

## INVESTIGATIVE REPORT

# Long-term Topical Oestrogen Treatment of Sun-exposed Facial Skin in Post-menopausal Women Does Not Improve Facial Wrinkles or Skin Elasticity, But Induces Matrix Metalloproteinase-1 Expression

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**It is controversial whether treatment with oestrogen stimulates collagen production or accumulation in sun-exposed skin. The aim of this study was to determine the effect of long-term treatment with topical oestrogen on photoaged facial skin, with regard to wrinkle severity, and expression of procollagen and matrix metalloproteinase-1 enzyme. Two groups of 40 post-menopausal women applied either 1 g of 1% oestrone or vehicle cream once daily to the face for 24 weeks. Visiometer R1–R5 values (skin wrinkles) and Cutometer values (skin elasticity) were not significantly improved in the oestrone group after 24 weeks of treatment. Type I procollagen immunostaining did not increase in the oestrone group compared with the control group. However, levels of matrix metalloproteinase-1 mRNA increased robustly (10.3 times) in oestrone-treated skin compared with vehicle-treated skin. Thus, treatment with topical oestrogen may be deleterious in ultraviolet-induced skin ageing, at least in part, through induction of matrix metalloproteinase-1 (MMP-1) expression in human skin. Key words: photoageing; oestrogen; skin; matrix metalloproteinase-1.**

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Oestrogen receptors are present in the skin (1, 2), and thus it is widely assumed that oestrogen affects the skin and skin function. During the peri-menopausal and post-menopausal periods, women may experience hot flushes, and vaginal and skin dryness subsequent to a significant decline in levels of serum oestrogen. Oestrogen replacement may ameliorate some of these effects (3). Oestrogen has long been thought to be an anti-ageing modality, which can improve skin thickness, collagen synthesis and wrinkles (3, 4). However, skin ageing is more complicated than ageing of other tissues, because of continuous exposure to the external stimulus, ultraviolet radiation.

It has been reported that systemic hormone replacement therapy increases skin collagen content in sun-protected areas, such as the thigh (5, 6) or abdomen (6,

7). Also, that topical application of oestrogen induces procollagen expression in the sun-protected skin of the buttocks (2, 8). However, limited evidence is available to support the anti-ageing properties of oestrogen in sun-exposed skin. Several studies that attempted to demonstrate the anti-ageing effect of oestrogen reported unclear results (9–12) and had shortcomings, such as the lack of a placebo group (9, 10), or no clinical end-point (10).

Only a few clinical trials have assessed wrinkle severity or elasticity with non-invasive objective devices that can evaluate the efficacy of oestrogen on facial wrinkles or elasticity. In addition, no study has investigated the histological and molecular changes in sun-exposed human skin after long-term application of oestrogen. One trial, which used non-invasive measures to assess the effect of oestrogen on wrinkles, reported a significant increase in wrinkle depth and numbers, as well as in skin thickness after application of a conjugated oestrogen cream (11). However, they did not assess collagen synthesis or any histological changes. A study that evaluated the effectiveness of topical oestradiol in stimulating collagen production in photoaged human skin found that topical application of oestrogen did not induce procollagen expression in sun-exposed skin of the forearms and face (2). In addition, 48-week low-dose systemic oestrogen treatment failed to demonstrate any significant effect on wrinkling and sagging in sun-exposed facial skin of post-menopausal women (13).

Therefore, it remains controversial whether oestrogen causes collagen accumulation in sun-exposed skin. We investigated whether treatment with topical oestrogen would improve photoaged facial skin, particularly with regard to wrinkle severity and skin elasticity, measured with an objective measurement device, and whether long-term application of topical oestrogen would improve procollagen expression and inhibit the collagen-degrading matrix metalloproteinase-1 (MMP-1) enzyme in sun-exposed facial skin.

## MATERIALS AND METHODS

### Subjects

The study group comprised 80 post-menopausal women, with a mean age of 55.2 years (standard deviation (SD) 2.2; age range

51–60 years), who were randomly assigned to the treatment or placebo group. The mean duration of amenorrhea was 4.7 years (SD 2.9; range 1–13 years). Recruitment criteria for the study were as follows: (i) no menstruation for at least 12 months; (ii) grade 2 or higher wrinkles (14); (iii) no hormonal treatment during the previous 3 months; (iv) no medical or cosmetic treatment during the previous 3 months; (v) no significant health problems; (vi) no visible skin disease that might be confused with a skin reaction from the test procedure or material, or interfere with the measurements. All subjects were required to provide written informed consent prior to entering the study. The study was approved by the Institutional Review Board at Seoul National University Hospital.

#### Study design and treatment

A prospective randomized, double-blind, vehicle-controlled study was conducted. Subjects applied either 1% oestrone cream (Esgen<sup>®</sup>, Myungmoon Pharma Ltd, Seoul, Korea) or vehicle cream once daily to the face for 24 weeks. Oestrone cream was used because topical oestradiol cream was not commercially available in Korea. During the study period, excessive exposure to sunlight was discouraged and all subjects were requested to use a sunscreen with a sun-protection factor (SPF) of at least 25. All participants had to apply 2 finger-tip units (15) (approximately 1 g) of the cream every evening to the entire face.

#### Non-invasive assessment

All measurements were performed in a controlled environment room with a constant temperature of 20–25°C and a constant humidity of 45–55% at the Clinical Research Institute, Seoul National University Hospital.

#### Wrinkle measurement

Facial wrinkles were measured in the crow's feet area using skin replicas and Visiometer SV 600 (Courage+Khazaka Electronic, Cologne, Germany) at baseline and after both 12 and 24 weeks. Visiometer is a computerized instrument that produces a skin microrelief from a replica using a light transmission method. It has 5 roughness parameters: depth of roughness (R1), mean depth of roughness (R2), maximum roughness (R3), depth of smoothness (R4), and arithmetic average roughness (R5). Visiometer R values (R1–R5) decrease as wrinkles diminish (16). R1, R2 and R3 represent deep wrinkles; R4 and R5 represent shallow wrinkles.

#### Skin elasticity measurement

Facial elasticity was measured in the crow's feet area with a non-invasive, *in vivo* suction skin elasticity meter, Cutometer MPA 580 (Courage + Khazaka Electronic). Cutometer takes measurements based on the principle of suction elongation, using an optical measuring unit. Of the measured and calculated R parameters taken with the Cutometer, certain ratios of parameters are biologically meaningful and do not depend on skin thickness; thus, they can be compared between sites and subjects (16, 17). In particular, R2 (gross elasticity), R5 (net elasticity) and R7 (elasticity/complete curve) are known indicators of skin elasticity; the closer each value to 1, the more elastic the skin (16).

#### Skin biopsy samples

A 2-mm, full-thickness skin biopsy sample was obtained at baseline and after 24 weeks of treatment in subjects who agreed ( $n = 6$ /group for immunohistochemical analysis;  $n = 7$ /group for

quantitative real-time PCR). All biopsy specimens were taken from the crow's feet area to ensure uniformity of sample sites and collection of specimens from a sun-exposed area. The site of the post-treatment biopsy, which was immediately adjacent to the pretreatment site, was selected to avoid the scar tissue.

Immediately after the biopsy, skin samples for real-time PCR analysis were snap-frozen in liquid nitrogen; specimens for immunohistochemical staining were embedded in low-temperature embedding medium (Tissue-Tek OCT compound, Miles, Naperville, IL, USA), frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ .

#### Quantitative real-time PCR

Total RNA was extracted from skin specimens using RNeasy Plus<sup>®</sup> reagent (Takara Bio Inc., Shiga, Japan) following the protocol recommended by the manufacturer. For mRNA quantification, cDNA was synthesized using 500 ng of total RNA through a reverse transcription reaction (First Strand cDNA Synthesis Kit; MBI Fermentas, Vilnius, Lithuania).

Using SYBR Green PCR Master Mix (Takara Bio Inc., Shiga, Japan), cDNA was amplified using a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA, USA). PCR conditions were 50°C for 2 min, 95°C for 2 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Expression levels of the genes were normalized to that of the 36B4 mRNA in each sample; to quantify the relative changes in gene expression between each sample, we used the comparative CT method, as previously described (18), in which the  $\Delta\text{CT}$  mean value obtained in baseline sample is 0 and the fold difference is 1 (16). The list of primer sequences in this experiment is summarized in Table S1<sup>1</sup>.

#### Immunohistochemical staining

Serial frozen sections (4  $\mu\text{m}$ ) were prepared from OCT-embedded skin biopsy specimens. Type I procollagen was stained as described previously (19), using SP1.D8 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA).

#### Statistical analysis

The primary end-point was the change from baseline in skin wrinkle depth measured by Visiometer at week 24. An independent *t*-test was used to compare the wrinkle severity (R1–R5) between the vehicle and oestrone groups at weeks 12 and 24. The treatment effects at weeks 12 and 24 were further analysed using the analysis of covariance (ANCOVA) with corresponding baseline Visiometer values as the covariate to adjust for potential confounding by baseline imbalance of photoageing severity.

In addition, the following end-points were analysed: (i) R2, R5 and R7 values measured by Cutometer (skin elasticity) between 2 groups were analysed using an independent *t*-test and further compared using ANCOVA to adjust for baseline corresponding values; (ii) the changes from baseline in relative mRNA expressions of procollagen type I, fibrillin-1, MMP-1, and Greb1 at week 24 were analysed using the Mann–Whitney *U* test. SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) was used for all analyses.

## RESULTS

Of 80 female volunteers who entered the study, 76 (38 in the oestrone group, and 38 in the vehicle-control

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group) completed the 24-week study period. The remaining 4 subjects withdrew because of failure to maintain the protocol.

#### Wrinkle measurements by Visiometer

No significant differences in facial wrinkles were observed between the vehicle and oestrone groups before treatment. R1–R3 values failed to demonstrate any significant differences between the 2 groups after 12 or 24 weeks of treatment. R4 values in the oestrone group were not changed at week 12 ( $p=0.163$ ), but significantly elevated (aggravated) after 24 weeks ( $p=0.023$ ) of treatment. R5 values in the oestrone group showed no significant differences compared with those in the vehicle group after both 12 ( $p=0.054$ ) and 24 weeks ( $p=0.057$ ) of treatment (Table I).

Since the differences between the 2 groups were very small and this study was a pilot study, we confirmed these results by adjusting the baseline values. We controlled the corresponding baseline R1–R5 values using ANCOVA to compare the 12- and 24-week R1–R5 values between the 2 groups. R4 values in the oestrone group showed no significant differences at week 12 ( $p=0.055$ ) and 24 ( $p=0.057$ ). In contrast, R5 values in the oestrone group showed no significant difference at week 12 ( $p=0.174$ ), but a significant increase (aggravation) at week 24 ( $p=0.025$ ) compared with those in the vehicle group when adjusted for the baseline corresponding values using ANCOVA (Table II).

#### Skin elasticity by Cutometer

Subjects in the vehicle and oestrogen group showed no improvement in skin elasticity (R2, R5, and R7) measured by Cutometer after both 12 and 24 weeks of

Table I. Facial wrinkles measured with Visiometer after treatment with 1% oestrone or vehicle cream in photoaged facial skin

	Vehicle (n=38) Mean $\pm$ SD	Oestrone 1% (n=38) Mean $\pm$ SD	p-value
Before treatment			
R1	1.26 $\pm$ 0.26	1.34 $\pm$ 0.31	0.267
R2	1.00 $\pm$ 0.23	1.03 $\pm$ 0.28	0.540
R3	0.63 $\pm$ 0.12	0.65 $\pm$ 0.14	0.417
R4	0.40 $\pm$ 0.12	0.41 $\pm$ 0.10	0.710
R5	0.16 $\pm$ 0.05	0.22 $\pm$ 0.30	0.239
12 weeks of treatment			
R1	1.30 $\pm$ 0.28	1.34 $\pm$ 0.33	0.586
R2	1.04 $\pm$ 0.23	1.01 $\pm$ 0.25	0.601
R3	0.64 $\pm$ 0.11	0.62 $\pm$ 0.13	0.649
R4	0.37 $\pm$ 0.10	0.41 $\pm$ 0.11	0.163
R5	0.16 $\pm$ 0.05	0.19 $\pm$ 0.07	0.054
24 weeks of treatment			
R1	1.25 $\pm$ 0.28	1.35 $\pm$ 0.34	0.180
R2	1.03 $\pm$ 0.20	1.01 $\pm$ 0.30	0.741
R3	0.62 $\pm$ 0.11	0.62 $\pm$ 0.14	0.993
R4	0.37 $\pm$ 0.12	0.43 $\pm$ 0.11	0.023
R5	0.16 $\pm$ 0.06	0.21 $\pm$ 0.17	0.057

treatment (Table SII<sup>1</sup>). Next, we performed additional analysis with corresponding baseline Cutometer values as the covariate using ANCOVA to compare the 12- and 24-week Cutometer values between the 2 groups. However, there was no significant differences between the 2 groups (data not shown).

#### Real-time PCR

We measured the induction of *GREB1* mRNA, an oestrogen-responsive gene (20). Topical oestrone treatment induced a 2.4-fold increase in *GREB1* mRNA levels compared with that of the vehicle-treated group (Fig. 1;  $p<0.05$ ), indicating that topical oestrone penetrated the skin and activated oestrogen receptor signals in facial skin. Type I procollagen, fibrillin-1, and MMP-1 gene expressions were compared using real-time PCR. Type I procollagen and fibrillin-1 mRNA levels increased significantly (5.0 and 3.0 times, respectively), compared with those of the vehicle group. However, MMP-1 mRNA in the oestrone-treated skin increased robustly by 10.3 times vs. vehicle-treated skin.

#### Immunohistochemical analysis

Immunohistochemical staining for type I procollagen demonstrated decreased expression in the dermoepidermal junction in 3 of 6 oestrone-treated subjects. Only one subject in the oestrone group showed increased procollagen expression compared with baseline level. However, no decrease in type I procollagen protein expression was observed in all vehicle-treated subjects (3/6 increase and 3/6 similar procollagen expression compared with baseline level; Fig. S1<sup>1</sup>).

## DISCUSSION

We evaluated the effects of oestrogen treatment on facial wrinkles, elasticity, and collagen production in

Table II. Facial wrinkles measured with Visiometer after treatment with 1% oestrone or vehicle cream in photoaged facial skin adjusted for corresponding baseline Visiometer values (estimated mean  $\pm$  SE)

Treatment duration	Vehicle (n=38) Mean $\pm$ SE	Oestrone 1% (n=38) Mean $\pm$ SE	p-value by ANCOVA
12 weeks			
R1	1.334 $\pm$ 0.030	1.309 $\pm$ 0.030	0.564
R2	1.054 $\pm$ 0.022	0.999 $\pm$ 0.022	0.285
R3	0.617 $\pm$ 0.011	0.615 $\pm$ 0.011	0.837
R4	0.162 $\pm$ 0.010	0.189 $\pm$ 0.010	0.055
R5	0.376 $\pm$ 0.016	0.407 $\pm$ 0.016	0.174
24 weeks			
R1	1.283 $\pm$ 0.036	1.323 $\pm$ 0.036	0.433
R2	1.040 $\pm$ 0.028	0.994 $\pm$ 0.028	0.253
R3	0.628 $\pm$ 0.015	0.611 $\pm$ 0.015	0.401
R4	0.158 $\pm$ 0.021	0.213 $\pm$ 0.021	0.057
R5	0.372 $\pm$ 0.018	0.431 $\pm$ 0.018	0.025

ANCOVA: analysis of covariance; SE: standard error.

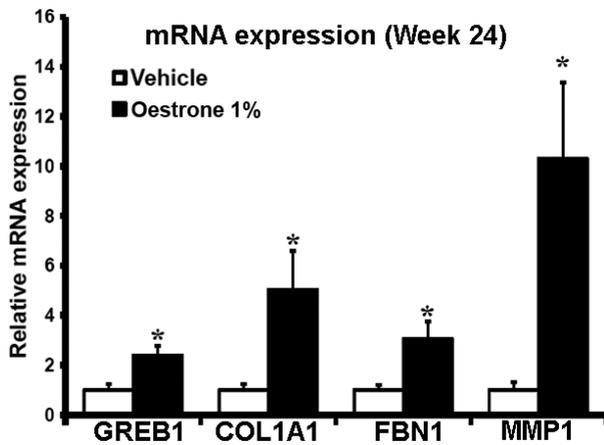


Fig. 1. Relative Greb1 (an oestrogen-responsive gene), fibrillin-1 (FBN1), type I procollagen (COL1A1) and matrix metalloproteinase-1 (MMP-1) mRNA levels as measured by real-time PCR in each group before and after 24 weeks of oestrogen treatment ( $n=7$ ). \*Significantly different vs. vehicle-treated skin by the Mann–Whitney  $U$  test ( $p<0.05$ ). Results shown as mean  $\pm$  SE.

photoaged facial skin of post-menopausal women. We found that 24 weeks of topical oestrogen treatment did not improve facial wrinkles or skin elasticity. Furthermore, the induction of procollagen type I protein was not observed after 24 weeks of oestrone treatment, with a significant increase in the collagen-degrading enzyme, MMP-1 mRNA. To the best of our knowledge, this is the first study that objectively assessed long-term changes in skin function over the course of topical oestrogen treatment, as well as evaluating the molecular changes in collagen in human facial skin.

Induction of type I procollagen protein was not observed after the 24-week oestrone treatment, regardless of the induction of procollagen mRNA. This discrepancy can be partially explained by robust induction of MMP-1 expression in oestrone-treated facial skin. MMP-1 is a key regulator of collagen degradation. As oestrogen suppresses MMP-1 expression in sun-protected skin *in vivo* (8), a different regulatory mechanism is likely to exist between sun-protected and sun-exposed skin. A few studies have shown that oestrogen upregulates collagenase expression induced by specific stimuli, such as interleukin-1 $\beta$  (21), parathyroid hormone (22), and relaxin (23). Further studies are needed to demonstrate the regulatory mechanism of oestrogen in UV-induced MMP-1 expression. Moreover, R4 and R5 values (representing shallow wrinkles) were aggravated in the oestrone-treated group compared with the vehicle-treated group; however, no difference was found in R1–R3 (representing deep wrinkles). These results suggest that another mechanism to maintain the extracellular matrix other than collagen might be functioning in oestrogen-treated skin. Oestrogen can stimulate several GAGs, including hyaluronic acid (24). These results demonstrate that complicated oestrogen modulation on ECM occurs under UV irradiation.

Our data agree with a previous report demonstrating the lack of an effect of short-term topical oestradiol treatment on procollagen protein production in photoaged skin (2). No significant changes in type I procollagen mRNA after short-term application of topical oestrogen were observed (2); however, long-term treatment with topical oestrogen induced type I procollagen I mRNA. This suggests that the potential collagen-stimulating effect of oestrogen on photoaged skin is partially maintained.

Induction of procollagen type I in immunohistochemical analysis was observed in the vehicle group. This finding can be explained by the regular use of sunscreens with an SPF during the trial period. Although direct clinical evidence is lacking, indirect evidence that sunscreens allow the repair of photodamaged skin has been reported by numerous clinical trials in which sunscreens are used in both control and treatment groups. For example, in one study use of sunscreen with an SPF of at least 15 produced an improvement in photo-damage compared with baseline after 24 weeks (25).

#### Study limitations

This study has some limitations. First, we used oestrone cream instead of oestradiol because oestradiol cream was not commercially available in Korea, where the trial was conducted. Oestrone is 1 of the 3 naturally occurring oestrogens: oestrone, oestradiol and oestriol. Oestrone is known to be a much weaker oestrogen in terms of genomic actions, but may be strong in the non-genomic signal pathways compared with oestradiol (26). Thus, we cannot exclude the differential effect of oestrone and oestradiol on skin collagen metabolism. However, *GREB1*, procollagen type I, and fibrillin-1 mRNA levels rose significantly indicating that penetration of topical oestrone and at least genomic response to oestrone were similar to oestradiol. Since procollagen type I gene promoter lacks oestrogen-response elements (27), non-genomic pathway or indirect mechanism might modulate the procollagen type I expression. The mechanism of how oestrogen signals regulate collagen production in human skin should be elucidated. Secondly, we only detected procollagen type I protein level by immunohistochemical staining using SP1. D8 antibody, which detects a marginal sub-fraction of procollagen I, below the dermoepidermal junction (28). Even though this is a widely used antibody to detect anti-ageing effects, another detection technique, such as immunoblot method, may give a slightly different result.

In conclusion, despite the limitations described above, the results of this study will be helpful for managing photoaged facial skin of post-menopausal women. The results indicate that topical oestrogen treatment has no additive effect compared with vehicle and sunscreen use, and may be deleterious to the UV-induced skin

ageing process, at least in part, through the induction of MMP-1 in human skin.

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*The authors declare no conflicts of interest.*

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