Effect of Atorvastatin & Hydroxy Appete on Osseointegration to Titanium Oxide Surface (Histological Study on Rabbits)

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ABSTRACT

Background: Atorvastatin elevates the expression of bone morphogenetic protein 2 (BMP-2) in osteoblasts, therefore it is important to investigate the effect of statins on bone formation, fracture healing and implant integration. Titanium is the most biocompatible materials utilized for implantation in the bone. It has a high biological response that there is no corrosion also high stability in body fluids and don’t stimulate allergic reaction or produce toxic effects of surrounding tissue. Some alteration at the surface, texture in which previous oxidation of titanium surface to produce titanium oxide TiO\textsubscript{2} enhances its biological response that elevates surface attachment to osteoblasts and elevate osteoblast proliferation and differentiation of its osteoblast precursor cells in the bone marrow with a slow elevation of osteocalcin level. A broad range of studies applied to improve the biological responses to titanium implant. The aim of the present study was to investigate the effect of Atorvastatin, locally applied on bone integration using Histological and histomorphometrical analyses.

Materials and methods: Twenty four rabbits were used for fixation of titanium screws of 5mm length and 2mm width in the femur bone of rabbit. 12 rabbits submitted to implant fixation alone as control groups. In the other 12 rabbits the cavities of implant filled with mixed powder of Atorvastatin and Hydroxyapatite before fixation of the implant as the experimental groups. Both experimental and control groups are studied in intervals of 1, 2, 3 and 4 weeks. Each group’s checked histomorphometrically under fluorescent microscope for undecalcified section without staining by using of oxytetracycline dihydrate as a fluorescent marker administered intravenously that assist counting of bone cells and bone trabecule 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 enhances specific visualization of osteoblast and new bone trabecula as oxytetracycline fluorescent markers have high affinity to calcium groups in osteoblast and bone matrix. This analysis, display significant elevation in the number of osteoblast adhered to the implant surface and new bone trabecula thickness in experimental group mainly at the end of the first week with the 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 the osteocalcin level in the serum that detected by ELISA test for all control and experimental groups.

Conclusion: This research was illustrated that the mixed powder of Atorvastatin & HAC promote osseointegration around the titanium implant by facilitating bone cell activation and differentiation also increase bone density due to its support extracellular matrix.

INTRODUCTION

Commonly known as statins, 3-Hydroxy 3-methylglutaryl coenzyme A (HMG Co-A) reductase inhibitors are routinely prescribed as cholesterol inhibiting drugs. The past decade, special attention has been focused on the potential effects of these mevalonate pathway inhibitors on hard tissue metabolism\textsuperscript{11}. Osseointegration process enhancements 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 branches of dentistry in addition to the wide range of materials used in this application with their varying degree of biocompatibility\textsuperscript{23}. Osseointegration or osseointegration 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 osseointegration process produces fusion of bone materials with implant coated layer lead to ankylosis\textsuperscript{33}. Titanium is the most biocompatible implant materials as it has excellent ability to withstand body fluids and tissue environments. This ability comes 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 cell differentiation and enhance bone remodeling. The physical properties of titanium oxide as if firmly bind to titanium surface and its impermeability in addition to its insolubility provides a strong barrier that inhibits the reaction of titanium material with surrounding tissues\textsuperscript{44}. The implant coating with the atorvastatin exhibited highest level of alkaline phosphatase enzyme activity among the other titanium implant cover by calcium phosphate and titanium modified by acid etched surface\textsuperscript{59}. Since the discovery titanium implant by Bränemark 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 (Ca\textsubscript{10} (PO\textsubscript{4})\textsubscript{6}(OH)\textsubscript{2}) has been used\textsuperscript{60}. HA is widely used as a biomaterial due to its excellent compatibility with bone. The HA material, usually produced commercially today in many forms like powder or crystals resorbable and unresorbable in addition to very slowly resorbable form\textsuperscript{59}.

**AIMS OF STUDY:**

A: Histological study enhanced by an immune fluorescent technique to evaluate effects of the Atorvastatin &HAC on the osseointegration process to the titanium oxide surface.

B: serological study of bone forming cell enzyme osteocalcin to support histomorphometric analysis of bone forming cells.

**MATERIALS AND METHODS:**

Pure titanium screw made in a 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 (TiO\textsubscript{2}) on the surface of implant. Mixed powder composed of Atorvastatin 50\% and synthetic bone graft hydroxyapatite crystals 50\%. Atorvastatin absorbed slowly over several weeks after helping with the initial shaping. The mixed powder was sterilized by gamma radiation. This material is moistened 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 standard weight of 1.5 kg divided into four groups 1, 2, 3 and 4 weeks. Each group consists of 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 it. Without any trauma to the muscles, the two muscles over the femur separated by artery forceps to expose 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 a round carbide bur. The second step makes a hole for the implant with 1.7 mm width taper drill and 5 mm 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 obtaining primary stability of the implant. No over driven done to prevent bone fracture. In the experimental groups the socket has standard sized fill completely with mixed powder of Atorvastatin and Hydroxyapatite after moisturizing it with distillate water before screw driving then the screw was driven over the material. The excess of material washed out with distillate water. This procedure insures the same amount of material to 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 tissues scarified from the bone, mainly around the head of the screw. The bone kept in buffer formalin 10\%. The bone cuts in the size of 1 cm around the screw to get a biopsy of 2 cm 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 L.A. 200mg. administered in a dose of 30mg/kg five days each. The first dose starts ten days before the operation that mean two doses before an operation to insure saturation of the body with this material from the first day of implantation. Third dose administered during operation and continuous every five days. Addition dose had been given 24 hours before sacrificerabbit also to insure the saturation of all new cells with marker.

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

**Ground section technique:**

1. After 5 days 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 (12μm) 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 a very sharp knife to cut the jugular 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 osteocalcin:** In this test used osteocalcin by the 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 1 liter 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 its 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 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 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 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 10 seconds 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 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 is 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 the fluorescent microscope in 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 graduation of graduate 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 biostatistical analysis.

Statistical 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 a relationship between groups and changes during four time 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 were markedly increased in the experimental groups (Atorvastatin 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 highly 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 a 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 still somewhat 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 of 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 rapidly increasing of osteocalcin in the first two weeks of experimental groups, and then stabilized in standard level but in control groups the level of osteocalcin raised gradually during the month to reach a 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 technique used graduated lens to measure the bone trabeculae thickness and the number of osteoblast associated with new bone formation. The osteoblast takes a fluorescent marker of bright yellow wavelength while bone trabeculae take marker with green wave length under fluorescent microscope.

1: Histomorphometric 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 the control group. The green color at the serration site of implant was the marker of mild new bone formation present in the experimental group with thin bone trabeculae formation (Fig. 1). In the control group there was a black field with very low acceptance of oxytetracycline marker. This gave an indication that the osteoblast nonfunctional in this stage (Fig. 2).

Biostatical analysis: The statistical analysis of both osteoblast and bone trabeculae show that the relation between experimental and control groups was significant (Table 5, 9) (Diagram 7, 13).

Histomorphometric analysis at the end of 2nd week: In the 2nd week there were obvious and rapid increases in the number of osteoblasts in the experimental group with the appearance of few osteocytes within lacunae in new bone
Trabeculae (Fig. 3). There was a mild gradual increase in the number of osteoblasts in the control group in 2nd week that were few in number and present on very thin new bone trabeculae (Fig. 4). The new bone trabeculae increased in thickness became more prominent and contain lacunae for osteocytes in the experimental group.

**Biostatistical Analysis:** The biostatistical analysis explains the significant relation of experimental groups with control group (Table 6, 10) (Diagram 8, 14).

**Histomorphometric analysis at the end of 3rd week:** During the third week, the number of osteoblasts in the experimental group stabilized at a nearly constant level with high increasing in the bone thickness. This stabilization in osteoblasts number was due to convert some of the cells to osteocytes (Fig. 5). The increasing in the number of osteoblast was obvious in the third week with gradual increase in the bone trabeculae thickness in control group (Fig. 6). The fluorescence of the bone in this stage will become more idealistic and contain bone trabeculae take market green color with osteoblasts and osteocytes had good brightness.

**Biostatistical analysis:** Biostatistical analysis show significant relation between experimental group and control group in both the number of osteoblasts and bone trabeculae 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 but the number of fluorescent dyes increased as the osteocyte also takes oxytetracycline marker. The bone thickness of all groups became prominent with very low differences among groups (Fig. 7, 8).

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

**DISCUSSION**

Titanium implant is a biocompatible material due to the presence of titanium oxide on its surface. Titanium dioxide TiO2 has higher biocompatibility and elevate bone adhesion to implant. The use of this surface coat induces osteoblast differentiations that have high levels of alkaline phosphatase and high level of osteoclin and produce an osteogenic environment (8). The retention of implant to the bone occurs in two ways, 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 (9). Studies have shown the osseoinductive effect of statins on stem cells in vitro. Maeda et al cultured MC3T3-E1 cells (a clonal pre-osteoblastic cell line derived from newborn mouse calvaria) in the presence of simvastatin, atorvastatin or cerivastatin for 4-24 days (10). So Titanium implant enveloped by Atorvastatin induce osseointegration significantly more than that of titanium implants alone (11).
(Table 1) Paired sample t. tests for osteocalcin levels at the end of 1st week.

<table>
<thead>
<tr>
<th>Paired Samples T Test</th>
<th>Sig. ≤ 0.05</th>
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<tr>
<td>Pair experimental group – control group</td>
<td>Significant 0.004</td>
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(Table 2) Paired sample t. tests for osteocalcin levels at the end of 2nd week.

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

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<td>Pair experimental group – control group</td>
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(Table 4) Paired sample t. tests for osteocalcin levels at the end of 4th week.

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<td>Pair experimental group – control group</td>
<td>Not Significant 0.066</td>
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(Table 5) Paired sample t. tests for osteoblast number at the end of 1st week.

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(Table 6) Paired sample t. tests for osteoblast number at the end of 2nd week.

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(Table 7) Paired sample t. tests for osteoblast number at the end of 3rd week.

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

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

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

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

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

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

(Fig. 2) Digital fluorescent micrograph of control group at the end of 1st week. No presence of bone trabeculae or osteoblast (Fluorescent, 400X).

(Fig. 3) Digital fluorescent micrograph of exp. Group at the end of 2nd week. OB: Osteoblast, BT: Bone Trabeculae, OC: Osteocyte. (Fluorescent, 400X)

(Fig. 4) Digital fluorescent micrograph of con. Group at the end of 2nd week. OB: Osteoblast, BT:Bone Trabeculae. (Fluorescent, 400X)

(Fig. 5) Digital fluorescent micrograph of exp. Group at the end of 3rd week. OB: Osteoblast, BT: Bone Trabeculae. OC: Osteocyte. (Fluorescent, 400X)

(Fig. 6) Digital fluorescent micrograph of con. Group at the end of 3rd week. OB: Osteoblast, BT:Bone Trabeculae. OC: Osteocyte. (Fluorescent, 400X)
REFERENCES


