Improvement of Skin Photoaging by Polysaccharide Extract Derived from *Tremella fuciformis* (White Jelly Mushroom)

Jae-Hwan Choi and Bora Kim*

1Creative and Innovation Center, IN2BIO Co., Ltd. Hwaseong, Gyeonggi 18471, Republic of Korea
2Department of Cosmetics and Beauty, College of Techno-Science, Mokwon University, Daejeon 35349, Republic of Korea

Abstract – Chronic ultraviolet (UV) radiation causes photoaging, which represents skin damage, disrupts skin barrier function, and promotes wrinkle formation. We investigated that the polysaccharide extract of an edible basidiomycetous white jelly mushroom, *Tremella fuciformis* (TF-Glucan®) exhibited statistically photoprotective activity by inhibiting matrix metalloproteases (MMPs) and increasing collagen synthesis, and an anti-inflammatory activity by inhibiting nitric oxide and pro-inflammatory cytokines at the concentrations of less than 1000μg/ml, which is not cytotoxic (p<0.05). Additionally, TF-Glucan® increased the expression of involucrin and filaggrin to prevent the disruption of UVB-induced barrier function (p<0.05). TF-Glucan® was assessed as a safe material by the human primary skin irritation (1, 3, 5%), human repeated insult patch test (no sensitization at 5%), 3T3 NRU phototoxicity assay (no phototoxicity, PIF<2, MPE<0.1), eye irritation test test by BCOP (no category, IVIS ≤3) and local lymph node assay (negative at 10, 25, 50%) for identifying potential skin sensitizing. These results suggest that TF-Glucan® may be useful as an anti-photoaging ingredient for developing cosmeceuticals.

Keywords – *Tremella Fuciformis*, Polysaccharide, UV, Skin photoaging, Cosmeceuticals

Introduction

The skin is an external organ that plays a role as an important barrier and defence system to prevent water loss, infection, and damage due to sunlight exposure and environmental pollution in the human body. UV irradiation causes reactive oxygen species (ROS) formation and inflammation in the skin; it also induces oxidative stress and inflammation, which occurs in extracellular matrix (ECM) degradation in the dermis and epidermal hyperplasia. UV irradiation enhances the signal transduction pathway involved in collagen degradation, which generates skin laxity and wrinkle formation. Therefore, the inhibition of UVB-induced MMP expression and its upstream regulation is significant for the treatment of skin photoaging. In addition to ECM damage, over exposure to UV irradiation can lead to the generation of pro-inflammatory molecules, stimulating skin inflammation. Interleukins (ILs) and nitric oxide (NO) play a crucial role in UV-induced skin inflammatory responses such as skin erythema, which is related to vascular expansion. These pro-inflammatory cytokines can weaken ceramide production in the whole epidermis; ceramide is mainly responsible for skin barrier function. Keratinocyte differentiation in the epidermis can be abnormal under certain stresses such as UV irradiation. Abnormal keratinocyte differentiation is a part of photoaging. Involucrin is a major cornified envelope protein rich in glutamine and lysine, which are essential for crosslinking by transglutaminase to build the cornified envelope during keratinocyte maturation. Filaggrin binds with keratin intermediate filaments, which combine with macrofibrils to form a tightly packed structure during terminal keratinocyte differentiation. In the stratum corneum, filaggrin is disassembled into free amino acids, referred to as natural moisturising factors (NMFs), which contribute to epidermal hydration. Chronic UV exposure downregulates filaggrin, filaggrin deficiency induces the disruption of skin barrier function. The photo-damage and photoaging may be associated with epidermis and dermis deformation.

In China, *Tremella fuciformis*, a species of fungus produces white, frond-like, gelatinous basidiocarps (fruiting bodies) and a type of edible mushroom, has been used in traditional medicine for centuries; it exhibits various

*Author for correspondence

Dr. Bora Kim, Department of Cosmetics and Beauty, Mokwon University 88, Doanbuk-ro, Seo-gu, Daejeon 35349, Republic of Korea.
Tel: +82-42-829-7565; E-mail: bora0507@mokwon.ac.kr
pharmacological functions, including immune-modulatory, anti-cancer, hypolipidemic, and hypoglycaemic effects. Many studies have reported that the active ingredients of *T. fuciformis* are polysaccharides. In addition, many studies have focused on the anti-oxidative and anti-inflammatory roles of *T. fuciformis* polysaccharides. The anti-phoetaging effects of *T. fuciformis* were evaluated using a 30-day UV-irradiated animal assay as food supplement not skin application. In particular, β-glucan, which is a natural cell wall polysaccharide found in fungi (including mushrooms), yeast, some bacteria, seaweeds, and cereals, is known as a representative polysaccharide with various effects in organisms. Natural β-glucans have been reported to possess many health-promotion effects on the human body, such as antioxidant, anti-aging, anti-photo protection, wound healing, moisturising, anti-tumour, anti-diabetes, anti-infection, blood cholesterol-lowering, and immune-modulating properties. *Tremella fuciformis* has already been utilized and there are many studies, but this study clearly suggested the basis for the development of cosmeceuticals by revealing the improvement of phoetaging by evaluating in vitro and clinical trials for its efficacy on the skin.

**Experimental**

**Sample preparation** – The *T. fuciformis* polysaccharides (Product name: TF-Glucan®) was obtained from 1 kg of dried *T. fuciformis* in 35 L distilled water at 100°C for reflux extraction for 8h, then filtered and freeze-dried. The yield of *T. fuciformis* polysaccharides reaches 80% by quantitative analysis.

**Analysis for quantification of polysaccharides** – The analysis of *T. fuciformis* polysaccharides was performed by using Bio LC systems (ICS-5000 with electrochemical detection, Dionex, Sunnyvale, CA, USA). The pretreatment conditions are 2.0 M trifluoroacetic acid for 4 hours. The mobile phase of 16 mM NaOH was eluted at 1.0 mL/min and the target substances were detected by ED® with integrate amperometry. Data analysis was performed by using PeakNet on-line software.

**Cell culture** – HaCaT keratinocyte cell line, normal human dermal fibroblasts and murine macrophage RAW 264.7 were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO₂ and were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), streptomycin (100 μg/mL) and penicillin (100 U/mL). For irradiation, the cells were exposed to UVB light at a dose of 20 mJ/cm² by using UV irradiation system (World Corporation, Korea). Cell cytotoxicity was determined by MTT assay.

**Collagen synthesis assay** – Human fibroblasts were treated at concentrations of 62.5–250 μg/mL of TF-Glucan®, and the supernatants were detected with procollagen Type I C-peptide (PIP) EIA kit (Takara, Japan) kit under the manufacturer’s manual. The absorbance at 450 nm was measured with microplate reader (Biotek Synergy-HT, Winooski, VT, USA).

**Determination of MMP-1, IL-6 and TNF-α by ELISA** – The secretion activity of MMP-1 and cytokines were measured by ELISA kit under the manufacturer’s manual (R&D system, Minneapolis, MN, USA). Cells were seeded in 96-well plate and treated with TF-Glucan®. After irradiation to UVB, the supernatant was collected and centrifuged at 1500 rpm for 15 min. The levels of MMP-1 and cytokines in culture supernatants were quantified by colorimetric analysis.

**Nitric oxide assay** – RAW 264.7 cells were treated with 0.1 μg/mL of Lipopolysaccharide (LPS) alone or in combination with different concentrations of TF-Glucan®. After 24 h, 100 μL of the medium was placed in a 96-well plate and an equal volume of Griess reagent (Sigma-Aldrich Co., St. Louis, MO, USA) was added. The amount of nitric oxide (NO) was calculated using sodium nitrite standard curve.

**RT-PCR** – Total RNA from HaCat cells was prepared using RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s manual. Total RNA was executed to reverse transcription and subsequent PCR to confirm changes in MMP-1, IL-6, TNF-α, involucrin and filaggrin mRNA levels observed. Total RNA was reverse-transcribed in a reaction mixture containing reverse transcriptase, dNTP mix, primers, and RNase free-water. PCR cycling conditions were chosen for cDNA to ensure that measurements were done during the exponential components of the reaction (Perkin-Elmer cycler 9700, Perkin-Elmer Applied, Waltham, MA, USA). The PCR products were separated on a 1.5% agarose gel.

**Western blot assay** – The cultured cells were washed in phosphate-buffered saline and lysed by a lysis buffer (Cell Signaling, Danvers, MA, USA). Protein amounts were determined using the Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins were resolved on a
sodium dodecyl sulfate polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Co., Billerica, MA, USA). After blocking the membrane with Tris-buffered saline and Tween 20 (TBS-T, 0.1% Tween 20), containing 5% non-fat dried milk, for 1 h at room temperature, membranes were washed with TBS-T and incubated with primary antibodies for 1 h at room temperature or overnight at 4°C. The primary antibodies were used: rabbit anti-involucrin, filaggrin (Santa Cruz Biotechnology, Carlsbad, CA, USA), and mouse anti-GAPDH (Sigma-Aldrich Co., St. Louis, MO, USA). The bands were detected by enhanced chemiluminescence reagent (Santa Cruz Biotechnology, Carlsbad, CA, USA). GAPDH was used as the standard for normalizing protein samples by ChemiDoc imaging system (Bio-Rad, Hercules, CA, USA).

Toxicological assessment for eye irritation, skin phototoxicity and sensitization – The Bovine Corneal Opacity and Permeability (BCOP) test for in vitro eye irritation test (OECD guideline 437) was utilized for its usefulness to identify chemicals inducing serious eye damage for the evaluation of the eye hazard potential of a test chemical, which is measured by its ability to induce opacity and increased permeability in an isolated bovine cornea. Phototoxicity is defined as a toxic response due to a substance applied to the body, which is either elicited or increased after subsequent exposure to light, or that is induced by skin irradiation after the systemic administration of a substance. The in vitro 3T3 NRU (Neutral red uptake) phototoxicity test (OECD guideline 432) is a non-radioactive modification of the LLNA method for identifying potential skin-sensitizing test substances and measuring the lymphocyte proliferation they induce in the auricular lymph nodes. The method described in mouse is based on the use of measuring the content of 5-bromo-2-deoxyuridine, an analogue of thymidine, as an indicator of cell proliferation they induce in the auricular lymph nodes. All tests were performed at KTR (Korea Testing & Research Institute) with guidance with GLP (Good laboratory practice).

Clinical study of safety evaluation – The clinical studies were performed by the established inclusion and exclusion criteria generally applied by the Ellead Co., Ltd. Skin Research Center. The experimental protocols were approved by the Institutional Review Board (IRB number: EL-IRB-170111041S016) in accordance with the Declaration of Helsinki. In, primary Skin irritation test, 31 female subjects (the average age 41.8 ± 8.5) between the ages of 20 and 52 were selected for the study according to the inclusion and exclusion criteria. TF-Glucan® 1.0, 3.0 and 5.0% (w/v (%) in distilled water) will be placed on Van der Bend chambers (Van der Bend, Brielle, the Netherlands). Then, they will be allowed to remain in direct skin contact for a period of 48 hours. Visual grading for skin irritation will be conducted at 30 minutes, 24 hours, and 120 h after the removal of patch, according to the ICDRG (International Contact Dermatitis Research Group). In human repeated insult patch test (HRIPT), 56 female subjects (the average age 46.6 ± 7.8) between the ages of 21 and 60 were selected for the study according to the inclusion and exclusion criteria. The study conducted by modified Shelanski method and valued skin sensitization potential of TF-Glucan® 3.0 % (w/v (%) in distilled water), after repeated 24 h epicutaneous applications under occlusive patch. The subjects visited the Research Facility for total of 14 times during 6 weeks. For both induction and challenge phases, the test products with IQ chambers (Chemotechnique Diagnostics, Sweden) were applied to each designated site of the back between the scapulae and the waist.

Statistical analysis – In vitro data was analyzed by the difference between means ± S.D. (standard deviation), and statistical significance was calculated by Fisher’s least significant difference or Mann-Whitney test at ** p < 0.01, and * p < 0.05 vs control group (IBM SPSS statistics version 21.0, Chicago, IL, USA).

Results and Discussion

The polysaccharide yield of T. fuciformis (about 500 KDa) reached 80% content by quantitative analysis; the polysaccharides comprised mannose, fucose, and low-molecular weight oligosaccharides, etc. (Fig. 1). These profiles of Fig. 1. show that three polysaccharides comprised the same sugars but in different ratios, and the carbon sources (β-glucan, mannose, fucose) affected the sugar ratios within the polysaccharides.

TF-Glucan® treatment at concentrations of 250 μg/mL led to a significant increase (p < 0.05) in collagen production by 20%, compared to control cells (Fig. 2B). TF-Glucan® treatment resulted in decreased MMP-1 mRNA expression, as observed by RT-PCR (Fig. 2C). At TF-Glucan® concentrations of 125, 250, and 500 μg/mL, MMP-1 expressions, as measured by ELISA, decreased significantly (p < 0.05), compared to UVB-exposed cells (Fig. 2D).

To evaluate the effects of TF-Glucan® on the UVB-induced production of inflammatory mediators, cells were treated with TF-Glucan® after UVB exposure. The TF-
Fig. 1. Bio-LC Analysis for quantification of sugars.

Fig. 2. Effects of TF-Glucan® on Type 1 procollagen synthesis and MMP-1 expression. (A) Cell viability of human fibroblast on TF-Glucan®, (B) Determination of production of procollagen Type 1 C-peptide (PIP), (C) MMP-1 mRNA and protein expression of TF-Glucan® treatment by UV irradiation (n = 3, means ± standard error of the mean; *p < 0.05; **p < 0.01 compared with the untreated control group).
Glucan® inhibited the LPS-induced production and secretion of NO (Fig. 3A) and pro-inflammatory cytokines such as IL-6, and TNF-α in RAW 264.7 cells. UVB significantly increased the mRNA and protein levels of IL-6 and TNF-α compared to the normal cells, but TF-Glucan® inhibited cytokine production (Fig. 3B-E). These data indicate that treatment with TF-Glucan® resulted in the down-regulation of mRNA levels of pro-inflammatory...
cytokines. We showed that the expression of involucrin and filaggrin was decreased by UVB. As shown in Fig. 4, mRNA and protein expressions of involucrin and filaggrin in UVB-exposed cells treated with TF-Glucan® were increased, as compared to the UVB non-exposed cells. We confirmed changes in the protein expression related to keratinocyte differentiation by determining the expressions of involucrin. As shown in Fig. 4B, the groups treated with TF-Glucan® showed high levels of involucrin expression, which was decreased by UVB exposure. The effect of TF-Glucan® on the expression of filaggrin was determined by RT-PCR and Western blotting (Fig. 4C, 4D). After TF-glucan® (undiluted) treatment, the opacity unit showed an average value of -1.1 ± 0.9, which belongs to the UN GHS No Category in BCOP test. In in vitro 3T3 NRU phototoxicity test, TF-Glucan concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 7.81 μg/mL were tested, and no phototoxicity was seen (PIF < 2, MPE < 0.1). TF-Glucan® was shown to be a non-skin sensitizer at concentrations of 10, 25, and 50% (v/v). TF-Glucan® (1.0, 3.0, 5.0%) were categorized as products in the safety zone, showing non-irritation response mean scores in the primary skin irritation test, in accordance with the human skin reaction evaluation criteria. To demonstrate the sensitization level of a chemical, there was no skin irritation or sensitization observed in any of the 56 subjects using HRIPT. These results indicate that 3.0% TF-Glucan® does not have any skin sensitization
potential.

This study shows that the beneficial effects of polysaccharides derived from *Tremella fuciformis* can be clarified by cell-based *in vitro* methods; their efficacy of photoaging improvement has been confirmed through clinical trials. β-Glucan is known as an effective polysaccharide in *T. fuciformis*, its mechanism of anti-photoaging, as well as its practical variety of therapeutic applications. We also confirmed collagen metabolism could potentially be very helpful in a (type I collagen) in the dermis. Thus, the control of fine wrinkles is the disappearance of structural proteins characteristic of skin photo-aging; the major causation of moisturization, in involucrin are important elements for maintaining skin homeostasis and keratinocyte differentiation. TF-Glucan was found to be promising as an active ingredient with anti-wrinkle activity and moisturising effects; it is also capable of maintaining skin barrier homeostasis. This study is meaningful for *T. fuciformis* research by proving its mechanism of anti-photoaging, as well as its practical application in cosmeceuticals.

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