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Inhibitory Effect of Galactooligosaccharide on Skin Pigmentation

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ABSTRACT: To investigate the effects of ingestion of galactooligosaccharide (GOS) on skin pigmentation, we conducted cell experiments and clinical trials. The effect of GOS on melanin accumulation was assessed *in vitro* using B16F10 cells. Moreover, melanin and erythema indexes following GOS consumption were explored during a double-blind, randomized, and placebo-controlled study, which included subjects divided by stratified block randomization to placebo or GOS. No cytotoxicity was observed at 70 mg/mL or lower GOS in B16F10 melanoma cells. Melanin accumulation was inhibited at 14 mg/mL or higher GOS. Upon ultraviolet B (UVB) irradiation, the survival of HaCaT cells (control) was reduced to 69.0% lower than baseline. A protective effect of GOS was observed upon treatment with 14∼35 mg/mL GOS; however at 70 mg/mL, cells showed 64% viability compared to control cells irradiated with UVB. Delta values (Δ melanin index), which indicate the difference from the baseline melanin level, were significantly different to placebo ($P<0.01$) after 8 weeks. In the GOS group, delta values (Δ erythema index), which indicate the difference from baseline erythema level, also significantly differed from the placebo group ($P<0.05$) after 8 weeks. Our results suggest that intake of prebiotic GOS inhibits skin pigmentation and may represent a novel nutritional approach for skin care.

Keywords: galactooligosaccharide, B16F10 cells, prebiotic, melanin, erythema

INTRODUCTION

Prebiotics in food or in oral formulations have been used to restore the microbial balance in the gastrointestinal tract (Saraswat and Richa, 2018). Intestinal microorganisms are particularly important since they are involved in physiological balance, intestinal development, and maturation of the host immune system (Floch, 2014). Prebiotics have been defined as "non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one, or a limiting number of, bacteria in the colon" (Gibson and Roberfroid, 1995). Prebiotics were originally developed for beneficial effects in the small intestine, but can also be applied to control skin microbial communities and to achieve beneficial effects for skin health (Al-Ghazzewi and Tester, 2014).

Recently, Arck et al. (2010) presented a hypothesis based on the gut-brain-skin axis. First, the regulation of the microbiome can exert profound beneficial effects on skin homeostasis, skin inflammation, hair growth, and peripheral tissue responses against stress. Second, oral administration of prebiotics can impart beneficial effects to the skin. Third, the intestine is the main immune system organ, and the intestinal mucosal immune system is linked to the immune system of the skin through the migration of immune cells. Finally, intake of prebiotics can regulate the immune system and can indirectly affect skin function.

In our previous studies (Hong et al., 2017), transepidermal water loss and moisture retention upon 2 g/d galactooligosaccharide (GOS) consumption significantly differed after 12 weeks vs. consumption of placebo. In addition, the wrinkle area was decreased significantly after 8 weeks of GOS ingestion. During previous clinical trials, GOS has been reported to aid digestive and immune systems (Vulevic et al., 2008). It is well known that GOS supports the growth of beneficial bacteria in the intestines (Nedaei et al., 2019). In general, GOS supplementation increases levels of lactic acid bacteria, *Bifidobacteria*, or both, and reduces levels of pathogenic microorganisms (Cardelle-Cobas et al., 2011; Davis et al., 2010). GOS, a

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stable soluble ingredient, is an ideal choice for improving intestinal microflora and improving immunity.

Therefore, in this study we evaluated the effects of GOS on pigmentation in skin cells through clinical tests. The inhibitory effect of GOS on melanin production was measured in B16F10 melanocytes. In addition, melanin and erythema indexes were measured in healthy adults who received GOS for 12 weeks.

MATERIALS AND METHODS

Materials

GOS, containing 74.9% galactooligosaccharide, 22.8% lactose, and 2.3% galactose, was obtained from Neo Cremar Co., Ltd. (Seoul, Korea). B16F10 cells were purchased from the Korean Cell Line Bank (Seoul, Korea). Cell culture reagents, such as Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics, were purchased from Gibco BRL (Grand Island, NY, USA). All other reagents were commercially obtained from standard sources.

Measurement of cell viability in B16F10 cells

Cell viability was measured according to the method of Yang et al. (1999). B16F10 melanoma cells were seeded into 96-well plates at a concentration of 1×10^4 cells/well, and allowed to stabilize at 37°C in a 5% CO_2 incubator for one day. In clinical trials, subjects consumed 2 g GOS/d for 12 weeks; therefore, GOS treatment concentration in skin cells were high, unlike conventional cell treatment concentrations. GOS was then added at concentrations ranging from 1.4 to 350 mg/mL, and cells were cultured for 48 h. After removing the culture medium, 100 μL of Ez-Cytox (DoGenBio, Seoul, Korea) solution dissolved in phosphate-buffered saline (PBS) at a concentration of 10% was added and cells were incubated for 1 h at 37°C and 5% CO_2. Cellular cytotoxicity was calculated as cell viability (%) of the inflammation-inducing substance control.

Inhibitory effect of GOS on melanogenesis in B16F10 cells

B16F10 melanoma cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 10% FBS and antibiotics. Cells were inoculated at a density of 2×10^5 cells/well in a 6-well plate and incubated for 24 h at 37°C in a 5% CO_2 incubator. The medium was removed and replaced with culture medium containing GOS at 1.4~350 mg/mL, and cultured for 48 h. The cells were washed twice with PBS, and then lysed with 100 μL of 100 mM sodium phosphate (pH 6.8) containing 1% (w/v) Triton X-100 at 5°C for 30 min. After cell lysis, the pellet was obtained by centrifugation at 12,000 rpm for 30 min and was used for melanin quantitation. In order to solubilize melanin, which is insoluble in water, an appropriate amount of 1 N NaOH containing 10% dimethyl sulfoxide was added, reacted at 65°C for 1 h and the absorbance was measured at 405 nm using a microplate reader (Hosoi et al., 1985).

Cell culture and ultraviolet (UV) B treatment of HaCaT cells

HaCaT keratinocytes (human skin keratinocyte line) were kindly provided by Ph.D. Kim (Kyung Hee University, Gyeonggi, Korea). The cells were grown in DMEM containing 10% FBS and 1% antibiotics at 37°C in a humidified atmosphere containing 5% CO_2. UV irradiation was performed using a UV lamp with a wavelength of 290~320 nm, and the amount of light was measured using UV-340A (Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan).

Viability and cytokines production in UVB-irradiated HaCaT cells

Normal human dermal keratinocytes were seeded at a density of 1×10^5 cells/well in 6-well culture dishes and were cultured in DMEM until 70% confluent. Cells were washed with PBS and were exposed to 20 mJ/cm^2 UVB irradiation. UVB-irradiated cells were cultured in serum-free DMEM with or without GOS for 24 h and the culture supernatant was obtained. Levels of human interleukin (IL)-8 and prostaglandin E (PGE) 2 were measured using a commercial enzyme-linked immunosorbent assay kits (BioSource International Inc., Camarillo, CA, USA) according to the manufacturer's protocols.

Subjects

A total of 84 healthy volunteers voluntarily listened to the purpose and content of the study, and then signed the clinical trial agreement. The clinical study was carried out according to the Functional Cosmetics Directive of Korea Food and Drug Administration. Eligible subjects were healthy Korean women aged 40~60 years who had no specific disease and were classified by the Fitzpatrick (1988) classification as having skin type II and III. The exclusion criteria were Fitzpatrick skin types I or IV, allergies, photosensitivity, tanning sunburns, infections, pregnancy, and breastfeeding. In addition, those who had lesions that interfere with accurate judgment due to erythema on the test site, and those who had skin peeling surgery, wrinkle removal, or who had a biopsy within 6 months of the study start were excluded.

Study protocol

The double-blind, randomized, and placebo-controlled study protocol was approved by Jeonju University, and all subjects gave written informed consent (jjIRB-160415-HR-2016-0608). Subjects were divided into two groups

(Placebo and GOS group) by stratified block randomization. For 12 weeks, the GOS group was asked to take GOS (1 g capsules) twice a day for a total daily dose of 2 g GOS. The placebo group consumed only the vehicle (100% dextrin), which was the same size and color tablet as the GOS capsules. GOS and dextrin capsules were provided by Neo Crema Co., Ltd. During the study, subjects were asked to not change their diet or lifestyle.

Measurement of melanin index (MI) and erythema index (EI)

Skin MI and EI were measured using a Mexameter MX18 (Courage+Khazaka electronic GmbH, Köln, Germany). Mexameter uses a narrow-band reflectance spectrophotometric measurement, which is characterized by a circular sensor array of 16 light sources with three different wavelength ranges corresponding to melanin and hemoglobin, suited for skin tone analysis. This machine has a built-in elastic spring in the sensor head to minimize measurement error caused by unnecessary excessive pressure during the process of measuring skin reflection color. The results were expressed as MI and EI within 1 s of contact with the skin.

Statistical analysis

All results were statistically analyzed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA), and were reported as mean ±standard deviation (SD) or standard error of the mean (SEM). The significances of the differences between conditions was compared using Duncan's multiple range tests. Differences between the two groups in the clinical test were statistically evaluated by *t*-tests. Repeated-measure ANOVA followed by Bonferroni-adjusted pairwise comparisons were used to assess differences in changes from baseline within groups. Values were considered statistically significant at $P<0.05$.

RESULTS

Effects of GOS on cell viability and melanin production in B16F10 cells

To investigate the effect of GOS on cell viability, cell viability was measured in B16F10 cells after treatment with GOS at concentrations from 1.4 to 350 mg/mL for 48 h. We did not observe any cytotoxicity at GOS concentrations of 70 mg/mL or less (Fig. 1).

Melanin production was measured at GOS concentrations of 70 mg/mL or less without increased cytotoxicity. Melanin production tended to decrease with increasing GOS concentration (Fig. 1). At GOS concentrations of 14 mg/mL and higher, the production of melanin was significantly lower than that in control cells ($P<0.05$). Melanin production decreased by about 25% upon treatment with 70 mg/mL GOS, compared to control cells ($P<0.05$). GOS therefore inhibited melanin production at high concentrations.

Effects of GOS on cell viability and cytokine production in UVB-irradiated HaCaT cells

To investigate the effect of GOS on cell viability in UVB-irradiated HaCaT cells, cells were exposed to 20 mJ/cm^2 UVB, and cell viability was measured after 24 h. Cell survival of the UVB-irradiated group (control) was reduced to 69.0% compared to the non-irradiated cells (Fig. 2). A protective effect of GOS was shown at GOS concentrations of 14~35 mg/mL; however, cells treated with 70 mg/mL GOS showed 64% viability of control cells irradiated with UVB (Fig. 2). This decrease in cell viability when treated with high concentrations of GOS may be due to osmotic pressure rather than GOS toxicity.

The amount of cytokine production in control cells irradiated with UVB increased from 2,082.6 pg/mL to 3,248.4 pg/mL. However, IL-8 production tended to decrease with increasing GOS concentration. Specifically, treatment with 35 mg/mL GOS lowered IL-8 produced to 1,123.3 pg/mL (Fig. 2). Production of PGE was also

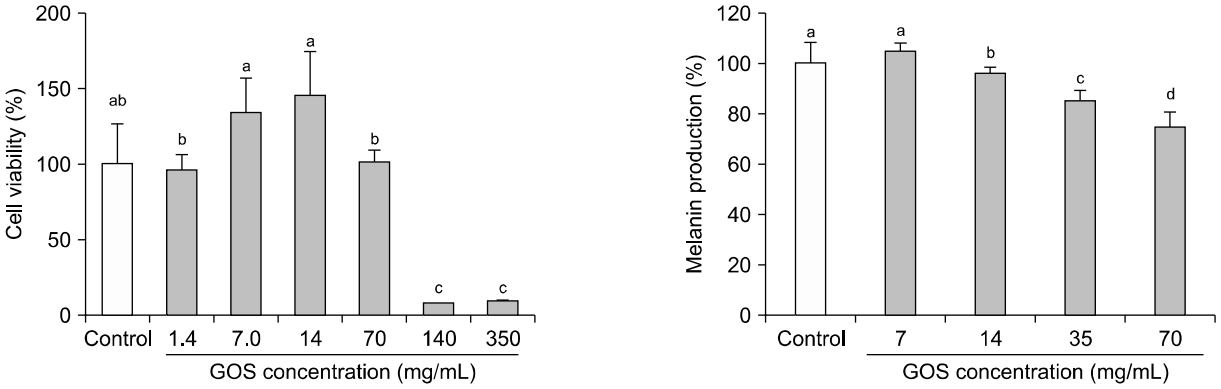

Fig. 1. Cell viability and melanin production in B16F10 melanoma cells treated with galactooligosaccharide (GOS). Values are mean± SD (n=3). Means with different letters (a-d) are significantly different at $P<0.05$ by Duncan's multiple range tests.

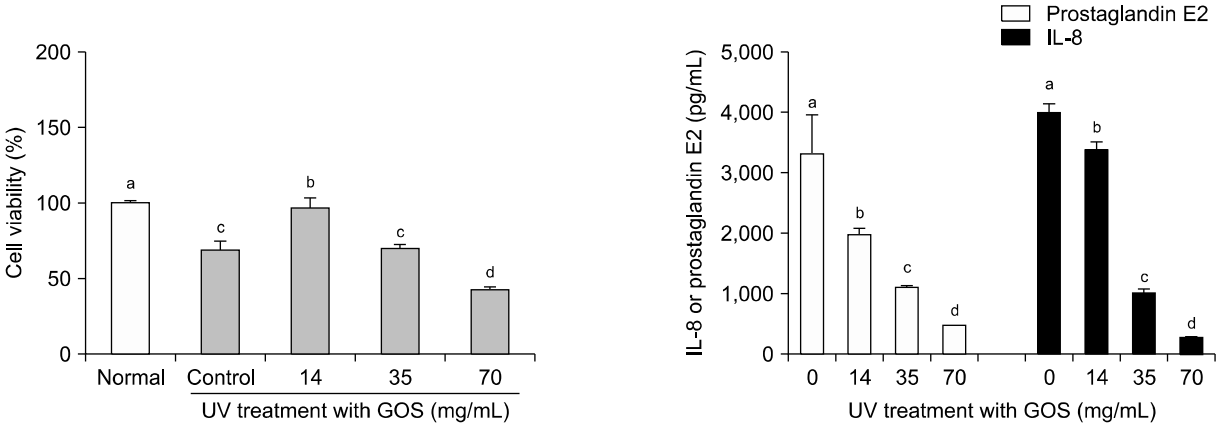

Fig. 2. Effects of galactooligosaccharide (GOS) treatment on cell viability and cytokine production in UVB-irradiated HaCaT cells. Values are mean±SD (n=3). Means with different letters (a-d) are significantly different at $P<0.05$ by Duncan's multiple range tests.

decreased with increasing GOS concentrations, as compared to controls (Fig. 2). Treatment with 70 mg/mL GOS strongly inhibited IL-8 and PGE production by UV light, and cell viability was decreased to 62.3% compared to control cells.

Subjects

A total of 79 patients were enrolled in this study. A total of 5 patients did not present at the 12-week measurement; the placebo group (mean age: 50.40 years) and the GOS group (mean age: 51.05 years) ultimately consisted of 40 and 39 subjects, respectively. The subjects did not experience side effects. The initial values of all indicators were not significantly different between the two groups.

Changes in MI during clinical test

Changes in the amount of melanin produced upon administering dextrin or GOS for 12 weeks are shown in Table 1. Melanin values for the GOS group were 161.84 MI at week 0, 158.42 MI at week 4, 145.85 MI at week 8 ($P<0.001$), and 145.55 MI ($P<0.001$) at week 12. In the placebo group, the MI tended to decrease from 4 to 12 weeks, but there were no significant differences from baseline. In the GOS group, delta values (ΔMI) indicated that differences from baseline melanin levels were significantly decreased after 8 weeks [15.99 MI at 8 weeks and 16.29 MI at 12 weeks ($P<0.01$)] compared to the placebo group.

Changes in EI during clinical test

Changes in erythema values after administration of dextrin or GOS are shown in Table 1. In the placebo group, EI values were 279.89 EI before administration, 270.56 EI at week 4, 270.13 EI at week 8, and 273.62 EI at week 12. In the GOS group, changes in erythema values were 282.85 EI before administration, 258.81 EI at week 4, 253.57 at week 8, and 253.85 EI at week 12. After 8 weeks, there was a significant decrease from baseline in the GOS group ($P<0.001$), and the delta values (ΔEI) for this group significantly differed from that of the placebo group ($P<0.05$).

DISCUSSION

The latest trend in skin care is to improve your appearance through dietary and oral supplements. Healthy skin can be obtained by orally ingesting substances, including vitamins and antioxidants, since skin appearance represents a condition of overall health. This approach to skin

Table 1. Changes in skin value indexes during 12 weeks of galactooligosaccharide (GOS) treatment

Variable	Group	Time (week)			
		Baseline	4	8	12
MI	Placebo (N=40)	160.08±4.28	159.53±4.05	154.72±3.92	155.65±4.13
	GOS (N=39)	161.84±5.57	158.42±4.48	145.85±4.94***††	145.55±5.03***††
EI	Placebo (N=40)	279.89±8.15	270.56±7.34	270.13±7.93	273.62±7.17
	GOS (N=39)	282.85±7.53	258.81±9.04***	253.57±9.30***†	253.85±8.11***†

Asterisk indicates a significant difference ($^{***}P<0.001$) between baseline and change from baseline at each week, as calculated by a repeated measure ANOVA followed by Bonferroni-adjusted pairwise comparisons within groups. Significant differences were indicated by daggers ($^{\dagger}P<0.05$ and $^{\dagger\dagger}P<0.01$ between changes in two groups at each week by *t*-test). Values are mean±SEM. MI, melanin index; EI, eythema index.

health is termed the "inside-out" approach because oral nutritional supplements and topical agents are synergistically combined to enhance efficacy (Draelos, 2010). The inside-out approach has led to development of a new category of product labeled "inner beauty". Inner beauty represents a synergy of oral and topical medications to improve skin appearance.

Prebiotics, food ingredients that are beneficial to the host by selectively stimulating the growth or activity of non-pathogenic intestinal bacteria, have been reported to prevent/treat allergic diseases (Tanabe and Hochi, 2010). Kukkonen et al. (2007) also reported that mixing four kinds of probiotic bacteria with the prebiotic GOS helps prevent allergic diseases. In this study, we measured the effect of GOS on inhibiting skin pigmentation.

Melanin is secreted between the epidermis and dermis of the skin by melanocytes present in the dermis to protect skin from ultraviolet rays. This melanin causes black pigmentation (Yoon et al., 2009). Melanogenesis in human skin is promoted by UVB irradiation, which results in a change in the color of the skin to brown (Murakami et al., 2009). We showed that GOS treatment inhibited melanin production *in vitro* (Fig. 2).

In our previous report (Hong et al., 2015), we showed that GOS intake increased the capacity of skin to retain water and prevented transepidermal water loss compared to controls in UV-irradiated hairless mice. In the GOS-treated group, erythema formation was reduced by 16.8% and CD44 gene expression was significantly increased compared to expression in the control group. These findings suggest that prebiotics, including GOS, are beneficial to both the gut and the skin, and that they have potential as nutritional strategies to prevent skin damage from UV rays. This effect of GOS appears to be due to increased CD44 expression. CD44 has been reported to play an important role in keratinocyte proliferation in response to extracellular stimuli (Kaya et al., 1997).

Erythema is a visible sign of UV-induced inflammation in mammalian skin (Clydesdale et al., 2001). These changes are accompanied or preceded by a variety of inflammatory mediators, including certain enzymes and cytokines. For example, when exposed to UV light, cyclooxygenase-2, an inflammatory enzyme that leads to PGE production, increased levels of a potent mediator of UV-induced erythema (Miller et al., 1994). Production of cytokines by UV irradiation plays a pivotal role in production of matrix metalloproteases (Wang and Bi, 2006) and in self-renewal and activation of inflammatory cells (Wlaschek et al., 1994), and contributes to pathological changes that occur in UV-damaged skin. External stimuli such as UV radiation play an important role in regulating immune and inflammatory responses by inducing secretion of various cytokines and inflammatory mediators, such as IL-1β, IL-6, IL-8, and PGE in dermal keratinocytes (Huang et al., 2010). In the present study, GOS treatment inhibited UV-induced production of cytokines IL-8 and PGE (Fig. 2). Quist et al. (2016) also observed a strong increase in monocyte chemoattractant protein-1, which is known to be readily released with IL-8 after exposure of keratinocytes to UVB *in vitro*, and which is also mediated by reactive oxygen species. These results can be expected to inhibit erythema, an inflammatory reaction caused by UV-induced cytokines.

In our clinical test, GOS intake reduced the MI and EI (Table 1). The decrease in MI seems to be due to the inhibitory effect of melanin formation, as shown in our *in vitro* melanocyte experiment (Fig. 2). The observed reduction in EI was similar to results of previous animal studies, which demonstrated photoprotective effects of GOS (Hong et al., 2015).

Prebiotics have been shown to stimulate the immune system through *in vitro* and *in vivo* experiments (Torrecillas et al., 2007). Prebiotics can also inhibit growth of pathogenic microorganisms, such as *Salmonella* Typhimurium, *Clostridium perfringens*, *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, and *Propionibacterium acnes*, and promote growth of probiotic species (Al-Ghazzewi and Tester, 2010; Elamir et al., 2008). Akiyama et al. (2002) reported that GOS can control *S. aureus* populations, which are often found in patients with atopic dermatitis. The effect of intestinal prebiotics can be reflected in/on other parts of the body, such as the skin, vagina, or bladder. Although the precise mechanisms underlying the effect of dietary fiber fermentation on specific immune systems in the intestine have not yet been fully established, it is assumed that fiber consumption influences intestinal microbiota. Glucomannans have also been reported to modulate skin bacterial proliferation and normalize barrier functionality in sensitive skin (Berardesca et al., 2009).

To date, antibiotics, probiotics, and prebiotics has been shown to affect the composition and functions of intestinal microbiota. GOS has been reported to selectively stimulate development of *Bifidobacteria* (Fanaro et al., 2005). In addition, consumption of GOS within infant formula has been shown to significantly increase amounts of *Bifidobacteria* and Lactobacilli compared with levels following maltodextrin consumption (Moro et al., 2002). Therefore, changes to intestinal microbiota due to GOS ingestion may have affected skin pigmentation.

In recent years, interest in the health effects of dietary nutrients has increased. Our results suggest that intake of the prebiotic GOS has an inhibitory effect on skin pigmentation, demonstrating the possibility of novel nutritional approaches for skin care. However, further research is needed to fully understand how prebiotics might influence skin through altering the intestinal microbiota.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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