Oil extracted from extremophile *Yarrowia lipolytica* alleviates the cold stress-induced skin aging

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Abstract

Cold exposure in humans is known to increase oxidative stress, and as the one of the critical factors of skin aging and skin barrier damage. In this study, we aimed to develop ca osmetic ingredient that can be able to overcome cold stress and improve the skin condition. To achieve this goal, we used the oil of from oleaginous *Y. lipolytica* living in the extreme cold environments. We used menthol to give a cold-stressed condition as an inducer to keratinocytes. The expression levels of superoxide dismutase 2 (SOD2) and collagen type XVII (COL17A) were down-regulated in menthol-induced human keratinocytes. *Y. lipolytica*-producing oil significantly increased the expression level of SOD2 and COL17A in menthol-induced keratinocyte. Moreover, the *Y. lipolytica* oil decreased the mRNA expression levels of matrix metalloproteinase-1 (MMP-1) and consequently increased the procollagen production even under the UVB irradiation. Furthermore, nitric oxide (NO) production in lipopolysaccharide (LPS)-treated cells was diminished under *Y. lipolytica* oil treatment in concentration-dependent

manner. Radical scavenging activity by DPPH compared to control was highly increased by the treatment of *Y. lipolytica* oil in concentration-dependent manner. Fatty acid of *Y. lipolytica* oil was consisted 9.7% of palmitic acid (C16:0), 2.2% of stearic acid (C18:0), 58.6% Oleic acid (C18:1), and 29.5% of linoleic acid (C18:2). The oil of oleaginous *Y. lipolytica* that alleviate the cold stress-induced skin damage and improve the skin condition would be a potent active cosmetic ingredient against cold-stress condition as well as anti-aging, anti-oxidation, and skin barrier.

Keywords: cold-stress; *yarrowia lipolytica*; biosynthetic oil; skin aging; oxidative stress

Introduction

The seasonal and climatic cold conditions would be the one of critical causative factors of skin aging and skin barrier abnormalities including the dry and sensation of itchy [1]. Especially for those who work in cold environments or where being outside in cold temperatures with lengthy exposures can cause dry skin conditions or aggravate common dermatoses, such as atopic dermatitis and psoriasis [2]. Cold conditions represent an environmental factor, where facial cooling usually occurs within minutes, contributing to skin sensitivity. Thus, cold weather appears to be a major concern of potential skin irritation [3]. These researches highlighted the importance of the understanding of the effects of cold condition on skin barrier and skin aging. Cold-inducible RNA-binding protein (CIRP) is identified as a cold-shock protein that is upregulated in response to hypoxia, oxidative stress as well as hypothermia stress. The CIRP is known to be activated by the cold temperature or menthol, a substitute that causes cold stress, via transient receptor potential melastatin 8 (TRPM8) channel [4, 5]. TRPM8 is known to mainly function for sensory responsiveness to cold temperature in neuron and also expressed in epidermal keratinocytes as functioning skin homeostasis [6].

Cold exposure in humans is also known to increase oxidative stress [7, 8]. Menthol was known to activate CIRP via TRPM 8 channel, a major sensor of environmental cold temperatures. In our previous study (not published yet), collagen type XVII (COL17), which was known to facilitate the keratinocyte adhesion and to be located at the basement membrane, which thins and flattens with increasing ages, were down-regulated under cold treatment mimicked by

menthol.

Oleaginous yeast *Yarrowia lipolytic*a (*Y. lipolytica*) has found in natural ecosystems as well as the extreme environments, such as arctic, antarctic sea, and so on [9]. This extremophile yeast has emerged both as a convenient microorganism for industrial application and as a model organism for investigating oil synthesis and accumulation in microbes and higher organisms [10]. Because it is recognized as a generally regarded as safe (GRAS) microorganism, their application field has been also expanded to cosmetic industry.

In this study, we aimed to develop the new cosmetic ingredients to prevent skin aging caused by cold stress.

Materials and Methods

1. Preparation oil sample of *Y. lipolytica*

Yarrowia Lipolytica strain was obtained from Seoul National University. These yeast cells were cultured in either YPD or YM media under 30℃ at 250 rpm for 24 to 48 hr (Sigma-Aldrich®, St. Quentin Fallavier Cedex, France). The medium was autoclaved at 121℃ for 15 min before use.

Oil was extracted from *Y. lipolytica* using a modified Bligh and Dyer method [11]. One hundred mg of yeasts cells was collected in glass tubes. A total of 7 mL of a chloroform/methanol mixture (1:1, v:v) was added and homogenized for 30 s with a vortex. Then, 2 mL of distilled water was added. The mixture was vigorously shaken for 15 min and then centrifuged for 15 min at 3000 rpm. The organic layer was collected in a new tube and kept at an ambient temperature. A second centrifugation was performed after 2 mL of chloroform/methanol (1:1; v:v) and 1 mL of NaCl 0.5 M were added. Finally, 2 mL of chloroform was added, and a final centrifugation and collection of organic layers were performed. The organic phase (lower) was recovered and stored at -80℃.

2. Human Keratinocyte Cell culture

Human epidermal keratinocytes (HaCaT) were purchased from the Cell Lines Service (CLS; Eppelheim, Germany) and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Logan, UT, USA) supplemented with 1% Antibiotic Antimycotic Solution and 10% fetal bovine serum in an atmosphere of 5% CO2 at 37°C. HaCaT keratinocytes were maintained until 80% confluence and then cells were treated with either 100 μM menthol or 25

mJ/cm^2 UVB irradiation in serum-free medium and incubated for 24 hr.

3. RT-PCR (Realtime-polymerase chain reaction)

Total RNA was isolated from HaCaT keratinocytes using TRIzol reagent according to the manufacturer's instruction (TaKaRa, Shiga, Japan). cDNA was synthesized from 1 μg of total RNA using Reverse Transcription Premix (Elpis-biotech, Daejeon, Korea) under the following reaction conditions: 45℃ for 45 min and 95℃ for 5 min. Gene expression signals were quantified with real-time PCR, and the data were analyzed using StepOne PlusTM system software (Applied Biosystems, Foster City, CA, USA). Real-time PCR amplification reactions were performed using SYBR Green PCR Master Mix with premixed ROX (Applied Biosystems, Foster City, CA, USA). Real time PCR was then performed using Applied Biosystems. The reaction conditions were as follows: initiation at 50℃ for 2 min and 95℃ for 10 min, followed by 40 cycles of 95℃ for 10 s and 60℃ for 1 min. The expression of β-actin was used as an internal control.

4. Procollagen quantification

Human keratinocyte HaCaT cells were irradiated with UVB and then treated with different concentration of *Y. lipolytica* oil for 24 hr. Procollagen type I contents in the cells were estimated using the procollagen I C-terminal peptide enzyme-linked immunosorbent assay (ELISA) kit (Takara, Shiga, Japan), following the manufacturer's protocols. Absorbance was measured at 590 nm using a microplate reader. The final procollagen type I levels were normalized to the total cellular protein content.

5. NO assay

HaCaT cells were seeded at a density of 5×10^5 cells/well in 24 well plates and incubated for 12 h at 37℃ and 5% CO_2. Then media of each well were aspirated and fresh FBS-free DMEM media were replaced. Cells were stimulated with 1 μg/mL of LPS (Lipopolysaccharide) for 24 hr. After that, different concentrations of *Y. lipolytica* oil were prepared in FBS-free DMEM to give a total volume of 500 μL in each well of a microtiter plate for 4 hr. The presence of nitrite was determined in cell culture media using commercial NO detection kit (iNtRON, Sungnam, South Korea). Protocols supplied with assay kit were used for the application of assay procedure. Briefly, 100 μL of cell culture medium with an equal volume of Griess reagent in a 96-well plate was incubated at room temperature for 10 min. Then the absorbance was

measured at 540 nm in a microplate reader (Biotek, Winooski, VT, USA). The amount of nitrite in the media was calculated from sodium nitrite ($NaNO_2$) standard curve.

6. DPPH scavenging assay

The scavenging activity with respect to free radical DPPH was determined by a method described by Wang et al. [12] with slight modifications. One hundred μL of different concentrations of test samples dissolved in DMSO were mixed with 900 μL of a 1mM DPPH solution in ethanol. The mixtures were well shaken and kept at room temperature for 30 min in dark condition. At the same time, the mixture without test sample was prepared as blank control. Then the absorbance was measured at 517 nm using a PerkinElmer multimode plate reader Victor X3 (PerkinElmer, Santa Clara, CA, USA). The percentage DPPH scavenging activity expressed as % scavenging was calculated by the equation (1- A_A/A_B) X 100, where A_A and A_B were the absorbance values of the test sample and blank, respectively. Ascorbic acid 1mg/ml sample was used for positive control.

7. Measurement of fatty acid methyl ester (FAME) content and composition

Lipids from aliquots of 10–20 mg of cells were converted into fatty acid methyl esters (FAME). First, 50 µg of triheptadecanoin (C17:0 TAG) was added as an internal standard for quantification. Then, 1 ml of 5% (v/v) H_2SO4 in methanol with 300 µl toluene as a co-solvent was added to the glass tube. Samples were incubated for 90 min at 90℃ to convert oils into their FAMEs. FAMEs were extracted with hexane and 1.5 ml of 0.9% KCl was added to enhance phase separation. FAMEs were quantified using gas chromatography-flame ionization detector (GC-FID) on a HP-INNOWAX capillary column (30m, 0.25mm, 0.25µm) with SHIMADZU GC-2010.

Results and Discussion

1. *Y. lipolytica* oil up-regulated the expression of SOD2 and COL17 genes in cold-stress induced human keratinocytes

Cold exposure mimicked by menthol decreased the expression levels of COL17 in human keratinocytes as known at our previous research. This stress condition elevated reactive oxygen (ROS) metabolites and the concomitant decrease of plasma antioxidants. To examine the effect of oil obtained from *Y. lipolytica* under the cold-stress condition, we checked the expression

levels of several marker genes related to skin aging. As shown in Figure 1, the mRNA expression level of SOD2 was declined upon menthol treatment at keratinocyte grown medium. In the presence of *Y. lipolytica* oil, the expression level of SOD2 was significantly increased in menthol-induced keratinocyte. In our previous study, we demonstrated that mRNA expression levels of COL17, which play a role in keratinocyte adhesion at the basement membrane bewteen epidermis and dermis, was decreased and therefore induced the skin-aging under the cold-stress condition. The treatment of menthol suppressed the mRNA expression of COL17 while *Y. lipolytica* oil rescue of COL17 mRNA levels (Figure 2). However, the expression level of TRPM8 was not altered in response to oil treatment in the medium of menthol-induced keratinocyte. These result suggested that oil extracted from *Y. lipolytica* alleviated the expressions of skin aging-related marker genes under cold-stress condition

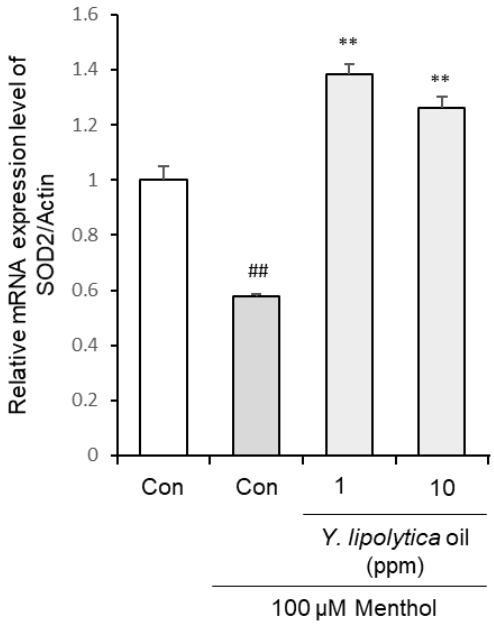

Figure 1. Relative expression level of SOD2 under the treatment of oil extracted from *Y. lipolytica* in human keratinocytes. The mRNA expression of SOD2 was measured by RT-qPCR. The means ± SEs are the average of three independent experiments. ##p<0.01 indicates a significant difference from the menthol-treated control. **p<0.01 indicates a significant difference from the control.

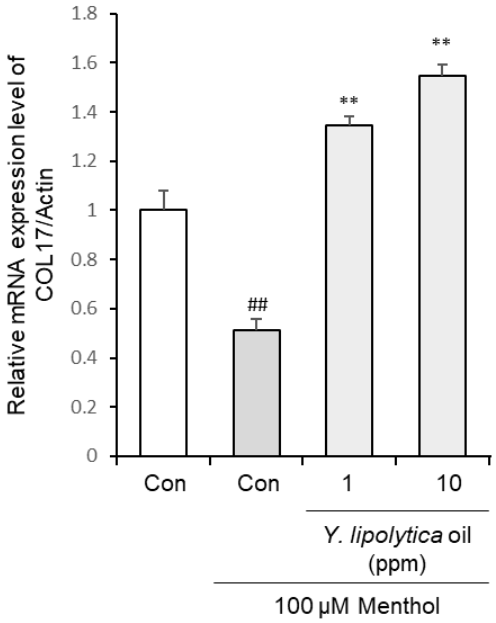

Figure 2. Relative expression level of COL17 in the presence of oil extracted from *Y. lipolytica* in human keratinocytes. The mRNA expression of COL17 was measured by RT-qPCR. The means ± SEs are the average of three independent experiments. ##$p<0.01$ indicates a significant difference from the menthol-treated control. **$p<0.01$ indicates a significant difference from the control.

2. *Y. lipolytica* oil alleviated the increase in MMP-1 expression and the decrease in procollagen synthesis by UVB irradiation in human keratinocytes.

Collagen synthesis is decreased and matrix metalloproteinase-1 (MMP-1) level is increased under the UVB irradiation (Figure 3, 4). The expression level of MMP-1 was down-regulated under the treatment of oil extracted from *Y. lipolytica* and Epigallocatechin gallate (EGCG) as a positive control (Figure 3). Moreover, procollagen type I contents in UVB-irradiated keratinocyte was increased by *Y. lipolytica* treatment in concentration-dependent manner (Figure 4). It indicated that *Y. lipolytica* oil improved the skin anti-aging markers *in vitro*.

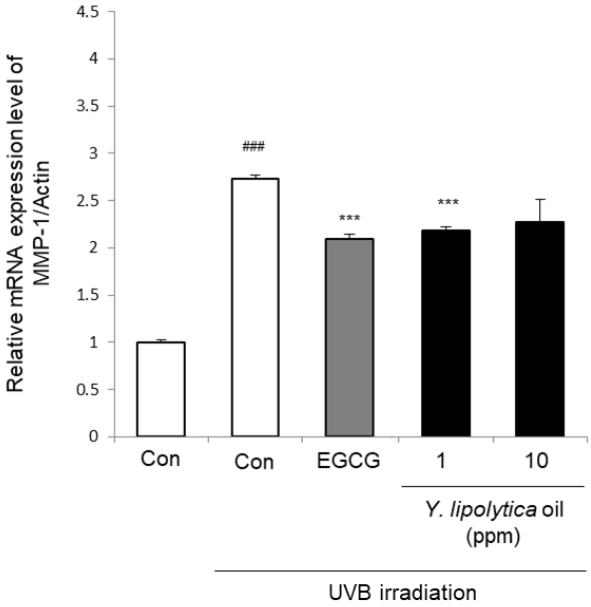

Figure 3. Relative expression level of MMP-1 in the presence of oil extracted from *Y. lipolytica* in human keratinocytes. The mRNA expression of MMP-1 was measured by RT-qPCR. The means ± SEs are the average of three independent experiments. EGCG was used for positive control. ###p<0.001 indicates a significant difference from the control. ***p<0.001 indicates a significant difference from the UVB-irradiated control.

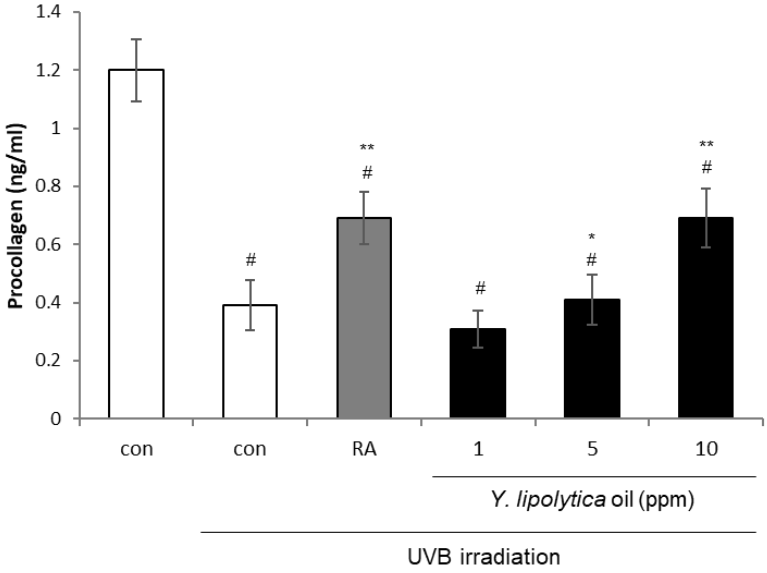

Figure 4. The contents of procollagen under the treatment of *Y. lipolytica* oil in UVB-irradiated human keratinocytes. The means ± SEs are the average of three independent experiments. RA was used for positive control. #p<0.01 indicates a significant difference from the control.

*p<0.05 and **p<0.01 indicate a significant difference from the UVB-irradiated control. RA, retinoic acid;

3. *Y. lipolytica* oil inhibited the oxidative stress in HaCaT cells

Since cold exposure was known to increase the oxidative stress, we tested whether *Y. lipolytica* oil has an anti-oxidation effect in keratinocytes [8]. Figure 5 showed that NO produced by LPS treatment was alleviated in *Y. lipolytica* oil-treated HaCaT cells with a concentration-dependent manner. Furthermore, free radical DPPH assay showed that ROS scavenging activity was significantly increased by the oil extracted from *Y. lipolytica* in dose-dependent manner (Figure 6). These results indicated that *Y. lipolytica* oil potentially improve the skin conditions by inhibiting free radical production under oxidative stress.

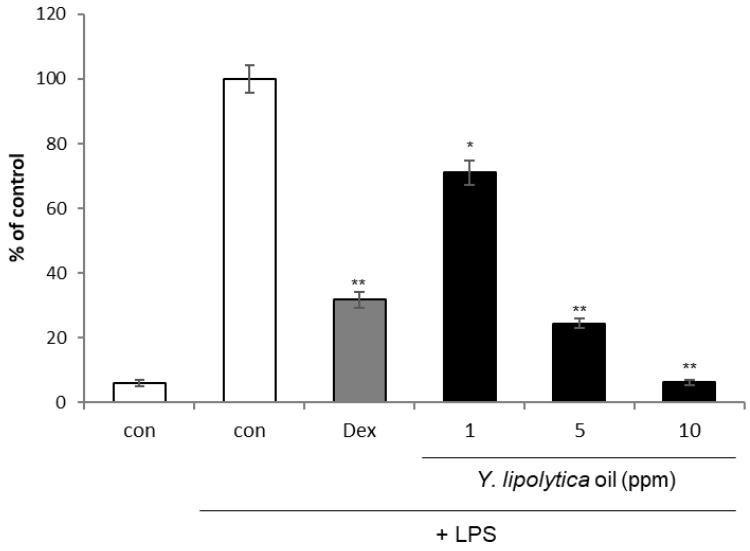

Figure 5. NO production by LPS treatment in HaCaT cells treated with different concentrations of *Y. lipolytica* oil. It was measured by NO detection kit. Dex was used for positive control. *p<0.05 and **p<0.01 indicate a significant difference from the LPS-treated control. Dex, dexamethasone;

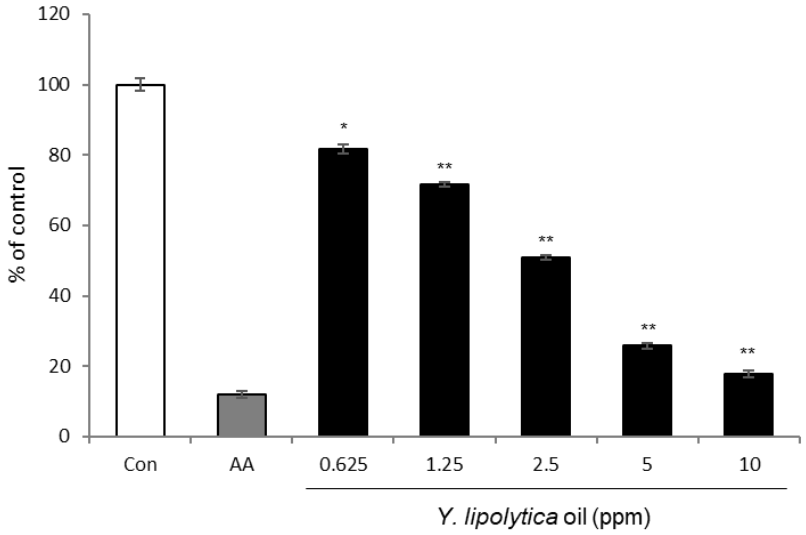

Figure 6. The scavenging activity of free radical DPPH in HaCaT cells in the presence of *Y. lipolytica* oil. One mg per ml of ascorbic acid sample was used for positive control. *p<0.05 and **p<0.01 indicates a significant difference from the control. AA, ascorbic acid;

4. *Y. lipolytica* oil is mainly consist of C16 and C18 fatty acids.

To further analyze the *Y. lipolytica* oil, we measured fatty acid content and composition using GC-FID. Total lipid from *Y. lipolytica* was consisted 9.7% of palmitic acid (C16:0), 2.2% of stearic acid (C18:0), 58.6% Oleic acid (C18:1), and 29.5% of linoleic acid (C18:2). Oleic acid (C18:1) was a major components of *Y. lipolytica* oil similar to data from Niehus X. et al. [13]. In our analysis, linolenic acid (C18:3) was not detected. Fatty acids in the stratum corneum lipid of human skin varied from C12 to C24 with C16 and C 18 predominating, according to several studies [13, 14]. Because *Y. lipolytica* oil is mostly consist of a C16 and C18 fatty acid species, it might be helpful to maintain the lipid of outermost layer of skin, consequently. Moreover, linoleic acid (C18:2) was known to have a role in the maintenance of skin barrier function and skin hydration. It might be able to have a positive effect to keep skin healthy by *Y. lipolytica* oil treatment.

Fatty acid	Content (% weight of total lipid)	
	Yarrowia lipolytica oil	Niehus X et al.,
C16:0	9.7	14.9
C18:0	2.2	11.1
C18:1	58.6	55.7
C18:2	29.5	18.5
C18:3	-	0.3

Table 1. Fatty acids contents of *Y. lipolytica* oil. Fatty acids were converted to FAME and then measured by GC-FID. Data from Niehus X. et al. was referred to compare the fatty acid composition with *Y. lipolytica* oil [13].

Conclusion.

In this study, cold stress induced by menthol treatment decreased expression levels of genes such as SOD2 and COL17. The oil extracted from *Y. lipolytica* increased expression level of these genes even in the menthol-induced keratinocytes. In addition, this oil alleviated the increase in MMP-1 expression and the decrease in procollagen synthesis by UVB irradiation in human keratinocytes. *Y. lipolytica* oil has a positive effect in skin anti-aging properties, suggesting that it would be a potent active cosmetic ingredient against a cold stress condition. Moreover, oxidative stress was diminished by *Y. lipolytica* oil in human keratinocytes. Fatty acids found in *Y. lipolytica* oil were similar to those of stratum corneum in human. It overall implied that *Y. lipolytica* oil can be able to use to improve skin condition both stress and normal condition.

Acknowledgments

We thanked to professor Seo's Lab at Seoul National University to provide the oleaginous yeast *Yarrowia lipolytica*.

Conflict of Interest Statement

None

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