Inhibition of advanced glycation end products in skin aging – a new target for *Citrus paradisi*

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Abstract

Skin aging is a multifactorial process in which the formation of advanced glycation end products (AGEs) is a major factor influencing extrinsic and intrinsic aging. AGEs are formed by non-enzymatic reactions between reducing sugars and amino acid residues in proteins such as collagen. These non-enzymatic Maillard reactions lead to the formation of autofluorescent protein crosslinks in the epidermis and dermis. The early stage of AGE formation is associated with cellular oxidative stress, inflammatory reactions and denaturation of essential metabolic enzymes. AGE accumulation in the skin could, in the long-term, be responsible for a reduction in its elasticity, an increase in deep wrinkles and the formation of dark age spots below the epidermis.

The goal of this project was to identify potent glycation inhibitors using a combination of fluorescence-based assays *in vitro* and non-invasive *in vivo* methods in human skin. The characteristic autofluorescence signals of AGEs were measured in a biochemical assay to analyse anti-glycation properties *in vitro*. In this assay, an extract of *Citrus paradisi* inhibited the formation of AGEs in a dose dependent manner, in comparison with a glycation inducer. The efficacy of the *Citrus paradisi* extract was further evaluated by two different methods *in vivo*. Dermal accumulation of AGEs increased the autofluorescence of skin. Therefore, in continuing work, fluorescence measurements of collagen and elastin crosslinks, using a fiber optic probe at excitation/emission wavelengths of 370/440 nm, will be used to directly detect changes in the AGE content of skin. Additionally, skin thickness and the density of the dermis containing echogenic collagen will be analysed using a high frequency ultrasound scanner (DermaScan® C).

In conclusion, a *Citrus paradisi* extract showed promising anti-glycation activity *in vitro*, whereas demonstration of its *in vivo* performance is still under investigation. Rational screening and a scientific approach to the testing of potent AGE inhibitors, *in vitro* and *in vivo*, requires a variety of well-established methods.
Introduction

In recent years, the role of cutaneous accumulation of advanced glycation end products (AGEs) has been discussed in relation to skin aging. AGEs originate in the physiological organism from slow non-enzymatic glycation reactions between reducing sugars and amino acid residues, nucleic acids or lipids. The so-called Maillard reaction is the first step in AGE formation, in which carbonyl groups of sugars react with proteins, lipids, or nucleic acids resulting in unstable Schiff bases [1]. Further rearrangements then lead to the formation of a more stable ketoamine, which is called an Amadori product. These early glycation end products are reversible reaction products but they can undergo non-oxidative rearrangements, hydrolysis or oxidation, leading to the formation of protein adducts or protein-crosslink intermediates in a second phase of the Maillard reaction [2]. In a late stage, these highly reactive intermediates react with lysine and arginine residues in proteins, resulting in the formation of a group of substances known as AGEs (see figure 1). These AGEs are often coloured, fluorescent and form crosslinks in proteins [2, 3].

Fig. 1: Overview of the Maillard reaction and AGE formation [4].

AGEs are irreversible post-translational modifications of proteins that accumulate with age in long-lived proteins such as tissue collagens [5]. AGE accumulation in slowly renewing tissue, like the collagen-containing dermis, could be responsible for its loss of elasticity during aging [3, 4, 6, 7].

The identification of potent glycation inhibitors has attracted great interest in the development of anti-aging cosmeceutical compounds. Therefore, an in vitro screening system for compounds with anti-AGE properties is necessary [8, 9]. To determine anti-glycation properties in vitro, we measured the intensity of AGE autofluorescence after a Maillard reaction [10]. Aminoguanidine, known to possess anti-glycation properties in vitro, was used as a control. [8, 11]. The anti-glycation properties of other substances of interest, in our case, an extract of Citrus paradisi, were compared with this glycation inhibitor. To transfer these observations in vitro to an in vivo system, skin thickness and dermal density due to alterations in collagen were measured using a non-invasive high frequency ultrasound scanner (DermaScan® C) [12]. Since alterations in collagen structure are indirect indicators of AGE reduction, it is necessary to analyse AGEs directly in skin. Fluorescence spectroscopy of human skin can therefore provide morphological and biochemical information. By measuring skin autofluorescence in vivo we have another non-invasive tool to directly detect alterations of AGEs in human skin [13, 14].
Material and Methods

Material

Bovine serum albumin (BSA) was purchased from Merck Millipore; D-(+)-glucose anhydrous, Dulbecco’s phosphate buffered saline (PBS) and aminoguanidine were purchased from Sigma Aldrich; Citrus paradisi extract (Cayoma® Grapefruit) was provided by Qenax AG, Waedenswil, Switzerland.

For a pilot study, standardized body lotions (KO-ML38) containing 0.5 % Citrus paradisi extract (Verum) or not containing the active ingredient (Placebo) were prepared.

INCI Declaration KO-ML38 B (Verum): Aqua, Olea Europaea (Olive) Fruit Oil, Glycerin, Squalane, Caprylic/Capric Triglyceride, Cetearyl Glucoside, Cetearyl Alcohol, Tocopheryl Acetate, Ethylhexylglycerin, Phenoxyethanol, Stearic Acid, Palmitic Acid, Xanthan Gum, Chondrus crispus (Carrageenan), Citrus paradisi Fruit and Peel Extract, Piroctone Olamine, Alcohol, Sodium Hydroxide, Parfum.

INCI Declaration KO-ML38 A (Placebo) : Aqua, Olea Europaea (Olive) Fruit Oil, Glycerin, Squalane, Caprylic/Capric Triglyceride, Cetearyl Glucoside, Cetearyl Alcohol, Tocopheryl Acetate, Ethylhexylglycerin, Phenoxyethanol, Stearic Acid, Palmitic Acid, Xanthan Gum, Chondrus crispus (Carrageenan), Piroctone Olamine, Sodium Hydroxide, Parfum.

In vitro Glycation Assay

The in vitro screening system for potent AGE inhibitors was adapted from the method of Matsuura and colleges [10]. A reaction mix was prepared with either 5 mg/ml BSA in PBS alone (control) or 5 mg/ml BSA in PBS in the presence of 500 mM glucose, which serves as a positive control for glycation. To test the inhibitory properties of the Citrus paradisi extract, the BSA/glucose mixture was added to 5 mM of the glycation inhibitor, aminoguanidine (AG control), or 1%, 0.5%, 0.1% or 0.01% Citrus paradisi extract. The reaction mixture was incubated at 60°C for 48 h. To remove interfering naturally fluorescent compounds, Citrus paradisi extract- containing samples were filtered through an ultra-centrifugal filter (Amicon® Merk Millipore). Fluorescence intensity (excitation wavelength 370 nm and emission wavelength 440 nm) was measured using an LS55 fluorescence spectrometer (Perkin Elmer; Massachusetts, USA) in a multi-well reader setup. Each sample was measured in triplicate.

In vivo pilot study

A double-blind placebo controlled pilot study was conducted with five female volunteers to investigate the anti-glycation properties of Citrus paradisi in vivo. Volunteers, aged between 45 and 55 years, had healthy normal to dry skin and were not suffering from either diabetes I or II, which would have had an influence on the study results. None of the volunteers suffered from an allergy. Each volunteer was informed about the aim of the study and the procedure before the start. The first ultrasonographic measurement was carried out before treatment start. All measurements were performed under controlled environmental conditions (T 22.±0,2°C; RH 31±2%).

Each volunteer received a set of verum and placebo lotions, neutrally coded in airless dispensers, and distribution was randomised among the volunteers. Test lotions were applied two-times daily for a period of 6 weeks on the inner forearms under standard conditions (controlled amounts and applications); references were placebo treated forearms.
Ultrasonographic measurement

The physical principle of a dermatological ultrasound scanner is the emission of high frequency ultrasound (>20 MHz) from a transducer. The sound emission is not continuous but pulsed, i.e., the equipment automatically and very rapidly switches between emission of sound and registration of the sound returning to the same transducer (the echo) from objects being studied. The time lag between emitted and reflected sound waves depends on the physical distance between the surface of the object and the different layers of the object that might reflect the sound. Using different modes of scanning, tissue parameters such as in vivo distance, in vivo cross-sectional area, and in vivo volume can be calculated on a strictly non-invasive basis. Parameters such as skin thickness (mm) and skin density (arbitrary units) can be acquired by a B-Scan. In B-mode scanning, the transducer is automatically moved tangentially over the object and a number of A scans are depicted and processed electronically, resulting in a cross-sectional image of the object in two dimensions (A-mode scan (upper half) and B-mode scan (lower half) of skin). Dermal echoes originate from the well-organized fiber network of the dermis, which is also responsible for the tensile properties of skin. Conditions that erode or disturb this network cause low reflectance. The normal regular fiber network of the dermis is a natural contrast medium in which different pathologies can be outlined if they cause low reflectance [12, 15, 16].

Skin measurements were performed using a DermaScan® C (Cortex, Denmark) and a supplemented image analysis program. For the measurement itself, the measuring head was placed vertically on the skin of the inner forearm, between elbow and wrist. It was important not to put pressure on the skin. The following settings were selected for each measurement: A and B-Scans, 20 MHz, 13 mm penetration depth and a velocity of 1580 m/s. For each of the measured pictures, skin intensity and thickness were quantified.

Skin autofluorescence measurements

The first test measurements to evaluate a reliable method for autofluorescence of skin were carried out using a LS55 fluorescence spectrometer with a remote fiber optic (Perkin Elmer; Massachusetts, USA). Measurements for each volunteer were performed at room temperature in a dark surrounding. The tip of the bifurcated fiber bundle was placed perpendicular to the skin with soft contact. Skin autofluorescence was induced by excitation wavelengths of 330 nm or 370 nm, which are related to collagen crosslinks [13, 14, 17, 18]. The selected emission wavelengths were between 300 nm and 600 nm, with a split of 10 nm.

Statistical Analysis

In vitro data were analysed by F- and T- tests using Microsoft Excel 2010. Pilot study data were analysed by the non-parametric Wilcoxon test for paired samples, using SPSS 22.
**Results and Discussion**

*Glycation inhibition of Citrus paradisi extract in vitro*

The anti-glycation properties of a *Citrus paradisi* extract was demonstrated *in vitro* using an autofluorescent AGE assay. The combination of BSA with glucose (positive control) caused a strong increase in autofluorescence due to AGE formation. The *Citrus paradisi* extract significantly inhibited the formation of AGE autofluorescence, in a dose-dependent manner, from 1% to 0.01% (see Fig. 2). In addition, inhibition of AGE formation was demonstrated using aminoguanidine (AG control) [8, 11]. The *Citrus paradisi* extract contains flavonoids such as naringenin, hesperidin and narirutin, which have been shown to inhibit AGE formation *in vitro* [19]. Our findings are consistent with this.

![Fig. 2: Inhibitory effects of Citrus paradisi extract on AGE autofluorescence in vitro compared to a BSA/glucose positive control and an aminoguanidine inhibitor control (AG control). Mean±SD of three replicates, **p<0.01](image-url)
In vivo pilot study with Citrus paradisi extract

Non-invasive DermaScan® C measurements of five volunteers indicated that the thickness of dermis declined whereas the intensity of signal increased after 6 to 7 weeks of treatment with the Citrus paradisi extract. Table 1 demonstrates that dermal thickness was slightly lower after 6 to 7 weeks on the Citrus paradisi-treated forearm compared to the placebo treated forearm that was not affected.

Table 1: Thickness of dermis at forearm before and after 2 and 6 - 7 weeks treatments. A significant reduction in dermal thickness could be detected after 6-7 weeks treatment with Citrus paradisi extract compared to the thickness before. A treatment with the placebo formulation had no influence on dermal thickness. Mean ± SD of five volunteers.

<table>
<thead>
<tr>
<th></th>
<th>Before treatment</th>
<th>after 2 weeks</th>
<th>p-value</th>
<th>after 6-7 weeks</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td><strong>Citrus paradisi</strong></td>
<td>1.167±0.111</td>
<td>1.153±0.129</td>
<td>0.500</td>
<td>1.074±0.113*</td>
<td>0.043*</td>
</tr>
<tr>
<td><strong>placebo</strong></td>
<td>1.151±0.126</td>
<td>1.129±0.054</td>
<td>0.893</td>
<td>1.091±0.131</td>
<td>0.345</td>
</tr>
</tbody>
</table>

*p<0.05

Moreover, skin intensities of placebo and Citrus paradisi-treated forearms were quantified in this small pilot study. After 6 to 7 weeks, a tendency to higher skin intensity was observed on the Citrus paradisi-treated forearm (green circles) compared to the placebo-treated forearm (black circles), as shown in the following scatter plot.

![Fig. 3: Scatter plot of skin intensity of forearms with five volunteers. Citrus paradisi-treated and placebo-treated forearms were determined before (week 0) and after 2 and 6 - 7 weeks of treatment.](image)

The slight reduction in dermal thickness, and the tendency to higher signal intensity could be due to an alteration of extracellular matrix proteins such as collagen. Differences between Citrus paradisi-treated and placebo-treated forearms were only detected after 6 to 7 weeks. After two weeks, neither dermal thickness nor skin intensity were affected; longer treatment periods were required in order to quantify properties of Citrus paradisi in vivo. However, these preliminary results provide the basis for a further in vivo evaluation with a higher number of volunteers, as well as longer treatment and observation periods.
Skin autofluorescence measurements

In contrast to ultrasonographic skin measurements, the determination of autofluorescence is a direct non-invasive method to investigate AGEs in the skin. There is evidence that emission spectra induced at 330 nm or 370 nm excitation are related to AGE induced collagen crosslinks [13, 14, 17, 18]. The following figure (4) shows the fluorescence spectra of skin at 370 nm and 330 nm excitation wavelengths. The emission signals at 440 nm (excitation 370 nm) and 375 nm (excitation 330 nm) correlates with the content of AGE pentosidine in human skin. We are conducting a further pilot study with *Citrus paradisi* based on skin autofluorescence at 330 nm or 370 nm.

Fig. 4: Autofluorescence of the skin was induced by an excitation wavelength of 330 nm (upper spectrum) or 370 nm (lower spectrum). The selected emission wavelengths were between 300 nm and 600 nm.
Conclusion

We have shown that a *Citrus paradisi* extract is an effective inhibitor of AGE formation *in vitro*. The first *in vivo* results of a small pilot study using ultrasonographic measurements of the skin indicate that there might be an effect of *Citrus paradisi* extract on skin thickness and intensity. It is therefore worth performing further high frequency ultrasonographic *in vivo* evaluations with more volunteers, as well as longer treatment and observation periods, to confirm our preliminary results. To determine whether alteration of skin morphology is due to AGE reduction, another non-invasive method *in vivo* would be necessary to directly detect AGEs in the skin. Therefore, skin autofluorescence measurements could be a promising method to analyse alterations in AGE-induced collagen crosslinks. To identify potent glycation inhibitors, a combination of *in vitro* assays and non-invasive *in vivo* methods in human skin is necessary.
References


