Effect of *in vivo* oxidized cellulose on *in vitro* growth of human respiratory mucosa and sub-mucosa during endoscopic skull base approaches

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ABSTRACT

Introduction: Despite major progress in closure techniques for endoscopic endonasal skull base approaches, cerebrospinal fluid (CSF) leakage remains a common complication. Because it simultaneously aids hemostasis and adheres the flap to the skull base, oxidized regenerated cellulose (ROC) (Surgicel™, Ethicon, USA) sheets have gained popularity as an adjunct to a vascularized nasoseptal flap for closure of large dural defects. However, evidence supporting its positive impact on the healing process is uncertain.

Methods: In 6 patients, a 1 cm² piece of ROC gauze was placed on the surface of the middle turbinate and sphenoid mucosa before they were resected as part of a standard endoscopic endonasal approach (EEA). After removal, mucosa treated with ROC was separated from untreated mucosa (control), and the two groups were compared. A histological examination of structural changes, cell viability, and growth capacity in in vitro cultures was performed.

Results: Compared to the unexposed controls, exposed tissues exhibited extensive necrosis including destruction of the endothelial lining of blood vessels. Viability techniques also showed extensive cell death in the exposed group. Finally, control specimens could be grown in cell cultures, whereas exposed specimens showed no growth.

Discussion: ROC was demonstrated to have deleterious effects on mucosal tissue viability. These results suggest that the use this material may actually inhibit rather than aid in effective dural repair. The use of ROC may therefore be relatively contraindicated in this clinical setting. Further studies which examine the impact of ROC in wound healing in an in vivo model are warranted.

INTRODUCTION

Endoscopic endonasal approaches (EEA)¹ to the skull base have increased in popularity over the past two decades and have become a major tool in skull base surgery. One of the factors contributing to its increased acceptance is the improvement of the previously high incidence of CSF leak.²⁻⁴ This complication is due to the difficulty of obtaining a watertight dural closure in these endonasal approaches. To address this issue, a number of different solutions have been developed⁵⁻⁷, decreasing its incidence to less than 10% in multiple large series.⁸,⁹ The most effective reconstructive technique is a vascularized nasal mucosal flap with a pedicle from the nasal septum⁵,¹⁰,¹¹ or the inferior turbinate.¹²,¹³ Vascularized nasal mucosa can reconstitute a skull base defect with its rapidly growing cells that can integrate with the overlying dural edge and bone.
In many centers it has become standard practice to use gauze impregnated with ROC (Surgicel™, Ethicon, USA) as an additional layer superficial to the vascularized nasoseptal flap in order to aid adherence to the adjacent mucosa, prevent the interposition of tissue glues, and achieve hemostasis.\textsuperscript{1,8,14,15} This material has been used to obtain hemostasis in a broad array of tissue types. In sinus surgery, in addition to hemostasis, it has been shown to produce an inflammatory reaction.\textsuperscript{1,8,14,15} This practice, although described by others\textsuperscript{1,16} and widely employed, has never been proven to encourage tissue healing on the cellular level after a surgical intervention.

ROC is a local hemostatic agent that works by lowering the pH of the surrounding tissue and thus activating the coagulation pathway.\textsuperscript{17} In a chronic wound environment such as diabetic or venous ulcer, oxidized cellulose promotes better conditions for healing.\textsuperscript{18,19} However, there is little data to support its use as a healing inductor after endonasal cranial base surgery.\textsuperscript{20–22} Our group has previously demonstrated using \textit{in vitro} experiments that ROC inhibits the growth of human dural fibroblasts\textsuperscript{20} which are essential to achieve healing. This study was performed in order to evaluate the effect of oxidized cellulose placed \textit{in vivo} on the middle turbinate and sphenoid mucosa and assess its influence on tissue structure, cell viability and growth capacity.

\textbf{METHODS}

\textit{Specimen collection}

Fragments of respiratory mucosa were obtained from the middle turbinate process and the sphenoid mucosa from 6 patients undergoing elective EEA surgery. These structures are routinely resected and discarded during a standard endoscopic endonasal approach to the sella and are not used for therapeutic or diagnostic purposes. The protocol was approved by the local IRB at Hospital Italiano de Buenos Aires, Buenos Aires, Argentina.

\textit{Treatment}

Before draping the patient, a fragment of a ROC gauze (1 cm\textsuperscript{2}) (Surgicel™, Ethicon, USA) was placed in the anterior-middle aspect of the middle turbinate process using endoscopic guidance. The patient was then prepped and draped in the usual sterile fashion. The middle turbinate was harvested on average 15 minutes after the application of ROC. The specimen was then sectioned to isolate the fragment with the ROC from the untreated area. A margin of at least 1 cm was present between treated and untreated tissue. The ROC gauze was removed before harvesting the tissue sample. Samples were washed with sterile, additive-free saline and immediately transported to the lab in a saline bath.
When the surgical approach reached the sphenoid sinus, the right sided mucosa was also treated with a 1 cm² gauze of ROC. After 30 seconds the mucosal layer of the sphenoid sinus was resected and treated in a similar fashion as the middle turbinate to obtain exposed and unexposed samples for further evaluation. Treated specimens and controls from both sites were analyzed by the same means and came from the same patient in every case.

**Explant culture**

Specimens were washed with Dulbecco’s modified Eagle’s medium (DMEM; Gibco, USA). The middle turbinate process was separated from its mucosal layer by sharp dissection with a #15 blade. Treated and untreated 5 mm² specimens were cultured separately on 30-mm culture plates (Nunc™, Thermo Fisher Scientific, USA) with 2 ml DMEM supplemented with 100 U/ml penicillin, 100 m g/ml streptomycin, 0.25 m g/ml amphotericin B (Gibco, USA), 200 mM alpha-glutamine (Gibco, USA), and 10% bovine fetal serum (Bioser, USA). Cultures were performed in a humidified atmosphere with 5% CO2 at 37°C. Cells migrating to the plate from the explant were documented with an inverted microscope (TMS™, Nikon, USA) until 5 days after seeding. Tissue fragments that were not used in this step were analyzed by common staining and immunohistochemical techniques as described below.

**Staining**

Fragments of treated and untreated sphenoid mucosa and middle turbinate process were analyzed by Hematoxicilin-Eosin (HE) and Masson’s trichromic (MT) histological techniques. Specimens were fixed with 10% paraformaldehyde, and the turbinate process was decalcified with EDTA for 24 hours. Both tissues were then embedded in paraffin and sectioned for histological examination.

**Immunohistochemistry**

Cells were characterized by staining with anti-vimentin and anti-cytokeratin primary antibodies (Millipore, USA) to examine for fibroblasts and epithelial cells, respectively. The protocol was conducted as follows. Cells were fixed in 4% paraformaldehyde for 10 minutes and then washed with phosphate-buffered saline (PBS) pH 7.2. Then they were treated with PBS with triton X-100 for 10 minutes and incubated with Powerblock™ (Biogenex, USA) for 5 minutes to block non-specific binding sites. The primary antibody was incubated overnight at 4µC in a moist chamber. The following day, they were washed with PBS and incubated with biotinylated (Multilink™; Biogenex, USA) secondary antibody for 30 minutes at room temperature and then
with fluorescein streptavidin (Vector Labs, USA) for 60 minutes. Finally, they were washed with PBS pH 8.2. Lastly, they were mounted and analyzed under optical microscope (Eclipse E400™, Nikon, USA). Nuclei were stained with Hoeschtl.

**Viability assay**

To evaluate for cell viability, a mixed solution containing acridine orange and ethidium bromide was added to the surface of the sphenoid mucosa and the middle turbinate, immediately after they were harvested. The tissue was next observed under the fluorescent microscope. Since live cells are not permeable to ethidium bromide and dead cells are permeable to both dyes, live cells appear green and dead cells appear red. To further assess for cell viability, cell growth onto Petri dishes from treated and untreated specimens was evaluated, as described.

In order to assess the role of acidity in the decrement of cellular viability shown by direct ROC gauze addition, sphenoidal mucosal-derived fibroblast subcultures were exposed to ROC extract, with and without medium pH neutralization. To prepare the extract, sterile gauze of ROC was incubated in the medium culture above described, in a 6 cm²/ml proportion, by 60 minutes. The solution was divided in two parts; one portion was used with the resultant pH while the other was neutralized by NaOH up to 7.40. Each portion was added to proliferating cultures as mentioned before. After 60 minutes of incubation, both plates were washed with sterile, additive-free saline. AO/EB staining was performed and plates were observed under fluorescent microscope. Average and percentage of viable and non-viable cells were analyzed by unpaired T-test with Welch’s correction. P<0.05 values were considered statistically significant. Stataal analysis was performed using GraphPad Prism version 6.00 for Windows, (GraphPad Software, La Jolla California USA).

**RESULTS**

**Histological Examination**

On histological examination, major structural changes to the tissue samples were seen after applying ROC. Fig. 1 demonstrates normal sphenoid mucosa. A pseudostratified (respiratory) epithelial lining covers the surface, and normal blood vessels are seen within the submucosal connective tissue. Tissue samples exposed to ROC showed a markedly diminished respiratory epithelium as well as extensive necrosis. Submucosal blood vessels in the treated mucosa exhibited endothelial destruction. Similar findings were evident on examination of the tissue samples from the middle turbinate. The superficial mucosal lining in the exposed group was either absent or showed evidence of necrosis, with preservation of the epithelium only of the submucosal glands (Fig. 2).
Cell culture

Untreated specimens from both the sphenoid and the middle turbinate process yielded a very large number of cells in all seeded samples (n=6). By day 3, the number of cells were too numerous to count. Active ciliary movement at the luminal end of the epithelial cells was evident under the inverted microscope. Two different kinds of cells could be seen. One cell type had long fusiform cytoplasm and grew in a typical fusiform pattern and the other one exhibited a polygonal shape with a chaotic intercellular arrangement, corresponding to mesenchymal and epithelial origins respectively (Fig. 3). In sharp contrast, specimens that were treated with ROC before resection did not show any growth from any seeded sample.

Immunohistochemistry

Cultured cells growing within the Petri dish were further characterized by immunofluorescence (Fig. 4). Two phenotypically different cell populations were present. Positive intermediate filaments marking vimentin were evident in some cell cytoplasm. However, some nuclei were present within the cultures that were negative for this marker, suggesting a different pattern of gene expression and suggesting that two different histological cell populations were present within the culture. Cytokeratin marking was positive in the remaining cells, consistent with their epithelial origin. Some cultured cells resembled epithelial disposition.

Cell Viability

Treatment with ROC induced cellular death in both samples and in every specimen. In the viability assays, the number of viable (green) nuclei were substantially diminished and replaced by non-viable (orange) ones (Fig. 5). As previously stated, no cellular growth was evident in any of the seeded samples, further supporting these findings of no cell viability.

The addition of ROC extract to sphenoidal mucosae-derived fibroblast cultures with and without pH neutralization was performed to determinate the role of the acidity in the ROC-induced cytotoxicity. Viability assay showed, in average, low amount of viable cell (8.18; 95% CI: 6.10-10.26 vs. 116.0; 95% CI: 99.39-132.6; p<0.0001) and more non-viable cell (68.25; 95% CI: 45.21-91.29 vs. 9.83, CI: 1.97-17.69; p=0.0001) in the non-neutralized ROC extract group than the neutralized one (Fig. 6). A clear implication of the acidity was also evidenced by the mean viable cell percentage within the non-neutralized extract group (93.38%; 95% CI: 91.89%-94.87%) than the neutralized extract group. (11.68%; 95% CI: 4.32%-19.03%; p<0.0001) (Fig. 7).
Even with current techniques, the incidence of post-operative CSF leaks after expanded endoscopic endonasal approaches is 10%. Understanding the healing process in these procedures is essential for developing techniques to diminish the rate of these leaks. Despite uncertainty about the effect of oxidized cellulose on mucosal healing, its use as an adjunct to skull base reconstruction has become widely accepted.

Wound healing is a complex process involving parenchymal cells as well as blood cells. The immune system plays a key role in the production of cytokines that induce fibroblast migration and extracellular matrix production. While the mechanisms of healing in skin defects have been well studied, they may differ from mechanisms in mucosal healing, which has not been well described to date.

The normal healing process results from a combination of a destructive inflammatory process and a regenerative response. In chronic wounds, the catalytic effect of proteases degrades extracellular matrices and growth factors inhibiting tissue regeneration. In this environment, oxidized cellulose inhibits metalloproteases, sequesters metal ions and inhibits bacterial growth, thus, promoting a regenerative process and slowing the catalytic process. ROC has been reported to be effective in improving healing in several skin models, specifically in chronic, contaminated ischemic wounds such as pressure, diabetic and venous ulcers.

The environment of wound healing after surgery differs from chronic wound healing in several regards. Surgical wounds have a brief, self-limited inflammatory phase that induces the migration of fibroblasts between tissue layers. This inflammatory state rapidly evolves to a regenerative one where the viability of adjacent fibroblast is key to achieve healing.

The current study describes how placement of gauze impregnated with oxidized cellulose induces cellular necrosis of the fibroblasts, disrupts the endothelium of blood vessels, and impairs cellular growth. Because cell viability and the vascular network are both essential in the healing process, these data argue against the healing-inductive properties of ROC, especially in the setting of a vascularized flap during EEA surgery.

Kazikdas et al. investigated the effect of oxidized cellulose on the viability of cartilage in vivo in rats. They found that the regeneration potential as well as the viability of chondrocytes was diminished compared to controls. Coskun et al. also demonstrated that cartilage transplanted into nasal septum showed tissue reabsorption when exposed to ROC. Liu et al. demonstrated in a rat model that ROC did not affect the healing process of the pharyngeal mucosa.
Our center previously reported that *in vitro* ROC induces cell necrosis in human dura mater cells by reducing the pH of the culture medium.\(^{21,22}\) However, *in vitro* studies may overestimate these effects due to the absence of buffer elements present in a living organism.

In this study, the *in vivo* acidic effect of ROC might actually be underestimated since pH returned to normal levels immediately after the samples were taken to the lab. It is reasonable to expect that the pH would have been lower if ROC is left on the mucosa until it is completely dissolved. This experiment demonstrated that even short-term contact between an acidic medium and mucosa can alter its structure and regenerative potential.

One of the limitations of this study is that the length of the exposure is not long enough to assess the effect of ROC on the deeper layers of the middle turbinate that might contribute to healing when this structure is used to reconstruct the cranial base. A better understanding of the full healing process would require harvest at multiple time points throughout healing. A second limitation of this study is that because the samples were taken 15 minutes and 30 seconds after exposure, the role of the immune system cannot be well assessed. The role of the immune system healing in initiating the healing cascade and the role of any chemical debridement secondary to ROC that might lead to a healing cascade is not well understood. However, these initial results suggest this debridement to be deleterious rather than restorative.

**CONCLUSION**

Although ROC has been routinely used as part of a layered closure for repair of skull base defects, this study suggests that ROC may actually inhibit the healing process essential for adequate reconstruction. ROC was demonstrated to have deleterious effects on mucosal tissue viability. The use of ROC may therefore be relatively contraindicated in this clinical setting. Further studies which examine the impact of ROC in wound healing in an *in vivo* model are warranted.
REFERENCES


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Figure 1. Normal human rinosinusal mucosa, HE (A) and MT (B) staining. Pseudostratified multilayer cells were present, with glandular and ciliated differentiation supported on the underlying submucosa (1, 40X), which provides vascular supply and mononuclear cells (2, 4X).
Figure 2. Human rinosinusal mucosa following ROC application, HE staining. Disruption of epithelial structure was evident (A, 40X). Submucosal capillary thrombosis was seen (B, 10X).

Figure 3. Cell culture from intact rinosinusal mucosa explants, optical microscopy (40X). Both fusiform- and polygonal-shaped cells were noted, suggesting mesenchymal and epithelial origins, respectively.
Figure 4. Rinosinusal mucosa-derived cell culture, immunofluorescence for cytoqueratin (green, 1) and vimentin (red, 2); and both merge with nuclear (blue) Hoeschl staining (3). Vimentin were evident in some cell cytoplasm, cytokeratin marking was positive in the remaining cells (A). Some cultured cells showed epithelial arrange (B).
Figure 5. Human rinosinal mucosa, acridine orange and ethidium bromide staining. Intact (A) and
in vivo exposed to ROC (B) fragments. The quantity of viable (green) versus unviable (orange) cells
was markedly different among both groups.

Figure 6. Cell viability with neutralized and non-neutralized ROC extract. The x-axis shows the
viable and non-viable cell count, respectively; non-neutralized (acid) ROC extract is associated to
diminished viable cell count and increased non-viable cell count. Columns represents means, bars
represents 95% CI.
Figure 7. Cell viability with neutralized and non-neutralized ROC extract. The x-axis shows the percentage of viable cells. Lesser viability percentage is associated to the non-neutralized (acid) ROC extract group than the neutralized group. Columns represents means, bars represents 95% CI.