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THE EFFECT OF SIMVASTATIN DRUG ON OSTEOGENESIS AROUND TITANIUM IMPLANT (RADIOGRAPHIC AND HISTOMORPHOMETRIC ANALYSIS)

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ABSTRACT

Understanding the feature of bone repair and osseointegration may aid in the development of therapeutics to improve implant outcomes. Statins are cholesterol-lowering drugs that have been reported to promote bone formation. The purpose of this investigation is to determine the effect of simvastatin drug on the enhancement of bone formation around titanium implants. Sixty male Sprague Dawely rats ranging in weight between 200-300 grams received pure titanium implants in the tibiae. The animals divided into A, B and C groups, A and B groups were intra-peritoneally administered 5 and 10 mg/ kg of simvastatin daily respectively. Animals of Group C were injected with isotonic saline. A, B and C groups were subdivided into 1, 2, 3 and 4 subgroups according to injection periods. After 4weeks, the animals were sacrificed, and specimens were prepared. Bone density in the medullary canal and percentage of cortical bone were obtained using cone-beam radiograph. Bone density of both groups A and B were significantly greater than those of the C group. Histomorphometric analysis to the specimens showed that statin increased bone formation in animals of group A and B more than those of group C. In conclusion, a simvastatin dose of 5 mg/ kg or higher increased medullary bone formation around the titanium implant.

INTRODUCTION

Implants made of commercially pure titanium (cpTi) are widely and successfully used in dentistry. For certain indications, diameter-reduced Ti alloy implants with improved mechanical strength are highly desirable. Tissue repair following surgical or trauma-related injuries remains a challenge in reconstruction. The healing process initiates an orderly but complex sequence of events that re-establish the integrity of the damaged tissues. If the result of the repair process is tissue that is structurally and functionally the same as the original tissue, then regeneration is said to have taken place. Despite rapid development in materials science and biotechnology, satisfactory bone regeneration and osseointegration remain major challenges for orthopedic and dental implants in cases of age-related, postmenopausal and other forms of secondary osteoporosis resulting from systemic diseases or pharmacological therapies. Different strategies have been proposed to improve implant–bone interfaces on mainstream titanium (Ti) and Ti alloy implants, To enhance the osseointegration of Ti implants in either normal or osteoporotic subjects, in some ways, it is important to induce and enhance the osteoblast functions more precisely and more effectively on the implant–bone interface. Thus, the studies of bone-targeting Ti implant–bone interface with bone-targeting effect have become a focus recently. Statins are widely used to lower blood cholesterol levels. Some studies have reported that statins can stimulate bone formation by stimulating the production of bone morphogenetic protein-2. Patients with: 1) poor bone quality, 2) inadequate bone, and 3) metabolic disease present challenging cases for dental implant procedures [(1)]. Hence; there is a specific need to improve bone anchorage through enhancing osseointegration. Osseointegrated implant therapy has been widely and successfully applied in dental rehabilitation for more than a decade with

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predictable long-term results[2]. Many significant studies have been performed to improve the bone implant interface. Nevertheless, further research is necessary to identify biomaterials able to induce alveolar bone for the purpose of implant placement in defective alveolar ridges, as well as to enhance osseointegration in unfavorable conditions[3]. Simvastatin, is a member of the lipid lowering statin family, it is a 3hydroxy 3methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor and has a potent effect [4].

MATERIALS AND METHODS

Experiment was approved by the Institutional Animal Care and Use of Mansoura University. Surgery was performed under sterile condition in a veterinary operating theatre. sixty male Sprague Dawley rats ranging in weight between 200-300 grams were divided into three groups (20 rats per group) according to simvastatin concentration dose: experimental group A (5mg/kg), experimental group B (10mg/kg) and control group C (saline). All animals have been housed in a specific-pathogen-free, temperature controlled room on a 12h alternating light-dark cycle, given food and water.

Implants and implantation:

Under systemic anesthesia using a combination of Ketamine (100mg/kg) and xylazine (10mg/kg body weight) injection, then the tibia area was shaved and disinfected with povidone iodine solution. An incision was created in the skin, and then reflection of the tibialis anterior muscle exposing the tibia. A hole of 1.0 mm in diameter was made with round dental bur (#1/2 & #1) below the knee joint by 15mm. In all steps during preparation of the implant site sufficient saline was irrigated for cooling and cleaning. Pure titanium implants of 1.0mm in diameter and 1.5mm long were sandblasted then sterilized by autoclaving. All rats used in this experiment received the implants into the diaphysis extending to the medullary canal of tibia. The assignment of implant to each hole was performed randomly. These assignments were conducted to rule out anatomic factors for osseointegration. The muscles were repositioned and sutured with chromic absorbable catgut, and the skin was closed by routine suturing material. The wound then was disinfected with povidone and wrapped with gauze and plaster. Each rat was caged individually and maintained on laboratory diet plus water, the rats received gentamicin 1mg/kg intramuscularly 3 times daily for 3 days. After implant placement and according to simvastatin concentration injections, rats were randomly divided into 3groups: 1- Experimental group A injected intrapretonially with 5mg/kg of simvastatin daily. 2- Experimental group B injected intrapretonially with 10mg/kg of simvastatin daily. 3- Control group C injected intrapretonially with isotonic saline daily.

Preparation and injection of simvastatin:

Simvastatin drug has been prepared for injection by mixing it with injectable vehicle. The vehicle composed of carboxy methyl cellulose sodium salt dissolved in normal saline and stored in dry cold place at 4c for 7days maximum. Each group of the three groups (A, B, C) was subdivided into 1,2,3&4 subgroups according to day of surgical operation and the first dose of simvastatin injection, e.g.:

- Subgroup 4 scarification period after four weeks of simvastatin injection.
- Subgroup 3 scarification period after three weeks of simvastatin injection.
- Subgroup 2 scarification period after two weeks of simvastatin injection.
- Subgroup 1 scarification period after one week of simvastatin injection.

Authorization and scarification was done in the same day for all groups. The tibiae were dissected out and fixed in 10% neutral phosphate-buffered formalin at 4c, pH 7.2.

Radiographic evaluation

To evaluate the bone density, cone beam computed tomography (CBCT) is a recommended procedure as it provides additional information and more accurate results than conventional radiographs. CBCT was obtained using an **I CAT** next generation (imaging science international / Hatfield, PA, USA). Exposure parameters SMA / 120kv / 14.7 sec 0.2mm voxel size. And densities were read using On Demand 3d software. CBCT examination was performed for the specimens of each subgroup alone. Specimens of subgroup 1 -of each group alone- were arranged in the CBCT machine in the form of arch shape (U-shape) and data were collected. Same procedures were done to the 2nd, 3rd, 4th subgroups. Three random readings around the implant at the cortical bone, medullary canal and bone in intimate contact with the implant

Histomorphometric examination and bone evaluation using of Masson's trichrome stain:

Specimens undergoes Fixation and Decalcification, Processing, Sectioning and Staining. The Masson's trichrome stained tissue sections were examined using light microscope to assess the prevalence of positive cases and the localization of staining within the tissues. In addition, image analysis computer system was used to assess area percentage of the distribution of collagen type I fibers. These areas were counted and the positive index (PI) was calculated by image analyzer computer system. The image analysis was performed using Leica QWIN V3 image analyzer computer system (Switzerland), the image analyzer consisted of a colored video camera, colored monitor and hard disc of Dell personal computer connected to the microscope and controlled by Leica QWIN V3 software. The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual micrometer units. This was done in the Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Cairo, Al-Azhar University. The investigated parameters were assessed using area distribution of

positive cells. It was measured in the form of area inside a standard measuring frame of area 11434.9 um per 5fields using a magnification (x200) by light microscopy transferred to the monitor. The selected fields had the most uniformly stained tissues for evaluation. These areas were masked by a blue binary color using the computer system for measurement. Mean values were obtained for the whole specimens in each group. All data were collected tabulated and statically analyzed using software SPSS program (statistical package for social sciences) Data was presented as mean ± SD and statistical significance was set at $\alpha = 0.05$.

RESULTS

Cone beam radiograph results

A-experimental group A:

1. At 1 week readings of bone density at the region of interest (ROI) was measured in Hatfield Unit (HU). The mean value was 534.16 ± 90.2
2. At 2 weeks readings of bone density at ROI. The mean value was 615.3 ±37.67
3. At 3weeks readings of bone density at ROI. The mean value was 706.18 ± 79.15
4. At 4 weeks readings of bone density at ROI. The mean value was 810.06 ±95.66

B- Experimental group B:

1. At 1 week readings of bone density at ROI. The mean value was 518.56 ± 82.63
2. At 2 weeks readings of bone density at ROI. The mean value was 620.74 ± 61.42
3. At 3weeks readings of bone density at ROI. The mean value was 714.52 ± 95.61

4. At 4 weeks readings of bone density at ROI. The mean value was 854.06 ± 59.48

C- Control group C:

1. At 1 week readings of bone densities at ROI. The mean value was 380.62 ±47.48
2. At 2 weeks readings of bone density at ROI. The mean values was 486.74 ± 56.48
3. At 3weeks readings of bone density at ROI. The mean value was 547.6 ± 74.89
4. At 4 weeks readings of bone density at ROI. The mean values was 676.5 ± 53.92

Histomorphometric results:

A- Experimental group A:

1. At 1 week: The MT stained tissue examination revealed positive expression of localized distribution of collagen type I fibers with mean value 8.66 ± 2.07
2. At 2 weeks: The MT stained tissue examination revealed positive expression of localized distribution of collagen type I fibers with mean value 11.63 ±3.497
3. At 3 weeks: The MT stained tissue examination find positive expression of wide distribution of collagen type I fibers with mean value 11.823±1.477
4. At 4 weeks: And the examination of MT stained tissue (fig 1) showed positive expression and well distribution of collagen fibers type I with mean value 14.23 ± 2.19

B- Experimental group B:

1. At 1 week: histomorphometric analysis showed positive expression of little amount of collagen type I fibers with mean value 9.75 ±3.38.
2. At 2 weeks: histomorphometric analysis showed positive expression of collagen type I fibers with mean value 12.36 ±4.16.
3. At 3 weeks: histomorphometric analysis showed positive expression of collagen type I fibers with mean value 15.96 ±2.20
4. At 4 weeks: The histomorphometric analysis showed positive expression of collagen type I fibers (fig 2) with mean value 16.93 ±3.25

C- Control group C:-

1. At 1 week: The histomorphometric analysis showed mean value 5.33 ±1.38
2. At 2 weeks: The histomorphometric analysis showed mean value 6.6 ±2.43
3. At 3 weeks: The histomorphometric analysis showed positive expression of collagen type I fibers with mean value 9.86 ±0.65

Table (1) Showing the P-values and difference between mean values, range and SD of each group

X-ray		Group A	Group B	Group C	F	P-value
1 week	Mean ± SD	534.16 ± 90.20	518.56 ± 82.63	380.62 ± 47.48	6.221	0.014
	Range	463.5 – 682.2	407 – 614.4	300.3 – 420.7		
2 weeks	Mean ± SD	615.30 ± 37.67	620.74 ± 61.42	486.74 ± 56.48	10.294	0.002
	Range	588.3 – 681.2	538 – 709	422 – 560.1		
3 weeks	Mean ± SD	706.18 ± 79.15	714.52 ± 95.61	547.60 ± 74.89	6.315	0.013
	Range	615.3 – 833.3	623.8 – 863.5	444.3 – 653.2		
4 weeks	Mean ± SD	810.06 ± 95.66	854.06 ± 59.48	676.50 ± 53.92	8.224	0.006
	Range	687.9 – 944.6	787 – 923.4	633 – 770		

4. At 4 weeks: histomorphometric analysis showed positive expression of collagen type I fibers (fig-3) with mean value 11.32 ±2.32

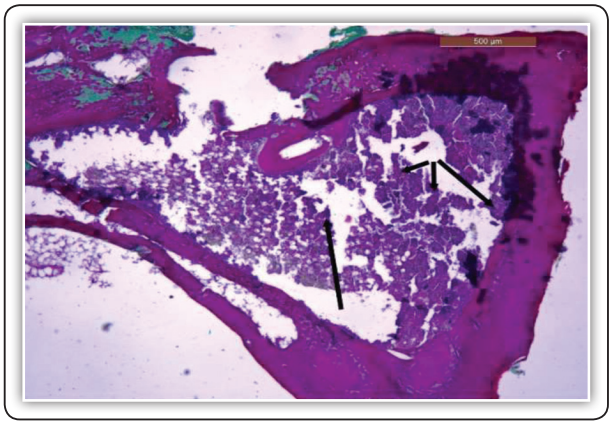

Fig .(1) photomicrograph of group A at 4 weeks showing: Positive expression of collagen type I fibers (MT stain, X40)

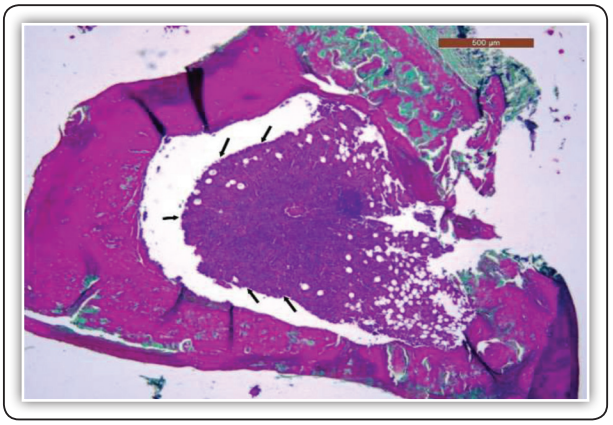

Fig. (3) Photomicrograph of control group C at 4 weeks showing: Positive expression of collagen type I fibers (MT stain, X40)

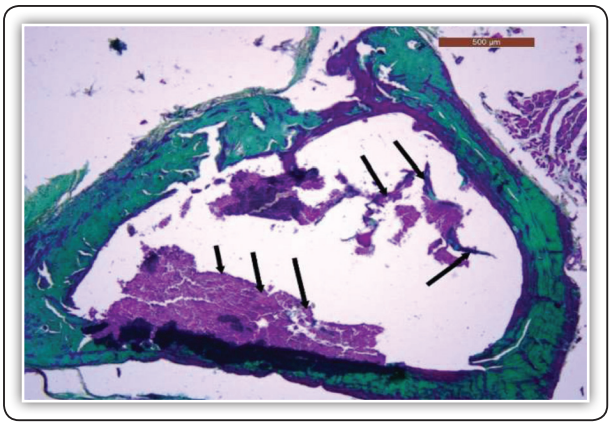

Fig. (2) Photomicrograph of group B at 4 weeks showing: Positive expression of collagen type I fibers (MT stain, X40)

TABLE (2) Showing the P-values and difference between mean values, range and SD of each group

Histology		Group A	Group B	Group C	F	P-value
1 week	Mean ± SD	8.66 ± 2.07	9.75 ± 3.38	5.33 ± 1.38	4.503	0.035
	Range	5.06 – 10.2	5.32 – 13.53	3.08 – 6.59		
2 weeks	Mean ± SD	11.63 ± 3.497	12.36 ± 4.16	6.60 ± 2.43	5.981	0.016
	Range	7.67 – 15.82	7.45 – 17.5	3.83 – 9.52		
3 weeks	Mean ± SD	11.823 ± 1.477	15.96 ± 2.20	9.86 ± 0.65	19.539	0.005
	Range	10.01 – 13.93	13.52 – 19.26	8.79 – 10.53		
4 weeks	Mean ± SD	14.23 ± 2.19	16.93± 3.25	11.32 ± 2.32	5.699	0.018
	Range	10.46 – 15.78	11.95 – 19.74	9.4 – 14.37		

DISCUSSION

Statins are specific inhibitors of 3-hydroxy 3methylglutaryl-coenzyme reductase that are traditionally used to inhibit the production of cholesterol in cardiovascular diseases [5-9]. However, statins have pleiotropic therapeutic effects including vasodilatory, antithrombotic, antioxidant, anti-inflammatory and immunosuppressive actions [10-12]. In 1999, Mundy G et al. [13] first reported that statins were potent stimulators of bone formation in vitro. In 2011, Moon HJ et al [14] proved that simvastatin acted as an osteoclastogenesis inhibitor by suppressing reactive oxygen speciesmediated signaling pathways. Therefore, simvastatin has both anticatabolic and anabolic effect on bone metabolism.

The current study was conducted to evaluate the effect of simvastatin drug on osteogenesis around titanium implants; radiographically and histomorphometric analysis.

Recently, various agents have been tried in attempts to locally enhance implant fixation, including bisphosphonates, bone cements and BMPs [15-17]. Bisphosphonates inhibit excessive bone resorption, but because bone resorption and bone formation are related, this inhibitory effect likely affects bone formation [18]. Bone cements physically, but not physiologically, improve fixation strength and poorly degraded, with number of limitation [19]. BMPs are not used widely in the clinical setting because of their short shelf life and high cost [20].

Simvastatin is a white to off-white, non-hygroscopic, crystalline powder that is practically insoluble in water, and freely soluble in chloroform, methanol and ethanol. Absorption of the ingested reductase inhibitors varies from 40 to 75% with the exception of fluvastatin, which is almost completely absorbed. All have high first-pass extraction by the liver [21]. In this study Simvastatin was prepared for injection by mixing the powder with injectable vehicle, the vehicle composed of carboxy methyl cellulose sodium salt dissolved in normal saline and stored in dry cold place at 4c for 7days maximum.

Recent studies showed beneficial effects of statins on bone mineral density [22] [13]. Simvastatin, a liposoluble statin, induces the expression of bone morphogenetic protein (BMP)-2 mRNA resulting in bone formation on the calvaria of mice following daily subcutaneous injections [23]. There are more investigations on statins metabolic effect. For instance, simvastatin improved cancellous bone mass and bone compressive strength by oral administration [24]. Ayukawa et al [25] confirmed that new bone tissue increased by topical application of statins. In addition, bone mineral density also increased using of statin by clinical investigations [26-27].

In this study examination of the bone density along the total length of the implant was obtained by CBCT using an **I CAT** next generation (imaging science international / Hatfield, PA, USA). Exposure parameters SMA / 120kv / 14.7 sec 0.2mm voxel size. Bone densities were recorded using On Demand 3d software.

CBCT examination was performed for the specimens of each subgroup of each group alone. Specimens were arranged in the CBCT machine in the form of arch shape (U-shape) and data were collected, same procedures were done to all subgroups. The collected data (housfield unit) revealed that the bone density reading in simvastatin treated group (10mg/kg) were significant in relation to treated group (5mg/ kg), and highly significant in relation to control group. These findings goes in accordance with Mundy G. et al [13] who found the positive function of simvastatin on bone tissue. Then, there were more investigations on statins. Systemic administration was described in many studies, including oral and intraperitoneal administration. Investigations also showed that

systemic administration improved osseointegration of pure titanium implants in normal or osteoporotic rats [28-29].

Ovariectomized rats, used as a model of postmenopausal women, were given oral statin medications in doses equivalent to those used in humans. The rats showed a 40%–90% increase in trabecular bone volume in the spine and femur [13]. N. Saulacic [30], in his experimental study explained the histological healing process around Ti implants.

In this study, the histomorphometric analysis - (using Masson's trichrome stain to detect the presence and concentration of the collagen fiber type I)- revealed the following results:

In subgroup 1 (7 days) of group B showed significant concentration of collagen fiber type I than in the same subgroup in group A and group C where the p-value was 0.035. In subgroup 2 (14 days) of group B showed significant concentration of collagen fiber type I than in group A and group C where the p-value was 0.016., while in subgroup 3 (21 days) of group B showed highly significant concentration of collagen fiber type I deeply blue stained than in group A and group C where the p-value 0.005. Finally in subgroup 4 (28 days) of group B it showed significant concentration of collagen fiber type I to form the trabecular structure than in group A and group C where the p-value 0.018. These results were in accordance with Issa JP et al 2015 [31] results.

Simvastatin is inexpensive and has been used safely for many years. Systemic administration of statins require a relatively high daily dose to counter hepatic clearance, which likely elicits adverse effects. Evidence for an anabolic effect on bone following local application of simvastatin has been shown in the mandible [32] and critical-sized calvarial bone defects [33]. Furthermore, local application of simvastatin in PGA gel, as a slow release carrier, has shown positive effects on bone around titanium implants in normal rats [34].

However, there have been some conflicting results. Anbinder et al [35] reported that simvastatin administration orally or subcutaneously did not improve bone repair of experimental defects and did not alter blood cholesterol levels in rats and simvastatin also failed to stimulate bone formation, despite the verification by liquid chromatography/ mass spectrometry of the active simvastatin beta-hydroxy acid metabolite in mouse serum.

This study was subjected to certain limitations. First, the animal model used here was developed to investigate the effect of simvastatin drug with two different concentration on implant osteogenesis avoiding mechanical load, which is an important factor in the clinical setting. Second, the number of animals (subgroup) and histological specimens were limited.

Histomorphometric and radiographic data were all evaluated in view of the small sample size. Despite these shortcomings, good osseointegration with the implant was observed.

In this study, the overall osseointegration in the simvastatin group was significantly higher than that of the control group. The successful use of simvastatin to promote bone formation in vivo depends on the local concentration, and there have been continuous efforts to find an appropriate delivery system. Different doses produce different effects and doses should be prescribed with caution considering benefits and risks [36].

Attempts have been made to escape the accumulation in the liver and to deliver the statins to the peripheral tissue by subcutaneous injection or transdermal patch [37].

Further research is needed to determine the optimal therapeutic threshold, mode of application and the effectiveness in humans for bone regeneration.

CONCLUSIONS

From the present study the following could be concluded:

- Simvastatin are well tolerated by the surrounding tissues with no evidence of inflammation.
- Simvastatin enhances bone regeneration.
- The administration of simvastatin increased the value of both bone contact to pure titanium implant and bone density around the implant installed in rat tibiae.
- The 10mg/kg concentration group and the four week follow up period exhibited better results.

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