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Evaluation of 3D Computer-Assisted Patient's Specific Scaffold Seeded with Autogenous Stem Cells and Platelet-Rich Fibrin in Alveolar Cleft Repair

Ahmad Abd El Azeem
Oro-dental Genetics Department, Human Genetics and Genome Research Division, National Research Centre, faroukahmad@hotmail.com

Hatem Al-Ahmady
Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine for girls Al-Azhar University, ebnmorr@gmail.com

Mostapha Abdul Rahman
Oro-dental Genetics Department, Human Genetics and Genome Research Division, National Research Centre, nermeenandel@gmail.com

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Evaluation of 3D Computer-Assisted Patient's Specific Scaffold Seeded with Autogenous Stem Cells and Platelet-Rich Fibrin in Alveolar Cleft Repair

Ahmad AbdeL-Azeem [1*], Hatem Al-Ahmady [2], Mustapha Abdul-Rahman [1]

Codex : 11/21.04

azhardentj@azhar.edu.eg

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ABSTRACT

Purpose: Evaluation of the treatment outcome of using patient-specific implantable scaffolds seeded with autogenous bone marrow mononuclear cell layer containing stem cells and platelet-rich fibrin in the treatment of alveolar cleft defect. **Materials and methods:** 12 alveolar cleft defects were divided into 2 groups; group A, six alveolar defects were treated using 3D printed patient's specific implantable PLLA scaffolds seeded with autogenous bone marrow mononuclear cell layer containing stem cells and platelet-rich fibrin. Group B, six alveolar defects were treated with standard anterior iliac crest cortico-cancellous alveolar bone grafting procedures. Clinical and radiographic assessments were done to compare between two groups. **Results:** The results Showed that after 6 months group A was radiographically significantly less dense than the autogenous grafting group and when compared to Bergland's scale there was no significant difference in bone loss after 6 months. **Conclusion:** Although this technique may not be the best choice regarding the density of bone formation, however within the limitations of this study, the results showed that this technique may be a new solution for transformation of the persistent cases of chronic oronasal fistula patients who underwent multiple failed autogenous bone grafting from a state of chronic fistulation to normal alveolar bone augmentation candidates.

KEYWORDS

Alveolar cleft, Stem cells, 3D printing, Scaffold

INTRODUCTION

As early as stereolithography initial development 1980s, multiple other Solid freeform fabrication (SFF) technologies have been emerging. It wasn't before been before 1990s that the first medical SFF was printed using a three-dimensional printing (3DP) machine. Since the 1990s, more commercially available technologies started

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1. Oro-dental Genetics Department, Human Genetics and Genome Research Division, National Research Centre
2. Department of Oral and Maxillofacial Surgery, Faculty of Dental Medicine for girls Al-Azhar University

* Corresponding author email: faroukahmad@hotmail.com

to appear commercially, such as fused deposition modeling, stereolithography (FDM), and selective laser sintering which have been utilized to fabricate scaffolds [1].

Designing and manufacturing, 3D biocompatible structures with spatial complexity is essential in tissue engineering, As can be elaborated by looking through the nature of defects in craniomaxillofacial area caused by cancer, trauma, and congenital defects. Valid rehabilitation of patients suffering from these damages requires functional nerves, vessels, muscles, ligaments, cartilage, bone, lymph nodes, and glands [2].

Poly (lactic acid) (PLA), till now, has been considered as one of the frequently researched and used biodegradable polymer in scientific history. This can be understood from the biocompatibility merit. PLA is a first choice biomaterial for multiple medical and industrial applications substituting other older polymers [3]. Both in vitro and in vivo in depth biocompatibility and efficacy studies have been performed on PLA, granting it U.S. Food and Drug Administration approval in number of various medical and drug delivery devices. As for the present time, PLA is considered as a compatible soft and hard tissue material [4-12].

Alveolar bone grafting is considered a major challenge in the integrated surgical management of oral clefts. Many merits mandate the process of alveolar cleft repair including maxillary arch stabilization, closure of the oronasal fistula, nasal base support, nasolabial soft tissue reconstruction, and creation of bony support for tooth eruption or dental implant placement [13]. Currently, the graft material of choice is autogenous bone graft obtained mainly surgically from the anterior iliac crest as an area of choice, knowing that autogenous bone grafting carries the significant risk of donor-site morbidity and carries an additional operative cost and scars[13].

The accessibility and easiness of gaining bone marrow stem cells caused increasing attention to its use in several studies. The running dilemma of whether the cells are to be inducted into the desired phenotype in vivo or in vitro and about the most suitable scaffold type and also the suitable growth factor and its amount all of this lead to advancement in the vision of the concepts regarding the fundamentals of clinical use of cell-based therapies [14,15].

Platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) are sets of autologous platelet products used to accelerate recovery from injury [16]. Several protocols and developments for the technique of preparations occurred, especially by Choukouron [17-21]. PRP and PRF seem to enhance bone formation in alveolar clefts when admixed with autologous bone chips harvested from the iliac crest as it leads to early bone formation, increased bone density, low infection rate, and least postoperative complications [22]. This study is concerned with the evaluation of the treatment outcome of using patient-specific implantable scaffolds seeded with autogenous bone marrow mononuclear cell layer containing stem cells and platelet-rich fibrin in the treatment of alveolar cleft defect.

SUBJECTS AND METHODS

The study comprises of a randomized controlled randomized controlled clinical trial on sample size consisting of 12 alveolar cleft defects. All protocols of the study were approved by the National Research Centre and Al-Azhar University and the Helsinki Declaration guidelines were strictly followed. All patients and their parents or guardians were informed about the nature of the procedures and possible complications that might occur. Informed consent was obtained from all subjects involved.

Patients included in the study were randomly selected from the outpatient clinic of the Department of Oral and Maxillofacial Surgery Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt and outpatient clinic of Oro-dental Genetics Department, Human Genetics Division, National Research Centre, Cairo, Egypt. Patients suffering from

alveolar cleft defects of both genders were included with the age range from 8 to 29 years old. Patients suffering from any systemic diseases, any known allergies, alcoholism, myasthenia gravis, pregnancy, or lactation were excluded from this study.

Complete dental and medical histories were obtained for all patients. Examination was performed for the residual defects at the central facio-lingual region of the alveolar bone graft site (i.e., a qualitative assessment of the amount of facio-lingual bone deficiencies at the central bone graft site between alveolar crest and root apex levels of the teeth on either side of the cleft).

Patients were then divided into 2 groups; group A, six alveolar defects were treated using 3D printed patient's specific implantable scaffold seeded with autogenous bone marrow mononuclear cell layer containing stem cells and platelet-rich fibrin. Group B, six alveolar defects were treated with standard anterior iliac crest cortico-cancellous alveolar bone grafting procedures.

Scaffold design

Multislice computerized tomography images were taken preoperatively for every patient obtaining DICOM data files which were used to create 3 dimensional printed scaffolds. The acquired data sets were then subsequently converted into stereolithography Interface Format (STL) files using Mimics version 10 Software (Materialise, Leuven, Belgium). The defects were mirror imaged and or segregated to produce STL models, via Rubick 3D, Egypt. Then the STL files were printed into patient-specific scaffolds with at least 65% porosity and 30% of its weights nano-hydroxyapatite powder (Sigma-Aldrich Co. LLC) using; Rubick 3D, Egypt, PLLA Implatent Line "pure polylactic filament".

The scaffolds were printed on Rubick bio FDM 3D printer then the scaffolds were sterilized through exposure to 35 kilos gray of radiation According to the standards of Egyptian Atomic Energy Authority (Fig. 1) [23-26].

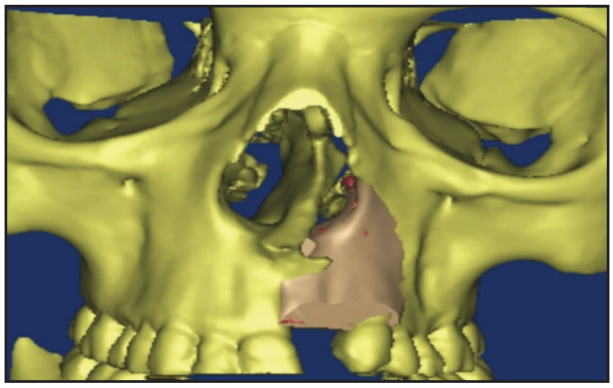

Figure (1): Scaffold designing and Computer planning

Separation of the mononuclear cell layer containing the bone marrow stem cells

In group A, under general anesthesia, iliac crest bone marrow aspirate was primarily collected using a gauge 13 bone marrow trocar, a puncture was made penetrating the anterior superior iliac spine with a watch wind movement. 20 ml of bone marrow aspirate was obtained in a heparin-treated 50 ml syringe . A minor repositioning of the trocar was done for every 10 ml to access different areas of cancellous bone marrow through the same cortical access hole.

Separation of the mononuclear cell layer containing the bone marrow stem cells:

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The aspirate was then centrifuged to separate the BMMNCs using the density gradient separation method. Briefly, Ficoll® Paque Plus (GE Healthcare, Buckinghamshire, UK) bottle was inverted several

times to ensure thorough mixing and 3ml of the media was added to a centrifuge tube. The bone marrow aspirate was diluted (1:1) with saline and then carefully layered onto the Ficoll gradient and then centrifuged at 2000 rpm for 20 minutes at room temperature using a multi-speed 4000 rpm vertical rotor.

The upper layer containing plasma and platelets was then collected using a sterile syringe, leaving the mononuclear cell layer undisturbed at the interface. The layer of mononuclear cells was then carefully transferred to a sterile centrifuge tube using a sterile syringe Cell isolate was then washed using balanced salt solution and centrifuged at 2000 rpm for 10 min, the supernatant was then removed, and the cell pellet was resuspended in 3 ml of the previously obtained platelet-poor plasma containing 01.ml of 80mg gentamycin and 01. ml of 8 mg dexamethasone .

Platelet-rich fibrin preparation

For group A, 20 ml of fresh venous blood was drawn from each patient and divided into two 10-ml glass Vacutainer test tubes without adding any anticoagulant and centrifuged immediately at 3000 rpm for 20 minutes. The resultant product consisted of the following three layers: the upper layer which was the acellular platelet-rich plasma (PRP); the middle layer which was the nontransparent platelet-rich fibrin (PRF) gel while the last layer was the RBCs Platelet-rich fibrin gel was picked up from the tube and clipped out using scissors to make it ready for use.

BMMNCs seeding on the 3D-printed scaffold

The isolated BMMNCs, in group A, were then seeded on 3D printed scaffolds, by injecting them through the scaffold using a 5ml syringe with gauge 14 sterile metal needle obtained from a cannula set. The seeded scaffold and the previously prepared two PRF pieces were put in a small dish and dusted with 1-2 grams of nano-hydroxyapatite powder according to the scaffold [18, 27, 28].

Surgical procedure

For group A, exposure of the defect area was carried on using a No.15 blade scalpel to incise the papillae corresponding to the teeth proximal and distal to the defect through a gingival incision. Also, vertical and horizontal incisions were made in the region of the defect allowing mobilization of sufficient mucosa in order to close the mucosa palatally and buccally. Afterward, dissection was performed subperiosteally using a mucoperiosteal elevator exposing the alveolar defect, the piriform aperture, and the anterior maxilla. The periosteum was then cut with surgical scissors to allow the mobilization of the tissues toward the cleft easing the tension-free closure. Careful dissection of both nasal and oral mucosa was performed separating the layers for proper anatomic reconstitution by latter nasal mucosa closure using interrupted inverted resorbable sutures. Oral mucosa closure was carried out afterward using everting interrupted resorbable sutures. The BMMNCs seeded scaffold with hydroxyapatite-PRF composite was packed into the alveolar cleft defect; fixed in place using membrane tag screws 10-16 mm length and, 1.5 mm diameter and closed with gingival muco-periosteal flaps. The two pieces of the PRF were placed; one towards the closed nasal side and the second towards the oral side, the closure of the papillary gingival incision was done via advancement of the buccal gingiva toward the cleft. The ratio of the total transplant to the defect was (1.25:1) (Fig. 2).

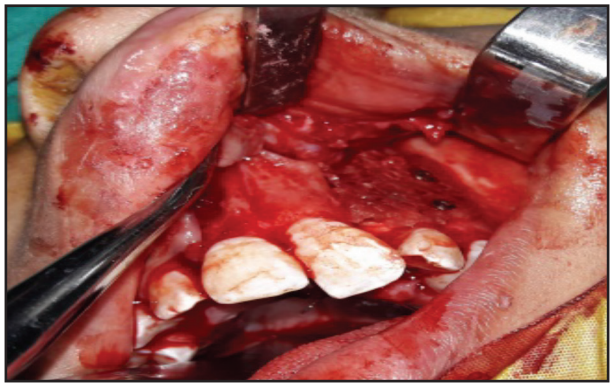

Figure (2)**:** Showing Scaffold fixed in place.

For group B, the intraoral surgical procedure was the same as that of group A. However, at the donor site, below the superior iliac crest, a 3-cm incision was made. When the iliac crest was exposed a piece of the cancellous bone was harvested which was then cut into small particles. These bone particles were used as a bone graft for the defect. After grafting, the cleft was then closed as described previously with the same ratios.

Postoperative care instructions; were clearly given to all patients and/or their guardians. These instructions included; ice packs, strict oral hygiene measures, eating soft food, mouth rinsing after every meal with an antiseptic mouthwash, analgesics, anti-inflammatory and prophylactic antibiotics were also prescribed.

Clinical assessment

Complications in both the donor site and alveolar cleft regions were observed. Postoperative follow up was carried out, at 1 day, 1 week, 3 weeks, 3 months and 6 months. Clinical evaluation included hip site infection, hip site scaring, wound dehiscence, bleeding, and swelling, exposure of graft, persistent Oro-nasal fistula, and postoperative pain.

Clinical criteria and complications were all measured by a well-trained clinician. Qualitative assessment of the amount of facio-lingual bone deficiencies at the central bone graft site between alveolar crest and root apex levels of the teeth on either side of the cleft was done. Pain measurements were done by a visual analog scale. The presence or absence of severe edema, signs of infection, dehiscence or severe tissue necrosis was detected. Moreover, the overall satisfaction with the result of the procedure was evaluated with a scale from zero to ten where one is the least satisfaction and ten is the most. A pain evaluation score; was composed of numerical scale score reporting pain intensity. The scores were recorded on a scale of 0 to 10 with ''0'' as ''no pain'' and ''10'' as ''bad as it could be''. This survey was used at follow-up time points, including postoperative day 1, week 1, week 3, month 3, and month 6.

Radiographic assessment

Radiographic assessment of the grafted sites was done using multislice computerized tomography, before grafting and six months later using (RadiAnt DICOM viewer, Poznań, Poland) software version 5.5, to asses, median height of the formed bone from the floor of the nose to the alveolar ridge, median bucco-lingual depth of the formed bone, median mesio-distal width of the formed bone, the remaining grafted bone using Bergland's classification, and the average density of the formed bone along with random different points of these readings.

Statistical analysis

All data were subjected to statistical analysis using descriptive and paired sample t-tests. Differences at $p < 0.05$ were considered significant. Calculations were performed using the SPSS statistical package (SPSS 17, SPSS Inc., Chicago, USA.)

RESULTS

A total of twelve patients were included in the study; eight females and 4 males. Overall the age of the patients ranged from 12 to 26 years old and the average volumes of the bone defects were 1070.5mm^2.

Evaluation of the whole surgical procedure

In group A, the operating time was calculated from the time of bone marrow aspiration to the end of the surgical procedure. The cellular processing for the isolation of BMMNCs was considered and the surgical procedure was included. Aspiration of the autologous bone marrow took 10-15 minutes. Isolation and purification of the mononuclear cells by centrifugation took about 40 min. PRF preparation took about 20 min. Compared to group B, the operative procedure wasn't prolonged because the BMMNCs isolation and PRF preparation run parallel to the surgery by additional trained personnel. On the contrary, the surgical procedure in group B was relatively longer than in group A.

Clinical assessment

In group A, no donor site complications were noted in all patients. None of the patients had sensory disturbances, infections, or problematic scarring. The patients were normally moving after the bone marrow aspiration next postoperative day. At the surgical site, all patients showed no excessive bleeding; there was no wound dehiscence and graft exposure except in two cases with no signs of infection. One of these cases lost part of the graft after 6 weeks with no signs of infection or oro-nasal communication while the other healed spontaneously. Successful healing left no fistula or oronasal communication in all patients.

Primary healing was observed in five out of six patients, four patients showed good soft tissue healing in the first week and one in three weeks and the remained case healed after 3 weeks; was no graft rejection in all cases or any sign of infection. Inflammation was noticeably minimal in all cases. Moreover, swelling was severe only on day one postoperatively in four out of six cases but became moderate on day three and totally subsided by the end of the first week, in two cases the swelling was moderate for 1week. Pain was notably minimal in all patients. On day one postoperatively, it was described by all patients as an average of seven on the pain scale. It was then dropped to five on day three and then to two after one week.

In group B, some donor site complications were noticed; pain and swelling were persistent for at least 3 days in all patients. Patients had to be hospitalized for at least 1 day before they were able to move normally. At the surgical site, failure of the oro-nasal fistula repair was noticed in one out of six patients. The overall soft tissue healing was average Moreover, swelling was severe for 3 days and became moderate after one week. It took almost 10 days for the swelling to totally subside in all patients. Pain was described by all patients as an average of eight on the pain scale for the first 3 days postoperatively. It was then dropped to five on day seven and then to two after one week.

Radiographic Bone assessment:

Bone assessment was performed through digital examination of the sites of alveolar clefts, alveolar ridges were continuous and hard with normal mucosal covering, Alveolar bone reunion was achieved in the majority of patients in both groups. In group A, Using RadiANT densitometer 9 readings of density were taken along the different lines of measurements of height, width, and depth, the mean density for each patient was then estimated, the average bone density was 163 Hounsfield unit (HU) which is equivalent to D4 bone type according to Misch's classification.

In group B, the average bone density was 283.33 which also equivalent to D4 bone type and significantly higher than group A (Table 1).

Table (1): *Showing the statistically significant difference between the density of the newly formed bone in group A (PLLA) and group B*

Bone Graft	**PLLA**	**ACBG**	**P-value**
	Mean (SD)	Mean (SD)	
	164.33 ± 59.91	283.33 ± 69.19	0.01*

** Statistically significant p-value ≤ 0.05*

When assessed using the Bergland method; by evaluating the Multi-slice C.Ts and pantomograms of the patients, it was found that alveolar bone reunion was achieved in the majority of patients in both groups.

In group A, the average percentage of bone loss was 50.83%, which can be classified as Type III on the Bergland scale at the 6 months follow up. In group B the average percentage of bone loss was 39.17 %, which can be classified as Type II on the Bergland scale at the 6 months follow up At the end of the six months follow-up period, though the difference in bone level loss between the two groups was not statistically significant, CT scan showed bony bridges in all patients. Bone graft consolidation

was apparent, however, differentiation between the newly formed bone and native bone at the cleft side was possible due to different bone densities and incomplete bone remodeling of the newly formed bone.

DISCUSSION

This work tackled ;since as early as its design phase the problem of critical size alveolar defects with relatively poor quality soft tissue due to previous multiple corrective surgical interventions defining the hardship journey of achieving normality of function via restoration of bone continuity functionally and esthetically in patients with cleft deformity. Authors here introduce a novel strategy for alveolar cleft reconstruction by combining BMMNCs seeded on a 3D computer-assisted patient's specific scaffold seeded with autogenous Stem cells and platelet-rich fibrin in alveolar cleft repair with PRF and Nano hydroxyapatite [29,30].

Being conducted on outpatients from cleft lip and palate referral centers, and due to ethical regulations regarding clinical research and patient right of informed consent ,most of the cleft patients in this study who joined based on detailed informed education about the procedure had relatively problematic history repairing their boney defects with iliac bone grafts ,with even multiple failures and chronic fistulas combined with donor site morbidty and scaring , all of these patients were seeking an alternative treatment for their cases not involving donor site and with a main aim of achieving oronasal fistula repair and aesthetically enhanced outcome [29,30]. The current study showed that Pre designing the shape and size of the grafts and even the fittability preoperatively using 3d printed models enhanced the overall surgical design and manipulation of soft tissue as it enlighten the surgical team antrospectively about the surgical scenario [2,3,9,16].

Also the long term stability of the implants lead to desirable long term endured results regarding the soft tissue of the nose and alar base yet this specific point might need further specific analysis and investigations to gain more solid grounded conclusion [6,13,30].

Evaluation method of the outcome of grafting procedure by relatively controlled reproducible method yet having surgeons' acceptance and usability is still a challenge, the current study combined a widely accepted gold standard method (Bergland scale) together with the newly standard computed tomography in order to enhance assessment and also evaluate the density of the grafted area, rendering deferential surgeon's logical and translational results in relation to quantitative and qualitative aspect ,also it could be revisable with fair amount of reproducibility [13,29,30].

By using this combined method results demonstrated relatively better over view on the change of both quantity and density of the residual bone over the follow up time, a similar assessment although without measuring densities was carried by other studies [30].

In bone tissue engineering, the use of autologous BMSCs, combined with a biodegradable scaffold, is the ideal technique. BMSCs have paracrine and immunomodulatory abilities.[31] For instance; a wide range of angiogenic factors was detected in the secretome of MSCs, which may be important in bone regeneration [32].

Moreover, the immunosuppressive effect of BMSCs has been reported in several studies that may prevent undesired immune reactions and provide support to repair injured tissue. Bone marrow mononuclear cells (BMMNCs) consists of a heterogeneous population of cells, including BMSCs, hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), hematopoietic progenitor cells (HPCs), monocytes, neutrophils, adipocytes, macrophages, and platelets. Earlier studies have documented therapeutic effects of BMMNCs in ischemic brain injury, myocardial infarction, spinal cord injury, and osteonecrosis of femoral head

Regarding bone regeneration, it was demonstrated that BMMNCs combined with tricalcium phosphate (TCP) had denser bone formation compared with autograft in an ovine lumbar spine fusion model[31,32].

In the current study, density gradient separation method technique was used to harvest BMMNCs. This method has been used to enrich the isolated mononuclear cell fraction and has been an essential part of several clinical procedures [29,30].

The density gradient separation/immediate transplantation method has several advantages when compared with in vitro expansion method. Foremost, no invasive enzymatic treatment is used, so, the isolated cells are considered minimally manipulated. Moreover, an immediate autologous transplantation of BMMNCs avoids complications related to the quality of the transplanted cells such as reduced viability, dedifferentiation or mutations that may associate in vitro-culture. In addition, the risk of contamination is reduced by decreasing the time of cell handling during culture [13,30].

Also within the scope and design of this study; it was found that the amounts of bone marrow previously reported although being practical but might be increased relatively with the age and the general health condition of the patient yielding more regenerative cells [11,30].

Moreover, we used Nano-hydroxyapatite (HA) powder, it was used to induce bone regeneration as it is biocompatible, osteoinductive, and even has an angioconductive potential compared with coarser crystals. Additionally, it was shown that BMSCs prepared with nano HA have greater cell viability and proliferation ability compared with traditional HA. Therefore, BMSCs/nano HA composite may contribute to bone regeneration [13,29,30].

One more component we used in our proposed technique and that was the PRF. We used PRF as a source of growth factors. PRF is a platelet concentrate, basically used to enhance soft and hard tissue healing [30].

PRF has been broadly used in plastic and maxillofacial surgery and many tissue engineering models. Its advantages include; ease of preparation, application, and absence of chemical alteration. A previous study showed that PRF growth factors were released in a time dependent manner; this releasing pattern may result in prolonged biological effects, which induce bone regeneration. Moreover, the fibrin network of the PRF in the regenerative site enables cell migration, particularly endothelial cells which are essential for the angiogenesis, neurogenesis, vascularization, and graft subsistence [11,13,29,30].

Moreover, in group A, postoperative pain and swelling decreased rapidly during the first week; while excessive bleeding was not detected except in two cases and we attribute that to the concentration of heparin used during the initial bone marrow aspiration. On the other hand, swelling and pain in group B patients was persistent for longer time [29,30].

CONCLUSION

Although this technique may not be the best choice regarding the density of bone formation, however within the limitations of this study, the results showed that this technique may be a new solution for transformation of the persistent cases of chronic oronasal fistula patients who underwent multiple failed autogenous bone grafting from a state of chronic fistulation to a normal alveolar bone augmentation candidates.

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