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Self-Nanoemulsifying System in the Accumulation of Resveratrol and *N*-Acetylcysteine in the Epidermis and Dermis

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Trans-resveratrol (RES) and *N*-acetylcysteine (NAC) have protective effects on biological processes; therefore, they are frequently included in food supplements. Their possible applications for the prevention of free radical-induced damage to the skin are of particular physiological relevance; however, their usefulness is limited by their metabolic fate and the unpredictability of their delivery to the skin following oral administration. In this work, we evaluated the concomitant and direct application of RES and NAC on the skin using a self-nanoemulsifying system we previously developed for the oral delivery of poorly absorbed food supplements. We evaluated the capability of this system to increase RES and NAC accumulation in porcine skin using permeation studies in Franz diffusion cells. The ascorbyl palmitate (ASP) self-nanoemulsifying system considerably increased RES and NAC accumulation in the epidermis and dermis, which peaked 6 h after application. This study reveals a new formulation strategy to improve the bioavailability of ingredients, which was previously used in the health supplements field, but has rarely been employed in dermatology because of its poor distribution in the skin.

Keywords: *trans*-Resveratrol, *N*-Acetylcysteine, Self-nanoemulsifying ASP, Skin accumulation.

The global incidence of ultraviolet (UV)-induced non-melanoma skin cancer has increased dramatically [1, 2]. Basal cell carcinomas (BCCs) and squamous cell carcinomas (SCCs), which are collectively known as non-melanoma skin cancer (NMSC), are the most common form of cancer in humans, accounting for approximately 80% and 16% of all skin cancers, respectively [3]. It is well known that topically applied antioxidants constitute an important group of agents that protect against UV-induced skin damage [4]. In our continuing investigations on the role of antioxidants in UV-mediated skin disorders [5], we focused our studies on *trans*-resveratrol (RES) and *N*-acetylcysteine (NAC), both of which are currently widely used antioxidants [6-9]. Previously published studies on RES permeation through excised human skin showed that this method of application may be a good alternative to the oral route [10,13]. Few topical applications of NAC have been described [14]. However, RES and NAC are able to substantially reduce reactive oxygen species (ROS)-induced damage and are used to treat diseases mediated by oxidative stress in human fibroblasts [15,16]. Based on these observations, we decided to explore a combination of these two antioxidants using auto-nanoemulsions to improve their accumulation in the skin. In this study, NAC was solubilized in water immediately prior to use. Furthermore, NAC was embedded in an oily surfactant system containing ASP to protect against and prevent oxidative phenomena. Recently, our group developed a new and unique association between active surface agents that is capable of creating very stable nanoemulsions with an average micellar diameter of approximately 100–150 nm, as well as a new method for the production of self-nanoemulsions for oral use [17,18]. In the current study, we adapted and explored the same extemporaneous self-nanoemulsions for topical application. Therefore, the aim of this work was to evaluate the capability of the formulations described in this study to improve RES and NAC accumulation in the skin using the ASP self-nanoemulsifying system (Mixture 1, more precisely 3.5 g of Mixture 1 were dissolved in 100 ml of distilled water). We compared the results obtained using Mixture 1 with the results obtained using a mixture that has the same composition but lacks caprylic/capric acid, polysorbate 80, and ascorbyl palmitate (Mixture 2, more precisely 3.5 g of Mixture 2 were dissolved in 100 ml of distilled water).

In the *ex vivo* experiments, RES and NAC accumulation in the porcine skin was determined using Franz cells. In the receptor compartment, RES was used at a concentration of 0.20 mg/ml and NAC at was used at a concentration of 4.3 mg/ml. As reported, higher concentrations of RES and NAC accumulated in the epidermis and dermis when MIX1 was used as the donor. In particular, the higher concentration of RES (Table 1) was observed with MIX1 after 6 h, when the concentrations that accumulated in the cut dermis was 17.9 μg/mg. In the same manner, the amount of NAC (Table 2) in the epidermis and dermis increased consistently throughout the time course (2, 4, and 6 h), reaching a peak level of 172 μg/mg in the epidermis and 122 μg/mg in the dermis after 6 h when MIX1 was used as donor. This finding agrees with recent reports [19,20]. The auto nanoemulsion system (MIX1) has the capability to significantly increase RES and NAC accumulation in dermis at 4 and 6 h in respect MIX2 that did not facilitate delivery. In fact, we observed the higher amount of RES accumulated in epidermis after 6h, reaching a peak level of 172 μg/mg, when MIX2 was used as donor (Table 1).

RES is a stilbenic, highly lipophilic molecule; therefore, it is very similar to the lipid layer surrounding the horny cells of the stratum corneum. The concomitant action of PS 80 and ASP induces a transitory alteration of the consistency of these lipid domains, which increases the epidermal flux of both lipophilic and hydrophilic molecules and thus improves their absorption.

Table 1: Accumulation of resveratrol (RES) in the epidermis and dermis at 2, 4 and 6 h. All values are expressed as μg of compound/mg of skin ± SEM. n = 6 for MIX1 and MIX2.

Time (h)	Conc. RES in the epidermis (μg/mg) for MIX1	Conc. RES in the epidermis (μg/mg) for MIX2	Conc. RES in the dermis (μg/mg) for MIX1	Conc. RES in the dermis (μg/mg) for MIX2
2	0.92 ± 0.32	17.3 ± 1.47	21.9 ± 2.27	0.92 ± 0.32
4	1.01 ± 0.35	22.3 ± 1.26*	14.7 ± 1.66*	1.01 ± 0.35
6	3.15 ± 0.96*	105.6 ± 5.78*	17.9 ± 2.38*	3.15 ± 0.96*

*$p < 0.05$, MIX1 vs. MIX2.

In addition to these considerations, the nano-sized dispersion of the system in water can explain the increased accumulation of RES in the dermis layer at 2, 4, and 6 h after application compared to MIX2. The molecules can freely dissolve in a large amount of water. A higher amount was delivered by MIX1, with the highest levels observed after 6 h; however, the difference between MIX1 and MIX2 was already evident 4 h after application

Table 2: Accumulation of *N*-acetylcysteine (NAC) in the epidermis and dermis at 2, 4 and 6 h. All values are expressed as μg of compound/mg of skin ± SEM. n = 6 for MIX1 and MIX2.

Time (h)	Conc. NAC in the epidermis (μg/mg) for MIX1	Conc. NAC in the epidermis (μg/mg) for MIX2	Conc. NAC in the dermis (μg/mg) for MIX1	Conc. NAC in the dermis (μg/mg) for MIX2
2	2.96 ± 0.45	1.97 ± 0.24	25.9 ± 1.97	37.0 ± 3.78
4	5.96 ± 0.83*	1.95 ± 0.54*	29.1 ± 0.98*	19.8 ± 2.66*
6	172.0 ± 7.99*	7.17 ± 0.60*	122.3 ± 17.3*	24.5 ± 0.32*

*$p < 0.05$, MIX1 vs. MIX2.

Based on the data presented here, we can conclude that this new delivery system shows promise for the delivery of antioxidant molecules through intact skin, resulting in the accumulation of an important reservoir to protect against oxidative stress.

To the best of our knowledge, the evidence showing that both NAC and RES can accumulate in the dermal layer only 2 h after application is unprecedented and offers new perspectives to extend an approach that has typically been used for dietary supplementation (i.e., a mixture of various ratios of triglycerides, ASP and PS 80) using the same emulsifier systems.

Moreover, RES is a molecule with proven phytoestrogenic activity [21], and NAC is a molecule that is capable of reducing skin ageing and modulating the expression of hyaluronidase and elastase in dermal fibroblasts [22,23]. Based on these observations, we believe that the results presented here represent a new and interesting dermo-cosmetic approach to reduce skin atrophy, time-related skin ageing during the course of menopause, and photo-ageing by increasing the concentration of these two molecules in the skin; the use of these compounds was previously limited by their poor absorption and bio-distribution. Further investigations utilizing this vehicle and non-invasive methods are currently on going in our laboratory to assess its efficacy *in vivo* and to better understand whether the increased absorption corresponds to a significant reduction in early ageing signs.

Experimental

General: The primary and secondary emulsifying agents ascorbyl palmitate (ASP), *trans*-RES (98% from *Polygonum cuspidatum* root) and *N*-acetylcysteine (NAC) were supplied by ACEF SPA (Fiorenzuola D'Arda, PC, Italy), and polysorbate 80 (PS 80; VEREMUL T 80) was obtained from Eigenmann & Veronelli SPA (Milan, Italy). The oily bulk was comprised of caprylic/capric glycerides (Delios V, Cognis, Monheim, Germany). Glycerol and the *Citrus limonum* essential oil were supplied by Esperis SPA (Milan, Italy). Potassium sorbate and citric acid were supplied by Faravelli SPA (Milan, Italy).

Preparation of the ASP self-nanoemulsions: Because ASP has the ability to create micellar dispersions in water, particularly under alkaline conditions; the extemporary nanoemulsion dispersion mixtures were prepared as follows:

Mixture 1 (MIX1):

- A total of 0.75 g of ASP was solubilized in a mixture of 3 g of Delios V, 0.3 g of tocopheryl acetate, and 4 g of PS 80. The solution was warmed to 50°C to completely solubilize the ASP in the mixture until a clear phase was achieved.
- Next, 0.5 g of RES was solubilized in this mixture under moderate stirring and maintained at 50°C until RES was completely solubilized (clear phase).
- After cooling to room temperature, 12 g of NAC were added to the mixture and mixed until a uniform, viscous suspension was achieved.
- The oily surfactant suspension phase was then adsorbed in a pre-mixed powder mixture consisting of 80 g of mannitol, 2 g of anhydrous citric acid, and 0.8 g of sucralose, while taking care to properly distribute the liquid phase into the solid phase. The mixture was mixed with a stirrer until a uniform powder was achieved.
- The final powder was then sifted through an 80-mesh sieve.

The resulting powder generated a uniform nanoemulsion upon dispersion in water, and a higher dispersion pH (> 7.5) lowers the average micellar diameter due to the ionization behaviour of ASP.

Mixture 2 (MIX2):

Mixture 2 was prepared as described above, but lacked caprylic/capric triglyceride, PS 80, and ascorbyl palmitate.

In vitro skin accumulation: Skin accumulation was determined using Franz-type diffusion cells (Ø 9 mm, 5-ml receptor compartment, SES GmbH—Analyse System, Bechenheim, Germany, 0.6 cm^2), and freshly excised porcine ear skin, a reliable model for human skin in terms of permeability, was used as a barrier [24]. The receptor compartment contained 4 ml of phosphate buffer, pH 7.4, whereas the donor compartment contained 1 ml of a mixture obtained by dissolving 3.65 g of MIX1 and MIX2 in 100 ml of water. The blank solution was obtained by dissolving 0.20 mg of RES in 100 ml of distilled water and 4.3 mg of NAC in 100 ml of distilled water.

Porcine ear skin (obtained from a local slaughter house) was removed from the inner portion of the ears by blunt dissection and mounted with the corneal side facing the donor compartment. The receptor compartment was maintained at 37°C and magnetically stirred to avoid any boundary effects. The experiment was stopped at fixed intervals (2, 4, and 6 h), the receptor solution was sampled, and the recovered skin was washed with phosphate buffer. A disc of tissue that fit the area covered by the donor compartment was cut, heated with hot air for 30 s, and then separated into the epidermis and dermis, according to the basal membrane, using a spatula. For resveratrol, we adapted the method described by Zillich et al. [25], but a new HPLC method using a reverse-phase Zorbax C8 column (250 x 4.60 mm, 5 μm, Finnigan System Thermo Electron Corporation) with a detection wavelength of 210 nm was developed for NAC. The mobile phase was a mixture of water with 1% formic acid (v/v) and 75/25 acetonitrile (v/v) at a flow rate of 1 ml/min. These conditions allowed for a good isolation of NAC (retention time 2.0 min, linear range). The injection volume was 200 μl in all cases. Good specificity (absence of interfering peaks derived from the skin samples) was observed. All accumulation experiments were repeated six times.

Statistical analysis: The results are expressed as the mean ± SEM of six experiments. Statistical significance was calculated using a one-way analysis of variance (ANOVA) with a Bonferroni-corrected *p*-value for multiple comparisons. The level of statistical significance was defined as $p < 0.05$.

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