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ORIGINAL CONTRIBUTION

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Anti-inflammatory and antiradical effects of a 2% diosmin cream in a human skin organ culture as model

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Summary

Background: Diosmin, a naturally occurring flavonoid, is considered as a vascular-protective agent and is used orally to treat chronic venous insufficiency. It exhibits anti-inflammatory and free radical scavenging properties, but, like many other flavonoids, it is poorly absorbed in the small intestine.

Objective: Our aim was to investigate the skin protective effects of a diosmin-based cream, using skin organ culture as model.

Methods: Fragments of human skin explants, cultured ex vivo, were allocated to four treatment groups: no cream, no cream + stress, placebo cream + stress, and 2% diosmin cream + stress. Stress was induced by exposure to either substance P (anti-inflammatory effects' assessment) or UVB irradiation (free radical scavenging effects' assessment). Vascular dilation and the pro-inflammatory mediator IL-8 release were determined in the first model, whereas hydrogen peroxide level and the number of cyclobutane pyrimidine-positive cells were evaluated in the second model.

Results: In the substance P-induced inflammation model, 2% diosmin cream exhibited significant vasoconstrictive (proportion of dilated capillaries: −29%, capillary luminal area: −49% vs no cream + stress) and anti-inflammatory (IL-8 release: −36% vs no cream + stress) effects. In the UVB irradiation model, 2% diosmin cream significantly reduced hydrogen peroxide production and cyclobutane pyrimidine dimer formation (−45% and −36% vs no cream + stress, respectively). These effects were not observed with placebo cream.

Conclusion: Diosmin administered topically may protect skin against the biological effects of various exogenous or endogenous stresses, such as those involved in chronic venous disease.

KEYWORDS
anti-inflammatory, antiradical, cream, diosmin, skin explant

1 | INTRODUCTION

Chronic venous insufficiency is a condition that affects the venous system of the lower extremities with venous hypertension causing various symptoms including pain, swelling, edema, skin changes (pigment dermatitis), and ulcerations.[1] Pain, the chief complaint associated with chronic venous disease (CVD), is thought to be related to a local inflammatory reaction resulting from venous stasis and

hypoxia, localized release of pro-inflammatory mediators playing a key role in the activation of venous and perivenous nociceptors.[2] However, dilated cutaneous veins, such as telangiectasias and reticular veins, and varicose veins are also common visible and cosmetically bothersome signs of CVD.[1]

Phlebotonics are often used as pharmacological agents to manage CVD.[3] Among the main classes of orally administered phlebotonics, gamma-benzopyrones, also known as flavonoids, are of particular interest. Flavonoids, including diosmin, form a large group of polyphenolic compounds found in plants, sharing the same basic chemical structure, namely a three-ringed molecule with multiple hydroxyl (OH) groups attached. These substances—or their metabolites—exhibit a wide spectrum of activities in mammals including antioxidant effects, by scavenging reactive oxygen species, and anti-inflammatory activity. Anti-allergic, antibacterial, estrogenic, anticancer, hepatoprotective, and antithrombotic and antiviral activities have also been reported.[4]

The biological mechanisms underlying the beneficial symptomatic effects of diosmin in patients with CVD consistently include inhibition of inflammatory reactions through potent reduction in prostaglandin E2 and thromboxane A2 synthesis, and inhibition of leukocyte activation/migration and adhesion.[5] Studies have shown that diosmin improves capillary permeability, thus protecting the microcirculation, increases venous tone, and facilitates lymphatic drainage.[6,7]

When administered orally, diosmin shows limited systemic bioavailability due to its poor aqueous solubility. In this context, the cutaneous route of administration may be an advantageous option, especially as the local application of antioxidants to the skin has the added benefit of directly targeting these to the dermal site needing protection.[8] A 2% diosmin-based cosmetic cream (Phlebodia® Cream gel) was therefore formulated to protect against microcirculatory skin damage, especially in the context of CVD.[1] A small amount of menthol was added to provide a pleasant cooling sensation. When applied topically on the legs twice a day, this cream is also claimed to reduce leg discomfort and to help relieve feelings of heavy legs and tiredness.

The purpose of this study was to investigate the anti-inflammatory and free radical scavenging activities of this 2% diosmin-based cream, using human skin samples kept alive in organ culture as an alternative to the use of animal models.[9] The two experimental models used in our study were based on inhibition of substance P-induced inflammation and free radical release caused by ultraviolet B (UVB) irradiation, respectively. Substance P (SP), the main neuropeptide triggering inflammatory responses in the skin, induces a composite neurogenic inflammation process involving NK_1 receptors and histamine release from mast cells and leading to vasodilation, edema, and pro-inflammatory mediator release.[10] UVB, directly absorbed by DNA, causes molecular rearrangements forming specific photoproducts, the major type being cyclobutane pyrimidine dimers (CPDs) involving two adjacent pyrimidine nucleotides on the same strand of DNA. CPDs are recognized to be the principal cause of non-melanoma skin cancer[11] UVB also generates reactive oxygen species such as hydrogen peroxide (H_2O_2) creating an oxidative environment favoring CVD.[12] We describe here our initial findings based on these models.

2 | MATERIALS AND METHODS

2.1 | Culture of human skin explants

Full-thickness human skin explants were obtained from eight adult Caucasian donors undergoing plastic surgery (mammoplasty, n = 4; abdominoplasty, n = 4; mean age: 48 ± 14.3 years). Informed consent was obtained from each donor prior to study initiation.

Within 1 hour after excision, the skin explants were rinsed with an antibiotic-containing phosphate buffer solution and divided into fragments allocated to the different experimental conditions. The fragments were placed with the epidermis facing upward on the 3-µm pore polycarbonate membrane of tissue culture inserts set in wells 12 mm in diameter in 12-well culture plates (Costar, VWR, Fontenay-sous-Bois, France). The culture medium (Dulbecco's modified minimal essential medium [Gibco DMEM]; Life Technologies, Courtaboeuf, France) enriched in antibiotics (penicillin 100 µg/mL, streptomycin 100 µg/mL, and amphotericin B 250 µg/mL; Life Technologies), bovine pituitary extract (Life Technologies), L-glutamine (200 µg/mL; Sigma, Saint Quentin Fallavier, France), and fetal calf serum (DAP, Neuf-Brisach, France) was added at the bottom of the wells, allowing diffusion between the two compartments separated by the porous membrane.[9] The culture plates were placed in a humidified incubator in a 5% CO_2 atmosphere at 37°C. In each experimental model, the control and test conditions were compared between skin fragments from the same donors.

The skin fragments were kept alive ex vivo for 3 days, corresponding to the time during which they were in contact with no cream, or the test or placebo cream according to the experimental protocols described in the section "Models" below.

2.2 | Test product

The test product was a 2% diosmin-containing cream (Phlebodia® Cream gel, Innotech, Arcueil, France; batch number A78273, expiry date: February 2020). This was compared to a diosmin-free placebo cream of strictly identical composition except that diosmin was replaced by water.

2.3 | Models

The anti-inflammatory and antioxidant effects of 2% diosmin cream were assessed using two models, a substance P-induced inflammation model and a UVB-induced free radical release model. In both models, four experimental conditions were compared: no exposure to the stress factor and no cream application, exposure to the stress factor with no cream application, exposure to the stress factor with the application of 2% diosmin cream, and exposure to the stress factor with the application of placebo cream. Separate skin fragments from each donor explant were subjected to the four experimental conditions.

2.3.1 | Substance P-induced inflammation model

For assessment of the anti-inflammatory effect of the test product, substance P (SP), the main neuropeptide triggering inflammatory responses in the skin, was used to reproduce the neurogenic inflammation process in ex vivo cultured skin fragments. Concomitant vascular changes (edema, vasodilation), and pro-inflammatory cytokine release, were assessed as previously reported.[10] This model of skin inflammation was used to mimic the local inflammatory process described as being involved in pain related to CVD.

After re-equilibration of the skin explants for one hour, 10 µM SP was added to the culture medium.[10] The culture medium was renewed at D1 with the addition of 10 µM SP as at D0. The 2% diosmin or placebo cream was applied topically to the epidermis (2 mg/cm^2) of the skin fragments, twice daily at D0, D1, and D2, the first daily application immediately following the addition of SP to the culture medium. At D3, the skin fragments were removed from the culture inserts, fixed in formol, and embedded in paraffin. Serial sections of 4 µm in thickness were cut using a standard microtome and placed on albumin-coated glass slides. After hematoxylin-eosin staining, vascular modifications were measured by digital image analysis. Only blood capillaries were taken into account. The slices were observed using an Olympus® BX41 microscope (Olympus France, Rungis, France) and photographed with a QImaging Retiga SP 2000R camera (QImaging, Evry, France) directly linked to the microscope. The surface of the light of the vessels (in µm^2) was measured using an image analyzer (16 fields of vision at 40× magnification) at different levels of the skin (in the superficial dermis and in the upper part of the dermis). The percentage of dilated capillaries was also determined.

Lastly, levels of the pro-inflammatory mediator interleukin 8 (IL8) was determined in cell culture supernatants collected at D3 and frozen at −32°C pending analysis, using an ELISA assay kit (Bio-Techne, Minneapolis, MN).

2.3.2 | UV-induced skin damage model

The free radical scavenging effects of 2% diosmin cream were tested in a UV-induced skin damage model.

Skin explants were irradiated at D0 with a UVB source (Vilber Lourmat simulator, emission peak: 312 nm, composed of Vilber Lourmat T-20.L-312 mercury vapor tubes, low pressure and hot cathodes with a Vilber Lourmat RMX-365/312 radiometer; Vilber Lourmat, Marne-la-Vallée, France), at a dose of 6 J/cm^2.[13] The 2% diosmin cream or placebo cream (at 2 mg/cm^2) was applied twice daily at D0, D1, and D2, the first application taking place immediately after irradiation of the skin fragments at D0.

At D3, the skin fragments were removed from the culture inserts then fixed in formol solution and embedded in paraffin.

The cyclobutane pyrimidine dimer (CPD) activity in nuclear cells of the irradiated skin explants was detected by immunofluorescence using an antithymine dimer antibody (Abcam, Cambridge, UK; mouse monoclonal, Clone H3, Ref ab10347). The number of positive cells in the epidermis was determined, and the percentage of CPD-positive cells was calculated.

Hydrogen peroxide was assayed on skin fragments disintegrated in 0.5 M Tris-HCl buffer at pH 7.6, with 0.1% Triton ×100. The assay method used is based on the oxidation of ferrous ions to ferric ions by hydrogen peroxide and the reaction of these ions with xylenol orange to produce a blue-purple complex quantifiable by spectrophotometry at 560 nm. The quantity of hydrogen peroxide present in the sample was determined by comparison with a standard curve. The results were expressed in µmoles of hydrogen peroxide per mg of tissue.

2.4 | Statistical analysis

The results obtained for each parameter were expressed as the mean ± SEM of the individual values determined on the eight skin fragments in each treatment group. The mean values calculated for untreated skin fragments (control skin), skin fragments exposed to SP or UVB without cream application, and skin fragments exposed to SP or UVB and treated with 2% diosmin cream or placebo cream were compared. The statistical significance of differences between treatment groups was determined using Student's *t* test with reduced distribution or the paired sample *t* test. The threshold of statistical significance was set at $P < 0.05$.

3 | RESULTS

3.1 | Anti-inflammatory effects of diosmin cream after exposure of skin fragments to substance P

SP-induced neurogenic inflammation resulted in a significant increase in vasodilation, the mean surface of the light of capillaries in skin fragments exposed to SP in the absence of any cream application being 88.2 ± 23.6 µm^2 vs 58.6 ± 27.9 µm^2 in unstimulated control skin fragments ($P = 0.0004$; Figure 1). Topical application of 2% diosmin cream resulted in a significant 49.0% reduction in the mean surface of the light of dilated capillaries (45.0 ± 10.5 µm^2 vs 88.2 ± 23.6 µm^2 in skin explants treated by SP without cream application; $P = 0.0011$), whereas the placebo cream did not significantly affect this parameter (mean area: 78.6 ± 28.4 µm^2). In addition, the percentage of dilated vessels after application of 2% diosmin cream was reduced by 28.8% compared with the SP-treated skin (53.6% ± 7.4% of dilated vessels vs 75.2% ± 6.2%; $P = 0.0001$). Compared with skin treated by placebo cream, the percentage of dilated vessels after application of 2% diosmin cream was reduced by 26.0% (53.6% ± 7.4% of dilated vessels vs 72.37% ± 8.89%; $P = 0.006$; Figure 2).

After stimulation of the skin fragments by SP, a significant increase in IL8 release was observed, reaching a mean of 7666.5 ± 2290.4 pg/mL as compared to 3871.1 ± 1282.2 pg/mL in unstimulated control skin fragments ($P = 0.0004$; Figure 3). A statistically significant 36.4% decrease in mean IL8 release was observed with application of 2% diosmin cream as compared to SP-stimulated

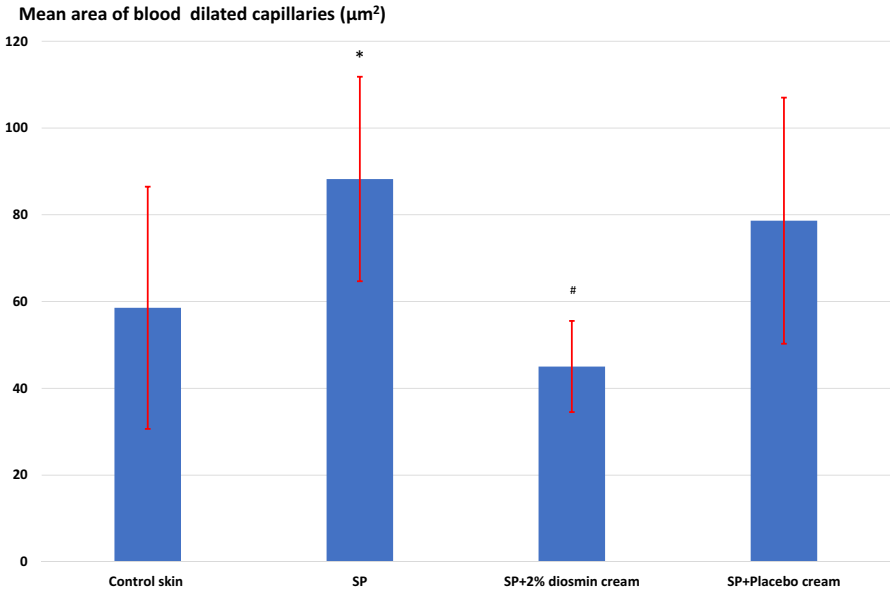

FIGURE 1 Analysis of the mean surface of the light of dilated vessels (in μm^2) determined on fragments of skin explants from eight donors (n = 8 fragments per group). SP, substance P. $^*P < 0.05$ compared to the control skin group. $^{\#}P < 0.05$ compared to the SP group (SP-stimulated skin with no cream application)

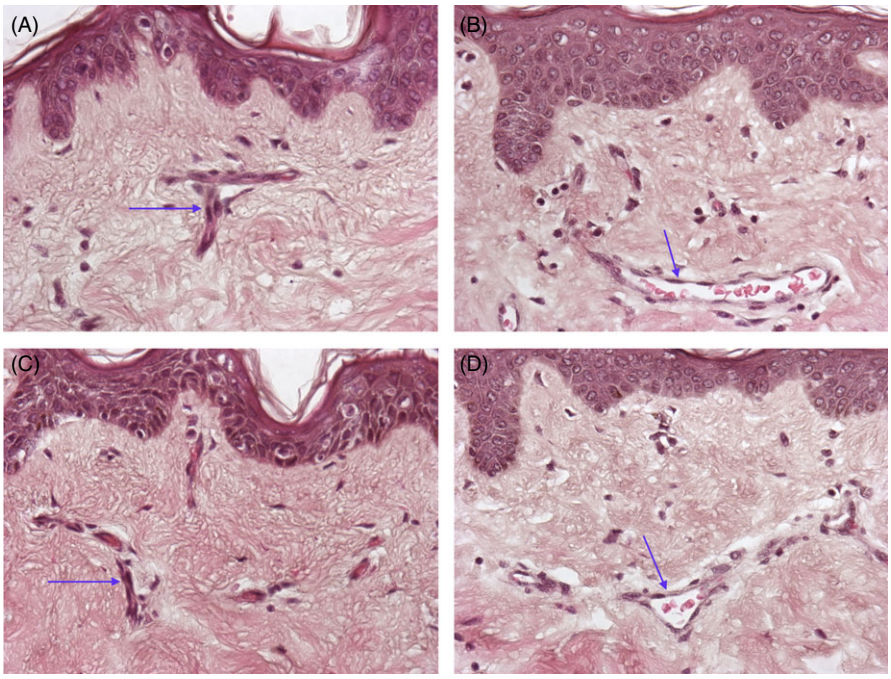

FIGURE 2 Representative micrographs showing capillary dilation observed in human skin explants after staining with hematoxylin-eosin (×400): (A) Control skin: no SP stimulation, no cream application, (B) SP-stimulated skin with very important vasodilation, (C) SP-stimulated skin and 2% diosmin cream: important vasodilation decrease compared with the SP-stimulated skin, and (D) SP-stimulated skin and placebo cream: very important vasodilation. SP, Substance P

skin fragments receiving no cream application (4877.8 ± 1813.6 pg/mL vs 7666.5 ± 2290.4 pg/mL, $P = 0.003$) and a 32.3% decrease as compared to SP-stimulated skin fragments on which placebo cream had been applied (4877.8 ± 1813.6 pg/mL vs 7201.2 ± 1363.9 pg/mL, $P = 0.0153$).

3.2 | Protection against free radical release after UVB-induced skin damage

There was no evidence of CPD release in control, nonirradiated skin fragments. The mean percentage of cells expressing CPD after UVB irradiation was 22.0% ± 6.9%. Application of 2% diosmin cream reduced the percentage of CPD-positive epidermal cells to a mean of 14.1% ± 5.1%, corresponding to a decrease of 35.9% compared to the percentage of CPD-positive epidermal cells in UVB-irradiated skin explants receiving no cream application ($P = 0.0015$; Figure 4). Placebo cream did not protect skin fragments from UVB damage, the mean percentage of CPD-positive epidermal cells in these fragments being 24.9% ± 6.9% ($P = 0.0001$ vs skin fragments treated with 2% diosmin cream).

A significant increase in hydrogen peroxide levels was seen after UVB-induced oxidative stress: 336.0 ± 204.7 nmol/mg as compared to 152.7 ± 85.3 nmol/mg in nonirradiated control skin fragments ($P = 0.005$; Figure 5). In skin fragments on which 2% diosmin cream had been applied, a statistically significant decrease of 44.8% in hydrogen peroxide levels was observed compared to the levels

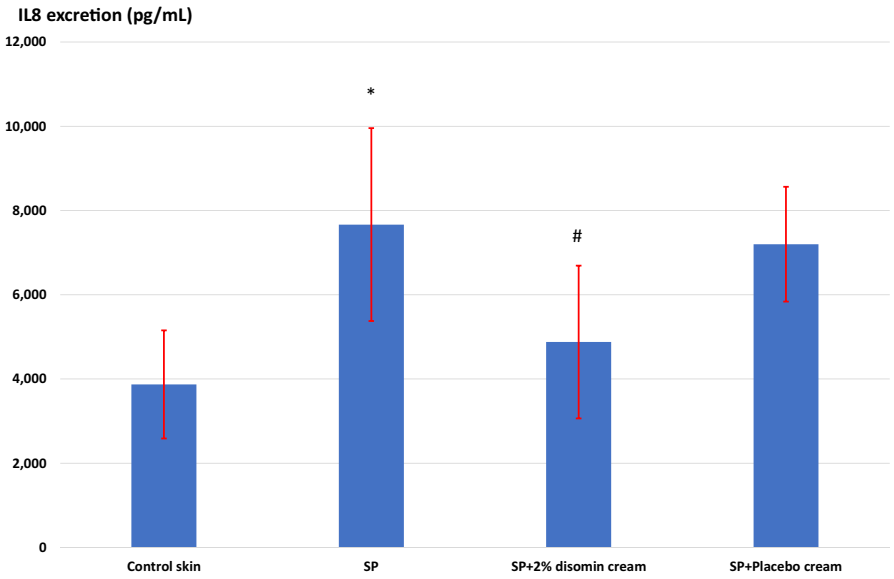

FIGURE 3 Pro-inflammatory cytokine assay: IL8 concentrations in pg/mL determined in the cell culture supernatants of skin fragments (n = 8 fragments per group). SP, substance P. $^{*}P < 0.05$ compared to the control skin group. $^{\#}P < 0.05$ compared to the SP (SP-stimulated skin with no cream application)

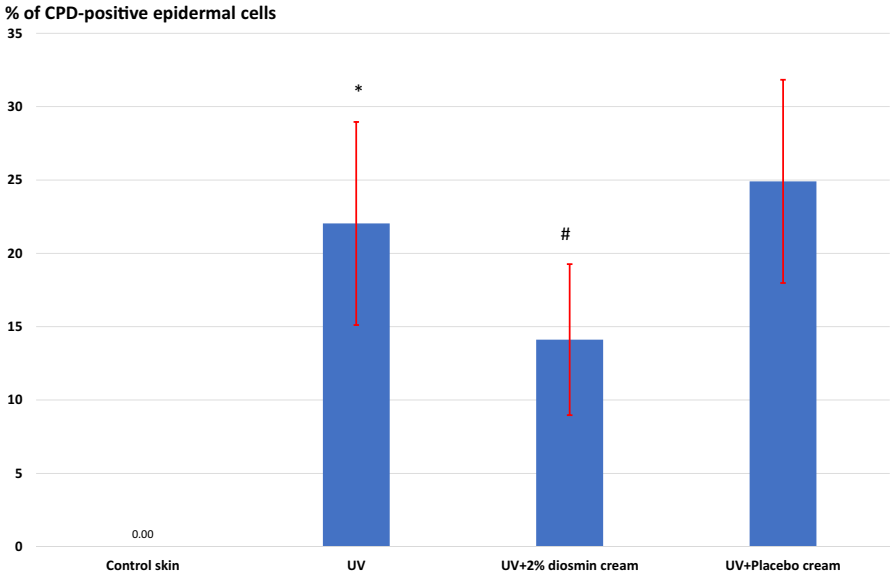

FIGURE 4 Immunohistochemical detection of CPD-positive cells (expressed as a percentage of total epidermal cells) in fragments of skin explants from eight donors (n = 8 fragments per group). $^{*}P < 0.05$ compared to the control skin group. $^{\#}P < 0.05$ compared to the UVB-treated group (UVB-irradiated skin with no cream application)

measured in UV-irradiated skin fragments receiving no cream application (185.3 ± 98.0 nmol/mg vs 336.0 ± 204.7 nmol/mg, $P = 0.007$). The placebo cream did not protect skin fragments from the UVB-induced increase in hydrogen peroxide level (305.6 ± 170.6 nmol/mg; $P = 0.01$ vs skin fragments treated with 2% diosmin cream).

4 | DISCUSSION/CONCLUSION

The purpose of this study was to investigate the local effects of diosmin applied directly on the skin using skin organ culture as a model. The skin organ culture model allows the integrity of skin structure to be maintained and is deemed to better reflect in vivo human skin responses. A correlation between ex vivo and in vivo effects was in fact established in a previous study.[14] The disappearance or improvement of mucosal lesions observed clinically in patients with oral lichen planus or oral leukoplakia treated with a 0.1% retinaldehyde gel was corroborated ex vivo by the disappearance of keratinization in surviving treated mucosal biopsies.[14] In our study, we reproduced two skin modifications known to be involved in the pathogenesis of chronic venous disease,[2,12] namely changes triggered by an oxidative environment, resulting from ultraviolet radiation, and SP-induced local inflammation.

Two-percent diosmin cream applied topically to human skin explants was shown to exert a positive vasoconstrictive effect counteracting SP-induced vasodilation. It concomitantly decreased the release of the pro-inflammatory cytokine IL8, testifying to its anti-inflammatory properties. Under the oxidative conditions induced by UVB irradiation, 2% diosmin cream exhibited a protective effect, as shown by the decrease in CPD and hydrogen peroxide levels. In contrast, the matching placebo cream exerted no significant protective effect, demonstrating that these anti-inflammatory and antioxidant properties were specific to diosmin and that the excipients included in the licensed formulation exerted no pharmacological effects.

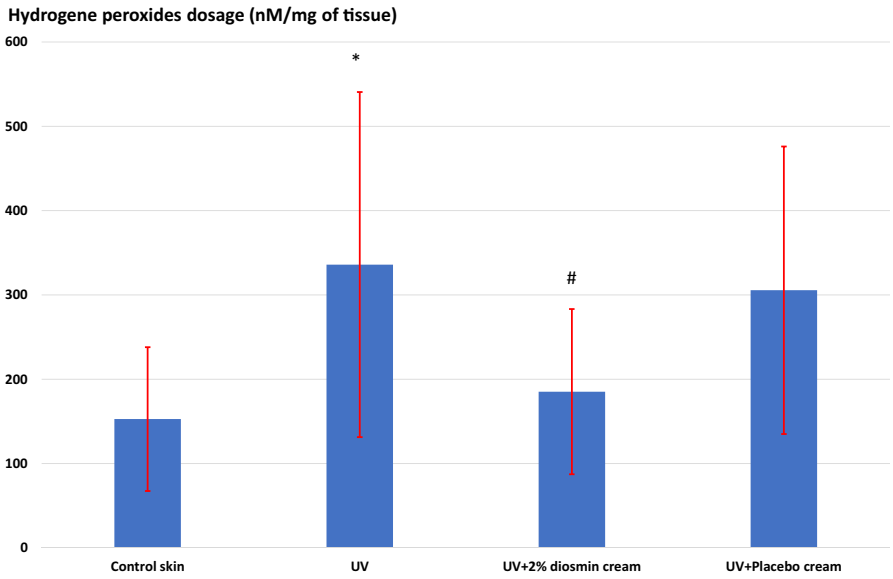

FIGURE 5 UVB-induced hydrogen peroxide levels in nmol/mg of tissue measured in fragments of skin explants from eight donors (n = 8 fragments per group). $^{*}P < 0.05$ compared to the control skin group. $^{\#}P < 0.05$ compared to the UVB-treated group (UVB-irradiated skin with no cream application)

These findings confirm the protective properties of diosmin previously demonstrated on epithelia. Thus, in a combined culture of human umbilical vein endothelial cells (HUVEC) and granulocytes, diosmin was shown to inhibit the IL1ß-induced inflammatory effects by decreasing the secretion of inflammatory mediators such as PGE2 (prostaglandin E2) and 15-HETE (15-Hydroxyeicosatetraenoic acid) and by inhibiting the adhesion of granulocytes to HUVEC, within a narrow range of concentrations (0.1–1 μM).[15] Diosmin also inhibited IL1β-induced enzymatic stimulation of arachidonic acid metabolism (eg, by phospholipase sand lipoxygenases) and expression of adhesion proteins such as intercellular adhesion molecule 1 (ICAM1). Diosmin acts on IL1β-mediated effects either directly, by preventing the binding of IL1β to receptors, or indirectly at the level of IL1β-induced protein synthesis.[15]

Flavonoids are generally considered to exert a beneficial effect on the skin by modulating blood vessel permeability and fragility.[16] Several studies have documented the anti-inflammatory and UV-protective properties of certain polyphenols.[17,18] Thus, topical application of green tea polyphenols enriched in catechins was shown to protect human skin against UV damage (CPD formation), and consequently against local inflammation, and to have a potentially promising photoprotective effect.[17] However, the anti-inflammatory and antiradical properties of topically applied flavonoids, especially flavonoid heterosides, have been poorly documented in experimental studies on human tissues. To the best of our knowledge, only animal studies and in vitro studies on reconstructed human skin models have been performed. Furthermore, these studies focused on pro-inflammatory mediators and/or oxidative damage without analyzing morphological effects on microcapillaries.

For example, hesperidin methyl chalcone (1% in solution)—another flavone glycoside also used as an oral treatment for chronic venous insufficiency—was shown to exhibit topical anti-inflammatory properties in a mouse model of skin oxidative stress and inflammation induced by UV irradiation, as suggested by its inhibition of various inflammatory and oxidative markers (TNF-α, IL-1β, IL-6, lipid peroxidation, etc) and its positive effects on endogenous antioxidant systems (catalase activity and GSH levels). In this model, decrease in skin edema was demonstrated by a loss of skin weight.[19] Hesperidin (12.5–100 μM) was shown to protect cultured human keratinocytes from UVB radiation-induced oxidative damage by scavenging reactive oxygen species, absorbing UVB radiation, restoring mitochondrial membrane depolarization, and regulating apoptotic proteins.[20] Consistently, in UVB-exposed mice epidermis, topical hesperidin application (1% in solution) promoted DNA photodamage repair by decreasing the quantity of epidermal CPD generated after UVB irradiation.[21] Studies on animal or human skin using models based on reconstructed epidermis have shown other polyphenols to be capable of preventing the formation of UVB-induced pyrimidine dimers in DNA following local application and to be promising candidates for the prevention of cutaneous carcinogenesis.[17,18]

Several hypotheses may be considered with respect to the mechanisms underlying the antiradical and anti-inflammatory effects of 2% diosmin cream. First of all, as for other flavonoids, there is evidence that diosmin physiologically blocks the penetration of UV radiation, thus superficially reducing oxidative damage. Free radical scavenging properties could be responsible for its indirect effects by decreasing skin inflammatory level and by inducing, via messenger diffusion, vasoconstriction of blood microcapillaries. Second, despite its high molecular weight, diosmin applied directly to the skin might permeate through the stratum corneum to reach the viable layers of the epidermis and dermis and thereby exert pharmacological effects within the skin. The precise mechanisms by which diosmin protects skin remain to be investigated and additional studies (for example a penetration study) would be of interest to better understand its mode of action in the skin.

The topical route is of particular interest because it bypasses the gastrointestinal tract. This 2% diosmin cream exerting antioxidant and anti-inflammatory effects could be used to protect skin integrity

from the adverse biological effects of exogenous stress factors (solar irradiation, pollution, etc) and as a complement to oral diosmin treatment for the protection of skin against the endogenous stress factors associated with CVD. Topical treatment with 2% diosmin cream might therefore help to further reduce the leg discomfort of patients suffering from CVD.

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