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Osseo integration enhancement to titanium oxide surface by osteon^{TM} (collagen type I and hydroxyapatite crystals) (Histological study on rabbits)

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ABSTRACT

Background: Titanium is the most biocompatible materials used for implantation in the bone. It has high biological response that there is no corrosion and high stability in body fluids and don't induce allergic reaction or produce toxic effects to surrounding tissue. Some modification on the surface texture in which previous oxidation of titanium surface to produce titanium oxide TiO2 enhances its biological response that increase surface attachment to osteoblasts and increase osteoblast proliferation and differentiation from its osteoblast precursor cells in the bone marrow with gradual elevation of osteocalcin level. Wide range of studies applied to improve the biological responses to titanium implant. As the collagen fiber type I is the first type of fiber precipitate during bone healing process and the hydroxyapatite crystals HAC is the main component of extracellular bone matrix, we study the biological activities of these materials in the enhancement of osseointegration process.

Aim of the study: This study was carried out to evaluate the histological and biological activities of combination of collagen type I and hydroxyapatite crystals (HAC) on the osseointegration process around titanium oxide layer coated titanium implant.

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 osteon^{TM} (COLLAGEN I & HAC) before fixation of implant as experimental groups. Both experimental and control groups are studed in intervals of 1, 2, 3 and 4 weeks. Each group's examined histomorphometrically under fluorescent microscope for undecalcified section without staining by using of oxytetracycline dihydrate as fluorescent marker administered intravenously that assist counting of bone cells and bone trabeculea thickness at the end of each four period's intervals post operatively supported by examination of osteocalcin serum level in each time intervals for all groups by ELISA test.

Results: histomorphometric analysis of fluorescent undecalcified sections enhance specific visualization of osteoblast and new bone trabeculea as oxytetracycline fluorescent markers have high affinity to calcium groups in osteoblast and bone matrix. This 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. The elevation in the number of osteoblast combined with elevation of osteocalcin level in the serum that deticted by ELISA test for all control and experimental groups.

Conclusion: This study was illustrated that osteon^{TM} (collagen I & HAC) enhanced osseointigration around titanium implant by facilitate bone cells activation and differentiation in addition to increase bone density due to it support extracellular matrix.

INTRODUCTION

Osseointegration process enhancement to the dental implants will be the most challenge to the dental biologists. The importance of this field comes from increase requirements of artificial tissue replacement in all branch of dentistry in addition to the wide range of materials used in this application with their varying degree of biocompatibility. Osseointegration 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 osseoinduction 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 undesirable tissue reactions were reduced by using of biocompatible enhancing materials such as bone grafting, osteogenic biological coatings and biophysical stimulation (3).

Type-I collagen, is the first and most abundant type of bone matrix protein to be appeared during bone healing and regeneration. Collagen coating on titanium implants show earlier and higher expression of bone cells migration and differentiation with acceleration of bone remodeling and enhancement of bone matrix protein perspiration on the collagen implant coating (4). Early bone healing around titanium implant very accelerate by addition of extracellular matrix component mainly collagen fiber type I lead to farther bone formation around implant (5). The implant coating with collagen fibers exhibited highest level of alkaline phosphatase enzyme activity among the other titanium implant cover by calcium phosphate and titanium modified by acid etched surface (6).

Since the discovery titanium implant by Brånemark in 1958, wide has been opened to improve the properties of titanium implants. One method is to coat the implant with a material that has similar properties as the mineral component of bone. For this purpose hydroxyapatite (HA), a calcium phosphate with the formula (Ca10)(PO4)6(OH)2 has been used (7). HA is widely used as a biomaterial due to its excellent compatibility with bone. The HA material usually produced commercially today in many form like powder or crystals resorbable and unresorbable in addition to very slowly resorbable form (8, 9).

AIMS OF STUDY:

A: Histological study enhanced by immune fluorescent technique to evaluate effects of the osteon ^{TM} (collagen I & HAC) on the osseintegration process to the titanium oxide surface.
B: serological study of bane forming cells enzyme osteocalcin 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. Osteon^{TM} II collagen is aboven bone filler composed of synthetic bone graft hydroxyapatite crystals 25% and bovine type one I collagen 75%. Osteon^{TM}II collagen absorbed slowly over several weeks after helping the initial shaping. Osteon^{TM}II collagen is a trimmable and moldable thanks to collagen coating. Osteon^{TM}II collagen was sterilized by gamma radiation. This material is moistening with blood or saline solution before use. This makes the application easy and can take any shape of sites of application. Rabbit osteocalcin 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 osteom ™ after moisturizing it with distillate water before screw driving then the screw was drove over the material. The excess of osteon ™ washed out by distillate water. This procedure insures the same amount of material 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.

Administration of fluorescent marker: Oxytetracycline dihydrate used as a fluorescent marker of osteoblast. Alamycin LA. 200mg. administered in a dose of 30mg/kg each five days. The first dose starts ten days before operation that mean two doses before operation to insure saturation of the body with this material from first day of implantation. Third dose administered during operation and continuous every five days. Addition dose had been given 24 hours before sacrifice rabbit also to insure the saturation of all new cells with marker.

Undecalcifide section preparation: In order to preserved oxytetracycline marker for osteoblast that removed by the acid of decalcified section, we used this type of section to examined the bones rabbit under fluorescent microscope.

Ground section technique: 1. After 5 day of fixation, the bone containing implant impression bed was thoroughly washed with distilled water. **2.** Each bone specimen stored individually in a vial contains normal saline and then mounted in acrylic in vertical position with exposed end of implant bed to allow for cutting. **3.** Ground sections of bone were prepared by cutting through implant impression bed in cross plane, about (12.µm) using low speed saw of a hard-tissue minitome. **4.** The prepared sections were then dehydrated by immersing them in ascending concentrations of alcohol (70%, 80%, and 90% for 1 min. intervals). **5.** Then mounted on glass slides and photographed under fluorescent 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 ostecalcin: In this tests used osteocalcin 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 fluorescent microscope: The histological examination was performed to the four groups by vertical undecalcified section under fluorescent 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.

Statical 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: Osteocalcin examination by ELISA test: ELISA test applied to the three groups for each time intervals (Time intervals include four steps along one month, One week for each interval).

1: Analysis of the ELISA test results for osteocalcin at the end of 1st week: The levels of the osteocalcin was markedly increased in the experimental groups (Osteon and HAC) but the increasing of its level in the control group was very low (Diagram 1).

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

2: Analysis of the ELISA test results for osteocalcin at the end of 2nd week: The rising in the levels of osteocalcin in experimental groups was continuous with high aspect while the control groups show gradual elevation of its level (Diagram 2).

Biostatical analysis: The relation still significant between experimental groups and control groups (Table 2).

3: Analysis of the ELISA test results for osteocalcin at the end of 3rd week: We noticed marked increase in the level of osteocalcin in control group lead to the reduction of variation in the levels between two groups (Diagram 3).

Biostatical analysis: The relations not significant between two groups in 3rd week (Table 3).

4: Analysis of the ELISA test results for osteocalcin at the end of 4th week: There were high reduction in the differences and fixation the levels of osteocalcin in all groups (Diagram 4).
Biostatical analysis: The relations not significant between groups (Table 4)

5: Analysis of ELISA test results of osteocalcin levels for each group through the month of study.

We show rapped increasing of osteocalcin in first two week of experimental groups then stabilized in standard level but in control group the level of ostecalcin raised gradually during month until reach fixed level (Diagram 5, 6)**.**

B: Histomorphometric analyses by fluorescent microscope: In this examination used ground (undecalcified) section without stain for fluorescent microscope. In this techniques used graduated lens to measure the bone trabeculea thickness and the number of osteoblast associated with new bone formation. The osteoblast takes fluorescent marker of bright yellow wave length while bone trabeculea take marker with green wave length under fluorescent microscope.

1: Hestomorphometric analysis at the end of 1st week: The oxytetracycline marker accumulates at the site of new bone formation and binned to the functional osteoblasts and calcium phosphate molecules in bone trabiculea. The numbers of fluorescent spots or osteoblasts in the experimental group were higher than that of control group. The green color at serration site of implant was the marker of mild new bone formation present in experimental group with thin bone trabeculea formation (Fig. 2). In the control group there was black filed with very low acceptance of oxytetracycline marker. This gave indication that the osteoblast nonfunctional in this stage (Fig. 1).

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 obvious and rapped increases in number of osteoblasts in experimental group with appearance of few osteocytes within lacunae in new bone trabeculea. There was mild gradual increase in number 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 became more prominent and contain lacunae for osteocytes in the experimental group (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. This stabilization in osteoblasts No. was due to conversion some of cell to osteocytes. 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). The fluorescence of the bone in this stage will become more ideal and contain bone trabiculea take market green color with osteoblasts and osteocytes had good brightness.

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 but the No. of fluorescent dyes increased as the osteocyte also take oxytetracycline marker. The bone thickness of all groups became prominent with very low differences among groups (Fig. 7, 6).
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).

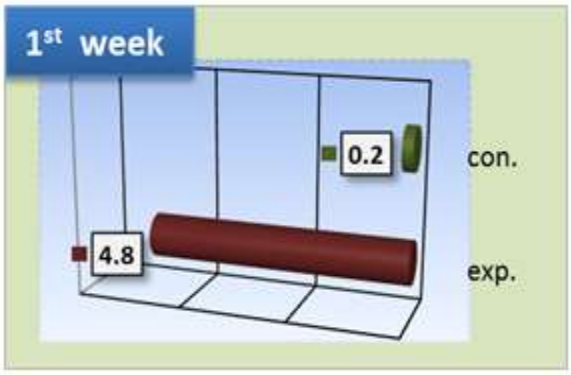
Discussion: Titanium implant is a biocompatible material due to of present titanium oxide on its surface. Titanium dioxide TiO2 has higher biocompatibility and increase bone adhesion to implant. The use of this surface coat induce osteoblast differentiation that have high level of alkaline phosphatase and high level of osteoclcin and produce osteogenic environment (10).

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 (11). Titanium implant covered by collagen fiber type I induce osseointigration significantly more than that of titanium implants alone (12).

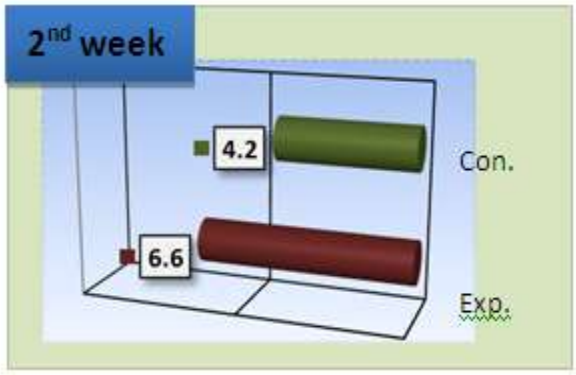
The cell growth of osteoblast are continuous around titanium implant with presence of collagen type I fibers and osteonectin. He said also that titanium not toxic and it biocompatible with osteoblast. The presence of collagen type I facilitate adhesion of implant to surrounding tissue and prevent growth of granulation tissue and enhance osteoblast accumulation on the titanium implant (13).

Many study compared the effect collagen I coating implant with titanium implant alone. The titanium implant coated by collagen type I increase osseointegration by enhance initial osteoblasts adhesion to surface of implant. This authors give an agreement of our study about that collagen type I increased osseointegration to the titanium implant and increase number of osteoblasts on the surface of its (14).

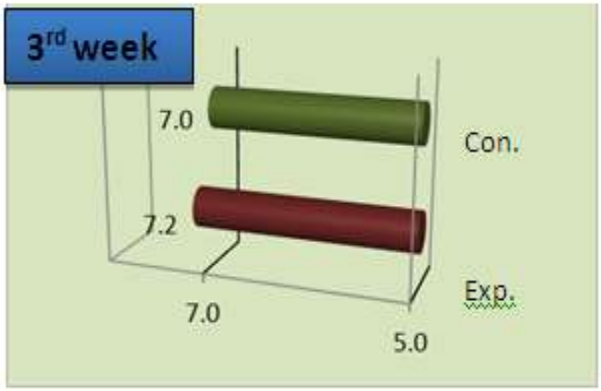
Early bone healing around titanium implant very accelerate by addition of extracellular matrix component mainly collagen fiber type I and HAC lead to farther bone formation around implant (5).The implant coating with collagen fibers exhibited highest level of alkaline phosphatase and osteocalcin enzyme activity among the other titanium implant cover by calcium phosphate and titanium modified by acid etched surface (6). These results is in agreement with our study in which that addition of collagen fiber type I on the surface of titanium implant accelerate bone implant secondary fixation by increase osteoblast differentiation and attachment to the surface of implant lead to enhance oseintegration.



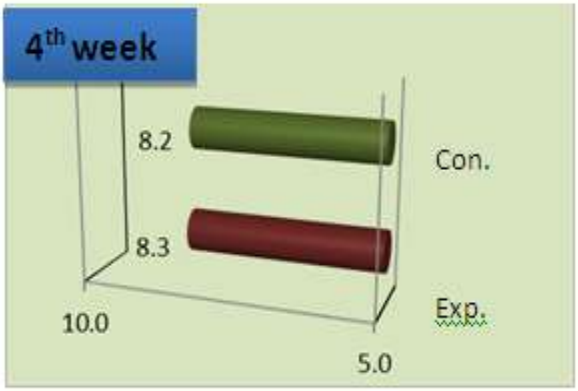
(Diagram 1) Osteocalcin levels at the end of 1st week.



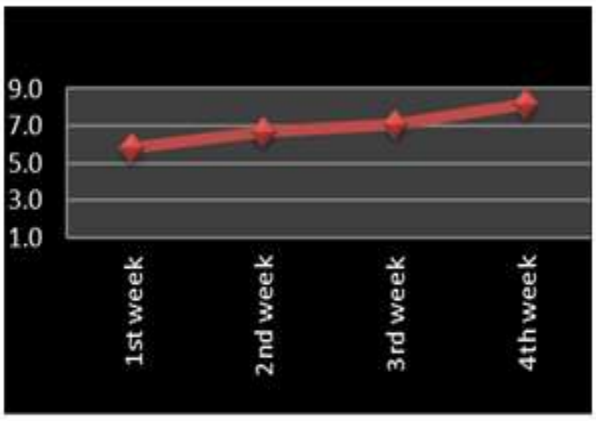
(Diagram 2) Osteocalcin levels at the end of 2nd week.



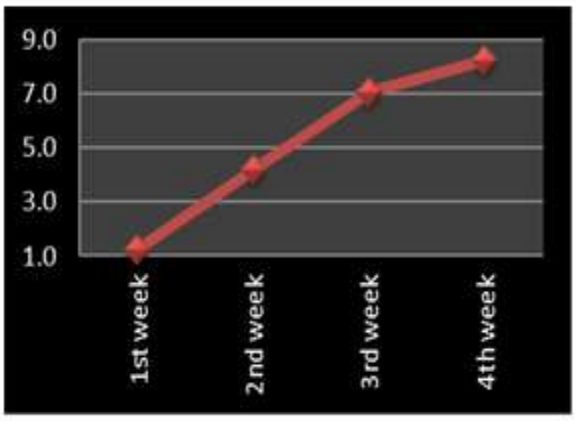
(Diagram 3) Osteocalcin levels at the end of 3rd week.



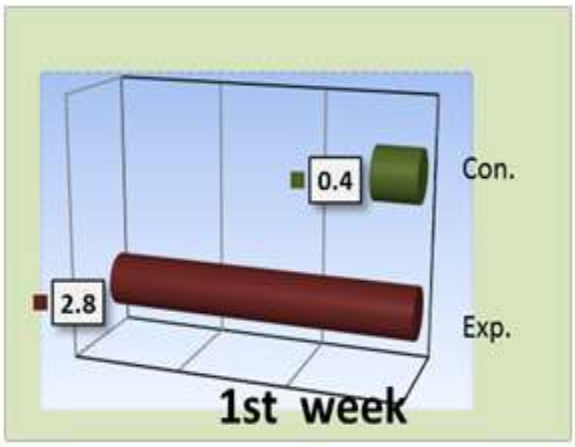
(Diagram 4) Osteocalcin levels at the end of 4th week.



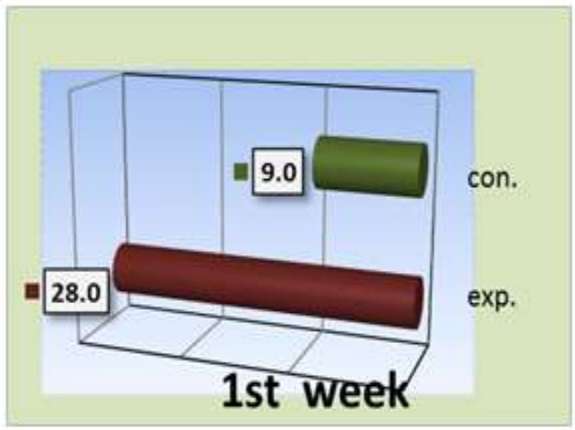
(Diagram 5) Osteocalcin level during month in exp. group



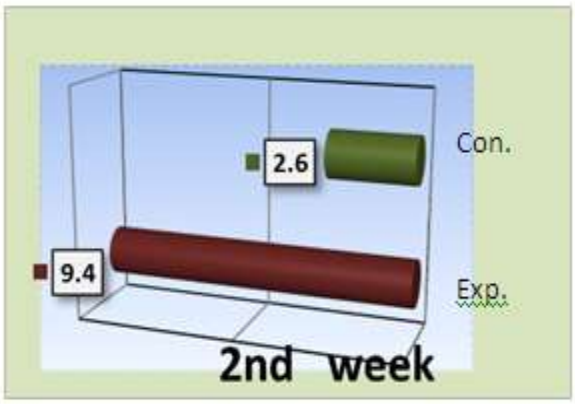
(Diagram 6) Osteocalcin level during month in con. group



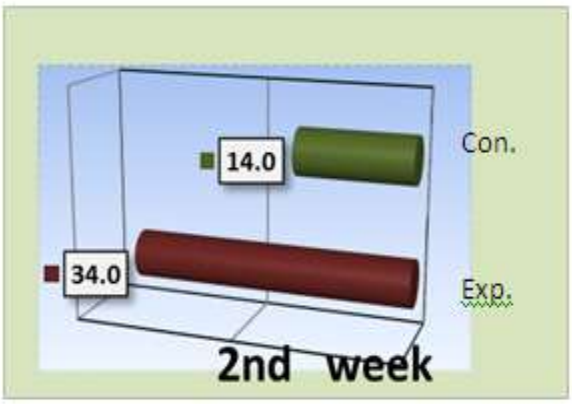
(Diagram 7) bone thickness at end of 1st week



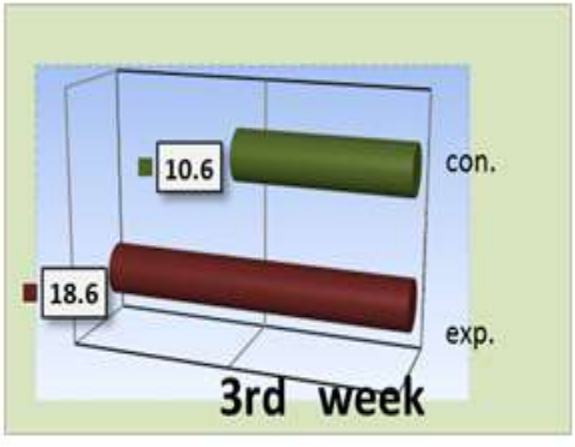
(Diagram 8) Osteoblasts No. at end of 1ST week



(Diagram 9) bone thickness at end of 2nd week

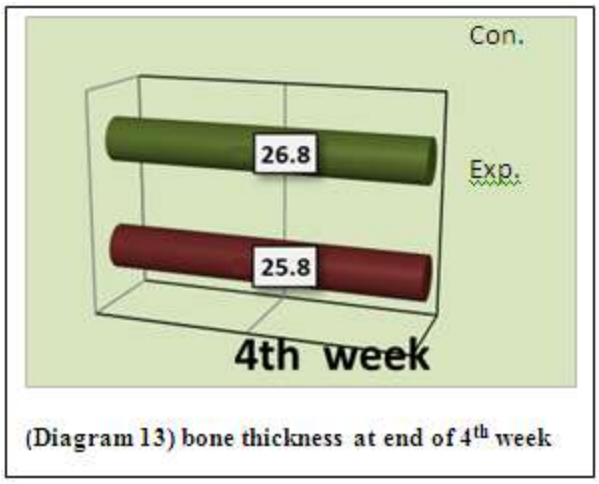


(Diagram 10) Osteoblasts No. at end of 2nd week

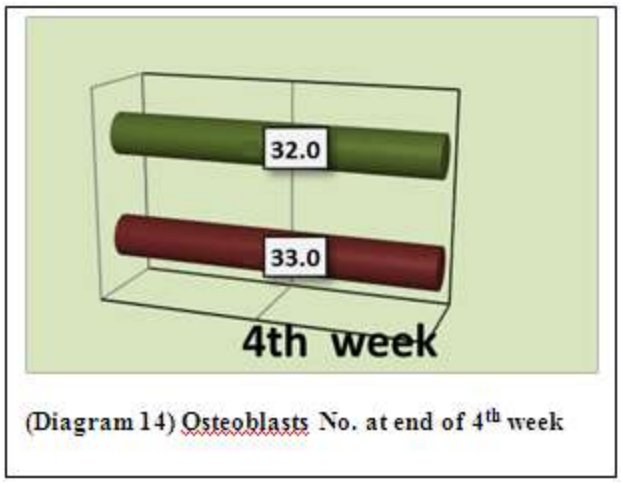


(Diagram 11) bone thickness at end of 3rd week

(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 osteocalcin levels at the end of 1st week.

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).003

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

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).02

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

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(significant).004

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

Paired Samples T Test	Sig. ≤ 0.05
Pair experimental group - control group	(not significant).076

(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).02

(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).028

(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).070

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

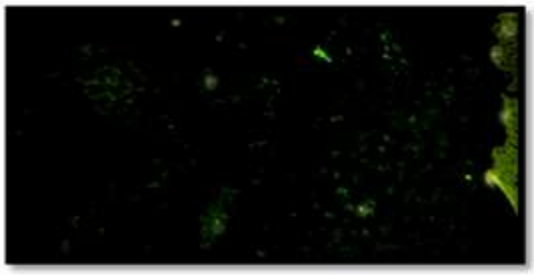
Paired Samples T Test	Sig. ≤ 0.05
pair experimental group - control group	(significant).003

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

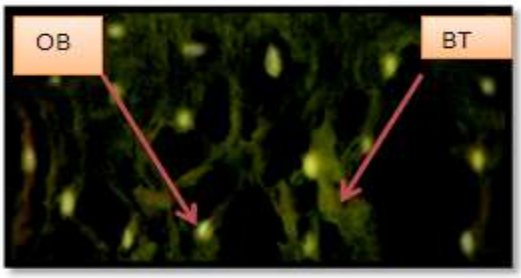
Paired Samples Test	Sig. ≤ 0.05
Pair experimental group - control group	(not significant).08

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

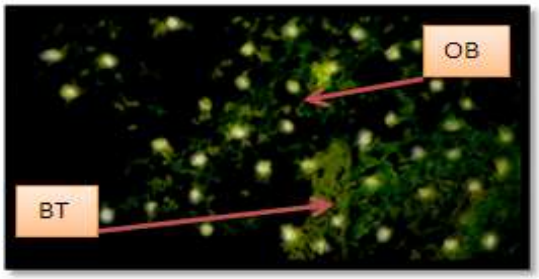
Paired Samples Test	Sig. ≤ 0.05
Pair experimental group - control group	(not significant).95



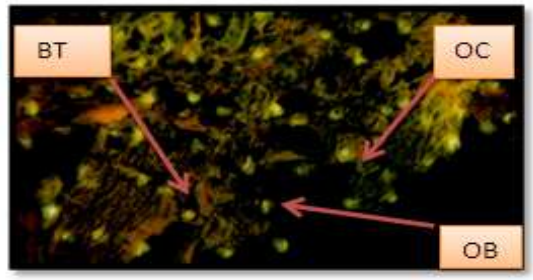
(Fig. 1) Digital fluorescent micrographs of control group at the end of 1st week No presence of osteoblast or bone trabeculea. (Fluorescent, 400X).



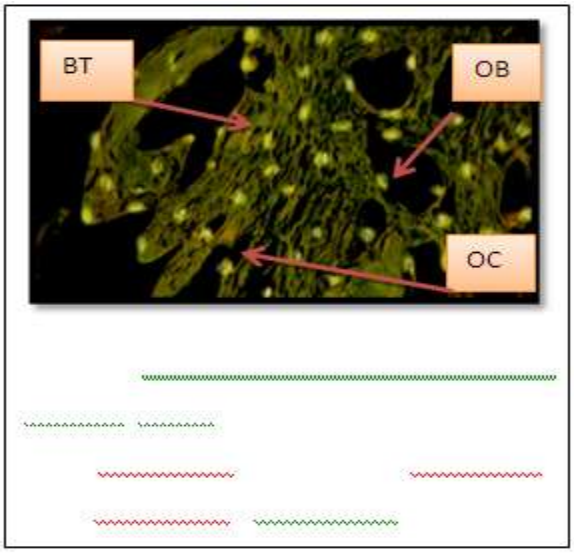
(Fig. 2) Digital fluorescent micrographs of exp. group at the end of 1st week. OB.: Osteoblast. BT.: Bone trabeculea. (Fluorescent, 400X).

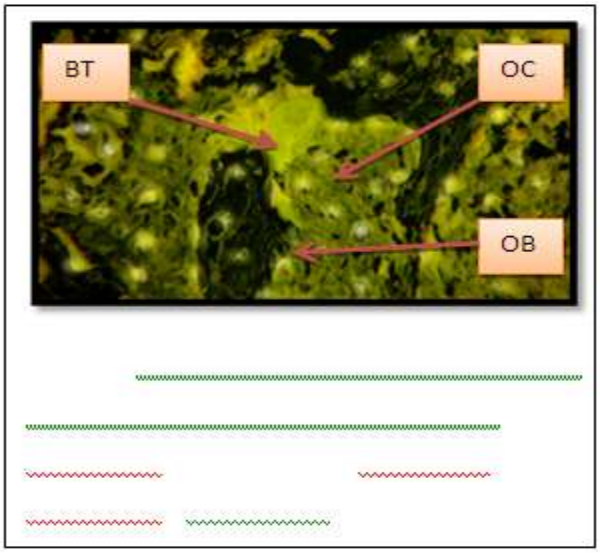


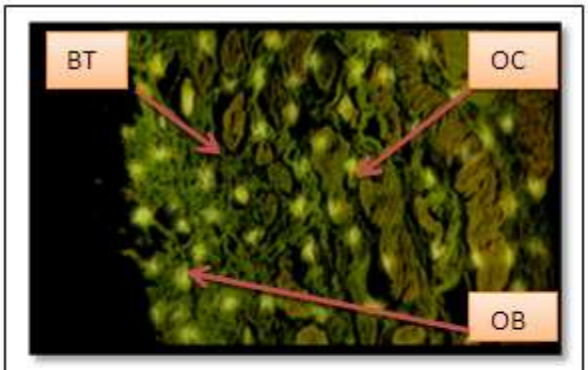
(Fig.3) Digital fluorescent micrograph of cont. group at the end of 2nd week. OB.: Osteoblast. BT.: Bone trabeculea. (Fluorescent, 400X).



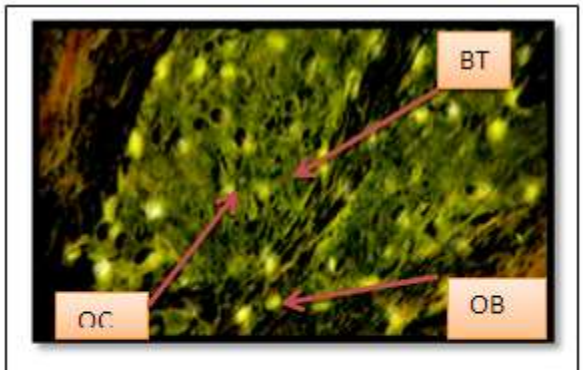
(Fig. 4) Digital fluorescent micrographs of exp. group at the end of week. OB.: Osteoblast. BT.: Bone trabeculea. OC.: osteocytes. (Fluorescent, 400X)







(Fig. 7) Digital fluorescent micrograph of 3rd group at the end of 4th week. OB.: Osteoblast. BT.: Bone trabecon. Ulea. OC.: Osteocytes. (Fluorescent 400X)



(Fig. 8) Digital Fluorescent Micrograph Of Exp. At The End Of 4th Week. OB.: Osteoblast. BT.: Bone Trabeculea. OC.: Osteocytes. (Fluorescent 400X)

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