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Effect of *Bacillus subtilis* natto–fermented *Radix astragali* on collagen production in human skin fibroblasts

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ABSTRACT

Radix astragali was fermented with *Bacillus subtilis* natto, and the effect of fermented products (HQB) and non-fermented products (HQNB) on collagen production in human dermal fibroblasts (HDF) were investigated. It was found that HQNB significantly improved cell growth and proliferation of HDF cells. However, the enzyme-linked immunosorbent assay and Western blot analysis demonstrated that HQB, but not HQNB, significantly and dose-dependently stimulated the biosynthesis of type I procollagen in both aged (81 y) and young (22 y) HDF cells. Real-time reverse transcription-polymerase chain reaction revealed that expression of type I, type III procollagen and transforming growth factor β1 (TGF-β1) mRNA was significantly stronger in HQB-treated HDF cells than that of HQNB-treated and un-treated HDF cells, suggesting that there was a close correlation between the mRNA expression and protein production in the fibroblasts response to HQB stimulation. The results also suggested that HQB could stimulate the collagen biosynthesis in human dermal fibroblasts, which is, at least in part, associated with the regulation of procollagen biosynthesis resulting from HQB-induced TGF-β1 expression and the mitogenic activity in HDF cells, and therefore, is expected to reduce the age-dependent loss of extracellular matrix proteins.

1. Introduction

Skin aging is a complicated biological phenomenon. The biochemical changes associated with skin aging include reduced expression of type I collagen messenger ribonucleic acid (mRNA), overexpression of matrix metalloproteinase (MMPs), and decreased synthesis of extracellular matrix (ECM), which results in increased collagen degradation and leads to skin laxity, atrophy, wrinkles, dryness, and other clinical manifestations of skin [1,2]. Type I collagen is the main component of the ECM of skin dermis; it provides strength and maintains the structure of the skin dermis. The quantity and quality of collagen are determined by the balance between degradation and synthesis [3]. Collagen degradation is mainly regulated by MMPs, while collagen synthesis is mediated by both transcriptional and post-translational processes. Therefore, it is generally believed that aging process of skin can be improved by promoting collagen synthesis and reducing MMPs activities in the skin.

In recent years, the use of traditional herbal medicines in skin care cosmetics has claimed tremendous attention. Chinese herbs are of particular interest because they have been applied in skincare products for more than 5000 years. It has been reported that some components in herbal extracts, such as isoflavones, polyphenols, asiaticoside and gallated catechins could enhance collagen production due to their hormone-like or antioxidant effects [4–7]. *Radix astragali* (root of *Astragalus*, known as Huangqi in Chinese; family Leguminosae) is one of the most popular Chinese herbs, which is used traditionally to boost energy, strengthen the immune system, and promote skin growth. The components that are most often associated with the health-promoting activities of *R. astragali* are isoflavonoids, triterpene saponins and polysaccharides [8,9]. Recent studies showed that *R. astragali* has significant mitogenic activity and can enhance cellular metabolism and increase the life span of cells [10–13]. Therefore, there is a great deal of interest in studying the role of *R. astragali* preparations in skincare function.

Chinese herbs have long been processed via microbial fermentation. Studies have demonstrated that fermentation not only alters the original bioactivities of Chinese herbs, resulting in new treatment effects, but also enhances the original treatment efficacy [14–16]. It is known that β-glucosidase from *Bacillus subtilis* natto can convert isoflavone glucosides to their aglycones [17]. Numerous studies have revealed that the biological effects of isoflavone stem from their aglycones [18,19], and isoflavone aglycones are more

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easily and rapidly absorbed in the intestines [20]. Since *R. astragali* has been traditionally used for skincare, and its active components, such as isoflavones and saponins, are glucosides, it is of great interest to know and compare the effects of non-fermented and fermented preparations of *R. astragali* on collagen production in human skin cells. Therefore, the objectives of this study were to investigate the effect of *B. subtilis* natto–fermented *Radix astragali* (HQB) on collagen synthesis in the cultures of human dermal fibroblasts, and their inhibitory effects on the matrix-degrading enzymes (collagenase, elastase, and gelatinase).

2. Materials and methods

2.1. Plant material

Astragalus membranaceus (Fisch.) Bge. var. *mongholicus* (Bge.) Hsiao, a commonly used species of *Radix astragali* supplied by Microbio Co. Ltd. (Taipei, Taiwan) was used in this study, and the authenticity was confirmed by Pharmaconosy Pharmaceutical Technology Division, Biomedical Engineering Research Laboratories, Industrial Technology Research Institute (Hsinchu, Taiwan). The specimens were deposited in the Institute of Food Science and Technology, National Taiwan University, Taipei, Taiwan.

2.2. Microorganism and culture

Bacillus subtilis natto (ATCC 7059) was maintained and subcultured in nutrient broth (NB) flasks. *A. membranaceus* var. *mongholicus* was milled with a grinder and used as the fermentation medium. Seed culture of *B. subtilis* natto was prepared by transferring 0.1 mL of bacteria from the NB flask into a 300-mL Erelenmeyer flask containing 100 mL of NB medium and cultured in shaker at 37 °C, 120 rpm for 12 h. The fermentation medium contained 10 g *A. membranaceus* var. *mongholicus* powder and 90 g water and was inoculated with 0.1% of the seed culture and then cultivated at 37 °C, 120 rpm for 48 h. As the control, the 10% *A. membranaceus* var. *mongholicus* medium was also incubated at the same condition without inoculation. After fermentation or incubation, the broth was centrifuged at 10,000 rpm for 25 min. The supernatant was filtered by Whatman no. 42 paper, freeze-dried, and stored at −20 °C for further experiments. All fermentation experiments were performed in triplicate to ensure the reproducibility. Normally, 2.92 g of dried powder can be obtained from 10 g of *A. membranaceus* var. *mongholicus*.

2.3. Assay for enzyme inhibition activities

Collagenase, gelatinase, and elastase inhibition activities were measured with EnzChek® Collagenase/gelatinase and Elastase Assay Kits (Invitrogen Life Technologies Inc., USA) according to the manufacturer's recommendations. In brief, the reaction mixture was prepared by mixing 80 μL of the sample or provided inhibitor, 20 μL of the substrate solution (collagen or gelatin or elastin), and 100 μL of the enzyme. The reaction mixture was incubated at room temperature for an appropriate time and then fluorescence intensity measured by a fluorescence microplate reader (TECAN Spectrofluor Plus, Maennedorf, Switzerland) set for excitation at 485 nm and emission detection at 535 nm. The inhibition rate was calculated following the formula provided by the manufacturer.

2.4. Cell culture

Primary cultures of two normal human dermal fibroblasts (HDF, Cat. C-013-5C, Cascade Biologics, Inc., Portland, OR) derived from adult skin were purchased from Cascade Biologics Company. Both primary fibroblasts were from female donors, 22 y (HDF_{22y}) and 81 y (HDF_{81y}), respectively. They were cultured in DMEM medium (Invitrogen Life Technologies Inc., USA) supplemented with 10% FBS in 95% air and 5% CO_2 humidified atmosphere at 37 °C.

2.5. Assay of cell viability

The MTT colorimetric method [21] was used to determine the cell cytotoxicity and proliferation. Briefly, cells were cultured in 24-well plates at 1×10^5 cells/well for cytotoxicity determination, and at 4×10^4 cells/well for proliferation study. After the cells were incubated for 24 h, various concentrations of samples were added and incubated for additional time periods. At the end of incubation, tetrazolium dye was added as an indicator in order to convert tetrazolium salts to a colored product, formazan. The formazan concentration was measured by spectrophotometer at 570 nm.

2.6. Dertermination of type I procollagen synthesis

To measure type I procollagen biosynthesis specifically, human dermal fibroblasts were plated in 24-well plates (4×10^4 cells/well). When the cells reached subconfluence in the subculture after 72 h, cells were incubated in the absence or presence of the sample. As positive control, 1 ng/mL transforming growth factor beta (TGF-β1) was used. At the end of incubation, cell culture supernatants were collected and analyzed for type I procollagen by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (Procollagen Type I C-Peptide EIA kit, TaKaRa Biomedicals Inc., Japan) [7]. Data were normalized to the protein content (Pierce Biotechnology Inc., USA) of the respective cell culture supernatant. Procollagen content of the control group is set to 100%.

Type I procollagen protein levels were assessed by Western blot analysis using the protocol described elsewhere [22] with slight modification. In brief, 25 μg of protein was electroblotted onto a nitrocellulose membrane (Amersham Biosciences, UK), following separation using 7.5% SDS-polyacrylamide gel electrophoresis. The transblotted membranes were blocked with 5% nonfat milk in Tris-buffer plus 0.1% Tween-20 for 1 h at room temperature. The membranes were incubated overnight at 4 °C with a monoclonal antiprocollagen type I aminoterminal extension peptide (SP1.D8) antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa), and then incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology Inc., USA) for 1 h at room temperature. β-Actin was used as internal control. The immunoreactive bands were detected by an ECL Plus detection kit (Pierce Biotechnology Inc., USA), and visualized and quantified by SYNGENE G:BOX fluorescence and chemiluminescence system (Cambridge, UK).

2.7. Total RNA isolation and quantitative reverse transcription-PCR

Total RNA was isolated from cultured fibroblasts using TRIzol Reagent (Invitrogen Life Technologies Inc., USA) following the protocol provided by the manufacturer. RNA was dissolved in RNase free water for RNA quality determination by UV spectrophotometer at the ratio of 260/280. Reverse transcription was performed with Omniscript® Reverse Transcription Kit (Qiagen GmbH, Germany) including Omniscript Reverse Transcriptase, 10× RT-PCR Buffer, 5 mM dNTP Mix and Oligo $d(T)_{18}$. Quantitative real-time PCR for type I, type III procollagen, TGF-β1 and connective tissue growth factor (CTGF) was performed using ABI PRISM® 7300 Sequence Detection System (Applied Biosystems, CA, USA). Aliquot of 2 μL of the oligo dT-primed cDNA was amplified by PCR in a 20 μL reaction mixture containing 0.5 μL of 10 μM of primers and 10 μL SYBR® Green PCR Master Mix (SYBR Green 1 Dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, Passive Reference 1, and buffer components). The primers used for amplification were listed in Table 1. PCR was performed using the thermal cycle protocol listed as follows: stage 1: 50 °C for 2 min; stage 2: 95 °C for 10 min; stage 3: 40 repetitions of 95 °C for 15 s, 58 °C for 30 s, 72 °C for 45 s; stage 4: 95 °C for 15 s, 60 °C for 1 min.

2.8. Constituents analysis

Extraction and HPLC analysis of isoflavonoids were performed as described by Ma et al. [23] with some modification. Five gram of the freeze-dried sample was extracted three times with 100 mL of aqueous methanol for 2 h. The combined MeOH extract was filtered and evaporated to dryness under vacuum. The viscous residue was stirred in 25 mL of hot water, and the suspension was partitioned with 10, 7.5, and then 5 mL of ethyl acetate. The ethyl acetate containing isoflavonoids was evaporated to dryness. The viscous residues was dissolved in 1 mL of MeOH and filtered through a Millipore filter unit before analyzed by HPLC. The HPLC was performed on a Finnigan SpectraSYSTEM liquid chromatography system (Thermo Scientific, USA). The system included a quaternary pump, an UV detector, an autosampler, and was controlled by ChromQuest chromatography software. An Inertsil ODS-2 column (250 mm × 4.6 mm i.d; 5 μm) was used for the separation. The mobile phases consisted of water (A) and acetonitrile (B) each containing a

Table 1
Real-time RT-PCR primer sequence of target genes designed by the software of Applied Bioscience Prism 7300 sequence detection system.

Target gene (H)	GenBank identifier	Forward primer	Reverse primer	Amplicon size (bp)
β-Actin	NM_001101.2	GCTCCTCCTGAGCGCAAG	CATCTGCTGGAAGGTGGACA	75
Type I procollagen	AF017178	GAACGCGTGTCATCCCTTGT	GAACGAGGTAGTCTTTCAGCAACA	94
Type III procollagen	X14420	AACACGCAAGGCTGTGAGACT	GCCAACGTCCACACCAAATT	88
TGF-β1	NM_000660	ACAATTCCTGGCGATACCTCA	GGCGAAAGCCCTCAATTTC	129
CTGF	NM_001901	ACTCCCAAAATCTCCAAGCCTAT	ATCGGCCGTCGGTACATACT	100

volume fraction of 0.1% glacial acetic acid, and the gradient elution program used was: 5–70% B (v/v) at 0–30 min, 70–85% B at 30–45 min, 85–5% B at 45–55 min, and the re-equilibration time of gradient elution was 10 min. Elution was performed at a flow rate of 1.0 mL/min at 25 °C and detection at 280 nm. Ononin, calycosin, and formononetin (ChromaDex, Santa Ana, USA) were identified by comparison of the HPLC retention time and the UV spectra of the authentic compounds. The calibration curve obtained by the authentic compounds was used for determining the concentration of isoflavonoids in the sample.

2.9. Data and statistical analysis

All data are means of at least triplicates ± standard derivation (S.D.). Statistical analysis was performed using ANOVA, Duncan and Dunnett test (SAS Institute Inc., Cary, NC, USA) to determine significant differences among means and *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Effect of HQB and HQNB on collagenase, gelatinase, and elastase inhibition

In the process of skin aging, expression of MMPs increases, causing more collagen degradation and resulting in aged skin with wrinkle and skin laxity [24,25]. MMPs are a family of zinc-requiring endoproteases capable of degrading the components of extracellular matrix. At present, the MMPs family can be divided into several groups, such as MMP-1 (collagenase), MMP-2 (gelatinases), MMP-3 (stromelysins) and MMP-12 (metalloelastase), according to their substrate specificity and primary structure [26]. This study examined the abilities of fermented (HQB) and non-fermented (HQNB) preparations to inhibit the activities of collagenase, gelatinase, and elastase (Fig. 1). The inhibition rates of HQB for collagenase, gelatinase, and elastase at a concentration of 800 μg/mL were 26.55%, 24.87%, and 12.58%, respectively. The inhibition rates of HQNB were 28.95%, 24.45%, and 7.25% for those three enzymes at the same levels, respectively. The inhibitory effects of both HQB and HQNB on collagenase and gelatinase were higher than that on elastase. There were significant difference between the inhibitory effects of HQB and HQNB on both collagenase and elastase ($p < 0.05$).

3.2. Effect of HQB and HQNB on cell viability

Even though *R. astragali* is considered as a top-grade herb (the herb without adverse effect) in traditional Chinese medicine, this study still needed to verify that HQB and HQNB are of low toxicity.

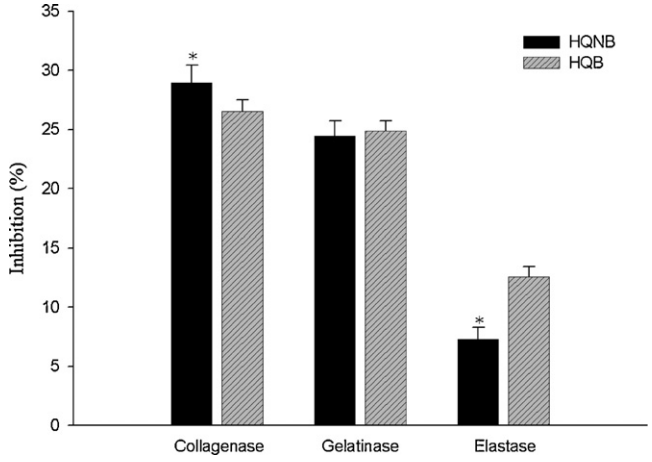

Fig. 1. Inhibition effects of HQNB and HQB (800 μg/mL) on collagenase, gelatinase, and elastase. Results are expressed as mean ± S.D., $^{*}p < 0.05$: significant differences between HQB and HQNB for each enzyme. The results were verified by the repetition of three independent experiments, each in triplicate.

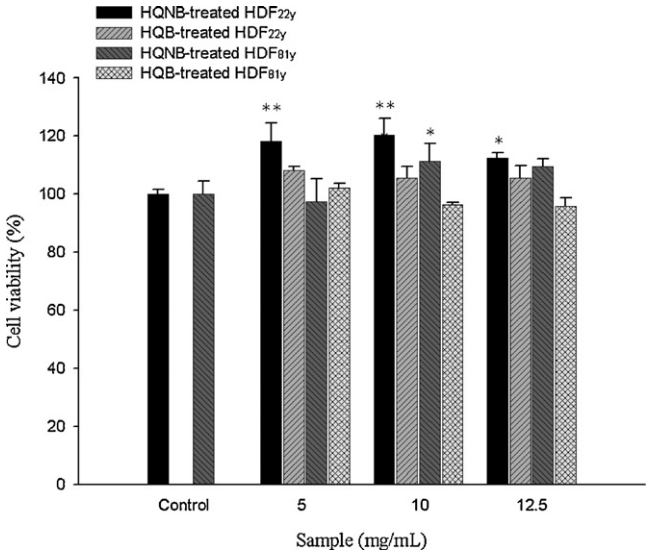

Fig. 2. The cytotoxicity of human dermal fibroblasts (HDF_{22y} and HDF_{81y}) treated with various concentrations of HQNB or HQB for 72 h. Cell viability was determined by MTT assay as described in Section 2. The results are expressed as mean ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$: compared with control group (culture medium only). The results were verified by the repetition of three independent experiments, each in triplicate.

The MTT assay was performed to evaluate cytotoxic effects of HQB and HQNB on human dermal fibroblasts (HDF). HQB and HQNB showed relatively low cytotoxicity, the cell viability was above 95% with a concentration of 12.5 mg/mL (Fig. 2).

The effects of culture time and dosage of HQB and HQNB on proliferation of the two HDF cells were shown in Fig. 3. Compared with un-treated cells, HQB- and HQNB-treated cells showed greater potential of growth and proliferation during the culture period ($p < 0.05$). It is interesting to note that both HQB and HQNB showed a higher potency on the growth of HDF_{22y} cells than that on HDF_{81y} cells, and HQNB-treated HDF_{22y} cells showed the highest proliferation rate among all treated cells ($p < 0.05$) (Fig. 3A). The effects of various concentrations (100, 500, and 2000 μg/mL) of HQB and HQNB on the proliferation of both HDF cells were depicted in Fig. 3B. After 72 h of incubation, both HQB and HQNB enhanced HDF cells proliferation in a dose-dependent manner. At the concentration of 500 μg/mL, the growth rate of HQNB-treated HDF_{22y} and HDF_{81y} cells increased 26% and 15%, respectively.

3.3. Effect of HQB and HQNB on type I procollagen biosynthesis activity in cultured human dermal fibroblasts

Collagen in the skin is synthesized by dermal fibroblasts. When the procollagen transcribed from collagen mRNA is secreted outside the cell, the propeptide at the carboxy or amino terminals of the procollagen is excised by a protease, which results in pN or pC collagen with only an amino or carboxy terminal. Because both pN and pC collagen are precursor molecules of mature collagen, the amount of propeptide reflects the level of collagen biosynthesis [27,28].

We used an enzyme-linked immunosorbent assay (ELISA) to evaluate the potential effects of HQB and HQNB on the type I procollagen production in HDF cells. As shown in Fig. 4A, HQB (500 μg/mL) significantly enhanced type I procollagen production in both HDF cells in a time-dependent manner, and its stimulating effect was more pronounced than those of HQNB-treated or un-treated cells ($p < 0.05$). For dose response (50, 100, 500, and

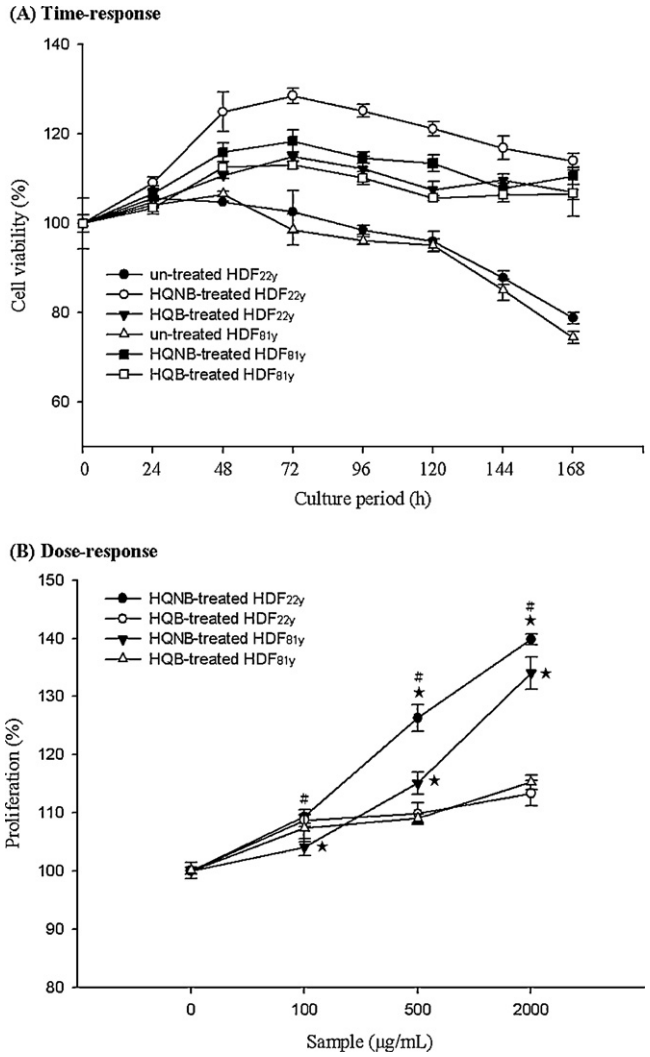

Fig. 3. Effects of HQNB or HQB on proliferation of human dermal fibroblasts (HDF_{22y} and HDF_{81y}) in MTT assay. (A) For time response studies, cells were treated with HQNB or HQB (500 μg/mL) for different time periods. (B) For dose response studies, cells were treated with various concentrations of HQNB or HQB for 72 h. The results are expressed as mean ± S.D., (★)$p < 0.05$: significant differences between HQNB- and HQB-treated of each HDF cell type. $^{\#}p < 0.05$: significant differences between HDF_{22y} and HDF_{81y} cells in HQNB-treated groups. The results were verified by the repetition of three independent experiments, each in triplicate.

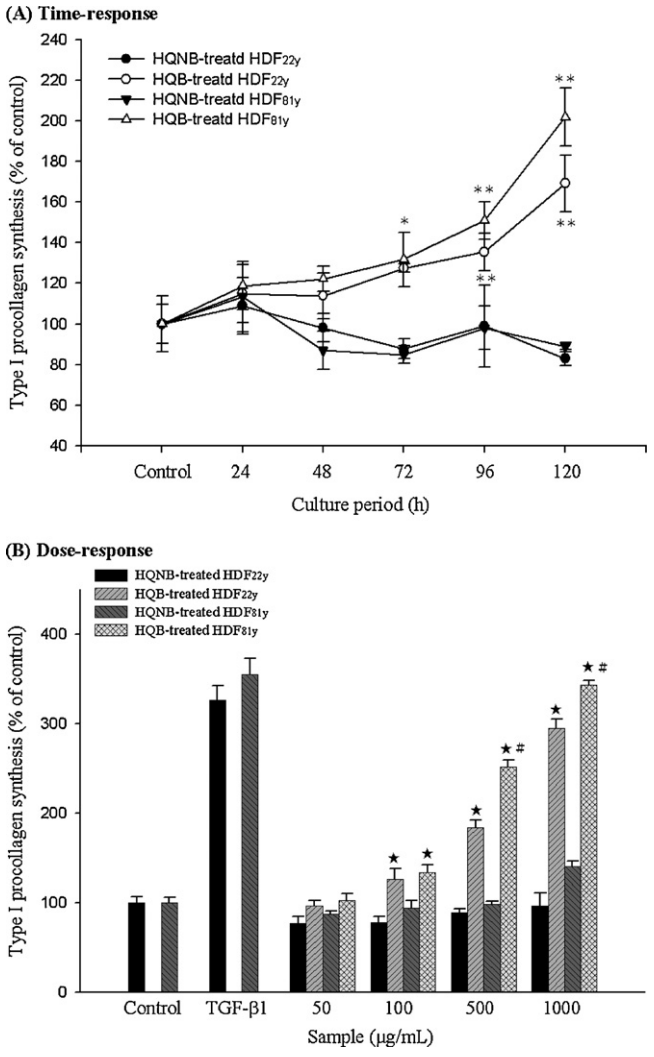

Fig. 4. Effects of HQNB or HQB on type I procollagen production in human dermal fibroblasts (HDF_{22y} and HDF_{81y}). (A) For time response studies, cells were treated with HQNB or HQB (500 μg/mL) and then the culture supernatant was collected at different time points. (B) For dose response studies, cells were treated with various concentrations of HQNB or HQB for 120 h and then the culture supernatant was collected. Type I procollagen synthesis was determined by ELISA kit as described in Section 2. The results are expressed as mean ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$: compared with control group (culture medium only). (★)$p < 0.05$: significant differences between HQNB- and HQB-treated of each HDF cell type. $^{\#}p < 0.05$: significant differences between HDF_{22y} and HDF_{81y} cells in HQB-treated groups. The results were verified by the repetition of three independent experiments, each in triplicate.

1000 μg/mL) studies, HQB exhibited a stimulating effect on the production of type I procollagen in both HDF cells in a concentration-dependent manner (Fig. 4B). In addition, the type I procollagen enhancing effect of HQB was significantly greater in HDF_{81y} cells than in HDF_{22y} cells ($p < 0.05$). The HQB treatment (500 μg/mL) increased the type I procollagen production by 152% and 84% in HDF_{81y} cells and in HDF_{22y} cells, respectively, as compared with the un-treated cells. Furthermore, the stimulation potential of HQB on HDF_{81y} cells was about 2.6-fold that of HQNB at the same concentration.

Type I procollagen protein levels in both HDF cells were examined using Western blot analysis. Cells were treated with different concentrations of HQB and HQNB (100, 500, and 1000 μg/mL) for 120 h to study dose response effect. Similarly, HQB had a more pronounced effect on enhancing the type I procollagen protein expression than the HQNB. In addition, the concentration-dependent effect on both HDF cells was observed for HQB. Also, the expression of type I procollagen protein enhanced by HQB was more pronounced in HDF_{81y} cells than in HDF_{22y} cells ($p < 0.05$). The HQB treatment (1000 μg/mL) increased the expression of type I procollagen protein in HDF_{81y} and HDF_{22y} cells by 73% and 58%, respectively ($p < 0.05$), as compared with the un-treated cells (Fig. 5A and B).

3.4. Effect of HQB and HQNB on type I and type III procollagen mRNA expression in cultured human dermal fibroblasts

To further establish whether HQB enhanced type I procollagen biosynthesis via regulation at transcriptional or post-translational level, more experiments were necessary. Both HDF cells were treated with 500 μg/mL of HQB or HQNB separately, and the total RNA of the cells was extracted after incubation for 48 h. The expression of type I procollagen and type III procollagen mRNA in cells was determined using real-time reverse transcription-polymerase chain reaction

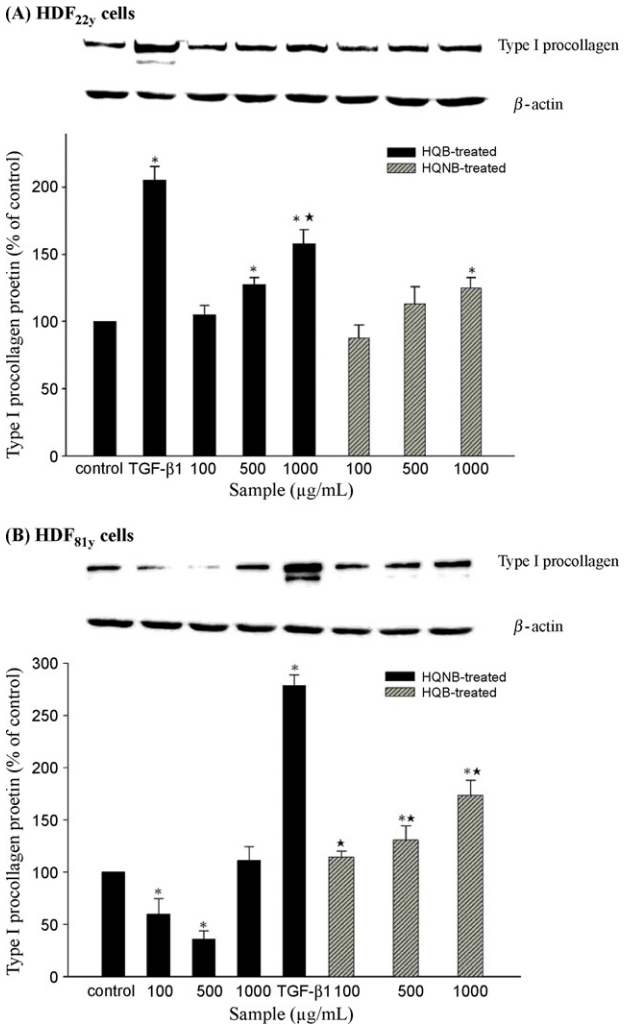

Fig. 5. Effects of HQNB or HQB on type I procollagen protein expression in human dermal fibroblasts (HDF_{22y} and HDF_{81y}). Cells were treated with various concentrations of HQB or HQNB for 120 h, after which cells were harvested and total cell lysates were prepared for Western blot analysis, as detailed in Section 2. β-Actin was used as a loading control. (A) HDF_{22y} cells and (B) HDF_{81y} cells. The results are expressed as mean ± S.D., $^{*}p < 0.05$: compared with control group (culture medium only). (★)$p < 0.05$: significant differences between HQNB- and HQB-treated cells. The results were verified by the repetition of three independent experiments, each in triplicate. The autoradiographys in the upper show representative experiments.

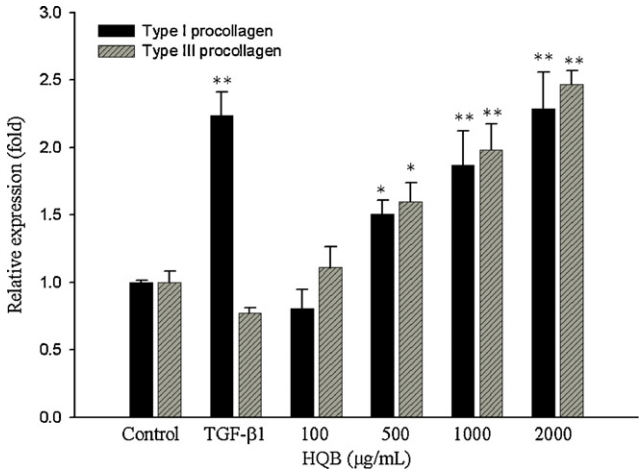

Fig. 6. Dose response of HQB on type I and type III procollagen mRNA expression in human dermal fibroblasts (HDF_{81y}). HDF_{81y} cells were treated with various concentrations of HQB for 48 h. The comparative changes in gene expression of type I and type III procollagen were determined by real-time RT-PCR analysis, as described in Section 2. Values were normalized using β-actin mRNA levels as a reference. The results are expressed as mean ± S.D., $^{*}p < 0.05$, $^{**}p < 0.01$: compared with control group (culture medium only). The results were verified by the repetition of three independent experiments, each in triplicate.

(RT-PCR). It was found that HQB significantly up-regulated type I and type III procollagen mRNA expression of HDF_{81y} cells ($p < 0.05$) (Table 2). In addition, the expression of type III procollagen was slightly higher than that of type I procollagen in both HQB-treated cells. In contrast, there was no marked expression of these two kinds of procollagen mRNA in HQNB-treated cells. Furthermore, HQB showed dose-dependent effects on the expression of type I and type III procollagen mRNA in HDF_{81y} cells ($p < 0.05$) (Fig. 6).

3.5. Effect of HQB and HQNB on TGF-β1 and CTGF mRNA expression in cultured human dermal fibroblasts

To study the up-regulation mechanism of HQB-induced procollagen expression, the effects of fermented and non-fermented samples on the expression of TGF-β1 and connective tissue growth factor (CTGF) in both HDF cells were investigated. Quantitative RT-PCR analysis revealed that HQB (500 μg/mL) significantly increased TGF-β1 mRNA expression in HDF_{81y} cells after 12 h of incubation ($p < 0.05$) (Table 2). However, similar to

Table 2
Effects of HQNB or HQB on type I, type III procollagen, TGF-β1, and CTGF mRNA expression in human dermal fibroblasts (HDF_{81y}).

	Cell treatment[1]		
	TGF-β	HQNB	HQB
	Relative expression over control group[2]		
Target gene/culture time	12 h	48 h	48 h
Type I procollagen	$1.56 \pm 0.06^{*}$	0.98 ± 0.11^{b}	$1.73 \pm 0.11^{*a}$
Type III procollagen	0.96 ± 0.07	0.92 ± 0.15^{b}	$1.90 \pm 0.08^{*a}$
	3 h	12 h	12 h
TGF-β1	–	1.02 ± 0.12^{b}	$1.39 \pm 0.07^{*a}$
CTGF	$2.13 \pm 0.15^{*}$	0.83 ± 0.05^{a}	0.74 ± 0.08^{a}

[1] HDF cells were treated with 500 μg/mL of HQNB or HQB or 10 ng/mL of TGF-β (positive control).
[2] The comparative changes in gene expression of type I, type III procollagen, TGF-β1, and CTGF were determined by real-time RT-PCR analysis, as described in Section 2. Values were normalized using β-actin mRNA levels as a reference. The results are expressed as mean ± S.D.
* $p < 0.05$: compared with control group (culture medium only). Mean with different letters (a, b) within the same culture time are significantly different among HQNB- and HQB-treated groups for each target gene. The results were verified by the repetition of three independent experiments, each in triplicate.

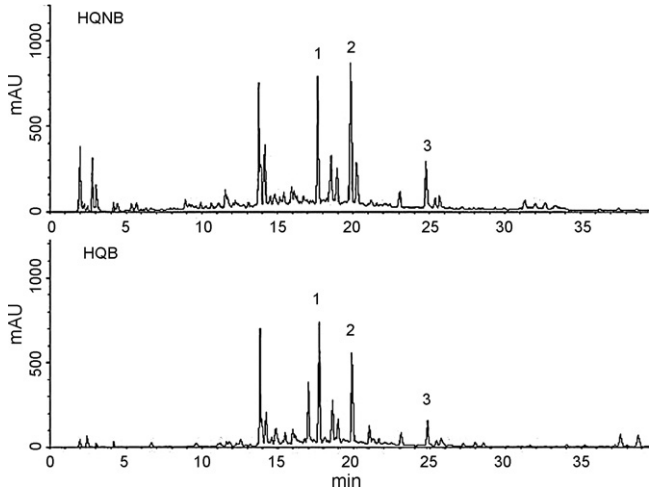

Fig. 7. The HPLC profiles of isoflavonoids in HQB and HQNB. (1) ononin; (2) calycosin; (3) formononetin.

Table 3
Contents of isoflavonoids in HQNB and HQB.

Samples	Isoflavonoids (μg/g) ($n = 3$)		
	Ononin	Calycosin	Formononetin
HQNB	194.08 ± 7.78^{a}	99.62 ± 1.76^{a}	30.71 ± 2.81^{a}
HQB	173.46 ± 8.25^{b}	59.85 ± 3.01^{b}	7.68 ± 0.37^{b}

[a,b]Means with different letters within a column are significantly different ($p < 0.05$).

the observations on the expression of type I and type III procollagen mRNA, HQNB was not able to induce TGF-β1 mRNA expression in both HDF cells. Also shown in Table 2, TGF-β1 significantly elevated the levels of CTGF mRNA in HDF_{81y} cells ($p < 0.05$) after 3 h of incubation. However, both of the fermented and non-fermented samples did not significantly affect the levels of CTGF mRNA in both HDF cells ($p > 0.05$).

3.6. Changes of isoflavonoids in R. astragali after fermentation

It has been revealed that isoflavonoids is one of the major bioactive components in *R. astragali* responsible for pharmacological activities and therapeutic efficacy [8,9]. Hence, the isoflavonoids compositions of HQB and HQNB were determined by HPLC, and their chromatographic profiles were shown in Fig. 7. There were three major active isoflavonoids, identified as ononin, calycosin, and formononetin found in *R. astragali*. After fermentation, all of these three compounds were significantly reduced as shown in Table 3 ($p < 0.05$).

4. Discussion

Chinese herbs have been used in clinical applications for many centuries. Driven by rising consumer interest and demand for natural products, there has been great interest and attention in the application of Chinese herbs in the development of skincare cosmetics [29]. Currently, various Chinese herbs are used in skin care cosmetics and claimed that they can improve the physical appearance of aged skin. To our knowledge, however, few claims are based on substantial scientific evidence. Since the ethnobotanical data revealed that *R. astragali* has been used clinically as skin applications [30], and microbial fermentation might be a useful tool to enhance the skin care function of Chinese herbs, this study investigated the effects of the non-fermented and fermented *R. astragali* on the biosynthesis of collagen in human dermal fibroblasts.

Skin, like all other organs, ages with time progresses. It is known that the mechanism for skin photoaging is due to the production of reactive oxygen species (ROS) induced by UV irradiation. The activation of ROS leads to a series of signal transduction and stimulate the gene transcription of matrix-degrading enzymes, such as MMP-1 (collagenase) and MMP-9 (gelatinase) [1,31]. On the other hand, the mechanism for chronological aging is not as well understood as that for photoaging. It has been reported that chronological aging is at least partially due to ROS-induced MMPs activation and to the slow proliferation of fibroblasts [32–34]. Nevertheless, both chronological aging and photoaging involve the expression and degradation of several molecules participating in the metabolism of the connective tissue, including changes in the amount of ECM components (e.g. collagen and elastin), and in MMPs activity.

The balance of collagen synthesis and degradation is crucial to the synthesis of collagen in human skin. Our study showed that HQB and HQNB (800 μg/mL) inhibited enzyme activities of collagenase, gelatinase and elastase, and the inhibitory effects on collagenase and gelatinase (24–28%) were significantly higher than that on elastase (12–7%). An et al. [6] reported that 100 and 500 μg/mL of persimmon leaf extract inhibited collagenase and elastase activity by 15.2% and 78.1%, respectively. Kim et al. [35] demonstrated that even 10 ppm of soy isoflavones could inhibit the UVB-induced expression of MMP-1 in fibroblasts. Oral feeding of polyphenols (0.2%) extracted from green tea to SKH-1 hairless mice also resulted in inhibition of UVB-induced expression of matrix-degrading MMPs, such as MMP-2 (67%) and MMP-3 (63%) [36]. It appeared that the inhibitory effects of HQB on collagenase, gelatinase, and elastase activities were not as prominent as the samples investigated in the above-mentioned studies. Hence, it appeared that the role of HQB in the skincare function is largely rely on its effect on the collagen synthesis, but not on its inhibitory effect of collagen degradation.

It has been reported that the growth potential of fibroblasts isolated from tissue fragments of different donor age declined with increasing age, but increased by 3-fold after topical application of 1% retinol on 80+ y old individuals for 7 d [37]. Our study showed that the non-fermented *R. astragali* (HQNB, 500 μg/mL) increased the growth of HDF_{22y} and HDF_{81y} cells by 1.26- and 1.15-fold, respectively. Chinese herbs generally contain amino acids, trace elements, polyphenols, flavonoids, saponins, and other bioactive ingredients which have been demonstrated to be able to enhance proliferation, DNA expression, protein synthesis and metabolism rate of cells [38,39]. The 4-hydroxy-5-hydroxymethyl-[1,3]dioxolan-2,6-spirane-5,6,7,8-tetrahydro-indolizine-3-carbaldehyde (HDTIC) extracted from *R. astragali* showed the senescence-delaying effect on human fibroblasts by its potentials of proliferation improvement, inhibitory effect of advanced glycation end products formation, and its antioxidant activity [13]. Together, these findings and the results of our study suggested that *R. astragali* was one of the effective anti-aging Chinese herbs.

In the culture of human dermal fibroblasts, fermented (HQB), but not non-fermented *R. astragali* (HQNB), significantly and dose-dependently (50–1000 μg/mL) elevated the expression of mRNA and protein for type I and type III procollagen. Contrary to the cell proliferation effect, HQB showed a greater enhancing activities on type I procollagen mRNA expression and protein synthesis in HDF_{81y} cells, compared with HDF_{22y} cells. Although the type I collagen is the predominant collagen found in skin (ca. 80%), type III collagen (15%) is the major collagen responsible for the elastic properties in human skin. Interestingly, we found that HQB had

more pronounced effect on the expression of type III procollagen mRNA in both HDF cells than that on type I procollagen mRNA. Furthermore, the results of real-time RT-PCR analysis revealed that the effect of HQB on procollagen production in HDF cells was by transcriptional regulation. On the other hand, HQNB did not induce the expression of procollagen mRNA and protein, even though it showed a significant proliferation effect on both HDF cells. These results demonstrated that fermentation is indeed a potential method for enhancing the skincare function of traditional Chinese herbal medicines.

It is well known that TGF-β1 promotes collagen synthesis [40,41]. Much evidence indicates that the inductive effects of TGF-β1 on procollagen production are mediated by CTGF [42,43]. Here we demonstrated that 500 μg/mL of HQB significantly increased the levels of TGF-β1 mRNA in HDF_{81y} cells, but had no effect on CTGF expression. These observations indicated that TGF-β1 transcripts induced by HQB might be partially responsible for the stimulation of procollagen expression in cultured HDF cells. Holmes et al. [44] have identified a functional Smad binding site in the *CTGF* promoter. Induction of *CTGF* by TGF-β1 is dependent on Smad3 and Smad4 but not Smad2. In the mean time, studies have shown that TGF-β-induced expression of type I collagen is mediated via the Smad signal transduction pathway as well as phosphatidylinositol-3 (PI-3) kinase/Akt/mTOR pathway in human dermal fibroblasts [45–47]. Akt activation, however, suppresses activation of Smad3 in an Akt kinase-dependent manner through mTOR [48]. Therefore, we suspected that TGF-β1-induced expression of procollagen in HQB-stimulated fibroblasts might be through PI-3 kinase/Akt/mTOR-dependent pathway, which suppress CTGF expression.

As shown in Table 2, HQNB had little effect on TGF-β1 mRNA expression, but showed potent mitogenic activity in HDF cells (Fig. 3A, B). Recent studies indicated that estrogen- (17β-estradiol) and phytoestrogen- (genistein, ginsenoside Rg1) stimulated mitogenesis in human breast cancer cells (MCF-7) could also be mediated by the enhancement of IGF-1 receptor (IGF-1R) signaling pathway [49–52]. It was supported by the up-regulation of IGF-1R and insulin-receptor substrate expressions which appeared to require estrogen receptor in MCF-7 cells treated with estrogen or phytoestrogen. In addition, formononetin, an isoflavonoid phytoestrogen, can induce the expression of the estrogen-responsive reporter gene in MCF-7 cells, as well as the proliferation of MCF-7 cells and mammary gland [53,54]. In this study, there were three major active isoflavonoids, identified as ononin, calycosin, and formononetin found in HQNB (Table 3). Since earlier investigations have shown that estrogen receptor does exist in the culture of human skin fibroblasts [55], therefore, we speculated that the mitogenic activity of HQNB in fibroblasts might be mediated by the activation of crosstalk between estrogen receptor and IGF-1R signaling cascade. In the mean time, we cannot yet exclude the possibility that other signaling pathway is also involved in the proliferative effect of HQNB on HDF cells. Future study is needed to elucidate the detailed mechanism as well as to provide insights for understanding the complex actions of both HQB and HQNB in HDF cells.

Hormone replacement therapy is one of the current available therapeutic approaches for aging skin [56]. It has been reported that genistein [7,57,58], asiaticoside [5,59], and ginsenosides metabolites [60] enhanced the biosynthesis of collagen and hyaluronic acid in human skin cells. The flavonoids found in *R. astragali* mainly consist of isoflavones, of which calycosin, formononetin, and ononin are three known major components. In this study, we found that the content of calycosin, formononetin and ononin in HQB was much less than that in HQNB, suggesting that β-glucosidase from *B. subtilis* natto might have converted these isoflavones to aglycones during fermentation, and the produced aglycones from their respective glycosides might be the bioactive components, at least partially, responsible for the skincare functions found in this study. However, due to the complexity of the constituents in the fermentation broth, further investigation is needed in order to identify all of the components in the fermented product which are responsible for the enhancing activities of HQB on procollagen synthesis in HDF cells.

5. Conclusion

This study provides positive and strong evidence in support of the enhancing activities of HQB on procollagen biosynthesis in human dermal fibroblasts isolated from human skin of two ages (22 y and 81 y). We demonstrated that the collagen biosynthesis enhancement is, at least in part, due to the mitogenic activity of HQB in HDF cells and the regulation of procollagen biosynthesis resulting from HQB-induced TGF-β1 expression. Given the low toxicity and the stimulating effects on collagen production of HQB, we expect that HQB may have a promising skincare application for internal and external uses. Further studies are required to identify the active components in HQB that are responsible for the positive effects on skin and the mechanisms of their skincare functions.

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