

The effects of the stem cell on ciliary regeneration of injured rabbit sinonasal epithelium

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Abstract Defects in mucosal healing after sinonasal surgery cause infection, scar formation causing obstruction, relapse of the disease within a shorter period and revision surgery. The present study aimed to create a functional ciliated epithelium using a stem cell and stem cell sheet of adipose tissue origin and to show such regeneration ultra-structurally on experimentally injured rabbit nasal epithelium. This was an experimental animal study and basic research. A total of 18 white New Zealand rabbits were divided into three groups. The medial wall of the maxillary sinus of the subjects was peeled off bilaterally. No additional procedure was applied to the subjects in Group 1. In Group 2, adipose tissue-derived mesenchymal stem cell was implanted on the wound edges of the subjects. In Group 3, a stem cell sheet of three layers was laid onto the defect area. All subjects were killed after 3 weeks. The presence of the stem cell stained with bromo-deoxyuridine

was assessed with a light microscope, whereas cilia density, ciliated orientation and cilia structure were evaluated with a scanning electron microscope. Ciliary densities in Group 2 and Group 3 were statistically superior compared to the control group ($p < 0.001$, $p = 0.007$). Cilia morphology in Group 2 and Group 3 was also better than the control group ($p < 0.01$, $p = 0.048$). Ciliary orientation in Group 2 was scored highest ($p < 0.01$). The ratio of BrDu-stained cells was observed to be 27% in Group 3 and 8% in Group 2. Sub-epithelial recovery was observed to be better in Group 3. Adipose tissue-derived mesenchymal stem cell increased the healing of the injured maxillary sinus mucosa of the rabbits in terms of cilia presence, density and morphology regardless of the implementation technique.

Level of evidence NA

Keywords Adipose tissue-derived mesenchymal stem cell · Sinus surgery · Stem cell sheet · Rabbit · Sinonasal epithelium

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Introduction

The purpose of functional endoscopic sinus surgery (FESS) is to restore sinus aeration by preserving the mucociliary clearance and to preserve the physiological functions of the paranasal sinuses. Sinonasal cavities, especially maxillary and frontal sinuses have different mucociliary transport paths which should be protected [1]. FESS is a minimally invasive procedure and is based on the removal of the pathological mucosa and bone and the preservation of as much normal tissue as possible. The success rate of FESS has been reported to be between 80 and 90% [2]. However, defects in mucosal healing after surgery causes infection, scar formation causing

obstruction, relapse of the disease within a shorter period and revision surgery.

There have been few studies on the post-surgical regeneration of paranasal sinus mucosa, and how mucociliary clearance and sinus functions are affected [3–5]. Such studies have shown that irreversible modifications and decreased mucociliary clearance in regenerated sinus mucosa cause disruption to the sinus functions [4, 5].

The effect of stem cells on wound healing through the secretion of cytokines and growth hormones and the activation of other stem cells in the tissue is well-known [6, 7]. Although bone marrow-derived mesenchymal stem cells have been considered the main source of stem cells for many years, adipose tissue-derived stem cells are easily derived, are plentiful and have higher proliferation and differentiation potential than bone marrow and cartilage-derived mesenchymal stem cells [8].

Beyond the conventional method where stem cell suspension is implanted onto the defective area, the development of stem cell technology has enabled the use of stem cell sheets in some areas such as the skin [9], corneal epithelium [10], and urinary bladder epithelium [11] to provide adherence of appropriate size, width and shape onto the defect area. Furthermore, the application of a stratified stem cell sheet created without the use of a scaffold has been reported to provide better original tissue healing and less fibrosis [12].

The aim of the present study was to maintain maximum nasal functions through the recovery of nasal mucosa injury with minimum scarring and fibrosis together with rapid formation of ciliated epithelium using stem cells and stem cell sheet.

Material and method

Approval for the study was granted by the Ethics Committee of the Ministry of Health Ankara Training and Research Hospital. The study was conducted in the Experimental Animal Research Laboratory of the Ministry of Health Ankara Training and Research Hospital.

Derivation and characterization of mesenchymal stem cells from adipose tissue

The inguinal region of two New Zealand rabbits were shaved and cleaned under deep anesthesia with xylazine and ketamine. The adipose fat pad was removed, and then the adipose material was placed in Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Belgium) and cut into small pieces using the explant method. These small pieces were placed into T25 flasks and kept in a 5% CO₂ incubator for 15 min. A medium of 20% fetal bovine serum

(Lonza, Belgium), 2% of L-Glutamine (Lonza, Belgium), 1% of Penicillin Streptomycin, Amphotericin (Biological Industries, Israel) and 77% of Dulbecco's Modified Eagle Medium (DMEM) (Lonza, Belgium) was added. The medium was replaced every three days. Passage was performed by considering cell development after 15 days. At the end of the second passage, the cells were identified first in a flow cytometry device (FACS Aria III, USA) with CD11b/c (BD, USA), CD49 (BD, USA), CD44 (BD, USA), CD45 (BD, USA) markers for the characterization of mesenchymal stem cells (Fig. 1). The cells after second passage were differentiated as adipocyte, osteocyte, chondrocyte cells through differentiation methods (Fig. 2). Cell count and vitality were observed using a Countess[®] Automated Cell Counter (Invitrogen, USA) device. The identified adipose tissue-derived stem cells were stained with bromo-deoxyuridine (BrDU) after the second passage to be able to monitor the stem cells after implantation to the rabbit sinus mucosa.

Mesenchymal stem cell BrDU staining method

A solution of 10 μM BrDU per 1 cc was prepared. The 10 μL BrDU solution was added to each cc to form 2.10⁶ cells in 1 cc. The solution was left for incubation for 2 h. At the end of the study, BrDU (+) cells were determined by averaging the BrDU (+)/H&E (+) staining ratios in three areas 20× magnification on each slide.

Stem cell sheet formation method

The stem cells derived through the explant method were planted on a heat sensitive culture container to create the stem cell sheet. A second cell sheet was planted when the first sheet covered 70% of the culture container and this procedure was continued until 3 sheets were formed. This procedure was performed by staining the cells with BrDU during each cell plantation. Thereafter, the heat-sensitive culture was taken out of the incubator and the cells were removed from the culture containers in sheets.

Surgical procedure

The present study used a total of 18 young adult, white New Zealand rabbits, each 15–18 weeks old and weighing 2.6–3.6 kg. The rabbits were kept under the same laboratory conditions with the same nutrition during the study period. The histopathological examination of the tissue samples derived from the rabbits was performed by the same pathologist. The electron microscope examination was performed by two researchers from the Anatomy Department of Hacettepe University. The photographs were taken with a Nikon Coolpix L330[®] camera.

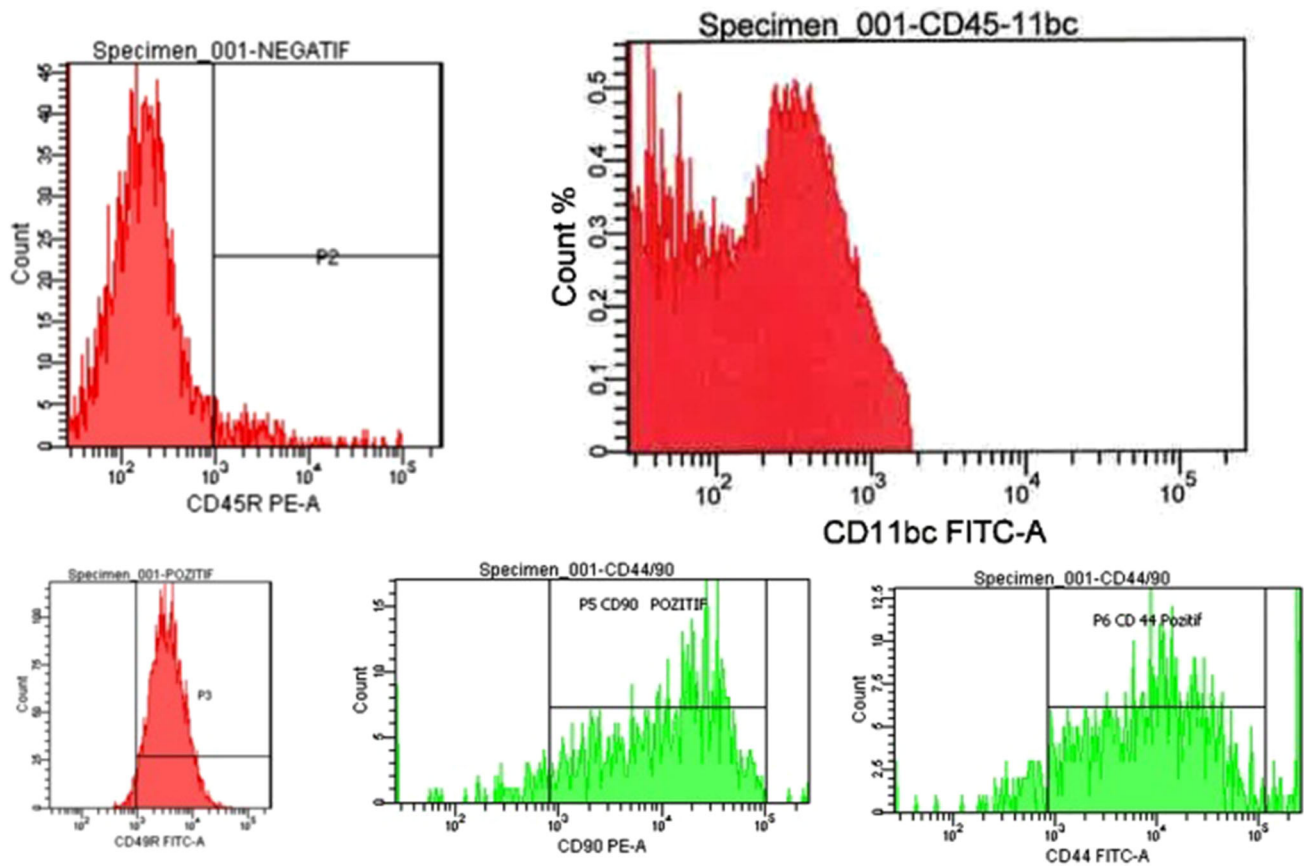
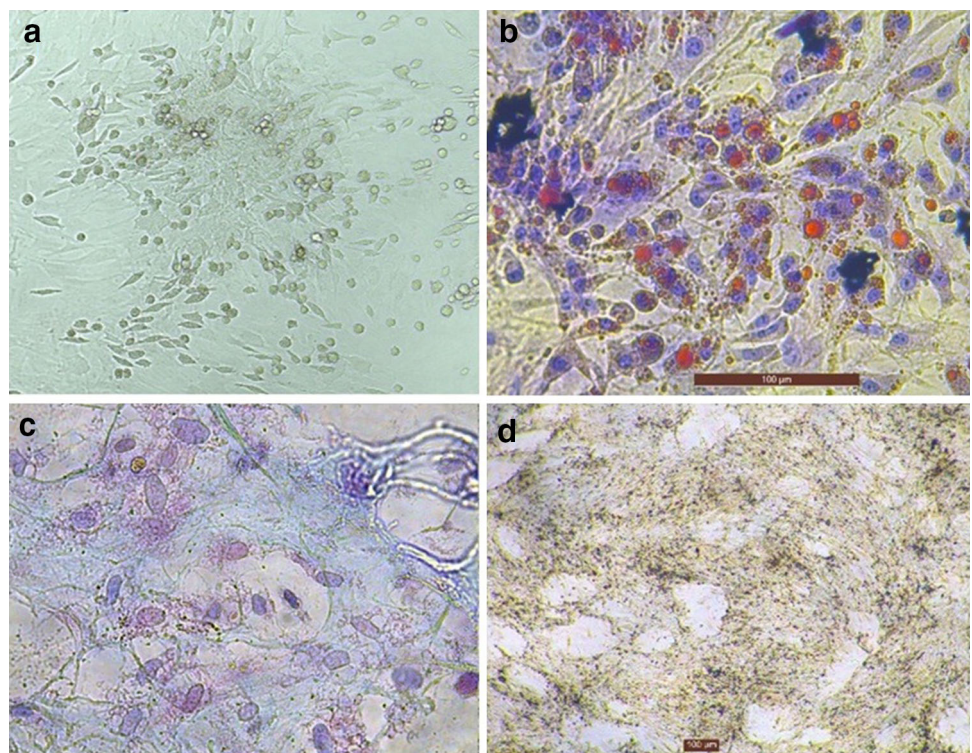


Fig. 1 The characterization of rabbit mesenchymal stem cells by flow cytometry. CD 45(-), Cd11b/c (-), CD49(+), CD 90 (+), CD44 (+)

Fig. 2 The differentiation of stem cells. **a** Rabbit adipose-derived mesenchymal stem cell (ADMSCs) colony forming unit fibroblasts (CFU-F) observed under light microscope on Day 14 (Leica D1000, ×10). **b** Showing the adipocyte differentiation of the ADMSCs with oil-red staining (Leica D1000, ×20). **c** Showing the chondrocyte differentiation of the ADMSCs with Alcian Blue (Leica D1000, ×40). **d** Showing the osteocyte differentiation of the ADMSCs with Von Kossa (Leica D1000, ×10)



The rabbits were randomly divided into three equal groups. One of the rabbits in the control group died after anesthesia.

Anesthesia was applied through intramuscular injection of 35 mg/kg ketamine hydrochloride and 5 mg/kg of xylazine. In addition, 2 ml of local anesthetic was infiltrated to the perinasal region (1% of lidocaine and 1:100,000 of adrenalin).

After shaving, the site was cleaned with povidine iodine. Then, a horizontal incision was made along the radix and a “T” shape incision extending from this incision to the nasal tip. The periosteum was elevated and the upper wall of the maxillary sinus was reached. The upper wall of the maxillary sinus was completely opened using a burr and Kerrison forceps and the maxillary sinuses were completely exposed. After identification of the ostium of the maxillary sinus bilaterally, the medial wall mucosa of the maxillary sinus starting from 1 to 2 mm peripheral to the ostium was peeled off widely using an elevator.

The subjects were randomly divided into three equal groups. In Group 1, the control group, no additional procedure was performed. In Group 2, the Suspension Stem Cell Group (SSCG), 2.10^6 stem cells were injected on the defect margins of both sides. In Group 3, the Stem Cell Sheet Group (SCSG), a 3-layer stem cell sheet, which had been previously prepared, was laid on the defective area. The incisions were sutured with 4/0 polyglycolic acid, applied with bacitracine cream and left to heal. An intramuscular injection of 30 mg/kg/day cefazoline was applied for 10 days during the postoperative period.

At the end of 3 weeks, the subjects were sedated with 35 mg/kg of ketamine hydrochloride and killed through intravenous injection of 500 mg phenobarbital. The medial wall of the maxillary sinus was revealed by elevating the

upper walls of the maxillary sinus and was removed from the operation site (Fig. 3).

Histopathological and ultrastructural examination

The specimens removed were divided into two pieces to examine one piece under a light microscope and other piece under an electron microscope.

The nasal tissue specimens removed for routine histopathological examination were fixed in 10% of Neutral Buffer formaldehyde. After the tissues were decalcified, sections of 5 μ thickness were taken and examined under light microscope after staining with hematoxylin and eosin (H&E).

The fresh specimens were immediately put into a 2.5% of glutaraldehyde solution, fixed for 24 h for screening, irrigated by phosphate buffer (pH 7.4), post-fixed by 1% of osmium tetroxide and dehydrated by gradually increasing alcohol concentrations for scanning electron microscope (SEM) examination. Following dehydration, the specimens were dried to a certain critical grade and put onto a metal plate using double-sided adhesive tape. The samples were exposed to scattering through a 150 Angström thick gold sheet in a BIO-RAD (Hercules, CA) scattering device. The images were obtained on JOEL SEM ASID-10 (Tokyo, Japan) and LEO 4.3 HVP SEM (Oberkochen, Germany) electron microscopes. Sample SEM images were obtained at 5–80 kV voltage and within $50\times$ – $5000\times$ magnification range. The specimens were assessed by two researchers who were blinded to the study in terms of 1-density (cilia coverage of the mucosa surface), 2-orientation (alignment and orientation), and 3-morphology (length, width and uniformity in detail structure). Scores were given from a maximum of 5 and the scores were averaged.

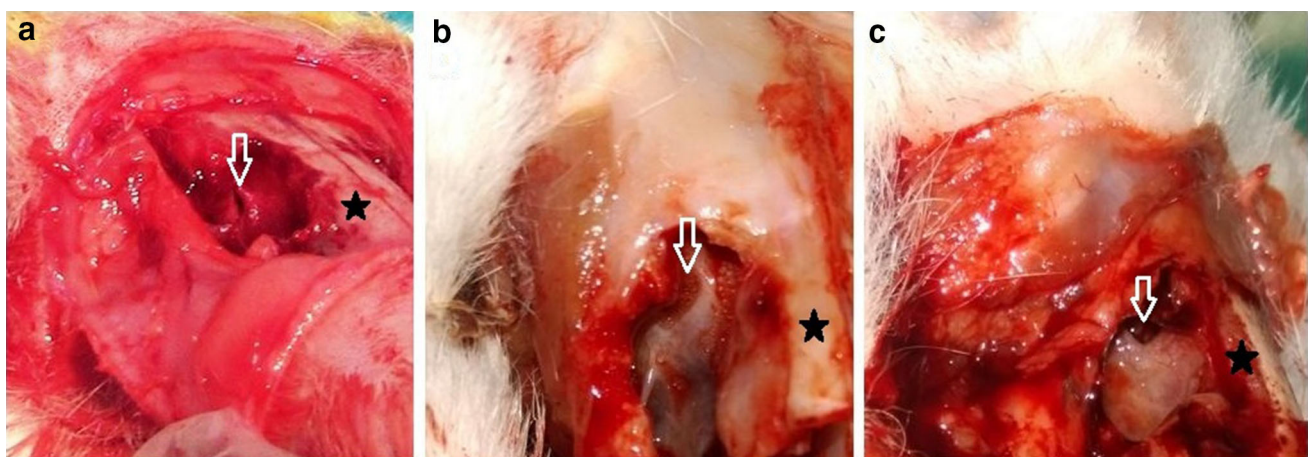


Fig. 3 Examples of maxillary sinus images after sacrifice from all three groups. **a** Control Group, **b** Suspension Stem Cell Group (SSCG), **c** Stem Cell Sheet Group (SCSG). *Arrows* indicate right maxillary sinus ostium, *stars* indicate right nasal bone

Data analysis was performed using SPSS 15.0 software (Statistical Package for Social Sciences, SPSS Inc., Chicago, IL, United States). The Kruskal–Wallis test was used for comparison of three groups. Binary comparisons were made using the Siegel Castellan test. A value of $p \leq 0.05$ was accepted as statistically significant.

Results

With the exception of one rabbit in the control group that died after anesthesia, all the other animals survived until killed. No signs of sinusitis were detected in the animals at the time of killing at the end of 3 weeks.

BrDU staining results

BrDu-stained stem cells (mostly the nucleus as well as some cells with brown stained cytoplasm) were detected in both the SSCG and the SCSG groups. The rate of BrDU stained cells was statistically significantly higher at 27% the SCSG compared to 8% in the SSCG ($p < 0.05$). Stratified, cubic, ciliated epithelium was detected in both procedures. In the stem cell sheet method, epithelial integrity and mucous acini in the sub-epithelial layer were observed more and goblet cells were observed everywhere in the epithelium (Fig. 4).

Scanning electron microscope (SEM)

As a result of the examination by SEM, cilia density was statistically significantly more in SSCG and SCSG than in the control group ($p < 0.001$, $p = 0.007$, respectively). Cilia morphology was statistically significantly better both in SSCG and SCSG than in the control group ($p < 0.01$, $p = 0.048$, respectively). Although ciliary orientation was scored better in SSCG and SCSG than in the control group, only the difference in SSCG was statistically significant (Table 1; Fig. 5).

Discussion

In the early 1980s, the success rate of FESS described by Messerklinger was considered to be over 90% [13]. However, 7.6–38% of patients need revision surgery due to ostial obstruction, synechia, or recurrent mucosal disease [14]. Healthy mucosa can also be affected and damaged by mechanical trauma during surgery. The quality of healing significantly influences the functional outcome after surgery. Some types of rhinitis are typically regarded as a reason for irreversible remodeling in the mucosa.

Wound healing is an extremely complex process of coordinated inflammation, cell proliferation and transformation, and matrix remodeling implied by various growth factors (GFs) [3]. Despite the scarcity of data in respect of a close resemblance between nasal and skin wound healing, nasal mucosal healing has remained a mystery [3, 4].

Nasal mucosa is formed by the respiratory epithelium covering the mucous membrane that lines the basal membrane and seromucinous glands which are interspersed among columnar cells and beneath the epithelium, and its basal membrane is a fibrous layer infiltrated with lymph corpuscles [3].

Recent insights into remodeling after nasal mucosal injuries have revealed the pivotal role played by GFs and cytokines, inflammation, cell proliferation, and matrix formation [15]. The early phase of wound healing (within the first 4 days) is essential for the normal function of epithelium cells, governed by fibroblast and epithelial proliferation, whereas in the late phase, fibroblast activation promotes fibrosis. Therefore, in a previous clinical study, nerve GF and keratinocyte GF were used to reduce fibroblast proliferation and adhesion formation and to enhance re-epithelization and neo-ciliogenesis [16]. In an experimental rabbit model study, epithelial regeneration was examined between groups of totally resected mucosa and partially resected and it was shown that in the totally resected group, the regenerated epithelium contained

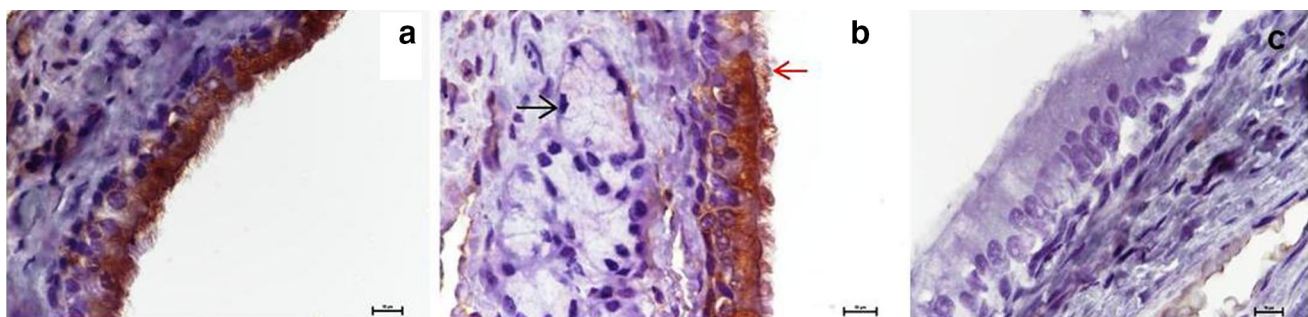


Fig. 4 **a** An image from the stem cell sheet group (100×10). Stem cells stained *brown* by BrDu and ciliated epithelium are observed. **b** An image from the stem cell sheet group (100×10). *Black arrow*

mucous acini; red arrow ciliated epithelium. **c** An image from the stem cell suspension group (100×10). Although ciliated epithelium can be seen, fewer stem cells stained by BrDu are observed

Table 1 Mean density, orientation and morphology of the cilia under electron microscope

	Density	Orientation	Morphology
Control group (<i>n</i> : 10)	1.75 ± 0.267	2.125 ± 0.443	1.625 ± 0.354
SSCG (<i>n</i> : 12)	3.625 ± 0.569	3,875 ± 0.311	4.125 ± 0.377
SCSG (<i>n</i> : 12)	3.125 ± 0.311	3.125 ± 0.377	3.00 ± 0.213
<i>p</i> ^a	<0.001	<0.001	<0.001
<i>p</i> ^b	0.007	0.048	0.057

SSCG Suspension Stem Cell Group, SCSG Stem Cell Sheet Group

^a Comparison of SSCG and Control group

^b Comparison of SCSG and Control group

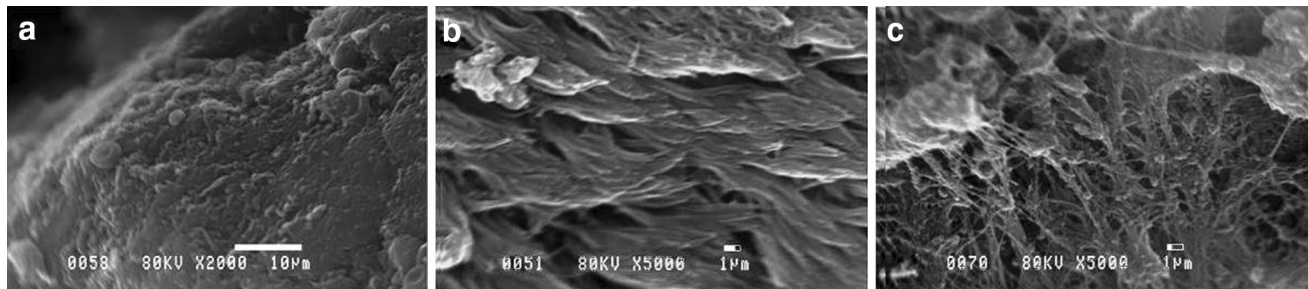


Fig. 5 **a** An image from the control group ($\times 2000$). Regular ciliary formation is not observed. **b** An image from the stem cell suspension group ($\times 5000$). Normal ciliary formations which partially exist as packaged and partially liberated covering the epithelial surface. **c** An

image from the stem cell sheet group ($\times 5000$). An image of the cilia which is similar to the stem cell suspension group for density, but poorer than the stem cell suspension group for orientation and morphology

atypical glands, more dense connective tissue in the lamina propria, and an absence of ciliary cells [4]. Another study evaluating cellular regeneration and recovery of the maxillary sinus mucosa after surgery, revealed regenerated epithelium displaying fibrosis and lacking serous glands with fewer ciliary cells [17]. In another experimental study of rabbit maxillary sinus mucosa, more increased levels of cytokines, interleukin (IL) 6 and 8 that induced collagen type I during healing were observed on postoperative day 14 [18].

To improve nasal wound recovery, different treatment modalities *in vivo*, *in vitro* and in animal models have been tested. In an animal model of sinonasal wound healing, chondroitin sulfate hydrogel revealed accelerated wound healing at the midpoint of 4 days, but no difference at the end of 14 days compared to the control group [19]. Topical dexamethasone application on a mucosal wound using a drug-releasing stent system decreases granulation formation [20] and in another rabbit model of sinus surgery, it was shown that topically delivered retinoic acid enhanced ciliogenesis [21]. In contrast to the above-mentioned previous studies, systemic dexamethasone after surgery has been reported to delay mucosal ciliary regeneration [22]. Hyaluronan also has a potential anti-scarring effect in the sinus ostium [23].

While bone marrow derived stem cells are known to contribute to wound repair resulting in the restoration of

tissue integrity and function by fibroblast and myofibroblast differentiation, stem cell technologies have also been promising areas of research for chronic skin wounds due to venous ulcer or diabetes, etc. for the last 30 years [24]. Mesenchymal stem cells (MSCs) applied to wounded skin can repair tissue defects by activation and differentiation of other stem cells and the production of growth hormones and cytokines [25]. The therapeutic efficacy of MSCs in the healing of fascial and cutaneous incisional wounds and the improvement of collagen formation by local injection has also been demonstrated in an experimental animal study [26]. Another role of MSCs in wound healing has been shown to be the accelerated time of wound repair through the effect of increased angiogenesis [27]. As a smaller number of stem cells have the ability to differentiate endothelial cells, expression of keratinocyte-specific markers and high levels of vascular endothelial growth factor and angiopoietin-1 in MSCs, this suggests that MSCs promote wound healing by differentiation and the release of proangiogenic factors [27]. MSCs neutralize reactive oxygen species that modulate the wound healing process resulting in scar formation and promote scar-free wound healing by increasing the stability of vascular angiogenesis [28].

Adipose tissue provides an important reservoir of mesenchymal stem cells, enabling adipose-derived mesenchymal stem cells (ADMSCs), as a pluripotent stem cell, to

develop into endoderm, mesoderm, or ectoderm. ADMSCs stimulate paracrine factors and proangiogenic growth factors for tissue regeneration, and they can be isolated in large amounts from various tissues. It is less invasive to harvest them rather than other types of stem cells so they are considered to be the ideal cell type for wound healing [29]. ADMSCs have been shown to significantly promote re-epithelialization and wound angiogenesis on the 14th day of wound healing via the secretion of collagen tissue [7]. Another study suggested that ADMSCs are constitutionally well suited for dermal wound healing by inducing dermal fibroblast proliferation [30]. Through the stimulation of the activation of dermal fibroblasts and keratinocytes, ADMSCs enhance the antioxidant effect in addition to wound healing [31].

Stem cell-based therapies for skin wound healing have been applied with favorable outcomes. Furthermore, stem cell-sheet technology has been used in experimental models of advanced retinal degeneration [32], corneal reconstruction [33], damaged myocardial muscle [34], and periodontal regeneration [35]. In wound healing models, 2- or 3-dimensional tissue engineering scaffolds are considered to be a valuable new therapeutic modality.

When the current study was started, there were no data on the effect of stem cell and stem cell sheet on nasal mucosal healing. However, during the study period, a paper came to our notice which evaluated the role of hepatocyte growth factor gene-modified mesenchymal stem cells, and reported a curative effect on damaged sinonasal mucosa, a reduction in fibrosis and the promotion of ciliogenesis on sinonasal wound healing [36]. In the current study the efficacy was evaluated of both suspension stem cell and stem cell sheet. BrDu labeled cells were detected more in SCSG than SSCG (27–8%, respectively). According to these data, stem cell sheet formation was shown to be more intense in the mucosal healing in the defect area. As stem cell sheet formation is high in the closure of large mucosal defects in particular, stem cell sheet can be considered superior to suspension stem cell. In SCSG with mucous acini in the sub-epithelial layer with goblet cells, almost normal respiratory epithelium was observed.

The normal ciliary appearance of the maxillary sinus mucosa in SEM has been shown in literature by Kim et al. [37]. In the current study design, the Group 1 (control) animals with peeled medial maxillary sinus mucosa are shown in Fig. 5a. This image shows that there is no ciliary process on the apical surface of the cells after the 3-week recovery period. On examination of Fig. 5b, which is an image of Group 2 (SSCG), healthy and well-organised ciliary processes according to density, orientation and morphology can be seen, as in the normal mucosa seen in previous studies [37, 38]. In Fig. 5c, which is an image of Group 3 (SCSG), the density of the ciliary processes is

similar to that of Group 2 but the alignment, orientation and morphology (length, width and unity of detail structure) are worse than in Group 2.

Greater ciliary density and better ciliary morphology were evident in both SSCG and SCSG compared to the control group, and ciliary orientation was superior in SSCG than in SCSG. This small difference between light microscopy and SEM was thought to be due to the different cross-sections taken. Despite the greater BrDu-stained stem cell intensity in SCSG, the better orientation scores in SSCG could be a sign of accelerated differentiation.

Conclusion

The results of this study showed that adipose tissue-derived mesenchymal stem cells increased healing of injured maxillary sinus mucosa in rabbits in terms of cilia presence, density and morphology regardless of the means of implementation. This study can be considered a strategic foresight for future stem cell studies investigating regeneration on injured epithelium of sinonasal mucosa due to surgery or sinonasal disease.

Compliance with ethical standards

Funding The study was financially supported by a Grant from Diskapı Yıldırım Beyazıt Training and Research Hospital Scientific Research Support Committee (47/3/2014).

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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