SPECIAL REPORT

A PROSPECTIVE OBSERVATIONAL STUDY OF THE YIELD OF OLFACTORY ENSHEATHING CELLS CULTURED FROM BIOPSIES OF SEPTAL NASAL MUCOSA

OBJECTIVE: We sought to study the yield of olfactory ensheathing cells from biopsies of the mucosa of the nasal septum. These specialized cells encourage regeneration of nerves of the central nervous system and may be of value for spinal cord and nerve injuries.

METHODS: We undertook a prospective observational study of biopsies of nasal mucosa by endonasal dissection of the mucosa of the nasal septum during the approach for routine transsphenoidal surgeries. Samples were cultured in the laboratory, and the yield of olfactory ensheathing cells was compared as to the location, size, and weight of the biopsies and the age of the patients.

RESULTS: A better yield of olfactory ensheathing cells was obtained from areas of the septum that were more superior and posterior in position. The yield was not related to the size of the biopsy or the patient's age.

CONCLUSION: Septal mucosa is a possible source of olfactory ensheathing cells, although the yield may be smaller than that which may be obtained from mucosa of the lateral nasal cavity and superior turbinate.

KEY WORDS: Biopsy, Culture, Mucosa, Olfactory ensheathing cells


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Received, July 18, 2007. Accepted, February 13, 2008.
We took biopsies from several different regions of the septal mucosa in patients who were undergoing routine pituitary surgery and studied the yield of OECs in the laboratory to assess the influence of biopsy position, size, weight, and patient age on the quality of the OECs that were cultured. The ability of OECs to facilitate neuronal repair is dependent on the presence of two cell subtypes: p75-expressing OECs and fibronectin-expressing olfactory nerve fibroblasts (12). Anti-p75 and anti-fibronectin antibodies were, therefore, used to evaluate the culture yields.

PATIENTS AND METHODS

Consecutive patients who presented to the National Hospital for Neurology and Neurosurgery were asked to participate in this observational study. Informed consent was obtained, and all procedures were performed with the approval of the hospital’s research ethics committee. During the endonasal approach to the sphenoid sinus, two or three small biopsies (3- to 4-mm cubes) were taken from the superoposterior aspect of the nasal septal mucosa and placed in physiological saline. An x-ray was obtained using an intraoperative image intensifier to document the position of the biopsy, which was mapped onto a diagram of the nasal septum with superimposed regional grids (Fig. 1). Patient data (age, sex, medical history, allergic history, smoking, and medications) were collected on a proforma. The specimen was immediately taken to the laboratory (average transit time, 10 min) for culture.

We adopted a grading system that was based on the microscopic assessment of cultures by two independent skilled observers (SL, DL). For each specimen, four to six plates (35 mm diameter) were cultured and assessed by both observers. An average of the plates was taken as the final grade, which was corroborated by the independent observers. Specimens containing no OECs were classified as Grade 0; those with one or two scattered cells were classified as Grade 1. If up to 10% of the cells on the plate were OECs, the specimen was classified as Grade 2; a specimen with between 10 and 25% OECs was classified as Grade 3; between 25 and 50%, Grade 4; and more than 50%, Grade 5 (Fig. 2). The percentage of OECs on each plate was estimated by gross microscopy, rather than direct cell-counting. We are presently investigating the possibility of fluorescence-activated cell sorting techniques, but this is presently limited by a degree of cell loss during counting.

Surgical Procedure

Surgery was performed by an experienced neurosurgeon (MP) who specializes in transsphenoidal approaches. The patient was placed supine with 15-degree head extension. An incision was made to raise the
mucosa off the nasal septum, and mucosal dissection was extended to the vomer with a “hockey-stick” dissector and microscope. With image-intensifier guidance, between one and three biopsies were taken using an Angel-James or Williams rongeur from the mucosa in the superior aspects of the operative field. The position of the biopsy was recorded with an x-ray photograph. After the biopsies had been taken, the standard transphenoidal operation was performed, followed by insertion of nasal tampons for 24 hours.

Culture Procedure

The specimens were transferred to Hanks’ Balanced Salt Solution (calcium and magnesium free; Invitrogen, Ltd., Paisley, UK) supplemented with 100 U/ml of penicillin and 100 µg/ml of streptomycin (Invitrogen) and separated from blood clots and debris. After removal of excess solution, each specimen was weighed on an analytical balance (Precisa 2x27MA-FR, readability 0.1–0.01 mg; Precisa Balances, Ltd., Milton Keynes, UK), and the greatest diameter was measured. Specimens were then transferred to 35-mm dishes filled with culture medium consisting of Dulbecco’s Modified Eagle Medium/Ham’s Nutrient Mixture (Logan, UT) F-12 (DMEM/F12) (1:1 DMEM/F12 with GlutaMAX [Invitrogen]), 1% insulin-transferrin-selenium (1.0 mg/ml insulin, 0.67 mg/ml transferrin, and 0.55 mg/ml selenium) (insulin-transferrin-selenium; Invitrogen), penicillin and streptomycin antibiotic solution, and 10% deactivated fetal calf serum (Invitrogen).

The specimens were then cut into small pieces (approximately 0.5 mm²), which were transferred and incubated in 2 ml of collagenase Type I solution (Sigma-Aldrich Company, Ltd., Gillingham, UK) 0.25% in DMEM/F12 medium at 37°C for 5 minutes. The pieces were trituration to dissociate the cells using a flame-polished Pasteur pipette, and the enzymatic reaction was stopped by adding 8 ml of Hanks’ Balanced Salt Solution. After centrifugation at 300 × g for 5 minutes and discarding of the supernatant, the tissue pellet was further trituration into cell suspension in a medium composed of DMEM/F12 with GlutaMAX supplemented with ITS, 10% fetal calf serum, and penicillin and streptomycin to a cell density of 2 to 2.5 × 10⁴/cm². The resulting cells were seeded on 35-mm culture dishes coated with poly-L-lysine 0.1 mg/ml (Sigma-Aldrich) and maintained in a humidified incubator enriched with 5% CO₂ for 14 days at 37°C. The culture medium was replaced every 3 days. The cell cultures were observed with daily light microscopy under aseptic conditions. Beyond 14 days of culture, fibroblast proliferation outstripped the growth rate of OECs, and at 14 days, the number of OECs was felt to be optimum.

Immunocytochemistry

After 14 days in culture, the cells were rinsed in 0.1 mol/L phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde in PBS (pH 7.4) for 30 minutes, washed in PBS, blocked in 1% skim milk powder (Merck Sharp & Dohme, Ltd., Herts, UK) in PBS for 30 minutes, and then incubated in primary antibody solution at 4°C overnight. After washing in PBS, the cultures were incubated with appropriate fluorophore-conjugated secondary antibodies in PBS for 1 hour at room temperature. The primary antibodies used to identify OECs were monoclonal mouse antihuman nerve growth factor receptor (p75; 1 µg/ml) (MAB5386; Chemicon Europe, Ltd., Hampshire, UK) and polyclonal rabbit anti-S100 (1:500) (Nr. Z 0311; Dako UK, Ltd., Cambridgeshire, UK) and, for olfactory nerve fibroblasts, polyclonal rabbit antihuman fibronectin (1:1000) (Nr. A 0245; Dako) was used. Cell proportions were assessed by comparing anti-p75 and anti-fibronectin staining with a cell nucleus background stain (Sytox; Molecular Probes, Eugene, OR). The secondary antibodies used were 1:400 Alexa-488 goat anti-mouse, 1:400 Alexa 488 goat anti-rabbit and 1:400 Alexa-546 goat anti-rabbit (Invitrogen). Cells were visualized, analyzed, and data were collected with a Leica laser scanning confocal microscope (Leica TCS-SP1; Leica Microsystems, Milton Keynes, UK). The quality of the OEC culture was assessed using a five-point scale that compared the number of OECs to the total numbers of cells on the culture dish. The effect of patient age, size, wet-weight of the biopsies, and position were assessed using scatter plots to determine any significant trends.

RESULTS

Biopsies were taken from 32 patients with their informed consent. The OEC yield was assessed by immunohistology and categorized into Grades 0 to 5, from no significant OECs seen (Grade 0) to a good yield (Grade 5) (Fig. 2). The majority of the OECs were triangular and multilayer or spindle-shaped and bipolar with tapering processes up to 200 µm long, but some OECs had a more flattened, multilayer appearance with short processes. In culture, the high-yield samples often segregated into parallel swaths of OECs. Olfactory nerve fibroblasts were typically flattened irregular shaped cells with much cytoplasm and were occasionally more spindle-shaped.

The numbers of biopsies from each region were as follows: none from Region 1; seven from Region 2; four from Region 3; two from Region 4; three from Region 5; eight from Region 6; five from Region 7; and one from Region 8. The mean grade of biopsy for each region was plotted (Fig. 3). Better yields were obtained when biopsies were taken higher on the nasal septum, but this was not statistically significant due to small sample sizes, as summarized in Figure 4.

Scatterplots of the sample weight and greatest dimension did not show any significant correlation with the yield of OECs, although there was a trend of larger samples producing more OECs (Fig. 5 for specimen size, r = 0.49, P > 0.05, and Fig. 6 for weight, r = 0.21, P > 0.05). There was no correlation with the patients’ ages (Fig. 7, r = –0.1). The yield did not improve significantly with successive patients, which suggests that better biopsies were not obtained with greater experience of the surgeon (Fig. 8, r = –0.2).
This study is the first to look at the submucosal septal approach for obtaining OECs from mucosal biopsies. Féron et al. (4) studied 23 patients who were biopsied with ethmoid forceps from within the nasal cavity and found between 40 and 76% yields of olfactory mucosa, with better results from more posterior biopsies. However, the authors studied the yield of mucosa obtained, rather than the yield of OECs, as in our study. Féron et al. went on to map the yield of OECs in different regions, but only studied three patients (1). We thought it was important to map the yield of OECs from different areas of mucosa and chose the septum as a readily accessible region that is straightforward to biopsy. In addition, bacterial contamination is minimized by our use of an endonasal technique. Our objective was to develop a technique for obtaining OECs from the nasal mucosa of a patient with a spinal cord or brachial plexus injury and, after a period of culture and purification, to perform an autologous transplant of the OECs, thereby avoiding the risks of immune rejection and cross-infection that would be associated with an allograft.

Our study shows that OEC yield from the mucosa of the nasal septum is better when taken from the more superior aspects of the septum, but at present this would not provide a
sufficiently reliable OEC yield to proceed with transplantation studies in patients with brachial plexus or spinal cord injuries. The grading system we used was based on the microscopic assessment of cultures by two independent skilled observers. We consider culture yields of Grade 4 and 5 to be sufficient to proceed with the treatment of patients. Building on this study, we are looking at alternative biopsy methods using different instruments and from different regions. The location of the biopsy is probably more important than the size, and we are currently studying biopsies from the lateral wall of the nasal cavity, above and around the superior turbinate, using an endoscopic technique with good preliminary results.

Our culture methods differ from those of other groups (1, 4, 11) in that we do not use nerve growth factors in our media. However, our method has been tried and tested previously by others (5, 13). We have avoided supplementary factors, which are usually derived from animal or human serum, to simplify our application for United Kingdom permissions from the Medicines and Healthcare Products Regulatory Agency. We rely on the natural tendency of OECs to grow into confluent cell layers at approximately 2 weeks of culture, with replating as necessary, and this technique has been optimized in animal models (7–10). Our standard protocol has been developed for culturing OECs from the olfactory bulb, and it is being adapted to culture cells from olfactory mucosa, because this source is more accessible.

However, in the olfactory bulb, there is a high proportion of OECs and very few fibroblasts, whereas the opposite is true in the mucosa. There is an initial burst of cell growth in the first week, which is attributable to fibroblast proliferation, whereas the OECs tend to multiply in the second week. By the third week, the fibroblasts become dominant in number. We are investigating methods to suppress the early multiplication of fibroblasts by using cytarabine and antibody-mediated complement killing. Another alternative would be to separate the cells using a fluorescent-automated cell sorter, culture-independently, then add the relevant cells together before transplantation. However, this technique would involve the loss of cells and might result in an insufficient number of cells for transplant.

**CONCLUSION**

OECs can be cultured from biopsy samples of the adult human septal nasal mucosa. The size of the yields would be limiting for obtaining sufficient cells for useful reparative transplants, and further sampling may identify areas giving higher yields.

**REFERENCES**


**Acknowledgment**

Sources of support: DePuy Spine, British Neurological Research Trust, Spinal Research. This work was undertaken at UCLH/UCL, which received a portion of funding from the Department of Health’s NIHR Biomedical Research Centre’s funding scheme.

**COMMENTS**

Along with bone marrow-derived cells, umbilical cord blood, and other mesenchymal sources, nasal septal mucosa may prove to be a source of neuronal cells for later research or clinical use. Much work needs to be done to characterize these cells (i.e., neurotrophin release), study growth patterns and promote growth in culture, and learn whether the cells are merely interesting or truly valuable. The authors have provided their preliminary efforts, and future studies could go in a myriad of different directions. From their results, it appears that to obtain an autologous cell source for transplantation one must first overcome the problem of limited cell numbers. If that can be achieved, evaluation in animal injury models would be warranted.

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The authors, who have been studying the ability of olfactory ensheathing cells (OECs) for neural regeneration, present their clinical experiences with 32 patients who underwent endoscopic harvesting. This study is notable in that few groups have been able to successfully and reproducibly obtain OECs for potential clinical use, whereas the authors have apparently been able to reliably obtain human OECs for cellular investigations. Their mapping of the nasal mucosa to determine the locations with the highest cellular yield is somewhat crude, but nevertheless, very practical as it is simply based on a lateral x-ray image that can be universally applied.
Ultimately, this approach may be a safe and valuable means for actuating autologous cellular therapies for the treatment of peripheral nerve or spinal cord disorders, and I look forward to seeing future reports from this group on the application of OECs for brachial plexus and spinal cord injuries.

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The transplantation of OECs has been shown to be promising in the treatment of patients with brachial plexus and spinal cord injuries, in part because the cells provide a supportive pathway for regenerating neurons. Any treatment that involves transplantation requires that a source of cells be available for transplantation. This article represents ongoing work by an established group of investigators on the isolation and culturing of OECs for possible transplantation. Previously, the nasal septum mucosa was identified as a possible source of OECs. Given that the nasal septal mucosa is readily harvested during transsphenoidal surgery, the authors have attempted to create a “map” of the nasal septum mucosa to identify particularly fertile areas for biopsy. The authors found that the posterior superior location provided better yields of OECs, but even in those regions, the yields were probably not adequate to produce enough cells to transplant. The authors suggest that the lateral nasal cavity may be more productive. Fortunately, the harvesting of OECs from the lateral nasal cavity and turbinates should pose no greater challenge for experienced neurosurgeons. The results of this article are important in guiding the future harvesting of OECs.

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