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Confocal Raman spectroscopy: *In vivo* biochemical changes in the human skin by topical formulations under UV radiation

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ABSTRACT

A new approach to the study of the effects on human skin of mycosporine-like amino acids (MAAs) and gadusol (Gad) incorporated in polymer gel is proposed in this work. The depth profile and photoprotector effects of Pluronic F127® gels containing each of the natural actives were evaluated by *in vivo* confocal Raman spectroscopy aiming at the analysis of the biochemical changes on human skin. Hierarchical cluster analysis (HCA) showed that the data corresponding to different depths of the skin, from surface to 4 μm, and from 6 to 16 μm, remained in the same cluster. *In vivo* Raman spectra, classified into five different layers of epidermis according to their similarities, indicated that the amount of Gad gel increased by about 26% in the outermost layer of the stratum corneum (SC) and that MAAs gel at 2 μm depth was 103.4% higher than in the outermost layer of the SC. Variations in the SC of urocanic acid at 1490–1515 cm^{-1} and 1652 cm^{-1} and histidine at 1318 cm^{-1} were calculated, before and after UV exposure with or without gels. With the application of gels the vibrational modes that correspond to lipids in *trans* conformation (1063 and 1128 cm^{-1}) increased with respect to normal skin, whereas *gauche* conformation (1085 cm^{-1}) disappeared. Our studies suggest that gels protected the skin against the stress of the natural defense mechanism caused by high levels of UV exposure.

1. Introduction

The skin provides the principal barrier to the external environment. It is exposed to oxidative stress both from endogenous and exogenous sources such as ultraviolet radiation (UV), which promotes wrinkle formation and loss of tissue elasticity. Most UV rays, which penetrate into the human skin, are absorbed by the chromophores of epidermis, and only the longer wavelengths are transmitted through the dermis. Several chromophores, which absorb the UV or visible radiation, initiate the biological responses including DNA, urocanic acid, amino acids, melanins and their precursors, and metabolites [1]. To induce immune suppression, the electromagnetic energy of UV radiation must be absorbed by an epidermal photoreceptor and converted into a biological recognizable signal [2]. There is evidence that UVB radiation (280–315 nm) directly damages DNA yielding photoproducts such as those formed by adjacent thymine bases, whereas chronic exposure to UVA light (315–400 nm) can cause premature aging and cancer of the skin by inducing the production of reactive oxygen species (ROS) [3,4]. ROS include singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($OH^{\bullet -}$) species that promote lipid, protein, DNA, and RNA oxidation. All these processes are involved in most of the biological effects caused by UV exposure, such as photocarcinogenesis, photoimmunosuppression, photoaging (development of both deep wrinkles and a marked loss of elasticity), sunburn, and photoallergy [5–7]. Urocanic acid (UCA), a major absorber of UV radiation, is produced in the upper layers of mammalian epidermis by the deaminating action of histidase on histidine (HIS) and is a major absorber of UV radiation [8,9]. Epidermal UCA is found predominantly as the *trans*-isomer. This isomer transforms to *cis*-UCA following exposure to UV light until a photostationary state is reached when the concentration of *cis*-UCA is about 60–70% of total UCA [10–12]. The quantum yield of the *trans* to *cis* ($\Phi_{t \rightarrow c}$) photoreaction is low, which generates an inefficient process of protection [12].

Wulf et al. suggested that natural photoprotection involves thickening of SC by sunlight and increased pigmentation [13]. The regular use of sunscreens have been found to exhibit protective effects against the different ROS involved in photoaging and photocarcinogenesis [14,15].

Recent studies have focused on marine organisms as a source of natural bioactive molecules with therapeutic properties. Porphyra-334 and shinorine, two mycosporine-like amino acids (MAAs) isolated from marine red algae (genus *Porphyra*), and gadusol (Gad), obtained from marine fish roes, are structurally related metabolites with photoprotective and antioxidant activities [16–18]. The photoprotective role of this family of natural compounds is supported by their strong absorption and high photostability in the UVA and UVB regions of solar radiation spectrum [17,18]. Moreover, Gad has been found to efficiently deactivate

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photosensitizers and is comparable to ascorbic acid towards reductive reactions of radical species [16,17]. Thus, potential application of these marine compounds has been suggested for prevention and therapeutic treatment of diseases related to the production of free radicals and UV irradiation of the skin. In this context, recent studies have assessed the protective effect of MAAs on human skin fibroblast cells [19]. The authors found that porphyra-334 is able to prevent photoaging by suppressing ROS production and inhibiting the expression of proteases involved in the photoaging of the skin under UV-A exposure [20,21]. In addition, *in vivo* evaluation of the cutaneous photoprotective ability of the MAA porphyra-334 and shinorine was carried out in mouse skin by de la Coba et al. [7]. According to their results, the mixture of MAAs prevented sunburn cell formation and structural and morphological alterations observed in biopsies of non-photoprotected skin. However, to the best of our knowledge, *in vivo* determinations of the effect natural actives based on MAAs and Gad on human skin have not been fully explored yet.

In vivo and non-invasive techniques in dermatology analysis are currently investigated to minimize the use of biopsies. Techniques such as videodermatoscopy, confocal microscopy, laser scanning microscopy, fluorescence lifetime imaging, dynamic infrared imaging, and optical coherence tomography provide fundamental knowledge about the layers of epidermis and superficial dermis, visualization of morphologic structures, and the effects of skin products [22–26]. Although the results are obtained in real time many methods present low specificity, and/or they require pigmented structures for analysis. Furthermore, the techniques do not provide details about the structure conformation of molecular constituents.

Raman spectroscopy has been widely used in recent years, especially for *in vivo* analysis, because it can offer direct molecular information of a sample without labeling, with the advantage of high spatial resolution (below 1 μm), and it is not affected by the interference of water signals. The Raman effect is based on inelastic scattering of light and was reported for the first time in liquids in 1928 by the Indian physicist Sir C.V. Raman [27]. A Raman spectrum is generally displayed by the wave number shift from incident laser to scattered photons against the scattering intensity, thus giving a unique characteristic for a specific chemical structure, comparable to a molecular fingerprint. For human skin, this technique has been applied for cancer diagnosis [28], analysis of physiological component distribution in skin tissue, permeation of cosmetic actives, skin decomposition [29,30], drug depth profiling [31] and biomechanical characterization of the SC [32,33].

In this study, we applied *in vivo* confocal Raman spectroscopy to investigate the biochemical effects of polymer gels containing Pluronic F-127® as delivery systems of MAAs and Gad in human skin. This triblock copolymer is commonly used in technological applications related to cosmetic and pharmaceutical industries [34,35]. Its biocompatibility and low toxicity make it suitable as a carrier for medical and pharmaceutical drug delivery [36,37].

2. Materials and Methods

2.1. MAA and Gadusol Extraction

MAAs were obtained from the red alga *Porphyra leucosticta* collected from the coast of Mar del Plata (Buenos Aires, Argentina) and stored at −20 °C until extraction with 50% ethanol:water. The extract was concentrated and treated with methanol in a sequence of evaporation, suspension, and centrifugation steps. The solid residue was dissolved in water and eluted with 50% methanol:water from an activated charcoal column. The fractions with maximal absorbance at 334 nm were collected and eluted with water from an ion exchange resin (Dowex 50W-X8). Finally, the purified samples were concentrated in a rotary evaporator and analyzed by High Performance Liquid Chromatography (HPLC) with an ODS (C18) column and aqueous mobile phase with 0.02% v/v acetic acid. Two major peaks were observed, and their retention times contrasted with standard samples, thus confirming the presence of the MAA known as shinorine and porphyra-334 at a concentration ratio of 1:4 [38].

Mature female gonads from the marine fish Argentine sandperch (*Pseudopercis semifasciata*) were used as the source of Gad. The extraction was carried out according to the procedure reported by Arbeloa et al. [18]. Briefly, the roes devoid of pelt were homogenized in ethanol and kept overnight at low temperature. The suspension was centrifuged, and after several cycles of filtering and new extraction with ethanol:water mixtures, the filtrates were combined and reduced in a rotary evaporator under vacuum. After successive washing steps with ethanol, chloroform, and water lots, the aqueous phase was concentrated and treated by ion-exchange chromatography on a Dowex 50W-X (8–400, H+ form) resin. The fractions containing gadusol were recognized by the reversible shift of the absorbance maximum from 268 to 296 nm on going from acid to neutral pH [39]. The presence of Gad was confirmed by HPLC analysis and contrasted with standard samples [18].

2.2. Gel Preparation

Gels containing the actives were prepared by using the "cold method" described by Schmolka [40]. A weighed amount of Pluronic F127® was slowly added to cold Milli-Q water (4–5 °C) by gentle mixing in order to obtain 8% w/v Pluronic 127® solutions. The samples were then frozen and kept overnight at 4 °C to form a macroscopically homogeneous and transparent solution. The actives were incorporated into different vials containing the aqueous phase gel up to 0.01% w/v of MAA and 0.01% w/v of Gad, respectively. The solutions were manually agitated and kept in the refrigerator.

2.3. Subjects

A total of 5 healthy subjects (females), age 20–30, with skin type II according to Fitzpatrick scale, without history of dermatological disease, participated in this study. The study was approved by the local ethics committee (CEP number 217948) and all subjects gave written informed consent. They were instructed to stop any topical application of cosmetics in the test area at least 12 h prior to the start of the study, and to withhold any exposure of the test area to artificial or natural UV-light at least 3 days before beginning the study. Raman spectra were recorded directly from the skin of the volar forearms.

2.4. Treatment

Three sites of 0.5 cm^2 each were delimited in the forearm to perform the Raman measurements. One area was used as a control, and each of other two were treated with 10 μL/cm^2 of the respective gel (MAA or Gad) formulation. UV exposure started 60 min after application of gel formulations. This treatment was repeated for 3 consecutive days.

2.5. UV radiation

UV irradiation was performed by using a Xenon lamp (Osram 24 V). A liquid filter was interposed to reduce the intensity within the IR wavelengths. The subjects were exposed for 15 min to approximately 1.3 J/cm^2 of UVA and UVB radiation [5,41]. The power of the radiation source was measured with a FM-GS Field Master meter (Coherent, USA). The dose used in this study is much smaller than 1 MED UVA (Minimal Erythema Doses) measured at 40° N Lat. during a full day in the summer [5].

2.6. Raman Spectroscopy

In vivo confocal Raman measurements were performed before and after UV exposure without and with gel applications. Before each test,