Porcine small intestine submucosal grafts improve remucosalization and progenitor cell recruitment to sites of upper airway tissue remodeling

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Background: **To better understand upper airway tissue regeneration, the exposed cartilage and bone at donor sites of tissue flaps may serve as in vivo "Petri dishes" for active wound healing. The pedicled nasoseptal flap (NSF) for skull-base reconstruction creates an exposed donor site within the nasal airway. The objective of this study is to evaluate whether grafting the donor site with a sinonasal repair cover graft is effective in promoting wound healing.**

Methods: **In this multicenter, prospective trial, subjects were randomized to intervention (graft) or control (no graft) intraoperatively after NSF elevation. Individuals were evaluated at 2, 6, and 12 weeks postintervention with endoscopic recordings. Videos were graded (Likert scale) by 3 otolaryngologists blinded to intervention on remucosalization, crusting, and edema. Scores were analyzed for inter-rater reliability and cohorts compared. Biopsy and immunohistochemistry at the leading edge of wound healing was performed in select cases.**

Results: **Twenty-one patients were randomized to intervention and 26 to control. Subjects receiving the graft had significantly greater overall remucosalization ($p = 0.01$) than controls over 12 weeks. Although crusting was less in the small intestine submucosa (SIS) group, this was not statistically significant ($p = 0.08$). There was no overall effect on nasal edema ($p = 0.2$). Immunohistochemistry demonstrated abundant upper airway basal cell progenitors in 2 intervention samples, suggesting that covering grafts may facilitate tissue proliferation via progenitor cell expansion.**

Conclusion: **This prospective, randomized, controlled trial indicates that a porcine SIS graft placed on exposed cartilage and bone within the upper airway confers improved remucosalization compared to current practice standards.**

Key Words:
nasoseptal flap; tissue repair; wound healing; remucosalization; upper airway; nasal septum; tissue graft; skull base surgery; basal cell

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Tissue repair in the human upper airway mucosa is poorly understood, despite numerous issues with tissue regeneration following a wide variety of endonasal sinus and skull-base procedures. Tissue grafting methods for skull-base defects have improved over several decades,[1–9] but studies on tissue repair/regeneration and/or ex vivo tissue engineering strategies are in their infancy for the nasal upper airway.[10,11] Evaluating methods to ameliorate

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Potential conflict of interest: COOK Medical provided research support and supplied the Biodesign™ porcine small intestine submucosa (SIS) grafts to both Stanford University and UAB for this study. All results were independently assessed and analyzed at both institutions respectively, without input from the sponsor. B.A.W. is a consultant for COOK Medical.

Public clinical trial registration: http://clinicaltrials.gov/show/NCT03159624. Use of Porcine Small Intestinal Submucosa (SIS) Graft to Aid in Nasal Septal Remucosalization and Tissue Healing in Patients Following Use of Nasoseptal Flap Elevation for Skull Base Surgery.

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wound healing for commonly used airway procedures are rational starting points for investigation.

The pedicled nasoseptal flap (NSF) is a widely used method of reconstruction for large skull-base defects, and has been shown to be a reliable method of repair in multiple series.[3,5,12–20] This versatile flap donates a robust, vascularized paddle of tissue based on the posterior septal artery to a new recipient site in the nasal cavity, but leaves behind a wide area of exposed cartilage and bone at the nasal septum donor site. The donor site is a frequent source of frustration for patients and endoscopic surgeons as a source of protracted wound healing. Patients require frequent office visits for repeated instrumentation and debridement of foul-smelling crusting until healed (Fig. 1A, B). de Almeida et al.[21] have previously quantified major and minor complication rates at the donor site in the nasal airway. Morbidity included nasal crusting (98%), discharge (49%), septal perforations (14.4%), and prolonged crusting >6 months (2.7%). In addition, time to complete remucosalization of the nasal septum donor site was determined to be approximately 90 days.[21,22]

Several methods have been suggested to improve healing at the donor site within the airway. Common practice includes insertion of a silastic sheet over the exposed cartilage/bone to prevent desiccation from nasal breathing/dry air exposure. Intranasal grafting is a consideration as various materials have been employed in the repair of skull base[7,23] and orbital wall defects,[24] closure of nasal septal perforations,[25] and total nasal reconstruction.[26] Free middle turbinate grafts[27] or contralateral septal rotation flaps[28] to cover the NSF donor site have been proposed, while another option includes the porcine small intestine submucosa (SIS) graft (Biodesign® Sinonasal Repair Graft; COOK Medical, Bloomington, IN), which is approved by the U.S. Food and Drug Administration for use in nasal/sinus soft tissue reconstruction. SIS has been successfully used as a repair graft in a number of body systems, including hernia repair, tendon repair, coronary valve replacement, diabetic wound care, and both open and endoscopic dural grafting.[29–31] This prepackaged, acellular mesentery maintains the natural separation between tissues or structures compromised by surgical manipulation, and available data suggests that this material appears to function as an adjunct to natural wound healing given the presence of indwelling growth factors within the graft matrix.[32–34] However, there is limited high-level data regarding the role of porcine SIS grafts in upper-airway tissue repair and its influence on aspects of remucosalization.[24,35,36]

We sought to evaluate under prospective, randomized, blinded trial conditions whether covering the NSF donor site with a sinonasal repair graft is efficacious in promoting remucosalization and healing time in the upper airway.

Patients and methods

This prospective, randomized, controlled study was approved by the institutional review boards (IRBs) at both Stanford University and University of Alabama at Birmingham (UAB). The study was registered with clinicaltrials.gov (NCT03159624).

In total, 47 patients undergoing endoscopic skull-base surgery requiring an NSF for reconstruction were recruited between both institutions. Patients were enrolled and consented between February 2015 and January 2016.

Patient demographics including age, gender, location, and etiology of skull-base defect, and complications were collected and recorded.

Surgical technique

Eligibility for enrollment in the study was as follows:

Inclusion criteria

1. Elective transnasal endoscopic skull-base surgery where closure with a large NSF was anticipated (exposure of >75% of the ipsilateral nasal septum bone/cartilage).
2. Generally healthy patients without nutritional compromise or otherwise debilitating conditions.

Exclusion criteria

1. Bilateral NSF placement in the same operative setting.
2. Patients without bone/cartilage exposure to incorporate an intact 2-cm × 3-cm covering graft.
3. Patients requiring 24-hour supplemental O_2 via nasal cannula.
4. Deconditioned, immunocompromised, or nutritionally-challenged patients.

Subjects were blinded to intervention and remained blinded throughout the study. Need for NSF reconstruction was determined intraoperatively by the neurosurgery and otolaryngology teams based on the skull-base defects created at the time of endoscopic skull-base surgery. Accordingly, 28 of the 75 patients screened/consented for the study were not enrolled when it was determined intraoperatively they did not meet criteria (eg, no NSF harvest, bilateral NSF harvest, small donor area). Patients were placed into a given cohort using a random number generator system at each institution by each coordinator. To eliminate any surgeon bias during NSF harvest, randomization to the covering graft intervention or control (silastic splint only) was determined intraoperatively by study coordinators after NSF elevation, at which point the graft or splint was unpackaged and used.

For the intervention group, a sterile, saline-soaked 2-cm × 3-cm SIS graft was placed as an intact single sheet directly onto the exposed nasal septum cartilage and bone. The grafts came sterilely packaged per manufacturer's standards. For uniformity and ease of analysis, no graft trimming was permitted by the operating surgeon, no suture or tissue glue was employed to secure the graft (which adequately adheres to the septum cartilage/bone by surface tension), and the bottom edge of the graft was placed parallel to and adjoining the freshly cut edge of the exposed

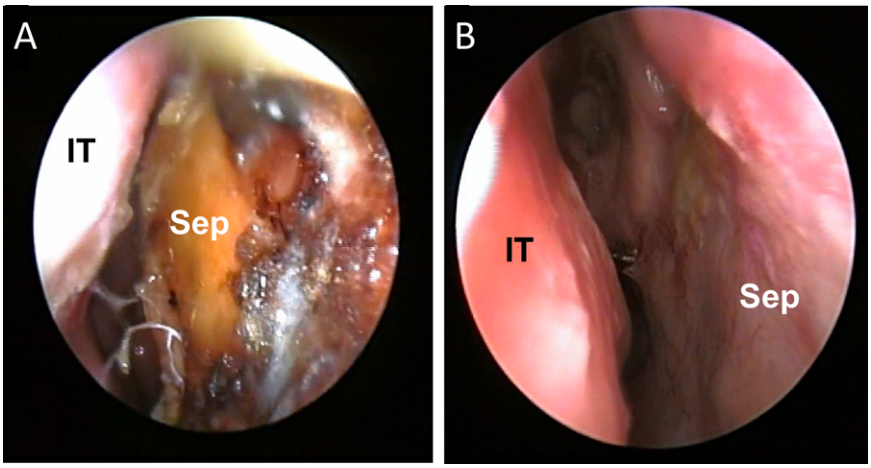

FIGURE 1. Endoscopy of the septum donor site following nasoseptal flap elevation in skull base surgery. (A) Profound crusting seen 2 weeks postoperatively in the right anterior nasal septum. (B) Healthy remucosalization of the same nasal septum site 6 months after surgery. IT = inferior turbinate; Sep = nasal septum.

nasal floor mucosa in all cases. For both cohorts, a standard silastic nasal splint (Medtronic, Jacksonville, FL) was placed over the covering graft or exposed nasal septum donor site, then secured to the anterior columella tissue with a single Prolene suture for 2 weeks.

Postoperative assessment and outcome measures

Subjects were evaluated in the office setting at 2, 6, and 12 weeks following randomization. Silastic splints were removed in all participants at 2 weeks postoperatively. Patients performed topical nasal rinses using 1 to 2 daily rounds of 250 mL isotonic saline over 12 weeks of this study without any additional medications or additives to the topical irrigations. At each clinic evaluation, routine rigid nasal endoscopies were video-documented prior to any crust or mucus debridement in the office (but after removal of silastic splint at the 2-week initial visit).

Scoring of videos

All patient videos were deidentified and numerically coded, and then condensed to 10-second to 20-second spliced video clips for each subject and interval time point to demonstrate the NSF donor site in its anterior-to-posterior extent. The 104 coded videos were randomized to both institution and time point of nasal endoscopy by the study coordinators. Scoring was performed by 3 blinded otolaryngologists (A.T., G.W.C., E.W.) with rhinology expertise who were not previously involved in the 47 procedures.

Likert scales were developed to score the postoperative video endoscopies, and were agreed upon by both institutions. These scales assessed the extent of remucosalization, amount of locoregional crusting, and severity of edema at the donor site on a 0 to 10 numerical scale. In order to normalize for internal bias and scoring range for each reviewer, 104 video clips were reviewed and scored twice by each of the 3 blinded reviewers. At the outset of the scoring period, 10 videos were randomly selected, and then viewed and discussed openly between all 3 reviewers to establish general scoring standards. Consecutive review of all videos was then completed by each evaluator in an independent fashion. At a later point (>3 weeks later), a second round of randomized review was conducted in the same manner with another randomized file of videos. Evaluators were blinded to all protected health information and institutional information regarding the enrolled patients, procedures conducted, or randomization, and no video contained identifying data.

Histology

Three patients (2 intervention, 1 control) were approved for office biopsy by the Stanford IRB as part of this study. Subjects were randomly selected by the research coordinator for biopsy at the leading edge of tissue healing of the donor site. After anesthesia with topical lidocaine, 2-mm to 3-mm tissue biopsies were obtained at the 6-week time point and placed in 4% paraformaldehyde. Six hours later, samples were dehydrated through a sucrose gradient, mounted in OCT tissue compound, and frozen sectioned with a microtome to 10 μm thickness onto frosted glass slides. Given the limited tissue sampling possible from this setting, immunohistochemical (IHC) staining for biomarkers of nasal airway basal cells (NBCs) was undertaken using 3 monoclonal antibodies against cytoplasmic cytokeratin 5 (Krt5) and cytokeratin 8 (Krt 8) or the nuclear transcription factor p63. Standard antigen-retrieval protocols for all tissue biopsies were performed on the same day to avoid confounders in technique or antibody titer differences. Slides were mounted in whole-mount medium, and fluorescent imaging was achieved using an Axiovert microscope (Zeiss Inc., Oberkochen, Germany).

Statistical analysis

All data items were examined using statistical summaries and graphics including smoothed plots of the outcomes

over time by study group. Intrarater reliability was assessed separately for each of the 3 raters and each outcome using Cohen's kappa statistic. Interrater reliability was estimated for each outcome by the intraclass correlation (ICC). Due to the complicated data structure of multiple raters, repeated ratings within rater (a total of 6 ratings per time point), and longitudinal observations; we averaged the 6 ratings at each time point to provide a single value for each measure per patient at each time point for the purposes of modeling. The effects of the graft type on the outcomes of remucosalization, crusting, and edema were estimated by linear models controlling for site and laterality accounting for the repeated measures per patient using generalized estimating equations with an unstructured correlation matrix. The postoperative time in weeks of each observation was included as a categorical fixed effect to allow for nonlinearity in time trends. A p value of <0.05 for the coefficient of graft type in the model was interpreted as evidence of a significant effect of graft type in the overall models and are considered to be the primary results. Additional models including the interaction between graft type and time were used to report predicted marginal means (also called ls-means [LSM]) and to test for differences between groups at each time point. These are primarily intended as an aid to interpreting the figures. All analyses were done using R 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria; https://www.r-project.org/)[37] including packages geepak[38] for modeling with generalized estimating equations (GEEs), LSM[39] for predicted marginal means, and ggplot2[40] for figures. Our sample size of >23 patients per group provided 80% power to detect a difference between groups of 0.85 standard deviations for a continuous outcome using a 2-sided Student t test with a significance level of $p < 0.05$.

Results

Seventy-five patients were screened and consented for the study, 47 of whom met criteria for randomization intraoperatively following NSF use; 21 were randomized to the intervention group (graft) and 26 were randomized to the control group. There were 26 whites, 11 African-Americans, 6 Hispanics, and 4 Asian-Americans enrolled. Average age for the intervention group was 50.5 years (range, 28–88 years) with 15 females and 6 males, while the control group age was 52.4 years (range, 30–74 years) with 15 females and 11 males. Two observations at 12 weeks were excluded because they were given identical labels. As not all subjects enrolled in the study completed follow-up at each requested time point, videos available for analysis were as follows: controls = 2 weeks (n = 16), 6 weeks (n = 19), and 12 weeks (n = 19) vs intervention = 2 weeks (n = 19), 6 weeks (n = 17), and 12 weeks (n = 14). In assessing for consistency in scoring, the ranges of within-reviewer correlations and ICC were as follows: remucosalization 0.69 to 0.80, ICC = 0.73; crusting 0.76 to 0.81, ICC = 0.79; and edema 0.38 to 0.59, ICC = 0.31 (all $p < 0.01$).

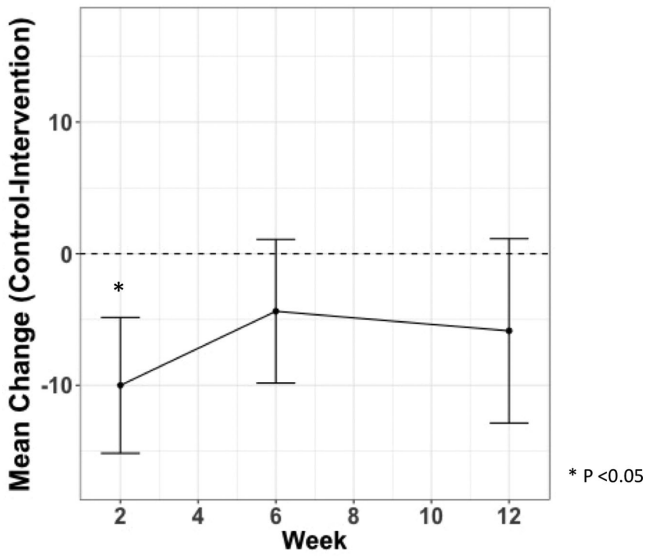

FIGURE 2. Mean change with standard error bars demonstrating the difference between the control and intervention (control-intervention) for remucosalization at time points 2, 6, and 12 weeks. Increased negativity favors the intervention group, which was noted to be significant at 2 weeks ($p = 0.045$) and on overall impact ($p = 0.01$).

Postoperative upper airway donor site remucosalization

Overall, the intervention group exhibited significantly more remucosalization when compared to the control group (LSM 57.1 [95% confidence interval {CI}, 52.1 to 62.2] vs 48.9 [95% CI, 45.1 to 52.7]), respectively with an overall difference of −8.13 between the 2 groups ($p = 0.01$). At 2 weeks, there was also statistically significant improvement in remucosalization following use of the covering graft in the intervention group (intervention 47.8 [95% CI, 40.3 to 55.3] vs control 37.8 [95% CI, 31.1 to 44.5], mean change −10.03; $p = 0.045$). Although not statistically significant, observed remucosalization was also improved in the intervention group at 6 weeks (intervention 49.7 [95% CI, 41.6 to 57.8] vs control 43.5 [95% CI, 36.7 to 50.3], mean change −6.17; $p = 0.26$) and 12 weeks (intervention 73.9 [95% CI, 63.8 to 83.9] vs control 65.3 [95% CI, 56.97 to 73.7], mean change −8.52; $p = 0.21$) (Fig. 2).

Postoperative upper airway donor site crusting

Mean observed crusting scores were lower with placement of the covering graft overall, and at each follow-up time point although none were statistically significant. Subjects in the intervention group had less crusting overall (intervention 3.45 [95% CI, 2.97 to 3.93] vs control 4.11 [95% CI, 3.63 to 4.59]) with a mean difference of 0.66 between the 2 groups ($p = 0.08$). The intervention group was noted to have less crusting at 2 weeks (intervention 3.50 [95% CI, 2.99 to 4.01] vs control 4.22 [95% CI, 3.50 to 4.94]; mean change 0.72, $p = 0.09$); 6 weeks (intervention 4.22 [95% CI, 3.03 to 5.41] vs control

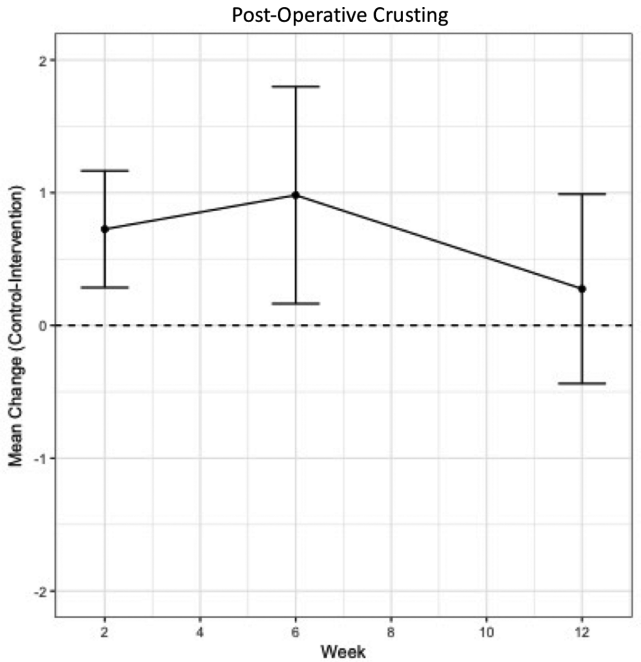

FIGURE 3. Mean change with standard error bars demonstrating the difference between the control and intervention (control-intervention) for crusting at time point 2, 6, and 12 weeks. Increased positivity favors the intervention group.

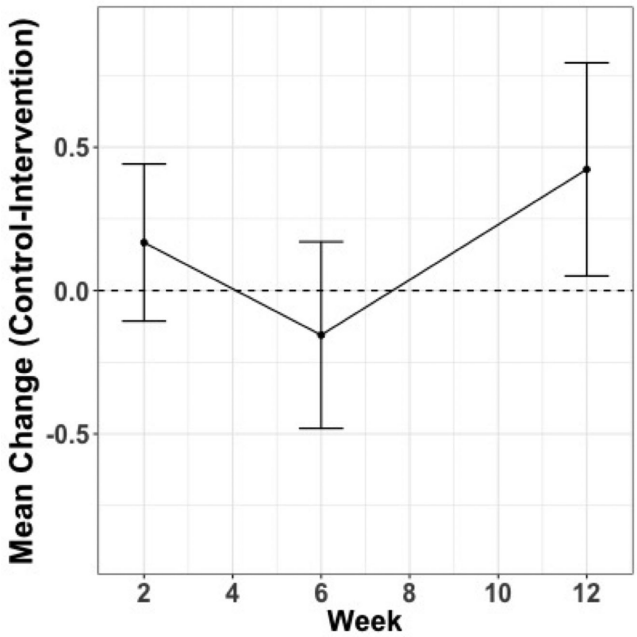

FIGURE 4. Mean change with standard error bars demonstrating the difference between the control and intervention (control-intervention) for edema at time points 2, 6, and 12 weeks. Increased positivity favors the intervention.

5.2 [95% CI, 4.17 to 6.24]; mean change 0.98; $p = 0.23$), and 12 weeks (intervention 2.64 [95% CI, 1.48 to 3.80] vs 2.92 [95% CI, 2.17 to 3.66]; mean change 0.28; $p = 0.70$) (Fig. 3).

Postoperative upper airway donor site edema

Although estimated edema scoring was slightly better overall with covering graft placement (intervention 2.95 [95% CI, 2.73 to 3.18] vs 3.11 [95% CI, 2.86 to 3.35]; mean difference 0.18; $p = 0.23$), this was not statistically significant. Edema scores were lower at 2 weeks (intervention 3.34 [95% CI, 2.94 to 3.75] vs control 3.52 [95% CI, 3.16 to 3.89]; mean difference 0.18; $p = 0.49$) and 12 weeks (intervention 2.26 [95% CI, 1.84 to 2.70] vs control 2.65 [95% CI, 2.11 to 3.19]; mean difference 0.38; $p = 0.28$), but higher at 6 weeks (intervention 3.25 [95% CI, 2.74 to 3.76) vs control 3.15 [95% CI, 2.75 to 3.55); mean difference −0.10; $p = 0.76$)) (Fig. 4).

Cellular composition of the epithelium sampled at the leading edge of upper airway wound healing

Office biopsy (Fig. 5A) of the healing nasal septum mucosa from a control patient at the 6-week time point revealed a fairly typical architecture to the nasal epithelium. This consisted of a single row of underlying NBCs and a denser, luminal layer of Krt8+ cells with overlying keratinaceous cell matrix and debris (Fig. 5B). By contrast, biopsies from both intervention patients demonstrated an abundance of stacked rows of NBCs with numerous p63+ nuclei and the limited presence of Krt8+ differentiated luminal cells at this time point (Fig. 5C, D). The NBCs were positive for expression of both nuclear p63 and cytoplasmic Krt5 (Fig. 5D). To the authors' knowledge, this is the first direct example of progenitor cell involvement in active tissue regeneration in the human upper or lower airway, which involves basal cells at the interface of wound healing.

Complications during course of prospective study

At each postoperative visit, patients were evaluated for any complications including septal perforation and development of transverse synechiae between the septum and the inferior turbinate. Four patients in the intervention group and 6 patients in the control group were noted to have minor septal perforations. Three patients were found to have synechiae in the intervention group vs 2 patients in the control group.

Discussion

Findings from the present randomized, controlled clinical trial provide evidence that covering the NSF donor site with a SIS graft promotes remucosalization and healing over the current standard of care (silastic splint alone) as well as early evidence of progenitor cell involvement in healing at the leading mucosal edge. While estimates demonstrated decreased donor-site crusting overall and at all follow-up periods, the difference was not significant. There also was no overall effect on edema. The benefits of faster healing time and remucosalization are numerous, including decreasing dried secretions, nasal blockage, rhinitis,

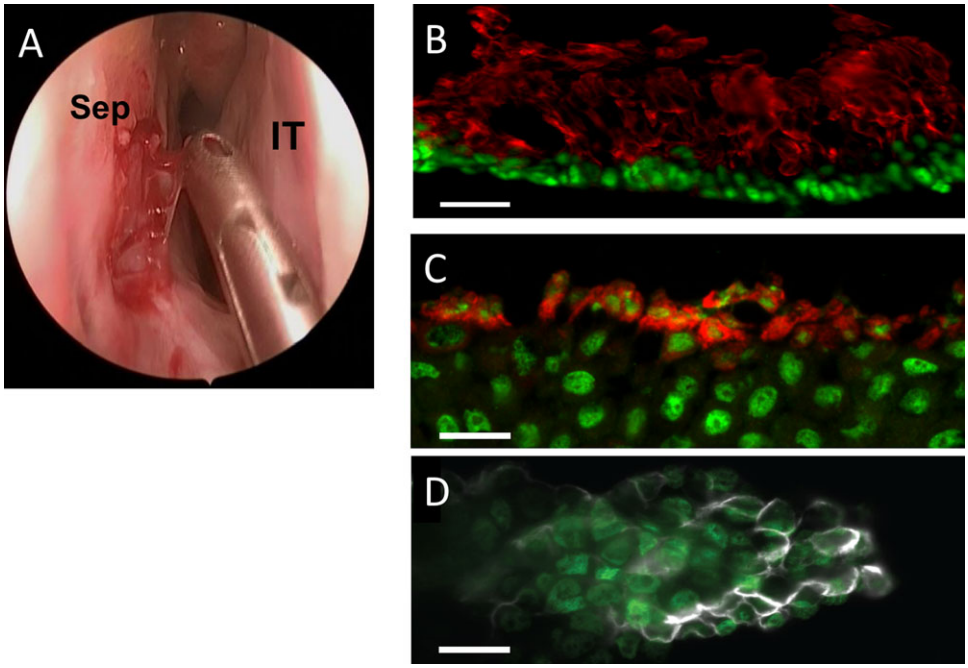

FIGURE 5. Office biopsy of the nasal septum donor site mucosa at the 6-week time point. (A) Forceps are used to endoscopically biopsy the leading wound edge of the septum. (B) Frozen cross-section of tissue biopsy stained for basal cells (using p63 nuclear stain in green) and differentiated cells (using Krt8 cytoplasmic stain in red). An overlying thick keratinaceous matrix was also seen. (C) Frozen cross-section of tissue biopsy from first patient in intervention group, showing an increased number of tiered basal progenitor cells (green nuclei) and fewer luminal cells that had undergone differentiation (red cytoplasm). (D) Frozen cross-section of tissue biopsy from second patient in intervention group, which also showed a similar predominance of basal progenitor cells, stained for nuclear p63 (green) and cytoplasmic Krt5 (white). All images are shown at ×20 magnification. Scale bar = 25 μm. IT = inferior turbinate; Sep = nasal septum.

and reduced need for uncomfortable office instrumentation and debridement. Since the donor site must heal from the free edges of the surrounding mucosa, the wound healing time is directly related to the size of the defect and the ability to optimize tissue proliferation in the postoperative setting. Our findings from this multi-institutional study support the use of SIS grafts to cover exposed airway bone and cartilage tissue beds to promote earlier remucosalization.

There is mounting evidence that nasal basal cells (NBCs) are the primary progenitor cells of upper airway epithelial cells.[41–43] Basal cells along the basement membrane express the biomarkers p63 as a nuclear transcription factor, and the cytoplasmic protein Krt5. In contrast, differentiated ciliated and secretory cells are progeny cells that reach the airway lumen, and convert from Krt5 expression to the cytoplasmic expression of cytokeratin 8 (Krt8).[41,44] The theoretical benefit of covering sites of exposed cartilage and bone in the upper airway with an SIS graft includes a scaffold for cellular ingrowth and growth factors to promote healing.[32–34] Biopsies of both intervention patients demonstrated an unexpected abundance of NBCs compared to the control biopsy at the leading wound edge at 6 weeks. These data may provide an important window into airway epithelial regeneration if the findings are reproduced in larger series. The significantly-improved wound repair seen with the SIS/sinonasal repair graft may occur at the native airway progenitor cell level, and growth factors present in the SIS acellular mesentery may be responsible for facilitating wound healing at this level and contributing to the expansion of the NBCs observed. These unexpected findings from tissue biopsies represent, to our knowledge, the first direct example of the presence/involvement of NBCs at the active site of tissue regeneration in the human airway. In previous studies, basal cells have been shown to have progenitor cell properties and are involved in healing of the local epithelium.[45,46] Further studies are of great interest between our groups to examine the incorporation of NBCs at sites of nasal airway mucosal repair to determine whether this particular type of SIS, vs coverage of donor sites with any covering matrix, is the primary basis for improved remucosalization.

The strengths of this study are founded in a robust prospective study design and selection criteria. Potentially eligible individuals were consented prior to the operation and then randomized to intervention or control by the study coordinator following NSF elevation intraoperatively using a random number generator. Participant ascertainment bias was eliminated by blinding the subjects to intervention and observer bias was minimized through blinding the video judges to treatment group. The primary limitation of this study was the small sample size as well as patients who were lost to follow-up and did not complete the study. Reasons cited for lack of follow-up included distance of travel from the treating institution and lack of nasal symptoms.

Conclusion

Results of this prospective, randomized, controlled trial indicate that an SIS/sinonasal repair covering graft placed on an exposed cartilage and bone donor-site tissue bed within the upper airway confers improved remucosalization and wound healing properties for patients undergoing nasal and/or skull-base procedures compared to current practice standards.

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