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Effect of simvastatin on the extraction socket healing in the mandible of prednisolone-treated albino rat

Asmaa Ahmed Fahem[1], Rania Mossad Hassan[2,3], Khaled El-Haddad[4,5]

Aim: This work aimed to evaluate the possible prophylactic effect of using Simvastatin as adjunctive treatment to ameliorate the effects of using prednisolone on the healing of the albino rat mandibular molar extraction socket.
Materials and Methods: 42 rats were equally divided into 3 groups (I: received distilled water, II received Prednisolone, III: received simvastatin + prednisolone via gastric tube). After 5 weeks, the mandibular 1st molar was extracted then each group was further subdivided into 2 subgroups depending on sacrifice time after extraction: (A: 10 days and B: 21 days). Mandibular molar areas were processed for histomorphometric and immunohistochemical examination using osteonectin marker. The cell counts and new bone area percentage were measured and statistically analyzed using T-test, ANOVA and post hoc tukey tests.
Results: Prednisolone significantly decreased the area of new bone, osteoblasts and osteocytes count. Simvastatin had positive effects in the active osteoblasts and recent osteocytes cell counts marked by osteonectin staining and the new bone formation to a comparable level to the control group. The significant effects of Simvastatin on wound healing were indicated by statistical analysis of different parameters.
Conclusions: Prednisolone adversely affected the extraction socket healing. Simvastatin leads to minimizing the effects of prednisolone by increasing the cellular count and bone formation.

Keywords: Simvastatin, Extraction Socket, Prednisolone, Rats

1- Assistant lecturer, Department of Oral Biology Faculty of Dentistry – Beni Suef University
2- Vice Dean and Professor of Oral Biology Faculty of Dentistry, Badr University in Cairo (BUC), Egypt.
3- Professor, Department of Oral Biology, Faculty of Dentistry, Ain Shams University (ASU), Cairo, Egypt.
4- Assistant Professor, Department of Basic Oral Medical Sciences, College of Dentistry, Qassim University, Al-Mulida, Kingdom of Saudi Arabia.
5- Assistant Professor, Department of Oral Biology, Faculty of Dentistry, Ain Shams University (ASU), Cairo, Egypt.
Corresponding author: Khaled El-Haddad, email: k.elhaddad@qu.edu.sa

Introduction

Corticosteroids are synthetic substances that simulate the steroid hormones secreted in the adrenal cortex and are involved in several physiological processes. Glucocorticoids are a category of corticosteroids that chiefly have anti-inflammatory and immunosuppressive actions in addition to vasoconstriction effects. Other types of steroids are mineralocorticoids, which control water and electrolyte balance via the control of the renal transport mechanisms. Synthetic corticosteroids have changing ratios of glucocorticoid and mineralocorticoid properties.[1]

Prednisone is a synthetic steroid that is transformed in the liver to prednisolone (PRED). It is used to manage rheumatic, dermatologic, respiratory, gastrointestinal, and other diseases.[2] Prednisone is one of the most common systemic corticosteroids with relatively higher glucocorticoid activity than mineralocorticoid, hence, it could act an anti-inflammatory and immunosuppressive role.[3]

Prednisolone has undesirable effect on bone configuration and increases fracture threat.[4] Steroid-induced osteoporosis arises from prolonged intake of glucocorticoids such as prednisone.[5] Furthermore, bone necrosis develops in long-term systemic or local injection glucocorticoid treatment patients, even with no reported osteoporosis.[6] There are clinical studies suggested impaired wound healing associated with preoperative prednisone treatment for more than thirty days with 40 mg/day dose or more.[7]

One of the common wounds in dentistry is the tooth extraction socket which induces several local changes in the hard and soft tissues leading to socket wound healing. The socket-healing could be categorized into three stages, inflammation, proliferation and remodeling.[8]

Animal experiments revealed that healing of extraction socket could be affected by the chronic corticosteroids administration with reduction of bone volume in the alveolar processes.[9] Additionally, human clinical trials reported that patients taking corticosteroids are subjected to the risk of delayed extraction wound healing and osteoradionecrosis in jaws.[10] The delayed extraction socket healing associated with prednisolone treatment to a dosage of at least 8.0 mg/day.[11]

The adverse effects of prednisolone on the wound healing might be minimized by the adjunctive treatments. One of the promising agents in the bone healing enhancement are statins (hydroxymethylglutaryl-coenzyme A "HMG-CoA" reductase inhibitors). They are used to decrease the cholesterol levels via blocking of the HMG-CoA reductase enzyme that controls the cholesterol production in the liver. Statins are either synthetic such as lovastatin or natural such as simvastatin (SMV).[12] Simvastatin reduces the triglycerides and the low density lipoprotein cholesterol in serum. It also improves the endothelial function and decreases the risk of atherosclerosis. It is considered safe but has adverse effects such as gastrointestinal upset and headache.[13]

Regarding the effects of statin on wound healing, histological evaluation of simvastatin therapy revealed excellent tissue regeneration by diminished inflammatory reaction and enhanced re-epithelialization, collagen formation and angiogenesis.[14] Furthermore, the sustained statin supply could significantly accelerate the fracture healing in rat femoral fracture.[15]

It inhibits the development of osteoclasts indicating the potential effectiveness of statins for osteoporosis and bone resorption control.[16] It was also reported that the simvastatin combined with calcium sulphate enhanced the bone regeneration for bone defect in rat.[15]

There are previous studies reported the efficiency of topical simvastatin application in the extraction socket wound healing and its positive effect in bone formation and reduction of the socket size.[17,18,19] However, to our knowledge, there are a few studies investigating the healing process of the extraction socket by systemic administration of simvastatin particularly in association of steroid treated animals. Thus, the aim of the present study was to evaluate the possible palliative efficacy of oral simvastatin administration on extraction socket healing in the rats treated with prednisolone.

Materials and methods

42 adult male Albino rats were utilized and housed in the animal house of "Medicine Ain Shams Research Institute (MASRI)". Rats were housed in proper ventilation and adequate stable diet and tap water. The Research Ethics Committee of faculty of dentistry, Ain Shams University has approved this study procedure (Approval no: FDASU-Rec PC 022462).

Grouping: The rats were equally divided into 3 groups (14 rats each):

Group I (Control group): Received 40 mg/kg distilled water daily via gastric tube.

Group II (PRED group): Received, by gastric tube, 40 mg/kg oral Prednisolone dissolved in distilled water three times per week. In the remaining days of the week, the rats received distilled water via gastric tube.[20]

Group III (SMV+PRED group): Received PRED and SMV three times per week via gastric tube. PRED was administered as in group II while SMV was of dose 20mg/kg/day.[21] (SMV tablets dissolved in distilled water with 10% concentration).

After 5 weeks from day one of drug administration, the rats were sedated (by ether inhalation) and the mandibular 1st molar was extracted. Then, each group was subdivided into 2 subgroups (7 rats each):

Subgroup A: Rats were sacrificed 10 days after extraction.

Subgroup B: Rats were sacrificed 21 days after extraction.

Sample preparation: At the specified date of each subgroup, rats were killed and the mandibular molar areas were dissected. Samples were rinsed thoroughly with tap water to eliminate blood and adhering tissues then fixed in 10% buffered formalin solution for not less than one week. Samples were decalcified in 12% Ethylene Diamine Tetra-acetic acid (EDTA) solution changed every other day for two weeks. The samples were then washed under running water. The specimens were dehydrated by immersing in increasing concentrations of alcohol, then transferred to xylol to clear the specimen from alcohol. The samples were then embedded in the center of paraffin wax blocks. The embedded specimens were sectioned to a thickness of 4-6 microns. The sections were shifted in decreasing concentrations of alcohol. Sections were stained with Hematoxylin and Eosin (H&E).

Sections were mounted on positive glass slides for immunohistochemical

staining with the monoclonal Osteonectin antibody (Lot No. 716061, LSL Co., Ltd., Tokyo. Japan), which is characterized by species reactivity to humans and rats. A labelled polymer reagent kit (Envision Kit/ HRP (DAB). Dako, Denmark) was used for immunostaining and the slides were counterstained with hematoxylin. Positive immunoreaction was detected as a brown color and the cellular localization of this antibody is cytoplasmic. Osteonectin is used for the demonstration of active osteoblasts and recent osteocytes.

Histomorphometric analysis: Image analysis was done using Image Analysis J Executable jar file Version 2015, Microsoft Windows 2013.

A- New bone area measurement: The area percentage of the new bone were measured by calculating the percentage of new bone in relation to the total extraction socket area in the samples stained with H&E with magnification (x160).

B- The cell count: In H&E stained sections (magnification x160), osteoclasts and osteocytes were counted in all subgroups killed after 21 days (subgroups B). In osteonectin stained sections (magnification x400), recent osteocytes and active osteoblasts were detected as immunopositive cells and counted in all subgroups.

Data were tabulated and statistically analyzed using t test for comparison between the 2 durations in each group. ANOVA test was performed to compare between the corresponding subgroups. Tukey post hoc Tuky test was used to compare between each pair in subgroups.

RESULTS

1- Histological results (H&E): Subgroup IA showed signs of the initiation of socket healing. Newly formed granulation tissue with minute bone fragments were detected (Fig. 1A). Osteoblasts were found lining the socket wall (Fig. 1B). Subgroup IB extraction sockets showed increased volume of newly formed bone with a trabecular pattern enclosing small marrow cavities and clearly detected osteocytes which appeared more mature than those in subgroup IA (Fig. 1C, D). The extraction sockets of subgroup IIA were filled with granulation tissue with less detected osteoblasts lining the socket walls. There were irregular bands of osteoid tissue (Fig. 1E, F). Subgroup IIB showed less dense bone formation with relatively wider marrow cavities than those of control subgroup IB. There were many osteoclasts found in Howship's lacunae and few number of osteocytes in the poorly organized bone trabeculae (Fig. 1G, H). Subgroup IIIA extraction socket was occupied by granulation tissue with osteoblasts lining the socket walls (Fig. 1I, J). Subgroup IIIB showed improved healing process where the socket was packed with moderately thick bone trabeculae. Many mature osteocytes were present in lacunae within the new bone trabeculae along with occasional osteoclast (Fig. 1K, L).

2- Immunohistochemical results (osteonectin): Subgroup IA showed many positively stained osteoblasts and osteocytes and few negatively stained inactive osteoblasts (Fig. 2A). This positive reaction was decreased in subgroup IB in the osteoblasts and osteocytes (Fig. 2B). Subgroup IIA showed few positive osteoblasts and osteocytes in relation to the control subgroups (Fig. 2C) while subgroup

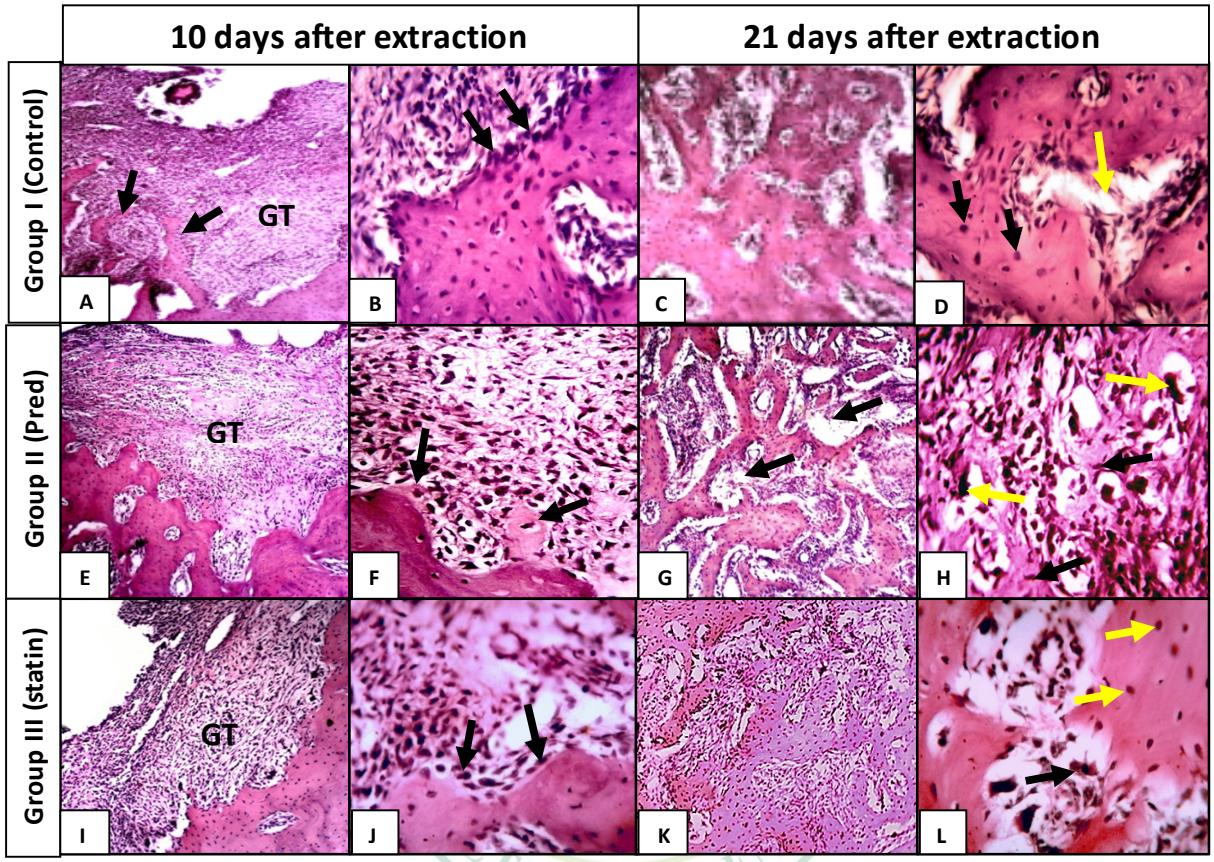

Figure (1): Photomicrograph showing: (A, B: Subgroup IA. C, D: Subgroup IB. E, F: Subgroup IIA. G, H: Subgroup IIB. I, J: Subgroup IIIA. K, L: Subgroup IIIB). (A): with formed granulation tissue (GT) and minute bone fragments were detected (arrows). (B): osteoblasts lining the socket wall (arrows). (C): increased volume of newly formed bone in a trabecular pattern. (D): relatively narrow marrow cavity (yellow arrow) and clearly detected osteocytes (black arrows). (E): socket filled with a granulation tissue (GT). (F): less detected osteoblasts lining and osteoid tissue with irregular thickness (arrows). (G): wide marrow cavities (arrows). (H): osteoclasts found in Howship's lacunae (yellow arrows) and few number of osteocytes (black arrows). (I): the sockets occupied by granulation tissue (GT). (J): osteoblasts lining the socket walls (arrows). (K): the socket is packed with bone trabeculae. (L): Osteocytes in lacunae (yellow arrows) and osteoclasts (black arrow). (H&E. A,C,E,G,I,K: x160. B,D,F,H,J,L: x400).

IIB showed slight increase in the immunopositive osteoblasts and osteocytes when compared to subgroup IIA but still less than control group (Fig. 2D). Subgroup IIIA and IIB showed positive reaction of many active osteoblasts and recent osteocytes comparable to subgroup IA (Fig. 2E, F).

3- Statistical results:

Area percentage of new bone: The statistical analysis revealed the least area % in the subgroup IIA treated with prednisolone (mean= 4.09) while the highest values were for subgroup IIIB treated with simvastatin and sacrificed 21 days after extraction (mean= 63.2).

Osteoclasts count in H&E stained sections: The statistical analysis showed that the lowest osteoclastic count was in the control subgroup IB (mean= 4) and the highest was of the SMV subgroup IIIB (mean= 26.8).

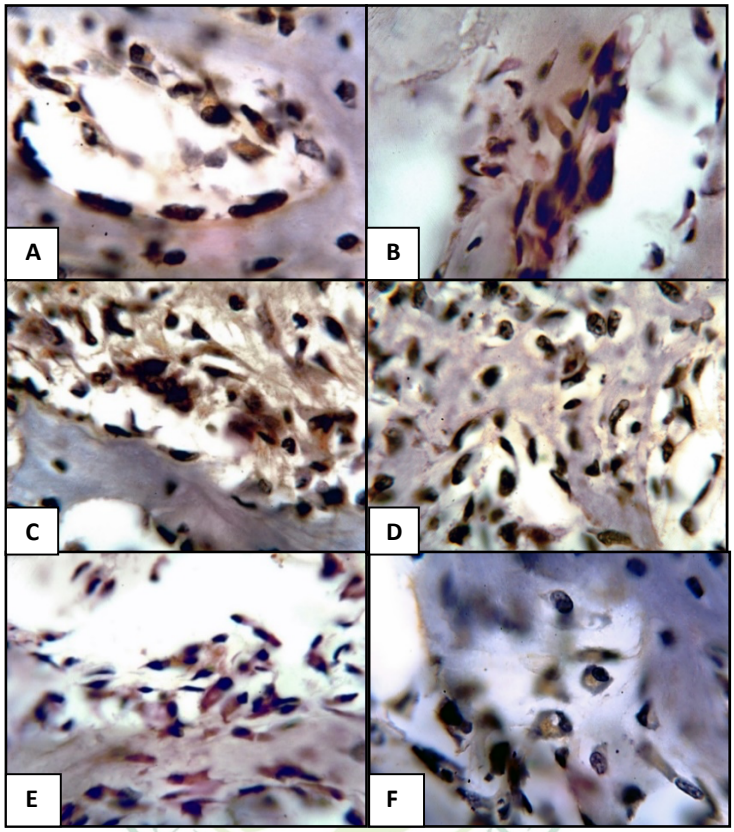

Figure (2): Photomicrograph showing positive immunoreaction in all groups (arrows) with more apparent positive cytoplasmic reaction in subgroups IA, IB, IIIA, IIIB (A,B,E,F) respectively. While the subgroups IIA (C) and IIB (D) revealed less observed positive reaction (Osteonectin. A,C,E,G,I,K: x160. B,D,F,H,J,L: x400).

Osteocytes count in H&E stained sections: This analysis was performed in subgroups sacrificed after 21 days only (IB, IIB and IIIB). The mean values were ordered in an ascending manner (4, 16.2 and 27.8 in the subgroups IIB, IIIB and IB respectively.

Osteoblasts and recent osteocyte count in osteonectin stained sections: Bothe cells had the least value in the prednisolone treated rats killed after 10 days while the highest value was of The control subgroup killed after 10 days (IA). The recent osteocytes mean values of subgroup IIIA equals that of subgroup IA. All mean and standard deviation values of all subgroups are illustrated in table (1) and the comparisons between subgroups are illustrated in (figure 3).

The comparison between each subgroup inside the group to evaluate the effect of time was performed by T-test. While ANOVA was used to compare between the three subgroups sacrifieced in the same time to assess the effect of the drugs. All these comparisons revealed statistically significant differences except for the comparison between the subgroups IB, IIB and IIIB in the recent osteocyte immunopositive cell count. Moreover, separate comparisons between each pair of subgroups was performed by post hoc Tuky test and revealed significant difference between all the compared pairs except some comparisons mainly when

comparing (IA with IIIA and IB with IIIB) (table 2).

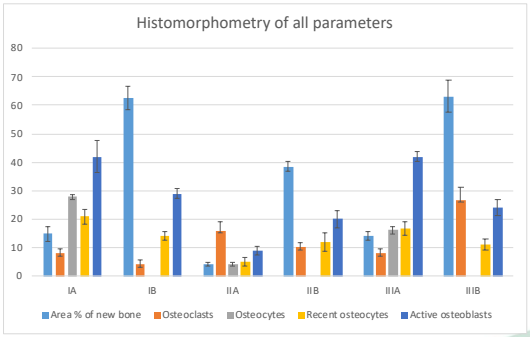

Figure (3): Bar chart illustrating the difference between all subgroups in the tested parameters.

Table (1): The mean and standard deviation of all subgroups in the tested parameters.

	Subg roup	Area % of new bone		Osteoclasts		Osteocytes		Recent osteocytes		Active osteoblasts	
		Mean	St.dev.	Mean	St.dev	Mean	St.dev	Mean	St.dev.	Mean	St.dev.
Mean and standard deviation values	IA	14.78	2.54	8.00	1.58	NA	NA	20.80	2.77	42.00	5.79
	IB	62.73	4.1	4.00	1.58	27.80	.84	14.00	1.58	29.00	1.58
	IIA	4.09	0.7	16.00	2.92	NA	NA	5.00	1.58	9.0	1.58
	IIB	38.53	1.76	10.00	1.58	4.00	.71	12.00	3.16	20.00	3.16
	IIIA	14.09	1.39	8.00	1.58	NA	NA	16.60	2.41	42.00	1.58
	IIIB	63.20	5.77	26.80	4.44	16.20	1.30	11.20	1.92	24.20	2.78

Table (2): The significance (p-value) of all comparisons between the tested subgroups by the used statistical tests.

Test	Subgroups	Area % of new bone	Osteoclasts count	Osteocytes	Recent osteocytes	Active osteoblasts
T-test (effect of time)	IA, IB	<0.001	0.004	NA	0.001	0.001
	IIA, IIB	<0.001	0.004	NA	0.002	<0.001
	IIIA, IIIB	<0.001	<0.001	NA	0.004	<0.001
ANOVA (effect of drugs)	IA, IIA, IIIA	<0.001	<0.001	NA	0.001	<0.001
	IB, IIB, IIIB	<0.001	<0.001	<0.001	0.19*	0.001
Post hock (pairs of subgroups)	IA, IIA	0.00	0.00	NA	0.00	0.00
	IA, IIIA	0.803*	1.00*	NA	0.035	1.00*
	IIA, IIIA	0.00	0.00	NA	0.000	0.00
	IB, IIB	0.00	0.016	0.00	<0.05	0.00
	IB, IIIB	0.983*	0.00	0.00	<0.05	.052*
	IIB, IIIB	0.00	0.00	0.00	<0.05	.060*

If p-value < 0.05, the difference is significant
* denotes the non-significant differences (p-value > 0.05).

DISCUSSION

The bone healing is an important process involved in the extraction socket healing. Many researches aimed to investigate several agents to promote the alveolar bone healing.[22,23] In the present study we aimed to evaluate the effect of prednisolone on the healing of the extraction socket of albino rat's mandibular first molar and to assess the possible protective effect of adjunctive Simvastatin treatment. We selected the prednisolone because corticosteroids are used nearly in all medical fields by various modes of administration.[24] We selected the oral route due to that prednisolone is sufficiently absorbed via oral administration.[25] The use of Simvastatin in order to counteract the effect of Prednisolone was supported by many researches that reported that Simvastatin promotes the osteoblastic activity, inhibits osteoclastic activity and enhances deposition of bone.[26]

In the present work, we evaluated the healing process of the extraction socket via routine histological examination via descriptive and histomorphometric parameters. Which is an essential evaluation method in bone healing.[27] Osteonectin was appropriate indicator for bone healing because its production is chiefly limited to areas undergoing repair or remodeling.[28] The use of the extraction socket of rat mandible provides a suitable model of the study of bone formation in rats, thus, it is considered a proper indicator of bone damage in the experimental conditions.[29,30]

In the present study the rats received Prednisolone revealed decreased extraction socket healing in comparison to the control rats in terms of low percentage of new bone formation, decreased osteoblasts cell count. These results were in agreement with Kim et al[31] who reported that steroids suppress bone formation, diminish the bone metabolism and impair thewound healing and bone strength. In addition, Kobza et al[4] reported that corticosteroids affect the bone healing by decreasing the bone formation in the extraction sockets.

In our results we found that the percentage of the new bone trabeculae in 21 days was significantly low and it was equal to 38% of the socket area in comparison with control group which was equal to 68% at the same period. This was in agreement with the previous findings of Kozai et al[20] and Osterhoff et al[32] who reported that corticosteroids are damaging to trabecular and cortical bone. Bone loss following corticosteroid administration is characterized by primary stage of fast bone resorption, with subsequent chronic stage in which bone is lost gradually.[20]

In our work, immunostaining with osteonectin revealed a statistically significant decrease in active osteoblasts number in

prednisolone treated rats compared with control rats. This may be explained by that corticosteroids administration led to a radical reduction in osteoblast and bone formation due to a decrease in osteoblast precursors and stimulation of osteoblastic apoptosis.[33] Moreover, Prednisolone leads to suppression of osteoblast which are the cells responsible for Osteonectin secretion.[34]

In the present study, Simvastatin had a positive effect on extraction socket healing particularly in the area percentage of new bone and active osteoblasts. Our results agreed with Venkatesan et al[35] who proved that Simvastatin promote bone formation via increase in osteoblasts numbers. Additionally, Yang et al[36] provided evidence of statin efficacy in wound healing therapy to be an efficient medicine in promoting wound healing.

Immunostaining with osteonectin to the rats treated with Simvastatin revealed that the number of active osteoblasts were increased in comparison with Prednisolone group, and it was nearly equal to the control group, these results were in agreement with Laçin et al[37] who stated that Simvastatin promotes the osteoblastic activity and enhances bone deposition. Maciel-Oliveira et al [38] also proved that Simvastatin enhances the production of osteoblasts.

Alam et al[39] suggested that statins elevate the gene expression of bone morphogenetic protein-2 which is a stimulating element for osteoblast differentiation. This clarifies the reason for the increase in the number of osteoblasts in the statin group. The authors also suggested the effect of statins involves enhancement of the osteoprogenitor cells' proliferation and differentiation and improvement in blood supply. Moreover, bone morphogenetic protein is involved in promoting the new bone formation in the extraction socket.[40]

Because of the great importance of angiogenesis in wound healing, one of the mechanisms could explain the positive role of statin in wound healing is that Simvastatin has a maintaining role for endothelium [(41)], which might lead to proper angiogenesis, promoting the wound healing process.

Conclusion

Prednisolone causes delayed healing of the extraction socket in both early and late stages. Simvastatin co-administration with Prednisolone enhances the socket healing by promoting bone formation and activating the osteoblastic differentiation. These findings propose the promising role of statin in post extraction healing.

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