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An in vivo confocal microscopy and impression cytology analysis of preserved and unpreserved levobunolol-induced conjunctival changes

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PURPOSE. *To provide an in vivo confocal microscopy (IVCM) and impression cytology analysis of preserved- and unpreserved levobunolol-induced changes of conjunctival epithelium.*
METHODS. *27 eyes of 27 patients were consecutively randomized to receive preserved or unpreserved levobunolol; all patients had a recent diagnosis of primary open angle glaucoma (POAG) or ocular hypertension and were not previously treated with topical medications. IVCM and impression cytology were performed before and after six months of therapy. Goblet cells density and a conjunctival epithelium regularity index were considered in the IVCM analysis, whereas impression cytology specimens were graded and scored in accordance with Nelson's method.*
RESULTS. *After six months of therapy, IVCM and impression cytology parameters showed significant differences with respect to baseline in both groups ($p<0.001$); significant differences were also found between the two groups ($p<0.001$). The IVCM analysis showed a goblet cells density reduction (61% and 17% from baseline, respectively in group 1 and 2) ($p<0.001$) and an higher index of epithelial regularity ($p<0.001$) in both groups; the impression cytology analysis showed an higher score in both groups ($p<0.001$).*
CONCLUSIONS. *All the IVCM and impression cytology parameters correlated well with the conjunctival modifications induced by the topical therapy, suggesting the less toxicity of unpreserved drugs (Eur J Ophthalmol 2008; 18: 400-7)*

KEY WORDS. *Antiglaucoma drugs, Impression citology, In vivo confocal microscopy, Levobunolol hydrochloride, Preservatives, Toxic Conjunctival modifications*

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INTRODUCTION

The toxicity of preservatives on conjunctival epithelium has been widely described in glaucomatous patients topically treated, suggesting both an indirect effect on the lacrimal film and a direct toxic action on epithelial cells (1). Previous studies indicate that long-term administration of preservative-containing eyedrops can lead to chronic conjunctival inflammation, ocular surface impairment, and to an increased risk for glaucoma surgery failure, due to the overexpression of several inflammation- or apoptosis-related molecules (2-4). Among all preservatives, benzalkonium chloride (BAC) is the most commonly used in the antiglaucoma preserved drugs preparation and its cellular toxicity after long term administration has been widely investigated and demonstrated (5, 6). Topical preserved and unpreserved beta-blockers are usually employed in the treatment of all types of glaucoma and ocular hypertension; many authors (7, 8) report that preservative-free beta-blockers are less toxic to the conjunctival epithelium than preserved forms, suggesting that the microscopically detectable ocular tissue alterations may be largely due to the presence of preservatives in the eyedrops. To date, only timolol maleate and levobunolol hydrochloride have been prepared and commercialized as preservative-free ophthalmic solutions. Several reports investigated and

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compared the conjunctival impression cytology findings in patients treated with preserved and unpreserved beta-blockers (9-11); however, no report has assessed the differences between the preserved and unpreserved levobunolol preparations on conjunctival epithelium using, simultaneously, laser scanning in vivo confocal microscopy (IVCM) or impression cytology. The aim of our study was to provide a combined approach in the analysis of the conjunctival changes induced by preserved and unpreserved levobunolol, by using both IVCM and impression cytology.

METHODS

A randomized single masked study was performed. This study was in agreement with the Declaration of Helsinki and an informed, written consent was obtained from all patients.

Twenty-seven white patients (12 with ocular hypertension and 15 with primary open-angle glaucoma [POAG], 14 male and 13 female, 27 eyes) who had not received any previous topical hypotensive medications, referred to the Ophthalmic Clinic of University Chieti-Pescara, Italy, from January to March 2006, were consecutively enrolled in this controlled clinical trial.

All hypertensive eyes were normal except for the intraocular pressure (IOP) values, which ranged from 22 to 27 mm Hg (mean of three measurements at 9 am, noon, and 4 pm); conversely, all glaucomatous eyes had IOP values at the time of diagnosis ranging from 22 to 34 mm Hg (mean of three measurements at 9 am, noon, and 4 pm), Humphrey 30–2 full-threshold visual field test showing at least three contiguous points on the total deviation probability plot at the less than 2% level, the Glaucoma Hemifield Test outside normal limits, and classic ophthalmoscopic signs of glaucomatous optic neuropathy (cupping, neural rim notching, saucerization).

Prior to enrollment in the study, a detailed history was recorded for each case in order to verify the adherence to the eligibility criteria: absence of systemic or external inflammatory eye diseases, no history of any topical or systemic therapy in the last 6 months that could significantly interact with the ocular surface tissues, discontinuation of contact lenses application. Each patient underwent a complete ophthalmic examination including visual acuity evaluation, noncontact tonometry, anterior segment biomicroscopy, and funduscopy.

Subsequently, digital confocal laser-scanning microscope (LSM) (HRT II Cornea Module, Heidelberg Engineering GmbH, Germany) and, after 24 hours (in order to avoid misinterpretation due to the technical execution), impression cytology was performed in all patients. Both examinations analyzed the temporal bulbar conjunctival epithelium.

After the examinations the patients were randomized to receive (by computer-generated randomization) preserved levobunolol hydrochloride 0.5% (Vistagan®) (Group 1, 14 patients) or preservative-free levobunolol hydrochloride 0.5% (Vistagan®) (Group 2, 13 patients) administrated once daily (between 7:00 and 9:00 am). If both eyes were eligible, one eye per subject was randomly included in the study; the nonstudy eye was treated as well as the study eye, but was not analyzed. In order to verify the IOP lowering efficacy of the therapy, at the first and third month of the study each patient underwent a safety check visit; if the IOP reduction was at least one-fourth with respect to baseline values, the patients continued the therapy unmodified. After 6 months of therapy, the conjunctiva were again evaluated by means of IVCM and impression cytology; both the examiners performing the two examinations were not informed of the identity of the drop received by the patients during the course of the study.

In vivo confocal microscopy

Confocal microscopy has been introduced in clinical practice with the aim to obtain noninvasive in vivo imaging of the ocular surface; the technical characteristics of the instrument and the details of conjunctival examination by using HRT II laser scanning confocal microscope have been previously described (12). For IVCM examination of temporal conjunctiva the patient was seated in front of the microscope, the head was set steady by the aid of a headrest, and the eye was properly aligned in order to obtain tangential optical section of upper and temporal bulbar conjunctiva, by using a dedicated target mobile bright red light provided with the instrument that the patient had to fix with the fellow eye. A digital camera furnished lateral view of the eye and objective lens in order to check for each scan the position of the objective lens on the surface of the eye. LSM objective was put gently in contact with the ocular surface separated by a PMMA contact cap and a drop of 0.2% polyacrylic gel (Viscotirs® Gel, CIBA Vision® Ophthalmics, Marcon, Venezia, Italy) served as coupling medium. Sequential images were derived from

automatic scans and manual frame acquisition throughout upper and temporal bulbar conjunctiva of each examined eye.

For the IVCM conjunctival epithelium assessment, we evaluated the density of presumed intraepithelial goblet cells (as described by Messmer et al [13]) and an arbitrary index of epithelial layer regularity. In order to define this index, we considered and analyzed for morphologic parameters cellular size, cellular shape, uniformity of cellular size, and uniformity of cellular shape.

The epithelial layer regularity index was finally calculated and graded according to the following arbitrary grading scale: epithelial cells small and round, high uniformity in cellular size and shape, (Grade 0) high regularity; epithelial cells slightly larger than those in grade 0, shape more polygonal, a slightly less uniformity in size and shape than those in grade 0, (Grade 1) moderate regularity; epithelial cells larger than those in grade 1, polygonal shape, moderate uniformity in size and shape, (Grade 2) low regularity; epithelial cells very large and clearly polygonal, low uniformity in size and shape, (Grade 3) absence of epithelial regularity.

All data were calculated by the examiner (M.L.) analyzing the best epithelial image obtained in a series of three images (300 x 300 µm in size), selected by the IVCM operator (M.N.).

Impression cytology

Impression cytology is a safe, minimally invasive and relatively easy to perform diagnostic tool for ocular surface pathology, allowing ex vivo cellular analysis and yielding reliable information about the conjunctival epithelial area sampled. After administration of a local anesthetic (oxybuprocaine hydrochloride) a Millicell Millipore nitrocellulose filter paper round strip (12 mm of diameter) was applied by the operator (R.C.) to temporal bulbar conjunctiva without the exertion of any pressure, the patient being asked to look at the opposite side. The specimens were therefore stored in 95% ethanol, stained according to Papanicolau's modification of Gill's technique, and finally examined and graded (ranging 0–3) under a light microscope in accordance with Nelson's method (14, 15) who proposed to grade the conjunctival impression cytology specimens based on the aspects of the epithelial cells and the numbers of goblet cells. We considered a specimen of small, round epithelial cells with large nuclei and more than 500 goblet cells/mm2 as grade 0, whereas another of large polygonal epithelial cells with small nuclei and less than 100 goblet cells/mm2 as grade 3. All specimens that were graded 2 or more were abnormal.

All data were calculated (and reported as a mean) analyzing 10 different areas (300 x 300µm in size) of each specimen, selected by a pathologist (V.F.).

Both the operators of IVCM and impression cytology were masked for the therapy of the patients, while the examiners were masked for clinically relevant data and also for the specimen information (baseline or 6 months).

Statistical analysis

Analysis was performed by SPSS® Advanced Statistical™ 13.0 Software and SPSS Sample Power Software (2005, Chicago, IL, USA). Mann-Whitney and Wilcoxon tests were made to analyze the goblet cells density; chi square or Fisher exact test were used to determine both the index of epithelial layer regularity and the impression cytology score of each group, before and after the therapy. p Values less than 0.05 were considered significant.

The sample size was established calculating a rate difference of at least 50% between groups for chi square or Fisher exact tests and of 52% for Mann-Whitney and Wilcoxon tests, for a power of 80%.

RESULTS

The demographic and clinical data of the two groups are shown in Table I; no significant differences were found in age (Student t test for unpaired data), gender, and type of disease (chi square test). At baseline, none of the IVCM or impression cytology conjunctival parameters examined showed significant differences between groups (Tab. I) (Figs. 1 and 2, A, B, E). At the safety check visits, all the enrolled patients showed at least a one-fourth reduction of IOP with respect to baseline values, reporting no significant side effects. After 6 months of therapy all parameters analyzed showed statistically significant differences with respect to baseline values in both groups ($p<0.001$); significant differences for all tested parameters were also found between the two groups (Tab. II).

Particularly, when analyzing the IVCM findings, we observed a significant decrease in density of presumed goblet cell in both groups: a density reduction of 61% (from 88.1±45.2 to 25.2±4.5 number per field, mean ± SE) and 17% (from 90.0±45.8 to 75.4±48.7 number per field,

mean ± SE) respectively was found in Groups 1 and 2 ($p<0.001$) (Figs, 1 and 2, C). The cumulative grading score of epithelial regularity was significantly higher in both groups, being greater in Group1 than in Group 2 (34 and 8, respectively, $p<0.001$) (Tab. II) (Figs. 1 and 2, D). Similarly, when analyzing the impression cytology parameters we found that the cumulative grading score was significantly higher in both groups, being greater in Group1 than in Group 2 (39 and 7, respectively, $p<0.001$) (Figs. 1 and 2, F).

DISCUSSION

Levobunolol hydrochloride is a nonselective β_1-β_2 adrenoceptor antagonist. Once or twice daily instillation have been reported to reduce IOP significantly in patients with POAG or ocular hypertension (OH) (16). Moreover, Freyler and associates (17) demonstrated that levobunolol 0.5% and timolol 0.5%, administered once and twice daily, respectively, are equally effective in the treatment of POAG and OH.

Levobunolol, like other beta-blockers, can lead to a series of systemic (18) and topical side effects (2, 19), respectively, due to the action of active compounds and preservatives.

The topical side effects of preservatives have been studied by evaluating either functional (noninvasive break-up time [NIBUT], meniscometry, fluorophotometry of the corneal fluorescein uptake [20,21]) or morphologic parameters (by means of impression cytology [22]). With regards to the morphologic features, Hong and associates (23) investigated the conjunctival modifications induced by preserved timolol maleate by using impression cytol-

TABLE I - CHARACTERISTICS OF THE PATIENTS AND GROUPS AND BASELINE IVCM AND IMPRESSION CYTOLOGY PARAMETERS

Demographic characteristics and baseline parameters	**Preserved levobunolol group (Group 1)**	**Unpreserved levobunolol group (Group 2)**
Age, yr, mean ± standard error	54±8.34	52±7.23
Gender (M/F)	8/6	6/7
POAG/OH	9/5	6/7
IVCM parameters		
Index of epithelial regularity (cumulative score)	3	4
Goblet cells density, mean ± standard error (number per field)	88.1±45.2	90.0±45.8
Impression cytology grading score (cumulative score)	7	9

IVCM = In vivo confocal microscopy; POAG = Primary open angle glaucoma; OH = Ocular hypertension

TABLE II - IVCM AND IMPRESSION CYTOLOGY PARAMETERS AFTER 6 MONTHS OF THERAPY

IVCM and impression cytology parameters	**Preserved levobunolol group (Group 1)**	**Unpreserved levobunolol group (Group 2)**
IVCM parameters		
Index of epithelial regularity (cumulative score)	34*	8*†
Goblet cells density, mean ± standard error (number per field)	25.2±4.5 (39%)*	75.4±48.7 (83%)*†
Goblet cells density decrease vs baseline	61%	17%
Impression cytology grading score (cumulative score)	39*	6*†

IVCM = In vivo confocal microscopy
* $p<0.001$ With respect to baseline.
†$p<0.001$ vs Group 1

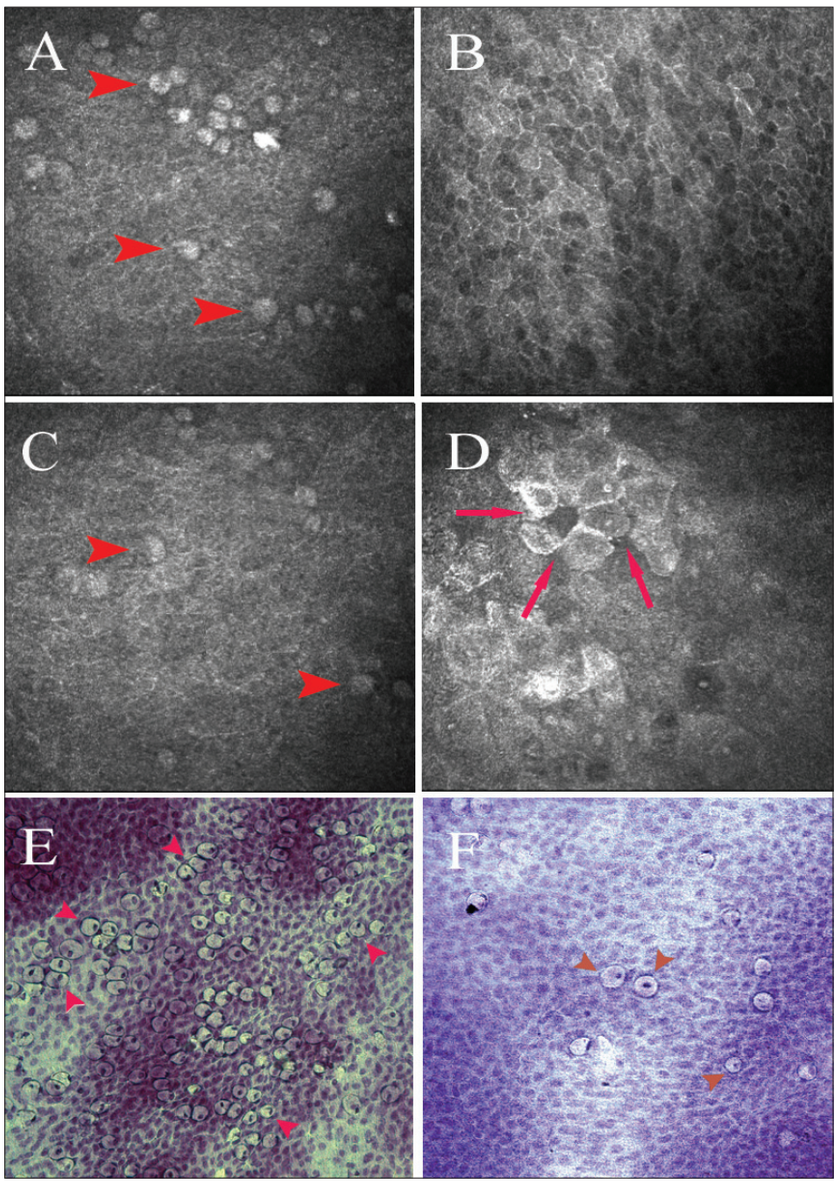

Fig. 1 - *Preserved levobunolol treated patients (Group 1).* **(A-D)** *In vivo confocal microscopy.* **(E, F)** *Impression cytology.* **(A, B, E)** *Goblet cells density (arrowheads) and conjunctival epithelium before therapy.* **(C, D, F)** *Marked reduction of the goblet cells density (arrowheads) and epithelial disruption (arrows) after 6 months of therapy.*

ogy, reporting goblet cells loss, squamous metaplasia, and stromal layer inflammatory cells infiltration. Such modifications progressively lead to chronic inflammation of conjunctiva, dry eye (1, 10), and to an increased risk of filtering surgery failure (24-26).

Nowadays, besides impression cytology, a morphologic examination of ocular surface can be achieved by the means of laser scanning in vivo confocal microscopy.

Laser scanning IVCM proves to be a powerful and well tolerated diagnostic tool in the diagnosis of various corneal and conjunctival diseases by performing noninvasive in vivo imaging and by providing essential information concerning the pathogenesis, the activity, the intensity, and extension within the tissue of most corneal and conjunctival diseases (19). Recently, the utility of this diagnostic method in ocular surface assessment has been demonstrated also in glaucomatous patients undergoing trabeculectomy, in which the post surgery IVCM analysis of conjunctival filtering blebs allows discrimination of successful from failed cases (27-29).

Labbè et al (30) studied the toxic effects of 0.5% BAC on Lewis rat cornea by means of IVCM, documenting flatten-

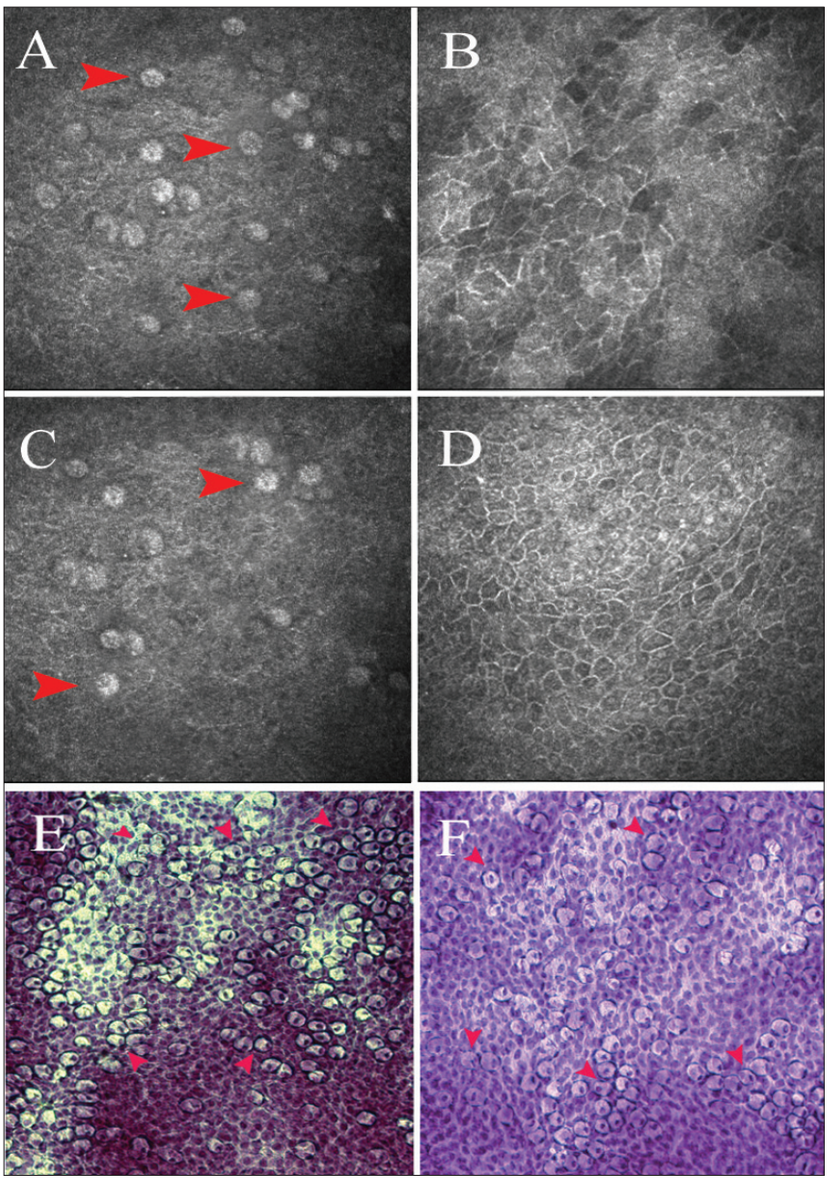

Fig. 2 - *Unpreserved levobunolol treated patients (Group 2).* **(A-D)** *In vivo confocal microscopy.* **(E, F)** *Impression cytology.* **(A, B, E)** *Goblet cells density (arrowheads) and conjunctival epithelium before therapy.* **(C, D, F)** *Mild reduction of the goblet cells density (arrowheads) and minimal changes of epithelial morphology after 6 months of therapy.*

ing and irregularity of basal epithelial cells, stromal neovascularization with inflammatory cells infiltration and endothelial guttae.

In our study we used IVCM to analyze the conjunctival changes induced by preserved and unpreserved levobunolol hydrochloride; when comparing the two groups of patients, we found statistically significant differences with respect to both conjunctival parameters examined, reporting a marked decrease of density of presumed goblet cells and a worse index of epithelial regularity (high score) in Group 1 with respect to Group 2. The present study also confirmed that impression cytology is a valuable method for the microscopic evaluation of the conjunctival changes induced by a long-term antiglaucoma therapy. In accordance with the literature (31, 32), goblet cells loss, large epithelial cells with irregular nuclei, and reduction of intercellular cohesiveness were more frequently observed in patients receiving preserved levobunolol eyedrops: an higher impression cytology score, alias a worse microscopic aspect, was more commonly found in patients treated with preserved levobunolol with respect to those treated with the unpre-

served form (from 2 to 3 and from 0 to 1, respectively).
Particularly, in Group 1 we found aspects of large and polygonal epithelial cells with basophilic-staining cytoplasm, small and pyknotic nuclei, and a nucleocytoplasmic ratio (NCR) range from 1:4 to 1:6; goblet cells were markedly reduced. Conversely, in Group 2, we documented opposite features as small and round epithelial cells with eosinophilic staining cytoplasm, large and basophilic nuclei, and NCR range from 1:2 to 1:3; goblet cells were mildly reduced. All these modifications emphasize the toxicity of preservatives on ocular surface tissues.
However, since statistically significant differences from baseline values were also found in Group 2, a toxic action of unpreserved levobunolol on conjunctival epithelium cells is a possible hypothesis, possibly due to an alteration of the tear system secretion (2, 19).
Furthermore, since the conjunctival epithelium changes become evident after 6 months of therapy, our results also suggest that preservative exerts its toxic and irreversible action in a short period of time.
The possibility to evaluate the same microscopic parameters (such as goblet cells density and epithelial regularity) with both examinations allows us to obtain the same information and to use equally an in vivo or an ex vivo analysis for the diagnosis of drug-induced conjunctival impairment.
However, when evaluating the pros and cons of the two techniques, IVCM presents some advantages over impression cytology since it allows a more comfortable and faster assessment of the ocular surface tissues preserving the integrity of conjunctival epithelium during the examination. Based on these facts, to microscopically assess drug-induced conjunctival impairment, an in vivo rather than an ex vivo analysis could be preferred, when possible.

None of the authors has a proprietary interest in the development or marketing of any of the products mentioned in this article.

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