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Pycnogenol® Effects on Skin Elasticity and Hydration Coincide with Increased Gene Expressions of Collagen Type I and Hyaluronic Acid Synthase in Women

A. Marini[a] S. Grether-Beck[a] T. Jaenicke[a] M. Weber[a] C. Burki[b] P. Formann[a]
H. Brenden[a] F. Schönlau[b] J. Krutmann[a]

[a]IUF – Leibniz Research Institute for Environmental Medicine, Düsseldorf, Germany;
[b]Horphag Research, Cointrin/Geneva, Switzerland

Key Words
Pycnogenol® · Pine bark extract · Skin hydration · Skin elasticity · Collagen expression · Hyaluronic acid synthase-1

Abstract
Introduction and Objectives: In recent years there has been an increasing interest in the use of nutritional supplements to benefit human skin. Molecular evidence substantiating such effects, however, is scarce. In the present study we investigated whether nutritional supplementation of women with the standardized pine bark extract Pycnogenol® will improve their cosmetic appearance and relate these effects to expression of corresponding molecular markers of their skin. ***Materials and Methods:*** For this purpose 20 healthy postmenopausal women were supplemented with Pycnogenol for 12 weeks. Before, during and after supplementation, their skin condition was assessed (i) by employing non-invasive, biophysical methods including corneometry, cutometry, visioscan and ultrasound analyses and (ii) by taking biopsies and subsequent PCR for gene expression analyses related to extracellular matrix homeostasis. ***Results:*** Pycnogenol supplementation was well tolerated in all volunteers. Pycnogenol significantly improved hydration and elasticity of skin. These effects were most pronounced in women presenting with dry skin conditions prior to the start of supplementation. The skin-physiological improvement was accompanied by a significant increase in the mRNA expression of hyaluronic acid synthase-1 (HAS-1), an enzyme critically involved in the synthesis of hyaluronic acid, and a noticeable increase in gene expression involved in collagen de novo synthesis. ***Conclusions:*** This study provides skin-physiological and for the first time molecular evidence that Pycnogenol supplementation benefits human skin by increasing skin hydration and skin elasticity. These effects are most likely due to an increased synthesis of extracellular matrix molecules such as hyaluronic acid and possibly collagen. Pycnogenol supplementation may thus be useful to counteract the clinical signs of skin aging.

Introduction

During the past years a growing number of nutritional supplements have been introduced which are intended to benefit human skin and to support cosmetic strategies

A.M. and S.G.-B. contributed equally to this work.

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Univ.-Prof. Dr. Jean Krutmann
IUF – Leibniz Research Institute for Environmental Medicine
Auf'm Henekamp 50
DE–40225 Düsseldorf (Germany)
Tel. +49 211 3389 225, E-Mail krutmann@uni-duesseldorf.de

Table 1. List of ingredients (mg/capsule)

Pycnogenol	25
Dicalcium phosphate	245
Microcrystalline cellulose	245
Magnesium stearate	10
Colouring ingredients	
Hydroxypropyl methylcellulose	
Microcrystalline cellulose	
Stearic acid	
Titanium dioxide (E171)	
Yellow iron oxide (E172)	
Red iron oxide (E172)	

aimed at defying skin aging. The vast majority of these claims are based either on in vitro studies with no or limited in vivo relevance or on in vivo studies in the absence of relevant molecular investigations. Skin aging, however, is accompanied and orchestrated by a number of very well-characterized molecular changes which are of established physiological relevance and, therefore, well-known targets for many effective cosmetic anti-aging strategies [1, 2]. It is currently not known whether nutritional supplements which are suggested to exert anti-skin aging effects can indeed exert beneficial effects in human skin at the molecular level. Demonstration of such effects, however, would provide a scientific rationale for any claimed skin anti-aging effects.

In the present human in vivo study, we have addressed this question by focusing on the nutritional supplement Pycnogenol®. Pycnogenol represents a standardized bark extract from the French maritime pine (*Pinus pinaster* Ait) in compliance with US pharmacopoeial requirements. We have chosen this product because the extract is standardized to contain 70 ± 5% procyanidins, oligomers of catechin and epicatechin subunits, taxifolin and a range of phenolic acids, derivatives of benzoic and cinnamic acids. In other words, Pycnogenol contains a variety of bio-active molecules which are known to exert beneficial effects on skin cells in vitro or in animal studies [3]. In addition, previous studies on Pycnogenol effects on human skin indicate that Pycnogenol supplementation improves human skin conditions including chronic venous insufficiency [4, 5] and skin inflammation [6, 7]. Pycnogenol protects against oxidative stress in several cell systems by doubling the intracellular synthesis of anti-oxidative enzymes and by acting as a potent scavenger of free radicals [3, 8]. In addition, Pycnogenol was reported to exert beneficial effects on organs other than the skin [for a review, see 3, 9, 10].

With regard to skin aging, it has been shown that Pycnogenol has a high physical affinity to extracellular matrix proteins which are rich in hydroxyprolines such as collagen and elastin [4, 11], i.e. two proteins which are critically involved in skin aging. This is also reflected by the clinical observation that in a double-blind, placebo-controlled trial with 62 women a complex oral formulation with Pycnogenol as leading active ingredient improved visible signs of skin aging, as well as skin elasticity and skin smoothness after 6 weeks [12].

In the present study we asked whether these and/or similar beneficial effects, which may result from Pycnogenol supplementation, may be reflected by concomitant changes at the molecular level. We were particularly interested in the in situ transcriptional expression of genes involved in the de novo synthesis of hyaluronic acid and collagen, as this would be of direct relevance for skin hydration, elasticity and firmness.

Materials and Methods

Materials

Pycnogenol capsules containing 25 mg of Pycnogenol were provided by Horphag Research, Geneva, Switzerland. The composition of the Pycnogenol capsule used in this study is shown in table 1.

Volunteers

Approval had been obtained from the Ethics Committee of the Heinrich Heine University Düsseldorf. The study was conducted according to the ethical rules stated in the Declaration of Helsinki Principles, and the ICH GCP guidelines were adhered to, as applicable. Twenty healthy postmenopausal women were enrolled after written informed consent. Their ages ranged from 55 to 68 years, and all individuals were non-smokers, had normal eating habits and no history of any skin disease.

Nutritional Supplementation

Figure 1 illustrates the study design. During the first visit after enrolment, demographic data were obtained and a nutrition questionnaire was filled out. After a wash-out phase of 8 days, all volunteers were supplemented with 3 × 25 mg Pycnogenol daily for a period of 12 weeks. At the beginning, after 6 (day 49) and 12 (day 91) weeks of supplementation, skin-physiological parameters were assessed as described below. In addition, at the beginning and after 12 weeks each time one 4-mm punch biopsy was obtained from buttock skin.

Skin-Physiological Measurements

All skin-physiological measurements were carried out by the same investigator in an air-conditioned room (room temperature 18–22°C, air humidity approx. 30–50%). To measure skin hydration, a Corneometer CM 825 (Courage-Khazaka Electronics GmbH, Cologne, Germany) was used. Skin elasticity was determined by means of a Cutometer MPA 580 (Courage-Khazaka

Table 2. Primer pairs for reverse-transcriptase PCR

Gene	Primer pair	Ref. No.
18S rRNA (housekeeping gene)	5′-GCCGCTAGAGGTGAAATTCTTG-3′ 5′-CATTCTTGGCAAATGCTTTCG-3′	15
COL1A1	5′-CCTGCGTGTACCCCACTCA-3′ 5′-ACCAGACATGCCTCTTGTCCTT-3′	16
COL1A2	5′-GATTGAGACCCTTCTTACTCCTGAA-3′ 5′-GGGTGGCTGAGTCTCAAGTCA-3′	17
HAS-1	5′-GCGGGCTTGTCAGAGCTACT-3′ 5′-AACTGCTGCAAGAGGTTATTCCTATAT-3′	18

Electronics GmbH). Skin topography was evaluated with the Visioscan® VC 98 (Courage-Khazaka Electronics GmbH). Echographic evaluations were carried out with a 20-MHz B Scanner (Dermascan C, Cortex Technology, Denmark). Assessments were made according to the EEMCO guidelines.

Assessment of Gene Expression in Skin Biopsies

Biopsies were snap frozen in liquid nitrogen and stored at –80°C until further analysis. For assessment of gene expression, total DNA was extracted from frozen biopsies and gene expression measured by semiquantitative reverse-transcriptase PCR (RT-PCR) as previously described [13]. In brief, for isolation of RNA from frozen skin biopsies, 600 μl lysis buffer from a PeqGold Total RNA Kit (PeqLab, Erlangen, Germany) was added, and the samples were disrupted in a MixerMill MM300 (Retsch, Haan, Germany) 3 times for 3 min with 30 Hz. Fifty nanograms total RNA were used for cDNA synthesis. PCR reactions were performed in an Opticon 1 (MJ Research, Waltham, Mass., USA) using Sybr QPCR Supermix w. Rox (Invitrogen, Karlsruhe, Germany). PCR conditions were as follows: activation of hot start Taq polymerase at 94°C for 15 min; denaturation at 95°C for 20 s; annealing at 55°C for 20 s; extension at 72°C for 30 s. Each sample was subjected to PCR in duplicate using the appropriate primer pairs for 45–50 cycles. For comparison of relative gene expression the $2^{-\Delta\Delta C_T}$ method was used [14]. Primer pairs used for RT-PCR are shown in table 2.

Statistical Analysis

The Wilcoxon signed-rank test as a non-parametric test for the comparison of differences between measurements as well as the t test were used for statistical analysis (PASW Statistics 18), and p values of less than 0.05 were considered statistically significant.

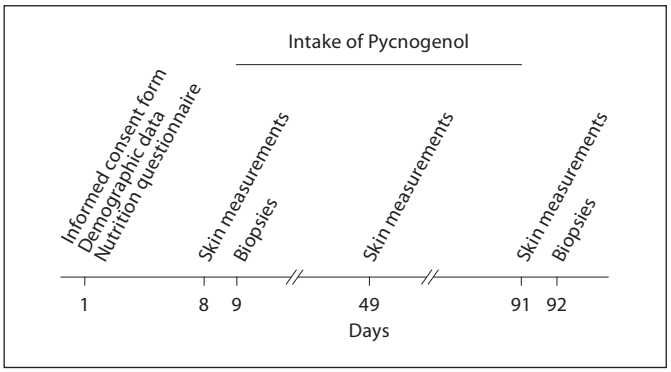

Fig. 1. Trial design testing the efficacy of the oral supplement. During the first visit after enrolment, demographic data were obtained and a nutrition questionnaire was filled out. After a washout phase of 8 days, all volunteers were supplemented with 3 × 25 mg Pycnogenol daily for a period of 12 weeks. At the beginning, after 6 (day 49) and 12 (day 91) weeks of supplementation, skin-physiological parameters were assessed as described in Materials and Methods. In addition, at the beginning and again after 12 weeks, a 4-mm punch biopsy was obtained from buttock skin for analysis of molecular markers.

Results

Pycnogenol supplementation was well tolerated by all volunteers. Skin hydration increased in the whole study population by 8% after 6, but not after 12 weeks (fig. 2a). This increase was even more pronounced, if volunteers with dry skin were analysed separately. In this subgroup (n = 13), a significant ($p < 0.05$), i.e. 21%, increase in skin hydration was observed (fig. 2b). In general, increased skin hydration corresponds to a loss of echogenicity of human skin, as can be demonstrated by high-frequency (20-MHz) sonography as a reduction in pixel numbers. Accordingly, in the present study the acoustic (= echo) density of skin decreased under Pycnogenol supplementation by 2% in all volunteers and by 2.8% in volunteers with dry skin ($p < 0.05$; data not shown).

Skin visco-elastic measurements confirmed the previous observation that Pycnogenol supplementation improves skin elasticity [12]. In the evaluation with the Cutometer, R2 and R7 values are the most important parameters, i.e. the closer they are to 1 (= 100%), the more

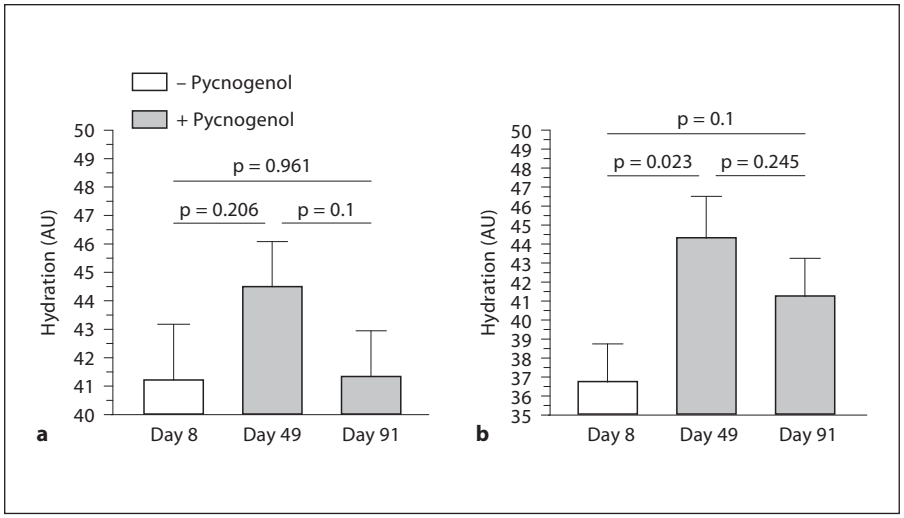

Fig. 2. Skin hydration. At study start and again after 6 (day 49) and 12 (day 91) weeks of Pycnogenol supplementation, skin hydration was assessed with a Corneometer CM 825 (Courage-Khazaka Electronics GmbH). Presented are the mean values ± SE of the study volunteers. The statistical significance was obtained by t test, and p values less than 0.05 were considered statistically significant. Skin hydration increased in the whole study population (n = 20) by 8% after 6, but not after 12 weeks (**a**). This increase was even more pronounced when volunteers with dry skin were analyzed separately (**b**). In this subgroup (n = 13), a significant ($p < 0.05$), i.e. 21%, increase in skin hydration after 6 weeks was observed.

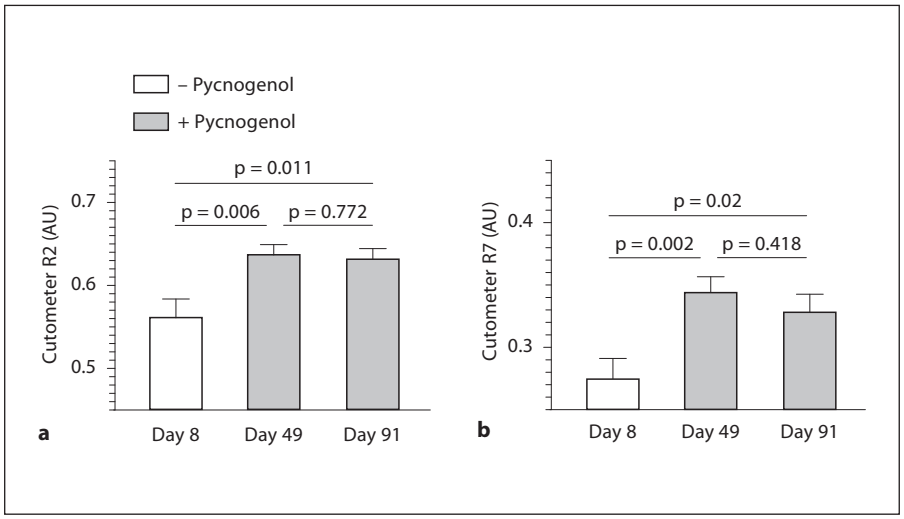

Fig. 3. Skin elasticity. Prior to the start, after 6 (day 49) and 12 (day 91) weeks of Pycnogenol supplementation, skin elasticity was measured with a Cutometer MPA 580 (Courage-Khazaka Electronics GmbH). Presented are the mean values ± SE of the most important parameters to evaluate the elasticity, R2 (**a**) and R7 (**b**). The closer they are to 1 (= 100%), the more elastic the skin is. The statistical significance was obtained by t test, and p values less than 0.05 were considered statistically significant. Both parameters significantly ($p < 0.05$) increased after 6 and 12 weeks of Pycnogenol supplementation in all volunteers.

elastic is the skin [19]. As shown in figure 3, both parameters significantly ($p < 0.05$) increased after 6 and 12 weeks of Pycnogenol supplementation in all volunteers. This was also reflected by a concomitant decrease in the R4 value, which is indicative of the fatigue phenomenon [20]. Accordingly, skin fatigue significantly ($p < 0.01$) decreased after 12 weeks of supplementation (fig. 4).

These skin biophysical improvements were accompanied by significant changes in mRNA expression levels for hyaluronic acid synthase-1 (HAS-1), i.e. HAS-1 mRNA expression increased by 44% 12 weeks after Pycnogenol supplementation ($p < 0.001$; fig. 5a). In addition, mRNA expression for COL1A1 and COL1A2 increased by 41 and 29%, respectively, but this trend did not reach statistical significance (fig. 5b).

Discussion

In the present study we provide for the first time molecular evidence that nutritional supplementation with Pycnogenol may benefit human skin. We are aware that the present study is limited by its uncontrolled design, which we have chosen for ethical reasons to limit the number of biopsies by leaving out a placebo group. A well-designed placebo-controlled study [21] with validated outcome variables such as hydration and skin elasticity could further substantiate the observed effects of Pycnogenol on human skin and its clinical relevance to treat symptoms of intrinsic aging such as xerosis, laxity and wrinkling but also environmentally induced detrimental changes of skin physiology [22, 23].

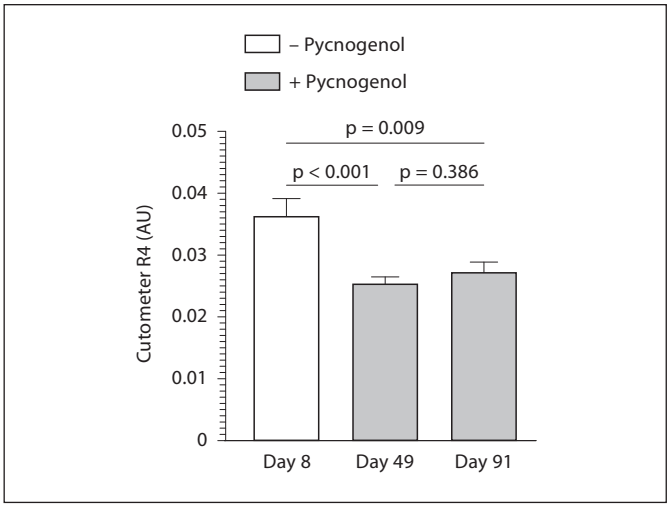

Fig. 4. Skin fatigue. The parameter R4 assessed by cutometry is indicative of the fatigue phenomenon of the skin. The smaller the value, the lower the fatigue. Presented are the mean values ± SE of the 20 subjects at the beginning, after 6 (day 49) and 12 (day 91) weeks of Pycnogenol supplementation. The statistical significance was obtained by t test, and p values less than 0.05 were considered statistically significant. Skin fatigue significantly decreased after 6 ($p < 0.001$) and 12 weeks of Pycnogenol intake ($p = 0.009$).

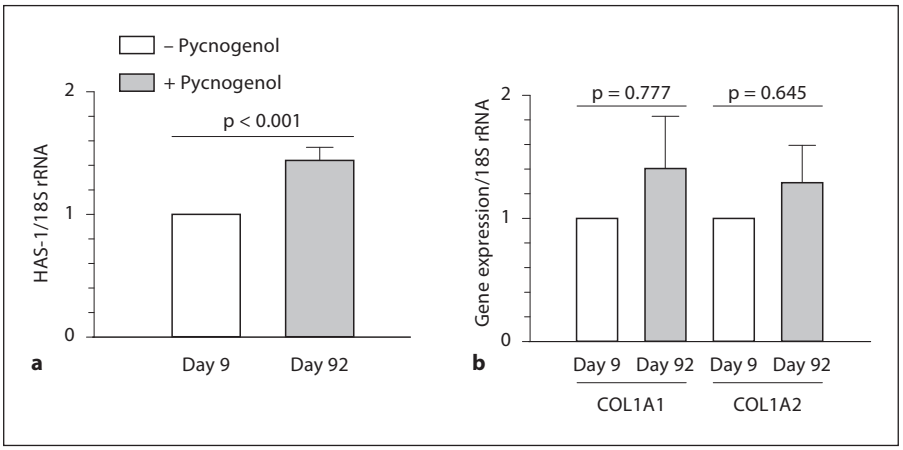

Fig. 5. Gene expression of HAS-1, collagen (COL) 1A1 and 1A2. Presented are mean values ± SE (n = 20), statistical significance was evaluated by Wilcoxon signed-rank test. At the beginning and again after 12 weeks of Pycnogenol supplementation, each time one 4-mm punch biopsy was obtained from buttock skin for the assessment of gene expression of HAS-1 (**a**) and COL1A1 and COL1A2 (**b**). mRNA expression levels for HAS-1 increased by 44% after Pycnogenol supplementation ($p < 0.001$; **a**), while mRNA expression for COL1A1 and COL1A2 increased by 41 and 29%, respectively, but this trend did not reach statistical significance (**b**).

Under the conditions of an uncontrolled design, we found that supplementing volunteers with Pycnogenol for 12 weeks increased the expression of HAS-1. The enzyme HAS-1 is critically involved in the de novo synthesis of hyaluronic acid and thereby profoundly contributes to skin hydration and skin elasticity. Hyaluronic acid is synthesized by keratinocytes and fibroblasts [24]. The synthesis of this macromolecular glycosaminoglycan is accomplished by 3 different HA synthase isoforms (HAS-1, HAS-2 and HAS-3) [25] and the mRNA expression is dependent on cell type and modulated by cell density and various growth factors [26]. HAS-1 mRNA is expressed by both dermal fibroblasts [27] and epidermal keratinocytes [27, 28], and, thus, Pycnogenol may stimulate hyaluronic acid synthase in both cell types.

Hyaluronic acid, also referred to as hyaluronan, is a widely distributed glycosaminoglycan and an essential component of the extracellular matrix. Hyaluronan is involved in a variety of biological processes, such as maintenance of tissue architecture, cell proliferation, migration, differentiation, angiogenesis, wound healing and tumorigenesis [29–31]. The skin contains about half of the total-body hyaluronan [29] which distributes to the dermis and epidermis [32]. Large quantities of hyaluronic acid reside in the dermal connective tissue; in the epidermis, it is strongly expressed around the basal and spinous cells, whereas the terminally differentiated cells of the stratum corneum usually lack hyaluronan [27, 33]. We have previously shown [24] that chronic, repetitive UVB irradiation caused marked loss of hyaluronan from the papillary dermis because of transcriptional downregula-

tion of HAS-1, HAS-2 and HAS-3. Pycnogenol may thus represent a novel strategy to counteract photo-aging of human skin.

The present study shows that Pycnogenol induces HAS-1 mRNA expression and thus provides a mechanistic explanation for the previous observation that Pycnogenol supplementation may increase skin elasticity [12], as demonstrated by skin biophysical measurements. We confirm this observation and additionally demonstrate that volunteers with dry skin preferentially profit from intake of this nutritional supplement. Why average skin hydration was lower after 12 weeks than it was after 6 weeks is difficult to interpret, but may result from seasonal changes occurring during the trial period in summer. Accordingly, patients with some skin conditions, such as atopic dermatitis, may markedly benefit from Pycnogenol supplementation. The precise mechanism by which Pycnogenol induces HAS-1 mRNA expression currently remains unknown.

In addition to HAS-1 mRNA expression, COL1A1 and COL1A2 mRNA levels were increased, indicating that Pycnogenol supplementation may stimulate collagen de novo synthesis in human skin. This effect did not reach statistical significance which is most likely due to the relatively low number of volunteers who were assessed in the present study. Nevertheless, we believe that the observed effect is real because (i) it has previously been reported that Pycnogenol supplementation increases skin elasticity and (ii) we have observed in the present study that Pycnogenol supplementation, as assessed by Visioscan, reduces skin wrinkles by 3% and increases skin smoothness by 6% (data not shown).

In conclusion the present study confirms at a molecular level the beneficial effects Pycnogenol supplementation may provide to human skin. Our study indicates that Pycnogenol supplementation improves skin hydration and elasticity by inducing the de novo synthesis of hyaluronic acid. In addition, we provide some evidence that collagen de novo synthesis may be stimulated. The latter observation should prompt further studies to more closely evaluate the potential of Pycnogenol supplementation to counteract human skin aging.

Disclosure Statement

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