学 位 論 文 の 要 旨

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学 位 論 文 題 名

Inhibitory effect of grape skin extracts on DNA damage caused by UV irradiation and development of cosmetics using the waste skin

(紫外線照射による DNA 損傷に対するブドウの皮の抽出物の抑制効果および廃棄果皮を用いた化粧品の開発)

Skin as the first protective barrier of people, has received the damage caused by ultraviolet (UV) radiation which is one of the most serious environment factors. Since absorption of UV light can bring the subsequent activation of factors, for instance, activated tumor suppressor factor P53 by UV leads to transition of C to T and CC to TT. These transitions produce two main types of DNA damage such as cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4PP). CPDs produced by UV have been recognized as a molecular causation for photocarcinogenesis. On the other hand, it is thought that the cause of cancer is an increase in reactive oxygen species (ROS). ROS has been often launched after expose to UV radiation, and increased ROS activated the intricate signaling pathways for apoptosis. In short, cytochrome c regulated by the expression of Bcl-2 protein family was released from the mitochondria by UV stimulation, consequent activated caspases, and further induced cell apoptosis.

In recent years, polyphenols have attracted attention as substances that suppress the production of ROS. Polyphenols was considered to indicate benefit effects of cardio-protective, anti-cancer, anti-diabetic, anti-Aging. Large amounts of polyphenols naturally consist in food, fruits, plants, and they maintain their natural characteristics during extraction follow with reduce the concern of side effects. Lot of attention is paid to the fruit, especially grape extract being one of them. Several studies demonstrated that grape extracts are a protective agent in skin damage induced by UV radiation on human keratinocytes. Grape extract were able to repair UV-induced CPDs in cell. In addition, it was indicated that grape extract inhibited experimental photocarcinogenesis *in vitro*, and grape extract reduced the probability of human skin cancer.

In the winemaking process a heavy load of byproducts and wastes are generate. Main wastes are grape pomaces and recommendation on effective use of its constituent (skin, seeds and pulp) are urgently needed to resolve the economic and environmental concerns. In generally, grape pomaces are given to feed for animal or stored for later use as compost. However, some of them finally rots away. Only minimum amounts of these wastes can be used for feed and compost. Therefore, the high content of polyphenols in grape pomaces will be expected to be consequently antioxidant advantage for human body which has been reported earlier. Half of the material weight of grape pomaces is grape skin, which has richly anthocyanin involved and famous for their beneficial property. However, most studies were explored in regard to bioactive compounds of grape such as

resveratrol, flavonoids, quercetin, and procyanidines and fewer topics were evaluation of the grape skin itself. In this study investigate optimal extraction condition of grape skins from winemaking press process, assess the effect of the extracts on intracellular damage induced by UV radiation in human keratinocyte, and then develop grape skin extract (GSE)'s practical application (Chapter 1).

This research was performed to confirm the damage caused by UV irradiation in NHEK cells inhibited by grape skin extract of wine-making residue, its potential mechanism, and exploring biomedical applications. Special emphasis was given to confirming the condition of the GSE extraction by evaluating the level of cyclobutane dimer generation, which is caused by DNA damage induced by different UV-irradiation wavelengths. Therefore, it was assumed that GSE from wine-making residue might have antioxidant capabilities similar to grape extract. In the Chapter 2, the toxicity of GSE (Zweigelt and Niagara) and the inhibitory effect of GSE in CPD generation via triggered DNA mutation in 2 different wavelengths of UV radiation (250 and 290 nm) was investigated. NHEK cells, as a human model cell line, were used in this study to further consider its applicability. Results showed the apparent decrease of CPD formation 1 h before and after treatment with two kinds of GSE by ELISA. However, there was no obvious difference in cell viability. This clarified the protective capability of GSE in cell survival against DNA damage caused by UV light, which means that the hypothesis was put forward early on was confirmed. However, a different result occurred in the 24 h case. More information is required to clearly understand the mechanism or relative factor of GSE's positive effect. Longevity gene Sirt 1 level was used as another index for reconfirmation. The data showed that GSE has a downwards sloping effect on the longevity gene Sirt 1 level (Fig. 1).

Hence, Chapter-3 discussed several factors of GSE involved in regulating its beneficial effects. They were the grape variety, extract condition, added timing, and mechanism. By analysis of data, 10 mJ/cm^2 at 250 nm, 10 mJ/cm^2 at 270 nm, 15 mJ/cm^2 at 290 nm, and 500 mJ/cm^2 at 310 nm equally provided energy to the NHEK cell by evaluating the amount of CPD formation. This condition was employed in subsequent experiments to clarify the beneficial effect of GSE on DNA damage caused by UV irradiation. The results showed that GSE had the best performance in the cell 1 h before or after treatment with UV radiation at 250 nm (10 mJ/cm^2) via examination of cell viability and CPD amounts. Then, extract conditions such as solvent, the concentration of solvent, temperature, and time were set, and the next step was to emphasize factors related to the final protective effect of GSE to avoid damage caused by UV light. In addition, no proof exists that states, RGZ (red grape Zweigelt) skin extract plays a stronger role than WGN (white grape Niagara) in mitigating negative effects of UV exposure, and that the most efficient condition in lessening CPD formation was RGZ extract with 80% ethanol, for 2 h at 60 ° C (Condition no. 2). CPD, which has been commonly regarded as the major photoproduct, and 6-4 PP, which is another photoproduct generated by DNA damage, should be investigated. The data indicate that GSE was more preventive (1 h before irradiation) than reparative (1 h after irradiation). Paired with GSE- HPLC data analysis, the inhibition of photoproduct formation was not attributed to resveratrol nor anthocyanin alone, but to a variety of compounds such as quercetin and proanthocyanidins. Such compounds have been found in the grape skin through different extraction conditions, and have been implicated in possessing anti-cancer activity. In addition, to clarify the motivational mechanism of GSE in cells after damage occurred through exposure to UV. The Bax/Bcl-2 ratio and cytochrome c levels were evaluated because the combination of these genes is an important standard that could direct the cell towards apoptosis or survival by influencing membrane permeability. Increasing Bcl-2 expression

and decreasing Bax expression leads to the downregulation of membrane permeability, which further prohibits cytochrome c release. It is very likely that apoptosis is stopped by GSE and results in cell survival, which is consistent with the cell viability data in the beginning. (Fig. 2)

Plants rich in antioxidants, such as grape, garner more attention as cosmetic ingredients, since they are known for their anti-inflammatory, anti-aging, and protective effects on the skin. It more actively used in cosmeceuticals owing to its characteristics, natural background, and biological activities. In chapter-4, this study stepped into developing GSE for application in cosmetic ingredients. GSE collected from wine-making factories was evaluated to be more refined. During the pressing process, GSE was collected from 0, 5, 8, and 10 days after pressing, and GEE was collected as a comparative group and were evaluated together. GEE showed better effects after pressing (Day 5), while GSE directly collected after pressing (Day 0) had the strongest ability to inhibit CPD formation and was better than GEE (Day 5). The CPD levels of GSE and GEE did not decrease as time increased. The expression level of Sirt 1 was measured and showed an increasing trend in GSE compared to the control group, but was lower than GEE. This supports that GSE from wine-making residue is indeed a practical, cheap resource against DNA damage induced by UV. Different concentrations of GSE were compared to clarify whether GSE from wine-making residues has benefits to the people, and is worth recycling to produce cosmetic face cream, results it indicated that the stock lotion of GSE was the best in reducing damage from UV. In addition, GSE has anti-UV capabilities similar to currently sold cosmetic products such as base cream and more than of peach lotion, which was sold as a kind of face water (Fig. 3).

Finally, a text cream using GSE from wine-making residue was manufactured (Fig. 4). For the preparation of the cream, the aqueous phase (*Vitis vinifera* (grape) peel extract, *Waltheria indica* leaf extract, Ferulic acid, Sodium gluconate, Artichoke extract, Hydrolyzed *Prunus domestica*, *Angelica acutiloba* root extract, Cnidium Officinale Rhizome Extract, Betula Platyphylla Japonica Juice, Arginine, Flavor) was formulated by adding hot water (75 ° C) until it was completely dissolved. At the same time, the oil phase (Propanediol, Olive oil, Shea butter, Glycerin, *Argania spinosa* kernel oil, Hydrogenated rapeseed oil alcohol, Cetearyl olivate, and Sorbitan olivate) was heated up to 75 °C. After that, the aqueous phase was slowly decanted into the oil phase (until wholly dissolved), and the mixture was kept under continuous stirring at room temperature. Caprylyl Glycol was added as a preservative when the mixture reached 40 °C. Then, Dextrin and Carbomer were added as thickeners during the cooling process. The pH was adjusted to neutral using Citric Acid and Sodium Citrate. Finally, the text cream was obtained.

In conclusion, in this research, GSE used in cosmetics acts as an inhibitor against DNA damage induced by UV radiation. It performed well in inhibiting photoproduct formation stimulated by DNA mutation, which was influenced by the effect of several compounds in GSE through the mitochondrial pathway of the cell. Finally, GSE prevents cell damage caused by UV and might be a natural cosmetic resource that is cheap and beneficial to the environment.

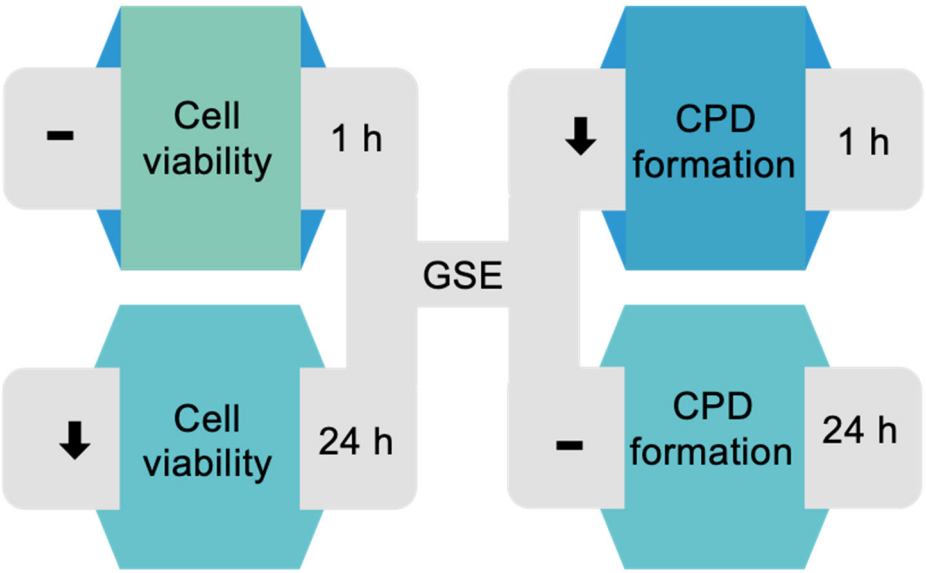

Fig. 1 Summary of results in chapter 2. "-" denote no significant change, " ↓ "denote significant difference compared to the NHEK cells added with EtOH.

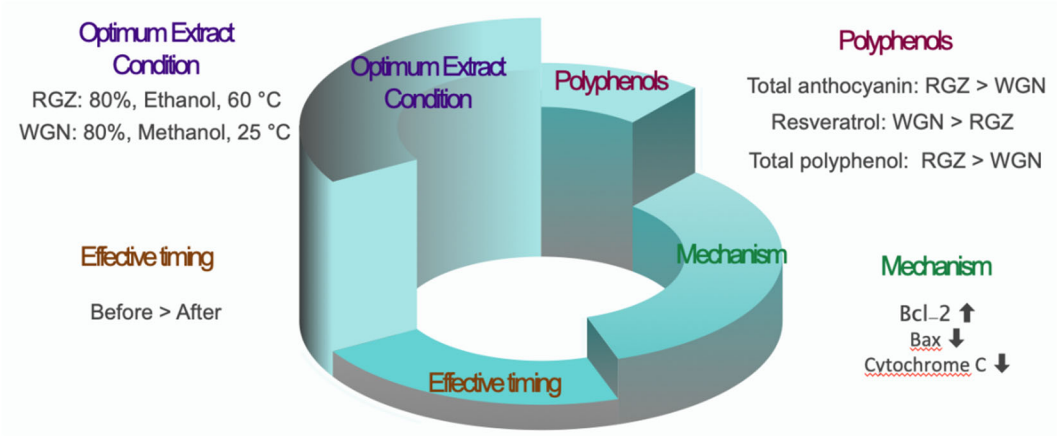

Fig. 2 Summary of results in chapter 3. " ↓ "denote significant difference compared to the NHEK cells added with EtOH.

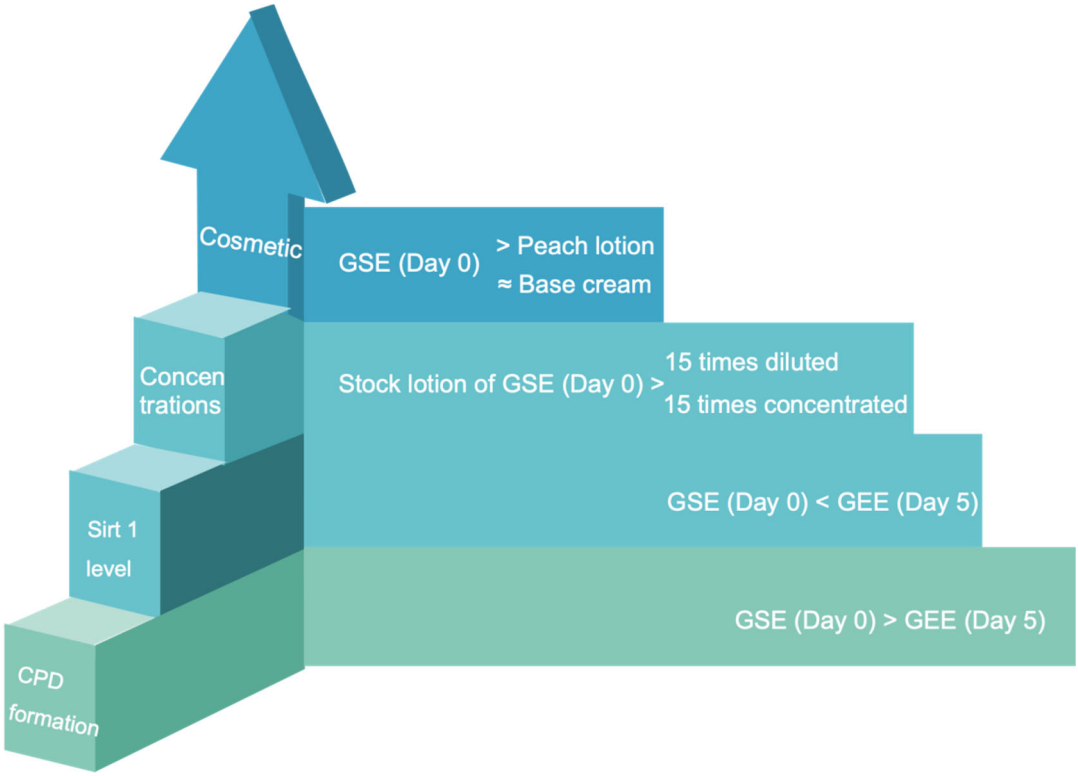

Fig.3　Summary of results in Chapter 4.

Fig.4 Picture of test specimen cream with GSE (Day 0)
(Extracted condition: 80% ethanol, for 2 h at 60 ˚ C).