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Research Article

MAPK and PI3K/Akt Signalling Pathways Potentially Modulate the Enhancing Effect of Black Tea Extracts on Endo180 Expression and Collagen Internalisation

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As the skin ages, collagen denatures into fragments that accumulate in the dermal matrix. Endo180 allows these fragments to reenter cells and be used for collagen recombination. While several studies have reported on the efficacy of green tea on skin health, studies on black tea have been limited. The aim of this study was to reveal the effects of black tea on skin health and the associated mechanisms. We estimated collagen internalisation using fluorescence-labelled gelatin and measured Endo180 expression following treatment with black tea extract (BTE). BTE at a concentration of 10 ug/ml increased Endo180 mRNA and protein expression by 1.67-fold ($p = 0.006$) and 1.46-fold ($p = 0.010$), respectively. In addition, collagen internalisation was increased by 1.29-fold ($p = 0.001$) in human skin fibroblasts compared with the untreated control group. These effects were reversed by five inhibitors of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways to the same levels as those of the untreated control group. These results suggest that BTE enhances collagen internalisation and upregulates Endo180 expression via the MAPK and PI3K/Akt signalling pathways in HDFs and that black tea could be used as a functional food for improving skin health.

1. Introduction

The skin, which is the largest organ in the human body [1], is vital for protecting muscles and other organs and also acts as a barrier to prevent external pathogens from entering the body. The skin consists of the epidermis, dermis, and subcutaneous layers [2]. The epidermis is the outermost and thinnest layer of the skin, mostly comprising keratinocytes. Keratinocytes are located in the deepest part of the epidermis layer, called the basal layer, and slowly move to the epidermal surface; when they reach the skin surface, they gradually disintegrate and are replaced by newer cells transported from the lower layer. The epidermis also contains melanocytes, which prevent the negative effects of ultraviolet (UV) rays, such as DNA damage, and Langerhans cells, which detect external substances and defend the human body from infection. The dermis is a thick layer located immediately below the epidermis. The dermis consists of collagen fibres and elastic tissues that provide flexibility and strength to the skin and comprises hair follicles, neurons, sebaceous glands, sweat glands, and blood vessels. The subcutaneous layer is the innermost layer of the skin, consisting of fat cells that protect the body from heat and cold and store energy. The depth of the subcutaneous layer varies substantially depending on the body part [3].

Collagen is the main component of human connective tissues distributed throughout the body, including the skin and bones [4]. The basic constituent unit of collagen is an alpha chain composed of approximately 1000 amino acids, wherein glycine, proline, and hydroxyproline appear repeatedly [5]; three strands of the alpha chain gather to form a triple helix, and crosslinking occurs between the triple helix to form collagen [6]. Collagen accounts for approximately 75% of the skin and is responsible for skin elasticity and moisturisation

[7]. The level of collagen in the body is maintained by balancing collagen decomposition and synthesis [8]. Collagen denaturation increases and synthesis decreases during aging or due to ultraviolet irradiation, resulting in reduced skin elasticity and increased wrinkling [7].

Matrix metalloproteinases (MMPs) and Endo180 (also known as MRC2, uPARAP, or CD280) are proteins that influence the collagen balance in the human body. MMP is an enzyme that catalyses collagen decomposition [8]. Of the several types of MMPs, MMP-1 can strongly influence collagen decomposition, as it initiates collagen breakdown [9–11]. Commonly, collagen fragments generated by MMP-1 accumulate in the dermal matrix and are then internalised into the cell for collagen resynthesis [12]. In this process, the protein involved in the internalisation of collagen fragments is Endo180 [12]. As intact collagen is integrated into fibroblasts without Endo180, while cleaved collagen is integrated into fibroblasts through Endo180 [13], Endo180 presumably helps remove denatured collagen from the skin matrix and reconstruct the skin matrix.

Black tea is prepared from the leaves or leaf buds of *Camellia sinensis* and is more oxidised than other teas prepared using this species. The name "black tea" is used in Western countries because of the black colour of the tea leaves; however, in Eastern countries, it is called "red tea" because of the red hue of the brewed tea. Both green tea and black tea originate from *C. sinensis*. However, the production process of green tea involves a sun-drying stage in which oxidase in the tea leaves is deactivated. In contrast, sun-drying is not required in the production process of black tea; therefore, the tea leaves turn dark as they are oxidised by oxidase [14]. Additionally, during this oxidation process, catechins in green tea are converted to theaflavin or thearubigin [15]. Green tea reportedly has a beneficial effect on photoaged skin. Kim et al. [16] reported that *C. sinensis* water extract inhibits melanin production by downregulating tyrosinase expression, and Klimczak and Gliszczyńska-Świgło [17] reported that (−)-epicatechin-3-gallate (ECG), epigallocatechin (EGC), epigallocatechin gallate (EGCG), and (−)-gallocatechin-3-gallate (GCG) coinhibit tyrosinase activity. In addition to these antimelanogenic activities, anti-wrinkling effects of green tea catechins have been reported [18, 19], including a collagen synthesis-boosting effect [20]. The activities of green tea catechins reportedly stem from their antioxidant or cell signalling-inhibitory activities [21]. Although the beneficial effect of green tea on the skin has been intensively studied using several methods, there are only a few studies on the beneficial effects of black tea on the skin [22–24]. However, studies have shown that black tea extract (BTE) and other components of black tea, theaflavin or thearubigin, exert beneficial effects on human health via antioxidant activity [25], cell signalling-inhibitory activity [26, 27], and regulatory activity of collagen in bones and teeth [28, 29]. Therefore, we hypothesised that BTE regulates collagen in human dermal fibroblasts (HDFs) and benefits human skin. To test this hypothesis, in this study, we aimed to determine whether BTE can regulate collagen in immortalised HDFs and the underlying mechanism.

2. Materials and Methods

2.1. Materials. Dulbecco's Modified Eagle's Medium (DMEM), foetal bovine serum (FBS), penicillin-streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Cell counting kit-8 (CCK-8) and lactate dehydrogenase (LDH) assay kits were purchased from Dojindo Molecular Technologies (Kumamoto, Japan). An E-64d cysteine protease inhibitor, gelatin, API2, LY294002, SB203580, SP600125, and U0126 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Oregon Green™ 488-conjugated gelatin (OG-gelatin), TaqMan universal master mix, and Endo180 TaqMan primers (HS00195862) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). An RNeasy kit was purchased from QIAGEN (Hilden, Germany), and a cDNA synthesis kit was purchased from Philekorea Technology (Daejeon, Korea). An Endo180 ELISA kit was purchased from MYBioSource (San Diego, CA). Passive lysis 5× buffer (PLB) was purchased from Promega Corporation (Madison, WI, USA).

2.2. Preparation of BTE. BTE was prepared as previously reported [30], with slight modifications. Darjeeling tea was purchased from Ambootia Tea Exports Pvt., Ltd. (Kolkata, India). Dried black tea leaves were ground to a coarse powder, and 50 g of the black tea leaf powder was immersed in 500 mL of water at 90°C for 30 min. Thereafter, the extract was filtered through a 75 μm mesh filter, and the filtrate was dried using a rotary evaporator R-100 (Buchi Labortechnik AG, Flawil, Switzerland) at 100 mbar and 60°C with low-pressure drying. After evaporation, we obtained BTE as a concentrate of approximately 50 Brix. The dry weight of the BTE concentrate was measured using a Halogen Moisture Analyzer (Mettler Toledo, Greifensee, Switzerland). Subsequently, the concentrate was dissolved in distilled water to reach 100 mg (dry weight)/mL and filter-sterilised for use in the experiments.

2.3. Cell Culture. Cells were cultured as previously reported [31]. Immortalised HDFs were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. The cells were incubated in a humidified chamber at 37°C with 5% CO_2. The cells were subcultured at a density of 1.5×10^6 cells/mL of growth media for 3 days or 1×10^6 cells/mL of growth media for 4 days.

2.4. CCK-8 Assay. The CCK-8 assay was performed by slightly modifying the method described previously [32]. A cell suspension (1×10^5 cells/mL) was prepared, and 500 μL of the cell suspension was placed in each well of a 24-well plate, which was then incubated in a humidified chamber at 37°C with 5% CO_2 for 1 day. The next day, the medium was replaced with serum-free DMEM, and the cells were incubated in the same humidified chamber for 1 day. The following day, the cells were treated with the indicated concentrations of BTE for 1 day. The next day, volume of the

CCK-8 solution adjusted to 10% of the total volume of the culture was added to each well, and the cells were incubated in the same humidified chamber for 1 h. The supernatant was then transferred to a 96-well plate, and the absorbance was measured at 450 nm.

2.5. LDH Assay. The LDH assay was performed according to the kit manufacturer's instructions, with modifications in the volume of reagents used. The experimental conditions were determined based on a previous study [32]. In brief, cell preparation for BTE treatment was performed in the same manner as that for the CCK-8 assay. The next day, the low control group, in which all cells were alive, and the high control group, in which all cells were dead, were prepared separately to set the criteria for cytotoxicity. Thereafter, 30 μL of HDF culture medium (including the low and high control groups) was transferred to a 96-well plate to measure absorbance at 490 nm. Equal amounts of LDH working solution were then added to the wells of a 96-well plate, and the plate was wrapped with aluminium foil and incubated for 30 min. Subsequently, 15 μL of the stop solution was added to the wells of the 96-well plate, and the absorbance of the samples was measured at 490 nm, using an Epoch microplate spectrophotometer (Biotek, Winooski, VT, USA). Cytotoxicity was calculated using the following two steps. First, to remove the bias caused by the colour of BTE, the absorbance measured first was subtracted from the absorbance measured second. Second, the cytotoxicities of the low and high control groups were set to 0% and 100%, respectively, using the following equation:

$$\frac{\text{Absorbance of target well} - \text{Absorbance of low control}}{\text{Absorbance of high control} - \text{Absorbance of low control}} \times 100. \tag{1}$$

2.6. Collagen Internalisation Measurement. Collagen internalisation was measured as previously reported [12], with slight modifications. Cell preparation for BTE treatment was performed in the same manner as that for the CCK-8 assay. The next day, the cells were treated with 20 μM E-64d cysteine protease inhibitor for 1 h, followed by treatment with OG-gelatin (2.5 M) for 2 h. After the treatment, the fibroblasts were washed once with cold PBS and then incubated with 0.4% trypan blue for 5 min in the dark to quench extracellular fluorescence. The cells were then washed twice with cold PBS, and 250 μL of 1× PLB was added to each well. The plate was wrapped with aluminium foil and shaken at 300 rpm for 5 min; the contents of the wells were added into a black 96-well plate with a transparent bottom to measure fluorescence at an excitation wavelength of 490 nm and emission wavelength of 520 nm, using an Epoch microplate spectrophotometer. Collagen internalisation of the nonlabelled control and labelled control was set to 0% and 100%, respectively, using the following equation:

$$\frac{\text{Fluorescence of target well} - \text{Fluorescence of nonlabeled control}}{\text{Fluorescence of labeled control} - \text{Fluorescence of nonlabeled control}} \times 100. \tag{2}$$

2.7. Real-Time PCR. Real-time PCR was performed per the standard method [12], with slight modifications. In brief, 1 mL of cell suspension was added to each well of a 12-well plate instead of adding 500 μL of cell suspension to each well of a 24-well plate. Cell preparation for BTE treatment was performed in the same manner as that for the CCK-8 assay. Subsequently, the fibroblasts were washed once with cold PBS, and RNA was extracted using the RNeasy kit (QIAGEN). cDNA was then synthesised using 1 μg of the extracted RNA as a template. The synthesised cDNA, TaqMan primers, and TaqMan universal master mix were mixed, and real-time PCR was performed using the StepOne™ Real-Time PCR System (Thermo Fisher Scientific).

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). An ELISA was performed according to the manufacturer's instructions. The experimental conditions were determined by referring to a previous study [32] with slight modifications. Cell preparation for BTE treatment was performed in the same manner as that for the CCK-8 assay. On the day BTE was treated, immunoplates were coated with the Endo180 to capture antibody diluted in PBS. The immunoplates were incubated overnight at 25°C. The next day, the immunoplates were washed with an ELISA wash buffer three times to remove the unbound capture antibody, followed by incubation for 1 h with 1× reagent diluent 2 to block the unbound space. During the blocking step, the fibroblast culture medium was collected to measure the concentration of Endo180. The immunoplates were washed thrice with the ELISA wash buffer and incubated with Endo180 standard solution of a known concentration or HDF culture medium for 2 h. The immunoplates were then washed thrice with the ELISA wash buffer, and the plate was incubated for 2 h with the Endo180 detection antibody. Next, the immunoplates were wrapped with aluminium foil and incubated for 20 min with streptavidin-HRP. Finally, the plates were washed thrice with the ELISA wash buffer, wrapped with aluminium foil, and shaken at 300 rpm for 15 min with the substrate. The stop solution was then added to the immunoplates, and

the absorbance of the samples at 450 and 570 nm was measured using an Epoch microplate spectrophotometer. After subtracting the absorbance at 570 nm from that at 450 nm, the absorbance of the unknown sample was substituted in the standard curve to calculate the Endo180 concentration in the unknown sample.

2.9. Statistical Analysis. Results are expressed as mean ± standard deviation. For comparison between the groups, a one-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc test using the SPSS Statistics 25.0 (Statistical Package for Social Sciences) program. Results with $p < 0.05$ were considered significant.

3. Results

3.1. BTE Does Not Exert Cytotoxicity at up to 10 μg/mL. First, we evaluated the effect of BTE on HDF viability. The results showed that 10 μg/mL BTE reduced cell viability to approximately 85% (Figure 1(a), $p = 0.001$). To verify whether this reduction resulted from the cytotoxicity of BTE, we performed the LDH assay. We found that BTE did not exert toxicity on HDFs at concentrations of up to 10 μg/mL (Figure 1(b), $p = 0.016$).

3.2. Collagen Internalisation Measured with OG-Gelatin. We used OG-gelatin to trace the internalised collagen. To determine the appropriate concentration of OG-gelatin at which an increase in collagen internalisation could be detected, we measured intracellular fluorescence at various concentrations of OG-gelatin. The results showed that intracellular fluorescence linearly increased with increasing OG-gelatin concentrations up to 6.3 μg/mL; however, the increase in intracellular fluorescence substantially decreased when the OG-gelatin concentration was above 6.3 μg/mL (Figure 2(a)). Based on this result, we set the concentration of OG-gelatin to 2.5 μg/mL in the subsequent experiments.

We then performed a gelatin competition assay to prove that the increased intracellular fluorescence originated from the gelatin uptake. We fixed the OG-gelatin concentration at 2.5 μg/mL and used gelatin at various concentrations. The results of the gelatin competition assay confirmed that intracellular fluorescence decreased with increasing gelatin concentration (Figure 2(b)). Based on these results, we inferred that this method could be used to measure collagen internalisation in HDFs.

3.3. BTE Enhances Collagen Internalisation and Endo180 Expression. Next, we evaluated the effect of BTE on collagen internalisation in HDFs. We treated BTE for 1 day and then measured the fluorescence of the cell lysate. BTE increased OG-gelatin uptake in a dose-dependent manner between 2 and 10 μg/mL and increased collagen uptake by approximately 1.29-fold at 10 μg/mL (Figure 3, $p = 0.001$). As Endo180 reportedly mediates collagen fragment internalisation, we examined the effect of BTE on Endo180 expression in HDFs. The real-time PCR and ELISA results showed that 10 μg/mL BTE upregulated both mRNA and protein expression of Endo180 by 1.67- and 1.46-fold, respectively, in HDFs (Figure 4(a), $p = 0.006$; Figure 4(b), $p = 0.010$).

3.4. BTE Increases Collagen Internalisation and Endo180 Expression through the MAPK and PI3K/Akt Signalling Pathways. BTE reportedly contains a large amount of theaflavin, which regulates the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signalling pathways. These signalling pathways reportedly mediate various cellular responses; however, it is not clear how these signalling pathways are involved in collagen internalisation. We examined whether collagen internalisation, which is increased by BTE, is inhibited by various inhibitors of the MAPK and PI3K/Akt signalling pathways. BTE at a concentration of 10 μg/mL increased collagen internalisation by 1.45-fold, and LY294002, API2, U0126, SB203580, and SP600125 blocked the effect of BTE on collagen internalisation in HDFs (Figure 5). Cotreatment with inhibitors and BTE increased collagen internalisation by 1.15- (LY294002, $p = 0.002$), 1.08- (U0126, $p = 0.001$), 1.19- (SB203580, $p = 0.004$), and 1.17-fold (SP60012, $p = 0.001$), whereas API2 reduced collagen internalisation by 0.91-fold ($p = 6 \times 10^{-5}$), compared with that in the untreated control group. However, in the absence of BTE, none of the inhibitors affected collagen internalisation in HDFs (Figure 5). Because Endo180 is deeply involved in collagen internalisation [12], we further assessed whether MAPK or PI3K/Akt influenced Endo180 expression. The results showed that all inhibitors of the MAPK and PI3K/Akt signalling pathways downregulated BTE-induced Endo180 protein expression (Figure 6). BTE increased Endo180 protein expression by 3.11-fold, and cotreatment with LY294002, API2, U0126, SB203580, and SP600125 decreased Endo180 protein expression by 0.91- ($p = 0.011$), 0.91- ($p = 0.009$), 0.86- ($p = 0.008$), 0.91- ($p = 0.007$), and 0.81-fold ($p = 0.006$), respectively, compared with that in the untreated control group. However, in the absence of BTE, none of the inhibitors affected Endo180 protein expression in HDFs (Figure 6).

4. Discussion

In this study, we showed through in vitro experiments that black tea can benefit skin health. The results suggested that BTE enhances collagen internalisation by upregulating Endo180 expression and that the MAPK and PI3K/Akt signalling pathways can influence this process. To the best of our knowledge, the relationship between the MAPK and PI3K/Akt signalling pathways and collagen internalisation or Endo180 expression has not been studied. However, several studies have reported that BTE activates the MAPK and PI3K/Akt signalling pathways [33–37]. Anter et al. [33] reported that black tea polyphenols activate endothelial nitric oxide synthase via the phosphorylation of PI3K/Akt and p38 MAPK. Bhattacharya et al. [34] reported that theaflavins and thearubigins, the active components of black tea, activate JNK

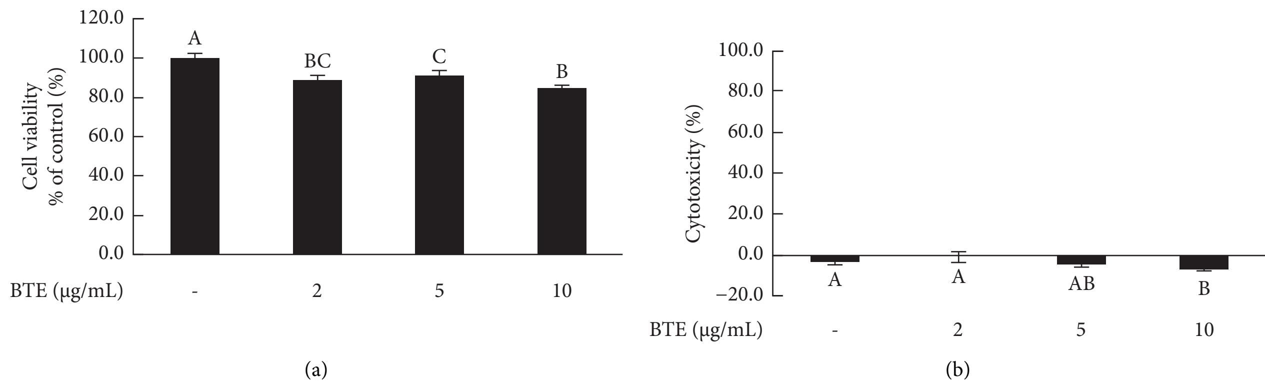

Figure 1: Effect of black tea extract (BTE) on cell viability and toxicity of BTE in human dermal fibroblasts (HDFs). (a) BTE reduced the viability of HDFs. HDFs were treated with the indicated concentrations of BTE for 1 day. Cell viability was measured as described in the "CCK-8 Assay" section. Cell viability in each treatment group is calculated as a percentage compared with the value of the untreated control group and is expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences. (b) BTE did not exert toxicity on HDFs. HDFs were treated with the indicated concentrations of BTE for 1 day. Cytotoxicity was measured and calculated as described in the "LDH Assay" section. The results are expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$.

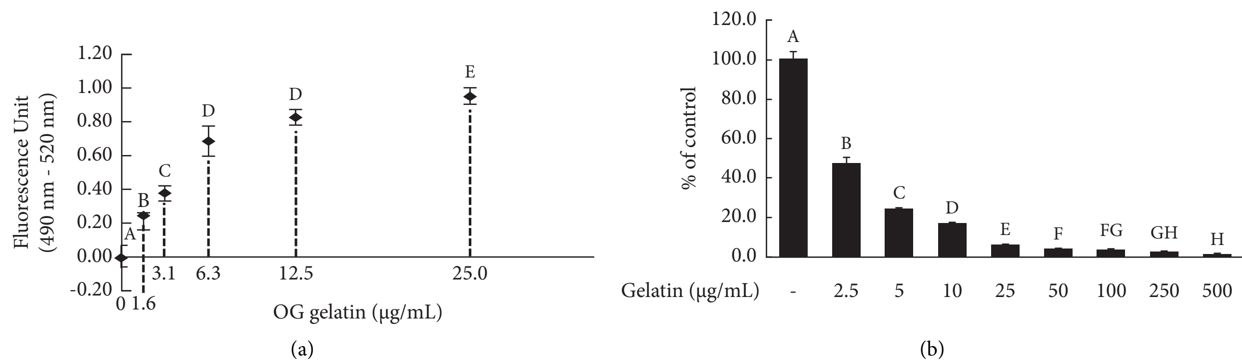

Figure 2: Correlation between Oregon Green™ 488-conjugated gelatin (OG-gelatin) concentration and fluorescence intensity and competitive inhibitory effect of gelatin on cellular internalisation of OG-gelatin. (a) The increase in fluorescence intensity slowed when the OG-gelatin concentration was above 6.3 μg/mL. Human dermal fibroblasts (HDFs) were treated with the indicated concentration of OG-gelatin for 2 h. OG-gelatin internalisation was measured as described in the "Collagen Internalisation Measurement" section. Results are expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$. (b) Gelatin competitively inhibited cellular internalisation of OG-gelatin in HDFs. HDFs were treated with a fixed concentration of OG-gelatin and the indicated concentrations of gelatin for 2 h. OG-gelatin internalisation was measured and calculated as described in the "Collagen Internalisation Measurement" section. Results are expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$.

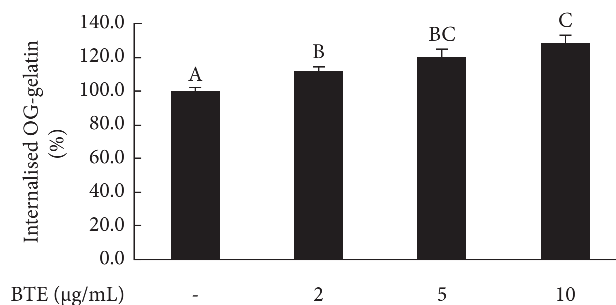

Figure 3: Effect of black tea extract (BTE) on collagen internalisation in human dermal fibroblasts (HDFs). BTE enhanced collagen internalisation in HDFs. HDFs were treated with the indicated concentrations of BTE for 1 day. Oregon Green™ 488-conjugated gelatin (OG-gelatin) internalisation was measured and calculated as described in the "Collagen Internalisation Measurement" section. Results are expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$.

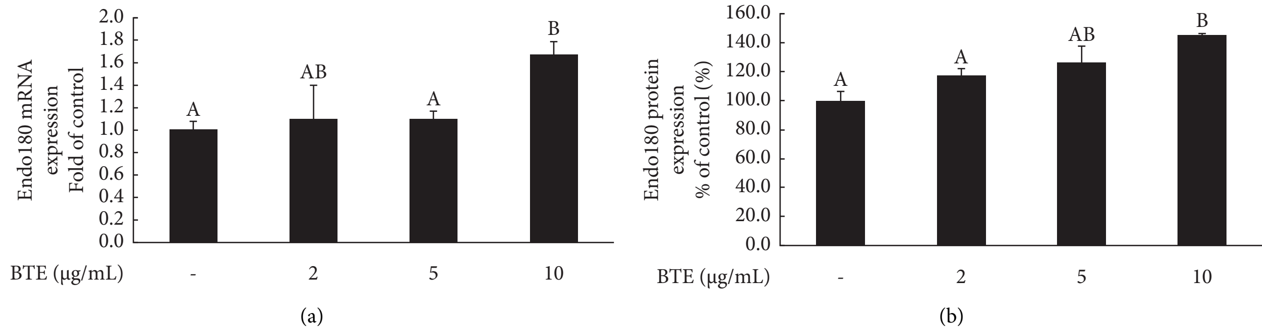

Figure 4: Effects of black tea extract (BTE) on Endo180 expression in human dermal fibroblasts (HDFs). (a) BTE increased Endo180 mRNA expression in HDFs. HDFs were treated with the indicated concentrations of BTE for 1 day. Quantitative real-time PCR was conducted as described in the "Real-Time PCR" section. Endo180 mRNA expression in each treatment group was calculated based on that in the untreated control group and is expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$. (b) BTE induces Endo180 protein expression in HDFs. HDFs were treated with the indicated concentrations of BTE for 1 day. Enzyme-linked immunosorbent assay was conducted as described in the "Enzyme-Linked Immunosorbent Assay (ELISA)" section. Endo180 protein expression in each treatment group is calculated as a percentage compared with that in the untreated control group and is expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$.

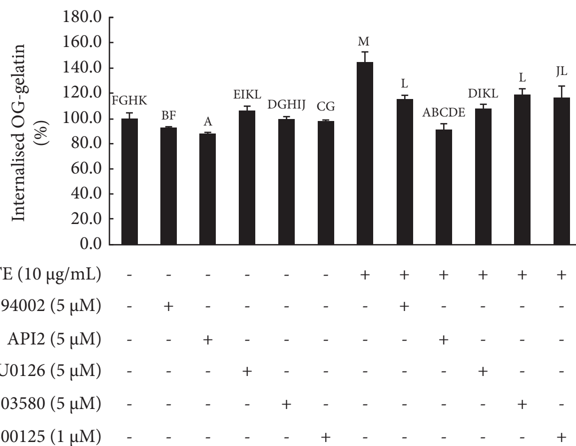

Figure 5: Effects of mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) inhibitors on black tea extract- (BTE-) induced collagen internalisation in human dermal fibroblasts (HDFs). LY294002, API2, U0126, SB203580, and SP600125 decreased BTE-induced collagen internalisation in HDFs. HDFs were treated with these inhibitors at the indicated concentrations for 1 h and then with BTE at 10 μg/mL for 1 day. Oregon Green™ 488-conjugated gelatin (OG-gelatin) internalisation was measured and calculated as described in the "Collagen Internalisation Measurement" section. The results are expressed as mean ± standard deviation of three independent experiments. Different letters indicate significant differences at $p < 0.05$.

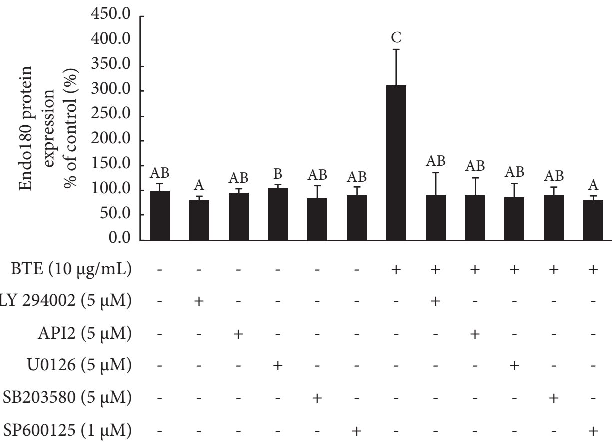

Figure 6: Effects of mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) inhibitors on black tea extract- (BTE-) induced Endo180 protein expression in human dermal fibroblasts (HDFs). LY294002, API2, U0126, SB203580, and SP600125 decreased BTE-induced Endo180 protein expression in HDFs. HDFs were treated with these inhibitors at the indicated concentrations for 1 h and then with BTE at 10 μg/mL for 1 day. An enzyme-linked immunosorbent assay was conducted as described in the "Enzyme-Linked Immunosorbent Assay (ELISA)" section. Endo180 protein expression in each treatment group is calculated as a percentage compared with that in the untreated control group and is expressed as mean ± standard deviation of three independent experiments. Different letters denote significant differences at $p < 0.05$.

and p38 MAPK during the apoptosis of melanoma cells. In addition, BTE has been recently reported to improve metabolic syndrome by activating the PI3K/Akt signalling cascade [35–37]. These findings prompted us to examine the relationship between the MAPK and PI3K/Akt signalling pathways and collagen internalisation. We showed that the inhibitors of MAPK or PI3K/Akt blocked collagen internalisation. These inhibitors did not affect collagen internalisation in the absence of BTE but blocked BTE-enhanced collagen internalisation (Figure 5). We further investigated the effect of the MAPK and PI3K/Akt signalling pathways on the expression of Endo180, as previous studies have implicated Endo180 in collagen internalisation [12, 38]. Our findings revealed that inhibitors of the MAPK or PI3K/Akt signalling pathways downregulate BTE-induced Endo180 expression (Figure 6). These results suggest that BTE activates the MAPK and PI3K/Akt pathways to upregulate Endo180

expression and collagen internalisation. However, the experimental results showed that the increase in collagen internalisation by BTE is not Endo180 dependent. Endo180 protein expression decreased to the level of the untreated control group upon exposure to all MAPK and PI3K/Akt inhibitors (Figure 6). However, collagen internalisation was not completely inhibited by LY294002, SB203580, and SP600125 (Figure 5). This discrepancy in the results suggests that mechanisms other than those mediated by Endo180 may be involved in regulating collagen internalisation. Following this finding, Elango et al. [38] reported the presence of several types of collagen receptors such as integrins, discoidin domain receptor, glycoprotein VI, osteoclast-associated receptor, leukocyte-associated immunoglobulin-like receptor 1, fibronectin, and vitronectin. Among these receptors, fibronectin, which has a higher affinity for denatured gelatin than triple-helix collagen and is expressed in skin cells, is likely to be involved in collagen internalisation. The report that collagen I matrix turnover is regulated by fibronectin polymerisation [39] also supports this possibility.

The content of collagen is determined by the balance between the decomposition and synthesis of collagen [8]. Kisling et al. [8] suggested that black tea may increase the amount of skin collagen by increasing collagen synthesis or inhibiting collagen decomposition. Therefore, to determine whether black tea can effectively increase collagen content in human skin, it is necessary to check how it affects collagen decomposition. Ratnasooriya et al. [40] reported the anti-collagenase activity of black tea *in vitro*. They reported that the half maximal inhibitory concentration (IC_{50}) of black tea brew and EGCG, a representative component of black tea, for collagenase was 80.04 and 112.12 μg/mL, respectively. Considering that the content of EGCG contained in black tea is 0.58 w/w%, the collagenase-inhibitory activity of black tea brew cannot be considered to originate from EGCG. However, Ratnasooriya et al.'s study [40] is meaningful as it showed the collagen decomposition-inhibitory effect of black tea. Furthermore, Lee et al. [41] reported the anti-wrinkle activity of black tea in SKH-1 hairless mice [41]. They compared three types of tea and found that black tea had the best skin wrinkle-reduction effect. In particular, the MMP-3activity-inhibitory effect of black tea is similar to that of retinoic acid, a well-known functional component for reducing skin wrinkles. Overall, it can be considered that black tea not only increases the synthesis of collagen but also inhibits the decomposition of collagen. However, to date, no study has revealed whether black tea increases the amount of collagen in the human body. Although research shows that black tea increases the amount of collagen to help wound healing [42, 43], it is still questionable whether black tea can increase the amount of collagen even under normal conditions, as this may result from a response to special environments such as wounds.

In order to reveal whether BTE increases collagen content in the human body and can be developed as a health functional food for promoting skin health, it is necessary to determine the amount of BTE that should be consumed. To estimate this concentration, we investigated the content of catechin, which is well known as a major ingredient in black tea. Du et al. [44] measured the catechin content of 12 types of black tea; they extracted 1 g of black tea raw material with 50 g of water and diluted it 100 times and reported an average catechin concentration of 44 mg/kg of BTE. In the present study, considering that 50 g of black tea raw material was extracted and concentrated to obtain 35.1 g of extract, our BTE was approximately 7122.5 times concentrated compared to the previous study. Thus, 1 kg of BTE used in this study contains approximately 313.39 g of catechin. In addition, clinical trials with catechin revealed that the catechin intake was at least 100 mg to a maximum of 1315 mg [45–47], which is equivalent to 0.32 to 4.20 g BTE. Therefore, a future clinical trial for skin health could be conducted considering the average BTE intake between 0.32 and 4.20 g.

The strength of our study is that it revealed for the first time that BTE activated collagen internalisation and Endo180 expression and suggested the possibility that the activating effect of BTE on collagen internalisation and Endo180 expression could be mediated by the MAPK and PI3K/Akt signalling cascades. However, a limitation of the study was that it did not confirm the possibility of whether the collagen fragment can enter the cell through a mechanism other than that mediated by Endo180 and if the collagen fragment that entered the cell was used for collagen synthesis. Overcoming these limitations through further research and confirming the effectiveness of BTE in the human body will provide evidence for the benefits of BTE as a functional food for skin health.

5. Conclusions

In the present study, for the first time, we proposed that BTE increases Endo180 expression and collagen internalisation and suggested the possibility that this effect could be achieved through MAPK and PI3K/Akt signalling cascades. These findings suggest the potential beneficial role of BTE for skin health because collagen fragments that enter the cell can be used for collagen resynthesis. However, further studies are required for BTE to be developed as a functional food for promoting skin health. Understanding the effects of BTE on the activity of collagen-degrading enzymes, one of the two important factors in collagen balance in the body, and validation of its efficacy to increase collagen content in clinical models would be essential to confirm the potency of BTE as a skin health-promoting functional food. These additional studies, as well as the present study, can be used as a basis for the future development of BTE as a functional food for improving skin health.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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