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Maltotetraose Activate Antioxidative Responses by NRF2 activation in human keratinocytes

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1. Introduction

In recent years, the prevalence of sensitive skin has been increasing due to external factors such as environmental changes and internal factors like emotional stress [1]. *Berardesca et al.* reported that approximately 70% of individuals consider themselves to have sensitive skin, while around 50% of them have experienced discomfort symptoms [2]. Sensitive skin symptoms include burning, stinging, itching, and tightness in response to low-level stimuli that would not typically affect normal skin [3]. This low epidermal barrier can cause various skin troubles, especially itching, which can aggravate the skin condition. Notably, itching can initiate the itch-scratch cycle where scratching to temporarily relieve the itch results in prolonged itching [4]. Therefore, effective treatment for sensitive skin requires not only moisturizing to improve skin barrier function, but also suppress itching.

Our research has identified that Maltotetraose (MTO), a plant-based ingredient derived from corn, possesses significant moisturizing and itch-relief properties [5]. MTO, consisting of glucose units with α-1,4-glycosidic bonds, has a higher number of hydroxy groups compared to traditional humectants like glycerin and butylene glycol, which enhance its skin moisturizing effect. Additionally, our previous studies have demonstrated that MTO suppresses chronic itching by inhibiting the activation of the neurokinin 1 receptor (NK1R), an important pathway in the itch response involving substance P and NK1R [6, 7]. Based on these findings, we developed itch relief moisturizers containing MTO and demonstrated their efficacy in skin moisturizing, skin barrier improvement, and itch relief through a clinical trial conducted on individuals with dry, itchy, and sensitive skin [8]. These results suggest the usefulness of MTO as a functional ingredient in cosmetic formulations. Furthermore, given the fact that MTO affects intracellular pathways involved in the itch response, it is expected that it may have further beneficial effects on the skin. However, such additional benefits of MTO for the skin have not been reported.

This study aimed to explore new functions of MTO using DNA microarray analysis and investigate its potential to enhance skin defense against oxidative stress through the activation of the transcription factor, Nuclear factor-erythroid 2-related factor (NRF2).

2. Materials and Methods

2.1 Cell Culture

Normal Human Epidermal Keratinocytes (PromoCell), NHEK cells were cultured in Keratinocyte Growth Medium 2 (PromoCell) containing SupplementMix and $CaCl_2$ (PromoCell). The cells were incubated at 37°C in a humidified incubator with 5% CO_2.

For all experiments, Hydrolyzed Corn Starch (HCS), whose main component is MTO, was used. HCS was diluted in the medium and treated for specific duration at 37°C in a humidified incubator with 5% CO_2.

2.2 RNA Extraction

Total RNA was isolated from the cultured cells using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. The RNA concentration and purity (Ab: 260 nm/280 nm) was analyzed using infinite® 200 PRO (TECAN). RNA was resuspended in nuclease free water and stored at -80°C.

2.3 DNA Microarray Analysis

DNA microarray analysis was conducted using Clariom™ S Array, human (Thermo Fisher Scientific) by Kurabo, and fluorescence signals were scanned with the Biosystems™ GeneChip Scanner 3000 7G System. Biosystems™ GeneChip Command Console Software ver. 3.2 was utilized to convert the array images into intensity values for each probe (CEL files).

2.4 Real-time PCR Analysis

Real-time PCR analysis was carried out using One Step TB Green® PrimeScript™ RT-PCR Kit II (Takara Bio). Sample were run under the following conditions: a reverse transcription step (42°C, 5 minutes), followed by an activation step (95°C, 10 seconds) and 40 cycles of an amplification step (95°C, 5 seconds; 61°C,30 seconds). The primer sequences were as follows: heme oxygenase 1(HMOX1), 5'-tcctgctcaacatccagctc-3' (forward) and 5'-cacatggcataaagccctac-3' (reverse); glutathione peroxidase 2 (GPX2), 5'-ttgatatcagtccccttcactg-3' (forward) and 5'-ctgccctttattggtctcttc-3' (reverse); NAD(P)H quinone oxidoreductase 1 (NQO1), 5'-agtcatctcattccactgttgg-3' (forward) and 5'-catctggtaaaggaggttttcc-3 (reverse)

2.5 Evaluations of ROS Reduction

The level of reactive oxygen species (ROS) was measured using ROS Assay Kit (DOJINDO) following the manufacturer's instructions. NHEK cells were seeded in 96-well plate and incubated in a humidified incubator with 5% CO_2 overnight. Cells were then treated with or without 4.0% HCS. After 48 hours, the cells were incubated with ROS detection fluorescent reagent for 30 minutes. After washing with Hank's Balanced Salt Solution (HBSS) (Thermo Fisher

Scientific), the cells were incubated with 200 μM H_2O_2 diluted in HBSS for 1 hour at 37°C in a humidified incubator with 5% CO_2. Following a wash with HBSS, ROS levels were measured using a fluorescence microplate reader (Ex: 490 nm, Em: 540 nm).

2.6 Western Blotting

NHEK cells were seeded in T75-flask and incubated at 37°C in a humidified incubator with 5% CO_2 overnight. Cells were then treated with or without 4.0% HCS. After 2 hours, cells were harvested, and nuclear and cytoplasmic proteins were extracted separately according to the instruction for the NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). Protein concentration was quantified using a bicinchoninic acid (BCA) assay kit (Takara Bio) and adjusted to an equal concentration. Each protein sample was separated on an electrophoresis gel (Invitrogen) and transferred to a PVDF membranes. The membrane was treated with 5% skim milk diluted in Tris Buffered Saline with Tween 20 (TBS-T) (FUJIFILM Wako Chemicals) at room temperature for 1 hour. Then, the membrane was incubated with primary antibodies of NRF2 (Abcam, 1:500) overnight at 4°C. The membrane was washed with TBS-T and treated with the HRP-conjugated secondary antibody (Abcam,1:20000) for 1 hour at room temperature. After washing with TBS-T, target proteins were detected by the ECL select WB detection reagent (Cytiva) and Amersham ImageQuant 800 (Cytiva). The target protein was quantified using ImageJ and each protein amount was normalized to the amount of β-actin.

2.7 Immunostaining

NHEK cells were seeded in a 6-well plate and incubated at 37°C in a humidified incubator with 5% CO_2 overnight. Cells were then treated with or without 4.0% HCS. After 2 hours, the cells were fixed with 4% formaldehyde (Pierce) diluted in Phosphate-buffered saline (PBS) (FUJI-FILM Wako Chemicals) for 15 minutes. After washing with PBS, 0.5% Triton-X (Sigma Aldrich) diluted in PBS was added to 6-well plate and incubated at room temperature for 15 minutes. Following another PBS wash, 3% Bovine serum albumin (BSA) (FUJIFILM Wako Chemicals) diluted in PBS was added to 6-well plate and incubated at room temperature for 1 hour. The cells were then incubated with a primary antibody of NRF2 (Proteintech, 1:250) overnight at 4°C. After washing with PBS, the cells were incubated with the secondary antibody conjugated with Alexa Fluor® 488 (Abcam, 1:300) at room temperature for 30 minutes. The cells were then washed with PBS and NucBlue™ Live ReadyProbes™ Reagent (Invitrogen) was added to it. After incubating at room temperature for 20 minutes, test cells were observed under a fluorescent microscope (Ex: 495 nm, Em: 519 nm for NRF2, and Ex 360 nm, Em: 460 nm for nuclear).

2.8 Statistics

Statistical analysis was conducted using JMP® statistical software (JMP Statistical Discovery LLC). The statistical significance between the HCS treatment groups and control groups was determined using Student's T-test and Dunnett's test. Data are presented as mean ± standard

deviation (SD), and “n” indicates the number of biological replicates in each experiment. P-values <0.05 were considered significant.

3. Results

3.1 Gene Expression Response to MTO

To examine the shifts in gene expression induced by MTO, a DNA microarray analysis was conducted under the condition of 2.0% HCS treatment for 24 hours. When comparing HCS-treated cells with untreated ones, genes showing a 2.0-fold or greater were judged to be up-regulated, while those with less than 0.5-fold change were judged to be down-regulated. Table 1 lists the 35 genes that were up-regulated and down-regulated with a signal intensity ranging from 20% to 100%. Among these genes, HMOX1, AKR1C1, AKR1C2, AKR1C3, ABCC2, GPX2, CYP4F11, NQO1, SLC7A11, and CHML are known to be related to oxidative stress response. Additionally, Gene Ontology (GO) analysis revealed that the effects of HCS treatment significantly involved the oxidation reduction process (GO:0016491) (Data not shown). To confirm the reproducibility of the microarray data indicating that genes related to oxidative stress were up-regulated by HCS treatment, the relative expression levels of HMOX1 mRNA, GPX2 mRNA and NQO1 mRNA in NHEK cells were measured by real-time PCR (Fig. 1). These genes are antioxidant responsive genes that are commonly regulated by NRF2, the key regulator of the skin’s defense against oxidative stress [9]. The findings indicated a concentration-dependent increase in mRNA expression when treated with 1.0%, 2.0%, or 4.0% HCS for 24 hours. Consequently, the antioxidative response of MTO was also evaluated.

Table 1. Identification of up-regulated or down-regulated genes by HCS using DNA microarray analysis. (a) Up-regulated genes with a fold change greater than 2.0. (b) Down-regulated genes with a fold change less than 0.5.

(a)

Genes	Fold Change
HMOX1	4.28
AKR1C1	3.45
TMPRSS11F	3.08
AKR1C2	2.82
ABCC2	2.51
GPX2	2.43
AKR1C3	2.35
KLC4	2.34
ZPLD1	2.30
CYP4F11	2.21
LRRC28	2.15
NQO1	2.14
MMP3	2.13
MMP1	2.07
SLC7A11	2.06
ZNF679	2.04
DHRSX	2.03
SYTL2	2.00

(b)

Genes	Fold Change
PRSS22	0.34
HIST1H2BM	0.37
SPATA5	0.37
MX2	0.39
MANF	0.40
KRTAP2-3	0.43
CRELD2	0.43
ANXA6	0.44
USP41	0.45
CAMK1D	0.48
RFX3	0.48
KRTAP2-2	0.49
OCLM	0.49
IFIT1	0.49
LAMA1	0.49
CHML	0.49
APOL3	0.49

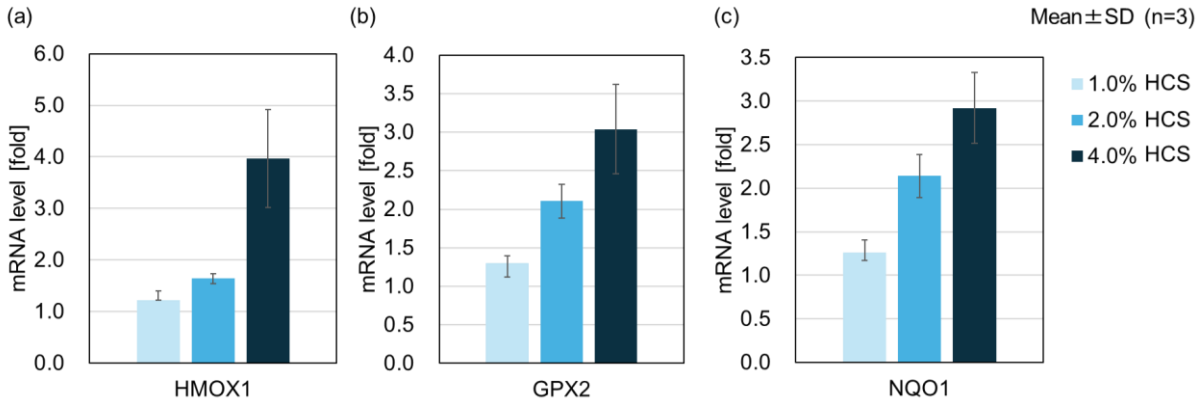

Fig. 1 Changes in expression levels of antioxidant responsive genes by HCS. The mRNA level of (a) HMOX1, (b) GPX2, (c) NQO1 were measured by real-time PCR. The results are shown relative to the corresponding mRNA levels of control (medium only) set to 1.0. The data were normalized to mRNA level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

3.2 The Cellular Antioxidant Activity of MTO

To evaluate the antioxidative response of MTO, intracellular ROS levels following HCS treatment were measured using the fluorescent probe DCFH-DA (Fig. 2). Exposure to H_2O_2 (200 µM for 1 hour) resulted in a significant increase in ROS levels in the cells. On the other hand, pre-treatment with 4.0% HCS for 48 hours significantly reduced the ROS levels induced by H_2O_2 exposure. These findings indicate that treatment with HCS, a main component of which is MTO, effectively inhibits ROS production.

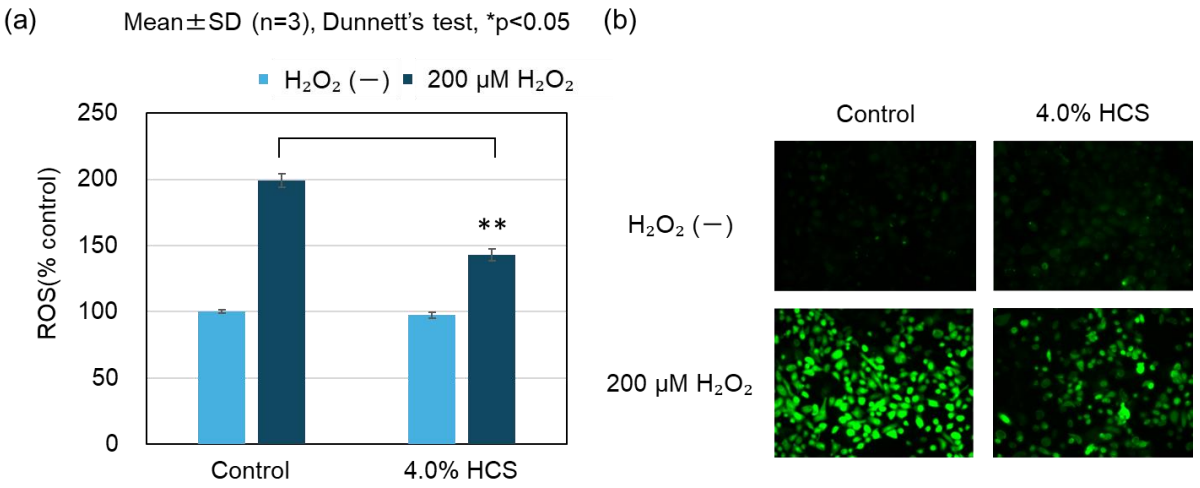

Fig. 2 HCS effect on intracellular ROS reduction. (a) ROS level evaluated by the fluorescence of the cells labeled with DCFH-DA. (b) Images of cells fluorescing due to the oxidation of DCFH-DA by ROS.

3.3 NRF2 Activity

To elucidate the mechanism underlying the antioxidative response induced by MTO, the role of transcription factor NRF2, which regulates the expression of antioxidant responsive genes such as HMOX1, GPX2, and NQO1, was investigated. Under non-stressed conditions, NRF2 resides in the cytosol where it binds to Kelch-like ECH-associated protein (KEAP1). This interaction results in the polyubiquitination of NRF2 by the Cullin 3 (Cul3) E3 ubiquitin ligase, targeting it for proteasomal degradation. Upon oxidative stress, the binding between NRF2 and KEAP1 is disrupted, leading to the stabilization and accumulation of NRF2. Subsequently, NRF2 translocates into the nucleus, where it activates the expression of its antioxidant responsive genes (Fig. 3) [9]. Therefore, the up-regulation of gene expression by NRF2 requires its localization in the nucleus, and the level of transcription should depend on its localization state. The intracellular behavior of NRF2 following HCS treatment was assessed using western blotting and immunoblotting. In western blotting, the levels of nuclear and cytoplasmic NRF2 protein were analyzed separately (Fig. 4). The nuclear NRF2 protein, which plays a role in

activating the expression of antioxidant responsive genes, showed a significant increase after treatment with 4.0% HCS for 2 hours. NRF2 nuclear translocation was determined by calculating the nuclear translocation rate (nuclear NRF2/cytoplasmic NRF2), normalized to β-actin. It was observed that the treatment with 4.0% HCS enhanced NRF2 nuclear translocation. Furthermore, immunostaining to verify the intracellular localization of NRF2 protein demonstrated that treatment with 4.0% HCS localized NRF2 into the nucleus (Fig. 5). These findings suggest that treatment with HCS, a main component of which is MTO, promotes the nuclear translocation of NRF2, leading to an increase in nuclear NRF2 levels and potentially activating the expression of antioxidant responsive genes.

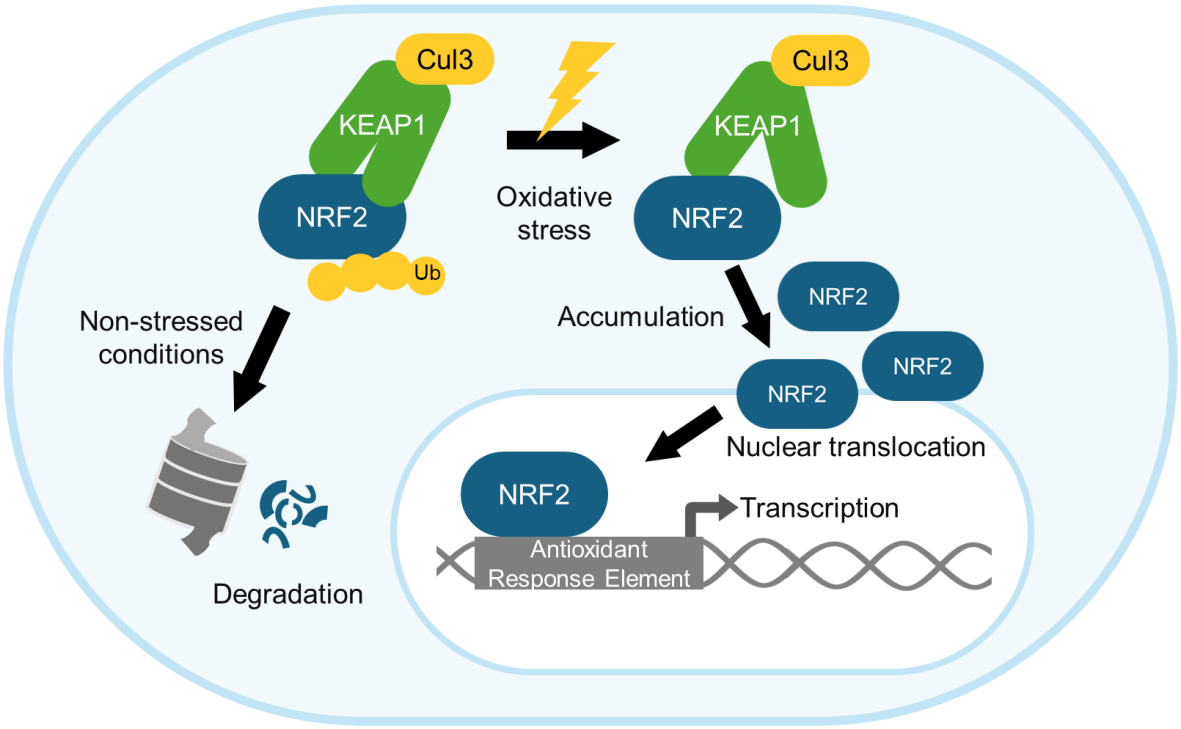

Fig. 3 Oxidative defense system by NRF2

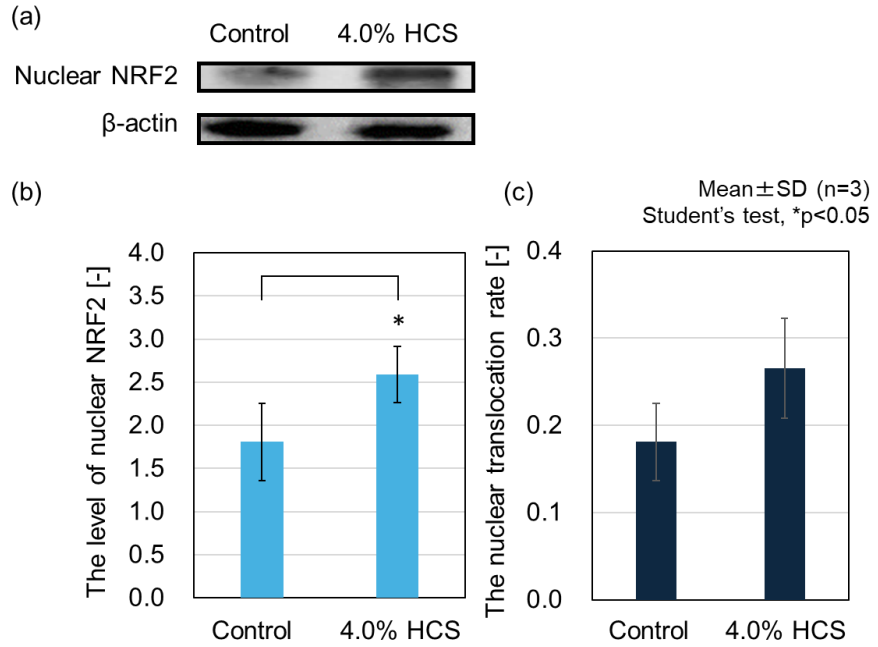

Fig. 4 HCS effect on NRF2 nuclear translocation. (a) The level of nuclear NRF2 protein analyzed by Westen Blotting. (b) The level of nuclear NRF2 was quantified by measuring the intensity of each band normalized to that of β-actin. (c) NRF2 nuclear translocation rate was calculated as the ratio of nuclear NRF2 to cytoplasmic NRF2 normalized to β-actin.

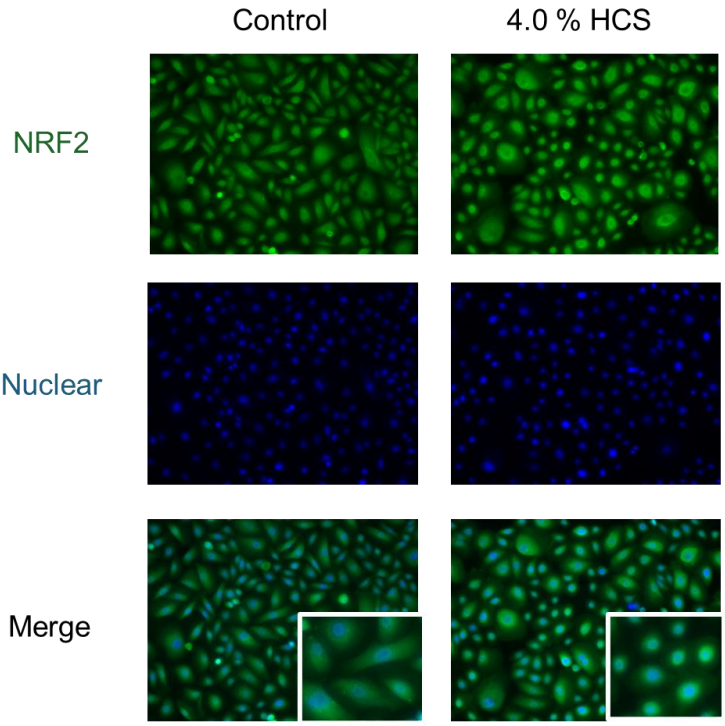

Fig. 5 Intracellular behavior of NRF2 by HCS treatment. The intracellular localization of NRF2 was evaluated by immunoblotting. The merged images of NRF2 fluorescence staining (Alexa Fluor® 488) and nuclear staining (Hoechst) were analyzed to assess the localization.

4. Discussion

The skin is subjected to oxidative stress caused by external stimuli such as solar ultraviolet radiation, infrared and visible light, environmental pollution. Excessive exposure to ROS results in oxidative stress, leading to skin pigmentation and aging, which further causes uneven skin tone, pigmentary disorders, skin roughness, and wrinkles [9, 10]. This study suggests that MTO has the potential to reduce intracellular ROS, indicating MTO may offer promising anti-aging and skin brightening benefits, expanding its application beyond moisturizing and itch relief.

Moreover, some reports suggest that oxidative stress is one of the inducers of sensitive skin development [11]. In particular, scratching due to itching causes pigmentation, suggesting that MTO may not only suppress itching but also inhibit post-scratch pigmentation, thereby providing new value in the treatment of sensitive skin.

Additionally, this study further indicates that MTO might bolster the skin defense against oxidative stress by the expression of antioxidant responsive genes such as HMOX1, GPX2, and NQO1 through NRF2 activation. These genes produce antioxidant-related enzymes that act through different mechanisms to provide sustained defense against oxidative stress [9, 12]. As a result, MTO is expected to offer a prolonged antioxidant effect compared to common antioxidants, such as vitamin C and vitamin E, that neutralize ROS directly through radical reactions. Moreover, NRF2 has been known to suppress inflammation by inhibiting the expression of cytokine genes such as interleukin 6 (IL-6) and interleukin 1β (IL-1β) [13]. Thus, MTO may have multiple beneficial functions in the skin.

5. Conclusion

In this study, we explored the novel function of Maltotetraose (MTO) by examining the intracellular effects of Hydrolyzed Corn Starch (HCS) primarily containing MTO. The results demonstrated that HCS can enhance the expression of antioxidant responsive genes and reduce reactive oxygen species (ROS). Furthermore, we found that HCS promotes the nuclear localization of NRF2. These findings suggest that MTO can activate the antioxidative response through NRF2-mediated transcription activation.

6. References

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