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Effect of *Camellia japonica* oil on human type I procollagen production and skin barrier function

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Abstract

Type I collagen is the primary component of the skin dermis. Both the quantity and quality of extracellular collagen are primarily related to skin ageing. In this study, we investigated the possibility that *Camellia japonica* oil (CJ oil) may be introduced as an anit-wrinkle agent. As a first step to this end, human COL1A2 promoter luciferase assay was performed in human dermal fibroblast cells. CJ oil was determined to activate human COL1A2 promoter in a concentration-dependent manner. In consistency with this result, while matrix metalloproteinase (MMP)-1 activity was inhibited by CJ oil, human type I procollagen synthesis was also induced by CJ oil. These results suggest the possibility that CJ oil may be involved in the skin ageing. For the evaluation of CJ oil's safety and efficiency on human skin, human skin primary irritation test and trans-epidermal water loss (TEWL) were performed. Transepidermal water loss (TEWL) was measured before treatment then, 1 h and 2 h after treatment; the forearm site was selected to measure TEWL. Also, a human skin primary irritation test was performed on the normal skin (upper back) in 30 volunteers to see if a certain material included in CJ oil has irritation or sensitization potential. In these assays, CJ oil reduced trans-epidermal water loss (TEWL) and did not induce any adverse reactions. Therefore, based on these results, we suggest the possibility that CJ oil may be considered as possible wrinkle-reducing candidates for topical application.

Keywords: Human type I procollagen; COL1A2 promoter; *Camellia japonica*

1. Introduction

Ageing of the skin is primarily related to reductions in the levels of Type I collagen, which is the principal component of skin dermis. Type I collagen is the main structural component of the extracellular matrix (ECM), which is known to perform a pivotal function in the maintenance of the structure of the skin dermis. It has been known that the Smad pathway is involved in the activation of type I collagen gene expression. The Smads are a series of proteins which perform downstream functions from the serine/threonine kinase receptors of the TGF-β family, thereby transducing signal to the nucleus (Piek et al., 1999; Attisano and Wrana, 2000; Massagué and Wotton, 2000).

Camellia japonica oil (CJ oil) has a long history of cosmetic usage traditionally in the oriental region as a protectant to keep health of skin and hair, but it has received relatively little attention for its biological activity thus far. A recent study suggested that CJ oil has anti-inflammatory activity (Akihisa et al., 1997). However, there are no studies which report effects of CJ oil on skin ageing.

In this study, in order to discover anti-wrinkle effect of *Camellia japonica* oil (CJ oil), we investigated the wrinkle-reducing effect of CJ oil using in vitro assays including collagen production, matrix metalloproteinase (MMP)-1, and cell proliferation assays, as well as clinical studies such as transepidermal water loss (TEWL) and human skin irritation assays.

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2. Materials and methods

2.1. Materials

The COL1A2 luciferase reporter (COL1A2-Luc) plasmid (Lee et al., 2006b) was constructed through the fusion of human COL1A2 promoter region into pLuc vector (Stratagene).

2.2. Preparation of essential oil from Camellia japonica seed

Bulk wild *Camellia japonica* seeds was obtained from Jeju Island, Korea and voucher specimens have been deposited in the laboratory of Jeju Hi-Tech Industry Development Institute, in Jeju Do, Korea. *Camellia japonica* seeds (1 kg) were transferred to the mechanical press (Dongkwang Oil Co, Seoul, Korea) and then pressed to 600 kg/cm^2 for 20 min to obtain the *Camellia japonica* oil. The extracted *Camellia japonica* oil was filtered with cheesecloth under vacuum to remove particles. *Camellia japonica* oil had the following characteristics: specific activity 0.9443 (a); acid value 0.99; easter value 35.44; iodine value 80.22; boiling point 164 °C; optical rotation +5.045 (at 20 °C); reactive index 1.462–1.464 at 20 °C; and soluble in 0.1 volume of 90% alcohol. A preliminary phytochemical investigation showered the presence of terpenes and flavonoids in the oil. A solution of *Camellia japonica* oil was prepared by dissolving the oil in dimethyl sulphoxide (DMSO), 100 mg per ml, and was used for the studies.

2.3. Cell lines and cell culture

Human dermal fibroblast cells (derived from neonatal foreskin) were acquired from the Amore-Pacific Corporation R&D Center, which is located in Korea. The cells were then cultured in Dulbecco's Modified Eagles Medium (Gibco, MD) containing 10% fetal bovine serum (GibCo, MD), and penicillin-streptomycin at 37 °C, in a humidified atmosphere containing 95% air/5% CO_2.

2.4. Transfection and luciferase reporter gene assay

Human dermal fibroblast cells were transiently transfected with 2 μg of the firefly luciferase reporter gene under the control of COL1A2 responsible elements and 0.2 μg of Renilla luciferase expression vector driven by thymidine kinase promoter (Promega) by superfect reagent (Invitrogen) (Lee et al., 2005). The transfected cells were transferred to 6 well plates and incubated for 24 h at a density of 8×10^5 cells per ml. After 24 h, the cells were further cultured in the presence of or absence of *Camellia japonica* oil for 5 h. Luciferase activity were determined using a Dual Luciferase Assay system (Promega) and a LB953 luminometer (Berthold, Germany) and were expressed as a ratio of COL1A2-dependent firefly luciferase activity divided by control thymidine kinase Renilla luciferase activity (relative luciferase unit). Results were confirmed by three independent transfections.

2.5. Quantitative detection of type I collagen

The quantity of type I collagen in the cells was determined using a commercially available kit (Takara Bio Inc., Japan). This kit is capable of detecting procollagen type I carboxy-terminal peptide (PIP) using polyclonal antibodies, rather than directly measuring collagen. Human dermal fibroblast cells were then incubated in either the presence or the absence of *Camellia japonica* oil or TGF-β for 24 h, and then the culture supernatants were harvested and measured with a sandwich immunoassay kit, which was utilized in accordance with manufacturer's instructions (Takara Bio Inc., Japan). The measurement was performed with a microplate at 450 nm (Lee et al., 2006c).

2.6. Quantification of MMP-1 activity

Human dermal fibroblast cells were incubated in either the presence or the absence of *Camellia japonica* oil or TNF-α for 24 h, and then the culture supernatants were harvested and measured with a sandwich immunoassay kit (Amersham Biosciences Corp, Piscataway, NJ), which was utilized in accordance with manufacturer's instructions. Active MMP (matrix metalloproteinase)-1 was measured by the Biotrak MMP-1 activity assay system, which can provide a precise quantitative determination of active MMP-1. In this system, the MMP-activated detection enzyme (modified urokinase) was measured using a specific chromogenic substrate (S-2444^{TM}). To measure the total MMP activity, MMPs were fully activated using *p*-aminophenylmercuric acid (APMA).

2.7. Cytotoxicity assay

Human dermal fibroblast cells were cultured in DMEM including 10% fetal bovine serum and 1x antibiotic solution. Cells were incubated at 37 °C in a 5% CO_2 incubator. Cells were seeded on 24-well plates and drug treatment began 24 h after seeding. General viability of cultured cells was determined by reduction of 3-(4,5-dimety-2-thiazoyl)-2,5-dipheny-2H-ltetrazolium bromide (MTT) to formazan (Mosmann, 1983). After incubation of human dermal fibroblast cells treated with various concentrations of *Camellia japonica* oil, for 24 h at 37 °C in 5% CO_2 atmosphere, MTT assay was performed.MTT (1 mg/ml in PBS) was added to each well, 1/10 volume of media. Cells were incubated at 37 °C for 3 h, and harvested by centrifugation. After then, dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm with a spectrophotometer (Power Wave, Bio-tek Inc.).

2.8. Human skin primary irritation test

Thirty healthy Korean subjects were selected on the basis of inclusion and exclusion criteria, and written consent was obtained in each case. The average age was 25.1 years (range 20–29: all females). The subjects had no history of allergic contact dermatitis, nor had they used topical or systemic irritant preparations in the previous 1 month. The human skin primary

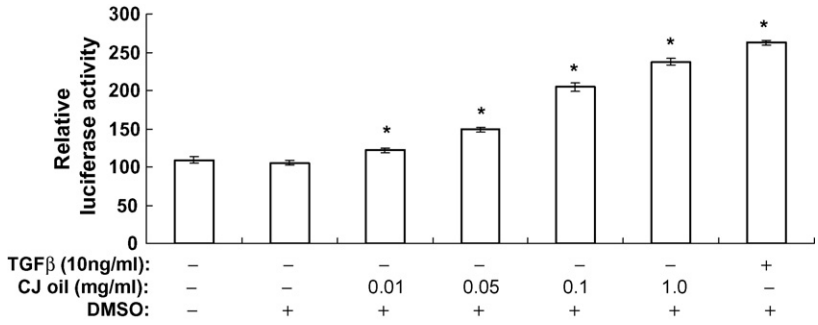

Fig. 1. . *Camellia japonica* oil activates human COL1A2 promoter activation. Human dermal fibroblast cells were transfected with COL1A2-Luc using superfect™. After incubation for 24 h, cells were stimulated for 14 h by indicated concentrations of *Camellia japonica* oil, harvested and lysed. Supernatants were assayed for luciferase activity which is the amount of light produced through conversion of luciferin substrate into oxyluciferin by luciferase. Relative luciferase activity, calculated as a relation of luciferase activity to the quantity of cells and to the efficiency of the transfection, was determined three times in duplicate for each experiment and the standard deviation is indicated as a bar. All values were significant ($^{*}p < 0.05$) compared with values for untreated control. CJ oil: *Camellia japonica* oil.

irritation test was performed using a Finn Chamber® secured to the back site with Scanpore tape. These chambers are made of inflexible aluminum, and have a diameter of 8mm and a depth of 0.5 mm. The round border of the chamber was placed firmly against the skin, causing a tight occlusion of the test materials. *Camellia japonica* oil formulated with squalene was prepared and applied. The patches (chambers) stayed in place for 48 h. The subjects abstained from showering or performing any work or exercise that might wet or loosen the patches. Once the patches were removed, a reading was done after 48 and the 72 h later, a reading was scored according to the criteria of the International Contact Dermatitis Research Group (ICDRG).

2.9. Measurement of trans-epidermal water loss (TEWL)

The effects on TEWL were measured with a TEWA-meter A TM 210 (Courage + Khazaka Electronic GmbH, Koln, Germany). The results were expressed in g/hm^2. The measurements were performed in an acclimatized room with a mean relative humidity of $50.2 \pm 6.9\%$ and a mean room temperature of 21.6 ± 0.6^{o}C. They were carried out under standardized conditions as described earlier (Rogiers, 1995).

2.10. Statistical analysis

Non-parametric one-way analysis of variance (Kruskal–Wallis test) and Mann–Whitney test were used for statistical analysis. Differences between groups were considered significant at $p < 0.05$ (*).

3. Results

3.1. COL1A2 promoter activation is induced by Camellia japonica oil

Significant progress has been made in understanding the expression of human α2(I) collagen (COL1A2) gene and its transcriptional regulation by cytokines and growth factors. As a preliminary step to determine if *Camellia japonica* oil affects collagen production, we performed COL1A2 luciferase assay. As shown in Fig. 1, *Camellia japonica* oil increased COL1A2 reporter activity by three-folds in a concentration-dependent manner. This result suggests the possibility that *Camellia japonica* oil may be involved in the production of human α2(I) collagen.

3.2. Effects of Camellia japonica oil on type I procollagen synthesis

We further studied the effect of *Camellia japonica* oil on the production of type I procollagen synthesis. As shown in Fig. 2, consistent with the finding in Fig. 1, *Camellia japonica* oil significantly increased the production of type I procollagen dose-dependently, confirming that *Camellia japonica* oil induces production of type I procollagen and also suggesting the collagen production-inducing function of *Camellia japonica* oil through activation of COL1A2 promoter. TGF-β was employed as a positive control.

3.3. Effects of Camellia japonica oil on TNF α-induced MMP-1 secretion

Matrix metalloproteinase (MMP-1) is termed fibroblast-type or interstitial collagenase, and is secreted from fibroblasts,

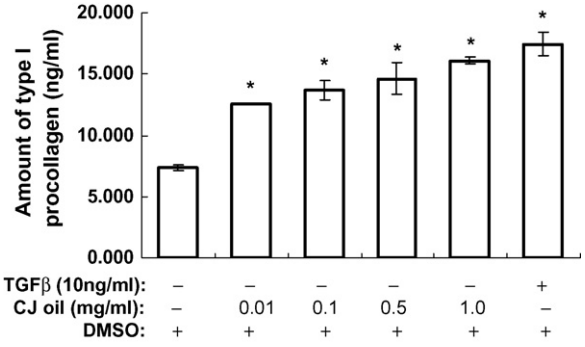

Fig. 2. Effects of *Camellia japonica* oil on type I procollagen synthesis, as determined with a sandwich immunoassay kit (Takara Bio Inc., Japan). Data are expressed as the means ± S.D., $^{*}p < 0.05$ compared with controls. The results were verified by the repetition of three experiments, each in triplicate. CJ oil: *Camellia japonica* oil.

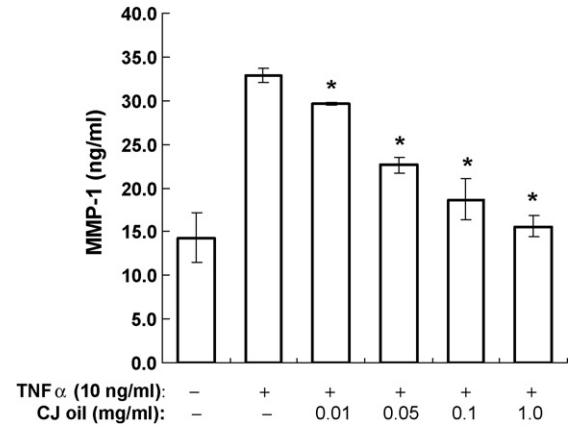

Fig. 3. Effects of *Camellia japonica* oil on TNF α-induced MMP-1 secretion. Human dermal fibroblast cells were incubated in either the presence or the absence of *Camellia japonica* oil or TNF-α for 24 h, and then the culture supernatants were harvested and measured with a sandwich immunoassay kit (Amersham Biosciences Corp, Piscataway, NJ), which was utilized in accordance with manufacturer's instructions. Data are expressed as the means ± S.D., $*p < 0.05$ compared with TNF α-treated control. The results were verified by the repetition of four experiments, each of which was performed in triplicate. CJ oil: *Camellia japonica* oil.

keratinocytes, and macrophages by TNF-α (Lee et al., 2006a; Borden and Heller, 1997). MMP-1 degrades collagen types I, II and III (Borden and Heller, 1997). We conducted an MMP-1 activity assay to investigate an effect of *Camellia japonica* oil on TNF α-induced MMP-1 secretion in human dermal fibroblast cells. Our MMP-1 activity test revealed that *Camellia japonica* oil inhibits significantly TNF α-induced MMP-1 secretion (Fig. 3). To measure the total MMP-1 activity in this study, MMP-1 was fully activated using *p*-aminophenylmercuric acid (APMA).

3.4. Effect of Camellia japonica oil on transepidermal water loss (TEWL)

As previously mentioned, we have found that there is a possibility that *Camellia japonica* oil may be involved in the blocking of skin ageing through both the activation of type I procollagen and the inhibition of MMP-1 secretion. In order to further confirm this, TEWL assays were performed. As shown in Fig. 4, lotion containing 1% *Camellia japonica* oil significantly reduced transepidermal water loss (TEWL), compared to control (lotion only) (Fig. 4).

3.5. Camellia japonica oil has relatively low cytotoxic property in human dermal fibroblast cells

We examined the cytotoxic effects of *Camellia japonica* oil on human dermal fibroblast cells (Fig. 5). While *Camellia japonica* oil at the lower concentrations than 100 μg/ml showed almost 100% cell viability, *Camellia japonica* oil at 1 mg/ml showed over 95% cell viability of human dermal fibroblast cells. In HaCaT cells, human keratiinocyte cell line, like human dermal fibroblast cells, *Camellia japonica* oil did not show significant cytotoxic effect (data not shown). These data suggest that *Camellia japonica* oil has low cytotoxic property against mammalian cells.

3.6. Human skin primary irritation test of Camellia japonica oil

To evaluate the irritation effect of *Camellia japonica* oil for clinical applications to human skin, a patch test was performed. In our study, as shown in Table 1, none of the 30 subjects experienced a reaction based on the 48 and 72 h readings. Specifically, we did not observe any adverse reactions such as erythema, burning or pruritus in the study subjects that was related to the topical treatment of *Camellia japonica* oil.

4. Discussion

To the best of our knowledge, this study is the first to attempt to elucidate the possibility that *Camellia japonica* oil may be considered as possible wrinkle-reducing candidates for topical application.

Although *Camellia japonica* oil (CJ oil) has a long history of cosmetic usage traditionally in the oriental region as a protectant to keep health of skin and hair, it has received relatively

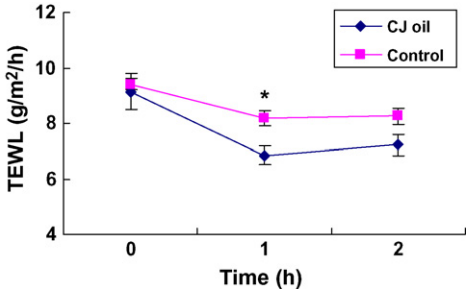

Fig. 4. . Effects of *Camellia japonica* oil on transepidermal water loss (TEWL). TEWL values ($n = 12$; mean age 58 ± 9 yrs) as measured before and application of *Camellia japonica* oil on the left forearm. Data are expressed as the means ± standard error, $*p < 0.05$ compared with lotion-treated control. CJ oil: *Camellia japonica* oil.

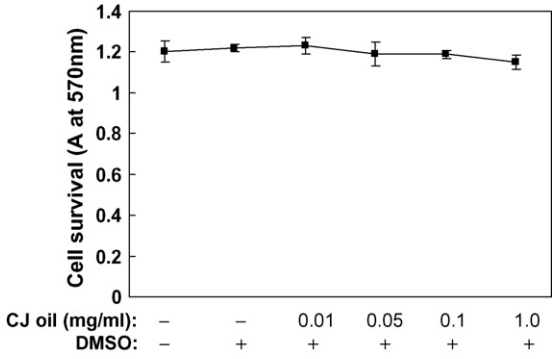

Fig. 5. Cytotoxity of *Camellia japonica* oil against human dermal fibroblast cells. Human dermal fibroblast cells were cultured for 24 h in medium with either presence or absence of *Camellia japonica* oil. The cellular cytotoxicity was determined according to a rapid colorimetric MTT assay. Data are expressed as the means ± S.D., $*p < 0.05$ compared with controls. The results were verified by the repetition of three experiments, each in triplicate. CJ oil: *Camellia japonica* oil.

Table 1
The results of human skin primary irritation test ($n = 30$)

No	Test material	48 h					72 h					Reaction grade[a]		
		±	1+	2+	3+	4+	±	1+	2+	3+	4+	48 h	72 h	Mean
1	Squalene	–[b]	–	–	–	–	–	–	–	–	–	0	0	0
2	CJ oil[c] (0.01 g/ml)	–	–	–	–	–	–	–	–	–	–	0	0	0
3	CJ oil (0.03 g/ml)	–	–	–	–	–	–	–	–	–	–	0	0	0

[a] Reaction grade = Σ[{Grade × no. of responders}/{4 (maximum grade) ×30 (total subjects)}] ×100 ×(1/2).
[b] No reaction.
[c] CJ oil: *Camellia japonica* oil.

little attention for its biological activity thus far and its effect on skin ageing has never been also reported. Therefore, we have attempted to elucidate effect of *Camellia japonica* oil on type I collagen which is the primary component of the skin dermis and principally related to skin ageing. As an initial step, we have attempted to determine whether or not *Camellia japonica* oil can activate COL1A2 promoter in human dermal fibroblast cells. In this study, we found that *Camellia japonica* oil induces COL1A2 promoter activation (Fig. 1). In a consistent with Fig. 1, after *Camellia japonica* oil treatment, we observed human type I procollagen synthesis was induced by *Camellia japonica* oil (Fig. 2). In addition, *Camellia japonica* oil inhibited TNF α-induced secretion of MMP-1 (Fig. 3). These results suggest that *Camellia japonica* oil may be involved in the blocking of skin ageing.

Skin dryness associated with a rough and scaly appearance is one of the major problems of ageing skin (Ghadially et al., 1995). The characteristic dry feature is often correlated with impaired barrier, decreased stratum corneum (SC) hydration and increased incidence of irritant contact dermatitis (ICD) (Conti et al., 1995). It is a common phenomenon that the skin becomes drier with ageing. Therefore, we examined effect of *Camellia japonica* oil on transepidermal water loss (TEWL) of human skin. In this study, we confirmed that CJ oil reduces transepidermal water loss (TEWL). In addition, according to patch test, CJ oil did not induce any adverse reactions, indicating that CJ oil is safe to use.

In conclusion, the data acquired in this study demonstrate that *Camellia japonica* oil can induce the synthesis of type I collagen, has high moisturizing effect, and is safe to use. These results suggest that *Camellia japonica* oil might be introduced as a possible anti-wrinkle agent for the management of skin ageing.

Acknowledgement

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