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Research paper

Quercetin in w/o microemulsion: *In vitro* and *in vivo* skin penetration and efficacy against UVB-induced skin damages evaluated *in vivo*

Fabiana T.M.C. Vicentini [a], Thaís R.M. Simi [a], José O. Del Ciampo [a], Nilce O. Wolga [b], Dimitrius L. Pitol [b], Mamie M. Iyomasa [b], M. Vitória L.B. Bentley [a], Maria J.V. Fonseca [a,*]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil*
[b] *Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, São Paulo, Brazil*

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Abstract

The present study evaluated the potential of a w/o microemulsion as a topical carrier system for delivery of the antioxidant quercetin. Topical and transdermal delivery of quercetin were evaluated *in vitro* using porcine ear skin mounted on a Franz diffusion cell and *in vivo* on hairless-skin mice. Skin irritation by topical application of the microemulsion containing quercetin, and the protective effect of the formulation on UVB-induced decrease of endogenous reduced glutathione levels and increase of cutaneous proteinase secretion/activity were also investigated. The w/o microemulsion increased the penetration of quercetin into the stratum corneum and epidermis plus dermis at 3, 6, 9 and 12 h post-application *in vitro* and *in vivo* at 6 h post-application. No transdermal delivery of quercetin occurred. By evaluating established endpoints of skin irritation (erythema formation, epidermis thickening and infiltration of inflammatory cells), the study demonstrated that the daily application of the w/o microemulsion for up to 2 days did not cause skin irritation. W/o microemulsion containing quercetin significantly prevented the UVB irradiation-induced GSH depletion and secretion/activity of metalloproteinases.
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Keywords: Microemulsion; Quercetin; Skin penetration; Skin irritation; UVB radiation

1. Introduction

Skin, a biological environmental interface having a barrier function, is a potential target organ for oxidative stress by external offenders, such as UV irradiation, ozone, ionizing radiation, various toxic chemicals, etc [1]. The organ does possess a wide range of interlinked antioxidant defense mechanisms to protect itself from damage by reactive oxygen species (ROS), but the capacity of these systems is limited and may be overwhelmed by excessive exposure to ROS. Then, supporting the cutaneous antioxidant defense systems with exogenous antioxidants could thus prevent or diminish ROS-mediated damage in the skin [2].

Topical antioxidants, such as the flavonoid quercetin, have been shown to diminish UV radiation-mediated oxidative damage to the skin [1,3]. Nevertheless, suitable percutaneous absorption is known to be an essential requirement for satisfactory topically applied photoprotective agents. Although lipid-soluble substances are usually considered to penetrate the stratum corneum fairly rapidly, the absolute water-insolubility of quercetin might justify its poor capability to permeate through excised human skin once skin absorption of a drug is determined not only by its partition coefficient, but also by other physicochemical properties, including water solubility, molecular size and diffusivity [1,4]. Therefore, strategies known to increase

* Corresponding author. Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, Av. do Café s/n, CEP 14040-903, Ribeirão Preto, São Paulo, Brazil. Tel.: +55 16 3602 4726, +55 16 3602 4433; fax: +55 16 3602 4879.
E-mail address: magika@fcfrp.usp.br (M.J.V. Fonseca).

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drug penetration into the skin have to be devised, so that topically applied quercetin might be employed to prevent oxidative skin damage [1].

Microemulsions and related systems represent pharmaceutically versatile formulations for various applications, including drug delivery to and through the skin [5]. They are defined as dispersions consisting of an oil phase, a water phase, surfactant/s and a co-surfactant, which are single optically isotropic and thermodynamically stable liquid solutions with a droplet diameter usually within the range of 10–100 nm [6–8].

Outstanding microemulsion properties such as thermodynamic stability, simple technology of preparation, transparency, low viscosity, and high solubilizing power allow incorporation of large amounts of poorly soluble compounds [2,9,10]. However, in spite of numerous advantages in comparison with other colloidal vehicles, microemulsions often require a high content of surfactant that can lead to skin irritation [9].

The present study was aimed to investigate the potential application of a w/o microemulsion as a topical carrier system for delivery of the antioxidant quercetin. As a first step, the *in vitro* and *in vivo* penetration of quercetin was investigated when the microemulsion system was topically applied and whether it caused skin irritation. In addition, the protective effect of w/o microemulsion containing quercetin was evaluated by measuring the UVB damage-induced decrease of endogenous reduced glutathione (GSH) levels and the increase of cutaneous proteinase secretion/activity.

2. Materials and methods

2.1. Materials

Quercetin dihydrate 99% ($C_{15}H_{10}O_7{\cdot}2H_2O$, $M_w = 338.26$) was purchased from Acros Organics (New Jersey, USA), propylene glycol and polyoxyethylene (80) sorbitan monolaurate (Tween 80®) from Synth (Diadema, SP, Brazil), polyoxyethylene (20) sorbitan monolaurate (Tween 20®) from Vetec (Rio de Janeiro, RJ, Brazil), sorbitan monolaurate (Span 80®), 2,2-diphenyl-1-picryl-hydrazyl (DPPH•) and glutaraldehyde 25%, ethylene glycol bis (-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), *o*-phthalaldehyde (OPT), sodium dodecyl sulphate (SDS) and acrylamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol (MeOH) and glacial acetic acid, both of high-performance liquid chromatography (HPLC) grade were from J.T. Baker (USA) and Merck (Darmstadt, Germany), respectively. All other chemicals were of reagent grade and were used without further purification.

2.2. Microemulsion containing quercetin

Microemulsion was obtained by adding the following components to the final stated percentages (w/w): 15% of a mixture of propylene glycol and water (3:1) as water phase, 46.75% of a mixture of Span 80® and Tween 80® (3:1) as the surfactant/co-surfactant system and 38.25% of canola oil as the external phase. The construction of the pseudo-ternary phase diagram in which this microemulsion was obtained was by F. Rossetti (unpublished data).

Oil phase dissolved quercetin (0.3%, w/w) was added to the mixture of surfactant and co-surfactant followed by the aqueous phase. After vortex mixing microemulsions formed spontaneously.

2.3. In vitro skin penetration and percutaneous delivery

The skin penetration of quercetin and its percutaneous delivery were assessed in an *in vitro* model of porcine ear skin, as previously described [11]. Briefly, the skin from the outer surface of a freshly excised porcine ear was carefully dissected and dermatomized, stored at −20 °C, and used within a month. On the day of the experiment, the skin was thawed and mounted on a Franz diffusion cell (diffusion area of 1.77 cm^2; Hanson Instruments, Chatsworth, CA), with the stratum corneum facing the donor compartment (where the formulation was applied) and the dermis facing the receptor compartment. The latter compartment was filled with 150 mM phosphate buffer (pH 7.2) containing Tween 20® (0.5%), which increased quercetin solubility to 27.93 ± 0.26 μg/mL (F.T.M.C. Vicentini, unpublished data). The receptor phase was under constant stirring and maintained at 37 ± 0.5 °C.

One hundred milligrams of the w/o microemulsion containing quercetin or controls were applied to the surface of the stratum corneum. Solutions of quercetin (0.3% w/w) in propylene glycol, in canola oil and in a micellar system, containing 45% of canola oil and 55% of the mixture Span 80®: Tween 80® (3:1), were used as control formulations.

At 3, 6, 9 or 12 h post-application of w/o microemulsion and propylene glycol solution and 12 h post-application of canola oil solution and micellar system, skin surfaces were thoroughly washed with distilled water and wiped with a cotton swab to remove excess formulation. To separate the stratum corneum (SC) from the remaining epidermis (E) and dermis (D), skin sections were subjected to tape stripping. The skin was stripped with 15 pieces of adhesive tape, the first one was discarded, and the other ones containing the SC were immersed in 4 mL methanol, vortex stirred for 1 min, bath sonicated for 15 min and the methanolic phase filtered using a 0.45 μm membrane. The evaporated filtrate residue was suspended in 500 μL of methanol and quercetin was assayed by measuring its antioxidant activity by the DPPH• assay, as described below.

The remaining [E + D] were cut in small pieces, vortex mixed for 2 min in 4 mL of methanol, and bath sonicated for 30 min. The resulting mixture was then filtered using a 0.45 μm membrane, and quercetin was assayed in the filtrate by HPLC as described below.

The extraction method was previously validated and absolute recovery of quercetin from skin tissue determined by spiking skin sections (area of 1.77 cm^2) with quercetin solutions in methanol (50, 100 and 250 μg/mL). The spiked skin sections ($n = 3$ for each concentration) were allowed to rest for 20 min, and quercetin extracted from SC and [E + D] was quantified by the stable free radical 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) assay and HPLC, respectively. Recovery of quercetin from spiked skin (SC + [E + D]) was 93.8%, 100.4% and 89.9% from the lower to the higher concentrations, respectively (F.T.M.C. Vicentini, unpublished data).

Samples of receptor solution were collected at 3, 6, 9 or 12 h post-application and treated with 5 M HCl to a final pH of 4.0. Five hundred microlitres of this solution were withdrawn and quercetin was extracted from each sample using 3 mL of dichloromethane. After the dichloromethane phase was evaporated, the residue was suspended in 100 μL of a methanol:water (60:40 v/v) mixture containing 2% acetic acid and quercetin was assayed by HPLC as described below. The average recovery in this procedure was approximately 96% when six different concentrations of quercetin were added to the receptor phase (F.T.M.C. Vicentini, unpublished data).

The amounts of quercetin detected in SC and in [E + D] are indicative of its penetration in the skin, whereas the amount in the receptor phase is indicative of its percutaneous delivery.

2.4. In vivo skin penetration

Sex-matched hairless mice (HRS/J) were housed in a temperature-controlled room, with access to water and food *ad libitum* until use. All experiments were conducted in accordance with National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Pharmaceutical Science of Ribeirao Preto (University of Sao Paulo, Ribeirao Preto, SP, Brazil).

At the day of the experiment, 100 mg of formulation added with quercetin (0.3% w/w) or control formulation (quercetin in propylene glycol at the same percentage) was applied on a limited area (~2 cm^2) on the back skin of each mouse. At 6 h post-application, the animals were killed with an overdose of carbon dioxide, and the treated skin area dissected, tape stripped (as described in experiments *in vitro*) and the amount of quercetin in SC and [E + D] determined.

2.5. Quercetin determinations

2.5.1. HPLC method

Quercetin concentration in the [E + D] and receptor phase samples (transdermal delivery) were determined by UV-HPLC analysis using a Shimadzu (Kyoto, Japan) liquid chromatograph, equipped with an LC-10 AT VP solvent pump unit and an SPD-10A VP UV–Visible detector. Samples were injected manually through a 20 μL loop with a Rheodyne injector. The separation was performed in a C18 Hypersyl BDS-CPS ciano (5 μm), 250 × 4.6 mm column with a mobile phase of methanol:water (60:40 v/v) containing 2% acetic acid (flow rate of 1 mL/min) and quercetin detected at 254 nm. Data were collected using a Chromatopac CR8A integrator (Shimadzu, Kyoto, Japan). Values obtained with standard methanolic quercetin solutions showed linearity over the concentration range of 0.1–200 μg/mL with a correlation coefficient (r) of 0.999. The quantification limit in the HPLC assay was 0.03 μg/mL and the average for relative standard variation and error was no more than 4.68% in all concentrations tested, which is considered adequate for analytical assays [12]. No unidentified peaks were seen in the HPLC chromatograms.

2.5.2. Antioxidant activity by the $DPPH^{\bullet}$ assay

Stratum corneum quercetin was measured by its antioxidant activity in the $DPPH^{\bullet}$ assay due to HPLC lack of selective.

The quercetin H-donor ability was evaluated using an ethanol solution of $DPPH^{\bullet}$, a stable nitrogen-centered free radical. Briefly, for radical scavenging measurements, 1 mL of 0.1 M acetate buffer (pH 5.5), 1 mL of ethanol and 0.5 mL of 250 μM ethanolic solution of $DPPH^{\bullet}$ were mixed with 50 μL of the test sample and the light absorbance measured after 10 min at 517 nm [13]. Positive controls were prepared using control skin sections (treated with quercetin-free formulations), which indicated the maximum odd $DPPH^{\bullet}$ electrons, considered as 100% free radicals in the solution and used to calculate the hydrogen-donating ability of quercetin (%). To determine SC extracted quercetin, the % H-donating ability was transformed in quercetin concentrations (μg/mL) using the regression equation obtained by plotting concentrations of quercetin against hydrogen-donating ability (%) for each concentration. The blank was prepared from the reaction mixture without $DPPH^{\bullet}$ solution and all measurements were performed in triplicate.

Values obtained for standard methanolic quercetin showed linearity over the concentration range of 0.1–2.0 μg/mL with a correlation coefficient (r) of 0.996. The average for relative standard variation and error was no more than 6.43% in all concentrations tested, in agreement with literature recommendations [12].

2.6. Evaluation of skin irritation

The microemulsion containing quercetin (100 mg) was applied topically and non-occlusively on a limited area of the dorsal skin of hairless mice once a day for 2 days. This regimen simulated a short-term treatment [11]. Skin irritation was evaluated according to established endpoints: erythema formation, epidermis thickening (basale and spinosum layers), and infiltration of inflammatory cells.

Skins of untreated animals or treated with saline were used as controls.

Skin redness measured with a Chroma Meter CR 200 (Minolta Instrument Systems) at different intervals of time during the treatment evaluated erythema formation. The a^* values were used to measure skin erythema.

For histological analysis, the animals were killed on the third day with an overdose of carbon dioxide and the skin treated area dissected and fixed by immersion into 2.5% glutaraldehyde in cacodilate buffer solution at room temperature for 24 h. Following dehydration and inclusion in paraffin wax, 6 μm thick sections were cut and stained with haematoxylin and eosin (H&E). Skin sections were examined by light microscopy (Leica DMLB2) and thicknesses of basal and spinosum layers of epidermis measured by stereological analysis using the test-system composed of cycloid arcs [14].

2.7. Efficacy of the w/o microemulsion as an in vivo protective agent against damage induced by UVB irradiation

2.7.1. Animals and experimental protocol

In vivo experiments were performed on 3-month-old sex-matched hairless mice of the HRS/J. Randomly chosen animals were divided into groups of 3–5 and topically treated on the dorsal surface with 300 mg of w/o microemulsion containing 0.3% quercetin or 300 mg of the control formulation (w/o microemulsion without quercetin). The formulations were applied 1 h and 5 min before irradiation of the dorsal surface and after irradiation as previously described [3]. The untreated control groups irradiated and non-irradiated were included in the experiments. The results are representative of three separated experiments.

2.7.2. Irradiation

The UVB source of irradiation consisted of a Philips TL40W/12 RS lamp (Medical-Holand), mounted 20 cm above the mice-supporting apparatus and emitting a continuous light spectrum between 270 and 400 nm with a peak emission at 313 nm. UVB output (78% of the total UV radiation) was measured using a model IL-1700 Research Radiometer (International Light, USA; calibrated by IL service staff) with a radiometer sensor for UV (SED005) and UVB (SED240). The UVB irradiation rate was 0.27 mW/cm^2 and the doses used were 0.96–2.87 J/cm^2. The animals were killed with an overdose of carbon dioxide 6 h after the start of UVB exposure, and full dorsal skins were removed and stored at −80 °C for further analysis.

2.7.3. Protective effect

The increase in skin erythema, after irradiation (3 h) and before the animal sacrifice (6 h), was measured using the Chroma Meter CR 200 (Minolta Instrument Systems). Redness increase was expressed as the difference between basal a^* (before irradiation) and irradiated skin values.

2.7.4. GSH assay

GSH skin levels were determined using a fluorescence assay as previously described [15]. The total skin of hairless mice (1:3, w/w dilution) was homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA using a Turrax TE-102 (Turratec). Whole homogenates were treated with 30% trichloroacetic acid, centrifuged at 1900g for 6 min at 4 °C and the fluorescence of the resulting supernatant measured in a Hitachi F-4500 fluorescence spectrophotometer. Briefly, 100 μL of sample supernatant was mixed with 1 mL of 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA and 100 μL of OPT (1 mg/mL in methanol). The fluorescence was determined after 15 min ($\lambda_{exc} = 350$ nm; $\lambda_{em} = 420$ nm) and the values referred to a standard curve prepared with 0–40 μM GSH. The results are presented as nmols of GSH per mg of skin.

2.7.5. Qualitative analyses of skin proteinases by substrate-embedded enzymography

SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate-embedded enzymography (zymography) was used to detect enzymes with gelatinase activity. Assays were carried out as previously reported [16]. Total skin of hairless mice (1:3, w/w dilution) were homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 0.1% SDS in a Turrax TE-102 (Turratec). Whole homogenates were centrifuged at 12,000g for 10 min at 4 °C, 400 μL supernatant aliquots mixed with 50 μL of glycerol and 30 μL of the mixture used in electrophoresis. SDS−PAGE was performed using 13.5% acrylamide gels containing 0.2% gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h with constant shaking, incubated overnight in 50 mM Tris–HCl (pH 7.4) 5 mM $CaCl_2$ and 0.02% sodium azide at 37 °C and stained the following day with Coomassie Blue 350-R (Phast gel blue R-Pharmacia Biotech). After destaining in 20% acetic acid, zones of enzyme activity were detected as regions of negative staining against a dark background. The proteolytic activity was qualitatively analyzed by comparing controls and microemulsion–quercetin treated animals. The Lowry method was used to measure protein levels in skin homogenates [17].

2.8. Statistical analyses

Data were statistically analyzed by one-way ANOVA, followed by Bonferroni's multiple comparison t-test. The level of significance was set at $p < 0.05$.

3. Results

3.1. In vitro skin penetration and percutaneous delivery

Compared to propylene glycol (control), canola oil and micellar formulations, the w/o microemulsion formulation significantly enhanced quercetin skin penetration 12 h after application (Table 1).

Table 1
In vitro skin penetration of 0.3% quercetin incorporated in carriers w/o microemulsion, propylene glycol, canola oil and micellar system, 12 h after topical application

Formulations	Quercetin (μg/cm^2)	
	SC (μg/cm^2)	[E + D] (μg/cm^2)
Propylene glycol	9.40 ± 0.57	0.13 ± 0.03
Canola oil	5.06 ± 0.24	–
Micellar system	7.16 ± 0.44	–
W/O microemulsion	16.94 ± 0.84*	2.51 ± 0.12*

Results are represented by means ± S.D. ($n = 5$–6). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. *Significant statistical difference compared to control (propylene glycol formulation) ($p < 0.05$).

The time-course of the *in vitro* quercetin skin penetration (Fig. 1) showed that when quercetin was incorporated in the w/o microemulsion, its concentration in SC was significantly enhanced at 3 h, 6 h, 9 h and 12 h ($p < 0.001$) post-application. Similarly, quercetin concentration in the [E + D] was significantly enhanced after 3 h ($p < 0.01$), 6 h, 9 h and 12 h ($p < 0.001$). By applying control formulation, maximal concentrations of quercetin were detected at 12 h post-application, respectively, 9.40 ± 0.57 μg/cm^2 and 0.13 ± 0.03 μg/cm^2 for SC and [E + D]. The maximal concentrations of quercetin in the same compartments were, respectively, ~2-fold and 20-fold higher when the carrier was w/o microemulsion. On the other hand, no quercetin was detected in the receptor phase after 12 h application of control or w/o microemulsion formulations.

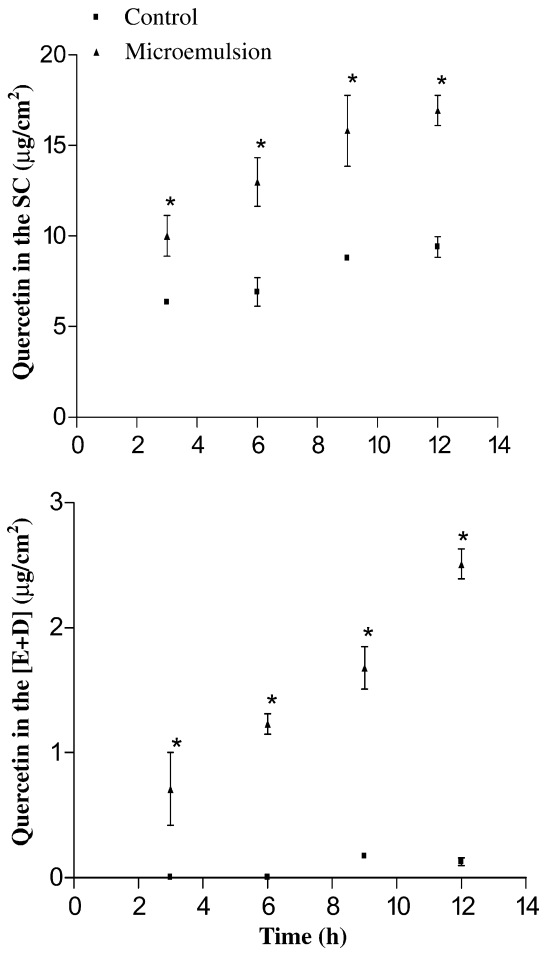

Fig. 1. Time-course of the *in vitro* skin penetration of quercetin incorporated in the w/o microemulsion or control formulation. SC, stratum corneum, [E + D]: epidermis (without SC) plus dermis. Results are represented by means ± S.D. ($n = 5$–6). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. *Significant statistical difference compared to control (propylene glycol formulation) ($p < 0.05$).

3.2. In vivo skin penetration

In vivo penetration of quercetin in SC and [E + D] was evaluated using hairless mice. Similarly to the *in vitro* observations, w/o microemulsion significantly ($p < 0.001$) enhanced quercetin skin penetration *in vivo* at 6 h post-application when compared with the control formulation (Fig. 2). Quercetin levels in SC and [E + D] were 14.31 ± 1.53 and 5.63 ± 0.01 μg/cm^2, respectively, when the flavonoid was contained in the control formulation. Concentrations, respectively, 1.5 and 2 times higher were detected when quercetin was incorporated in the microemulsion.

3.3. Skin irritation tests

Chromametry measurements of skin redness in hairless mice did not change during the full treatment, indicating that erythema formation was not induced (data not shown).

No histopathological alterations in the skin of animals treated with saline or w/o microemulsion were seen by light microscopy, as compared to the untreated animals

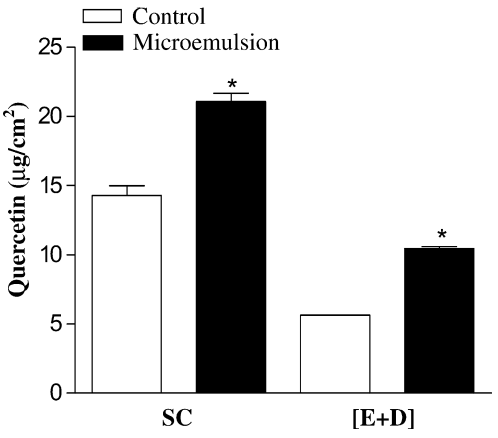

Fig. 2. I*n vivo* skin penetration of quercetin at 6 h following its topical application using the w/o microemulsion or control formulation. SC, stratum corneum, [E + D]: epidermis (without SC) plus dermis. Results are represented by means ± S.D. ($n = 5$). Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. *Significant statistical difference compared to control (propylene glycol formulation) ($p < 0.05$).

(Fig. 3A–C). Thicknesses of basale and spinosum layers of epidermis in topically microemulsion-treated, saline-treated, and untreated animals were not significantly different (Fig. 4).

3.4. Efficacy of the w/o microemulsion as an in vivo protective agent against damage induced by UVB irradiation

3.4.1. Protective effect

A formal comparison of UV-induced skin reddening of unloaded and quercetin-loaded microemulsion-treated skin to untreated skin was performed. Neither unloaded nor quercetin-loaded microemulsion pretreatment conferred protection against UV-induced skin reddening (data not shown). Thus, the protective effect of microemulsion evaluation demonstrated that its effects on UV-induced responses reported below are not secondary to interference of UV transmission.

3.4.2. GSH assay

Preliminary tests determined that UVB irradiation induced a dose-dependent decrease in GSH levels (0.96–2.87 J/cm^2). Hairless-skin mice were treated with dosages of UVB radiation in this range and the results compared to controls (untreated–unexposed group). There was no significant statistical difference when the lower dose was tested (0.96 J/cm^2). Higher doses produced significantly different decreases of GSH when compared to the control group, and the effect of the higher dose (2.87 J/cm^2) was significantly different from the smaller ones, 0.96 and 1.91 J/cm^2 (data not shown). Thus, the dose of 2.87 J/cm^2 was chosen to test the efficacy of w/o microemulsion as a carrier of antioxidant drugs.

W/O microemulsion containing quercetin (0.3%) inhibited the UVB irradiation-induced depletion of GSH, maintaining its levels near to the ones in untreated–unexposed controls (Fig. 5).

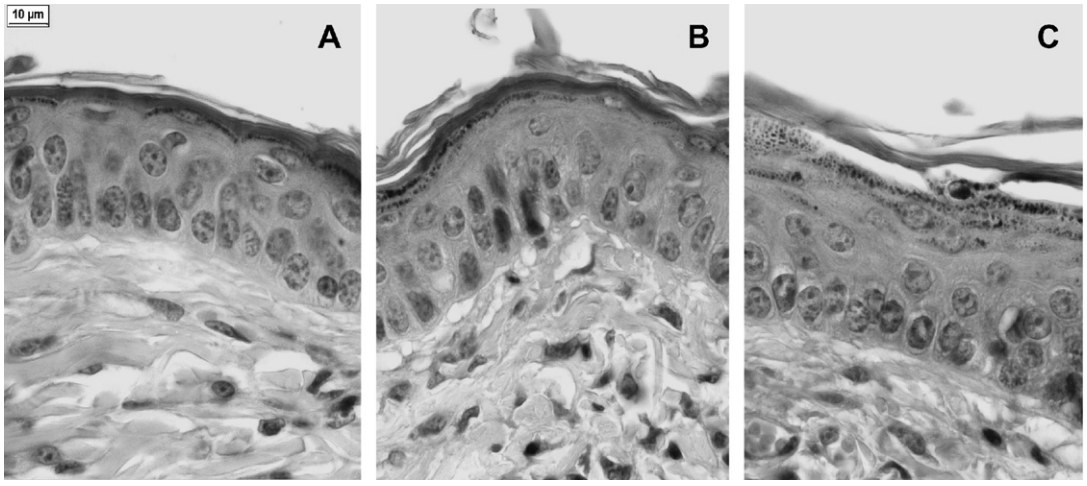

Fig. 3. Photomicrographs of skin sections of untreated animals (A), animals treated with saline (B) and w/o microemulsion (C). Sections were visualized by conventional light microscopy through a 100× objective.

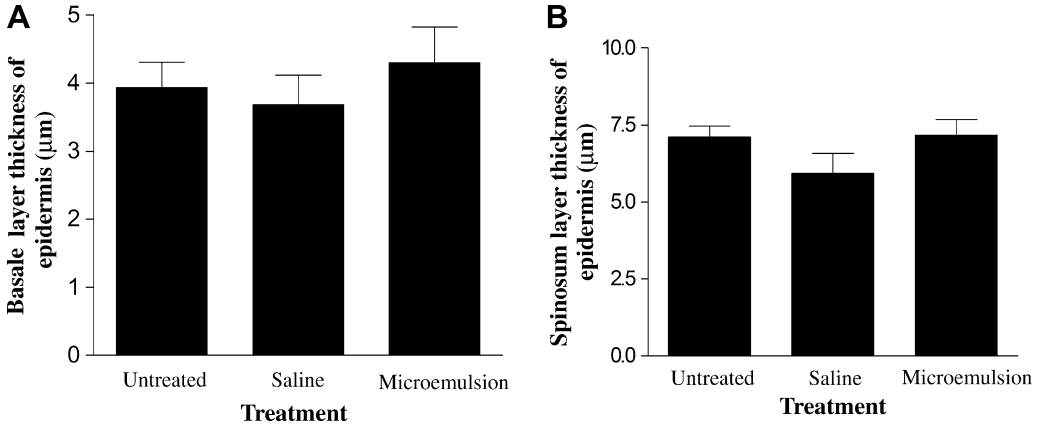

Fig. 4. Thickness of the basale (A) and spinosum (B) layers of epidermis of untreated animals or treated with saline or w/o microemulsion. Results are represented by means ± S.D. ($n = 5$). No statistical significant difference was detected. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons ($p < 0.05$).

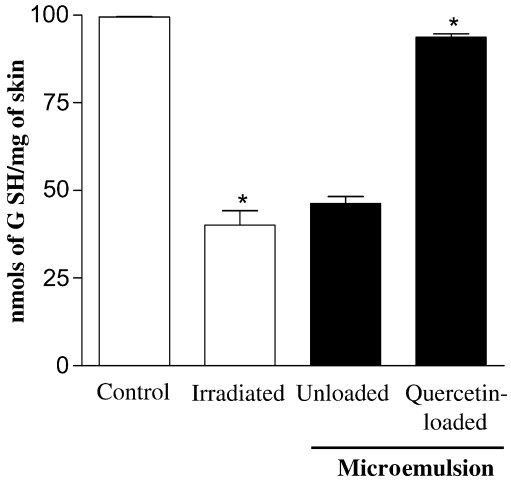

Fig. 5. Effect of w/o microemulsion containing quercetin on the decrease of the endogenous antioxidant GSH system induced by UVB irradiation. Bars represent means ± S.E.M. of three separated experiments, 3–5 animals per group. Statistical analysis was performed by one-way ANOVA followed by Bonferroni's test of multiple comparisons. $^{*}p < 0.05$ compared to the respective control (untreated–unexposed or irradiated treated with unloaded microemulsion groups).

3.4.3. Qualitative analyses of skin proteinases by substrate-embedded enzymography

As described above for GSH levels, UVB irradiation dosages capable of increasing proteinase activity were evaluated. Fig. 6A shows an increased intensity in proteinase activity bands in a dose-dependent manner (0.96–2.87 J/cm^2). The dose of 2.87 J/cm^2 was used to test the microemulsion efficacy. SDS–PAGE zymography detected inhibition of proteinase secretion/activity increase induced by UVB irradiation when quercetin-loaded microemulsion was applied (Fig. 6B). Furthermore, supporting these results, the control dosage of total proteins in the skin detected no significant statistical difference among the samples (data not shown).

4. Discussion

The present study describes the potential application of w/o microemulsion as a topical carrier system for delivery of the antioxidant quercetin. The ability of w/o microemulsion to improve quercetin skin penetration was initially investigated. An increased penetration of quercetin in SC and [E + D] *in vitro* was observed 3, 6, 9 and 12 h following application of the w/o microemulsion. No quercetin was detected in the receptor phase, which is an advantage, because the aim is the topical (not transdermal) delivery of quercetin. Like *in vitro* observations, *in vivo* skin penetration experiments revealed that incorporation of quercetin in the w/o microemulsion also results in increased skin penetration of the flavonoid at 6 h post-application.

The skin models used in the *in vitro* and *in vivo* experiments present distinct characteristics, and thus, caution should be taken in comparing results. Mouse skin used in the *in vivo* is more permeable than the porcine skin used in *in vitro* experiments; thus, skin penetration might be faster in the mouse. Additionally, the lack of blood flow in the dermis of porcine skin *in vitro* may artificially hinder absorption of lipophilic compounds in this water-rich environment [18]. The skin models used are different, but results obtained *in vivo* confirmed *in vitro* observations that w/o microemulsion enhances skin penetration of quercetin. Taken together, the results demonstrate that the microemulsion promotes the increased penetration of quercetin in the skin as compared to a control formulation without affecting the transdermal delivery, and thus, can be a useful strategy to improve the topical delivery of the lipophilic flavonoid quercetin.

Although not investigated, some speculation may be made about the mechanism by which w/o microemulsion influences skin penetration of quercetin.

Drug thermodynamic activity in the formulation is a significant driving force for its release and penetration into the

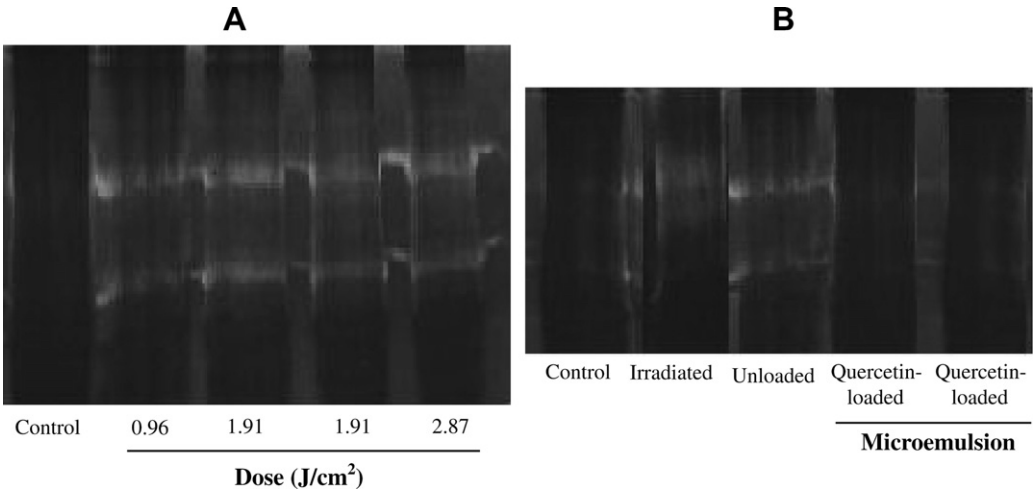

Fig. 6. Zymographic patterns of metalloproteinase secretion/activity induced by UVB irradiation in hairless mice skin. (A) Dose-dependent increase of metalloproteinase activity induced by UVB irradiation. (B) Effect of w/o microemulsion containing quercetin on the increase of metalloproteinase activity induced by UVB irradiation. The data are representative of three separated experiments, 3–5 animals per group.

skin, so that saturated solutions should have the highest thermodynamic activity and consequently higher permeation rate [7]. The w/o microemulsion used in the present study was saturated with quercetin (0.3%), which might be one of the factors responsible for the increase in skin penetration after topical application.

Furthermore, microemulsions generally have ultra-low interfacial tension, which ensures an excellent contact surface between the skin and the vehicle over the entire application area [10].

In addition, the surfactant, co-surfactant or oil molecules in the microemulsion may diffuse on the skin surface and act as enhancers, either by disrupting the lipid structure of the stratum corneum, facilitating diffusion through the barrier phase, or by increasing the solubility of the drug in the skin, i.e., increasing the partition coefficient of the drug between the skin and the vehicle. It is likely that with the microemulsion tested, the vehicles act as enhancers by means of increasing the partition of the drug into the skin, thus mainly increasing dermal drug delivery [10]. The reduced quercetin penetration from the canola oil or micellar system relative to that presented by the w/o microemulsion vehicle, suggests that the combined effects of hydrophilic and lipophilic components of the microemulsion enhanced the activity of the whole system, which significantly contributed to the penetration of quercetin.

Last but not least, due to the small size, droplets settle down to close contact with the skin which leads to a considerable increase of surface area and hence improved absorption. It has been found that the microemulsion structure plays an important role in the rate of drug release [19].

The potentiality of a topical formulation to be used as a delivery system should be evaluated not only in terms of carrier capacity and percutaneous drug absorption, but also in terms of its tolerability and toxicity [20]. As microemulsions typically are composed of large amounts of surfactants and oil, it is particularly important to consider potential skin irritation and toxicological reactions resulting from topical application [10].

By evaluating established endpoints of skin irritation (erythema formation, epidermis thickening and infiltration of inflammatory cells), the present study demonstrated that the daily application of the w/o microemulsion for up to 2 days did not cause skin irritation.

Sunlight UV rays penetrate the skin as a function of their wavelengths. Short wave length radiation (UVB, 290–320 nm) is mostly absorbed in the epidermis and interacts predominantly with keratinocytes while UVA, 320–400 nm penetrates deeper, affecting the epidermal and dermal cells. Convolution of the spectra with biological damage action shows that, despite the significantly greater incidence of UVA radiation (95% of the UV reaching the Earth), predominant acute and chronic damages to the skin are associated with the UVB portion of the solar spectrum [21]. Chronic exposure of unprotected human skin to UVB irradiation is known to induce an array of adverse reactions, probably through formation of ROS, including premature skin ageing, erythema, inflammation and photo-carcinogenesis [22].

One of the most important endogenous defense mechanisms against UV-induced ROS is glutathione, a cysteine containing tripeptide with reducing and nucleophilic properties. This tripeptide exists in either a reduced (GSH) or oxidized (GSSG) form and in unstressed cells, the majority (99%) of this redox regulator is in its reduced form [23]. It directly scavenges radicals by hydrogen transferring, acts as cofactor for the enzyme GSH-peroxidase, which scavenges peroxides, and finally regenerates vitamins E and C [22].

Fuchs et al. [24] demonstrated that the glutathione system is strongly challenged by photooxidative (UVB) stress. Therefore, the glutathione redox status has been confirmed as an early and sensitive sensor of UVB-induced epidermal oxidative stress, suitable for testing the protective antioxidant effect of a substance [25].

Corroborating with Casagrande et al. [3], a dose-dependent depletion of GSH in the skin after UVB irradiation was detected in the present study. The UVB dose of 2.87 J/cm^2 induced approximately 2-fold decrease in the GSH levels compared to non-irradiated control levels.

Topical treatment with quercetin-loaded microemulsion maintained GSH levels close to non-irradiated control, which demonstrate that this formulation significantly prevented the UVB irradiation-induced GSH depletion. The results are in agreement with Skaper et al. [26] who demonstrated that quercetin protects GSH depletion induced by buthionine sulfoximine in cultured human skin fibroblasts and keratinocytes and with Casagrande et al. [3] who demonstrated that topical formulations (non-ionic emulsion and anionic emulsion) containing quercetin (1%, w/w) were able to prevent the UVB irradiation-induced GSH depletion.

It has been demonstrated that ultraviolet exposure leads to sustained elevation of matrix metalloproteinases (MMPs), a family of proteolytic enzymes which contribute to skin photoaging and in skin carcinoma to the spreading of metastatic cells by specifically degrading skin collagen and elastin [22]. The effectiveness of the w/o microemulsion containing quercetin in inhibiting the proteinase secretion/activity increase induced by UVB irradiation was also investigated in this study. The dose-dependent induction of secretion/activity of these proteinases by UV irradiation demonstrated by zymography corroborated with Onoue et al. [16] and Kim et al. [27]. The similarities of the eletrophoretic profiles in the present work with those reported by these authors suggest that the different UVB irradiation doses employed increased the secretion/activity of MMP-2 and MMP-9. Gelatinase A (MMP-2, 72 kDa gelatinase) is expressed in a variety of normal and transformed cells, including fibroblasts, keratinocytes, endothelial cells and chondrocytes. Gelatinase B (MMP-9, 92 kDa gelatinase) is produced by keratinocytes, monocytes, macrophages and many malignant cells [28].

Molecular mechanisms underlying the induction of MMPs after exposure to UV irradiation have been proposed. Fisher et al. suggested that UV radiation activates growth factor receptors, which induce the activation of protein kinase cascades, like the MAPK cascade [29]. The activation would be followed by an increase in the expression of the c-Jun and c-Fos and formation the AP-1 complex, a MMP transcription regulator [30].

ROS generation plays a critical role in the MAP kinase-mediated signal transduction triggered by UV, a rapid and significant inducer of increases in the concentration of hydrogen peroxide and other reactive oxygen species which in turn participate in the triggering of the MAP kinase cascade. Topical treatment with antioxidants may interrupt the activation of MAP kinase pathways, and thus inhibit UV-induced MMP expression in human skin [30,31]. This was demonstrated in several studies reporting effects of antioxidant topical treatments on the inhibition of increased metalloproteinase expression induced by UV irradiation [30,32–35].

The quercetin-loaded microemulsion inhibited the secretion/activity of metalloproteinases (possibly MMP-2 and 9) induced by UVB irradiation, as demonstrated by zymography. In addition to the antioxidant activity, it is likely that quercetin may exert its protective effect via MMP inhibition [36]. This suggestion might be supported by the observation that polyphenols tend to bind to proline-rich structures, which are highly abundant in matrix proteins [37]. However, further studies must be conducted in order to investigate the fine signaling pathways affected by quercetin and whether or not the effects found are linked to its antioxidant properties.

Therefore, this study confirms results by Casagrande et al. [3] who reported the effectiveness of topical formulations containing quercetin against damage induced by UVB radiation exposure. However, incorporation into w/o microemulsion optimized the effects of the flavonoid since a dosage approximately 6-fold smaller produced the same *in vivo* results obtained with non-ionic emulsion containing quercetin. The optimization is probably due to the significant increase in quercetin skin penetration caused by its incorporation into w/o microemulsion.

In conclusion, the present study demonstrates that the w/o microemulsion increased the skin penetration of quercetin both *in vitro* and *in vivo*, and did not cause skin irritation. Furthermore, this formulation was effective against UVB damage-induced decrease of GSH levels and increase of cutaneous proteinase secretion/activity.

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