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How to Predict AGEs Accumulation Slowdown Effect of a Cosmetic Ingredient? Two Steps *In-Vitro* System for Evaluating the Anti-AGE Impact of a New Blend

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Abstract

Advanced Glycation End-Products (AGEs), play a crucial part in advancing the process of cellular skin aging and its link to chronological age was re-assessed. AGEs accumulation alters cell structure and function of most types of skin cells, affecting skin's mechanical and physiological properties, following the molecular transformations. Slowdown AGEs accumulation rate in skin, although a potent anti-aging strategy, is difficult and tricky. The lack of working methods for *in-vivo* and *in-vitro* measuring AGEs level complicates the evaluation and prediction of active ingredients' ability to affect cellular AGEs accumulation. A two-step *in-vitro* systematic screening method is proposed and three different cosmetic active ingredients were selected for its demonstration, using BSA-Glucose and Collagen-Glucose predicting models. Candidates' effects on AGEs accumulation were evaluated as standalone, and when formulated in a blend. Additionally, the potency of non-invasive auto-fluorescence *in-vivo* measurement to detect AGEs levels among subjects of different ages was demonstrated. The results are presented in this work and the potential contribution of the proposed system to assist the desired inhibition of AGEs accumulation in skin is discussed.

Keywords

Advanced Glycation End-Products (AGEs), Cellular Skin Aging, Anti-Aging,

AGEs Accumulation, Predicting Models

1. Introduction

AGEs' Accumulation Phenomenon and the Methods for Its Measurement

The aging hypothesis of Advanced Glycation End-products (AGEs) was raised with the assertion that the accumulation of AGEs plays a crucial part in advancing the process of cellular aging [1] [2] [3]. AGEs are modified organic molecules, mainly proteins, lipids, and nucleic acids, that had passed non-enzymatic oxidizing glycation, with the active involvement of sugars [4]. Throughout life's passing years, AGEs gradually accumulate in cells, resulting in a chronic failure to preserve the balanced ratio of AGEs formation and their degradation [5]. Two main biochemistry pathways are involved in the cellular AGEs formation, one is based on the reaction of reducing sugars, especially aldoses and ketoses, and the other involves the impact of reactive carbonyl species with nucleophilic protein sites, catalyzed by metal ions [4].

Skin's chronic exposure to external oxidative stress, originated by UV radiation, cigarettes smoke, and urban pollution, was proven to dramatically boost the formation and accumulation of AGEs within skin cells. In addition, Intrinsic factors related to modern urban lifestyle, namely diets of highly processed and sugar-rich food, and little physical activity, are believed to increase AGEs accumulation [3] [6].

AGEs accumulation alters cell structure and function, affecting skin's mechanical and physiological properties, following the molecular transformations [2] [3]. Almost all skin cell types and components suffer from AGEs accumulation, keratinocytes, fibroblasts, immune cells, melanocytes, and extracellular matrix proteins (e.g. collagen and elastin) [7].

More than 20 different types of AGEs have been identified in the human body [8]. Due to their great heterogeneity, there is no one standard test for AGE measurement. Chromatographic, colorimetric, spectroscopic, mass spectroscopic and serological methods are used to determine AGEs content in biological samples [5] [9].

Simple *in-vitro* lab methods are needed, as a working tool, when searching ingredients, which affect AGEs accumulation in skin. The desired *in-vitro* method could predict the real glycation within human skin, on the one hand, and be easily performed, *i.e.*, simple to apply, relatively short and not too expensive.

Our work investigates the prospect of achieving reduced AGEs accumulation within the skin, via active cosmetic ingredients, using two complementary *in-vitro* methods, BSA-Glucose and Collagen-Glucose for measuring AGEs accumulation, based on auto-fluorescence measurements [10] [11]. Additionally, we demonstrate the potency of non-invasive auto-fluorescence *in-vivo* measurement to detect AGEs levels among subjects of different ages.

2. Materials and Methods

2.1. Study Population

All measurements were conducted on healthy human subjects from Hadassah Medical center, Israel (age ranged 26 - 82) men and women, according to the Fitzpatrick scale with skin type II or III and with no visible skin abnormalities. All studies were carried out following informed consent. The study was approved by the Institutional and Ministry of Health Ethics Committee.

2.2. Skin Auto-Fluorescence Measurements

In vivo Fluorescence spectroscopy was performed using a spectrofluorimeter (Fluoro-Max 4, JY Horiba, Edison, NJ, USA). The excitation source was a Xenon arc lamp. Measurements were performed by placing the fibreoptic probe in contact with the skin site of interest in human volunteers. Acquisition of emission spectra was the preferred method of measuring *in vivo* skin auto-fluorescence. Serial fluorescence emission spectra were measured from healthy human volunteers (n = 11 - 16). All measurements were performed on the abdomen.

The fluorophores were detected: tryptophan moieties, pepsin-digestible collagen cross-links (PDCCL), collagenase-digestible collagen cross-links (CDCCL) and elastin cross-links (ECL). Serial fluorescence emission spectra were used [12], for pepsin-digestible collagen cross-links (PDCCL), excitation was set at 335 nm, and emission was scanned from 360 nm to 450 nm (maximum at 380 nm). for collagenase-digestible collagen cross-links (CDCCL), excitation was set at 370 nm, and emission was scanned from 420 nm to 550 nm (maximum at 460 nm); for elastin cross-links (ECL), excitation was set at 420 nm, and emission was scanned from 480 nm to 550 nm (maximum at 500 nm).

2.3. Data Analysis

Correlations between age and fluorescent intensity values were calculated using Spearman's rho. A probability of $p < 0.05$ was considered statistically significant.

2.4. Chemicals and Materials

Myrrh resin extract, *Commiphora abyssinica* (Glycerolat® of Myrrh, Solabia, France). Osmoter, Dead Sea water extract (DSW) (AHAVA the Dead Sea Laboratories, Israel). Silybum extract, *Silybum marianum* seeds extract (ACTIPHYTE™ Milk Thistle, Lipotec, Spain). A blend mixture of 0.5% (v/v) Myrrh resin extract, 0.2% (v/v) DSW and 1.0% (v/v) Silybum extract. in double-distilled water (DDW). Bovine serum albumin (BSA) (Sigma Aldrich, U.S.A) and Collagen solution (6 mg/ml) from bovine skin (Sigma Aldrich, U.S.A).

2.5. *In-Vitro* AGEs Accumulation Chemical Models

In-vitro AGEs accumulation was evaluated using two models:

2.5.1. BSA-Glucose AGEs Accumulation Model

AGE-BSA was prepared by incubating a final concentration of 6 mg/ml BSA with 0.2 M D-glucose (Sigma Aldrich) in 50 mM PBS pH 7.4 in glass vials. Samples of tested ingredients were added to the glass vials, protected from light, and incubated at 37˚C for 14 days before auto-measurements.

2.5.2. Collagen-Glucose AGEs Accumulation Model

AGE-Collagen was prepared by incubating a final concentration of 1 mg/ml collagen (Sigma Aldrich) with 0.2 M D-glucose (Sigma Aldrich, U.S.A) in 50 mM PBS pH 7.4 in glass vials. Samples of tested ingredients were added to the glass vials, protected from light, and incubated at 37˚C for 14 days before auto-measurements.

2.6. Auto-Fluorescence Measurement

After incubation, samples were placed in a UV 96 wells dish and measured at excitation/emission 350/450nm. The samples were measured at T0 and after 14 days. The results were calculated as relative fluorescence units (RFU) by:

RFU for BSA was calculated by = [RFU (BSA + glucose + Ext.) – RFU (glucose + Ext)].

RFU for collagen was calculated by = [RFU (collagen + glucose + Ext.) – RFU (glucose + Ext)].

Final RFU after 14 days incubation were calculated as = [RFU T_{14days} – RFU T_0].

2.7. Data Analysis

In-vivo measurements on human subjects: Correlations between age and fluorescent intensity values were calculated using Spearman's rho. A probability of $p < 0.05$ was considered statistically significant.

In-vitro measurements: Four replicates experiments were analyzed unless otherwise noted. T-test assay was performed for statistical significance. A probability of $p < 0.05$ was considered statistically significant.

3. Results

3.1. *In-Vivo* Evaluation on Human Subjects of AGEs Related Biomarkers Expression in Skin

Correlations between skin's content of AGE-affected vital molecules and chronological age were investigated on healthy volunteers aged 26 to 82 years. Special probes were attached to volunteers' abdomen skin. The contents of cross-linked collagen and elastin in the dermis were detected, using an auto-fluorescence intensity reader in specific emission spectra. As shown in **Figure 1**, a significant correlation between tested volunteers' age and the detected intensity of fluorescence was established in three tested AGE affected biomarkers, including pepsin and collagenase-digestible collagen cross-links (PDCCL, CDCCL), and cross-linked elastin (ECL) ($p < 0.05$ for PDCCL and CDCCL and $p < 0.001$ for ECL).

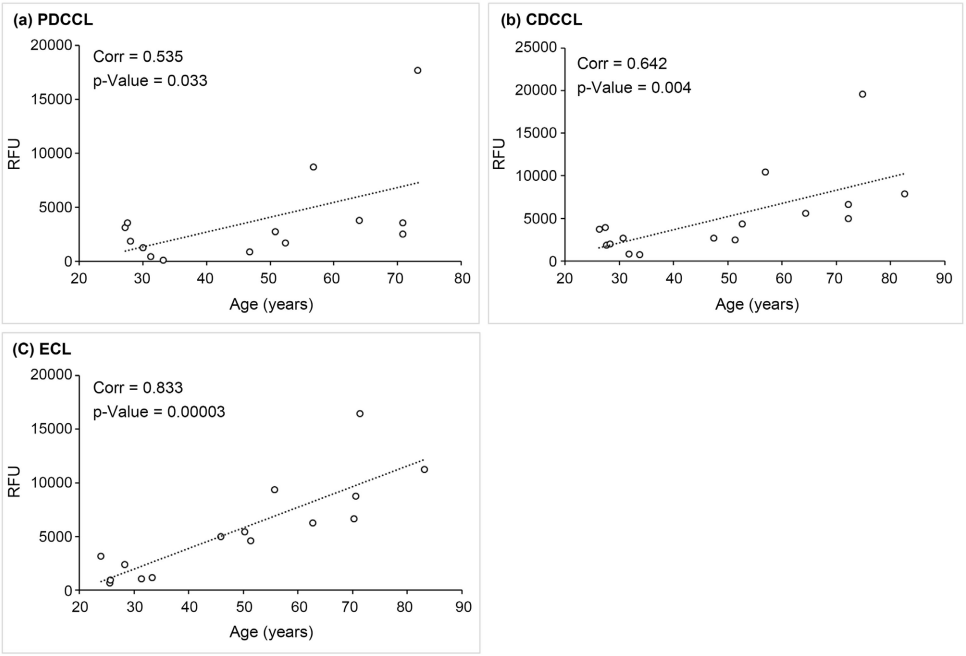

Figure 1. Cross-linked collagen and elastin increase with age in human skin. Skin auto-fluorescence was assessed on healthy volunteers (n = 15 - 16) of different ages by measuring the emission spectra on the abdomen of PDCCL (a), CDCCL (b), and ECL (c) as described in Methods. Correlations between age and fluorescent intensity values were calculated using Spearman's rho.

3.2. *In-Vitro* Evaluation of Protein-Glucose Models as Indicators for Slowing Down AGEs Accumulation, Following Treatments with Active Ingredients

Two *in-vitro* protein-glucose bio-reactivity models were used, one with BSA and the other with collagen as proteins. These BSA-Glucose and Collagen-Glucose models were served as acceleration models, enabling prediction of the possible slowdown effect of active ingredients in a few days, which generally takes years.

As described in Methods, BSA, usually representing non-specific protein, was incubated with 0.2M glucose in pH-7.4 solution with Myrrh resin extract, Dead Sea Water (DSW), and Silybum extract or blended mixture.

In our work, three cosmetic active ingredients were collected and tested on BSA-Glucose and Collagen-Glucose accelerating models. Their AGEs accumulation was evaluated as standalone and when formulated in a blend, composed of three different ingredients.

Based on preliminary screening, Myrrh resin extract, Silybum extract, and Dead Sea water were selected and incubated with the BSA-Glucose model to measure AGEs accumulation. In addition, a blend comprised of three selected ingredients, was prepared and its effect on AGEs accumulation was tested using BSA-Glucose and Collagen-Glucose *in-vitro* models.

As shown in Figure 2, After 14 days of incubation, the auto-fluorescence signal of the control sample contains BSA and Glucose, had elevated by 200 folds compared to the sample contained BSA only. When BSA-Glucose model was incubated with Silybum extract or DSW, the AGEs signals were significantly re-

duced, compared to the BSA-Glucose control. Incubation of BSA-Glucose model with the blend mixture led to a reduced level of AGEs by 56% compared to the BSA-Glucose control.

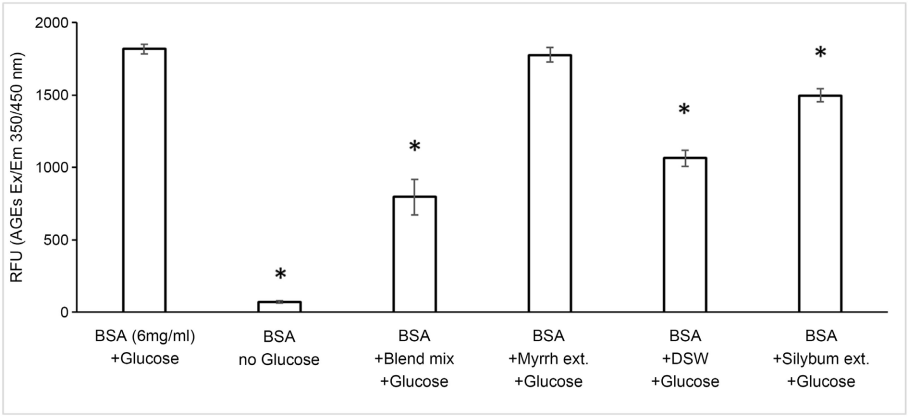

Figure 2. Auto-fluorescence of AGEs generation on BSA after Myrrh resin extract, DSW, and Silybum extract exposure. Each sample contained 6 mg/ml BSA, 0.2 M D-glucose and 0.5% (v/v) Myrrh resin extract or 0.2% (v/v) DSW 1.0% or 1.0% (v/v) Silybum extract or (v/v) or blended mixture. Negative Control was used as 6 mg/ml BSA without D-glucose. Samples were incubated at 37˚C for 14 days. Auto-fluorescence was measure as Em/Ex 350/450nm. A probability of $p < 0.001$ was considered statistically significant vs (BSA + glucose) sample.

The blended mixture was tested with a collagen solution derived from bovine skin. The results showed that the blended mix reduced the AGEs accumulation by 81% compared to the untreated control, as illustrated in **Figure 3**.

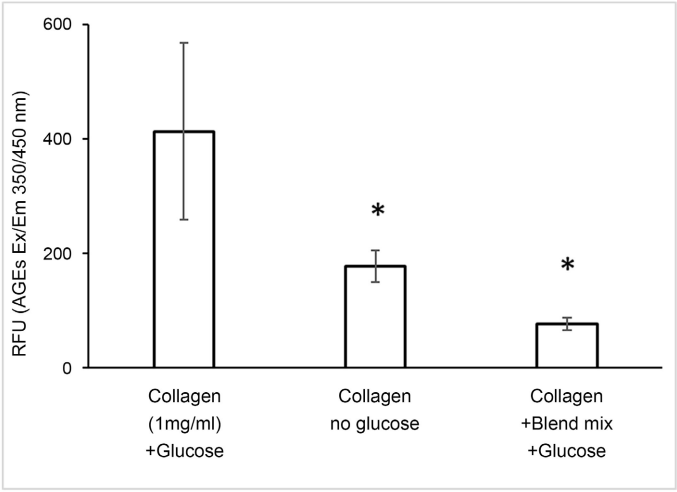

Figure 3. Auto-fluorescence of AGEs generation on collagen after exposure to a blended mixture of Myrrh ext., DSW and Silybum ext. Each sample contained 1 mg/ml Collagen, 0.2 M D-glucose. Blended mixture was composed by 0.5% (v/v) Myrrh resin extract, 0.2% (v/v) Osmoter and 1.0% (v/v) Silybum seeds extract in DDW. Negative control was used as 1 mg/ml Collagen without D-glucose. Samples were incubated at 37˚C for 14 days. Auto-fluorescence was measure as Em/Ex 350/450nm. A probability of $p < 0.05$ was considered statistically significant vs. (Collagen + glucose) sample.

4. Discussion

AGEs formation is a prolonged process, and therefore, long-lived proteins are its most vulnerable target molecules [3] [6]. In skin, a healthy and young appearance is highly dependent on the good functioning of these long-lived proteins, namely collagen, elastin, laminin, and fibronectin. The direct link between chronological age and cross-linked collagen and elastin, were re-assessed in this work. The accumulation of pepsin and collagenase-digestible collagen cross-links and cross-linked elastin were detected and found in a high correlation with the age of the volunteers, as presented in **Figure 1**. Reducing the formation rate of cross-linked collagen and elastin within the dermis is, therefore, one of the most valuable objectives, while aiming to affect age-related physiological skin changes. Consequently, a proactive anti-aging strategy is proposed to slow down the unavoidable accumulation of AGEs within the skin. The AGEs accumulation slowdown strategy is associated with the protective and preventive approach, allocating most efforts to decelerate the process of biological skin aging, rather than fighting its undesired symptoms, when they appear.

Once synthesized within skin, AGE molecules tend to remain stable, and hence, the choice to enhance AGEs degradation ratio is not a real effective option. Several approaches are considered for inhibiting the synthesis rate of new AGE molecules, according to the different reactions, leading to AGEs creation, involving various inducers, precursors, and intermediates. Among the AGEs inhibition approaches, one may find metal chelators, reactive carbonyl species quenching molecules, and anti-oxidants [4]. Many plants contain different antioxidants, such as tea polyphenols, pomegranate polyphenols, spirulina polysaccharides, and blueberry anthocyanins. All can scavenge free radicals and protect skin from damage, caused by free radicals, and thereby may attenuate AGEs related skin damages, including the formation of cross-linked collagen. Formulating plant extracts, and other ingredients, known for their antioxidative activity in the formulae of anti-AGEs, anti-aging skincare products could be considered a reasonable choice.

The lack of standard clinical tests for *in-vivo* measurements of AGEs accumulation within the skin, raises the need to find working methods for evaluating AGEs *via* monitoring its affected molecules. Detecting the level of AGE related biomarkers, using attached to skin probe, connected to a fluorescence spectroscopy instrument, is proposed in our work as an uncomplicated answer to the need for *in-vivo* assessment of skin's AGEs level. Moreover, these measurements demonstrate clearly the correlation between Skin's AGEs levels and chronological aging.

In order to develop skincare products, aiming to effectively treat AGEs accumulation, there is a need for a simple lab method, which could supply predicting data, enabling cosmetic researchers to screen various actives, and select the most promising ones. Furthermore, the desired method, is expected to help establish marketing anti AGEs claims, proving the AGEs attenuation effect of the tested

active ingredient, as standalone, mixed in a blend with other actives, or when formulated in a final cosmetic product. Predicting AGEs' accumulation rate in the laboratory is a challenging task, since the process of their accumulation in skin is relatively slow, naturally involving chronic exposure, which takes months and years. Several accelerating lab methods have been proposed, using *in-vitro* protein-sugar biochemical models [5].

In our work, following early screening, three cosmetic active ingredients were selected and tested: *Silybum marianum*, Myrrh resin and Dead Sea water.

Several scientific studies report that the plant *Silybum marianum*, which contains the active ingredient, silymarin, has a proven anti-AGEs capability [13]. Silymarin was found as an effective molecule to reduce the level of glycated albumin in plasma of diabetic rats and decrease the content of total level of AGEs [14] [15].

Myrrh is a bush of desert flora, known to synthesize secondary metabolites, enabling its survival in the extreme conditions of the desert. Furthermore, *Commiphora myrrha* extract had presented anti-diabetic capabilities, when added as a food supplement to induced diabetic rats [16].

Dead Sea water, containing high levels of minerals, in addition to established therapeutic impacts to heal skin diseases, was proven for its contribution to improving skin smoothness, skin moisturizing level, and protecting skin against premature aging [17] [18].

To predict the capacity to slowdown the process of AGEs accumulation, the three selected cosmetic ingredients were tested, using two protein-glucose accelerating models, described in Methods, suggesting a distinct capability for each tested ingredient to contribute to the desired effect. A complex of the three selected ingredients had been blended, and its effect on AGEs accumulation rate was predicted, using both BSA-Glucose and Collagen-Glucose *in-vitro* models. Based on the results, partially described in **Figure 2** and **Figure 3**, an anti-AGEs patent was applied [19].

Due to its relative simplicity, performing BSA-Glucose tests is proposed as an initial filtering tool, enabling to perform a quick and inexpensive wide screening. This is because Albumin is very abundand and represents general and water-soluble proteins. Collagen-Glucose is suggested as a complementary step, to be performed on a more precised list of candidates. We suppose that the Collagen-Glucose model could be considered as better representing the actual AGEs accumulation process that occurs in dermis, with the resulting crossed linked collagen.

5. Conclusions

The combination of both BSA-glucose and collagen-glucose methods is suggested as a two steps complementary system, starting with a wide screening step, on simple BSA-Glucose method and followed by more relevant to skin Collagen-Glucose model, allowing to predict anti-AGEs activity.

Further work is needed to better understand the biological mode of activation of AGEs accumulation, and the involved steps, which may support slowing its rate within the skin.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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