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Research Article

Immunohistochemical expression of transforming growth factor -β in bone regeneration in rats after application of simvastatin

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Abstract

The purpose of this study was to evaluate histologically and immunohistochemically the bone defects regeneration after topical application of simvastatin in tibia of rats. The experimental part was carried out on 30 adult male albino rats (220-250 gm). The animals were grouped into 2 equal groups. The animals were scarified after 7, 14 and 28 days alternatively post operatively. The simvastatin obtained in solid form and prepared into a solution form with the recommended concentration by centrifugation with distilled water for 1 hour. Under general anaesthesia, full thickness flap created at medial aspect of the tibia. Circular bony defects of 4 mm diameter was created. The bony cavity in control group (group1) lifted without any graft, the bony cavity in thesimvastatin group (group 2) received a collagen sponge soaked in simvastatin solution. After scarification of the animals by intra cardiac anaesthetic injection, the tibia was surgically removed, fixed in formalin 10% for five days, then the specimens were prepared and examined histologically by H&E, Masson trichrome stain and immunohistochemically for the expression of transforming growth factor-β (TGF-β). Histological results were revealed that, the evidence of new bone formation was very weak in group 1(control group), while advanced new bone formation and the best healing and bone regeneration occurred in the simvastatin group which was markedly increased along the intervals of the study. TGF-β expression was more intense in group 2(simvastatin group)in the ossifying centers, marrow cavities between the newly formed bone trabeculae and in the wall of blood vessels.

Keywords: Boneregeneration, Simvastatin, transforming growth factor-β.

Introduction

Extensive bony defects represent a complex reconstructive problem. Different grafting materials have been used for obliteration of osseous defects of different causes. These materials are applied for osteoconductive and/or osteoinductive purposes.

Over the last decade, statins or 3-hydroxy 3-methylglutaryl coenzyme A reductase (HMG-CoA reductase) inhibitors have been widely used for the treatment of hypercholesterolemia through regulation of production of microsomal HMG-CoA reductase and low-density lipoprotein (LDL) cell surface receptors **(Han et al, 2011)**. Researches have demonstrated the anabolic effects of statins on bone regeneration through increasing expression of bone morphogenetic protein-2 (BMP-2**) (Maeda et al, 2001)** and reduction of signal proteins regulating osteoclasts activity **(VanBeek et al, 1999).** Statins can induce and accelerate bone formation locally, and trigger the early expression of growth factors that regulate angiogenesis, differentiation of bone cells and o*steogenesis* **(Wong and Rabie, 2005).** Others reported that statins stimulated significant new bone formation and improved the healing of fractures in **rodents (Mundy et al, 1999**). Risk of fractures is markedly reduced in people using statins. Since statins

are hepatoselective and mainly degraded in the liver, their effect locally is too low**(Jadhav et al, 2006**). To make possible the use of statins as therapy for bone pathologies, it is important to find a way of using these drugs that maximize their action on bone. In previous studies local delivery type of simvastatin was mostly multiple injections **(Seto et al, 2008).**

Injection method is less invasive and more flexible for clinical applications and multiple injection method is preferred due to accumulative effect in new bone formation. However, this form of delivery is not acceptable for patients. To be acceptable, the statin would need to be delivered locally as a single dose in a manner similar to the current administration of BMP-2 **(Garrett et al, 2007).**

Local application has a therapeutic advantage by preventing systemic side-effects and focusing the drug dose. Simvastatin was chosen from different statins as lovstatin and pravastatin because it exerts greater effects **(Mundy, 1999).** Absorbable collagen sponge is biocompatible and acts as a scaffold that replaces the missing bone. It is porous, which facilitates bony ingrowth and allows osteoconduction while it is absorbed. It also has a haemostatic effect, producing almost instant haemostasis when implanted into the defects. Absorbable collagen sponge had been used successfully as a carrier for BMP-2 to induce bone formation in animals and in humans **(Nevins and Kicker, 1996).**During the healing phases interactions among many different cells via various growth factors, cytokines, receptors and intermediate signaling molecules take place.Although several growth factors are involved in the repair process, TGF-β is produced in the fracture site by platelets,inflammatorycells, macrophages, osteoblasts, osteoclasts and chondrocytes **(Mundy, 2000).**

The purpose of this study is to perform histological and immunohistochemical evaluation of bone defect regeneration after topical application of simvastatin in tibia of rats.

Materials and Methods

The present studywas carried out on 30adultmalealbino rats, their weight approximately 220-250 gm. The experiment was carried out in the Department of Zoology, Faculty of Science Damanhour University. The animals were examined to rule out the presence of any disease. The animals were housed in separate cages, supplied with food and water tanks and allowed to live in optimal conditions.

Animals grouping

The animals randomly assigned to two groups according to local bone defect treatment.

Group (I), Control group received no graft and the defect lifted empty.

Group (2),Simvastatin group which received collagen sponge soaked in simvastatin solution.

Collagen sponge (carrier preparation)

Collagen sponge in the form of sheet (septocoll® E 20, lot no 33MO10702, Biomet, Berlin-Germany) were sectioned into disks with diameter 4 mm using tissue punch with the same diameter in order to be used in the created defects to fulfill the whole defect.

Simvastatin solution preparation

The simvastatin solutions were prepared 1 day before the surgical procedures. The salt (lot no. 101M4743V, Sigma-Aldrich, Germany) was dissolved to a concentration of 0.5 mg/50 µl in distilled water under centrifugation for 1 hour. The solutions were refrigerated at 13^{o}C until 1 h before use **(Stein et al,2005).**

Collagen sponges were used as a carrier for simvastatin solution. The sponges were soaked in simvastatin solution in Group 2 (simvastatin group).

Surgical procedures

The animals were weighed and anaesthetized intramuscularly with a combination of 2% xylazine in a dose of 13 mg/kg (ADWIA, Egypt) and ketamine in a dose of 33 mg/ kg (ROTEXMEDICA, Germany). The overlying skin of the tibia was shaved and disinfected with iodated alcohol. An incision about 1.5 cm was then made in the medial aspect of the tibia, Full thickness flap, the skin, subcutaneous tissue, muscular layer and periosteum were reflected, exposing the tibial bone. A circular 4-mm diameter defect created using carbide rose head surgical bur mounted in a dental hand piece connected to a micro-

motor with speed of 2,000 rpm, a metal template with a round cavity 4 mm in diameter had been used to standardize the defect site and size. The process of defect creation done under copious irrigation with warm saline to avoid bone burning and to maintain the vitality of bone cells around the defect.

The defect in each group of animals treated according to the previously prescribed manner. The flap repositioned, the muscular layer was sutured with resorbable #4.0 catgut and the skin was sutured with interrupted #4.0 silk sutures.

The animals grouping, scarification and processing

The thirty animals which used in this study were divided into 2 group equally, 15 for each. 5animals from each group were scarified after 7, 14, 28 days alternatively post operatively. The animals were scarified with an intracardiac anesthetic injection. The tibiae were removed and immediately placed in 10% neutral buffered formalin for fixation up to 5dayes at room temperature. Decalcification was performed by immersion of the bony specimens in buffered 10% EDTA.After decalcification the specimens were processed for paraffin imperegnation.Paraffin sections(5-7um)were stained with haematoxylin&eosin and Masson"s trichrome stain **(Drury &Wallington,1980).**

Immunohistochemical detection of TGF-β

Immunohistochemistry was performed onparaffin sections, and mounted on coated glass slides. Antigen was retrieved in citrate buffer (pH 6.0)microwave digestion (2 cycles of 12 minute each).Endogenous peroxidase was blocked with 0.05%hydrogen peroxide for 30 min. After incubation with a1:20 dilution of normal horse serum, the slides wereincubated over night at 4 0C with primary antibodies(Dako, 1:50). Secondary antibodies associated with astreptavidin-biotin-peroxidase method were applied(Dako A/S). Diaminobenzidine was used aschromogen. All sections were counter-stained withhaematoxylin. The sections were washed withphosphate buffered saline after each step. Negativecontrols were used using non-immune serum insteadof the primary or secondary antibodies. The methodused was outlinedaccording to **(Ramos-Vara 2005).**

Results

Histological results

Control group (I)

After 7 days post surgery the defect is filled with granulation tissue with a notable new vascularization (proliferating endothelial cells providing new capillaries), fibroblasts, prominent inflammatory infiltration (macrophages, neutrophils). In some specimens sequestrated bone chips of old bone as a result of drilling observed surrounded by osteoclasts to undergo resorption. The old bone at the periphery of the defect has the normal structure as a lamellar bone. **Fig.(1).**

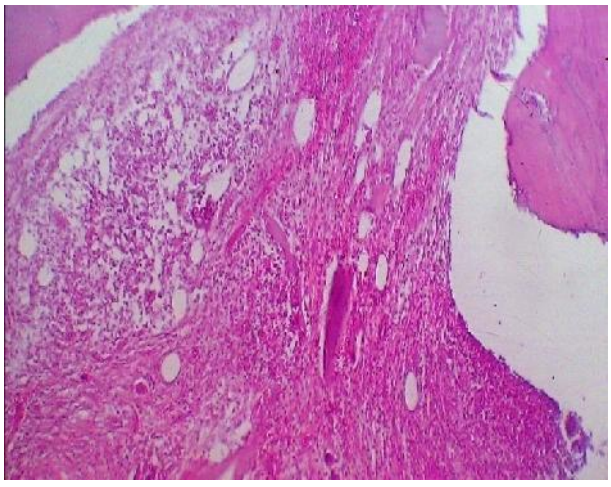


Fig. (1) : Photomicrograph of, group I (control) 7 days showing organized granulation tissue, inflammatory cells,blood vessels and old cortical bone.H&E x100

After 14 days post surgery the defect is surrounded by collagen fibers at the sides and base of the defect arranged in a perpendicular direction on the old bone as an attempt of woven bone formation. **Fig.(2)**.

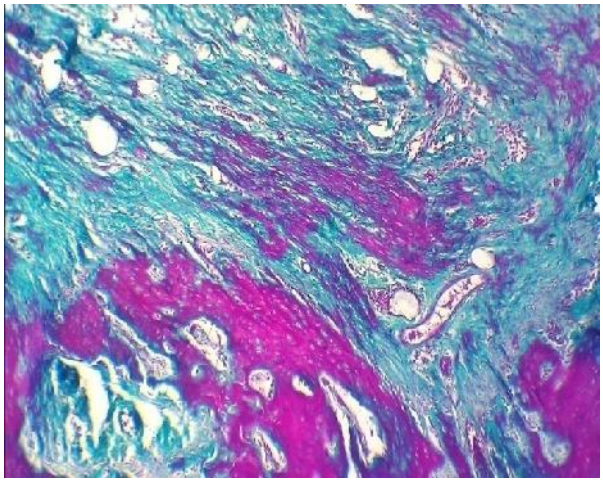


Fig. (2) : Photomicrograph of group I (control) 14 days showing abundant collagen (green), little new bone (red) , old bone (red) . Masson trichrome stain,X 100.

Prominent inflammatory cells infiltration, numerous blood vessels, and fibroblasts filling the center of the defect, the reversal line appeared faintly separating the defect from the old bone. **Fig (3).**

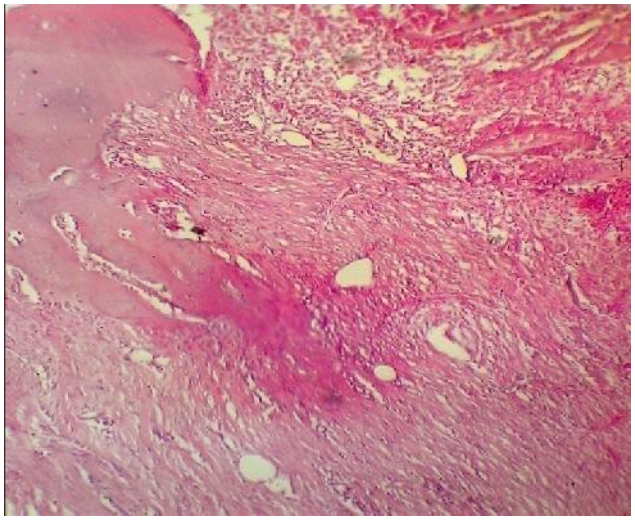


Fig. (3) : Photomicrograph of the group I (control) 14 days showing,old cortical bone ,proliferating blood vessel. H&E X 100.

At 28 day post surgery demonstrated that initial healing by new bone formation at the base and periphery of the defect to make a bridge of bone isolating the bone marrow from the outer tissues. The newly formed bone has regular bone trabeculae with wide marrow spaces and numerous large osteocytes with wide osteocytes spaces, a rim of osteoblasts lining the newly formed bone.**Fig.(4)** a layer of woven bone rich in osteoblasts and encircling the remaining defect which was filled with well oriented collagen fibers interspersed with numerous fibroblasts and blood vessels. The collagen fibers at the periphery were tightly packed and parallel to the bone surface while the central fibers were less dense and more oblique. Chronic inflammatory cells and congested blood vessels noticed in the center of the defect, increased invasion of blood vessels from the surrounding bone at the base as well as periphery. **Fig. (5).**

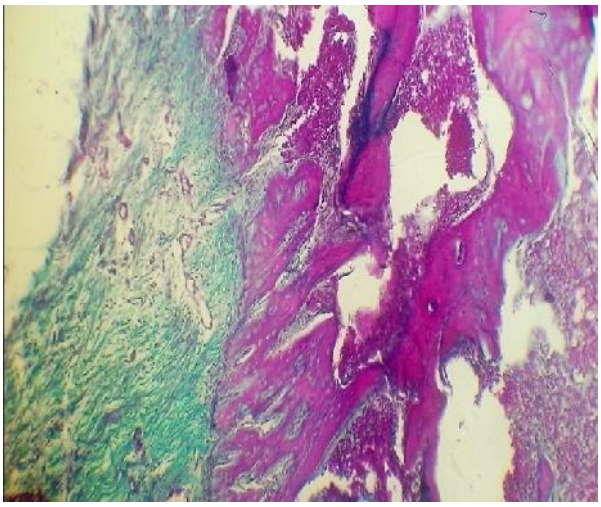


Fig. (4) : Photomicrograph of group I (control) 28 days showing bone trabeculae with variable thickness (red), abundant collagen occupying the majority of the defect (green) . Masson trichrome stain,X 100.

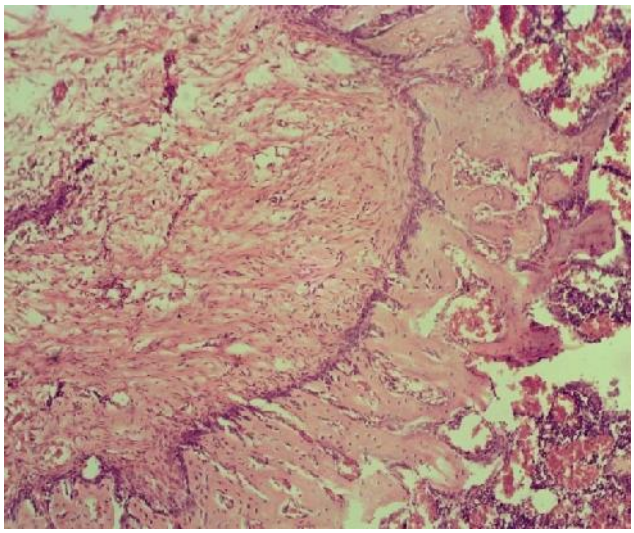


Fig. (5) : Photomicrograph of the group I (control) 28 days showing, new bone(NB) at the base and sides of the defect ,wide bone marrow spaces (b), osteoblast layer (arrow heads), H&E X 100.

Simvastatin group (2)

After 7 days post surgery the histological examination of simvastatin treated group demonstrated that the defect was filled with collagen sponge with inflammatory infiltration surrounded by stabilized blood clot which contains inflammatory cells and rich in vascular network, presence of coalescent proliferating blood vessels in different manner and number greatly more than that of the control groups. More condensed, tightly arranged collagen fibers interspersed by well differentiated osteoblasts on the top of woven bone formation. New abundant bone formation**. Fig.(6)** with thin irregular bone trabeculae formed at the sides and base of the defect, sheet of condensed osteoblasts lining the surface of the newly formed bone and reversal line separating the newly formed bone from the old bone. **Fig. (7).**

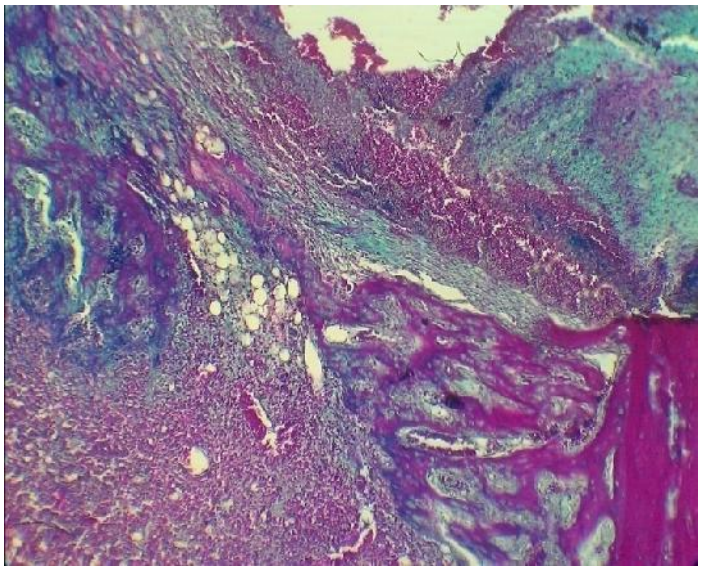


Fig. (6) : Photomicrograph of group 2 (simvastatin) 7 days showing intense red color of thenew bone trabeculae with different thickness, green color of collagen .Masson trichrome stain X 100).

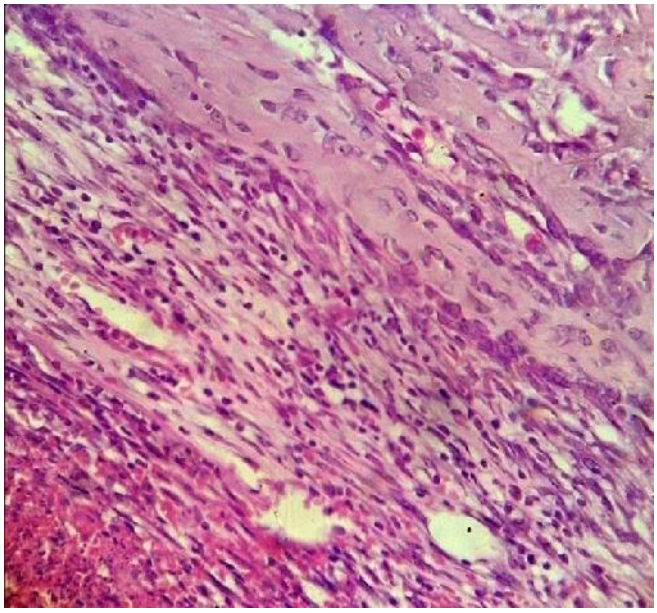


Fig (7): Photomicrograph of, group 2 (simvastatin) 7 days showing ,numerous blood vessels .H&E x100

After 14 days post surgery the histological examination of the defect demonstrated that new immature lamellar bone had been formed and approximately fills the whole defect, remnants of collagen sponge located at the center of the defect infiltrated by inflammatory cells and undifferentiated mesenchymal cells. The newly formed bone contains osteocytes with narrower spaces and smaller marrow spaces . Marrow cavities of the newly formed bone were lined by osteoblasts. **Fig. (8)**.

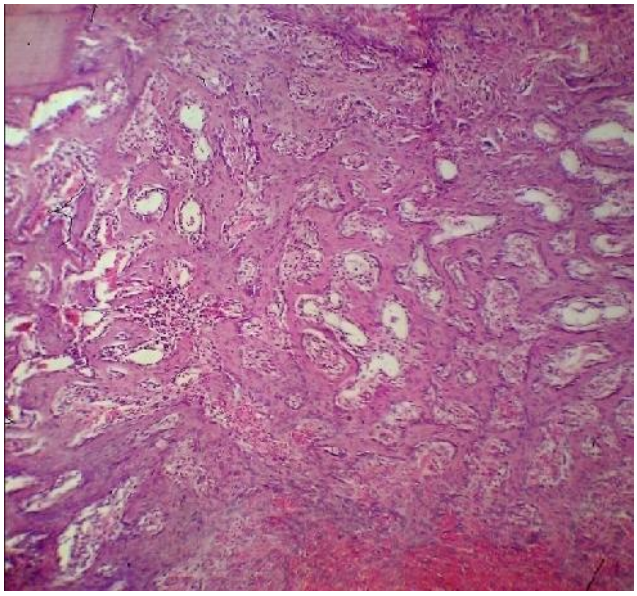


Fig. (8) : Photomicrograph of the group 2 (simvastatin) 14 days showing, abundant new bone formation with thick bone trabeculae, bone marrow spaces containing haemopiotic tissue and lined by osteoblasts . H&E (original magnification) X 100.

Histological examination at 28th day post surgery shown complete closure of the defect with a new mature lamellar bone, mature collagen bundles is approximately absent **Fig.(9).**The defect now is healed with well formedHaversian system composed of concentric bone lamellae enclosing central Haversian canals, Small size osteocytes with narrow osteocyte spaces arranged in a regular Haversian pattern, faint blue reversal line demarcating the old cortical bone from the newly formed bone. Complete absence of collagen sponge. The new bone trabeculae are perpendicular to the long axis of the old bone. **Fig. (10).**

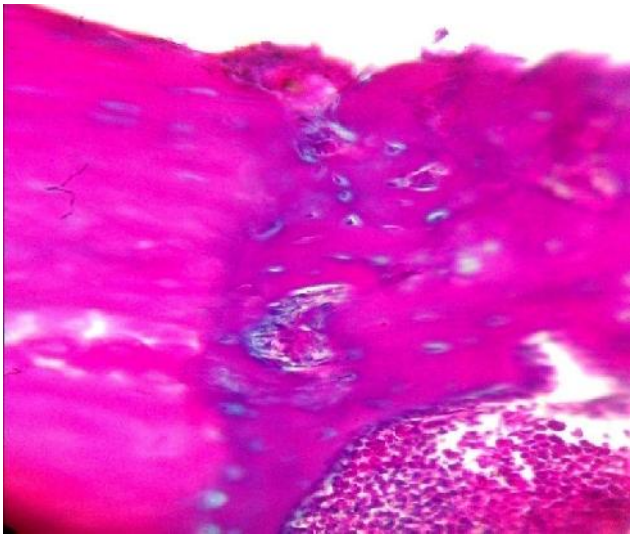


Fig. (9) :Photomicrograph of group 2(simvastatin) 28 days showing new mature lamellar bone (NB), old cortical bone (OB).Masson trichrome stain, X 400).

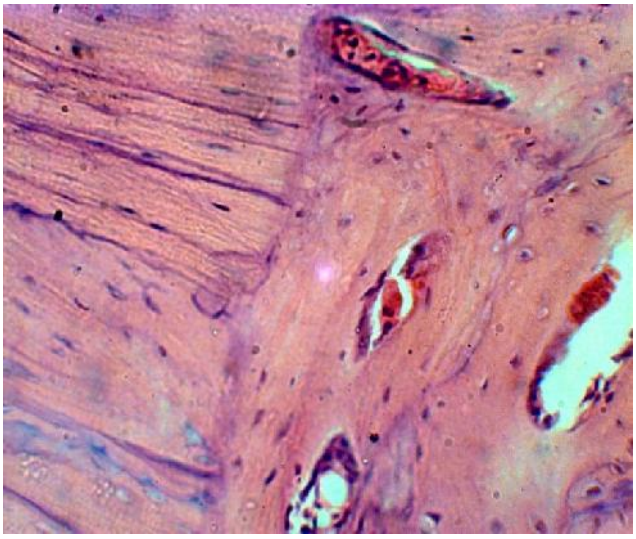


Fig. (10) : Photomicrograph of the group 2 (simvastatin) 28 days showing, mature lamellar new bone perpendicular to the long axis of the cortex , with narrow bone marrow cavities ,reversal line separating the newly formed bone from the old bone . H&E X 100.

Immunohistochemical results

Immunohistochemical examination of group 1 (control group) revealed moderate positive immunoreactivities for TGF-β in the wall of blood vessels ,extracellular matrix of the granulation tissue, ossifying centers and bone marrow spaces. Negative immunoreactions were noticed in the rest of chondroid tissue**.(Fig.11 & 12).**

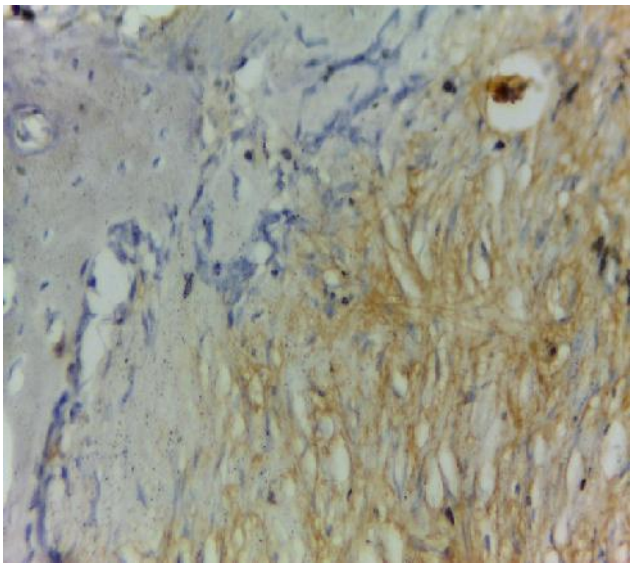
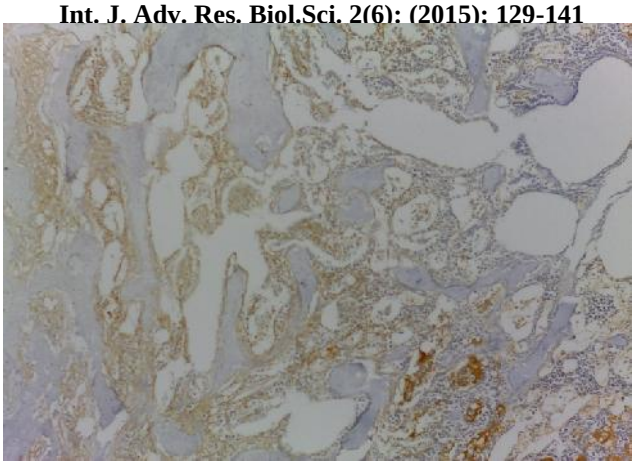
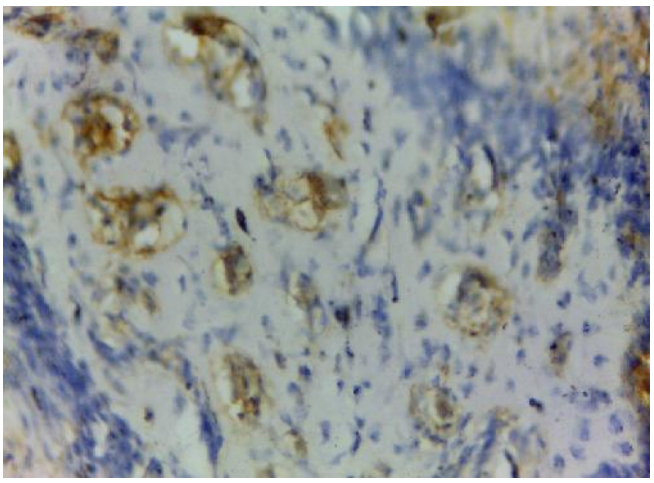


Fig.(11) : Photomicrograph of the group I (control) 14 days showing moderate immunoexpression of TGF-B in the granulation tissue. TGF-β X100

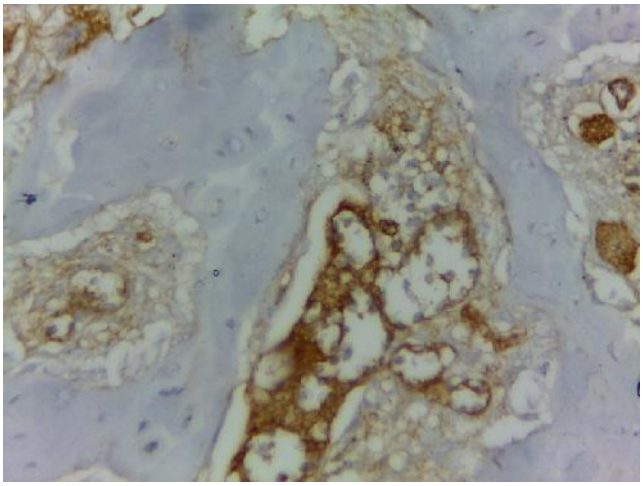


Fig(12) : Photomicrograph of the group I (control) 28 days showing moderate immunoexpression of TGF-B in bone marrow cavities. TGF-β X100

Simvastatin group revealed more intense immunoreaction of TGF-β in bone marrow cavities between the newly formed bone trabeculae,on the lining surfaces of the new trabeculae and the walls of blood vessels **(Fig.13 & 14).**



Fig(13): Photomicrograph of, group 2 (simvastatin) 7 days showing intense reaction in ossifying centers and newly formed blood vessels. TGF-β X100



Fig(14): Photomicrograph of, group 2 (simvastatin) 28 days showing intense reaction in bone marrow spaces. TGFβ X400

Discussion

Bone regeneration has attained a great interest in researches, many aids has been used to improve the bone regeneration capacity either as a scaffold or an inductor for bone formative cells. Simvastatin which is a member of statins group has been used for many years ago as hypolipidemic material, recently used in many researches as a bone anabolic material since it is discovered by **Mundy et al(1999).**

In vitro studies have shown that simvastatin is among the most potent in stimulating bone growth**(Mundy et al,1999).**simvastatin stimulates BMP-2, endothelial nitric oxide synthase (eNOS) and alkaline phosphatase**(Maeda et al,2001).**

The histological findings in the present experiment corroborate with the findings of earlier in vitro and in vivo studies that confirm the positive anabolic effect of simvastatin on bone. Increase in bone formation rate in simvastatin group compared to the control group which was obvious in the two weeks period, and lead to complete closure of the defect by new mature bone in the 28 days period make our results agree **with Maeda et al (2001**), who stated that Simvastatin markedly increased mRNA expression for bone morphogenetic protein-2, vascular endothelial growth factor (VEGF), alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin (OCN) in MC3T3-E1 cells, while suppressing gene expression for collagenase-1, and collagenase-3**(Maeda et al, 2001**). Also**Mukazawa et al (2011**), used simvastatin in rat bone marrow stromal cells culture and observed that simvastatin enhanced matrix calcification and increased alkaline phosphatase (ALP) activity.

Absorbable collagen sponge was used in this study because it had been used successfully as a carrier for BMP-2 to induce bone formation in animals and in humans 19**(Nevin et al,1996, Li St 1996 and Boyne et al,1997). Bouxsein et al (2001**) assessed the retention time of 125IrhBMP-2 in absorbable collagen sponge using gamma scientigraphy and showed that about 37% of the initial dose remained at the site 1 week after surgery, and 8% remained after 2 weeks. We thought that it is possible that statins could have been retained by the absorbable collagen sponge and released over time to exert their effect that led to such a significant increase in osteogenesis. Absorbable collagen sponge is biocompatible **(Chay et al,2000)** and acts as a scaffold that replaces the missing bone. It is porous, which facilitates bony ingrowth and allows osteoconduction while it is absorbed. It also has a haemostatic effect, producing almost instant haemostasis when implanted into the defects.

Healing process of bone involves the chemotaxis of mesenchymal cells, their proliferation and differentiation into osteoblastic cells and the production and mineralization of extra- cellular matrix (ECM) by osteoblasts. It has been found that these critical cellular events are tightly regulated by appropriate signal molecules, such as growth factors and cytokines**(Gregg et al,1991**).Some growth factors, such as TGF-β, BMP-2 and VEGF, are signaling proteins secreted by cells recruited to the bone defect which control osteogenesis and angiogenesis by autocrine and paracrine effects in the local **area (Marie,1995).**

Owing to the competent ability of these growth factors in promoting bone formation, local administration of simvastatin carried by PLGA could significantly increase new bone formation in the tooth extraction socket without obvious side effects on the healing process **(Wu Z et al,2007)**which go together with our results In the present study at where simvastatin produced greater cellular proliferation and differentiation. This mitogenetic ability appears to enhance bone healing and regeneration at early stage. Furthermore Wong et al. supported the hypothesis that simvastatin could act as a local regulator of bone metabolism during early healing**(Wong etal,2005**).) The previous findings were in agreement with those concluded through the present study as there was a significance difference between experimental and control after 7 days postoperatively.

Although several studies have shown a beneficial effect of statins on bone **(Monjo et al,2010**), this subject is still controversial. Other studies could not confirm the positive effect of simvastatin on bone formation**. Van Staa et al (2000)** analyzed the medical records of 81,880 patients who had fractures and concluded that the use of statins at dosages prescribed in clinical practice was not associated with a reduction in risk fracture **(Gerber et al, 1999). Maritz et al, 2001)** reported that simvastatin was unable to prevent bone loss caused by ovariectomy in rats and suggested a permissive effect for estrogens in statin action on bone. According to this, the contrasting results

obtained in the last studies and those **of Mundy et al (1999)** may be explained by differences in statins, dosages, methods of administration, experimental animal model and design. On the other hand, another study analyzed mandibular bone defect regeneration in ovariectomized and non-ovariectomized rats and found that simvastatin aided new bone formation only in ovariectomizedanimals **(Deekers et al, 2000).**

Our finding at one week postoperatively showed that, there were significant histological differences between the two groups, in all groups the bone defect were filled with granulation tissue consists of large number of fibroblasts, inflammatory cells, and proliferating blood vessels, as a normal reaction against the surgical procedures, which correlate with the results **of Wu.Z et al (2007).**

Presence of coalescent numerous proliferating blood vessels in simvastatin group in compare to the control group may explain one of the major roles of simvastatin in new bone formation which is angiogenesis. Proliferating blood vessels budded from the bone marrow carrying the undifferentiated mesenchymal cells which differentiate into osteoblasts under the effect of ligands as bone morphogeneic proteins (BMPs), osteogenic growth peptides (OGP) and vascular endothelial growth factor (VEGF) all of which arised from the platelet destruction and inflammatory response**(Lalani et al , 2003)** these results coordinate with **Maeda et al (2001**),who stated that simvastatin increase angiogenesis through enhancement of vascular endothelial growth factor (VEGF). Other studies have shown that VEGF acts through an autocrine/paracrine factor in bone, promoting angiogenesis, ossification, and bone turnover **(Gerber et al, 1999 and Deckers et al, 2000).**

After 14 days post surgery we could see that there were more fibrous arrangements in the control group with differentiated scanty osteoblasts, the diminished number of osteoblasts may explain the indolent bone formation. This is in agree with Marie (1995), who stated that Bone formation depends mainly on the number of osteoblastic cells rather than the activity of the osteoblasts**(Ferreira et al,2014).**This is also coordinate with the others in that collagen sponge acts as a scaffold for cell migration, proliferation and blood vessels formation**(Nevin et al,1996 and Wong et al,2005).** Abundant new bone formation all around the margins of the defect is evidenced in the simvastatin group after 14 days post surgery which correlates with the results of **Wong et al (2005).**

After 28 days postoperatively, absolute closure of the defect with new bone formation was observed in the simvastatin group, the newly formed bone showed a lamellar pattern but in a direction perpendicular to the lamella of the old cortical bone with narrow marrow spaces and osteocytes well arranged in Haversian pattern which coordinate with the results **of Frederic (2008)** in that the bone formed originates from marrow cells which a rise through bone marrow vessels and is aligned at right angles to the long axis of the bone according to the perforating vessels direction.

In our results the osteoblasts lining the marrow spaces hadn't ceased in activity but still active cells forming bone lamella inside the marrow spaces aiming to narrow it until only occupied by a blood vessel. It was obvious in the simvastatin group after 28 days that new bone is formed inside the marrow space causing narrowing of it, this is what we called maturation of the Haversian system or osteonal conformation.

TGF-β expression was more intense in ossifying centers,marrow cavities ,surfaces of newly formed bone trabeculae and blood vessels in simvastatin group than that of control group.

TGF-β is produced in the fracture site by platelets,inflammatorycells,macrophages,osteoblast and chondrocytes **(Cisneros et al, 2007).**TGF-β promoting angiogenesis which is essential for orderly fracture repair,stimulating bone formation by inducing differentiation of periosteal mesenchymal cells into chondroblasts and osteoblasts **(Joyce,1999)** which in accordance with our results.

These results implied that the prominent new bone formation in the simvastatin group could only be attributed to the osteoinductive character of simvastatin. In the simvastatin group, the constant release of simvastatin was thought to up-regulate the expression of a series of growth factors in osteoblasts which would enhance matrix production and calcification**(Cochran et al,1999 and Maeda et al,2001).**

These findings suggest that local administration of simvastatin could accelerate the bone healing process.

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