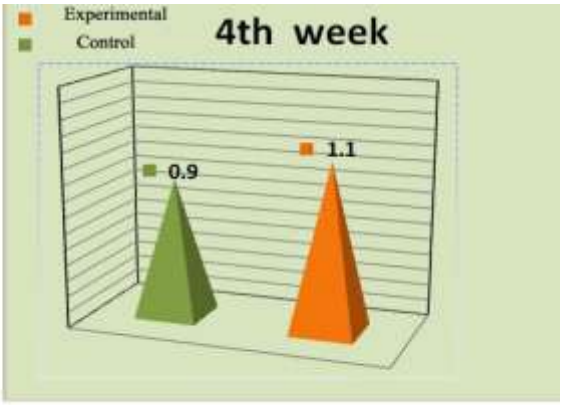
(Diagram 1) APL level at the end of 1st week



(Diagram 2) APL level at the end of 2nd week



(Diagram 3) APL level at the end of 3rd week

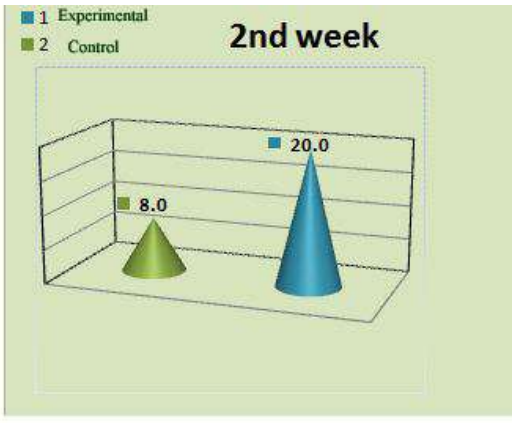


(Diagram 4) APL level at the end of 4th week

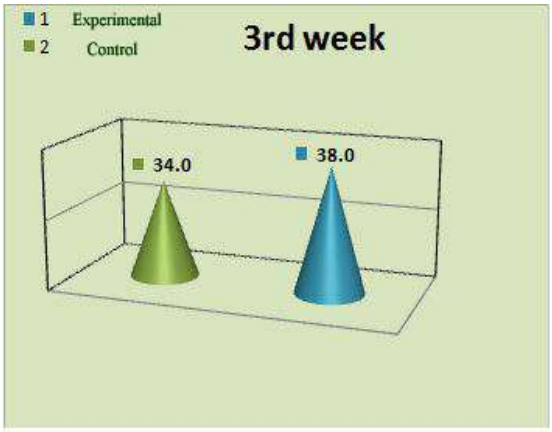
(Diagram 5) ALP level during month in exp. group

(Diagram 6) ALP level during month in cont. group

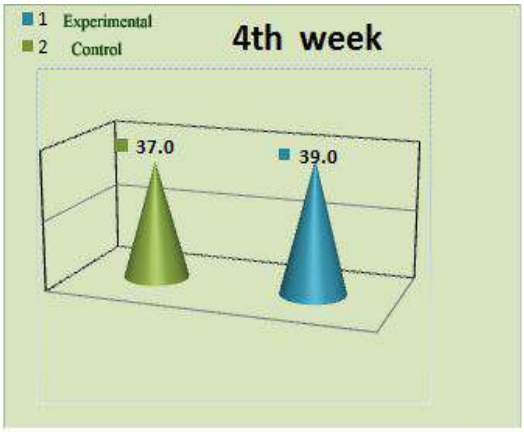
(Diagram 7) Osteoblast No. at the end of 1st week



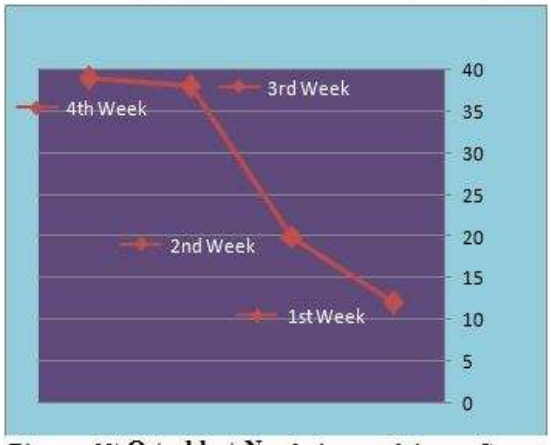
(Diagram 8) Osteoblast No. at the end of 2nd week



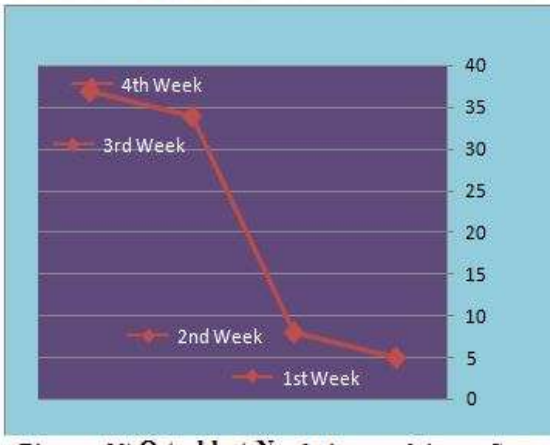
(Diagram 9) Osteoblast No. at the end of 3rd week



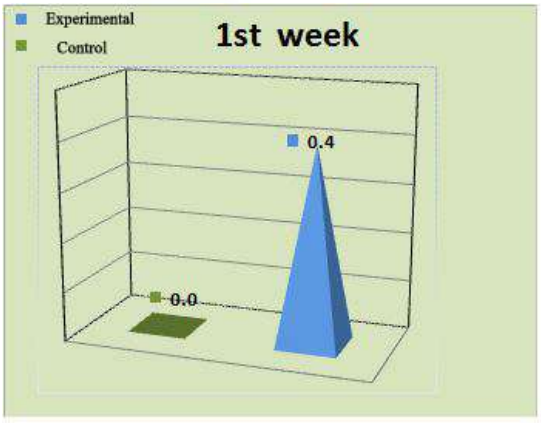
(Diagram 10) Osteoblast No. at the end of 4th week



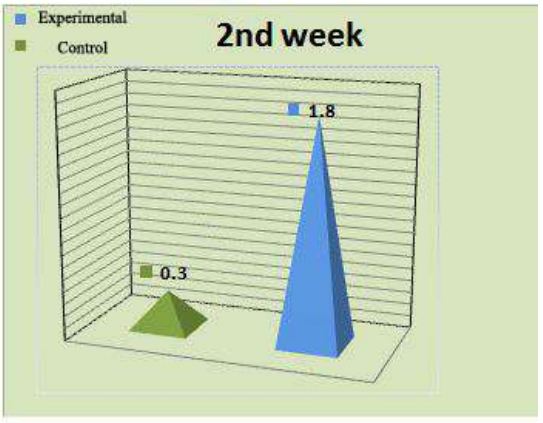
(Diagram 11) Osteoblast No. during month in exp. Group



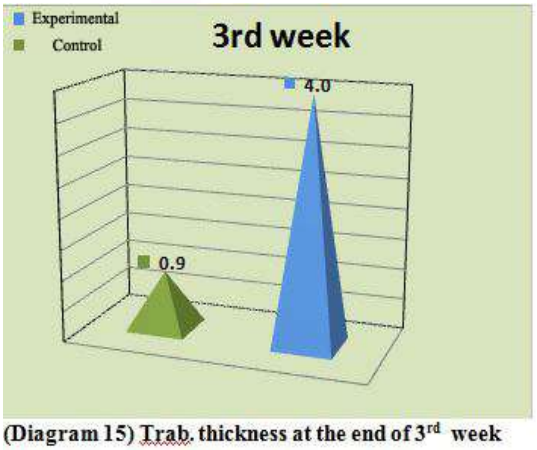
(Diagram 12) Osteoblast No. during month in con. Group



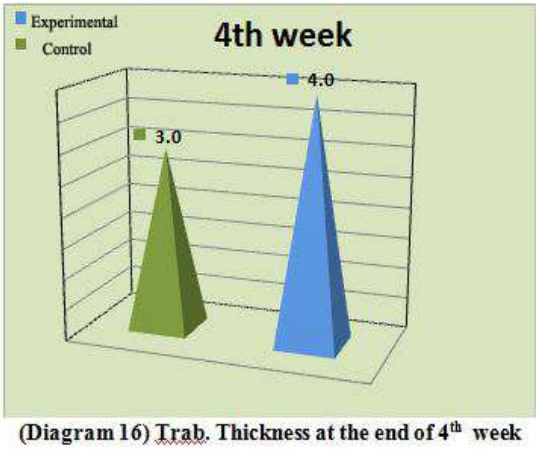
(Diagram 13) Trab. thickness at the end of 1st week



(Diagram 14) Trab. Thickness at the end of 2nd week



(Diagram 15) Trab. thickness at the end of 3rd week



(Diagram 16) Trab. Thickness at the end of 4th week

(Diagram 17)Trab. Thickness during month in exp. Group

(Diagram 18) Trab. Thickness during month in con. Group

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

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Significant 0.004

(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 0.005

(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 0.02

(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 0.066

(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	Significant 0.003

(Table 6) 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 0.007

(Table 7) 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 0.05

(Table 8) Paired sample t. tests for osteoblast number at the end of 4th week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Not Significant 0.07

(Table 9) Paired sample t. tests for Trabecular thickness at the end of 1st week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Significant 0.004

(Table 10) Paired sample t. tests for Trabecular thickness at the end of 2nd week.

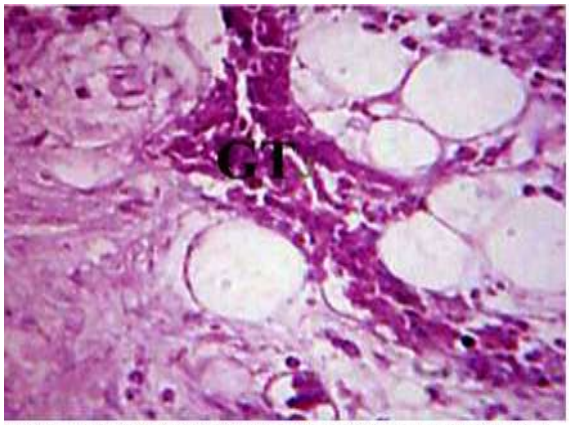
Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Significant 0.005

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

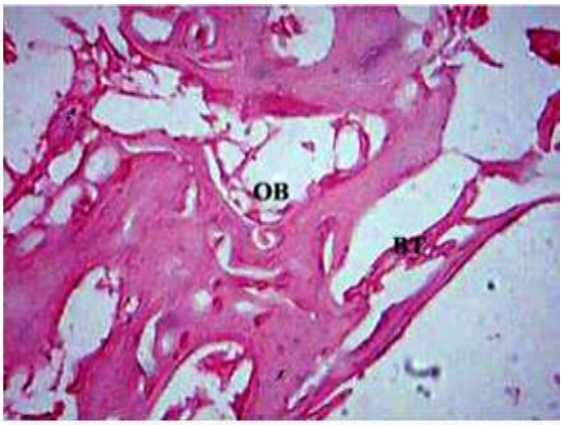
Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Significant 0.02

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

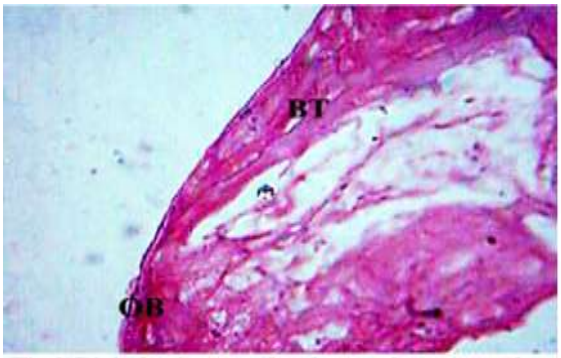
Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group – control group	Not Significant 0.05



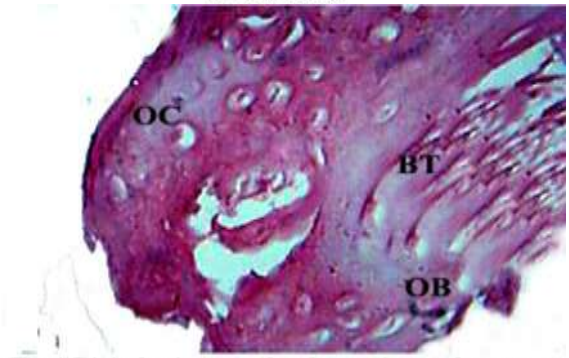
(Fig. 1) Digital micrograph of control group at the end of 1st week. GT.: granulation tissue. (H&E, 400X).



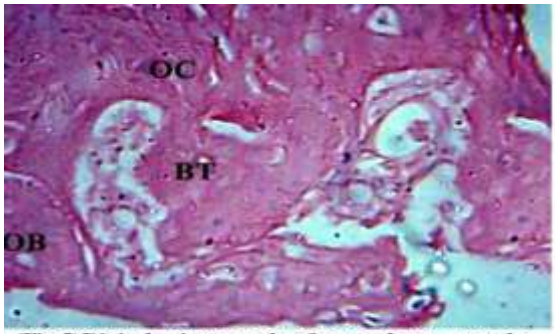
(Fig. 2)Digital micrograph of experimental group at the end of 1st week. BT: Bone trabeculea. OB: Osteoblast. (H&E, 400X).



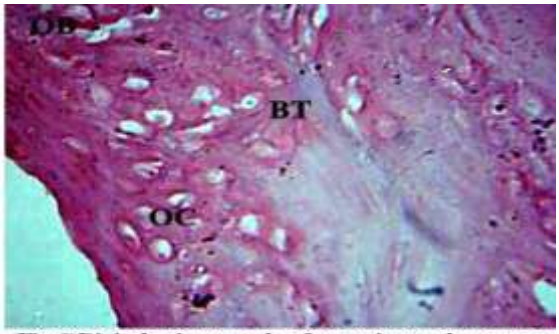
(Fig.3)Digital micrograph of control group at the end of 2nd week BT: Bone trabeculea. OB: Osteoblasts (H&E 400X).



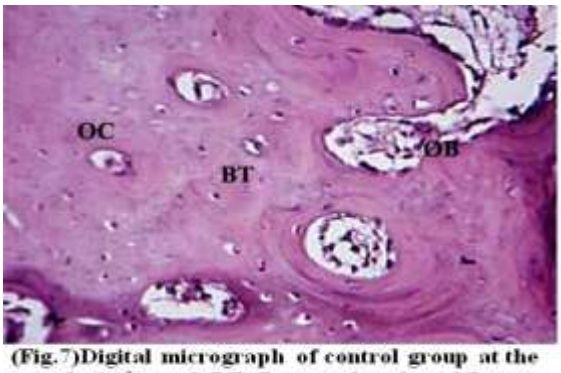
(Fig.4)Digital micrograph of experimental group at the end of 2nd week. BT: Bone trabeculea. OB: Osteoblasts. OC: ostecyts (H&E 400X).



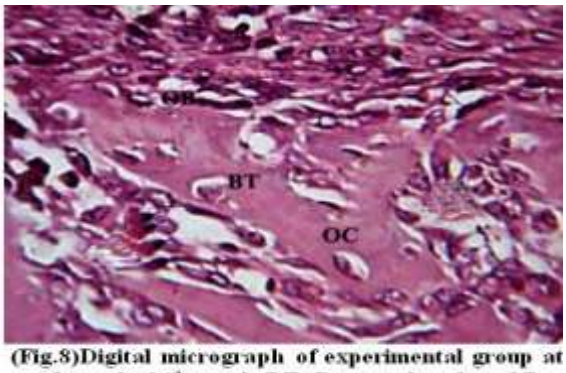
(Fig.5)Digital micrograph of control group at the end of 3rd week.BT: Bone trabeculea. OB: Osteoblasts (H&E 400X).



(Fig.6)Digital micrograph of experimental group at the end of 3rd week. BT: Bone trabeculea. OB: Osteoblasts. OC: ostecyts (H&E 400X).



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



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

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