Review Article

The influence of Simvastatin carried by Chitosan nanoparticle on bone regeneration using Masson's Trichrome histochemical stain

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Abstract: Background: Due to the complicated and time-consuming physiological procedure of bone healing, certain graft materials have been frequently used to enhance the reconstruction of the normal bone architecture. However, owing to the limitations of these graft materials, some pharmaceutical alternatives are considered instead. Chitosan is a biopolymer with many distinguishing characteristics that make it one of the best materials to be used as a drug delivery system for simvastatin. Simvastatin is a cholesterol lowering drug, and an influencer in bone formation process, because it stimulates osteoblasts differentiation, bone morphogenic protein 2, and vascular endothelial growth factor. Objectives: histological, histochemical and histomorphometrical analyses were carried out to evaluate the effect of local application of chitosan simvastatin nanoparticles (ChSimN) on bone healing. Materials and Methods: New Zealand rabbits (n=14) were used in this study. Two defects were made: one on the right side (the experimental side) that received ChSimN and the other one on the left side (the control side), which left to heal spontaneously. Seven rabbits were sacrificed after 2 weeks of the experiments, while the others after 4 weeks. Bone samples were taken for histological and histomorphometric study after the sacrifice. Results: The histological study, using both H&E and Masson's Trichrome stain, revealed that the ChSimN group recorded an increased amount of bone formation at both time points. Histomorphometrical analysis recorded a significant increment in bone marrow and trabecular areas in the ChSimN group. Conclusion: ChSimN had a pronounced effect on bone formation.

Keywords: simvastatin, Chitosan, Bone Regeneration, Nanoparticles, Drug Delivery Systems.

Introduction

In routine clinical practice, bone-repair mechanisms are crucial as bone healing is necessary for dental procedures including tooth extraction, dental implant placement. Guided bone regeneration techniques, in addition to many diseases like periodontal disease, tumors, and accidents, may cause bone defects, while pharmaceutical intervention could be useful as a stimulus for bone repair [1].

Chitosan is a natural, non-toxic, and biodegradable biopolymer with great biocompatibility and little immunological responses, and mucoadhesive properties. It is a linear polysaccharide with a random distribution of N-acetyl-D-glucosamine (acetylated unit) and D-glucosamine (deacetylated unit), separated by a (1 → 4) link, with molecular weight (MW) ranging from 50 to 1000 kD and degree of deacetylation (DD) ranging from 30 to 95 percent (MW and DD are the two key chitosan characteristics that have a significant impact on the effectiveness of drug encapsulation). The presence of reactive functional groups, such as amino groups at the C2 position and OH groups at the C3 and C6 locations allow chitosan to be chemically modified through electrostatic interactions, to overcome the drawbacks of using pure chitosan, as well as making chitosan more soluble. Additionally, the number of amino groups reflected in DD values is inversely correlated with the rate of degradation in the body, meaning that the higher the DD value (>85%) the slower the degradation. It is available in diverse forms and has many different applications[2]. Chitosan is frequently employed in drug delivery systems (DDS) because of its

advantageous features, which include enhanced biodistribution, higher specificity, and sensitivity, as well as decreased pharmacological toxicity [3].

DDS made of chitosan has been employed extensively in NPs, hydrogels, and polymeric hydrogel membranes. NPs are the most promising chitosan-based DDS formulations for application in pharmaceuticals. The properties of polymers that have undergone natural or chemical modification are shared by chitosan nanoparticles (ChNPs). Due to the chemical characteristics of chitosan, which make it soluble in acidic aqueous solutions at room temperature without the need for harmful organic solvents or heat, the manufacture of ChNPs can be done under benign conditions [2].

Simvastatin belongs to the statin family and is a lipid lowering drug that acts on the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A by preventing its conversion to L-mevalonate so the pathway of cholesterol formulation is stopped[4]. Statins are suited for bone development stimulation, as they are known to inhibit osteoclast activity while promoting osteoblast development and bone formation. According to clinical in vivo and in vitro investigations, they specifically stimulate the expression of vascular endothelial growth factor (VEGF) and bone morphogenetic protein-2 (BMP-2) and, because of these qualities, they are thought to be a significant therapeutic option for the treatment of osteoporosis, fractures, and bone abnormalities. Statins are more effective on skeletal tissues when they are applied locally, directly to the site of a bone defect, utilizing suitable grafted materials that can produce a regulated and progressive release of the medication[5,6]. The first study to document the in vivo stimulation of bone formation by statin treatment was published by Mundy et al.[7].

A review of previous studies indicated that most studies focused on the effect of simvastatin or chitosan alone on bone healing and inadequate data were available concerning their combined effect. Therefore, this study has focused on evaluating the efficiency of chitosan nanoparticles as a carrier for simvastatin in the process of bone healing and Masson's Trichrome stain was used to detect the degree of mineralization.

Methods

Study design and setting

Fourteen mature male New Zealand rabbits with an average weight of 1.5 to 2 kg and an age range of 6 to 8 months were employed in this investigation. All the ethical guidelines established by the College of Dentistry University of Baghdad were followed in this investigation (reference number 767). The animals were kept at a private animal house where their overall health was monitored. For each rabbit, two intrabony holes with dimensions of 3 mm in diameter and 4 mm in depth were created on the right and left buccal surfaces of the upper diastema. Following a split-mouth design, the experimental material, namely simvastatin chitosan nanoparticles (ChSimN), was used to fill the created defect on the right side while leaving the defect on the left side to heal on its own and treating it as a control (Co). Seven rabbits were sacrificed at 2 weeks and the remaining rabbits were sacrificed at 4 weeks.

Interventions

SimChN was made utilizing the ionic gelation technique that uses TPP as a crosslinking agent[8]. Saturated Sim ethanolic solution (Sigma-Aldrich Chemical Co., St. Louis, USA), 5 mg 0.5ml was added to a 9 ml solution of chitosan (degree of deacetylation ≥90%, Glentham Life Science, United Kingdom) in 1% v/v acetic acid (Thomas Baker, India) (pH 3.5). The TPP (DIDACTIC, Barcelona, Espana) solution 1 ml was then added drop by drop, homogenized for 8 minutes at 12,000 rpm, and stirred for an additional 2 hours at 600 rpm. The generated nanoparticles were separated using centrifugation at 18,000 rpm for 20 minutes.

Surgical procedure

A general anesthetic solution containing 50mg of ketamine HCL (20mg/kg BW) and 20mg of xylazine (2%) (0.2ml/kg BW) was given intramuscularly after all surgical tools and towels had been sterilized. Using a vernier caliper, the natural edentulous space which is located between the centrals and premolars was measured. Two surgical incisions were made exactly in the midline of the space to create a two-sided flap. After utilizing a vernier caliper to determine the necessary depth, a bur stopper was put on the surgical bur[9], a fissure bur (#010) was used after drilling with a tiny round bur size (#010), with the sustaining of aggressive irrigation throughout the drilling process. 0.1 mg ChSimN was weighed and applied in the created bony defect using a spoon excavator, followed by suturing. In order to obtain the bone specimens, the animals were sacrificed using an overdose of anesthetic solution. The premaxilla was separated using surgical disc accompanied by vigorous irrigation. Cutting the bone at least 5mm away from the surgical site allowed for the collection of the samples, which were then fixed in 10% fresh formalin for two days before being decalcified in a solution of formic acid sodium citrate (125cc of 90% formic acid, 125cc of distilled water, 50mg sodium citrate, and 250cc of distilled water)[10]. The specimens were checked using a needle and ease of inserting the needle was used as an indicator of good decalcification[11]. This was followed by dehydration in ascending concentrations of alcohol, clearing in two different changes of xylene, and blocking. Sectioning in buccopalatal direction was performed to produce 4 um sections, half of which were stained with H&E stain and the other half with Masson's Trichrome.

Outcomes

Field emission scanning electron microscopy (Fe-sem) was used to analyze the particle size and form of the chemically created substance, ChSimN. The ChSimN pictures showed uniform spherical-shaped particles with a particle size of approximately 39 nm (Figure 1). Additionally, the material's Zeta potential was evaluated. The ChSimN particles were thought to be stable based on their zeta potential, which ranged from 24.6 to 28.5 mv.

Osteoblasts (OB), osteocytes (OC), and osteoclasts (OCL) were counted and the means determined in histological sections stained with H&E at two different healing intervals (2 and 4 weeks). The National Institutes of Health's ImageJ software was used to quantify the size of the trabecular region (TA), the bone marrow area (BMA), and the number of trabeculae (TN)[12].

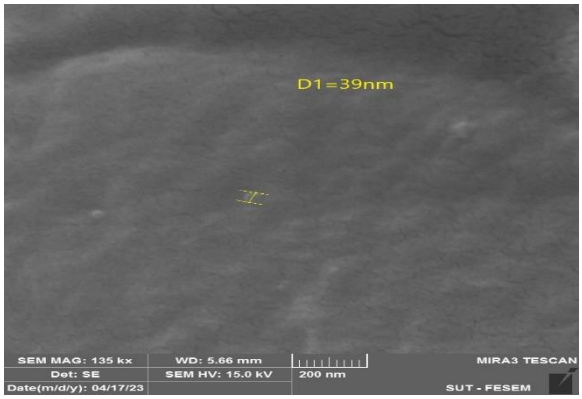

Figure 1: particle size of the SimChN under Fe-sem.

Statical analysis

The Statistical Package for Social Sciences (SPSS) version 26 was used to process the data. The descriptive data analysis was represented by means, standard deviation (SD), and range. In the independent t-test $p < 0.05$ was considered as the statistically significant level in all the inferential data analyses, also paired t-test was used.

Results

Fourteen rabbits were involved in this study. The histological findings on the bony defect in the Co group showed a number of OB along the borders of the bone trabeculae after two weeks of healing. A number of OC were trapped in these trabeculae, with a few OCL on the border, and blood vessels that were filling the BMA (Figure 2A).

While ChSimN exhibited additional new bone trabeculae surrounding the BMA, which was occupied by OC, with many OB and OCL on the boundary (Figure 2B).

The Co group's histological findings after four weeks healing showed dense trabecular bone with small, evenly spaced OC, OB lining the boundary of the trabeculae, and a few OCL had also formed in some locations (Figure 2C). The ChSimN group displayed mature bone that could be distinguished by an extensive amount of osteons, OC grouped in a ring-like pattern around the Haversian canals, and OB lining these canals (Figure 2D).

Histochemical analysis

The sections were stained using Masson's Trichrome stain to evaluate the amount of mineralized bone and the osteoid tissue, Co group after two weeks sections showed a great amount of newly formed osteoid with dispersed mineralized tissue arranged in newly formed trabeculae containing few osteocytes with few osteoblasts on the periphery. ChSimN group showed a greater amount of mineralized tissue with very few amounts of osteoid tissue containing within them a great number of OC with OB on the periphery.

After four weeks, Co showed more thick bone trabeculae with an increase in the amount of mineralization compared to 2 weeks with remaining of a great amount of tissue that is still not fully mineralized (Figure 3C) while ChSimN revealed complete mineralized tissue with osteocytes arranged around the haversian canal and the appearance of few osteoid tissue around the haversian canal (Figure 3D).

Histomorphometrical analysis

The analysis of the mean of the TA and BMA revealed a significant difference between the two groups at both intervals (2 and 4 weeks) (Table 1,2). The ChSimN group had significantly higher TN than Co at 2 weeks (Table 1). Meanwhile, no significant difference was observed in OB, OC, OCL at either interval (Table 1,2).

The intergroup comparison between 2 and 4 weeks showed that Co possessed a significantly higher number of OB TN and BMA at 2 weeks than at 4 weeks, while OC and TA were significantly higher at 4 weeks. ChSimN had significantly higher OC and TA at 4 weeks, while TN and BMA were higher at 2 weeks (Table 3).

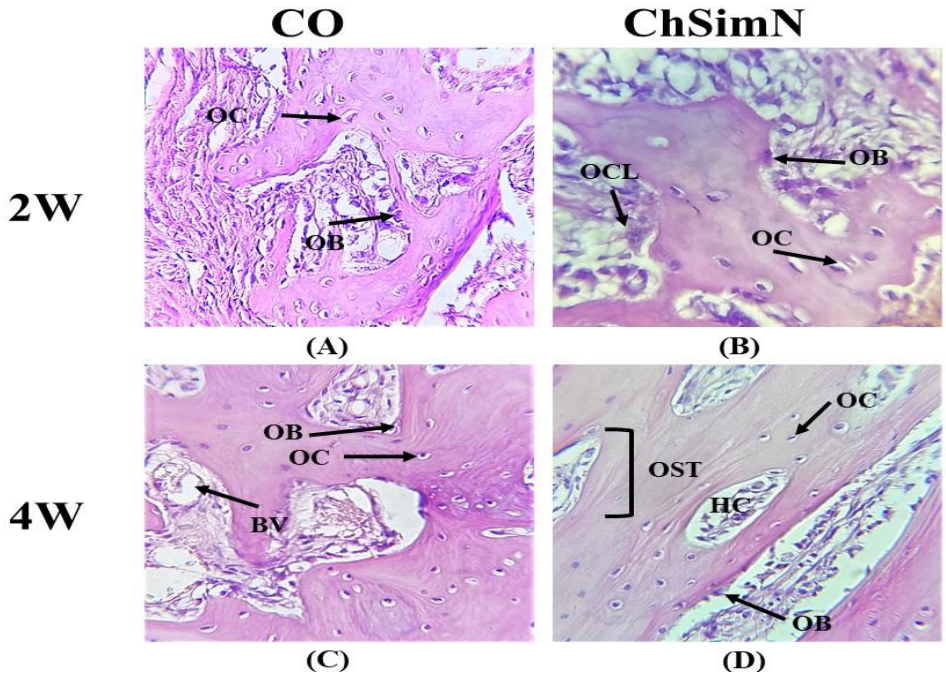

Figure 2: Histological section of the bone defect stained with H&E (A) control group after 2 weeks showing (OB) on the border of the trabeculae and (OC) embedded in the trabeculae. (B) Chitosan simvastatin nanoparticle after two weeks showing (OCL), (OB) on the border and (OC). (C) Control group after four weeks showing (OB), (OC) and blood vessel (BV) in the bone marrow area. (D) chitosan simvastatin nanoparticle after four weeks showed (OC) arranged around haversian canal (HC), (OB) and osteons (OST).H&E X40.

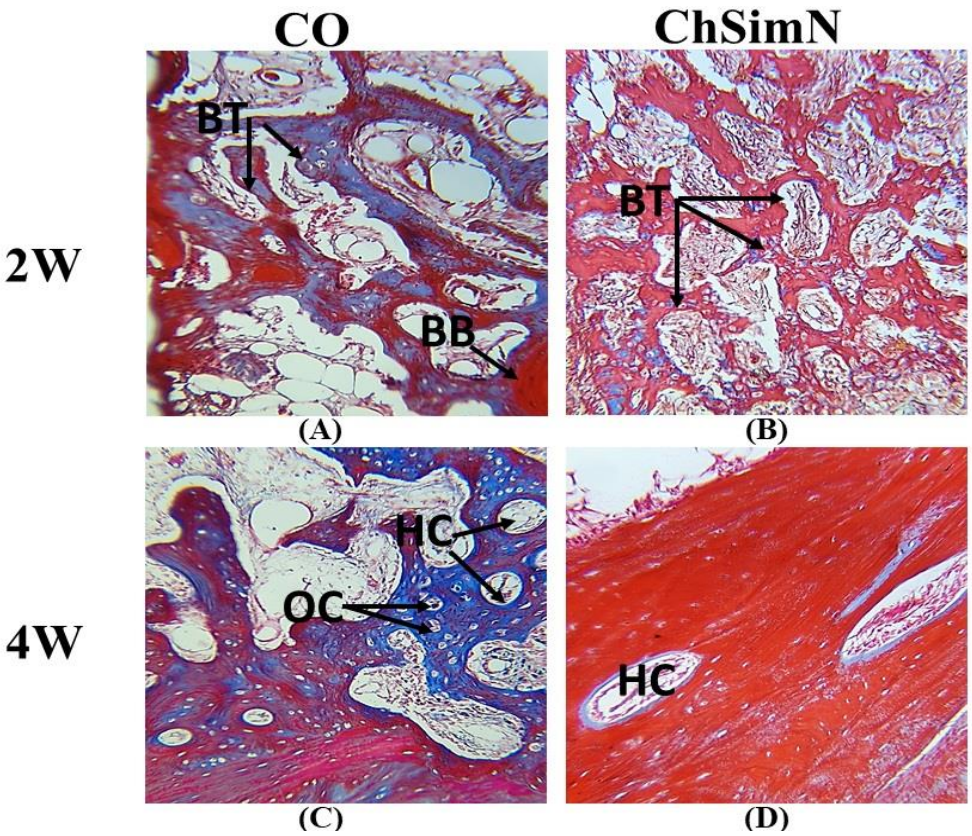

Figure 3: Histological section of the bone defect stained with Masson's Trichrome stain (A) Control group at two weeks showing new (BT) composed of osteoid tissue indicated by blue color and basal bone (BB) which is indicated by red color. (B) chitosan simvastatin nanoparticle group at two weeks showed new (BT) colored in major red and scant blue color indicating the presence of more mineralized tissue. (C) control group at four weeks showed (OC) embedded in new bone and arranged around (HC). (D) Chitosan simvastatin nanoparticle group at four weeks showed (OC) and (HC). Masson's Trichrome stain X10.

Table 1: Comparison in the mean of all bone parameters at 2 weeks.

		Mean ± SD	P-value
Osteoblasts	Co	89.57 ± 9.1	0.337
	SimChN	98.28 ± 20.7	
Osteocytes	Co	54.56 ± 10.2	0.277
	SimChN	61.71 ± 13.0	
Osteoclasts	Co	1.0 ± 0.81	0.207
	SimChN	1.57 ± 0.78	
Trabecular number	Co	11.42 ± 4.0	0.02
	SimChN	17.71 ± 4.7	
Trabecular area	Co	0.79 ± 0.52	0.007
	SimChN	2.04 ± 0.81	
Bone marrow area	Co	1.2 ± 0.5	0.001
	SimChN	2.91 ± 0.6	

Co: control, ChSimN: chitosan simvastatin nanoparticle.

Table 2: Comparison in the mean of all bone parameters at 4 weeks.

		Mean±SD	P-value
Osteoblasts	Co	49.18 ± 12.5	0.159
	SimChN	70.85 ± 34.5	
Osteocytes	Co	76.02 ± 15.7	0.548
	SimChN	80.85 ± 13.4	
Osteoclasts	Co	0.71 ± 0.48	0.338
	SimChN	1.0 ± 0.57	
Trabecular number	Co	3.85 ± 1.3	0.064
	SimChN	10.0 ± 7.2	
Trabecular area	Co	2.78 ± 0.47	0.002
	SimChN	3.87 ± 0.58	
Bone marrow area	Co	0.85 ± 0.36	0.039
	SimChN	0.4 ± 0.37	

Table 3: Intergroup comparison at both durations.

		Co Mean ±SD	ChSimN Mean ±SD
Osteoblasts	2 weeks	89.57 ± 9.1	98.28 ± 20.7
	4weeks	49.18 ± 12.5	70.85 ± 34.5
	p value	0.002	0.102
Osteocytes	2 weeks	54.56 ± 10.2	61.71 ± 13.0
	4weeks	76.02 ± 15.7	80.85 ± 13.4
	p value	0.045	0.019
Osteoclasts	2 weeks	1.0 ± 0.81	1.57 ± 0.78
	4weeks	0.71 ± 0.48	1.0 ± 0.57
	p value	0.356	0.15
Trabecular number	2 weeks	11.42 ± 4.0	17.71 ± 4.7
	4weeks	3.85 ± 1.3	10.0 ± 7.2
	p value	0.002	0.038
Trabecular area	2 weeks	0.79 ± 0.52	2.04 ± 0.81
	4weeks	2.78 ± 0.47	3.87 ± 0.58
	p value	0.001	0.001
Bone marrow area	2 weeks	1.2 ± 0.5	2.91 ± 0.6
	4weeks	0.85 ± 0.36	0.4 ± 0.37
	p value	0.161	0.001

Co: control, ChSimN: chitosan simvastatin nanoparticle

Discussion

The goal of the field of bone tissue engineering (BTE) is to successfully incorporate bone regeneration at the site of the host's defect without encountering any extra difficulties, such as donor site morbidity, immunogenicity, or poor vascularization. A biocompatible scaffold made of bioactive materials that mimic the ECM of bone, osteogenic cells that produce the bone tissue matrix, vascularization that provides mass transport of nutrients and wastes, and morphogenetic signals to direct the cells are the four main components of BTE[13,14]. A biomaterial's objective is to help the body's natural processes of tissue regeneration at the location of a defect and later resorption and replacement over time with newly formed bone tissue[15]. Because of its high biocompatibility, biodegradability, and osteogenic potential, chitosan (CS) has attracted a lot of interest in the development of scaffolds for biomedical purposes[16]. Furthermore, chitosan has been shown in numerous studies to stimulate osteoprogenitor cell differentiation and mineralization, in addition to having antibacterial and hemostatic properties. Due to these characteristics, chitosan has been researched in the fields of guided tissue and bone regeneration[17]. Simvastatin, on the other hand, has the power to upregulate the expression of BMP-2 in osteoblasts by inhibiting the inflammatory cytokine tumor necrosis factor-alpha (TNF-), which suppresses osteogenesis. It also increases the expression of the vascular endothelial growth factor (VEGF) in osteoblasts and decreases the activity of osteoclasts[18]. Rabbits were chosen for this investigation because of their low cost, short lifetime, and convenience of handling and they are considered the most widely used animals for pre-clinical studies[19]. The edentulous gap between the incisors and premolars was chosen as the experimental site because the mechanical tension to which this area is subjected during mastication meant that it would thoroughly reflect the repair of intraoral jaw-bone defects[20].

Histological and histomorphometric analysis revealed that ChSimN had a better effect in improving bone healing compared to the Co at both intervals, 2 and 4 weeks, considering the TA, BMA at both intervals and the TN at 2 weeks which indicated the increased amount of the bone that had formed when ChSimN was applied to the defect site. This finding is compatible with results of Delan and his colleagues (2020) who demonstrated the efficacy of ChSimN in improving the bone healing capability[21], and also agrees with the finding by Xue et al. that a scaffold containing both chitosan and simvastatin had a notable effect on accelerating the healing [22]. This great potential of ChSimN in the bone regeneration process is highly attributed to the combined effect of the two materials, chitosan and simvastatin. Experimentation by Al-Mashhadi and Al-Ghaban had already shown that chitosan had played an outstanding role when combined with β-Tricalcium Phosphate[23]. Chitosan had been proven to have a great effect in accelerating soft and hard tissue healing when mixed with other materials [24] and this notion is strongly supported by Amal et al. who concluded that the use of chitosan as a hemostatic agent had improved bone healing after sternotomy [25]. Moreover, studies have confirmed the effect of simvastatin on bone healing after extraction of the mandibular third molar [26]. Moshiri and his colleagues (2016) concluded in their systematic review that simvastatin had a valuable effect on bone healing [27]. Additionally, the use of chitosan in nanoparticle form to deliver simvastatin locally was found to ensure the sustained release and improve the solubility of simvastatin [28]. Simvastatin was also found to power the bone regeneration capability properties of chitosan by some investigators who attempted to address chitosan's low bone generation capability, especially at early steps of the healing process[29].

The typical sequence of bone healing was seen in all groups at varying rates. This process included the transformation of OB into OC after 4 weeks, when it became entrapped in its matrix. The total amount of OCL also decreased at week 4 and the newly created bony trabeculae fused together, reflecting the increase in the TA and decrease in the BMA.

The mechanism of action is credited to the use of chitosan nanoparticles as a carrier for delivering Simvastatin, with nanoparticles considered the most suitable choice in this system of drug delivery. Chitosan is employed in drug delivery systems (DDS) because of its advantageous features of enhanced biodistribution, higher specificity, and sensitivity, as well as decreased pharmacological toxicity[2,3].

Simvastatin acts in stimulating osteoblast activity while suppressing osteoclast activity, increasing vascular endothelial growth factor (VEGF) expression in osteoblasts through the BMPs' induction of osteoblast differentiation, increased alkaline phosphatase activity, increased expression of bone sialoprotein, osteocalcin, and type I collagen. In addition, Simvastatin has anti-inflammatory activity of lowering interleukin 6 (IL-6) and 8 (IL-8) production as it has the power to upregulate the expression of BMP-2 in osteoblasts and inhibit the inflammatory cytokine tumor necrosis factor-alpha (TNF-), which suppresses osteogenesis[18,30].

The healing intervals may be regarded as a limitation of this study and extending the intervals, potentially to six or eight weeks, would make the results more reliable. Further studies could be conducted to explore the effect on healing of the application of ChSimN to the socket of the mandibular third molar after extraction.

Conclusion

The total amount of bone tissue, including the trabecular and bone marrow area in addition to the trabecular number, was greater when ChSimN was applied at both durations 2 and 4 weeks, which proves that this material had a distinguished and remarkable effect in promoting bone healing compared to spontaneous healing and the efficacy of chitosan as a carrier to deliver simvastatin in a proper manner which expecting a bright future for ChSimN the field of bone tissue engineering.

Conflict of interest

The authors have no conflicts of interest to declare.

Author contributions

MAA and NMHA; study conception and design. MAA; data collection. MAA and NMHA; Methodology. AK and MAA; statistical analysis and interpretation of results. MAA; original draft manuscript preparation. NMHA and AK; Writing - review & editing. Supervision; NMHA. All authors reviewed the results and approved the final version of the manuscript to be published.

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تأثير عقار سيمفاستاتين الذي تحمله جسيمات الشيتوزان النانوية على تجديد العظام باستخدام صبغة ماسون الكيميانية النسيجية ثلاثية الألوان

منى علاء السعيد ، ندى محمد حسن الغبان , Adnan karaibrahimoğlu

المستخلص:

ملخص: الخلفية: نظرًا لأن شفاء العظام هو إجراء فسيولوجي معقد ويستغرق وقتًا طويلاً، فقد تم استخدام بعض المواد المطعومة في كثير من الأحيان لتعزيز إعادة بناء البنية العظمية الطبيعية. ومع ذلك، بسبب الاثار الجانبيه الناتجه من استخدام هذه المواد، مثل الرفض المناعي، تعتبر بعض المواد الصيدلانية أكثر ملاءمة كبديل. الشيتوزان عبارة عن بوليمر حيوي له العديد من الخصائص المميزة التي تجعله من أفضل المواد المستخدمة كنظام لتوصيل الدواء للسيمفاستاتين، وهو دواء خافض للكوليسترول وله تأثير ملحوظ على تكوين العظام لأنه يحفز تمايز الخلايا العظمية وتكوين العظام. البروتين 2، وعامل نمو بطانة الأوعية الدموية. الأهداف: التقييم النسيجي والكيميائي والنسيجي لتأثير التطبيق الموضعي لجسيمات الشيتوزان سيمفاستاتين النانويه على شفاء العظام. المواد والطرق: شارك في هذه الدراسة أربعة عشر أرنباً نيوزيلندياً. تم إجراء عيبين: أحدهما على الجانب الأيمن الذي تلقى المادة التجريبية، والآخر على الجانب الأيسر الذي ترك للشفاء تلقائيًا. تم التضحية بنصف الحيوانات عند أسبوعين والنصف الآخر عند 4 أسابيع وتم أخذ عينات العظام للدراسة النسيجية الهستومورفومتريه. النتائج: كشفت الدراسة النسيجية، باستخدام كل من الهيماتوكسلين ايوسين وصبغة ماسون ثلاثية الألوان، ان عينات العظام التي تم وضع مادة الشيتوزان سيمفاستاتين النانويه عليها اظهرت زيادة في كمية العظم المتكونه وكذلك فان مقاييس مساحة المنطقه العظميه الجديدة ونخاع العظم كانت اعلى في كلا الفترتين الزمنيه. الاستنتاج: كان لـ لمادة الشيتوزان سيمفاستاتين النانويه تأثير واضح على تكوين العظام.