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인간 진피모유두세포에서 서리태 검은콩 추출물의 항세포사멸 효과

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Anti-apoptotic Effect of Seoritae Extract on Human Follicle Dermal Papilla Cell

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ABSTRACT

Background: This study investigated the anti-apoptotic effects of an extract from seoritae, a black soybean cultivar, on human follicle dermal papilla cells (HFDPC) and assessed the underlying mechanisms to evaluate its potential as a cosmetic raw material.
Methods and Results: The antioxidant effects of the seoritae extract were evaluated using a 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity assay. In HFDPCs, the results of tests evaluating the enhancement of cell proliferation and protection against apoptosis induced by hydrogen peroxide or dihydrotestosterone (DHT) revealed that seoritae extract exhibited a skin-protective effect. The levels of β-catenin and Bax proteins, which are involved in cell survival and death, were measured to assess the underlying signaling mechanisms that enabled the extract to protect HFDPCs against DHT activity.
Conclusions: Our results collectively indicate that seoritae extract can enhance HFDPC proliferation and inhibit cell apoptosis, thus it has the potential to be developed as a cosmetic ingredient with a potential hair loss-alleviating effect on HFDPCs.

Key Words: Antioxidant, Anti-apoptosis, Human Follicle Dermal Papilla Cells, Proliferation, Seoritae Black Soybean

INTRODUCTION

Hair is a specialized derivative structure of skin and a defining characteristic of the human integumentary system. The hair root is buried inside the epidermis and enclosed within a hair follicle, which comprises epithelial components (the matrix and outer-root sheath) and dermal components (the dermal papilla and connective tissue sheath) (Rogers, 2003).

The dermal papilla (DP) is located at the base of the hair follicle and functions as a primary mesenchymal component of hair. It plays pivotal roles in the hair cycle by inducing the production of new hair follicles and helping maintain hair growth (Rho *et al.*, 2005).

DP cells (DPC) are a rich source of nutrients and secrete multiple growth factors that support the proliferation and growth of neighboring keratinocytes (Stenn and Paus, 2001).

The causes of alopecia (hair loss) include aging, nutritional deficiency, hormone imbalances, disease, and stress. Reactive oxygen species (ROS) are a key etiological factor for the senescence of DPC. Under stressful environmental conditions (e.g., ionizing radiation, ultraviolet (UV) radiation, drugs, and smoking), the levels of ROS, including hydrogen peroxide (H_2O_2), are significantly increased (Birben *et al.*, 2012).

Accumulating evidence supports the hypothesis that oxidative

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stress caused by H_2O_2 is a key factor in the onset and progression of hair loss, which is known as alopecia (Bahta *et al.,* 2008; Naito *et al.,* 2008; Trüeb, 2009; Seo *et al.*, 2012). In addition, dihydrotestosterone (DHT) is also known to inhibit human hair follicle proliferation and induce apoptosis in HFDPC (Winiarska *et al.,* 2006; Kang *et al.*, 2015; Jain *et al.*, 2016).

DHT inhibits hair growth by inhibiting insulin-like growth factor-I production in dermal papillae and suppresses matrix cell proliferation and promotes regression of hair follicles in mice (Zhao *et al*, 2011; Kwack *et al*., 2012).

5α-reductases irreversibly reduce testosterone to the more potent DHT. Thus, 5α-reductase inhibitors are useful treatments for DHT-dependent diseases, including androgenic alopecia, hair growth and acne (Srivilai *et al*., 2019).

Finasteride and minoxidil have been approved for treatment of hair loss by the US Food and Drug Administration (FDA). However, due to their limited efficacy and side effects, the development of natural (and therefore less toxic) hair growth promoters is an unmet need of the hour. Natural agents may promote hair growth by stimulating the proliferation of DPC and keratinocytes (Yoo *et al.,* 2007; Kang *et al.,* 2013; Jain *et al.*, 2016; Dhariwala and Ravikumar, 2019).

DPC strongly express β-catenin, an anti-apoptotic protein that may prevent apoptosis in these cells under physiological conditions. However, relatively little is known about how androgens influence the cell death and regulation of pro-apoptotic (Bax), anti-apoptotic (β-catenin), and caspase proteins in human DPC (Winiarska *et al.,* 2006; Bak *et al*., 2020).

Black beans, which are a common plant-derived food item are rich in antioxidant components, anthocyanins, and isoflavones, which can help prevent the skin effects of aging (Fonseca *et al.*, 2021). Furthermore, black bean has been reported to possess hair growth-promoting potential (Jeon, 2011). Moreover, the skin-benefiting effects of black bean extract has been continuously studied.

Seoritae is a kind of black soybean (Fig. 1A), which is harvested after the late autumn frost and has various physiological properties, such as anti-inflammatory and antioxidant properties (Min, 2009; Im *et al*., 2006; Rhim, 2020). However, the efficacy by which seoritae promotes hair growth and protects against cytotoxicity about have not been researched in detail. For this reason, we studied the effects of seoritae extract on *in vitro* cell proliferation, its protective effect against oxidative damage, and the potential involvement of β-catenin signaling in HFDPC.

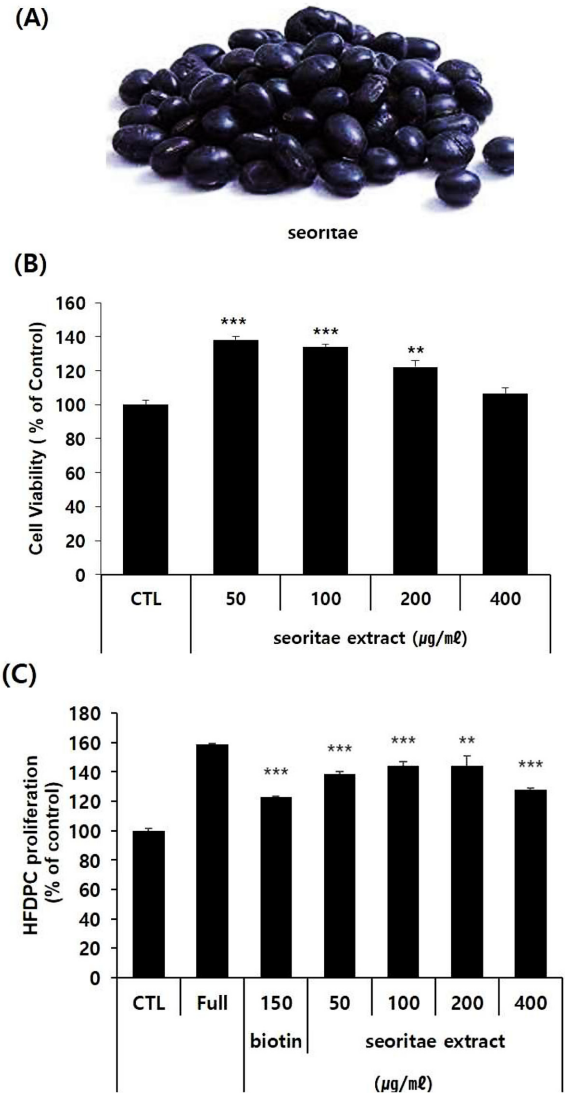

Fig. 1. Effects of seoritae extract on cytotoxicity and proliferation of HFDPC. (A) Photograph of seoritae. (B) Viability of HFDPC after exposure to the indicated doses of the seoritae extract. HFDPC were treated with the indicated concentrations of seoritae extract for 24 h and estimated using a colorimetric WST-1 assay kit. (C) HFDPC were treated with the indicated concentrations of seoritae extract for 72 h and cell proliferation was estimated using a colorimetric WST-1 assay kit. The results showed the percentage compared to control group (Means ± SD). CTL and treated group: 20% supplement, Full; 100% supplement, Positive control; biotin, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control (CTL).

Ultimately, our results collectively suggest that seoritae extract regulates cell proliferation and apoptosis through β-catenin and Bax proteins in HFDPC, and thus may have promise as a potential naturally derived hair loss-preventing material.

MATERIAL AND METHOD

1. Preparing the seoritae extract and materials

Seoritae was mixed with 70% ethanol in a ratio of 1 to 10 and extracted at room temperature for 24 h. The resulting extract was filtered through filter paper (AF-31H, HM, Seoul, Korea), and then concentrated in a rotary vacuum concentrator (EYELA N-2100, Tokyo Rikakikai Co., Tokyo, Japan).

Seoritae powder was dissolved in DMSO for the cell-based experiments. DHT, H_2O_2, and biotin were purchased from Sigma-Aldrich (St Louis, MO, USA). Human follicle dermal papilla cells (HFDPC) were purchased from PromoCell (C-12071, Heidelberg, Germany).

2. Cell culture and evaluation of cell viability

2.1. Cell culture

Cultured HFDPC (early-passage cells) were used for all experiments. HFDPC were grown in follicle dermal papilla cell growth medium (C-26501, Promocell, Heidelberg, Germany), supplemented with supplement mix (C-39625, Promocell, Heidelberg, Germany) and 1% penicillin-streptomycin (Lonza, Basel, Switzerland) in a T75 culture flask (SPL Life Science, Pocheon, Korea). Cells were cultured in a cell incubator (BB15 CO_2 Incubator, Thermo Fisher Scientific, Waltham, MA, USA) maintained at 37°C, 5% CO_2 conditions. When cell density at least 80% in the flask, cell sub-culture was performed.

2.2. Cell viability (WST-1 assay)

Cell viability was determined using the WST-1 assay (EZ-Cytox, DoGenBio, Seoul, Korea) according to the manufacturer's instructions. Briefly, cells were seeded in 96-well plates and cultured for 24 h.

Test samples were added at different concentrations, the cells were incubated for 24 h, 100 ㎕ volumes containing medium and 10 ㎕ WST (water soluble tetrazolium salt) solution were added to each well, and the plates were incubated for 1 h at 37℃.

The absorbance of each well at 450 ㎚ was measured using a microplate reader (Multiskan GO, Thermo-Fisher Scientific Inc., Waltham, MA, USA). Cell viability was determined by the equation: Cell viability (%) = (sample treatment group absorbance /control group absorbance) × 100.

3. Cell proliferation assay

Cell proliferation was assessed using the WST-1 assay (EZ-Cytox, DoGenBio, Seoul, Korea) according to the manufacturer's instructions. Briefly, WST-1 analysis was performed as described above. Unlike generally cell viability assays, cells were incubated for 72 h after treatment with test samples (50 ㎍/㎖ - 400 ㎍/㎖) and positive control biotin (150 ㎍/㎖) cell for proliferation assays.

4. Ability of seoritae extract to suppress DHT- or H_2O_2-induced apoptosis

To assess the ability of seoritae extract to protect HFDPC against DHT- or H_2O_2-induced apoptosis, we dispensed HFDPC to 96-well plates (0.6×10^4 cells in 100 ㎕/well), treated each well with 200 μM DHT or 400 μM H_2O_2 with various concentrations of seoritae extract (3.125 ㎍/㎖ - 50 ㎍/㎖), and incubated the plates for 24 h.

The supernatant was removed, WST-1 reagent was added to a final concentration of 10%, and the plate was further incubated at 37℃ with 5% CO_2. After 1 h, the absorbance was measured at 450 ㎚ using a microplate reader.

5. Western blot analysis

Cells in culture dishes were directly lysed with RIPA buffer (Thermo Fisher Scientific, MA, USA) supplemented with a proteinase and phosphatase inhibitor cocktail mixture (Thermo Fisher Scientific, Waltham, MA, USA). Cell lysates (30 ㎍) were resolved by 4% - 12% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Invitrogen, Waltham, MA, USA).

The blots were washed with Tris-buffered solution containing Tween-20 [TBST; 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween-20], blocked with 5% skimmed milk in TBST for 1 h at room temperature, and incubated for 12 h at 4℃ with primary antibodies against Bax, β-actin, and β-catenin (1 : 1000 dilutions, Cell Signaling Technology Inc., Danvers, MA, USA).

The membranes were washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (1 : 10,000 dilutions, Cell Signaling Technology Inc., Danvers, MA, USA) for 1 h at room temperature.

The bands were visualized using an enhanced chemiluminescence detection system (GE Healthcare, ORD, USA) according to the manufacturer's protocols.

6. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay

A DPPH discoloration assay was carried out to examine the

scavenging effect of the seoritae extract or ascorbic acid (positive control). Briefly, the seoritae extract or ascorbic acid and 1 mM DPPH solution (Sigma Aldrich, St Louis, MO, USA) were mixed at a ratio of 4 : 1 and incubated at room temperature for 30 min.

The absorbance of each sample at 517 ㎚ was measured by spectrophotometry. The DPPH-scavenging effect was expressed as percent inhibition, as follows: (%) = [(A0-A1)/A0] × 100, where A0 is the absorbance of DPPH and A1 is the absorbance of the test material.

7. Statistical analysis

All analytical values were obtained by repeating 3 times and expressed as mean and standard deviation (Means ± SD). Comparisons between samples were statistically processed based on Student's *t*-test *via* the Microsoft Office (Raymond, WA, USA) Excel T-TEST function and determined to be significant if the *p*-value was less than 0.05 ($p < 0.05$).

RESULTS AND DISCUSSION

1. Cytotoxicity of seoritae extract against HFDPC

To rule out the possibility that seoritae could have cytotoxic effects on HFDPC that would impact our experiments, we treated HFDPC with various concentrations of seoritae extract (50 ㎍/㎖ - 800 ㎍/㎖) and assessed cell viability using the WST reagent. We observed cell survival of 90% or more at concentrations of 400 ㎍/㎖ or less, indicating that these concentrations were not toxic to HFDPC. We performed our subsequent experiments in HFDPC using sub-toxic doses of seoritae extract.

Next, to assess the proliferative effect of seoritae extract on HFDPC, we applied sub-toxic concentrations of the extract to HFDPC, cultured the cells for 3 days, and examined cell viability. Indeed, seoritae extract effectively enhanced the cell proliferation of HFDPC (Fig. 1C). by 20 to 45% depending on the concentration of seoritae extract.

These results indicate that seoritae extract can enhance the proliferation of a human dermal papilla cell line, confirming its potential as a material for alleviating hair loss.

2. Effect of seoritae extract on DHT- or H_2O_2-induced apoptosis in HFDPC

To assess the ability of seoritae extract to suppress the DHT- or H_2O_2-induced apoptosis of HFDPC, we applied sub-toxic doses of the extract and assessed cell viability. Our results confirmed that the applied concentrations of H_2O_2 and DHT induced apoptosis in HFDPC under our experimental conditions (Fig. 2A).

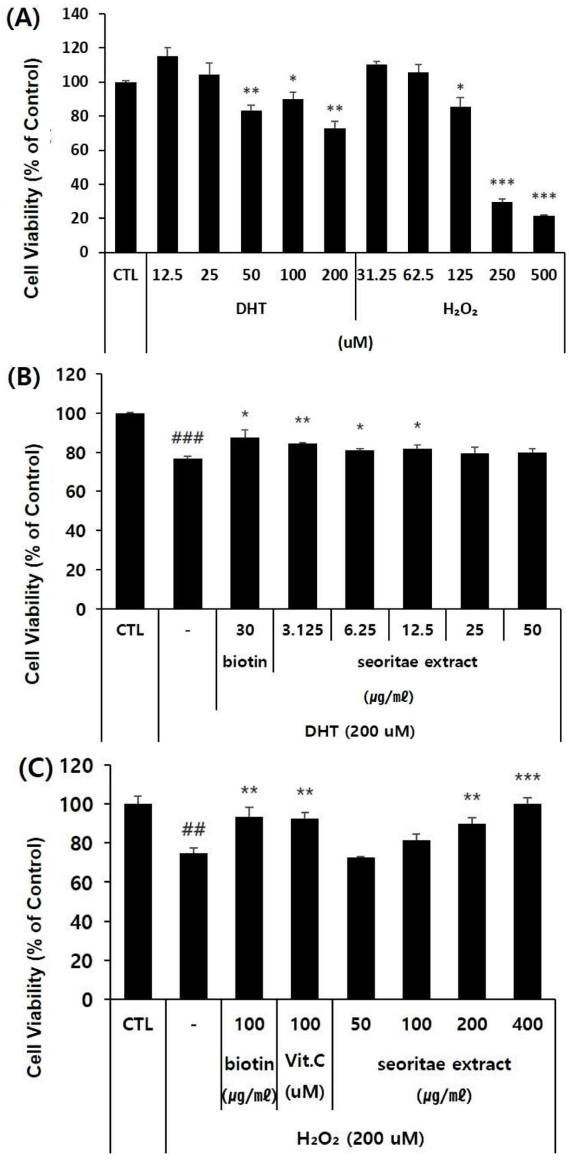

Fig. 2. The effect of seoritae extract on the H_2O_2- and DHT-induced apoptosis of HFDPC. (A) Optimized stimulators concentration by using cytotoxicity assay on HFDPC. (B) Anti-apoptotic effect of seoritae in HFDPC by DHT. (C) Anti-apoptotic effect of seoritae in HFDPC by H_2O_2. Cell viability was estimated using a colorimetric WST-1 assay kit. The results are given as the percentage compared to control group (Means ± SD). Positive controls: Vit. C and biotin. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the stimulator (DHT, H_2O_2). $^{\#\#}p < 0.005$, $^{\#\#\#}p < 0.001$ compared to the CTL.

The concentrations of DHT and H_2O_2 that maintain cell

viability at 70% were all about 200 μM, and this concentration was applied throughout this study. Then, the data showed that the seoritae extract suppressed apoptosis in our system, depending on the stimulant and applied concentration of extract (Fig. 2B and 2C). In DHT-treated HFDPC, searitae extract slightly recovered the cytotoxicity at relatively low concentrations. On the other side, searitae extract dramatically recovered the cytotoxicity at relatively high concentrations. Thus, we found that the anti-cytotoxic concentration of seoritae extract differed apparently, depending on the stimulant.

These results confirm that seoritae extract can suppress the H_2O_2- or DHT-induced apoptosis of HFDPC, and thus may be a hair loss-easing substance.

3. Western blot analysis of signaling molecules potentially involved with the anti-apoptotic effect of seoritae extract on HFDPC

Whereas a variety of growth factors and signaling pathways are involved in hair growth, the activation of wnt/β-catenin signaling plays a central role in hair follicle regeneration (Choi, 2020).

We determined if seoritae extract could induce the regulation of wnt/β-catenin signaling molecules in HFDPC under apoptotic conditions. The HFDPC were treated with the indicated concentrations of DHT and seoritae extract for 4 h, and the Western blot analysis of cell lysates was performed to evaluate the levels of selected proteins involved in β-catenin signaling (Bax, β-catenin).

As shown in Fig. 3, 200 ㎍/㎖ and 400 ㎍/㎖ seoritae extract increased β-catenin and suppresed Bax expression in DHT-treated HFDPC, although DHT (200 μM) did not affect on β-catenin and Bax expression in our experimetal system. These results suggest that seoritae extract modulates the levels of β-catenin and Bax proteins in human follicular dermal papilla cells even if the cells are under apoptotic conditions.

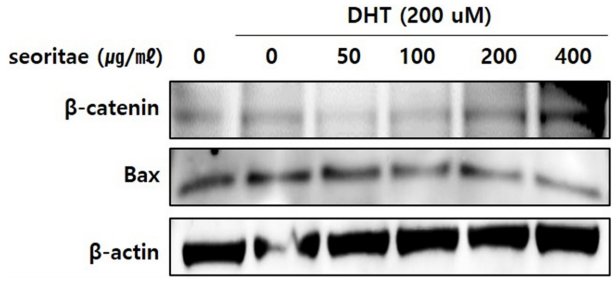

Fig. 3. The effects of seoritae extract on the protein expression levels of β-catenin and Bax in DHT-treated HFDPC cells. The cells were co-treated with DHT (200 μM) and seoritae (50, 100, 200, 400 ㎍/㎖) for 4 h, and whole-cell lysates were subjected to immunoblotting with antibodies against the indicated proteins.

4. DPPH radical-scavenging activity (antioxidant effect) of seoritae extract

Oxidative stress cause the apoptosis of hair follicle cells, resulting in the hair loss (Naito *et al*., 2009; Seo *et al*., 2012). Thus, we determined the anti-oxidative efficacy of seoritae extract.

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, which is commonly used to assess the antioxidant activity of plant extracts, was used to examine the radical-scavenging effect of seoritae extract. As shown in Fig. 4, the seoritae extract showed a significant DPPH scavenging effect.

In recent years, interest in appearance has spread to various fields. Hair loss is increasing not only in middle-aged and elderly people, but also in younger people. Agents capable of exerting cell-protective effects against aspects of skin inflammation and aging can be important contributors to ameliorating such conditions, and this may be extended to preventing hair loss. An ultimate solution for hair loss would be to address its most common cause by suppressing the synthesis of 5α-DHT.

Much research has been already done in this regard, but many of the well-known chemical agents for treating hair loss, such as minoxidil, cause side effects such as erythema and itching.

Currently, researchers are actively working to identify effective

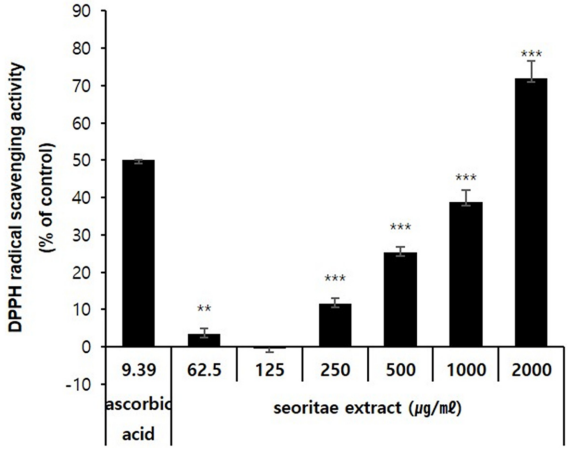

Fig. 4. DPPH radical-scavenging activity of seoritae extract. The given values represent the means ± SD of three independent measurements. Positive control: Ascorbic acid. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to the control.

natural product-derived substances that can address hair loss with few side effects (Park *et al*., 2015; Jain *et al.*, 2016; Dhariwala and Ravikumar, 2019; Jung, 2019), and to assess their underlying action mechanisms.

In this study, we show that seoritae extract has antioxidant, cell proliferation-enhancing, and anti-apoptotic effects in a human follicle dermal papilla cells. In addition, we further use protein analysis to show that alterations of β-catenin signaling may be a mechanism underlying this anti-apoptotic effect of seoritae extract, because there are multiple lines of evidence suggesting that various wnts promote hair cycling and regeneration via the activation of β-catenin signaling (Choi, 2020).

Based on these results, we propose that seoritae extract may be a candidate agent for effectively alleviating hair loss. Additional studies are needed to further explore the potential hair loss-relieving effect and underlying action mechanisms of seoritae extract. In addition, we consider that it is necessary to conduct further research to analyze the components of seoritae extract and to identify a single compound that exhibits efficacy.

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