INVESTIGATING MELANIN AND ITS DISTRIBUTION IN RECONSTRUCTED PIGMENTED EPIDERMIS MODEL BY FAST 2D XZ MULTIPHOTON IMAGING

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1 INTRODUCTION

Investigating melanin's density and z-epidermal distribution in skin is essential for medical and cosmetic applications. The in situ characterization of this skin pigment requires the use of label free non-invasive imaging methods such as multiphoton microscopy. Using endogenous autofluorescence signals from melanin, keratin, NAD(P)H and FAD metabolic coenzymes, multiphoton microscopy provides non-invasive 3D visualization of epidermal constituents and allows assessing melanin's distribution in vivo in human skin and in vitro in reconstructed pigmented epidermis (RPE) model.

In this study, we report on a new faster method for melanin assessment in RPE model, based on the acquisition of several transversal 2D XZ multiphoton images, equivalent to the ones provided by histology Fontana-Masson (FM) staining. We applied melanin fast 2D XZ multiphoton imaging method to the study of melanin modulations in RPE model upon application of different associations of Ferulic Acid, Niacinamide and Phenylethyl Resorcinol, and compared the results to control samples.

2 MATERIALS AND METHODS

1. Reconstructed pigmented epidermis (RPE) model

Normal human keratinocytes (NHK) and melanocytes (NHM) were seeded onto an insert with a bovine collagen matrix as dermal substitute.

2. Raw material preparation

For all the raw materials (RM) to be evaluated, we prepared the stock solutions in their appropriate solvents (DMSO, water).

Association 1: Phenylethyl Resorcinol+Niacinamide. Association 2: Phenylethyl Resorcinol+Niacinamade+Ferulic Acid.

3. Melanin quantification by Fontana-Masson histology analysis

The melanin present in RPE is stained with Fontana-Masson staining on 5μm-thick transverse sections, then quantified by image analysis. Every epidermis slide is scanned using Nanozoomer® system (HAMAMATSU, Japan).

4. Multiphoton microscopy analysis

Multiphoton imaging was performed with an up-right laser scanning microscope (Nikon A1RMP/FN1, Tokyo, Japan).

5. Multiphoton melanin distribution analysis

All 50 multiphoton transverse XZ images are saved as a stack in 'tiff' format. After that we need to use the cross section, which is alongside the Y axis, as the calculation layer to sum up the melanin pixels and obtain the distribution from the basal skin to the stratum cornuem. In this case, all work was implemented in Python using Numpy and OpenCV packages.
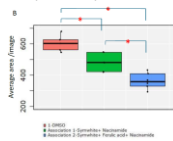
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3 RESULTS DISCUSSION

Melanin quantification by Fontana-Masson image analysis

From the Fontana-Masson image analysis results below (Fig 1), we can find that both associations of active ingredients showed significant depigmentation effect compared to DMSO. The depigmentation effect of the association of Symwhite (Phenylethyl Resorcinol), Niacinamide and Ferulic acid is significantly stronger than the association of Symwhite (Phenylethyl Resorcinol) and Niacinamide. (Table 1)

Fig1. Fontana-Masson image analysis

TOPICAL	Placebo	Lucinol 0.0005%	Association 1 (symwhite 0.0006%+Niacinamide 0.0019%)	Association 2 (symwhite 0.0006%+Niacinamide 0.0019%+Ferulic acid 0.001%)
Average area/image	604.12	480.9	483.12	363.23
P-value (Mann-Whitney)		0.026	0.018	0.006

Table 1. Statistical analysis results of melanin quantification by Fontana-Masson image analysis.

Melanin imaging and quantification by multiphoton microscopy

Fig 3 shows representative images of the melanocytes and melanin (green) present within the reconstructed skin as visualized by 3D multiphoton imaging, with the depigmenting effect of the associations 1 &2 clearly visible. This effect was also quantified based on melanin fluorescence with fast 2D XZ multiphoton imaging, similarly demonstrating a statistically-significant depigmentation in association-treated samples (Fig 2 & Table 2)
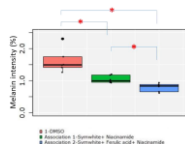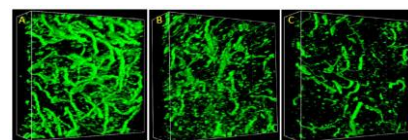

Fig2. Melanin quantification by MMP

Fig 3. Representatives multiphoton 3D images of RPE model treated with A: Solvent control (DMSO), B : Association 1, C: Association 2

TOPICAL	Placebo	Association 1 (symwhite 0.0006%+Niacinamide 0.0019%)	Association 2 (symwhite 0.0006%+Niacinamide 0.0019%+Ferulic acid 0.001%)
Melanin intensity (%)	1.619	1.042	0.792
P-value (Mann-Whitney)		0.004	0.004

Table 2. Statistical analysis results of melanin quantification by fast XZ multiphoton microscopy.

Melanin z-epidermal distribution results using the new image analysis algorithm

Since we got the melanin distribution curve, we can obtain the amount of melanin contained and analyze its changes by integrating it. After integrating, we can see that (Fig 4):

- In the shallow layer (pixel 0 to 40), the melanin content of Association 1 is 13.28% lower than that of Placebo, and Association 2 is more significantly lower compared to Placebo, of which the improvement reached 40.55%. While Association 2 is 31.44% lower compared to Association 1.
- In the deeper layer (pixel 40 – 256), as the same way, Association 1 is 2.33% lower than Placebo, while Association 2 is 26.49% lower. Meanwhile, in this skin layer, Association 2 decreased by 24.74% compared to Association 1.
- The aforementioned results demonstrated the effectiveness of our products in reducing melanin content in RPE model.
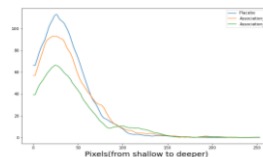

Fig 4. Melanin z-epidermal distribution results obtained by fast XZ multiphoton imaging

4 CONCLUSIONS

This study demonstrated that association 1 (Symwhite (Phenylethyl Resorcinol)and Niacinamide) and association 2 (Phenylethyl Resorcinol, Niacinamide and Ferulic acid) led to a decrease in melanin content in the reconstructed pigmented epidermis by fast 2D XZ multiphoton microscopy analysis, consistent with the results from Fontana-Masson image analysis. Using fast 2D XZ multiphoton imaging method, one can acquire representative images of melanin density within a larger skin area, and at an operational time 5 times faster compared to 3D imaging. Multiphoton XZ imaging combined with the newly developed algorithm for melanin distribution analysis allow for an optimized in vitro multiphoton melanin assessment workflow and it brings a great improvement not only for enhancing the research accuracy during the experiments, but also for significantly improved processing speed in large-scale image processing, strongly assisting experimental progress.

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