Effect of Local Simvastatin on the Healing of Surgically Created Critical-Sized Bone Defects: An experimental study on sheep

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Background and objective: the role of simvastatin in lowering serum cholesterol levels is well described. However, recent findings suggest they have a role in bone formation as well. The study aims to determine the effect of local simvastatin application on bone defect healing and compare the amount of new bone produced by a simvastatin-treated defect with that produced by a bone graft (biphasic calcium phosphate) and non-treated defects (left empty) histologically.

Methods: Forty-five critical-size defects were created (8mm in diameter and depth) in the iliac bone of 6 sheep. For the first three sheep (5 defects/ilium), the five defects on the right ilium were left empty as a Control group, while the five defects on the left ilium were filled with biphasic calcium phosphate as Test 1 group. For the other three sheep, 5 defects were created on the right ilium, the defects were filled with 10mg crushed simvastatin tablet with gelfoam (as a carrier) as Test 2 group. The animals were sacrificed over periods of 1, 2, and 3 months. Histopathological studies were done for all the samples. SPSS version 28 was used to analyze the results. The numerical variables were checked for normality using Smirnov – Kolmogorov test, then analyzed using ANOVA and unpaired t-test (p–values ≤ 0.05 were considered statistically significant).

Results: All 6 adult male sheep passed the scheduled periods uneventfully. During the wound healing period, there was no complication such as infection, excessive hematoma, or wound dehiscence. All 45 standardized iliac bone defects were included in the final analysis (n= 45). The histologic results showed that Test 2 group (defect filled with simvastatin) in the 1st, 2nd, and 3rd months had significantly higher bone formation at the surface and depth of the defects than Test 1 and Control group with P values (<0.0001) at all period intervals.

Conclusion: Simvastatin enhances bone formation and accelerates the healing process of the bony defect.

Keywords: Bone formation, Bone graft, Bone healing, Simvastatin, Statin.

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INTRODUCTION

Bone defects are created by different etiological factors, such as tumors, infections, and trauma.[1] Different bone grafts used for increasing the rate of bone formation and augmentation include autografts, allografts, xenografts, or alloplastic bone substitutes. Autogenous grafts are the gold standard for bone reconstruction because of their histocompatibility, osteoinductivity, osteogenicity, and low cost.[2]

But they have their disadvantages, donor site morbidity, limited availability, postoperative discomfort for the patient, and increased surgical time. In the case of allografts, the disadvantages are the possibility of immunological reactions and

disease transmission. [3]

Xenogeneic bone grafts are effectively acquired and fabricated, but they have patient acceptance problems and low osteoinductive capacities.[4] To overcome these drawbacks, many attempts have been developed to obtain synthetic bone substitutes or alloplastic graft that is osteoconductive and act as a scaffold for new bone and blood vessel formation.[5]

Biphasic calcium phosphate (BCP) is a mixture of hydroxyapatite (HA 30%) and β -tricalcium phosphate (β- TCP 70%) used widely to control the resorption rate and provide space maintenance. Alloplastic bone can be manufactured without restriction and poses no risks of infection or immune response, but its osteoinductive potential is poor.[6]

Simvastatin is a specific competitive inhibitor of 3-hydroxy-2-methyl-glutaryl coenzyme A (HMG-CoA) reductase and is a widely-used anti-hyperlipidemia drug.[7,8] Recent research data has demonstrated that the effect of statins is well investigated for their pleiotropic effects other than lowering cholesterol, including angiogenesis, osteogenesis, anti-inflammatory, nitric oxide bioavailability, and endothelial cell function. Mundy et al.[9] first reported that simvastatin in vivo enhanced bone formation in rodents and augment new bone volume in cultures from mouse calvaria.

Bone morphogenic proteins (BMPs) are essential regulators of osteogenic differentiation during bone repair. BMP-2 causes multipotent stem cells to differentiate into osteoblast-like cells.[10] Use of inexpensive pharmacologic compounds, such as statins, to stimulate autogenous bone growth factors could be a promising approach for bone regeneration. Gutierrez et al[11] first reported that statins increased BMP-2 expression in bone cells. Statins are frequently used to lower cholesterol levels. Statins induce neoangiogenesis, cause stimulation of osteoblasts, decrease vascular inflammation, are anti-thrombogenic, and increase the expression of BMP-2 along with many other osteoinductors.[12,13]

This experimental study aims to investigate the effect of local simvastatin on the healing of surgically created critical-sized defects of the iliac bone in sheep histologically.

Methods

In a comparative, prospective, experimental study, six adult local breed male sheep (rams), (2.5-3.5 years of age, 70 kg in weight) were included in study. All surgical procedures were performed under intramuscular sedation and local anesthesia at the Veterinary Theater (Qoshtapa Veterinary Center-Erbil-Iraq). The animals were sedated using ketamine hydrochloride 5mg/kg (KETALROM-50 ,S.C ROMVAC company, Ilfov,Romania) and xylazine0.2mg/kg (xyla; metaalweg 8,CG ventery, the Netherland). In the areas exposed to surgery, 5ml of (Lidocaine 2%) was injected for local anesthesia and hemostasis. The surgical area is shaved, washed, and disinfected with povidone-iodine (Betadine 10%, Iosept, Berat International Limited, Turkey).

An incision of the skin and subcutaneous tissues were made over the iliac crest, and the skin and the subcutaneous tissues were bluntly dissected from the underlying middle gluteal muscle. The periosteum was incised and elevated from the bone by a periosteal elevator.

Forty-five critical-size defects were created (8mm in diameter and depth) in the iliac bone of 6 sheep. For the first three sheep (5 defects/ilium), the five defects on the right ilium were left empty as a Control group, then covered with collagen membrane (Ceno Membrane 20mm×20mm, Tissue regeneration corporation TRC, Iran), while the five critical-sized defects on the left ilium were filled with biphasic calcium phosphate (osteon II, Dentium. Co. Ltd, Suwon, South Korea) as Test 1 group, then covered with a collagen membrane.

For the other three sheep, 5 defects were created on the right ilium, the defects were filled with 10mg crushed simvastatin tablet (Pharma International, Jordan) as Test 2 group (drug preparation of which is done before surgery crushing 10mg simvastatin tablet using a sterile bone crusher (Osung Bone Mill, China), Moist gelfoam with 2 ml of normal saline (Gelita Tampon 50 Tampons 1 x 1 x 1 cm B. Braun, Germany) and absorption crushed simvastatin by

moistened gelfoam, then placed inside the defects were created (Figure 1), finally the defects were covered with collagen membrane (Figure 2). The surgical sites were sutured in a layering approach, using resorbable (Vicryl sutures 2/0, DAMACRYL, GMD, Turkey).

To reduce the pain, diclofenac sodium 2.5mg/kg was intramuscular (IM) injected twice a day for 3 days.[14] Intramuscular injection of antibiotic oxytetracycline 10mg/kg once a day for five days.[14] The surgical site was monitored from 1st day up to 10 days to notice any swelling, pus discharge, infection, and wound dehiscence. The surgical site was washed with iodine solutions 2 times a day. No special feeding program. They were bred on a natural green farm in a rural area with natural food (grass and green fodder crops).

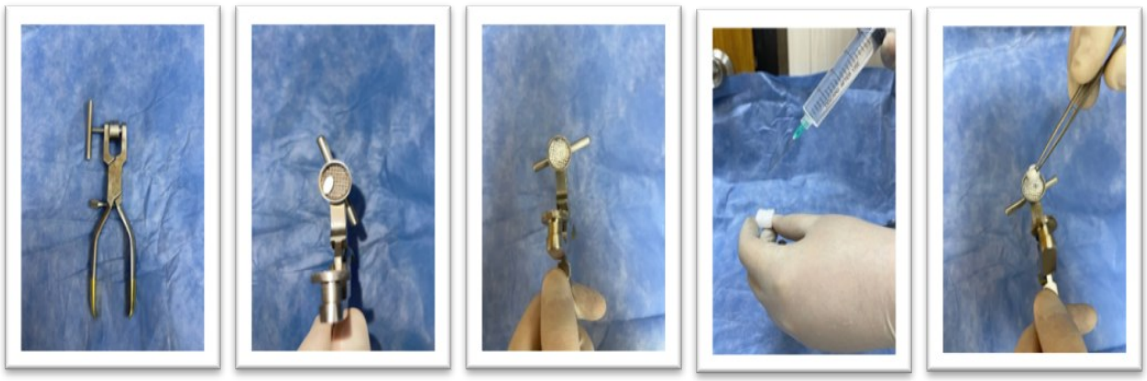

Figure 1: Simvastatin preparation

(A) Bone crusher (B) Placement of 10mg of simvastatin tablet into the bone crusher instrument (C) Crushing 10mg of simvastatin by a sterile bone crusher (D) Moist gelfoam with 2ml of normal saline (E) Absorption of crushed simvastatin by moistened gelfoam.

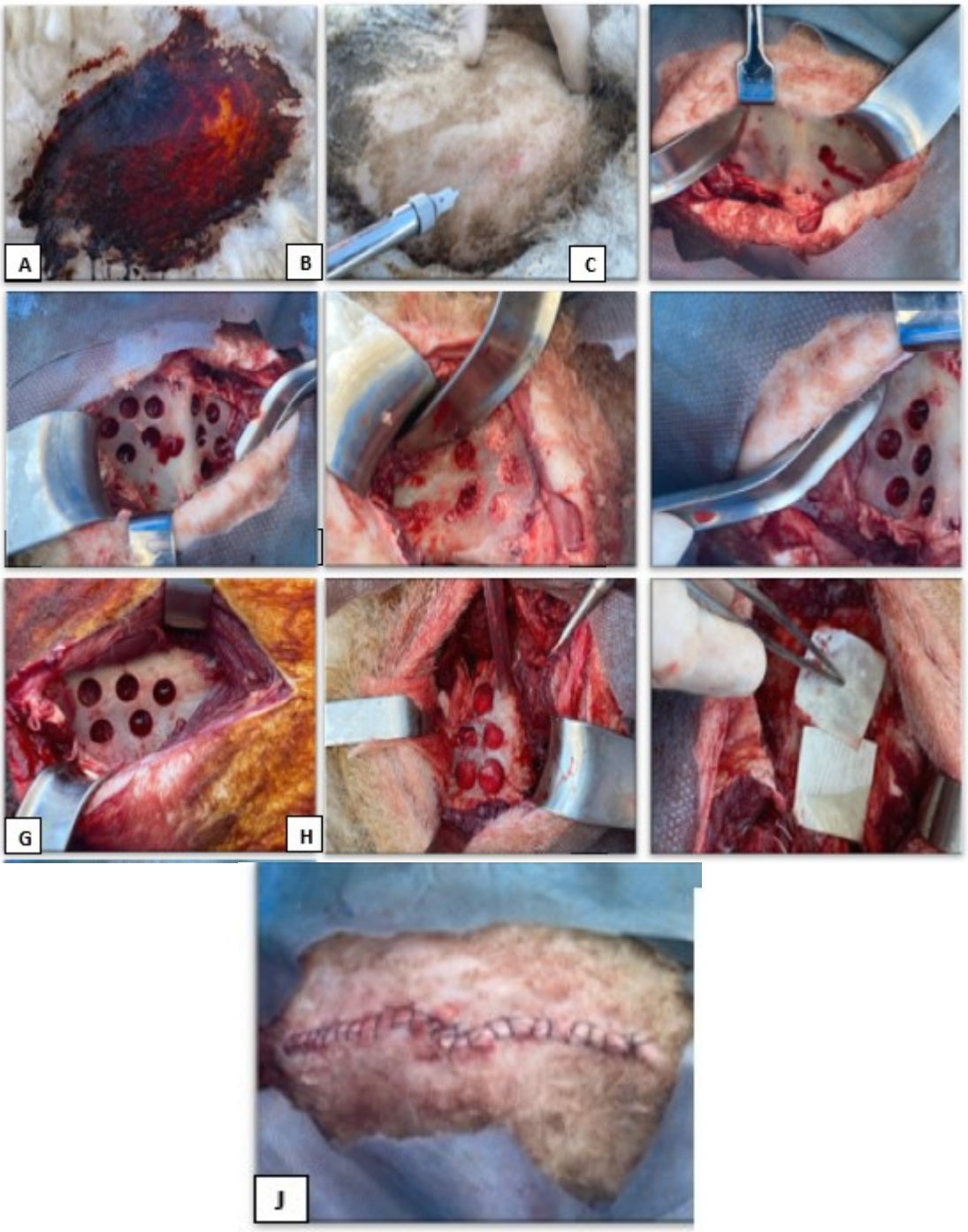

Figure 2: Step-by-step surgical procedures

(A) Shaved and disinfected surgical site (B) Anesthetized surgical site (C) Incision area and reflection of all overlying soft tissue (D) Ten prepared defects 8mm in diameter and depth in sheep ilium (E) Five defects of prepared sheep ilium filled with bone substitute (F) Other 5 holes left empty(G) Five prepared defects 8mm in diameter and depth in sheep ilium (H) Filling the defects with simvastatin(I) Membrane placement (J) Suturing in layers.

The first sheep with the biphasic calcium phosphate and non-treated defect (left empty) was sacrificed at the end of month 1 postoperatively, then the second one was sacrificed at the end of month 2, and finally, the third sheep were sacrificed at the end of month 3. The other three sheep with the simvastatin-treated defects were sacrificed at the same time interval as the previous three sheep. Each time after sacrificing, the iliac bone was isolated and stripped from all soft tissue. The iliac bone was cut into blocks containing an area of surgically created defects. They were immersed immediately in 10% neutral buffered formalin for fixation.

Histological examination and analysis

All 45 standardized iliac bone defects were included in the final analysis and they underwent processing for slide preparation and were stained with hematoxylin and eosin (H&E) and Masson trichrome stains step-by-step slide preparation. At three different periods (1, 2, and 3 months), the following histological parameter (percentage of osteoid (newly formed bone) at the surface and depth of defects) were evaluated.

Statistical Analysis

The statistical package for the social sciences program (SPSS, version 28) was used for data analysis. The numerical variables were checked for normality using Smirnov – Kolmogorov test, then analyzed using ANOVA and unpaired t-test (p–values ≤ 0.05 were considered statistically significant).

RESULTS

All 6 adult male sheep (2.5-3.5 years of age and 70 kg in weight) passed the scheduled periods uneventfully. During the wound healing period, there was no complication such as infection, excessive hematoma, or wound dehiscence. All 45 standardized iliac bone defects were included in the final analysis (n= 45).

The osteoid (newly formed bone) at the surface of defects was highly and significantly increased (p-value <0.001) in all groups with healing time progression at the end of months 1, 2, and 3 as shown in Table 1.

Table 1: Difference in the mean of osteoid (newly formed bone) at the surface of defects within 3 groups at the three-time intervals.

Groups	Healing time period	N	Mean (mm)	S.D	p-value	ANOVA Test
Control	One month	5	0.01	0.03	<0.001	Highly significant
	Two months	5	0.38	0.16		
	Three months	5	0.83	0.12		
Test 1	One month	5	0.59	0.07	<0.001	Highly significant
	Two months	5	0.97	0.03		
	Three months	5	1.68	0.08		
Test 2	One month	5	3.34	0.19	<0.001	Highly significant
	Two months	5	4.36	0.23		
	Three months	5	7.24	0.07		

The mean difference of osteoid between the control and test 1 groups was -0.5800, -0.5900, and -0.8500 at the end of months 1, 2, and 3 respectively with a highly significant difference p= <0.0001 at all period intervals. Similarly, the mean difference of the osteoid between control and test 2 groups was -3.3300, -3.9800, and -6.4100 at the end of months 1, 2, and 3 respectively with a highly significant difference p= <0.0001 at all period intervals. However, the mean difference between test 1 and test 2 groups was (-2.7500, -3.3900, and -5.5600 at the end of months 1, 2, and 3 respectively with highly significant difference p= <0.0001 at all period intervals as presented in Table 2 and (Figures 3, 4, and 5).

Table 2: Comparison of osteoid at the surface of defect between 3 groups at the three-time intervals (using unpaired t-test).

Healing time period	Group	Group	Mean difference (mm)	P- value	SE difference	95% confidence intervals	
						Lower bound	Upper bound
1 Month	Control	Test 1	-0.5800	<0.0001**	0.034	-0.6585	-0.5015
	Control	Test 2	-3.3300	<0.0001**	0.086	-3.5284	-3.1316
	Test 1	Test 2	-2.7500	<0.0001**	0.091	-2.9588	-2.5412
2 months	Control	Test 1	-0.5900	<0.0001**	0.073	-0.7579	-0.4221
	Control	Test 2	-3.9800	< 0.0001**	0.125	-4.2689	-3.6911
	Test 1	Test 2	-3.3900	<0.0001**	0.104	-3.6292	-3.1508
3 months	Control	Test 1	-0.8500	<0.0001**	0.064	-0.9987	-0.7013
	Control	Test 2	-6.4100	<0.0001**	0.062	-6.5533	-6.2667
	Test 1	Test 2	-5.5600	<0.0001**	0.048	-5.6696	-5.4504

*** The p-value is significant at ≤ 0.05**
**** The p-value is highly significant at ≤ 0.001**
ns. The p-value is non-significant > 0.05

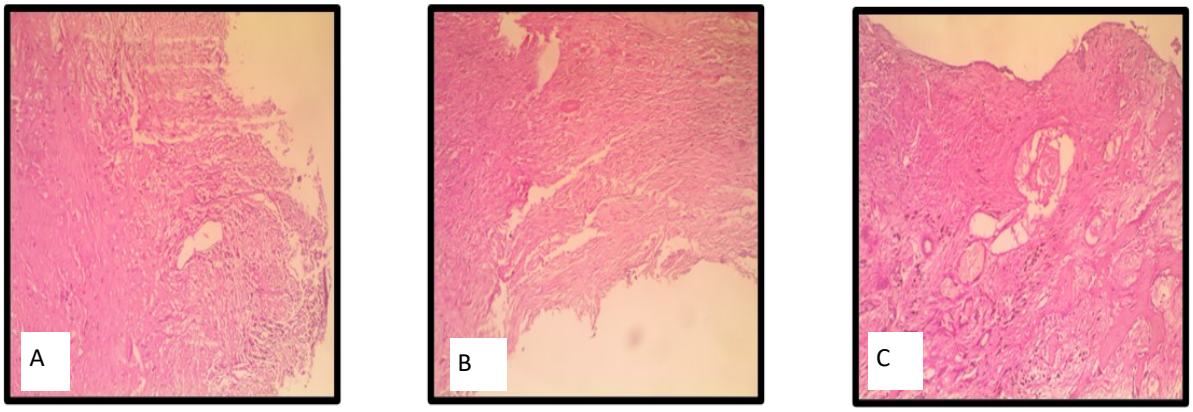

Figure 3: Light micrograph shows new osteoid at the surface of defect (A) Control group (H&E x100), (B) Test 1 group (H&E x100), and (C) Test 2 group (H&E x400) at the end of month 1.

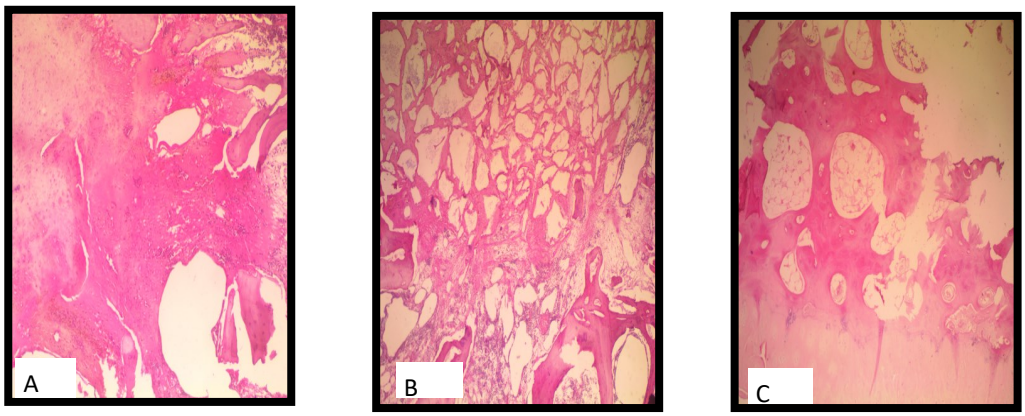

Figure 4: Light micrograph shows new osteoid at the surface of defect (A) Control group(H&E x400), (B) Test 1 group (H&E x100), and (C) Test 2 group(H&E x100) at the end of month 2.

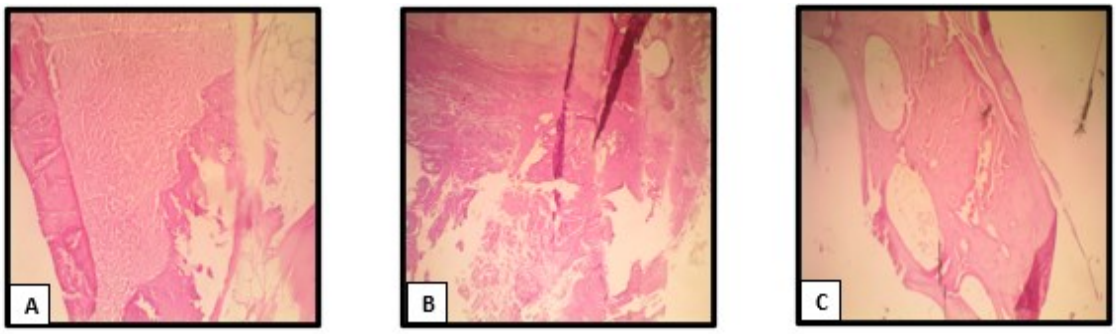

Figure 5: Light micrograph shows new osteoid at the surface of defect (A) Control group (H&E x400), (B) Test 1 group (H&E x100), and (C) Test 2 group (H&E x100) at the end of month 3.

The osteoid (newly formed bone) at the depth of defects was highly and significantly increased (p-value <0.001) in all groups with healing time progression at the end of months 1, 2, and 3 as shown in Table 3.

Table 3: Difference in the mean of osteoid (newly formed bone) at the depth of defects within 3 groups at the three-time intervals.

Groups	Healing time period	N	Mean (mm)	S.D	p-value	ANOVA Test
Control	One month	5	0.09	0.14	<0.001	Highly significant
	Two months	5	1.74	0.22		
	Three months	5	2.72	0.37		
Test 1	One month	5	1.04	0.11	<0.001	Highly significant
	Two months	5	2.00	0.32		
	Three months	5	3.44	0.27		
Test 2	One month	5	3.76	0.48	<0.001	Highly significant
	Two months	5	5.76	0.18		
	Three months	5	7.63	0.36		

The mean difference of osteoid between the control and test 1 groups was (-0.9500 P=<0.0001, -0.2600 P= 0.1727, and -0.7200 P=0.0079) at the end of months 1, 2, and 3 respectively. The mean difference of the osteoid between the control and test 2 groups was -3.6700, -4.0200, and -4.9100 at the end of months 1, 2, and 3 respectively with highly significant differences p= <0.0001 at all period intervals. However, the mean difference between test 1 and test 2 groups was -2.7200, -3.7600, and -4.1900 at the end of months 1, 2, and 3 respectively with highly significant difference p= <0.0001 at all period intervals as presented in Table 4 and Figures 6, 7, and 8.

Table 4: Comparison of osteoid at the depth of defect between 3 groups at the three-time intervals (using unpaired t-test).

Healing time period	Group	Group	Mean difference (mm)	P- value	SE difference	95% confidence intervals	
						Lower bound	Upper bound
1 Month	Control	Test 1	-0.9500	<0.0001**	0.080	-1.1336	-0.7664
	Control	Test 2	-3.6700	<0.0001**	0.224	-4.1856	-3.1544
	Test 1	Test 2	-2.7200	<0.0001**	0.220	-3.2278	-2.2122
2 months	Control	Test 1	-0.2600	0.1727 ns.	0.174	-0.6605	0.1405
	Control	Test 2	-4.0200	<0.0001**	0.127	-4.3131	-3.7269
	Test 1	Test 2	-3.7600	<0.0001**	0.164	-4.1386	-3.3814
3 months	Control	Test 1	-0.7200	0.0079*	0.205	-1.1924	-0.2476
	Control	Test 2	-4.9100	<0.0001**	0.231	-5.4424	-4.3776
	Test 1	Test 2	-4.1900	<0.0001**	0.201	-4.6541	-3.7259

*** The p-value is significant at ≤ 0.05**
**** The p-value is highly significant at ≤ 0.001**
ns. The p-value is non-significant > 0.05

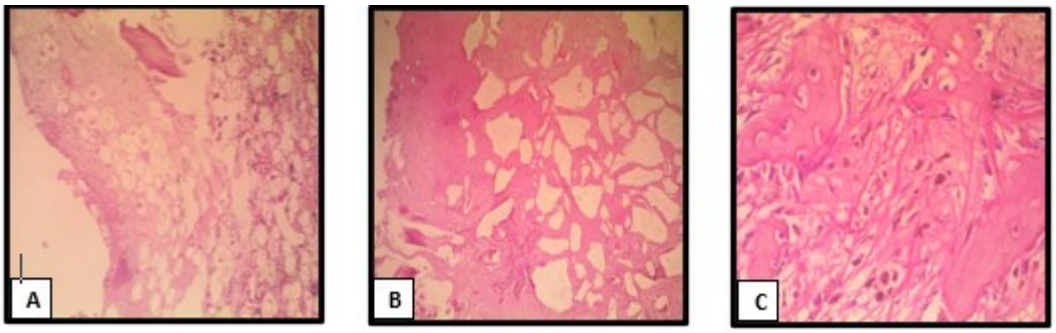

Figure 6: Light micrograph shows new osteoid at the depth of defect (A) Control group(H&E x100), (B) Test 1 group (H&E x100), and (C) Test 2 group (H&E x400) at the end of month 1.

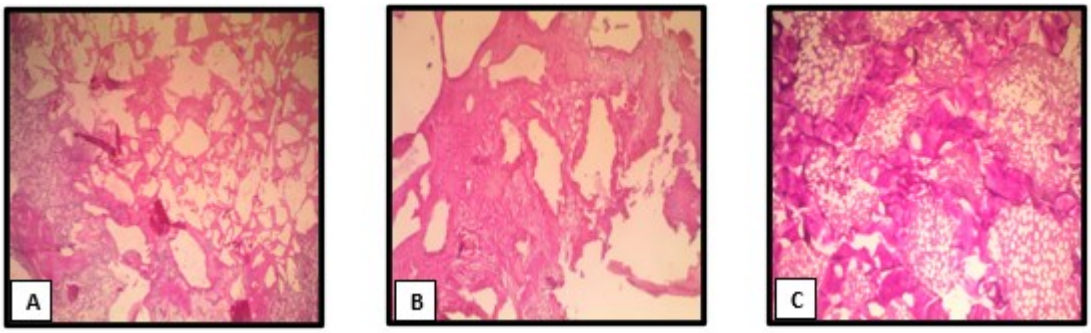

Figure 7: Light micrograph shows new osteoid at the depth of defect (A) Control group (H&E x100), (B) Test 1 group (H&E x100), and (C) Test 2 group (H&E x400) at the end of month 2.

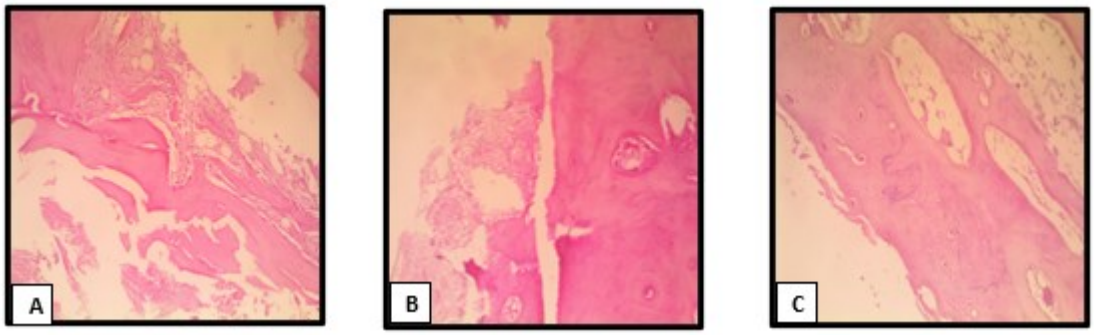

Figure 8: Light micrograph shows new osteoid at the depth of defect (A) Control group (H&E x400), (B) Test 1 group (H&E x100), and (C) Test 2 group (H&E x100) at the end of month 3.

DISCUSSION

Bone induction has a wide range of clinical applications; however, many bone induction techniques are still undergoing active research and have their shortcomings. In recent years, many researchers have investigated the utilization of statin, a drug that turns on the genes for bone formation, in bone grafting and found that this drug has a tremendous osteoinductive effect and great promise in routine use in ridge augmentation and bone grafting in the craniofacial region.[15,16]

Simvastatin, a specific competitive inhibitor of 3-hydroxy-2-methyl-glutaryl coenzyme A (HMG-CoA) reductase, The enzyme HMG-CoA reductase is one of the rate-limiting enzymes within the mevalonate pathway, through which cholesterol is biosynthesized. This enzyme is successfully inhibited by statins causing a reduction in blood cholesterol levels. Other products of the mevalonate pathway are also essential for the prenylation of some kinds of small guanosine triphosphate (GTP)ases. Since small prenylated GTPases are important both for activatingosteoclasts and inhibiting the synthesis of bone morphogenetic protein-2 (BMP-2), statins inhibit the prenylation of small GTPases and, as a result, they produce an anabolic effect on bone by inducing BMP-2 and create the anticatabolic effect by inhibition of osteoclast function.[17]

Simvastatin is suggested to be stimulating osteoblastic differentiation and mineralization in nontransformed osteoblastic cells in vitro and in vivo in rats and rabbits. It also markedly increases mRNA expression for alkaline phosphatase, type I collagen, bone sialoprotein, and osteocalcin in nontransformed osteoblastic cells and decreases gene expression for collagenase-1 and collagenase-3.[13,18,19] Some studies have found that simvastatin suppresses osteoclasts, thus promoting bone repair.[20] Vascular endothelial growth factor (VEGF) is not only an intense angiogenic factor but also a vital particle for bone development, suggesting that statin-actuated VEGF could assume a part in angiogenesis and osteo-

genesis.[10]

In this study, simvastatin has been administered because Gutierrez et al.[11] found that topical application of statin was 50 times more active on bone formation than oral administration(systemically). Statins undergo high first-pass metabolism and increasing the dose of systemic statins can cause liver failure, kidney disease, and rhabdomyolysis.[12] Conversely, local administration was suggested to deliver simvastatin directly in therapeutic concentrations for bone formation with virtually no side effects.[13,19]

Several studies have used simvastatin with carriers, such as the gelatin sponge[13,21] collagen sponge[22] collagen matrix,[23] poly (lactic-co-glycolic acid),[24,25] methylcellulose gel,[24,26] a-tricalcium phosphate rods,[13] and biodegradable hydrogel,[27] for successful local delivery. In this study, the gelatin sponge was the carrier of choice because of its biocompatibility. In addition, it is bioresorbable and conforms easily to the shape of bone defects.[13,19,28]

We selected 10 mg as a safe dose to insert in each critical-sized defect with a carrier gelfoam surrounding it. The powder cannot be directly inserted as it will cause severe inflammation in the surrounding soft tissues. The idea was to have the statin protected by gel foam and as the gelfoam resorbed slowly, was biocompatible, and easily adapted to the wall of defects, the powder would have a slow sustained release effect. We never got any adverse reaction to the drug in this way.[13,19,28]

To our knowledge, no previous studies in a similar manner were done in the sheep model using the local application of Simvastatin in bone defects.

In this study, sheep are used as an experimental animal model. Many studies prefer the sheep model over rabbit and rodents[29–31] due to being more readily available, compliant, and docile while being less controversial, sheep have also become a popular in vivo experimental animal model, mainly in orthopedic and dental research, due to the similarity of body weight, the presence of long bones and a bone regeneration time similar to humans. Their remodeling rate, lamellar bone structure, and primary bone healing characteristics are similar to humans.[32] Immune responses of larger animals are more similar to humans than those of rodents.[32–34]

The factors mentioned above which indicate the similarity of larger animals such as sheep and humans aided in selecting the dosage of simvastatin which is 10mg and it was similar to the previously done studies on humans.[13,19,28]

The results of the present study are in line with those of Mukozaw et al.[35] they demonstrated bone healing of critical-sized bone defects in rabbits by statins in different time intervals, using 2.5 mg/ml simvastatin, and the animals were divided into four groups and the groups were sacrificed at periods, 1, 2, 4, 8, and 12 weeks postoperatively. The histologic assessment reveal that more new formation bone in defects filled with simvastatin groups increased significantly at 2, 4, 8, and 12 weeks postoperatively in comparison with the control group.

The finding of this study agrees with the same findings of Ayukawa et al.[20] in their study, which demonstrated the effect of the local administration of simvastatin on the healing of artificially created bone defects. In the histologic and histomorphometric study, the local application of simvastatin successfully increased bone regeneration.

Mouhamed et al.[36] in their clinical study concluded that both digital radiological examination and histological analysis prove that adding simvastatin to tricalcium phosphate improves bone formation. Hassan et al.[37] concluded that the use of simvastatin accelerates bone graft healing, and maturation maintains its volume to a great extent and decreases its resorption. It also increases the density of the graft compared to a native bone or autogenous bone graft in human bone graft remodeling after ridge reconstruction, which is again in accordance with our study.

Our findings also come in agreement with the study of Rossili et al.[38] on eighteen rabbits using 1 mg of simvastatin on the femoral epiphysis 6mm prepared to defect, with scores for the new bone formation greater in the simvastatin group.

The results of the articles mentioned above are similar to this study in the aspect of enhancement of bone formation by simvastatin but this study differs from the previously mentioned studies in the animal selection, dosage, technique of application of simvastatin, and duration of the study. No article was found that contradicts the results of this study.

CONCLUSION

In this study, simvastatin enhances bone formation and accelerates the healing process of the bony defect.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relevant to this article.

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