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MALDI MSI analysis of lipid changes in living skin equivalents in response to emollient creams containing palmitoylethanolamide

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ABSTRACT

Mass spectrometry imaging (MSI) is a powerful tool for the study of intact tissue sections. The use of matrix-assisted laser desorption/ionisation (MALDI) MSI for the study of the distribution and effect of emollient treatment on sections of reconstructed living skin equivalents during their development and maturation is described. Living skin equivalent (LSE) samples were obtained at 14 days development, re-suspended in maintenance medium and incubated for 24 h after delivery. The medium was changed, the LSE treated with either Physiogel A.I.® or Oilatum Junior® emollients and then re-incubated and samples taken at 4, 6 and 24 h time points. Mass spectra and mass spectral images were recorded from 12 μm sections of the LSE taken at each time point for comparison using MALDI mass spectrometry (MS). It was possible to detect ions characteristic of each emollient in the LSE. In addition a number of lipid species previously reported as being significant in the maturation of the LSE were observable. At the 24 h time point, the images revealed what appeared to be differences in the organisation of the skin cells observed across the Physiogel A.I.® treatment group tissue sections when directly compared to the untreated tissue group.

1. Introduction

Whilst many immunohistochemistry, autoradiography and spectroscopic methods are routinely used in dermatological research, the application of MALDI MSI is still relatively new. One of the key features of MALDI MSI that makes its use appealing is the ability to detect and study the distribution of multiple compounds simultaneously in a label-free manner. Additionally by using tandem MS analysis, molecules can be identified directly on the tissue sections. In the initial proof of concept study which demonstrated MALDI MSI of biological tissue [1] the technique was introduced for the MS analysis of large biomolecules. Since then however it has been applied to the analysis of a wide range of pharmaceutical compounds in situations ranging from whole animal sections to drug eluting stents [2–4].

A fine line exists between medicinal and cosmetic skin care products. In light of the seventh amendment to the EU directive 76/768/EEC, (which proposes a ban on the testing of cosmetics ingredients and products on animals), the use of *in-vitro* skin models, often described as living skin equivalent models (LSE), has become increasingly important in toxicity testing. Such models are an attractive alternative to the use of both animals and *ex-vivo* human skin for a number of reasons; (i) they allow for the topical application and testing of products used in daily life and can overcome the ethical constraints through being utilised as a substitute to mammalian *ex-vivo* tissue and (ii) the metabolism of skin models during an experiment can be sustained over time if they nurtured in ideal conditions [5,6]. This is not always possible with excised mammalian tissue. Once mammalian tissue is removed from the host, the cells slowly lose their viability, unless metabolism is quenched immediately. One known issue with LSE is however a reduced barrier function compared to human skin.

Emollients are multifunctional formulations for which multiple claims of efficacy are often made. Examples of emollients include: skin-hydration agents, hygroscopic agents, skin permeation enhancers, skin protectants against the external environments, particle coating and suspension stabilizers and essential lipid supplements

Abbreviations: MALDI MSI, Matrix-Assisted Laser Desorption Ionisation Mass Spectrometry Imaging; PCA, Principal Component Analysis; PEA, Palmitoylethanolamide; LLP, Light liquid paraffin.

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for barrier restoration [7]. For chronic skin diseases, many of the emollient treatment constituents are directed towards restoring epidermal differentiation and barrier function via the passive and active functions that they exert. Oil-based formulations containing, *e.g.*, petrolatum, paraffin, or mineral oil (known for their passive functions) can form a water-impermeable film over the surface of skin to decrease evaporation of physiological water [8,9]. Whilst the improvements in skin barrier function arising from treatment with emollients/moisturisers are well documented [10] it is still the case that not much is known on the underlying pharmacodynamic/toxicodynamic effects of many commercially available emollient treatments. Palmitoylethanolamide (PEA) (the endogenous cannabinoid agonist) and light liquid paraffin (LLP) incorporated into emollient formulations have been shown to reduce the clinical signs and symptoms of atopic dermatitis (AD) in children and in adults [11,12]. A recent study carried out using Physiogel A.I.®, the same PEA-containing emollient studied here, supports a role of PEA in the enhancement of lipid production in the stratum granulosum. However, the mechanisms proposed for this effect are at present speculative [13].

In order to study the effects of emollients on skin an untargeted lipidomic study of skin equivalent tissues treated with emollients containing PEA or LLP via MALDI MSI analysis was performed. The aim of the study was to examine endogenous lipid responses to treatment over a time course and to observe treatment dispositions in the skin models. This study follows on from a previous investigation that explored changes within untreated skin models over time [14]. Changes in response to treatment were weighted via multivariate statistical analysis so that lipids contributing to the variances could be identified.

2. Materials and methods

2.1. Materials

Alpha cyano-4-hydroxycinnamic acid (CHCA), acetonitrile (ACN), trifluoroacetic acid (TFA), carboxymethylcellulose (CMC) haematoxylin, eosin and xylene were purchased from Sigma-Aldrich (Gillingham, UK). Dulbecco's phosphate-buffered saline and Dulbecco's modification of Eagle's medium, used for tissue washing and incubations of living skin equivalents, were purchased from Invitrogen (Paisley, UK). The active ingredient of Physiogel A. I.®, palmitoylethanolamide (PEA), of Oilatum Junior®, light liquid paraffin and the emollient treatments themselves were provided by Stiefel Laboratories (GSK, Stevenage, UK).

In addition to the active ingredient the formulated Oilatum Junior® was stated to contain macrogol 1000 monostearate (*i.e.* polyethylene glycol monostearate), cetostearyl alcohol, glycerol, potassium sorbate, benzyl alcohol, citric acid monohydrate, povidone and purified water. In addition to the active ingredient the Physiogel A. I.® formulation was stated to contain *Olea europaea*, glycerin, pentylene glycol, palm glycerides, Olus, squalane, betaine, palmitamide MEA (PEA), acetamide MEA, sarcosine and water.

Labskin™ living skin equivalent (LSE) samples were provided by Innovenn (York, England).

2.2. Treatment and preparation of tissue

LSE samples were provided after 14 days of development. They were delivered as 4.5 cm^2 surface area inserts within transport culture medium. On delivery the LSE samples were partially suspended in LabSkin™ maintenance medium so that the cells were nourished and subsequently incubated overnight for 24 h within 5% CO_2 at 37 °C to normalise their metabolism.

In the second experiment, three LSE samples were treated with the Oilatum Junior® cream and three were treated with the Physiogel A. I.® cream emollient (5 mg per cm^2). For the control group, three skin samples were left untreated. All of these samples were then incubated for 4 h, 6 h or 24 h to create a time course across the treatment groups.

After incubation, the surface of all the samples was carefully washed with deionised water to remove excess formulation and then left to dry in ambient temperature. All samples were then frozen to −80 °C using the Grant Asymptote EF600 Control Freezer and stored at −80 °C until ready for analysis.

Tissue sections with a thickness of 12 μm were cut using a cryostat (Leica 2000 UV, Leica Microsystems, Milton Keynes, UK) and thaw-mounted onto indium tin oxide glass slides for MALDI MS analysis. Sections from each treatment group (at one specific time point) were thaw-mounted onto the same glass slide, so that they could be directly compared. The mounted sections were carefully washed for 20 s with deionised water to remove any excess salts and other impurities. Excess water was taped off and the mounted tissue sections were left to dry at ambient temperature.

The emollients' active ingredients palmitoylethanolamide (PEA) and light liquid paraffin (LLP) were made up in 70% MeOH/30% water (PEA) and in water (LLP) as 500 ng/μl standards. Standards were spotted (1 μl) on the same tissue slide but slightly away from the tissue sections to act as positive controls for imaging and profiling experiments. The spots were left to dry in ambient temperatures.

2.3. MALDI matrix application

A matrix solution of 5 mg/ml CHCA dissolved in a 70% MeOH/30% water/0.2% TFA solution was made up. This was deposited onto other tissue sections and over the compound standards surface using an Image Prep® matrix application device (Bruker Daltonics, Bremen, Germany).

2.4. Mass spectrometry

Analyses were performed using an UltrafleXtreme time-of-flight MALDI mass spectrometer (Bruker Daltonics) with a high-repetition Smartbeam™ laser operated at 2 kHz. Prior to experimentation, the instrument was calibrated using mixed lipid calibration standards (Bruker Daltonics). The instrument was set to reflectron mode to reach its highest mass resolving power (40,000 FWHM). The laser spot diameter was set to 30 μm (as a compromise between spatial resolution and sensitivity with the instrument having a minimum possible spot size of 10 μm) and data was acquired at a spatial resolution of 30 μm × 30 μm, with the laser energy set to 20% arbitrary units. The data was then processed using FlexImaging™ 3 software (Bruker Daltonics). All images were normalised to the total ion count.

For MS/MS profiling, the collision energy was adjusted between 2 eV and 60 eV and the laser energy adjusted between 185 and 200 arbitrary units to produce a signal-to-noise ratio of >4:1 for parent ions and product ions respectively. MS and MS/MS and MSI experiments were conducted on the epidermis and dermis of the different samples within the cohort and were conducted on the spotted compound standards to support the tentative identification of small molecules.

2.5. Data processing

All spectra used for multivariate analysis were re-calibrated by aligning the measured CHCA $[M+H]^+$ mass with the expected mass (*m/z* 190.050). Spectral data lists from the main cohort of the study were examined by principal component analysis (PCA). To perform