New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

Keijser, S.

CHAPTER 5

IMPRESSION CYTOLOGY OF MELANOCYTIC CONJUNCTIVAL TUMOURS USING THE BIOPORE MEMBRANE

European Journal of Ophthalmology 2007;17:501-506

S. Keijser,[1] G.S. Missotten,[1] D. de Wolff - Rouendaal,[1]
S.L.J. Verbeke,[2] C.M. van Luijk,[1] M. Veselic-Charvat,[2]
R.J.W. de Keizer.[1]

1 Department of Ophthalmology, Leiden University Medical Center, The Netherlands
2 Department of Pathology, Leiden University Medical Center, The Netherlands

Abstract

Background: 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 histological 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 cyto-pathologists and graded into four different categories of atypia.

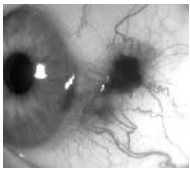
Results: Twenty-three out of 26 Biopores and 20 out of 24 for the exfoliative smears correlated with the corresponding histological sample. Biopore cytology resulted in higher numbers of cells with a greater density compared to exfoliative cytology.

Conclusions: Biopore cytology can be used for cytological 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, caruncle, and sampling of ocular material in children is difficult with the Biopore method, and exfoliative cytology seems to be the favourable test in those situations.

Introduction

Conjunctival melanoma is a rare malignant tumour, accounting for 2-3% of all ocular tumours.[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 cytological 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 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 cytological sampling between April 2003 and November 2004 (Table 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 histological 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, USA) 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

Table 1. Patient characteristics.

Characteristics	*Number*	*(percentage)*
Gender		
Male	33	(49)
Female	35	(51)
Clinical diagnosis		
Nevus	31	(46)
PAM	28	(41)
Melanoma	9	(13)
Location		
Caruncle	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

membrane, the device is placed in a slightly larger plastic tube. The eye is anaesthetized with 1-2 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, 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.

Cytological interpretation

Exfoliative cytology and Biopore membranes were all interpreted by two cyto-pathologists (MVC and SV). When there was disagreement between the two observers a third independent observed 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 cytoplasmatic 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 conjunctival cells with or without melanin pigment, reactive conjunctival cells as seen in in-

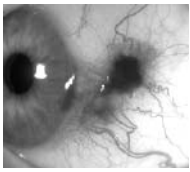
flammation, 2: melanocytes with mild atypia, 3: melanocytes with moderate atypia, 4: melanocytes with severe atypia.[11] The amount of cells collected (low, moderate, high, very high) was noted for all samples.

Statistics

Data was analysed in SPSS 11.0 (SPSS Inc, Chicago, USA). Differences in amount of cells harvested were calculated with a paired samples t-test. The Fisher's 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 histological diagnosis.

Results

Biopore provided a cytological diagnosis in 67 out of the 68 samples (99 %), where 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, and were 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. Of 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 one (2 %) Biopore was graded in a higher category than the corresponding exfoliative sample (Table II). Figure I shows exfoliative cytology, Biopore cytology, and histology for four different cases.



A corresponding histological sample was available for 26 Biopores and 24 exfoliative smears. The histological diagnosis was confirmed by the Biopore in 23 of 26 cases (88 %), and in 20 of 24 (83 %) exfoliative smears (p=0.697, Fisher's Exact Test) (Table III). We

Table 2. Cross table for Biopore and exfoliative cytology grading. Numbers within the dotted-lined squares represent the cases where Biopore and exfoliative cytology were graded similarly.

Biopore grading	Exfoliative grading					
	0	1	2	3	4	Total
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

Table 3. Correlation between histological diagnosis and cytological diagnosis of the same conjunctival melanocytic lesions.

	Correct correlation	
Histological diagnosis	Exfoliative smears % (numbers)	Biopore % (numbers)
Naevus	75 (12)	92 (13)
PAM without atypia	100 (2)	100 (2)
PAM with atypia	-	100 (1)
Melanoma	89 (9)	78 (9)
Pigmented piqueculum	100 (1)	100 (1)
Total	83 (24)	88 (26)

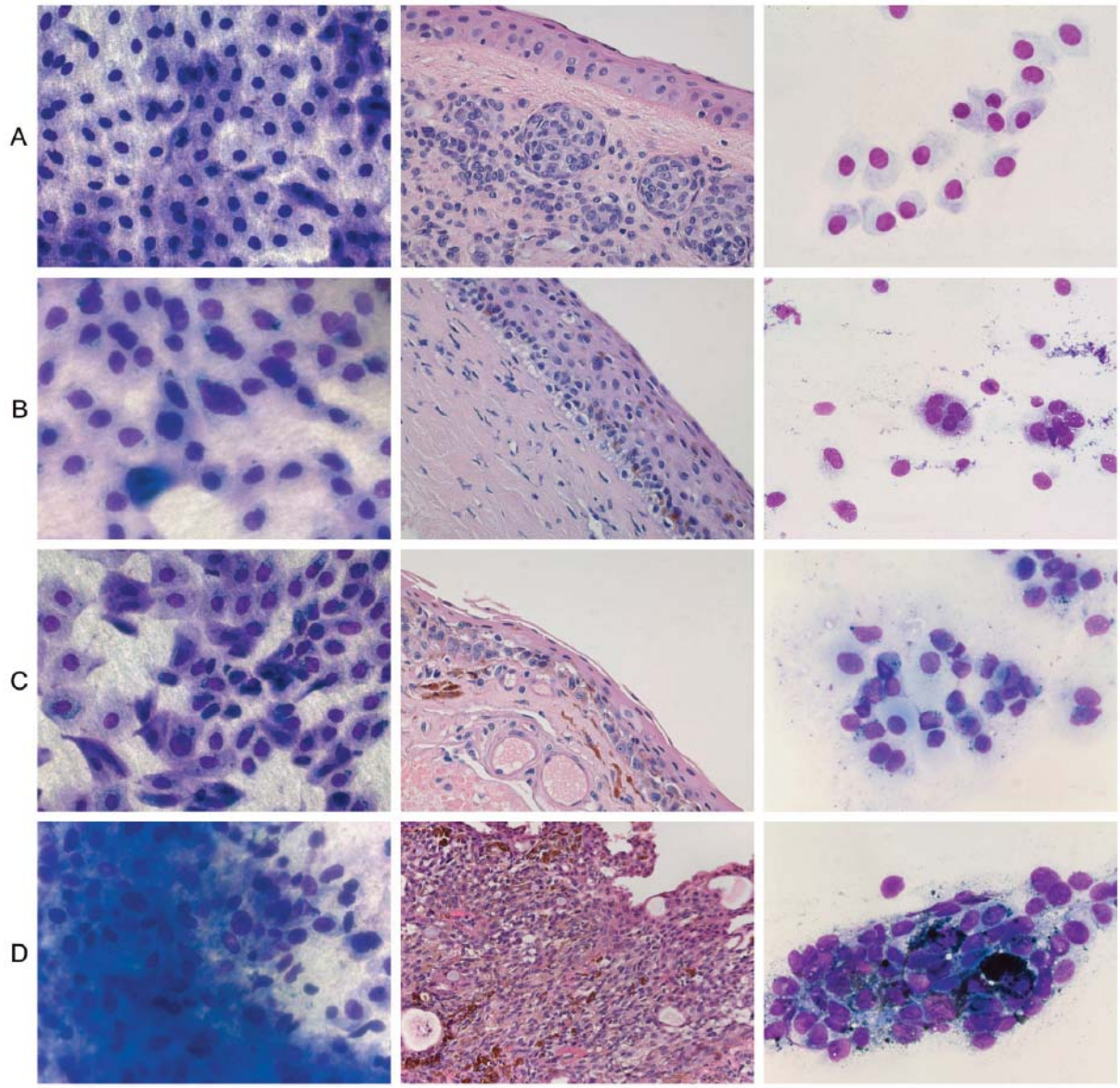
Table 4. Amount of cells

	Exfoliative smears numbers (%)	Biopore numbers (%)	p<0.0001 paired T-test
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)	

previously noted that atypia grade 3 and 4 should be considered as a positive clinical marker for conjunctival melanoma.[11] Of all histological samples, nine were conjunctival melanomas. In these, seven out of nine (78 %) Biopores and eight out of nine (89 %) exfoliative samples had an atypia grade 3 or 4 (p=1.0, Fisher's Exact Test).
Biopore sampled significantly more cells from the conjunctival surface than exfoliative cytology (p<0.001, paired sample T-test) (Table IV, Figure I). Fewer cells were collected with the Biopore when the lesion was situated in the caruncle (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 in 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



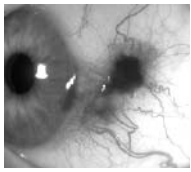


Figure 1. Biopore (left hand column), histology (middle column), and exfoliative cytology (right hand column) samples are shown for four different cases. Figure IA shows a subepithelial conjunctival nevus in a 51 year old female. Exfoliative and Biopore cytology sampled normal cells since the lesion is located underneath the epithelium. Figure IB represents PAM at the limbal region in a 23 year old female. Histology and both cytology methods showing mild atypia. Figure IC represents a PAM at the limbal region in a 57 year old male. Histology and both cytology methods showed PAM with moderate atypia. Note the fine pigmentation around the nucleus in the Biopore and exfoliative samples. Figure ID represents a conjunctival melanoma at the bulbar conjunctiva in a 83 year old female. Histology and cytology showing severe atypia.

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 was 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 caruncle, 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 histological 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 caruncle and fornices, we experienced that the relatively large Biopore is also difficult for sampling in young children.

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 mm2 as compared to exfoliative smears where a total surface of 900 mm2 has to be screened microscopically. The Biopore also had less 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 was not of major influence for the clinician.

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

Conclusion

Biopore can be used in cytology of melanocytic lesions and is easier and faster to interpret than exfoliative cytology. When a cytological 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 caruncle and fornix, and in young children.

References

1. Keller AZ. Histology, survivorship and related factors in the epidemiology of eye cancers. *Am J Epidemiol* 1973; 97: 386-93.
2. Char DH. The management of lid and conjunctival malignancies. *Surv Ophthalmol* 1980; 24: 679-89.
3. Seregard S. Conjunctival melanoma. *Surv Ophthalmol* 1998; 42: 321-50.
4. Tuomaala S, Aine E, Saari KM, Kivela T. Corneally displaced malignant conjunctival melanomas. *Ophthalmology* 2002; 109: 914-19.
5. Missotten GS, Keijser S, De Keizer RJ, Wolff-Rouendaal D. Conjunctival melanoma in the Netherlands: a nationwide study. *Invest Ophthalmol Vis Sci* 2005; 46: 75-82.
6. Lommatzsch PK, Lommatzsch RE, Kirsch I, Fuhrmann P. Therapeutic outcome of patients suffering from malignant melanomas of the conjunctiva. *Br J Ophthalmol* 1990; 74: 615-19.
7. Paridaens AD, Minassian DC, McCartney AC, Hungerford JL. Prognostic factors in primary malignant melanoma of the conjunctiva: a clinicopathological study of 256 cases. *Br J Ophthalmol* 1994; 78: 252-59.
8. Werschnik C, Lommatzsch PK. Long-term follow-up of patients with conjunctival melanoma. *Am J Clin Oncol* 2002; 25: 248-55.
9. de Potter P, Shields CL, Shields JA, Menduke H. Clinical predictive factors for development of recurrence and metastasis in conjunctival melanoma: a review of 68 cases. *Br J Ophthalmol* 1993; 77: 624-30.
10. Wolff-Rouendaal de D. Management of conjunctival tumours. In: Oosterhuis JA, editor. Ophthalmic tumours. Dordrecht: Dr Junk Publishers, 1985:159-71.
11. Keijser S, van Luijk CM, Missotten GS, Veselic-Charvat M, Wolff-Rouendaal D, de Keizer RJ. Predictive value of exfoliative cytology in pigmented conjunctival lesions. *Acta Ophthalmol Scand* 2006; 84: 188-91.
12. Lopez Cardozo P, Oosterhuis JA, de Wolff-Rouendaal D. Exfoliative cytology in the diagnosis of conjunctival tumours. *Ophthalmologica* 1981; 182: 157-64.
13. Egbert PR, Lauber S, Maurice DM. A simple conjunctival biopsy. *Am J Ophthalmol* 1977; 84: 798-801.
14. Thiel MA, Bossart W, Bernauer W. Improved impression cytology techniques for the immunopathological diagnosis of superficial viral infections. *Br J Ophthalmol* 1997; 81: 984-88.
15. Paridaens AD, McCartney AC, Curling OM, Lyons CJ, Hungerford JL. Impression cytology of conjunctival melanosis and melanoma. *Br J Ophthalmol* 1992; 76: 198-201.
16. Tole DM, McKelvie PA, Daniell M. Reliability of impression cytology for the diagnosis of ocular surface squamous neoplasia employing the biopore membrane. *Br J Ophthalmol* 2001; 85: 154-58.
17. McKelvie PA, Daniell M. Impression cytology following mitomycin C therapy for ocular surface squamous neoplasia. *Br J Ophthalmol* 2001; 85: 1115-19.
18. McLean IW, Burnier MN, Zimmerman LE, Jakobiec FA. Tumors of the conjunctiva. In: Rosai J, editor. Atlas of tumor pathology. Tumors of the eye and ocular adnexa. Washington DC: Armed Forces Institute of Pathology, 2003:72-88.
19. Singh R, Joseph A, Umapathy T, Tint NL, Dua HS. Impression cytology of the ocular surface. *Br J Ophthalmol* 2005; 89: 1655-59.
20. Wolff-Rouendaal de D. Conjunctival melanoma in the Netherlands: a clinico-pathological and follow-up study. Thesis. University of Leiden, Leiden, The Netherlands, 1990.