*EVELIO SKIN EXPLANTS UNDER NEURO-INFLAMMATORY STRESS: SYNERGISTIC PROTECTION BY ESCINE AND DEXTRAN SULFATE*

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**Summary**

Human eyelid skin explants can be maintained in an appropriate culture medium in vitro for several days while retaining most of the histological features of normal skin. It thus represents a valuable tool to investigate the potentially damaging effects of biological, chemical or even physical stress that might alter their integrity. It is also a useful model to study the ability of various ingredients and products to prevent such damages and/or improve the morphological integrity of the skin. We observed here that a multi-stress inducing preparation including Substance P, Arachidonic Acid and Tumor Necrosis Factor could alter eyelid skin morphology. Dilation of superficial plexus microvasculature of the epidermis and an increase in water retention between collagen bundles of the extracellular matrix were the most reproducible histological alterations observed. We furthermore evidenced that topical application of escine and dextran sulfate synergistically protected eyelid skin explants in culture against the neuro-inflammatory stress preparation when applied prior to neuro-inflammatory stress conditions. It is thus predictable that not only these two ingredients will be well tolerated in vivo but also that they may, to some extent, protect eye skin outline against the major environmental external insults encountered day to day.

**Riassunto**

La cute prelevata della palpebra può essere mantenuta in vitro in appropriato mezzo di coltura per parecchi giorni mantenendo le caratteristiche istologiche di una pelle normale. Questo metodo rappresenta quindi, un valido mezzo per verificare gli eventuali danni biologici, chimici e psicofisici da stress che possono alterare l’integrità del tessuto periculare. Inoltre rappresenta anche un utile modello per studiare e verificare come e quando i diversi ingredienti e prodotti possano prevenire determinati danni o migliorare l’integrità morfologica della pelle. Abbiamo osservato che uno stress multiplo indotto su una preparazione contenente sostanza P, acido
arachidonico e TNF (Tumor Necrosis Factor) altera la morfologia della cute delle palpebre. E' stata osservata una dilatazione del microcircolo cutaneo ed un incremento della capacità di trattenere acqua da parte delle fibre di collagene della matrice extracellulare a livello della quale si verificano la maggior parte delle alterazioni istologiche.

Inoltre, abbiamo potuto evidenziare che l'applicazione topica di escina e di destrano solfato proteggono in modo sinergico l'espianto della cute delle palpebre in coltura allo stress neuro-infiammatorio, se applicati prima che si verifichino le condizioni di stress sperimentali. Sembrerebbe, perciò, che questi due ingredienti non siano soltanto ben tollerati in vivo ma che possono proteggere in qualche modo la zona cutanea delle palpebre contro le aggressioni ambientali a cui è sottoposta tutti i giorni.
INTRODUCTION

Eyelid skin is of particular interest in both dermatological and cosmetic research. It is highly innervated mostly by sensitive c-fibers that goes up to the upper layers of the epidermis (1, 2). In addition, it undergoes frequent stress, either physical (UV radiations), chemical (pollutants), or even mechanical (as being frequently rubbed). Due to this high level of stimulation combined with dense innervation, it thus represents an alert part of the body, the appearance of which reflects the extent of sustained stress. Thus eyelid biopsies maintained in a survival medium represent a sensitive « skin alert tool » that might help to predict both deleterious or protective effects of any topically applied product. We investigated the effects of a multi-stress inducing preparation on this model in order to evaluate to what extent skin morphology might be affected. This stress “cocktail” was designed so that it could mimic both a neurogenic agression (Substance P, SP) and two distinct inflammatory pathways (Arachidonic Acid ; AA) as a lipidic mediator and Tumor Necrosis Factor-a (TNFα) as a classical pro-inflammatory cytokine mediator of stress. Under those conditions, as previously reported for SP alone (1), we observed that two representative histological parameters were reproducibly affected by such a stress. These are a vasodilatation of the superficial microvascularature of the skin and water retention within extracellular matrix. Then, in a second time, we attempted to protect the skin from the above stress-induced effects using a combination of escine and dextran sulfate. Furthermore, with a view to test the combination when formulated as a cosmetic product, it was evaluated using Lucas Spring Water as a vehicle since it was previously shown to have a protective effect against inflammation induced by SP alone on surviving skin (3).

MATERIALS AND METHODS

Cosmetic ingredients: dextran sulfate (MW 10000) was obtained from Pharmacia Biotech (Upsala, Sweden) and esine 3030000 was obtained from INDENA Co (Italie). Lucas Spring Water was obtained from a 50 ml cosmetic Spray “EAU THERMALE/ THERMAL SPA WATER” marketed by Laboratoires Vichy, Vichy-France.

Human eyelid skin explant culture: normal human eyelid skin samples were obtained after informed consent from volunteers undergoing plastic surgery (caucasian women from 25 to 35 years old). The method for growing human eyelid skin ex vivo was adapted from previously reported work by Boisnic et al. (4) on normal skin. Briefly, skin fragments were washed three times with antibiotics. Subcutaneous fat and lower dermis were mechanically removed under a stereomicroscope using a surgical scalpel and skin samples were cut into 0.5 cm² full thickness pieces. Dissected fragments were then placed with the epithelium uppermost and maintained at the air-liquid interface on culture inserts (filter pore size 12µm; Costar, Poly Labo Paul Block, France). These inserts were set on 12 well plates (Costar) for 24 hours at 37°C in an humidified incubator with 5% CO2. Dulbecco’s minimal essential medium supplemented with antibiotics (100U/ml penicillin ; 100µg/ml streptomycin), L-Glutamin (200µg/ml), bovine pituitary extract, growth factors (Gibco BRL, USA) and fetal calf serum (DAP, France) was added to the culture wells so that the surface of the medium was level with the filter as previously described; (5-8). Cohesion between skin and insert was obtained with polysiloxane vinyl seal so that neither skin retraction nor direct lateral passage of topically applied product towards the dermis could be possible (4).

Application of an irritant stress cocktail to the explants: a multi-stress reaction was induced for 2 hours by applying 25µl of a combination of substance P (SP ; 5µM solution; Bachem, France) together with Arachidonic Acid (A.A.;
40mg/ml solution; SIGMA, France) and Tumor Necrosis Factor-a (TNF-a; final concentration of 50ng/ml; Valbiotech, France) to the surface of normal eyelid pieces. Application of products: to assess their protective potential, both escine and dextran sulfate were applied topically, either alone or in combination at the indicated concentrations (see legend for figures), just before applying the «stress cocktail» to the surface of eyelid explants. Moreover, they were tested after dilution in Lucas Spring water.

Histological evaluation: after 2 hours of application, the stressed skin samples were fixed in Bouin’s liquid and embedded in paraffin prior to histological analysis (4). 5µm thick sections were stained with hematoxylin and eosine. Histological evaluation was performed on papillary dermis and on the upper part of reticula dermis. Sections were double-blind evaluated and histologically scored by two distinct operators using an Olympus light photomicroscope with a 10X objective and they were photographed using an Ektachrome 64t Kodak film.

Histological criteria were scored as follows:

a) superficial skin microvasculature vasodilation: the evaluation was made on luminal areas of blood capillaries which are easily detected by their endothelial cells (see fig 1). 0-no vasodilation; 1-slight vasodilation; 2-moderate vasodilation; 3-marked vasodilation; 4-severe vasodilation

b) Water retention in the intercellular spaces:
0-no retention; 1-slight retention; 2-moderate retention; 3-marked retention; 4-severe retention.

Intermediate values were attributed when the observed parameters were not homogeneous within the same section. For each sample of skin (3 different donors per condition), 4 slides were evaluated and scored. The results are expressed as the mean and SEM of these scores for each of those parameters analyzed.

Statistics: the Student’s t-test was used to analyze paired data. Significance was calculated with stressed eyelid skin as a reference and p<0.05 was considered as a statistically significant score.

RESULTS

Histological alterations following application of a «stress cocktail» on eyelid skin in culture ex vivo: the “stress cocktail” was designed so that it could mimic at the same time a neurogenic stimulation (SP), a lipid mediator stimulation (AA) and a pro-inflammatory cytokine stimulation (TNFa).

Figure 1a shows a normal eyelid skin explant incubated in culture medium. Vessels can be seen with a flat lumen and normal endothelial cells nuclei (see arrows) indicating the absence of any spontaneous vasodilation. In the dermis, collagen’s bundles are dense and homogeneous with no space between collagen fibers. No lymphocytes can be seen, indicating no evident sign of irritation of the skin in vitro (9).

By contrast, after 2 hours of incubation with the “stress cocktail”, one can clearly see a severe vasodilation (see fig.1b) as well as a severe increase of white spaces between collagen fibers (fig. 1c). These observations suggest a swelling of the skin isolated ex vivo, resembling an in vivo edematous reaction. As shown in table I, there is indeed a significant change in the scores of

Fig. 1a
vasodilation ranging from 0.37 (absent to slight vasodilation) for normal eyelid skin to 2.2 (moderate to marked vasodilation) for stressed skin. Similarly, the scores of water retention moved from 0.75 (absent to slight retention) in normal conditions to 3.0 (marked retention) under stress conditions (Exp.1; table I).

This experiment was performed on another set of 3 different donors (Exp.2; table I) and

| Table I |
| Scoring of the effects of the stress cocktail (SP+AA+TNFα) on human eyelids biopsies in culture in vitro. |

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<thead>
<tr>
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<th>EXP.1</th>
<th>EXP.1</th>
<th>EXP.2</th>
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<tbody>
<tr>
<td></td>
<td>Vasodilation</td>
<td>Interstitial retention</td>
<td>Vasodilation</td>
<td>Interstitial retention</td>
</tr>
<tr>
<td>Control eyelid</td>
<td>0.37 +/- 0.47</td>
<td>0.62 +/- 0.63</td>
<td>0.5 +/- 0.57</td>
<td>0.75 +/- 0.64</td>
</tr>
<tr>
<td>Stressed eyelid</td>
<td>2.2 +/- 0.57</td>
<td>2.8 +/- 0.76</td>
<td>2.5 +/- 1.0</td>
<td>3.0 +/- 0.61</td>
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showed again an increase in water retention from 0.62 for normal skin (absent to slight) to 2.8 (moderate to marked) for stressed skin. In both experiments, the difference between control skin and stressed skin was statistically significant (p<0.05). Similarly, vasodilation increased from 0.5 (absent to slight) to 2.5 (moderate to marked) after applying the “stress cocktail” suggesting a good reproducibility of the scoring method (table I).

Protective effect on the dilation score: As shown in figure 2, Dextran Sulfate alone could not protect eyelid skin from vasodilating in response to the “stress cocktail” treatment. Histological scores of stressed eyelid skin were ranging from 2.2 (+/- 0.57) without Dextran Sulfate to 1.8 unprotected skin; p<0.05).

Protective effects on the interstitial water retention score:

As shown in figure 2, as seen in the vasodilation score, dextran sulfate alone could not provide a statistically significant protection of the eyelid skin against interstitial water retention increase in response to the “stress cocktail”. The score were 2.8 (+/- 0.76) without dextran sulfate and 2.1 (+/- 1.04) after pre-treatment with dextran sulfate). By contrast, escine albeit inefficient to significantly protect against vasodilation increase showed a significant protective effect against interstitial water retention increase 1.17 (+/- 0.77) versus 2.8 +/- 0.76 in stressed unprotected skin (p<0.05). When combined together, dextran sulfate and escine produced even an enhanced protection compared to escine Alone (0.43 +/- 0.51 versus 2.8 +/- 0.76; p<0.05).

Dose-effect study for optimal protection: As shown in figure 3, in an attempt to define a minimal level of concentration of both ingredients for which a synergistic and significant activity could be still observed after topical application on the eyelid skin explant, we evaluated the protective activity of serial dilutions (in distilled water) of a concentrated solution prepared in
25% Vichy Water (Lucas Spring; Vichy, France). The experiment was performed using Vichy Water for two other reasons i) check whether Vichy water might interfere with the synergistic protective effect of the two combined ingredients. ii) determine the lowest concentrations still showing a significant protective effect.

The results indicate that the lowest concentration showing a protective effect on both vasodilation and interstitial water retention scores is 0.05% escine, 0.08% dextran sulfate and 2.5% Vichy Water. The best efficiency on both histological scores was however obtained with 0.1% Escine, 0.16% Dextran Sulfate and 5% Vichy Water. In both cases, the protective effect was statistically significant (p<0.05) on the two endpoints.

**DISCUSSION**

Our results show that eyelid explants can be maintained ex vivo using conditions culture similar to those used for normal skin explants from other parts of the body (1,3-8). Furthermore, eyelid skin explants in culture medium are responsive to a “stress cocktail” containing both a neurogenic stimulator (SP), a precursor of the inflammatory and chemotactic lipid mediators (AA) and a pro-inflammatory cytokine (TNF-a). Each of those factors has distinct but sometimes overlapping function in the skin. SP receptors are expressed on cutaneous Mastocyes, Langerhans Cells and keratinocytes (10). In response to SP, keratinocytes produce the pro-inflammatory cytokines IL-1 and TNFa (1,3, 10, 11).

In response to SP, an increase in IL-1 production has been recently evidenced in human skin explants in culture (1,3). TNFa is an other pro-inflammatory cytokine which, together with IL-1 (12, 13), but through distinct receptors, can directly induce the expression of several inflammatory genes by keratinocytes among which those of the so-called inflammatory cytokines such as Interleukin-8 or MCAF (13). The latter inflammatory secondary cytokines belong to the large family of chemokines, responsible for specific attraction of blood cells infiltrates into the epidermis (14). Apart from the cytokine cascade, Arachidonic Acid (AA) which is the common precursor for both Prostaglandins and Leukotrienes, and is commonly generated through the activity of cell membrane phospholipase A2 (15) represents a non-specific branch of the inflammatory process responsible (among other phenomenon) for vasodilation (prostaglandins) and even for non specific chimiotacti-
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sm (leukotrienes); (16). Altogether, these factors contribute to the so-called “inflammatory cascade” in the skin.

It is thus expected that the combination of the above stress factors might, to some extent, recapitulate most of the biological pathways that a normal skin is supposed to go through during usual stress condition. The cocktail was adjusted so that the response of the skin could not be histologically quoted as severe but rather displayed a moderate to marked stress which may model the reaction of normal skin under non-pathological life-conditions.

Using this stress model, we could evidence that some cosmetic ingredients, when applied topically on the eyelid skin in culture ex vivo could protect the skin, to some extent, from morphological changes induced by multiple stress. Synergistic combinations were found to provide a significant protection from multi-stress alterations ranging from moderate to marked (but never severe) on a wide range of concentrations. Vichy water from Lucas spring, which had previously been reported to inhibit the histological alterations induced by SP in a similar model (3) was used as a vehicle. It was shown not to interfere with the synergistic protective activity of escine and dextran sulfate in a range of concentration from 2.5% and 25%. Whether the combination product is active in vivo remains now to be established which is currently under investigation in our research laboratory.

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