Original Article

Evaluation of the Regenerative Potential of Human Gingival Mesenchymal Stem Cells' Secretome in Rabbits' Tibial Bone Defects

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ABSTRACT

Introduction: Bone defects might result in significant damage and require prolonged treatment durations. Consequently, the treatment of bone defects persists to be one of the greatest obstacles in clinical practice. Ideal bone graft material is not available as each one has its pros and cons, that is why tissue regeneration aims to find an effective modality.

Aim of the Work: This research aimed to evaluate the regenerative potential of human gingival mesenchymal stem cells' secretome (hGMSCs' secretome) in rabbits' tibial bone defects.

Materials and Methods: 22 male New Zealand rabbits were used. One defect in each tibia of 6 mm diameter size was created. Defects were classified into three groups: Non-interventional group (Gp1, the defects were left for spontaneous healing), Collagen group (Gp2, the defects were filled with only collagen sponge) and hGMSCs' secretome group (Gp3, the defects were filled with hGMSCs' secretome loaded on collagen sponge). Euthanization occurred after 3 and 6 weeks. Defect areas were extracted and processed for histological examination and histomorphometric analysis of the percent of the newly formed bone as well as mature bone percent.

Results: Bone defects of Gp 3 showed improved healing of the defects histologically as compared to the other two groups in both time intervals. The histomorphometric analysis revealed an increased new bone area percent as well as the amount of mature bone in group 3 as compared to the other two groups in both time intervals which was statistically significant.

Conclusion: hGMSCs' secretome resulted in bone regeneration in-*vivo* in rabbits' tibial bone defects by increasing both bone formation and maturation.

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Key Words: Bone defect, GMSCs, secretome, tissue regeneration.

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INTRODUCTION

Bone defects are serious pathological conditions that may result from trauma, tumor resection, infection, fractures, congenital malformation, surgery, as well as diseases. Bone defects represent a major clinical problem as they result in socioeconomic burdens and a decrease in quality of life[1]. Bone transplantation comes next to blood transplantation as the second most transplanted tissue[2]. Worldwide 4 million persons require bone replacement or transplantation surgery annually[3].

Autogenous bone grafts are the most effective treatment in clinical practice. However, their drawbacks include donor site morbidity, difficulty in obtaining tissue, and postoperative complications. Allografts and xenografts have the advantage of unlimited availability and no size restrictions which make them an alternative treatment. However, their limitations include the possibility of pathogens transfer and host immune rejection risk[4]. Therefore, bone regeneration that seeks to provide an effective alternative approach for bone healing has been a major goal for clinicians and researchers[5].

Gingival mesenchymal stem cells (GMSCs) are considered a mesenchymal stem cells (MSCs) subpopulation that is isolated from the gingival connective tissues' lamina propria. They were able to differentiate into osteoblasts, chondroblasts, and adipocytes[6]. In addition, GMSCs are easy to be isolated. Into the bargain, they proliferate faster than bone marrow mesenchymal stem cells (BM-MSCs). Over and above that, GMSCs exhibit stable morphology and MSCs characteristics after multiple cultures. GMSCs showed also a significantly higher expression of bone-specific markers compared with BM-MSCs[7].

It is believed that the main effect of MSCs is mediated through paracrine mechanisms rather than their direct differentiation, by releasing different factors. These factors without the presence of the stem cells themselves can cause tissue repair[8]. The word secretome is used to refer to these secreted factors which are found in the culture medium the stem cells are in. Therefore, the name conditioned medium (CM) is used[9]. Proteins, lipids, nucleic acids, and trophic factors such as growth factors, chemokines, cytokines, extracellular vesicles (EVs) and hormones are

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all found in the stem cells' secretome[10], which affect tissue homeostasis by promoting cellular migration, proliferation, and immunomodulation as well as tissue regeneration[11].

On the basis of this, cell-free regenerative medicine methods harnessing secretome from stem cells have developed as an alternative to cell-based therapies[12]. Advantages of using MSC's secretome over stem cells transplantation include; the possibility of malignant transformation upon stem cells usage[13,14], immune rejection of donor cells[15] and stem cells death that occurs after transplantation[16]. Secretome shows less immunogenicity and can be produced at higher rates leading to the production of more economical and practical products for clinical use that can be safely stored[8]. Therefore, this work aimed to evaluate the regenerative power of hGMSCs' secretome in treating bone defects.

MATERIALS AND METHODS

Secretome preparation

hGMSCs were obtained from the stem cells lab, at the National Research Center. For the preparation of hGMSCs' secretome, the cells derived from the 5th passage were cultivated until they reached a confluency of 60%. Subsequently, the culture medium was replaced with DMEM serum-free solution, and the cells were subjected to a period of 24 hours of nutrient deprivation. The collection and concentration of the medium were performed using an Amicon Ultra-15 centrifugal filter machine equipped with an ultracel-3 membrane (Millipore, located in Billerica, MA). The medium was concentrated approximately 40-fold. 10 µL/mL of Halt protease inhibitor cocktail (Thermo Scientific, USA) were added to the collected secretome. By using Easy protein quantitative kit (Bradford) (Thermo Scientific, USA), the protein concentration was measured and adjusted to 3 µg/ml.

Sample size calculation

According to a prior investigation[17], a sample size of 22 rabbits was determined to be enough for detecting an effect size of 0.74, with a power of 0.8, employing a two-sided hypothesis test and a significance threshold of 0.05. The calculation was performed using PS: Power and Sample Size Calculation Software Version 3.1.2, developed by Vanderbilt University in Nashville, Tennessee, USA.

Animals

The experiment used a sample of 22 male New Zealand white rabbits, all of whom were in good health and approximately weighing 2.5-3.5 kg. The study protocol received permission from the Institutional Animal Care and Use Committee (IACUC) at Cairo University, with the assigned approval number CU/III/F/57/19.

Surgical procedure

The surgical procedures were conducted in the animal facility of the Faculty of Medicine at Cairo University. The rabbits were anesthetized with an intramuscular injection of a mixture of Xyaline® 2% (Xyla-Ject®, PhoenixTM, Pharmaceutical Inc.) at a dosage of 5mg/kg and Ketamine Chlorhydrate® (Ketamine®, Amoun pharmaceutical company) at a dosage of 40mg/kg. In 20 rabbits, both tibiae were disinfected with betadine and shaved. A linear incision of 3-4 cm incision was performed just below the head of the tibia followed by skin, muscular tissues and periosteal reflection. A defect of 6 mm diameter size was performed at the medial surface of the tibia using a round surgical carbide bur (Meisinger, Germany) until reaching the medullary canal. The remaining 2 rabbits were exposed to only one defect to adjust the sample size.

Then the defects were evenly and randomly divided between the groups, each group consisted of 14 defects. In the non-interventional group (Gp1), the defects were left untreated. The collagen group (Gp2) received only collagen sponges (collacone®: Collagenic hemostat (cone)- botiss biomaterials- Germany). Finally, the hGMSCs' secretome group (Gp3), received collagen sponges that were previously immersed in 500 µl of previously prepared hGMSC's secretome for 5 minutes. In Gps 2 and 3 the collagen sponge was cut into small equal pieces to accommodate the defects size.

The reflected covering soft tissues were then repositioned and sutured. Immediate postoperative intramuscular injection of systemic antibiotic Amikacin® (Amoun pharmaceutical company) (1.5 mg/ kg) was administrated every 12 hours for 1 week[18]. Cataflam 10 mg/kg (Novartis, Egypt) was administered to alleviate postoperative discomfort. During the postoperative stage, free movement of the animals in their cages was allowed and no external support was used. Half of the animals (10 rabbits with two defects and one rabbit with one defect) were euthanized at 3 weeks postoperatively with an intra-peritoneal injection of an overdose of Ketamine/Xylazine mixture while the rest were euthanized after 6 weeks.

Histological and histomorphometry examination

The tibiae were dissected in order to remove all soft tissues. The defect-bearing regions were fixed for 48 hours in 10% calcium formol solution, rinsed, decalcified in 10% EDTA for 4–5 weeks[19], dehydrated, clarified in xylene, embedded in paraffin wax, and subsequently sectioned at 5-6 m thickness. Hematoxylin and Eosin (H&E) and Masson's Trichrome (MT) stains were applied in accordance with standard histological procedures. The area percent of newly formed bone between the edges of the defect was measured from H&E-stained sections. In addition, areas of mature bone identified by red color in MT-stained sections[20] were measured as well. Measurements were obtained using image J 1.50i (NIH) software.

Statistical analysis

The data collected were analyzed using IBM SPSS advanced statistics, specifically version 18 of the Statistical Package for Social Sciences (SPSS Inc., Chicago, IL). The data were presented in terms of the mean and standard

deviation (SD). The study employed a two-way analysis of variance (ANOVA) to evaluate the impact of various interventions on the percentage of newly formed bone area and the percentage of mature bone area over different time periods. Subsequently, Tukey's post hoc test was conducted to examine potential pairwise differences when the analysis of variance (ANOVA) yielded a statistically significant result. A *p-value* less than 0.05 was deemed to be significant statistically.

RESULTS

Histologic Evaluation

H&E- stained sections

The histological examination of both Gps 1 and 2 after 3 weeks postoperatively revealed the defects with granulation tissue filling their centers. A few newly formed bone trabeculae of woven type were detected extending from the indigenous bone at the edges of the defect (Figures 1A,B). However, in Gp 2, organization of collagen fibrils was detected in an attempt to close the defect area (Figure 1B). While in Gp 3, the defects showed incomplete bony closure with interconnected islands of newly formed bone trabeculae with large fibro-cellular marrow cavities in between them. The new bone was mainly of woven type, but lamellar bone and osteons were detected as well (Figure 1C).

At 6 weeks postoperatively, the defects in Gps 1 and 2 were still filled with granulation tissue in their centers. The amount of newly formed bone increased compared to week 3. The new bone was mainly of woven type with large marrow spaces (Figures 1D,E). Gp 3 defects, on the flip side, were filled with more organized and denser bone with small marrow cavities. Fewer woven bone areas and more lamellar bone as well as osteons were demonstrated (Figure 1F).

MT- stained sections

The immature woven bone was represented by blue color while the red color represented mature lamellar bone. At 3 weeks postoperatively both Gps 1 and 2 showed mostly immature bone with very thin areas of mature bone (Figures 2A,B). While Gp3 the mature bone detected was more (Figure 2C). At 6 weeks postoperatively, the amount of mature bone with its red color was increased in all groups compared to the results that were detected at 3 weeks (Figures 2D,E,F), especially in Gp3. This finding reflected the progressive maturation, faster bone remodelling and enhanced bone healing (Figure 2F).

Histomorphometric analysis

Area percent of the formed new bone

Statistical analysis of the formed new bone area percent showed that the highest mean value was in Gp 3 in both time intervals with a difference that was found to be statistically significant when compared to the other two groups ($P<0.05$). The difference between Gp 1 and 2 was statistically significant in week 6 postoperatively ($P<0.05$) and insignificant in week 3 postoperatively, with Gp 2 being higher (Figure 3).

All groups showed an increase in bone area percent between the two dates that was found to be statistically significant.

Mature bone percent from MT-stained sections

Two-way ANOVA revealed a statistically significantly higher mean value in Gp3 as compared to the other 2 groups within both timelines (3 and 6 weeks postoperatively) ($P<0.05$). Within the 3 weeks' time, pairwise comparison demonstrated a significantly higher mean area percent in Gp3 as compared to both Gp2 and Gp1, while the difference between Gp2 and Gp1 was not significant. Regarding the 6 weeks' time, a significantly higher mean area percent was recorded in Gp3 as compared to the other 2 groups, and also a higher mean value with statistically significant difference was recorded in Gp2 as compared to Gp1 (Figure 4).

An increase in the mature bone percent was detected in all groups between the two dates that again was found to be of statistical significance.

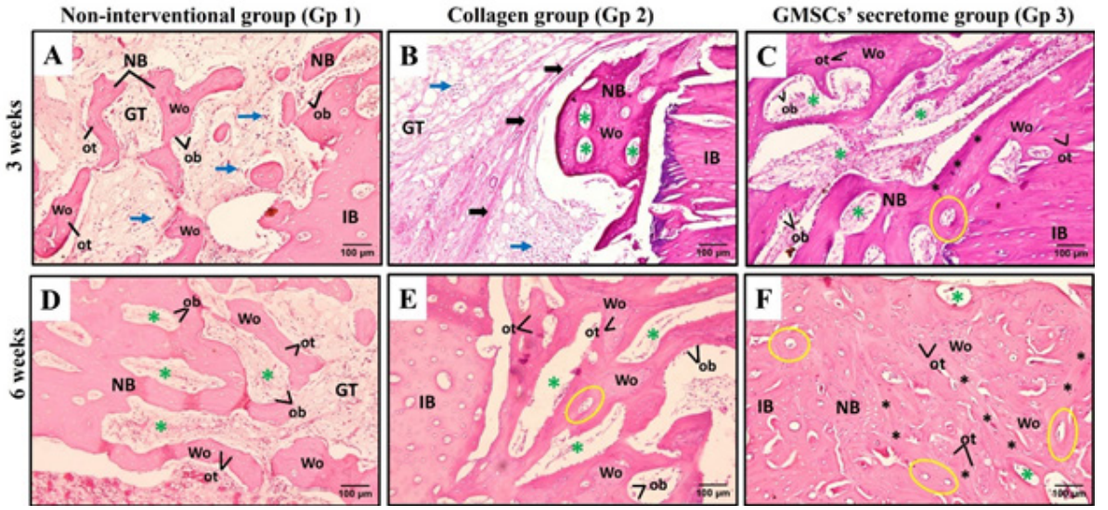

Fig. 1: A photomicrograph of the bony defects; (A, B and C) Gp1, Gp2 and Gp3 respectively at 3 weeks postoperatively, (D, E and F) Gp1, Gp2 and Gp3 at 6 weeks postoperatively showing; indigenous bone (IB), new bone (NB), woven bone (Wo), osteoblasts (ob), osteocytes (ot), marrow cavities (green asterisks), granulation tissue (GT), inflammatory cells (blue arrows), collagen fibrils (black arrows), osteons (yellow circles) and lamellar bone (black asterisks) (H & E stain) (original magnification x200, Scale bar 100µm).

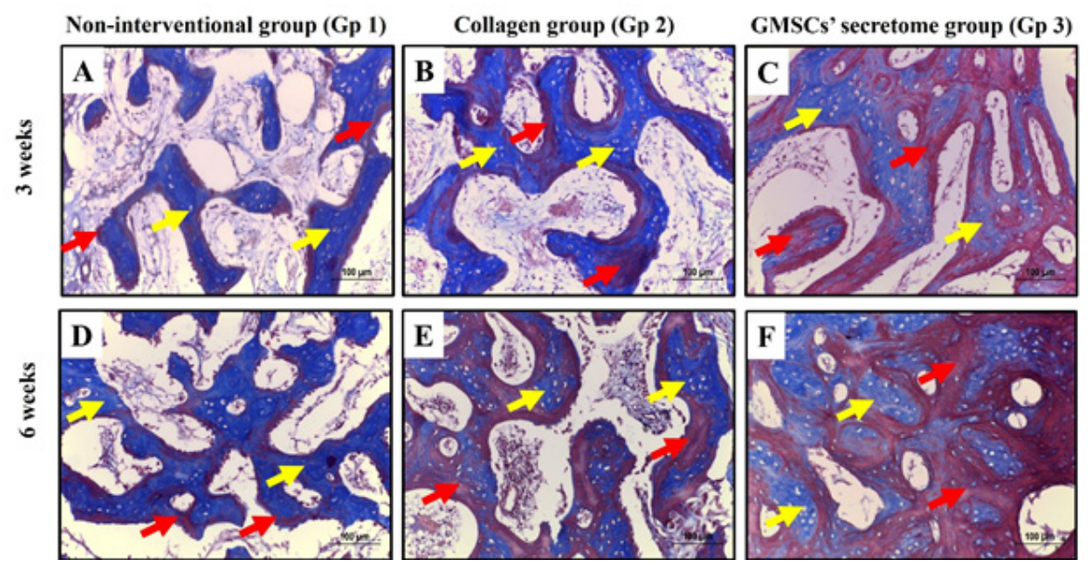

Fig. 2: A photomicrograph of the bony defects; (A, B and C) Gp1, Gp2 and Gp3 respectively at 3 weeks postoperatively, (D, E and F) Gp1, Gp2 and Gp3 at 6 weeks postoperatively showing; blue color indicating the newly formed woven bone (yellow arrows) and red color indicating areas of mature bone (red arrows) (MT stain) (original magnification x200, Scale bar 100µm).

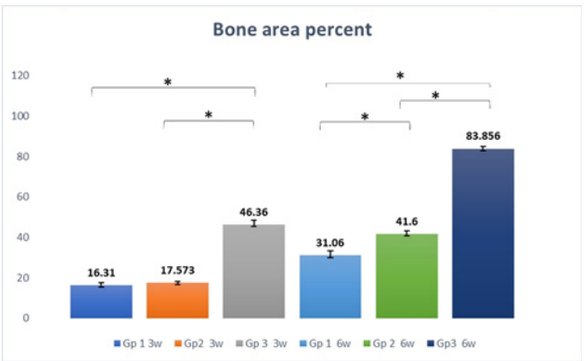

Fig. 3: showing column chart of bone area percent mean value of the 3 groups in both dates with SD error bars, * *P*< 0.05 (ANOVA test).

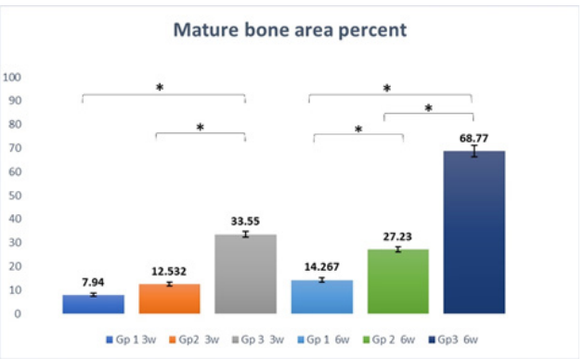

Fig. 4: showing column chart of mature bone area percent mean value of the 3 groups in both dates with SD error bars, * *P*< 0.05 (ANOVA test).

DISCUSSION

Bone regeneration for either medical or aesthetic reasons is highly required for craniofacial bone defects resulting from bone disease, congenital malformations, trauma, severe infection, or tumor resections[21]. Different regenerative modalities have been widely used throughout medical treatment history, including autogenic, allogenic, synthetic grafts and cell-based therapy. Each modality has its drawbacks. In an attempt to overcome these drawbacks, this study used the secretome derived from hGMSCs as a modality representing cell-free therapy. GMSCs' secretome was locally administered into the bone defect in a rabbit model to evaluate its regenerative potential.

Gp 1 and 2 demonstrated delayed healing defects with granulation tissue filling the center of the defects till 6 weeks postoperatively. The newly formed bone trabeculae were mainly of immature woven bone type. In agreement with our findings a control group with 6mm defects size in rabbits' tibiae were left empty and after 6 weeks they revealed only partial defect closure with the formed new bone trabeculae originating from the defect borders[22]. Moreover, another study reported that the control group that received collagen sponge only showed small areas of new bone 8 weeks postoperatively in a 5 mm in diameter calvarial defects in mice. They also reported that the collagen sponge was highly biocompatible, absorbable, and could be used for 3D cultures. This was similar to our finding as both Gp 2 and 3 showed no residual material of the collagen at 3 weeks[23]. In addition to that recently it was reported that the placement of collagen cone (Collacone®) soaked with saline at the osteotomy site in a rat femoral defect model was insufficient for bone healing[24].

In the ongoing study, Gp 3 (hGMSC's secretome group) showed a statistically significant increase in both area percent of the formed new bone and bone maturation in both dates when compared to the other two groups. The unique effect of hGMSC's secretome in bone tissue regeneration results from its components, especially EVs that contain transcripts for different proteins. EVs are capable of modifying cellular phenotypes and functions by controlling multiple cellular pathways. Additionally, they activate different regenerative mechanisms by transferring lipids, proteins, and nucleic acids to their receiving cells[25,26].

Transcriptomic analysis of EVs extracted from hGMSC's-CM showed that they can successfully assist therapeutic methods in regenerative medicine. Their work revealed the existence interleukins-19, -27 and -37 known for their anti-inflammatory effects. Moreover, the analysis showed the presence of different transcripts that encode for bone morphogenic proteins (BMPs) family. Many of them, such as BMP2, 4 and 7, induce ossification and chondrogenesis[27]. They are essential for bone repair and regeneration through the induction of osteoblastic differentiation of MSCs[28,29].

By increasing osteogenic markers genetic expression such as RUNX2, BMP2, and BMP4, EVs produced from hGMSCs planted on 3-dimension poly-lactide scaffold in *vitro* improved the osteogenic differentiation. They boosted the levels of BMP2 and BMP4 in rats with calvarial bone defects in *vivo* resulting in a complete repair of the defects[30]. Additionally, in a rat periodontal defect model, hGMSCs-CM stimulated the development of alveolar bone. According to their findings, the hGMSCs-CM and PDLSCs-CM groups expressed osteogenesis-related markers raising the levels of BSPII and Runx2 in periodontal defects[31].

Moreover, EVs from hGMSCs transcriptomic analysis revealed the presence of WNT4, 10B, 11 and 16, all belonging to the Wnt family[27]. They play an important role in bone development by induction of osteoblast differentiation as well as activation and suppression of their apoptosis[32]. Furthermore, the presence of different vascular endothelial and fibroblast growth factors was detected. These factors have role in different processes, as angiogenesis, cellular differentiation and proliferation[27]. The fact that hGMSCs-derived EVs include all these transcripts demonstrates their significance in bone regeneration. This is confirmed with the histomorphometric analysis of mature bone in the present study which revealed a statistically significantly higher mean value in Gp3 as compared to the other 2 groups within both timelines.

These data explain the better osteogenesis translated in higher bone area percent and bone maturation expressed in group 3 compared to the other two groups. The usage of hGMSC's secretome improved both new bone formation and mineralization processes.

CONCLUSIONS

hGMSC's secretome proved their ability to regenerate bone defects in *vivo* via noticeably increased bone area percent as well as the amount of mature bone. It can be used in bone regeneration and results in acceptable outcomes without using the stem cells themselves, overcoming their drawbacks and drawbacks associated with other bone grafts. hGMSC's secretome provides a promising therapy for safe clinical applications in the future. More studies are required to explore the possibility of clinical applications of hGMSCs' secretome.

CONFLICT OF INTERESTS

There are no conflicts of interests.

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الملخص العربى

تقييم إمكانيات التجديد لسيكريتوم الخلايا الجذعية الوسطية من لثة الأنسان في العيوب العظمية في قصبة عظم ساق الأرانب

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قسم بيولوجيا الفم، كلية طب الأسنان، [١]جامعة القاهرة، [٢]جامعة الجلالة، مصر

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مقدمة البحث: قد تؤدي عيوب العظام إلى تلف كبير وتتطلب فترات علاج طويلة. وبالتالي، لا يزال علاج عيوب العظام أحد أكبر العقبات في الممارسة السريرية. لا تتوفر مادة طعم العظام المثالية لأن كل واحدة لها إيجابياتها وسلبياتها، ولهذا السبب يهدف تجديد الأنسجة إلى إيجاد طريقة فعالة.

الهدف من البحث: يهدف البحث الحالي إلى تقييم الإمكانات التجديدية لسيكريتوم الخلايا الجذعية الوسطية من لثة الأنسان في العيوب العظمية في قصبة عظم ساق الأرانب.

المواد والطرق: استخدم ٢٢ من ذكور الأرانب البيضاء النيوزيلندية الصحية. تلقى كل أرنب عيبًا واحدًا في بحجم ٦ مم في كل قصبة. تم تقسيم العيوب بشكل عشوائي إلى ثلاث مجموعات: المجموعة الأولى المجموعة غير التدخلية (تم ترك الثقوب بدون معالجة)، المجموعة الثانية مجموعة الكولاجين (تم ملأ الثقوب بسقالة إسفنج الكولاجين) والمجموعة الثالثة مجموعة سيكريتوم الخلايا الجذعية الوسطية من اللثة (تم ملأ الثقوب بسقالة إسفنج الكولاجين المحمل بسيكريتوم الخلايا الجذعية الوسطية من اللثة). تم القتل الرحيم بعد ٣ و٦ أسابيع. تمت معالجة عينات العظام وفحصها هيستولوجيا وهيستومورفومتريا لتحديد نسبة العظام المكونة حديثا والعظام الناضجة.

النتائج: أظهرت عيوب العظام في المجموعة الثالثة تحسنًا في التئام العظام من الناحية النسيجية مقارنة بالمجموعتين الأخريين. كشف التحليل النسيجي أيضًا عن زيادة كبيرة ذات دلالة احصائية في نسبة العظام الجديدة بالإضافة إلى كمية العظام الناضجة في المجموعة الثالثة مقارنة بالمجموعتين الأخريين.

الخلاصة: أدى سيكريتوم الخلايا الجذعية الوسطية من لثة الانسان إلى تجدد العظام في عيوب قصبة عظام الساق في الأرانب عن طريق زيادة تكوين العظام ونضجها.