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Topical equol preparation improves structural and molecular skin parameters

U. Magnet*, C. Urbanek†, D. Gaisberger†, E. Tomeva*, E. Dum*, A. Pointner* and A.G. Haslberger*
*Department for Nutritional Research, University of Vienna, Vienna 1090, Austria and †HealthBioCare, Mooslackengasse 17, Vienna 1090, Austria

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Abstract

OBJECTIVE: Equol has been shown to improve skin health and regeneration, due to its antioxidative, phytoestrogenic and epigenetic characteristics. The effects of a topical intervention on skin structure, telomere length and epigenetic markers in skin cells were analysed.

METHODS: Sixty-four participants were divided in four groups and three of them treated topically with the following: emulsion with Equol powder (Isoflavandiol-E-55-RS®); emulsion with microencapsulated Equol (Vesisorb® Isoflavandiol-E-55-RS®) and an emulsion with lecithin (Vesisorb® placebo). A control group of 6 volunteers did not receive any intervention. The active compound was a 0.5% equol-racemate. For 58 participants, all samples were collected. Structural analysis, molecular analysis and questionnaires were performed at the start of the study and after 8 weeks of intervention, twice a day. Structural skin parameters were analysed by Visioscan® VC 98 and Cutometer® dual MPA 580. Molecular analyses from epidermal cells collected by skin stripping of the forehead included telomere length and LINE-1 methylation, following DNA extraction, bisulphite conversion and qPCR as well as high-resolution melting curve analysis. Effects of nutrition and lifestyle habits were evaluated with a standardized food and lifestyle questionnaire.

RESULTS AND DISCUSSION: The surface analysis showed significant improvements in skin roughness, skin texture and skin smoothness after both interventions. Cutometer® dual MPA 580 measurement revealed improvement of skin firmness and elasticity parameters for both preparations. A decrease in mean LINE-1 methylation (n.s.) and telomere length (sign. $P < 0.05$) was observed in the sample group with age. In the treated groups, significantly longer telomeres were observed after intervention. Whether changes in telomere length reflect changes in the regulation of telomerase, epigenetic interactions or turnover of keratinocytes needs further research. Stability and availability of preparations in skin seems to be high as not many significant differences in the activity of pure or encapsulated substances were seen.

CONCLUSION: The results of this study indicate that equol has beneficial effects on structural as well as molecular skin parameters and encourages further investigations to decipher the epigenetic regulation of skin ageing and interactions of equol.

Correspondence: Alexander Haslberger, Department for Nutritional Research, University of Vienna, 1090 Vienna, Austria. Tel.: +43 6991 2211212; e-mail: Alexander.haslberger@univie.ac.at

Résumé

OBJECTIF: Il a été démontré que l'équol améliore la santé et la régénération de la peau grâce à ses caractéristiques antioxydantes, phytoestrogéniques et épigénétiques. Les effets d'une intervention topique sur la structure de la peau, la longueur des télomères et des marqueurs épigénétiques dans les cellules de la peau ont été analysés.

PROCÉDÉS: Soixante-quatre participants ont été divisés en quatre groupes dont trois groupes ont reçu un traitement topique avec les préparations suivantes: une émulsion avec poudre d'équol (Isoflavandiol-E-55-RS); une émulsion avec équol microencapsulé (Vesisorb Isoflavandiol-E-55-RS) et une émulsion avec la lécithine (placebo Vesisorb). Un groupe de contrôle de 6 volontaires n'a reçu aucune intervention. Le composé actif était de l'équol-racémate à 0,5%. Pour 58 participants, tous les échantillons ont été collectés. L'analyse structurelle, l'analyse moléculaire et des questionnaires ont été menés au début de l'étude et 8 semaines après l'intervention, deux fois par jour. Les paramètres structurels de la peau ont été analyses à l'aide du Visioscan VC 98 et du Cutometer dual MPA 580. Les analyses moléculaires des cellules épidermiques collectées par pelliculage de la peau du front comprenaient la longueur des télomères, la méthylation LINE-1, suivant une extraction d'ADN, une conversion au bisulphite et le qPCR ainsi que l'analyse de la courbe de dénaturation haute-résolution. Les effets de la nutrition et des habitudes du style de vie ont été évalués avec un aliment standardisé et un questionnaire relatif au style de vie.

RÉSULTATS ET DISCUSSION: L'analyse de surface a démontré des améliorations significatives de la rugosité, de la texture et de la douceur de la peau après les deux interventions. La mesure au Cutometer dual MPA 580 a révélé une amélioration de la fermeté et de l'élasticité de la peau pour les deux préparations. Une diminution de l'hémi-méthylation LINE-1 (n.s.) et de la longueur des télomères (sign. $P < 0,05$) a été observée dans le groupe échantillon avec l'âge. Dans les groupes traités, des télomères significativement plus longs ont été observés après l'intervention. Des recherches supplémentaires s'imposent pour savoir si les changements dans la longueur de télomères sont dus à des changements dans la régulation de la télomérase, à des interactions épigénétiques ou au renouvellement de kératinocytes. La stabilité et la disponibilité des préparations dans la peau semblent être élevées, étant donné que peu de différences significatives dans l'action entre les substances pures et encapsulées ont été observées.

CONCLUSION: Les résultats de la présente étude indiquent que l'équol possède des effets bénéfiques sur les paramètres structurels et moléculaires de la peau et encouragent des investigations

supplémentaires pour décrypter l'épigénétique. 5 regulation of skin ageing and interactions of equol.

Introduction

Phytochemicals have been shown to affect skin health and ageing due to their antioxidative properties [1]. Even before the isoflavonoid 'equol', as an active ingredient has attracted the attention of scientists and the cosmetic industry [2].

At a fundamental level, ageing can be outlined as a progressive decline of the biological functions of human cells, ultimately resulting in senescence. Ageing processes, affected by environmental, nutritional and lifestyle factors, involve molecular mechanisms such as shortening of telomeres, epigenetic and mitochondrial changes, DNA instability and reduced rate of cell delivery from stem cell departments [3]. During intrinsic skin ageing, epidermis and dermis thin down, collagen content decreases and elastic tissue progressively disappears in the papillary dermis. Extrinsic skin ageing, especially photoageing, is marked by loss of elasticity, irregular pigmentation, deep wrinkling and increased roughness and dryness. It has been suggested that sun exposure causes 80% of facial ageing [4].

Equol

Equol belongs to the class of polyphenols and is a metabolite of the isoflavone daidzein. It is assumed that only 24–30% of the Western population is able to metabolize equol from soy daidzein, whereas in Asian countries, 50–60% of the population are 'equol producers'. The bacterial composition and intraluminal conditions in the gut are the major factors for these interindividual differences [5].

Equol and skin ageing

The isoflavonoid equol provides three main properties to improve skin health and to fight against skin ageing 1. antioxidative capacity, 2. phytoestrogenic activity and 3. epigenetic effects. Isoflavonoids, due to their phenolic structure, are able to reduce oxidative stress. Oxidative stress contributes to the skin ageing process. It results from an imbalance between the formation of free radicals and the capability of endogenous defence mechanisms [6]. In a study of Mitchell *et al.*, [7] equol showed a higher antioxidative efficacy, when compared to other isoflavonoids. Further Widyarini and colleagues found evidence in hairless mice that topically applied equol has photo-protective effects and thus is able to decrease inflammation, photoageing and carcinogenesis in skin [8]. As a phytoestrogen, equol has binding affinity for oestrogen receptors, which is significantly higher for oestrogen receptor-beta (ERβ). ERβ occurs to a high degree in keratinocytes of the epidermis and in the collagen-producing fibroblasts of the dermis. Therefore, equol as an ERβ agonist is able to induce positive oestrogen-like consequences in skin [6, 9]. Equol has several positive effects in improving skin health and regeneration including the stimulation of collagen and elastin gene expression. In 2013. Lephart compared the effects of equol isomers concerning skin ageing. They found that R-equol and racemic equol showed better effects on dermal gene expression than the S-isomer [10]. Isoflavones have been shown to affect all major epigenetic mechanisms [11]. Equol and other polyphenols are able to inhibit DNA methyltransferases (DNMTs) [12]. Improved structural skin characteristics due to topical equol treatment were reported before (SOFW, Global Ingredients & Formulations Guide, 2016).

DNA methylation, nutrition and ageing

DNA methylation is the most studied epigenetic mechanism and very important for the understanding of the phenotypic changes associated with human ageing [13]. It was confirmed in several species and tissues that the global DNA methylation level in the genome of mammals decreases with age. This age-dependent global hypomethylation depicts the demethylation in repetitive elements, which are abundant in CpGs [14].

Line 1

LINE 1 (long interspersed nuclear element 1) is a retrotransposable element and represents about 17% of the human genome [15]. Due to their high occurrence in the genome, repetitive sequences including LINE-1 can serve as markers for determining global genomic DNA methylation [16, 17]. Decreased LINE-1 methylation is linked to a higher risk for metabolic syndrome and other obesity relevant disorders and thus should be considered a metabolic parameter [18, 19]. In addition, global hypomethylation of LINE-1 has been described in many cancers [20]. A high LINE-1 methylation correlates with a healthier lifestyle (sports and not smoking), which was recently reported in the investigation of Marques-Rocha and colleagues [19]. Moreover, studies showed that alterations in repetitive elements such as LINE-1 are associated with age [21, 22].

Telomeres and ageing

Telomeres are nucleoprotein structures at the end of eukaryote chromosomes. Those structures have the ability to protect and stabilize the chromosomes. During the ageing process, telomeres become shorter with each cell division and are finally unable to sufficiently protect chromosome [23, 24].

With each cell division, telomeres decrease up to 150 base pairs, meaning telomere shorting acts like a biologic clock [25–27]. Specialized reverse transcriptase maintains telomere length and is called telomerase. Normal somatic cells lack telomerase resulting in consequent telomere shortening. [28]. Telomerase was detected in keratinocytes but not in skin fibroblasts [25]. When telomeres get a critically short length, the cell becomes senescent. Thereby, the cell irreversibly stops proliferation.

Various studies showed a decrease in telomere length with age, which varied between individuals and species of the same age. The average reduction rate of telomeres ranges from 19.8 bp per year up to 92 bp per year. Sugimoto *et al.* showed in epidermal, as well as in dermal ageing, associated telomere loss with a rate of 9 and 11 bp per year, respectively [27, 28].

Materials and methods

Subjects

The study investigated the effect of topically applied equol on the forehead area of women. The inclusion criteria were as follows: no apparent skin disorder (psoriasis, dermatitis, skin cancer), female at the age of 40–60 years. All subjects signed a written informed consent and confirmed thereby all participation criteria. Sixty-four subjects were recruited. Finally 58 female participants were randomly assigned to one of the following study groups:

- Group 1: emulsion with lecithin (placebo)
- Group 2: emulsion with equol powder
- Group 3: emulsion with microencapsulated equol
- Group 4: no intervention

To ensure a pleasant and hygienic application, the emulsions were packaged in a polypropylene roll-on. Roll-ons of group 1 to 3 had no differing external characteristics.

The groups of volunteers were sampled at specific time-points. During the treatment duration of 2 months, participants were asked to use no other skincare products in the forehead area. They also received detailed instructions, including how to apply the emulsion two times a day.

Study design

We analysed skin parameters before (T0) and after (T1) the treatment with scientifically certified skin analysis equipment provided by Courage and Khazaka Electronic GmbH (Köln, Germany). The UVA-light video camera Visioscan® VC 98 can take high-definition pictures of skin surface and determines a variety of different parameters. Cutometer® dual MPA 580 is an acknowledged standard device in dermatology and cosmetology to measure elasticity and firmness of the skin.

For molecular analysis, we removed skin cells with a sterile adhesive strip from the forehead of the study participants. We determined methylation (LINE-1) and absolute telomere length of skin cells before and after the treatment period.

A questionnaire for dietary and lifestyle habits was used to assess potential changes within the study period. Further, subjects had to state their skin parameters after the treatment through a self-assessment questionnaire.

Test substances

The active compound in test substances was a 0.5% equol-racemate, either as pure powder (Isoflavandiol-E-55-RS®); or microencapsulated (Vesisorb® Isoflavandiol-E-55-RS®) The placebo consisted only of lecithin.

Food and lifestyle questionnaire

Food and lifestyle affect our health and are positively associated with epigenetic mechanisms and telomere length. The questionnaire, used in the intervention study, included parts about anthropometry (BMI, age, weight, height), state of health (diseases, medication, physical activity, sport etc.) and eating habits (preferred food and portion size, liquid intake, coffee and alcohol consumption).

Self-assessment questionnaire

Subjective perception was assessed by means of self-assessment questionnaire. In this form, we asked participants about the improvement and difference of skin texture. It included questions about the following categories: skin smoothness, moisture, overall well-being, freshness, oiliness and general differences. The validation was categorized in yes (improvement), somewhat positive, somewhat negative and no (non-improvement).

Sample collection

We collected the DNA samples via stripping with sterile PCR films (Thermo Scientific ASF 6020/100) at baseline and after an intervention period of 8 weeks. PCR films were placed on the subjects' forehead and pressed down firmly before careful removal. The films were then put back onto the protective paper and stored in sterilized vessels in the laboratory refrigerator at −4°C.

Skin analysis devices

For the analysis of skin firmness and elasticity, we used the approved standard device Cutometer® dual MPA 580 (Courage and Khazaka, Germany). The ability of the skin to resist against suction (firmness) and to return into its original shape (elasticity) is displayed as curves.

To analyse visible changes in skin surface, we utilized the UVA light video camera Visioscan® VC 98 (Courage and Khazaka, Germany).

Skin analysis

Before the start of skin analysis, standardize room conditions for the measurements were controlled (time-point 0: Humidity: 29.5 ± 5.8% and temperature: 25.1°C ± 1.0°; time-point 1: humidity: 47.4 ± 5.6% and temperature 25.3 ± 1.3°C). To prevent biases, the subjects were instructed to avoid the use of any skincare or make-up products on the analysing days (at baseline and after intervention). The first step was to assess the centre of the forehead from the subjects, to examine the same measurement place at both time-points. We used a tape measure and recorded the middle of hairline horizontally and from hairline to eyebrows vertically in centimetre.

A picture of the skin surface with Visioscan® VC 98 was taken directly on the analysed measurement place followed by the calculation of structural parameters with the software. Additionally sebum production was analysed with the foil Sebufix® F16. Before picture exposure the foil has to stay on the skin for 20 s. Sebum of the skin penetrates into micropores of the foil and becomes visible through 'black spots'. The spots are calculated by the software taking into account quantity (distribution) and size. Thus, it is possible to determine skin grease content. We followed with elasticity and firmness measurement via Cutometer. For this, we put the probe on the centre of the subject's forehead with low but constant pressure. Live curves on the screen represented the measurement, and from them, structure parameters were derived via Cutometer® software. The measurements were performed in mode 1, which is mostly used in literature. In this mode, the skin is inserted into the probe with a constant vacuum. The measurement conditions were as follows: load: 450 mbar, aperture: 2 mm, on-time: 3 s, off-time: 3 s, pre-time: 0, repetitions: 3 (Courage and Khazaka, 2016).

Last step was the sample collection for molecular analysis. We obtained skin cells from the forehead, which we were able to purify and investigate about telomere length and DNA methylation of LINE-1.

DNA extraction

For DNA extraction from the skin cells, we used QIAamp® DNA Mini Kit (QIAGEN, Germany). After sample collection via stripping, we prepared the PCR films (Thermo Scientific ASF 6020/100) for purification. Small pieces were placed carefully with tweezers in 1.5-mL tubes prepared with 400 μL PBS buffer. Then, each tube was vortexed for a few seconds to cover the strips with the buffer. Further steps were performed following the original protocol 'DNA

Purification from Buccal Swabs' (QIAGEN, 2016). The purified DNA samples were stored in the freezer at −20°C.

Bisulphite conversion and high-resolution melting analysis

For methylation analysis of LINE-1 via high-resolution melting (HRM), first bisulphite treatment is performed to convert unmethylated cytosines to uracil. We used the EpiTect Bisulfite Kit (Qiagen, Germany) for complete bisulphite conversion according to the manufacturer's protocol: 'Sodium Bisulfite Conversion of unmethylated Cytosines in DNA'.

For HRM analysis, bisulphite converted DNA samples from the first and second adhesive strip from every participant and every time-point were used. 5 µL of bisulphite converted DNA from each tube was vortexed in a sterile 1.5-mL tube. A mix containing forward and reverse LINE-1 primer (10 pmol μL^{-1}) and methylated controls (25%, 50% and 75%) was prepared with 0% and 100% methylated standards for the standard curve. Two different reaction mixes, one for the standards and one for DNA templates, both containing HRM master mix (5 µL), primer mix (0.75 µL) and RNase-free water (template: 2.25 µL, standard: 3.25 µL) were prepared. Rotor-Gene Q (Qiagen, Germany) was used to perform qPCR and HRM analysis. PCR conditions were as follows: initial step/hold 1: 5 min at 95°C, 45 cycles: 30 s at 95°C, 45 s at 54°C, 30 s at 72°C, followed by two holding steps: hold 2: 1 min, 95°C, hold 3: 1 min, 45°C. During HRM analysis, temperature increased from 65 to 95°C in 0.1°C steps, every 2 s. Primer sequences and PCR conditions are listed in Appendix S1.

Telomere methodology

Absolute telomere length was measured according to a protocol from O'Callaghan and Fenech 2011. For telomere measurement, we used the skin samples after extraction and stored at −20°C. For better results, we mixed DNA elution from the first and second adhesive strip (6 µL elution from each tube). Furthermore, we prepared standard curves for telomeres and single copy gene 36B4, by attenuation of known quantities of oligomers. The master mix (LightCycler Master with SYBR Green Dye from Roche) solution was calculated for the samples and standards. Two master mix solutions were prepared, one for the telomere and the other for the single copy gene.

Accordingly, a qPCR with the single copy gene (acidic ribosomal phosphoprotein 36B4) control was used, with which we standardized the samples. Further relative values were established, while we set T0 to 1 and got a relative factor for F1. AB StepOnePlus™ was used to perform PCR under previously published conditions [29]. Primer sequences and PCR conditions are listed in Appendix S1.

Statistics

For statistical analysis, we used the software IBM SPSS Statistics. The significance was analysed by ANOVA, *t*-test, Pearson's and spearman's correlation, Kolmogorov–Smirnov test and general linear model. Values of $P < 0.05$ were assessed as statistically significant.

Results and discussion

Non-invasive measurements were used for our analysis. We investigated via Cutometer® and Visioscan® the effects of different emulsions (equol, encapsulated equol and lecithin), which were applied to the forehead. Parameters of roughness, smoothness, wrinkles, sebum content, firmness, elasticity, capability of regression, elastic regression and signs of fatigue were determined. Surface analysis with Visiocan device showed significant ($P < 0.05$) changes of all roughness parameters after both equol treatments (pure and microencapsulated). Roughness is defined as the asperity of the skin,

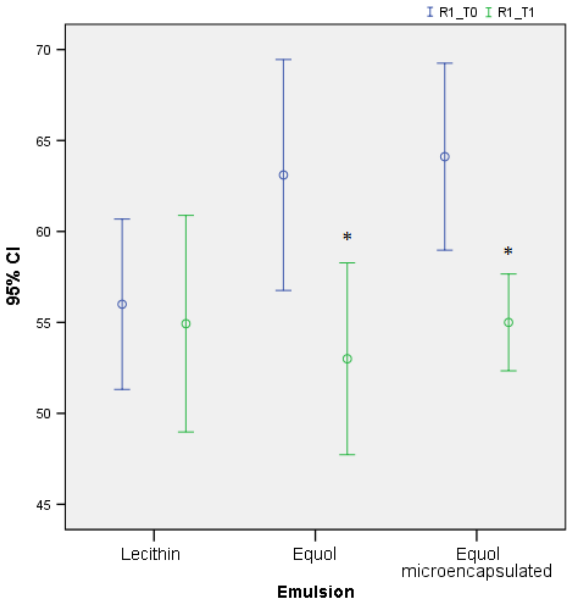

Figure 1 Roughness: Roughness was analysed before and after intervention according to methods using Visioscan® VC 98. Equol treatments using pure and encapsulated preparations show a significant improvement ($P < 0.05$).

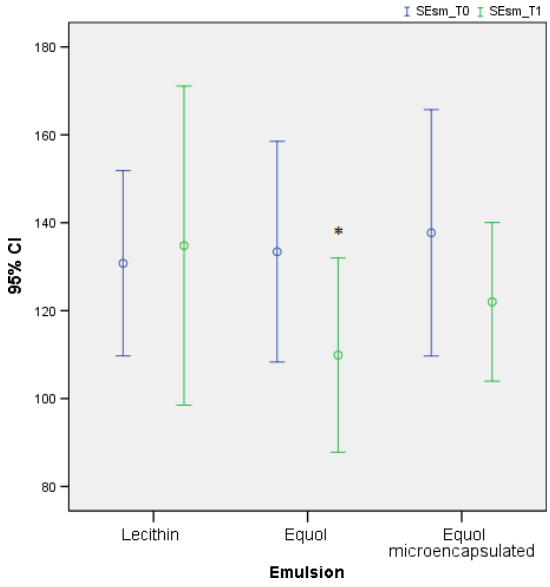

Figure 2 Smoothness (SEsm): Smoothness was analysed before and after intervention according to methods using Visioscan® VC 98. Pure Equol preparation showed a significant improvement ($P < 0.05$).

because primary and secondary lines characterize skin surface. Primary lines are characteristic for individuals, their age and anatomical position. Secondary lines are more affected by external influences such as temperature, cosmetics, humidity, UV-radiations and smoking. With increasing age, the fine lines disappear and deep wrinkles are formed instead [30]. Two SELS-parameters demonstrated significant ($P < 0.05$) improvement with equol (Isoflavandiol-E-55-RS®) or encapsulated equol treatment. SELS-parameter (SEr) showed significant improvement with both emulsions. Equol decreased roughness from 2.99 to 2.34 and encapsulated equol from 3.07 to 2.48. In conclusion, roughness improved in R-parameter, as well as in SEr. The smoothness value (SEsm) improved significantly with pure equol (133.4–109.9 $P < 0.5$). It may depend on the type of action of the anti-ageing treatment, if it fills up wrinkles and product particles remain on skin, the value is suspected to be higher. Thus, both skincare and make-up residues have an effect on SEsm and may explain different outcomes of the two equol treatments, Figs. 1 and 2 (lecithin, equol and microencapsulated equol shown), Appendix S1. Lower baseline roughness values of the lecithin control group compared to the intervention groups (T0) are presumably caused by a higher percentage of younger participants in the lecithin group despite random allocation of volunteers. However, this study focused on the alterations caused by the treatments and as there were no statistically significant differences between T0 and T1 in the lecithin group, the variance among baseline values is negligible.

Equol (Isoflavandiol-E-55-RS®) significantly enhances texture parameter like energy, contrast, variance and homogeneity. Energy indicates the homogeneity of a picture taken by Visioscan® and reflects a general overview on skin conditions. It is important to mention that after treatment with an anti-ageing product, the energy value is expected to increase. All three treatments improved the energy value and therefore skin conditions, whereas another parameter for skin condition 'contrast', showed only improvements with equol (Isoflavandiol-E-55-RS®) intervention. Furthermore, variance and homogenity decreased significantly with equol (Isoflavandiol-E-55-RS®). Those parameters represent roughness and hydration. The ability of equol to bind to the oestrogen receptor is the reason, why it replaces oestrogen and increases epidermal hydration [9]. Surprisingly, only lecithin enhanced entropy values from 1.6 to 1.56. Young skin with a network of many fine lines in all direction forms many corners and results in high corner density. Equol increases corner density from 10.62 to 11.55 significantly ($P < 0.05$). This means equol, but not encapsulated equol, refined skin wrinkles, which is also confirmed through numbers of in-section lines, which increased after equol intervention. Similarly, equol enhanced the number of cells from 644.42 to 681.42. Young skin has a greater number of cells in smaller categories. This is confirmed due to an increase in the small cell class 3 (50–99 pixel) that equol showed and a decrease in higher cell class 5 (>200 pixel). Cell class 4 (100–199 pixel) showed an increase from 173.79 to 187.95. At this point, it is unclear whether this category can be placed among smaller or higher cell classes. Encapsulated equol showed the same result, an increase in cell class 4 and a decrease in cell class 5. With regard to anisotropy and cell parameters, only equol showed good results. Skin sebum increased with encapsulated equol intervention and decreased with lecithin and equol intervention. However, sebum demonstrated no significant correlation with equol intervention, Figs. 3 and 4 (lecithin, equol and microencapsulated equol shown).

Cutometer® measurement was used to evaluate skin parameters of firmness, elasticity, capability of regression and signs of fatigue. After 8 weeks of treatment with equol ($N = 19$), six skin parameters were improved (R0, R1, R3, R5, F0 and F1). Firmness (R0) was significantly ($P < 0.05$) decreased from 0.23 to 0.18. This could be due

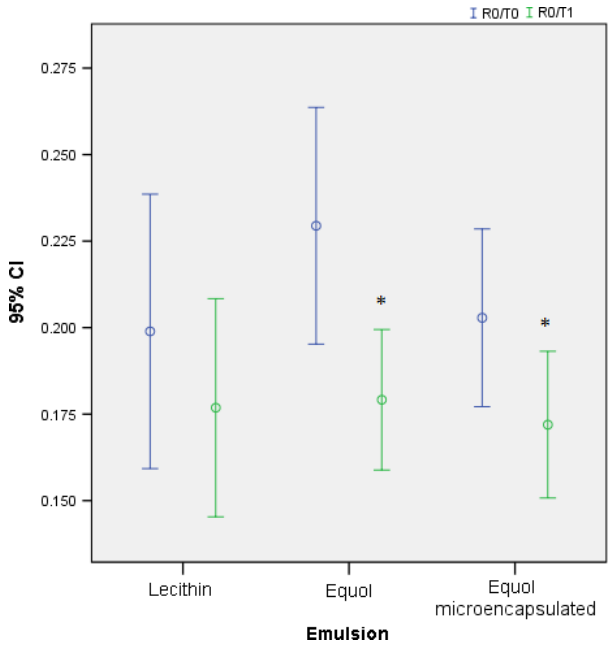

Figure 3 Firmness: Firmness was analysed before and after intervention according to methods using Cutometer® dual MPA 580. Equol treatments using pure and encapsulated preparations show a significant. improvement ($P < 0.05$).

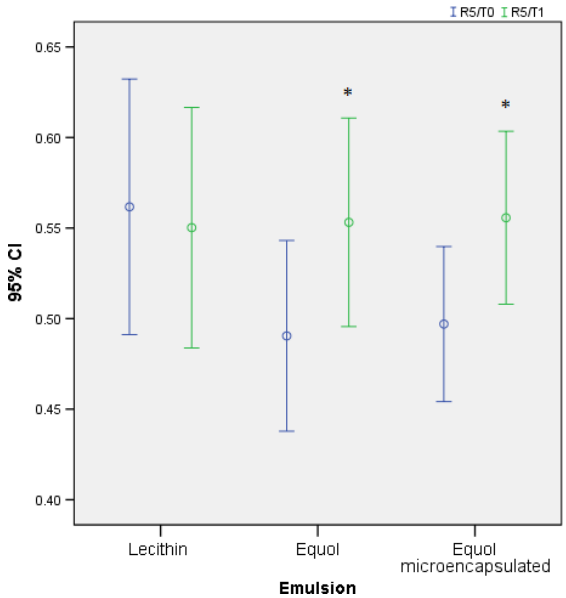

Figure 4 Elasticity: Elasticity was analysed before and after intervention according to methods using Cutometer® dual MPA 580. Equol treatments using pure and encapsulated preparations show a significant improvement ($P < 0.05$).

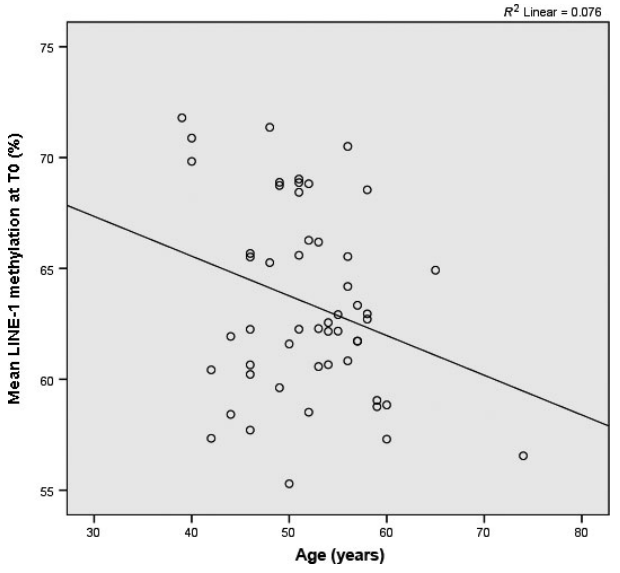

Figure 5 LINE-1 methylation: Methylation of CpGs in Line 1 was analysed using skin stripping, DNA extraction, bisulfite conversion and HRM- analysis before and after intervention according to methods. The mean methylation of LINE-1 methylation decreased with age.

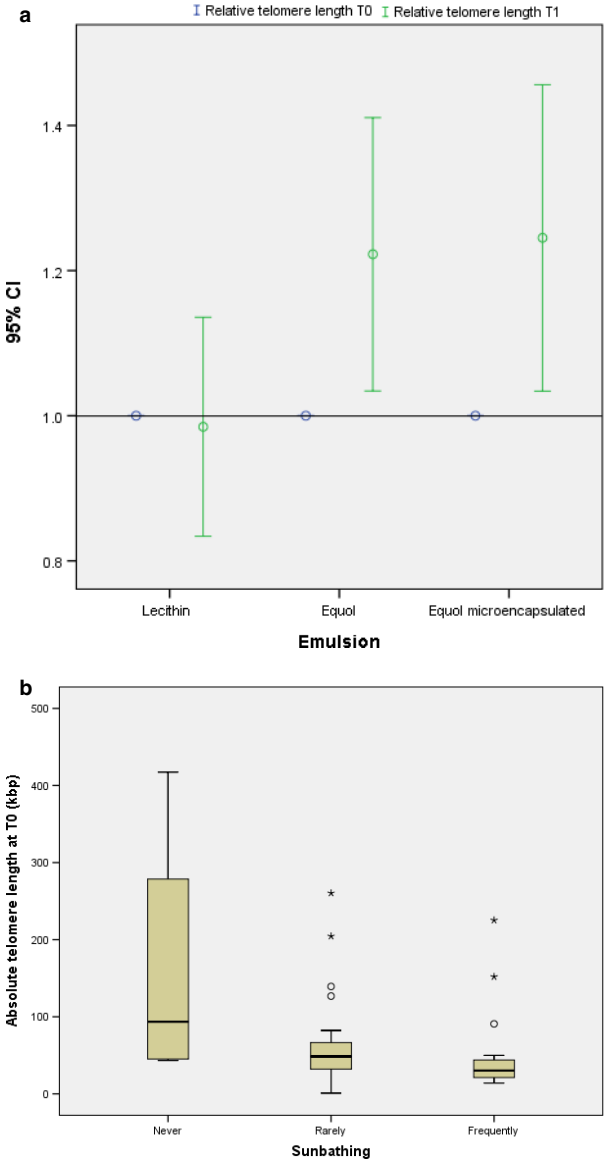

Figure 6 (a) Telomere length and equol treatment: Telomere length was analysed from DNA obtained with skin stripping using a qPCR protocol described in methods. Telomere length is shown relative to control. A significant longer relative telomere length was seen after treatment with equol and microencapsulated equol. (b) Telomere length and Sunbathing: Telomere length was analysed from DNA obtained with skin stripping using a qPCR protocol described in methods. Telomere length is expressed in kb. Subjects reporting frequent sunbathing show significantly shorter telomeres lengths.

to the fact, that equol stimulates collagen gene expression. Moreover, this effect may also be responsible for the significant betterment of fatigue signs (R3). Equol improved the skin parameters R3 from 0.27 to 0.21 ($P < 0.05$). Equol preparation improved elasticity in three skin parameters (R5, F0, F1). Elasticity enhancement may be explained by stimulation of elastin expression. This elasticity improvement was also underlined by surface parameter F0 and F1. Intervention with encapsulated equol ($N = 18$) displayed, besides significant ($P < 0.05$) parameters (R0, R1 R3, R4, R5 and F0) we discussed above, additional skin parameters like R7. R7 represents elastic regression. It may also be based on higher elastin expression through encapsulated equol. In conclusion, Cutometer® based results suggest that equol emulsion, as well as encapsulated equol emulsion, significantly improved elasticity and firmness parameters.

Structural parameters were also analysed in connection with lifestyle and nutrition factors. Thereby physical activity showed a correlation with anisotropy. With higher physical activity level, anisotropy values improved significantly ($P < 0.05$). Beside this, there were no significant connections with other lifestyle and nutrition factors.

LINE-1 methylation represents a marker for global DNA methylation. A higher methylation of these repetitive element has been reported to correlate with a healthier lifestyle, whereas a global hypomethylation was shown to be present in some cancers, metabolic syndrome and ageing [19–22].

Differences in LINE-1 methylation of skin cells before and after intervention were determined with HRM analysis. Statistical analysis of mean LINE-1 methylation levels revealed no significant changes after the equol and lecithin treatment. Age-related and sun exposure-related shifts in global DNA methylation have already been shown in epidermis samples [31]. There was a significant correlation of mean LINE-1 methylation at T0 with age ($P < 0.05$), showing a decrease in methylation with age. A demethylation of repetitive elements with age has been previously described in literature [21]. Moreover, age-associated global DNA hypomethylation has been shown in several mammalian tissues. Other repetitive elements, including Alu repeats, may also be important for the analysis of overall DNA methylation, in particular for age-relevant effects [14] Fig. 5.

After intervention of 8 weeks' treatment with equol and encapsulated equol relative telomere length was significantly higher ($P < 0.05$) compared to lecithin control, Fig. 6a. The reason for

this effect is not clear. Possibly increased activity of telomerase is responsible. Many studies investigated the effect of telomerase on increasing telomere length or other approaches to TERT and TERC expression. Telomerase was detected in keratinocytes of epidermis, which can express low levels of TERT. So with high expression of exogenous TERC, keratinocytes have the ability to activate telomerase and maintain telomeres. Fibroblasts in dermis have no active telomerase and do not express TERT [25]. Alternatively changes in the turnover of keratinocytes from stem cells to desquamation may contribute to changes in the length of telomeres.

UV irradiation leads to photoageing and is known to induce telomere shortening [28]. In the present study, a correlation between absolute telomere length and sunbathing was found, Fig. 6b.

The subjective changes during intervention period were assessed via a self-assessment questionnaire, which the participants completed at time-point 1. Participants, who received lecithin reported a better overall skin well-being compared to equol groups. Whereas subjects with equol intervention indicated only improved skin moisture by contrast with encapsulated equol and lecithin treatment. Volunteers, who received emulsion containing encapsulated equol reported better skin texture, charisma, freshness, smoothness and fewer oily shine, compared to equol and lecithin groups (n.s.).

The small control group without intervention showed no significant changes in any of the assessed parameters (results not shown).

Methodological limitations: To our knowledge, the present study is one of the first studies tackling molecular parameters such as epigenetic methylation and telomere length from skin strips. A detailed comparison of results obtained from cells derived from skin stripping with results from histological analysis would be desirable. Certainly also the statistical power to investigate differences between the subgroups was limited by a small study size due to time-intensive molecular analysis. Possible variations of structure parameters could come from the climate because skin parameter analysis devices are very sensitive to external conditions, like temperature, light and humidity. Analyses were started in April (lower humidity) and finished in June (higher humidity); however, no significant changes were seen in the control group without intervention.

Conclusion

Results of this intervention study showed a strong effect of equol preparations on several skin parameters especially skin roughness, skin texture, skin smoothness, elasticity and firmness. Molecular parameters of ageing were also affected by equol treatment. Molecular analysis including epigenetic mechanisms may strongly increase understanding of skin ageing.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Supporting information including detailed info on skin structure.