

Effects of Exopolysaccharides on the regulation of FTH1 protein expression and cell death of HaCaT cells under ultraviolet irradiation

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Abstract: Previous research works indicate that natural exopolysaccharide (EPS) produced by lactic acid bacteria *Lactobacillus plantarum* HY7714 might be effective at anti-photoaging and due to its function as antioxidants against reactive oxygen species (ROS). Through regulating the expression level of FTH1, intracellular iron dyshomeostasis induced by UV irradiation might be relieved. This research paper investigates on the regulation of exopolysaccharides on FTH1 expression, ferritin concentration and level of lipid peroxidation in HaCaT cells under UVA+UVB exposure. In our research, we found that HaCaT cell viability is higher when treated with exopolysaccharides, which indicates that exopolysaccharides could effectively improve cell viability under UV radiation. Also, the presence of EPS and FTH1 are shown to decrease lipid peroxidation level and reduce intracellular ferritin concentration, which demonstrates that EPS could upregulate the FTH1 expression level to mediate the ferritin concentration in the HaCaT cells which would further balance iron homeostasis. Ultimately, EPS are proved to decrease cell death under UV radiation through modulating FTH1 protein expression.

1. Introduction

Skin is the largest organ that covers and protects human body, and the health condition of skin could be affected by several factors, including bacteria contact, genetics, allergic triggers and sun exposure [1]. In particular, excessive exposure to ultraviolet (UV) light might cause serious and irreversible effects to skin cells. Since UV irradiation could cause skin cancer and photoaging in extreme cases, it is critically important to protect skin from UV exposure [7]. The most popular ways of protecting skin from the Sun are staying under an umbrella, wearing long-sleeved shirts, covering faces with hats, and using sunscreen or sunblock products [9]. Even though clothing might be considered as the most effective form of sun protection, sunscreen products offer protection against UV radiation at certain skin surfaces that could not be typically covered by clothing, such as faces, calves and hands. Therefore, investigating in improving the sunscreen is significant to human skin health. Different sunscreen products can protect skin from different types of UV radiation [8]. Typically, the UV irradiation could have three subdivisions: UVA, UVB and UVC. Nevertheless, because ozone in the atmosphere absorbs UVC, surrounding solar light is mostly UVA and UVB, which makes up of 90-95% and 5-10% of UV irradiation respectively [2]. More specifically, the effects on skin cells caused by UVA and UVB differed in the depth of penetration and location of effects. UVA with a relatively longer wavelength could penetrate deeply into

the dermis, while UVB is pretty much entirely absorbed by the epidermis (a shallower layer of skin than dermis). UVA resulted in excessive production of reactive oxygen species (ROS) whereas UVB had more influence on DNA, the molecular level [6].

Recent research conducted at the R&BD Center demonstrates that exopolysaccharide (EPS) produced by lactic acid bacteria *Lactobacillus plantarum* HY7714 might be able to slow photoaging processes and have potential against UVB-induced damages to skin cells. However, mechanisms of how EPS and other probiotics protect skin cells are not clarified yet [3]. It is now being showed that exopolysaccharides produced by lactic acid bacteria could be utilized as effective antioxidant defenses which are essential for maintaining redox homeostasis through neutralizing excessive ROS that are likely to be generated by UV irradiation [5][6].

Photoaging and many skin diseases are potentially caused by ferroptosis, which is predominantly affected by lipid peroxidation damages and iron accumulation [4]. In cellular iron regulation pathways, the concentration of ferritin present in the cell is significantly important for the occurrence of ferroptosis. Recent research presents that redundant ferritin in cells might lead to ferroptosis. The amount of ferritin in cells is related to the iron-storage protein, includes ferritin heavy chain (FTH1). Limited amounts of FTH1 in the cell will result in excess release of ferritin which may eventually lead to ferroptosis [10]. Hence, the expression of FTH1 is critical for photoaging and other skin cell diseases. Based on former researches, EPS is likely to regulate FTH1 expression and thus

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influence ferritin concentration and lipid peroxidation in cells, which further limit the skin aging and ferroptosis effects brought by UVA+UVB irradiation [3].

2. Methods and Materials

Cellular photoaging modeling: Photoaging Modeling Cells were grown to 30%-50% confluence, and the culture medium was removed. In the control group and modeling group, PBS was added, and in the polysaccharide treatment group, PBS and polysaccharides were added to achieve a final polysaccharide concentration of 100µg/mL. For 6-well plates, the solution volume per well was 1mL, and for 96-well plates, the solution volume per well was 50µL. After changing the medium, cells were irradiated with UV (UVB+UVA, 2mW/cm²) for 3-4 hours. During the irradiation process, the control group was shielded with aluminum foil. MTT Assay for Cell Viability After UV irradiation, the solution in the 96-well plate was removed, and after washing with PBS twice, 100µL of MTT culture medium (5mg/mL MTT mixed with culture medium at a ratio of 1:9) was added to each well. The plate was then incubated at 37°C in the dark for 4-6 hours. After removing the supernatant, 150µL of DMSO was added to each well, and the absorbance was measured at 570nm after shaking for 10 minutes.

Cell Protein Extraction: Use ProteoPrep® Protein Extraction Kit to extract total protein.

BCA Assay Protein Measurement: Follow the Pierce™ BCA Protein Assay Kit handling instructions to detect and quantify protein.

Western Blot: GAPDH and FTH1 Primary Antibody, and Goat anti-Rabbit IgG (H+L) Secondary Antibody are used to detect FTH1 protein expression level.

Cell Lipid Peroxidation Fluorescence Staining and Ferrous Ion Measurement: Measurement of Intracellular Iron Ion Content After UV irradiation: after UV irradiation, discard the liquid from the 6-well plates, wash with PBS twice, and then digest and collect the cells. Use C11 BODIPY 581/591 Lipid Peroxidation Sensor to measure the cell lipid peroxidation degree.

siRNA Transfection Experiment: Observe the growth status of cells in a 6-well plate. When cell confluence reaches 30-60%, begin the siRNA transfection experiment. **Transfection Reagent Preparation:** Prepare two centrifuge tubes. Add 125µL of serum-free culture medium to each tube. In one tube, add 5µL of Lipo6000, and in the other tube, add 5µL of 20µM siRNA-FTH1. Let them sit at room temperature for 5 minutes, then mix the contents of both tubes and let them sit for an additional 5 minutes. Before transfection, discard the culture medium from the 6-well plate containing the cells. Wash the cells twice with PBS. Add 2mL of serum-free culture medium to each well, followed by the addition of 250µL of the prepared transfection reagent. Gently mix and incubate at 37°C for 6 hours. Afterward, replace the medium with fresh culture medium containing serum and antibiotics. Validate the knockdown effect through a Western blot experiment after 48-72 hours. (For a 96-well plate, a similar procedure is followed with proportionate addition of the transfection

reagent.) **Control Group:** Serum-free culture medium without transfection reagents.

3. Results and Discussion

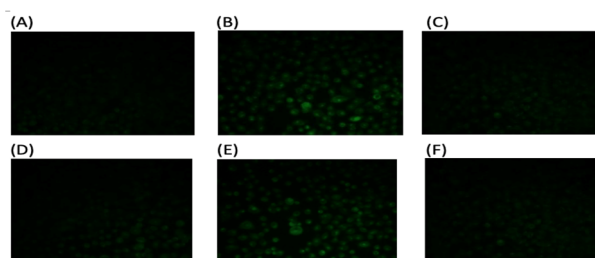


Figure 1. Detection of lipid peroxidation levels in the control group, model group, and polysaccharide group with or without FTH1 silencing (A and D: Control group with no UV irradiation or exopolysaccharide treatment, B and E: Model group with UV irradiation but no exopolysaccharide treatment, C and F: Exopolysaccharide group with both UV irradiation and exopolysaccharide treatment. A, B and C groups do not have FTH1 gene silencing, whereas D, E and F groups have FTH1 gene silencing).

Based on the data from Figure 1, in terms of inter-group comparisons, the group with FTH1 silencing generally has higher lipid peroxidation levels compared to the non-silenced groups. Specifically, the silenced groups exhibit more and brighter green fluorescence. This is because FTH1 has a function in storing iron ions. If FTH1 is silenced, it cannot store iron ions, and the iron ions originally stored in FTH1 will be released into the cell, leading to an increase in the cell's iron ion concentration. For intra-group comparisons, both the silenced and non-silenced groups within the control group show the lowest lipid peroxidation levels, characterized by the least and dimmest green fluorescence. The model group exhibits the highest lipid peroxidation levels. In the polysaccharide group, the lipid peroxidation levels are higher than in the control group but much lower than in the model group. This is because the control group did not undergo UV irradiation, resulting in lower lipid peroxidation levels. The polysaccharide group, while subjected to UV irradiation, also received protective polysaccharide treatment, leading to lower lipid peroxidation levels compared to the model group, which experienced UV irradiation without polysaccharide protection.

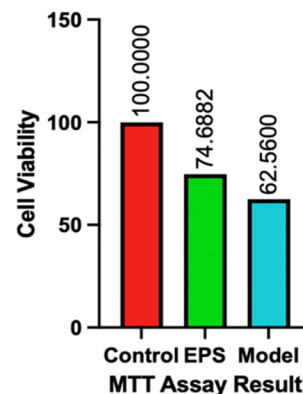


Figure 2. Standardized and simplified data

Table 1. Dataset of Figure 2

C	C	C	M	M	M	M	M	E	E
0.2711	0.2612	0.2646	0.2404	0.1899	0.1648	0.146	0.1455	0.174	0.1653
0.2986	0.2786	0.2398	0.1925	0.1841	0.1715	0.1514	0.2385	0.1692	0.1661
0.2848	0.2656	0.2417	0.1984	0.1842	0.1742	0.1591	0.1657	0.1876	0.1655
0.2929	0.2831	0.2773	0.2459	0.1999	0.169	0.1659	0.1664	0.1834	0.1714
0.2788	0.2585	0.2722	0.2362	0.2042	0.1686	0.1656	1613	0.17	0.1716
0.2203	0.2809	0.2482	0.2144	0.2	0.1713	0.1606	0.1543	0.1635	0.1624

Figure 3 (Standardized and simplified data of Fe²⁺ Concentration in 6 groups of HaCaT cells. 1st. C group without FTH1 silencing, 2nd. C group with FTH1 silencing, 3rd. M group without FTH1 silencing, 4th. M group with FTH1 silencing, 5th. E group without FTH1 silencing, 6th E group with FTH1 silencing):

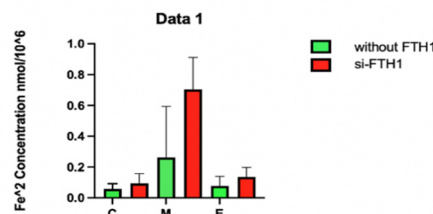


Figure 3. The Fe² Concentration (nmol/10⁶)

Table 2. Dataset of Figure 3.

C	1.025521	0.158083	0.473515	0.631231	0.867805
M	0.828376	4.626703	0.052939	6.138148	0.210655
E	2.799826	0.302656	1.603813	1.078093	0.27637
siC	0.460372	0.749518	3.838123	0.14494	-0.052205
siM	3.56212	0.250084	4.271842	2.313535	1.012378
siE	1.06495	0.80209	1.485526	0.841519	0.315799
C	0.142433472	0.02195597	0.06576597	0.08767097	0.12052847
M	0.115052222	0.64259764	0.00735264	0.85252056	0.02925764
E	0.388864722	0.04203556	0.22275181	0.14973514	0.03838472
siC	0.095910833	0.15614958	0.79960896	0.03019583	-0.010876
siM	0.742108333	0.05210083	0.88996708	0.48198646	0.21091208
siE	0.221864583	0.16710208	0.30948458	0.17531646	0.06579146
C	84.89034944	13.0857594	39.1965194	52.2518994	71.8349694
M	37.62207667	210.129428	2.40431292	278.774222	9.56724792
E	46.66376667	5.04426667	26.7302167	17.9682167	4.60616667
siC	5.115244444	8.32797778	42.6458111	1.61044444	-0.5800556
siM	39.57911111	2.77871111	47.4649111	25.7059444	11.2486444
siE	11.83277778	8.91211111	16.5058444	9.35021111	3.50887778

Based on figure 3 and table 2, In terms of inter-group comparisons, overall, the group with FTH1 silencing has higher iron ion concentrations compared to the non-silenced groups. For example, the FTH1-silenced control group has a concentration 0.05 nmol/10⁶ higher than the non-silenced control group, the FTH1-silenced model group has a concentration 0.45 nmol/10⁶ higher than the non-silenced model group, and the FTH1-silenced polysaccharide group has a concentration 0.08 nmol/10⁶ higher than the non-silenced polysaccharide group.

Based on the data in Table 2, for intragroup comparisons, both the FTH1-silenced and non-silenced groups have the lowest iron ion concentrations in the control group, with concentrations of 0.05 nmol/10⁶ and 0.1 nmol/10⁶, respectively. The model group has the highest concentration, with concentrations of 0.25 nmol/10⁶ and 0.7 nmol/10⁶ for silenced and non-silenced groups, respectively. However, the iron ion concentration in the polysaccharide group is lower than

the model group but higher than the control group. For example, the FTH1-silenced polysaccharide group has a concentration 0.6 nmol/10⁶ lower than the FTH1-silenced model group, and the non-silenced polysaccharide group has a concentration 0.2 nmol/10⁶ lower than the non-silenced model group. This is consistent with the results of other studies (Hou W, 2016).

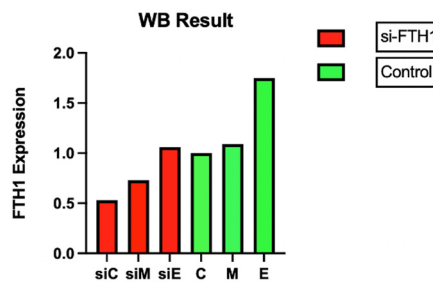


Figure 4. The FTH1 expression level through Western Blot testing

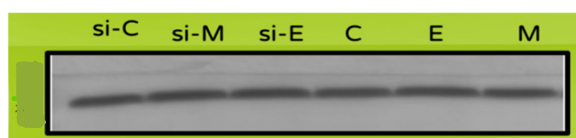


Figure 5. WB result of In-house reference GADPH

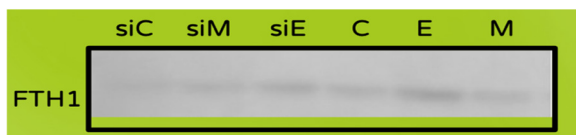


Figure 6. WB result of FTH1 expression level with and without silencing

According to figure 2 and table 1, the MTT assay results showed that the control group had the highest cell viability. The model group had the lowest cell viability, with only 62.56% of the control group. The polysaccharide group had lower cell viability than the control group but higher than the model group by 12.2%, reaching 74.69%. The highest cell viability in the control group can be attributed to the fact that UVA and UVB ultraviolet irradiation can cause cell death, while the control group was not subjected to ultraviolet irradiation, resulting in higher cell survival rates. The lowest cell viability in the model group is because the cells in the model group were exposed to 6 hours of UVA and UVB irradiation without polysaccharide treatment. Therefore, the cells in the model group were neither protected by extracellular polysaccharides nor exposed to UV irradiation, resulting in the lowest cell viability. The higher cell viability in the polysaccharide group can be attributed to the fact that polysaccharides can mitigate the oxidative damage caused by UV irradiation, which is consistent with the results from other studies (Lin & Baek, 2020).

From figure 4, In terms of inter-group comparisons, the groups with FTH1 silencing generally have lower FTH1 protein expression levels compared to the non-silenced groups. For example, the FTH1-silenced control group has a 0.5 decrease compared to the non-silenced control group, the FTH1-silenced model group has a 0.6 decrease compared to the non-silenced model group, and the FTH1-silenced polysaccharide group has a nearly 1 decrease compared to the non-silenced polysaccharide group. This indicates the successful silencing of FTH1. For intra-group comparisons, the control group has the lowest FTH1 protein expression levels, with values of only 0.5 and 1 for silenced and non-silenced groups, respectively. The model group follows, but the FTH1 protein expression levels in the polysaccharide group are the highest. In the non-silenced polysaccharide group, the FTH1 expression level reaches 1.75. This may be due to the fact that there are multiple proteins involved in regulating iron ions, not just FTH1. Therefore, the increase in iron ion concentration is not solely caused by the downregulation of FTH1, but the decrease in iron ion concentration is regulated by the upregulation of exopolysaccharides. This phenomenon can be explained by figures from other literature [6].

4. Conclusion

The results of the MTT Assay Result indicate that exopolysaccharide treatment can enhance cell viability under UVA+UVB irradiation, thereby repairing cellular function. The inter-group comparison results of the experiment on iron ion concentration indicate that the downregulation of FTH1 increases the concentration of iron ions inside the cells. The intra-group comparison results indicate that exopolysaccharides upregulate FTH1, thereby reducing the concentration of iron ions inside the cells. This aligns with research findings from other studies. Based on the result shown in Figure 5 and Figure 6, the Western Blot (WB) results show that the exopolysaccharide group has the highest FTH1 protein expression level. This may be because there are multiple proteins involved in regulating iron ions, not just FTH1. Therefore, the increase in iron ion concentration is not solely caused by the downregulation of FTH1, but the decrease in iron ion concentration is regulated by the upregulation of exopolysaccharides, consistent with descriptions in other literature. In summary, exopolysaccharides can be effectively used in sunscreens and cosmetics to repair cell viability under UV radiation. Additionally, exopolysaccharides can upregulate FTH1, reducing the concentration of iron ions inside the cells and minimizing cell death caused by UV radiation.

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