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Assessing the Efficacy of Endoscopic Office Olfactory Biopsy Sites to Produce Neural Progenitor Cell Cultures for the Study of Neuropsychiatric Disorders

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Abstract

Introduction—The olfactory region is capable of continuous neurogenesis. Situated on the cribriform plate and segments of the superior septum and both superior and middle turbinates, it is accessible through office-based biopsy and can be used to generate neural progenitor cells to study molecular abnormalities associated with neuropsychiatric disorders.

The purpose of the study was to evaluate the efficacy of the endoscopic office olfactory biopsy from middle turbinate and superior-posterior septum to produce the neural progenitor cells.

Methods—Endoscopic office-based biopsy samples were collected and cultured neuronal cells derived from olfactory neuroepithelium (CNON) were established from 40 healthy individuals and 40 schizophrenia patients. All patients underwent biopsies of both the middle turbinate and the superior-posterior septum. Specific culture conditions promoted the growth of neural progenitor cells from these biopsy sites. CNON cultures were established from such outgrowing neuronal cells. The study was IRB approved and informed consent was obtained.

Results—Cultures were successfully developed from 98.8% of participants. No complications were observed. The single, unsuccessful specimen failed to grow any cell types due to tissue mishandling. Overall, we have observed no significant difference in the effectiveness of biopsy from middle turbinate and superior-posterior septum to produce neural progenitor cells.

Conclusions—The middle turbinate biopsies contain viable neural progenitor cells capable of generating neuronal cell cultures. Thus technically more simple biopsy of the middle turbinate can be used to propagate neural progenitor cells.

The olfactory neuroepithelium (ON) is an accessible source of extracranial neural stem cells and progenitor cells (1, 2). Biopsies of ON can be obtained within 12 hours postmortem or from living patients. Biopsies obtained from living patients have the advantage of correlation of the patient's clinical records (e.g. psychiatric evaluation) with the genetic and molecular studies of the neural progenitor cells.

Various techniques of ON collection and methods of neural stem cell cultures have been described over recent years (3, 4). Biopsies of ON have been obtained in cadavers and in patients during routine endoscopic sinus surgery. The success rate of harvesting and culturing these cells has been reported between 10–90 % (3). Factors implicated in decreased culture rate include the culture method, site of biopsy, type of biopsy forceps utilized and concurrent sinonasal inflammatory disease. Additionally, general anesthesia is required in patients undergoing biopsies during sinus/nasal surgery. A minimally invasive,

office-based procedure to retrieve ON for cultures of neuronal cells would be optimal for studying large groups of patients.

Numerous studies have determined that the superior turbinate and superior-posterior septum have the highest probability of containing olfactory neuroepithelium (3, 5). The studies of Nibu (6) *et al* and Feron *et al* (3) reported the presence of functionally mature olfactory receptor neurons in the anterior and medial part of the middle turbinate. Leopold *et al* (7) found olfactory positive biopsies in this region as well as positive electro-olfactogram (EOG) responses from sites above and below the anterior middle turbinate insertion.

The superior turbinate remains an excellent source for ON biopsy but is limited to collection in the operating room under general anesthesia. It is not technically possible to biopsy the superior turbinate in an office based procedure. The superior posterior septum has been consistently reported as reliable site for ON biopsy and can be accessed in the office procedure, although in some patients an anatomical variations can make obtaining biopsy from this site challenging.

In a previously reported pilot study (8) we described a method to obtain ON for neuroprogenitor cell cultures from four control subjects undergoing routine septoplasty in the operating room and then from 8 control and 8 patients with schizophrenia (SZ) as an in-office procedure. Multiple sites of the septum and superior turbinate were tested in the operating room to determine the best region for biopsy to produce successful neural cell cultures. The superior turbinate followed by superior-posterior septum were the most reliable source of cultured neuronal cells. The superior turbinate is however not accessible for in-office biopsy, so for the office-based procedure group the sample were taken from posterior-superior septum and from the superior-medial aspect of the middle turbinate. Both the posterior-superior septum and middle turbinate were accessible for in-office biopsy and contained ON capable of culturing neuroprogenitor cells.

The purpose of this study is to compare the efficacy of the endoscopic office-based ON biopsy from the middle turbinate and from the superior-posterior septum to produce the neuronal progenitor cells.

PATIENTS and METHODS

Patients recruitment

The study was approved by Institutional Board Review (IRB) at the University of Southern California. The patients were recruited through Los Angeles County/University of Southern California outpatient psychiatric clinic or Department of Psychiatry and the Behavioral Sciences. All patients met the criteria for SZ confirmed by the senior board certified psychiatrist. Additionally all patients had no active allergies or history of sinus disease. The control group was recruited from healthy volunteers with no history of sinus disease or active nasal allergies and no history of psychiatric disorders. The study group included 40 patients with SZ and 40 healthy volunteers as a control group. Informed consent was obtained from all participants of the study.

Biopsy Method

All participant underwent office-based biopsy and tissue samples were obtained from superior-posterior septum and superior medial aspect of the middle turbinate (Figure 1).

First, a diagnostic nasal endoscopy was performed to assess for structural abnormalities such as septal deviation. Based on the anatomy, the most accessible nasal cavity was addressed to obtain tissue. The mucosa was anesthetized beginning by spraying the nasal cavities with a

mixture of 1% lidocaine and 0.05% oxymethazoline, then strategically placing cottonoid pledgets soaked in the same mixture between the middle turbinate and septum. After 5 minutes, 1cc of 1% lidocaine and 1:100,000 epinephrine was injected into the target mucosa under direct visualization with either a 0° or 30° 4mm endoscope. Cupped forceps 2 mm were used to obtain 1–2 mm biopsy specimens (Figure 2).

Culture Technique

The culture technique was described previously in detail in Evgrafov et al (8). Specifically, each biopsy sample was dissected into 2–3 pieces, approximately 1mm³ in size, placed on a culture dish coated with Matrigel basement membrane matrix (BD Biosciences), reconstituted in Coon's medium 1:2, and each piece of tissue was covered by undiluted Matrigel matrix. After incubation of the culture dish at 37°C for 20 minutes, when the Matrigel matrix forms a gel, growth medium was added. Both epithelial and neuronal cells typically start growing from the biopsy pieces inside Matrigel matrix within several days. In 1–4 weeks neuronal cells grew through the Matrigel matrix layer and spread onto the surface of the culture dish. Cloning cylinders were then used to facilitate the isolation of cells with a neuronal phenotype. Cells collected from within these cylinders were washed again with Coon's medium and plated onto another tissue culture dish for further propagation.

Genetic Analysis

Purified RNA samples were extracted from cells using QuickGene RNA cultured cell HC kit S (Fujifilm) according to the manufacturer's protocol and stored at –80°C. RNA-Seq v4 protocol (Illumina) was used for sample preparation of 26 samples. DNA libraries for 9 samples were prepared using TruSeq RNA sample preparation kits. DNA libraries were clustered on cBOT (Illumina) using either cBOT Single Read Cluster generation kit (26 samples) or TruSeq Cluster kit v.3 (9 samples). Samples have been sequenced on either Genome Analyzer II or HiSeq2000 (both instruments are from Illumina, Inc.) for either 36 bp (first 26 samples) or 100 bp (last 9 samples) per fragment. On average, 14.3 million high quality reads per sample have been obtained from sequencing and analyzed using CASAVA 1.8 (Illumina). This software provides expression value for every annotated gene as RPKM - Reads Per Kilobase of gene model per Million of total number of reads for the sample. Immunostaining with antibodies to general neuronal marker PGP9.5, marker of differentiated neuronal cells NEFM, marker of neural stem and neural progenitor cells nestin, marker of glial cells GFAP, was performed to validate expression profiling data and evaluate expression of corresponding genes in cells (Figure 3) Real-time quantitative PCR was then carried out to verify expression.

RESULTS

Olfactory neural progenitor cells were successfully isolated from 79 out of 80 subjects (98.5%). The single, unsuccessful specimen failed to grow any cell types due to tissue mishandling. Typically only one cell culture per individual either from the middle turbinate or posterior superior septum was developed. In every case we observed neuronal cells growing from tissue of both origins. There was no difference in the number of cell lines originated from the posterior superior septum and the middle turbinate.

Microscopic examination of neuronal cells growing inside the Matrigel matrix (3D culture) revealed branching patterns consistent with neuronal cells (Fig. 4. top). As soon as the cells grew out of the Matrigel matrix block (2D culture), their morphology changed, but still showed elongated branches in many cells (Fig. 4, middle). Phenotypically, they are very distinct from epithelial cells. (Fig. 4, bottom) shows neuronal cells reaching monolayer. These changes in cell morphology are very consistent from sample to sample.

Measurement of expression of all genes in expression profiling studies allows very specific classification of cell type. Expression profiling was performed on 34 CNON cell lines, derived both from the posterior-superior septum and the middle turbinate. They also included samples from SZ patients and from the control group. Table 1 shows expression of genes, which expression is characteristic for particular cell types. CNON cells express general neuronal markers at a high level. No expression of glial or epithelial cells clearly classify the cells as neuronal. Low or no expression of markers of terminally differentiated neuronal cells, expression of nestin and lack of expression of markers specific for stem cells define CNON as neural/neuronal progenitor cells. Immunostaining confirms that conclusion. PGP9.5 shows high fluorescence of all cells, GFAP did not stain any cells, NEFM stains ~1% of all cells, and nestin was found in a majority of cells, but the intensity of fluorescence differs substantially between cells. Although there were individual variations in gene expression, expression profiling from every sample demonstrates exactly the same cell type specificity thus proving that developed cell cultures are indeed neural progenitor cells.

DISCUSSION

Early olfactory biopsy research of neuropsychiatric disorders was based on post-mortem tissue samples. Although it was found that viable olfactory neuroepithelium could be isolated within 2–12 hours post-mortem, factors such as decreased tissue pH, loss of RNA integrity, and coexisting medical conditions contribute to significant limitations of this approach.

In 1982, Lovell et al (9) developed a biopsy instrument and technique for obtaining olfactory neuroepithelium specimens for electron microscopy. Biopsies were performed in a total of twelve patients, some with local anesthesia and some in patients undergoing procedures with general anesthesia. The nasal mucosa was prepared with topical anesthetic and decongestant and specifically-designed biopsy forceps were advanced to the superior posterior septum to obtain the specimen. This technique was further modified by Lowry and Pribitkin (10) several years later. The authors outline the materials and methods involved in obtaining biopsies of the superior septum and the adjacent medial surface of the middle turbinate in patients undergoing endoscopic sinus surgery and biopsies taken in the office with local anesthesia. The specimens were then prepared for histological evaluation. The efficacy of the methods and success rate in obtaining the olfactory neuroepithelium was not reported. Feron *et al* (3) obtained 97 biopsy specimens from 33 patients undergoing septoplasty and/or turbinate surgery. Six areas of the olfactory neuroepithelium were biopsied: dorsomedial and dorsoposterior superior turbinate, dorsoanterior and ventroposterior middle turbinate, and the dorsomedial and dorsoposterior septum. Each specimen was divided so that one half was submitted for immunohistochemistry or electron microscopy and the other half for culture. The specimen set aside for culture was further divided to be grown on various media. The probability of identifying olfactory epithelium in biopsy specimens ranged from 30–76% depending on biopsy site. The dorsoposterior region of the septum and superior turbinate gave the highest yield of such cells. One method of culturing described, the “slice culture technique”, resulted in an improved success rate of 10–90% for generating neuronal cell culture. Winstead *et al* (4) performed a study involving endoscopic nasal biopsies at the completion of endoscopic sinus surgery. Samples were obtained from the dorsoposterior septum, medial wall of superior turbinate and posterior aspect of the middle turbinate. Overall, progenitor cells emerged in 50% of 42 enrolled patients, and the superior turbinate gave the highest yield of progenitor cells. The success rate of isolating progenitor cells increased from 23 to 76% with a change in the type of forceps used. In the latter phase of the study, forward-biting giraffe cupped forceps were replaced by through-cutting punch forceps. A limitation of this study cited is coincident chronic rhinosinusitis present in the patients. Girard *et al* (11) proposed a protocol for the collection of olfactory mucosa and

culture methods for isolating olfactory stem cells in rats and humans. The biopsy technique described involves the use of local anesthesia and through-cutting forceps. A literature search failed to identify studies exclusively focused on office-based olfactory biopsies and we are not aware of any investigation into the effectiveness of various biopsy sites to successfully isolate and propagate neural progenitor cells.

In the previous report (8) we obtained biopsies in the operating room under general anesthesia and then progressed to exclusively in-office based procedure. The biopsies in the operating room were performed from superior turbinate and several sites on the septum and confirmed previous reports that superior turbinate, followed by posterior-superior septum were the most reliable sources of ON for neural cell cultures. In the same study, we reported a first pilot group of in-office biopsies from the middle turbinate with ON capable of producing viable progenitor cells cultures.

For in-office procedure middle turbinate is an ideal site of biopsy due to accessibility. Since the biopsy is performed from the medial aspect of the middle turbinate there is no concern of compromising the middle meatus or causing scarring of the middle meatal region.

Our hypothesis that the middle turbinate would be a reliable biopsy site was confirmed by establishing cultures in all but one subject. All of these cultures produced proliferating neural progenitor cells. There were individual variations in time to initiation of neuronal cell growth between individuals, between biopsies taken from the superior turbinate and superior posterior nasal septum for every individual, and between tissue pieces from the same biopsy piece. These variations are partially explained by the variation in size of biopsy pieces. The sizes of tissue pieces after dissecting the biopsy samples also varied in size. Smaller pieces possibly provided better conditions for internal neuronal cells to grow outside the tissue. In some cases, neuronal cell growth was detected only after more than 3 weeks of incubation, although in most cases, outgrowing neuronal cells could be found within a week after plating. All of these variables make quantitative assessment impossible. However, we have not observed any clear overall difference in efficiency of neuronal cell growing between biopsy samples from either site. We believe that single source of biopsy material either from either regions would result in cell culture development in over 90% of the biopsies.

For the purpose of providing tissue for culture of neural progenitor cells we found that the biopsy of ON from the middle turbinate is as effective as the superior turbinate and superior posterior septum.

In this study we did not perform histological evaluation of ON. There is no implication that the middle turbinate is as effective as the superior turbinate or posterior-superior septum in providing samples of ON for histological evaluation.

The results suggest that the in-office endoscopic biopsy of ON method could frequently replace the method of intraoperative tissue collection given the ease of obtaining tissue from the middle turbinate region. The high success rate of positive cell cultures (over 90%) in our study compare to similar past studies, may be due to absence of sinonasal disease in all our participants. The inclusion criteria for both control and study group was no history chronic rhinosinusitis or active allergic rhinitis. Since chronic sinonasal inflammation may lead to loss of olfactory neural elements (Kern, 12), the presence of inflammatory changes of the nasal mucosa in general, may impede the ability to produce neural progenitor cells when compared to healthy individuals. If biopsy needs to be performed on patients with history of chronic rhinosinusitis or inhalant allergies, the factors like accessibility of biopsy site, severity of inflammation, presence of structural abnormalities will be all influencing the decision which biopsy site to target. Possibly lower success rate can be anticipated in the presence of sinonasal inflammatory disorders.

The high positive cell cultures in our study are also due to effective tissue handling and culture methods employed.

Variety of instruments has been used for similar studies, and a higher success rate of culture growth with through-cutting forceps has been cited (4). On the other hand studies concentrated on immunohistologic analysis of ON, have suggested that cupped forceps may provide better specimen than specifically designed through cutting forceps (13, 14). We used only cupped forceps for in-office biopsy in all participants. We found that cupped forceps were easier to use without necessitating deep bites of tissue and in our pilot study biopsies obtained with cupped forceps resulted in 100 % neuroprogenitor cell growth.

CONCLUSION

The in-office endoscopic biopsy technique and culture methods described provide an efficient method for obtaining ON and isolating neural progenitor cells. We have found no difference in the ability of the different biopsy sites to produce neural progenitor cells from the superior medial aspect of the middle turbinate and posterior-superior septum. The middle turbinate is easily accessible for an in-office endoscopy and should be consider as one of the primary sites for retrieval of ON for neuronal cell cultures.

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Figure 1.
Endoscopic view of the right nasal cavity showing biopsy sites: medial aspect of the middle turbinate and adjacent superior septum.

Figure 2.
Biopsy of medial aspect of the middle turbinate with cupped forceps.

Figure 3.
Immunostaining with antibodies to a number of neuronal markers

Figure 4. Cultured neuronal cells derived from neuroepithelium. Top: Neuronal and epithelium cells growing from biopsy sample inside Matrigel matrix. Middle: Neuronal cells growing on the surface of culture dish. Bottom: Neuronal cells reaching monolayer on the susurface of culture dish.

Table 1

Expression of different cell-type specific markers in CNON cells (in RPKM).

	Gene/marker	RPKM
Glial markers	S100B	0
	GFAP	0
	OLIG2	0
Epithelial markers	KRT5	0
	EPCAM (TACSTD1)	0
	CDH1	0
Neuronal markers	UCHL1	359.22
	MAP1A	53.028
	MAP1B	109.48
	L1CAM	17.106
	TUBB3	10.263
	CDH2	11.974
	INA	6.8423
Markers of differentiated neurons	OMP	0
	GNAL	0
	NEFL	0
	NEFM	3.42
	NEFH	0
	MAP2	8.55
Stemness markers	NES	34.21
	POU5F1	0
	SOX2	0
	NANOG	0
Other markers	NGFR	0
	NRP1	138.56
	BEX1	417.38
	MKI67	30.79
	NCAM1	0.68
	TERT	0
	ACTB	2095.4