European Journal of Ophthalmology / Vol. 17 no. 4, 2007 / pp. 501-506

Impression cytology of melanocytic conjunctival tumors using the Biopore membrane

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PURPOSE. *To compare a new Biopore membrane impression cytology method with the routinely used exfoliative cytology in patients with a melanocytic lesion of the conjunctiva.*
METHODS. *Sixty-eight consecutive patients with a conjunctival melanocytic lesion underwent Biopore membrane impression cytology as well as exfoliative cytology. A histologic sample was also available in 26 cases. All Biopore samples were stained immediately with RAL 555. Both Biopore and exfoliative cytology samples were assessed by two cytopathologists and graded into four different categories of atypia.*
RESULTS. *Twenty-three out of 26 Biopores and 20 out of 24 for the exfoliative smears correlated with the corresponding histologic sample. Biopore cytology resulted in higher numbers of cells with a greater density compared to exfoliative cytology.*
CONCLUSIONS. *Biopore cytology can be used for cytologic sampling of conjunctival melanocytic lesions. Because of the larger amount and higher density of cells obtained with the Biopore membrane, interpretation by a pathologist is easier and faster. Sampling of the fornix, caruncula, and ocular material in children is difficult with the Biopore method, and exfoliative cytology seems to be the favorable test in those situations. (Eur J Ophthalmol 2007; 17: 501-6)*

KEY WORDS. *Biopore, Conjunctival melanoma, Cytology, Diagnostic, Histology*

Accepted: February 20, 2007

INTRODUCTION

Conjunctival melanoma is a rare malignant tumor, accounting for 2 to 3% of all ocular tumors (1, 2). The incidence of conjunctival melanoma in Caucasians is 0.02–0.08 per 100,000 inhabitants (3-6). Conjunctival primary acquired melanosis (PAM) is the most frequently reported precursor of conjunctival melanoma and in general affects the limbal and bulbar conjunctiva, although some conjunctival melanomas evolve from pre-existing nevi or develop de novo (3-9). Clinically, the differentiation between PAM and a nevus with or without progression to melanoma is often difficult (10), and a biopsy for histologic examination can be obtained. Cytology could be an alternative to diagnostic biopsies, and is a minimally invasive diagnostic tool, which can also specify the risk of the lesion developing into a conjunctival melanoma without the need for biopsy, as especially the severe atypia is correlated with the presence of a conjunctival melanoma (11). Cytology can therefore help the ophthalmologist in the diagnosis and subsequent treatment of conjunctival pigmented lesions and follow-up after observation or mitomycin-C treatment.

Exfoliative cytology and impression cytology are two different techniques to acquire cells for cytologic analysis. In exfoliative cytology, cells are collected with a cotton-wool swab and mounted on glass slides (12). Impression cytology is either done with cellulose acetate filters or by use of a Biopore membrane (13, 14); the cellulose acetate filters have already been tested on conjunctival melanocytic

 1120-6721/501-06$15.00/0

lesions (15). The Biopore membrane has already been used in patients with superficial viral infections, and in case of ocular surface squamous neoplasia (14, 16, 17). Biopore impression cytology is a newer technique that provides a relatively large surface, and can therefore strip off a high amount of cells, still in their original configuration. In this study we investigated whether the Biopore can be used to interpret a melanocytic lesion, and compared the advantages and disadvantages with exfoliative cytology.

PATIENTS AND METHODS

Patients

Sixty-eight patients with a pigmented conjunctival lesion underwent both Biopore and exfoliative cytologic sampling between April 2003 and November 2004 (Tab. I).
There were 33 men with a mean age of 42 years (SD: 22.9, range 8 to 87), and 35 women with a mean age of 49 years (SD 26.4, range 8 to 92). All patients came from the outpatient clinic of the Department of Ophthalmology at Leiden University Medical Centre, Leiden, The Netherlands. Of 26 of the 68 patients, a histologic sample was available. The study was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants.

Technique

The eye with the melanocytic lesion was first sampled with the Biopore impression cytology method. The Biopore (Millicell-CM 0.4 µm PICM 012550, Millipore Corp, Bedford, MA) is an 8-mm round membrane disc, which is placed in a plastic ring. Before sampling, three plastic legs are removed from the plastic ring. To obtain a firmer grip on the Biopore membrane, the device is placed in a slightly larger plastic tube. The eye is anesthetized with one to two drops of 0.4% oxybuprocaine (mono free, Théa Pharma, Ukkel, Belgium), and the eyelids are opened for a few seconds to dry the conjunctiva to improve the adherence of cells onto the Biopore membrane. The Biopore membrane is pressed gently onto the conjunctiva, after 3–5 seconds the Biopore is removed and immediately fixed and stained with RAL 555 (555-FIX-RAL, 555-Eosin-RAL, 555-Blue-RAL, Reactifs RAL Bordeaux Technopols, Martillac, France). The Biopore membrane is submerged in each of the three RAL 555 solutions (methanol, eosin, methylene blue) for approximately 10 seconds. After staining, the membrane is cut out with a 15-degree knife and fixed with mounting medium on a glass slide for microscopic evaluation.
After Biopore sampling, the same lesions are swabbed with a cotton-wool tip for exfoliative cytology. The cells on the cotton-wool tip are than transferred to several glass slides; the procedure is repeated three times to acquire more cells for analysis. The glass slides of the exfoliative cytology are processed under standard protocol used in our hospital.

Cytologic interpretation

Exfoliative cytology and Biopore membranes were all interpreted by two cytopathologists (M.V.C. and S.V.). When there was disagreement between the two observers a third independent observer made the final decision. All Biopore samples were numbered, and bias through prior knowledge of the exfoliative cytology was therefore excluded. Both exfoliative smears as well as Biopore samples were graded by a standard grading system used in our hospital. In brief, the samples were screened for nuclear size, nuclear to cytoplasmic ratio, irregular nucleus, irregular nuclear chromatin pattern, and prominent nucleoli, and subsequently graded into four different stages: 0: insufficient material for diagnosis; 1: normal epithelial

TABLE I - PATIENT CHARACTERISTICS

Characteristics	No. (%)
Gender	
Male	33 (49)
Female	35 (51)
Clinical diagnosis	
Nevus	31 (46)
PAM	28 (41)
Melanoma	9 (13)
Location	
Caruncula	10 (15)
Fornix	3 (4)
Bulbus	55 (81)
Limbal*	36 (65)

*Number of bulbar lesions that were located at the limbus.
PAM = Primary acquired melanosis

TABLE II - CROSS TABLE FOR BIOPORE AND EXFOLIATIVE CYTOLOGY GRADING

		Exfoliative grading					
		0	1	2	3	4	Total
Biopore grading	0		1				1
	1	1	20	13		4	38
	2		1	11	3	1	16
	3	1			5		6
	4	1				6	7
	Total	3	22	24	8	11	68

Numbers within the dotted-lined squares represent the cases where Biopore and exfoliative cytology were graded similarly

conjunctival cells with or without melanin pigment, reactive conjunctival cells as seen in inflammation; 2: melanocytes with mild atypia; 3: melanocytes with moderate atypia; 4: melanocytes with severe atypia (11). The amount of cells collected (low, moderate, high, very high) was noted for all samples.

Statistics

Data were analyzed in SPSS 11.0 (SPSS Inc., Chicago, IL, USA). Differences in amount of cells harvested were calculated with a paired samples *t*-test. Fisher exact test was used to calculate the significance of the differences in percentage of conjunctival melanomas detected by both methods. It was also used to calculate the differences in correlation between both methods and the histologic diagnosis.

TABLE III - CORRELATION BETWEEN HISTOLOGIC DIAGNOSIS AND CYTOLOGIC DIAGNOSIS OF THE SAME CONJUNCTIVAL MELANOCYTIC LESIONS

	Correct correlation	
Histologic diagnosis	**Exfoliative smears, n (%)**	**Biopore, n (%)**
Nevus	12 (75)	13 (92)
PAM without atypia	2 (100)	2 (100)
PAM with atypia	—	1 (100)
Melanoma	9 (89)	9 (78)
Pigmented pingueculum	1 (100)	1 (100)
Total	24 (83)	26 (88)

PAM = Primary acquired melanosis

RESULTS

Biopore provided a cytologic diagnosis in 67 out of the 68 samples (99%), whereas exfoliative cytology was able to give a diagnosis in 65 out of the 68 samples (96%). In all four cases (one Biopore, three exfoliative smears) this was due to a very low cell count, therefore graded in category 0. There was concordance between the two observers in 58 out of 68 Biopores (85%) and 47 out of 68 exfoliative smears (69%). In 9 of the 10 Biopore disagreements and 19 of the 21 exfoliative disagreements there was only one grade of difference in atypia. In 64 patients both a Biopore and an exfoliative sample of the same lesion were graded. In 42 of these 64 patients (66%), Biopore and exfoliation were graded in the same category, 25 (33%) of the Biopores were graded in a lower category, and 1 (2%) Biopore was graded in a higher category than the corresponding exfoliative sample (Tab. II). Figure 1 shows exfoliative cytology, Biopore cytology, and histology for four different cases.

A corresponding histologic sample was available for 26 Biopores and 24 exfoliative smears. The histologic diagnosis was confirmed by the Biopore in 23 of 26 cases (88%), and in 20 of 24 (83%) exfoliative smears (p=0.697, Fisher exact test) (Tab. III). We previously noted that atypia grade 3 and 4 should be considered as a positive clinical marker for conjunctival melanoma (11). Of all histologic samples, 9 were conjunctival melanomas. Of these, 7 out of 9 (78%) Biopores and 8 out of 9 (89%) exfoliative samples had an atypia grade 3 or 4 (p=1.0, Fisher exact test).

Biopore sampled significantly more cells from the con-

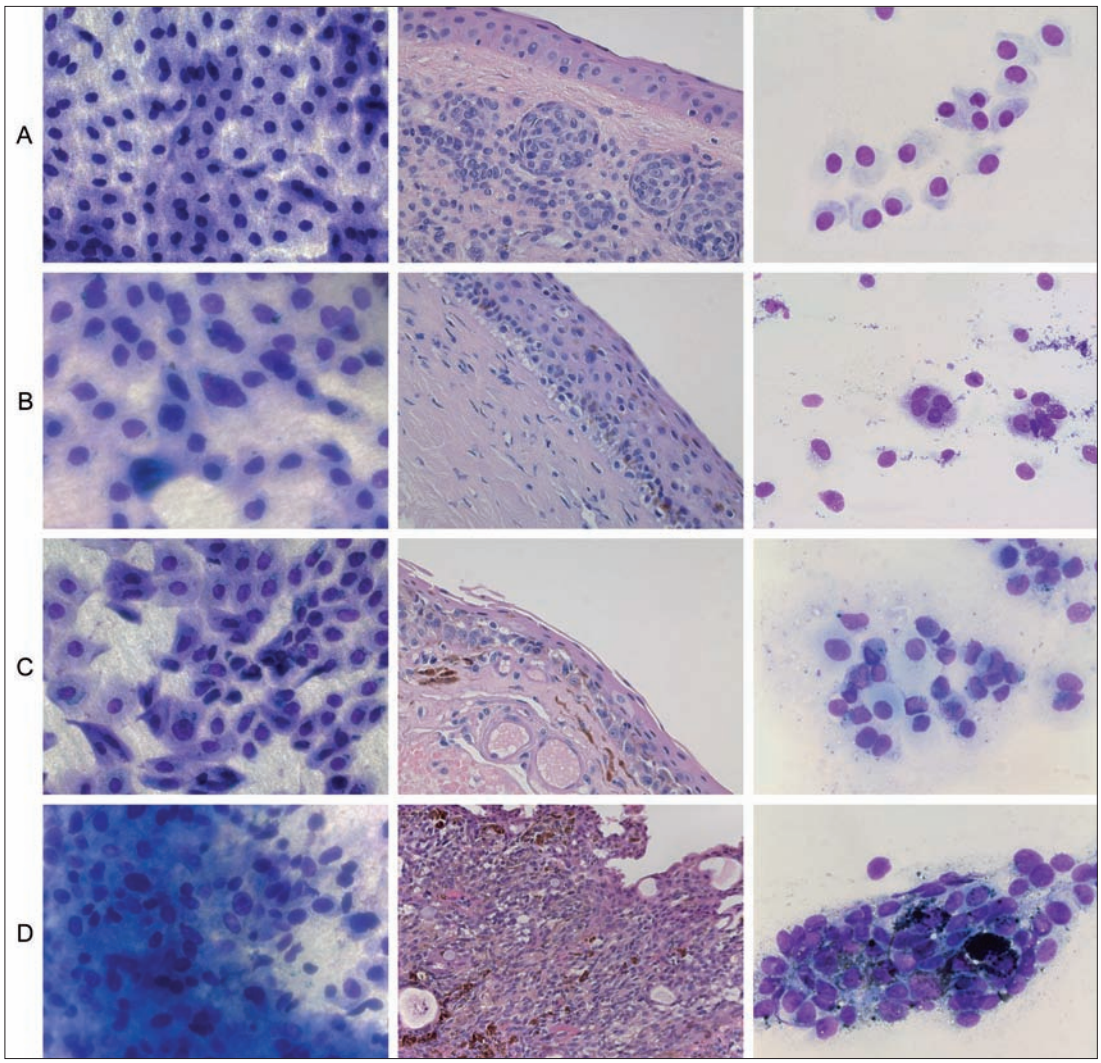

Fig. 1 - *Biopore (left column), histology (middle column), and exfoliative cytology (right column) samples are shown for four different cases.* **(A)** *Subepithelial conjunctival nevus in a 51-year-old woman. Exfoliative and Biopore cytology sampled normal cells since the lesion is located underneath the epithelium.* **(B)** *Primary acquired melanosis (PAM) at the limbal region in a 23-year-old woman. Histology and both cytology methods show mild atypia.* **(C)** *PAM at the limbal region in a 57-year-old man. Histology and both cytology methods showed PAM with moderate atypia. Note the fine pigmentation around the nucleus in the Biopore and exfoliative samples.* **(D)** *Conjunctival melanoma at the bulbar conjunctiva in an 83-year-old woman. Histology and cytology show severe atypia.*

TABLE IV - AMOUNT OF CELLS

	Biopore, n (%)	Exfoliation p<0.0001 paired t-test, n (%)
Low amount of cells	9 (14)	14 (21)
Medium amount of cells	16 (24)	27 (40)
High amount of cells	15 (23)	26 (38)
Very high amount of cells	28 (39)	1 (1)
Total	68 (100)	68 (100)

junctival surface than exfoliative cytology (p<0.001, paired sample *t*-test) (Tab. IV, Fig. 1). Fewer cells were collected with the Biopore when the lesion was situated in the caruncula (p=0.03, *t*-test), primarily because the relatively larger Biopore was not able to reach the lesion properly.

DISCUSSION

A variety of pigmented lesions can exist in the conjunctiva, and can be clinically and histologically divided into nevi, melanosis, and malignant melanoma. All these lesions can be further histologically subdivided. In only a part of the melanocytic lesions, melanocytes will arise to the epithelial surface, such as in juvenile intraepithelial nevi, compound nevi, adult onset PAM with moderate and severe atypia, and conjunctival melanoma (18). Since some melanocytic lesions will be covered with one or more layers of normal epithelium, cytology can only give a realistic picture of a lesion when it is able to sample deeper than the most superficial layer of epithelial cells. Exfoliative cytology is able to sample more than one epithelial layer since the lesion is rubbed three times on the same

spot. Biopore, however, will sample only the first layer of cells on the conjunctiva, unless the Biopore is repeated several times to acquire cells of deeper layers. Similarly, impression cytology with cellulose acetate filters is able to sample deeper layers of the conjunctiva when performed repeatedly (19). This could probably explain why 33% of the Biopores were graded in a lower category than the corresponding exfoliative smear, since in most cases only one Biopore was sampled. Further studies need to prove whether the Biopore is able to sample deeper layers when performed repeatedly.

The most important task for cytology is to detect conjunctival melanomas. Exfoliative cytology was able to detect 89% of the melanomas and Biopore was able to detect 78%. One of the missed conjunctival melanomas with the Biopore technique was situated in the caruncula, which is a difficult location to sample with the relatively large and flat Biopore. The second conjunctival melanoma was situated under the conjunctival epithelium (local in-transit-metastasis), and could therefore not be reached by both Biopore and exfoliative method. However, when all histologic samples were taken into account, Biopore correctly predicted the outcome in 88% of the lesions, and exfoliative cytology in 83%. Other authors also found similar correlations between cytology and histology (15, 16, 20). Besides the difficulty of the Biopore to sample the caruncula and fornices, we experienced that the relatively large Biopore is also difficult for sampling in young children.

An advantage of the Biopore is the high yield of cells that are collected on a relatively small surface. The high density of cells makes interpretation also faster when compared to exfoliative smears. With Biopore, the pathologist only has to screen approximately 50 mm^2 as compared to exfoliative smears where a total surface of 900 mm^2 has to be screened microscopically. The Biopore also had fewer disagreements in atypia classification between the two observers than the exfoliative samples. Since most of the high risk samples (grade 3 and 4) remained in this category, the disagreements between the two observers were not of major influence for the clinician.

Recently, Singh et al recommended the introduction of impression cytology for routine clinical practice in major ophthalmic centers (19). We agree that impression cytology (Biopore or cellulose acetate filters) and/or exfoliative cytology should be available to ophthalmologists in major centers, since these minimally invasive techniques can help the ophthalmologist in the diagnosis of a variety of ocular surface diseases.

CONCLUSIONS

Biopore can be used in cytology of melanocytic lesions and is easier and faster to interpret than exfoliative cytology. When a cytologic test is indicated, the Biopore can be used complementary to exfoliative smears on bulbar lesions, while exfoliative cytology alone is preferable on lesions situated in the caruncula and fornix, and in young children.

Proprietary interest: None.

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