Keratinocyte Proliferative and Wound Healing Effects of Edible Bird’s Nest Extract on Human Skin

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ABSTRACT

Edible bird’s nest is a delicacy of Chinese cuisine. Its major components include mucin and sialic acid in addition to epidermal growth factor-like substances. However, its effects on skin cells and skin barrier function currently remain unknown. Therefore, we herein examined the effects of edible bird’s nest extract (EBN) on skin cell proliferation, wound healing, and tight junction proteins as well as human skin barrier function. The results obtained showed that EBN (0.1%) enhanced the proliferation of keratinocytes and fibroblasts as well as the migration and tight junction protein expression by keratinocytes. The oral ingestion of EBN (70 mg/day) standardized by sialic acid for 1 month reduced transepidermal water loss, the shallow wrinkle area, and dermal thickness. These results suggest that EBN exerts epidermal protective and smoothing effects through keratinocytes. Thus, EBN is a valuable ingredient for maintaining healthy skin. (Int J Biomed Sci 2020; 16 (4): 43-51)

Keywords: Edible bird’s nest; keratinocyte; wound healing; tight junction; claudin; transepidermal water loss

INTRODUCTION

Edible bird’s nest, so-called swallow’s nest, is produced from the nests of swiftlets in South East Asian countries and Southern China. It is formed by solidified interwoven salivary secretions from male swiftlets during the breeding period. Edible bird’s nest is a delicacy in Asian countries (1) and is also used for medicinal purposes (2), including wound healing. According to ancient Chinese literature, bird’s nest exerts healing effects on tuberculosis, hematemesis, chronic diarrhea, chronic malaria, and pulmonary infections. Bird’s nest is regarded as an important food ingredient in Chinese cuisine for the maintenance of health and youthful facial skin. Its components include amino acids (3), peptides (4), minerals, sialic acid derivatives (5, 6), and glycoproteins (7), such as chondroitin (8).

Edible bird’s nest exerts a number of biological effects. It has been shown to enhance corneal keratocyte proliferation (9) and mitogenic responses in lymphocytes (10). It also exhibits epidermis growth factor (EGF)-like activity (11) and development of uterine structure (12) and its function (13). Moreover, it has been shown to suppress high fat diet-induced oxidative stress (14), procoagulant activity...
(15), and insulin resistance (16). However, even though it exhibits epidermal growth factor (EGF)-like activity (11), the effects of edible bird’s nest extract (EBN) on skin cells remain unknown. Therefore, we herein investigated the effects of EBN on skin cells, particularly facial skin, as well as its wound healing effects.

MATERIALS AND METHODS

Preparation of EBN

EBN was manufactured by Toyo Koatsu Inc. (Hiroshima, Japan). Dried edible bird’s nest was powdered by grinding and then treated using a patented (Japanese Patent No. 3475328) enzymatic digestion method under high pressure conditions (http://www.toyokoatsu.co.jp/toyoko-c2/m_extract.htm). After filtration, the solution was concentrated under reduced pressure to obtain EBN. Bird’s Nest Extract-P was prepared by Oryza Oil & Fat Chemical Co., Ltd. by mixing EBN with dextrin in a 1:4 ratio. The contents of sialic acid in EBN and Bird’s Nest Extract-P were 7.5 and 1.5%.

Cells

Normal human keratinocytes (Product No. KK-4009) were purchased from Kurabo Co., Ltd. (Neyagawa, Japan). Cells were maintained in keratinocyte growth medium (Product No. KK-2150S, HuMedia-KG2, Kurabo). TIG-108 (JCRB0537), a normal human skin fibroblast cell line (diploid cells established from a Japanese woman aged 40 years old), was obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were pre-cultured in Dulbecco’s modified Eagle’s medium (D-MEM) supplemented with a 10% fetal bovine serum (FBS), penicillin (100 units/mL), and streptomycin (100 μg/mL) mixture.

Cell proliferation

Keratinocytes (5 × 10^4 cells/100 μL/well) suspended in HuMedia-KG2 were seeded on 96-well culture plates. After cell adhesion, the medium was replaced to supplement- and serum-free culture medium. EBN solution was added and cells were cultured for 24 hr. Cell proliferation was assessed using the N’-anilino-N’-[4,5-dimethyl-1,3-thiazol-2-yl]iminobenzene carbboximidamide (MTT) assay. TIG-108 (5 × 10^3 cells/100 μL/well) cells were seeded on 96-well culture plates. After the confirmation of cell adhesion, wells were washed with phosphate-buffered saline (PBS). FBS-free D-MEM was added to the well and EBN solution was then added (final concentration: 0.05-0.5%). Cells were cultured for 24 hr and cell proliferation was evaluated by the MTT assay. Cell proliferation rate was calculated by the following formula.

In vitro wound healing model

The experiment was described by Wu et al. (17). Keratinocytes (2 × 10^5 cells/mL/well) were seeded on 12-well culture plates. After the confirmation of cell attachment, the medium was replaced with fresh supplement- and serum-free culture medium. Cells were cultured for 24 hr. The cell layer was scratched with a micropipette tip (200 μL) and cell images were captured microscopically. Cells were cultured for another 24 hr and wound images were captured again. The keratinocyte migration area was analyzed by ImageJ 1.52 V (NIH, USA) and indicated as pixel values.

RT-PCR of claudins, tight junction (TJ) proteins

Keratinocytes (5 × 10^5 cells/500 μL/well) were seeded on 24-well culture plates. After the confirmation of cell attachment to the bottoms of the plates, medium was replaced with fresh supplement- and serum-free culture medium and then cultured overnight. The medium was removed and replaced with fresh medium (500 μL) containing EBN. Cells were cultured for 12 hr and then lysed using the solution included in the RNA extraction kit (NucleoSpin RNAII, Takara Bio Science, Kusatsu, Japan). Total RNA was extracted and cDNA was synthesized by a routine procedure. A real-time PCR analysis was performed using SYBR Premix EX Taq (Takara Bio Science) and the following primers: GAPDH: forward 5’-AAGGTGAAGGTCGGAGTCAC-3’, reverse 5’-GGGGTCATTGATGGCAACAATA-3’, claudin-1: forward 5’-GCTCTAGAATTCCGAGCGAGTCATGGC-3’, reverse 5’-GCTCTAGAATTCCGAGCGAGTCATGGC-3’, claudin-4: forward 5’-GCTCTAGAATTCCGAGCGAGTCATGGC-3’, reverse 5’-GCTCTAGAATTCCGAGCGAGTCATGGC-3’. Western blotting of claudins

Human normal keratinocytes (3 × 10^6 cells/2 mL/well) in HuMedia-KG2 were seeded on 6-well culture plates. After the confirmation of cell attachment on the bottoms of the plates, the medium was replaced with fresh supplement- and serum-free culture medium and then cultured overnight. The medium was removed and fresh medium (2 mL) containing EBN (0.05, 0.1, and 0.5%) was added to the wells. Cells were cultured for 72 hr and collected using radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher Scientific). The following primary antibodies were used in the Western blotting analysis: a claudin-1 antibody
(Santa Cruz Biotechnology Inc., ×1,000 dilution), claudin-4 antibody (Santa Cruz Biotechnology, Inc., ×1,000 dilution), and β-actin antibody (SIGMA ALDRICH, ×5,000 dilution).

**Immunostaining of claudin-4**

Normal human keratinocytes (3 × 10⁵ cells/2 mL/well) in HuMedia-KG2 were seeded on cover glasses (Matsunami glass, Kishiwada, Japan) in 6-well culture plates. After the confirmation of cell attachment on the cover glass, the medium was replaced with fresh supplement- and serum-free culture medium and then cultured overnight. The medium was removed and fresh medium (2 mL) containing EBN (0.1%) was added to the wells. Cells were cultured for 96 hr and fixed by 4% paraformaldehyde solution. Then cell surface was washed with PBS and reacted with signal enhancer solution (Image-iT FX Signal Enhancer, Life Technologies) for 30 min. Blocking was performed with 1% bovine serum albumin (BSA) for 1 hr. The preparation was washed with PBS and then soaked in anti-claudin-4 antibody solution (×100 dilution) at 4°C overnight. After washing with PBS, the preparation was soaked in a fluorescence secondary antibody (Alexa Fluor® 546 Donkey Anti-goat IgG, Life Technologies) solution (×400 dilution) for 1 hour. After washing with PBS and embedding, fluorescence images of claudin-4 were captured under a fluorescence microscope.

**Clinical trial**

The pilot clinical trial was performed according to the Declaration of Helsinki (2013 revision, Fortaleza, Brazil) and conducted in conformity with ethical considerations. The Ethics Committee of Oryza Oil & Fat Chemical Co., Ltd. was convened to assess the ethicality and appropriateness of the study protocol. The present study was implemented according to the protocol approved by the Ethics Committee, and any substantial protocol deviations required authorization by the committee.

Five female employees in our company aged between 27 and 57 years were recruited. Exclusion criteria were as follows: 1) current use of medication to treat chronic diseases, 2) allergy to the test product, 3) atopic dermatitis, and 4) the use of oral or topical medications. After a full explanation of the study protocol, including the purpose, methods, and test product, as well as the voluntary nature of participation and the right to refuse to participate without penalty, written consent was obtained from all subjects. Subjects were asked to maintain a regular lifestyle during the study period. The test product (brown capsules containing EBN) was prepared by Oryza Oil & Fat Chemical Co., Ltd. Capsules comprised 70 mg of Bird’s Nest Extract-P (standardized EBN, Oryza Oil & Fat Chemical Co., Ltd.), which contained 1.5% sialic acid.

The present study was conducted as an uncontrolled before and after study (18, 19). Subjects took one capsule daily with water after breakfast for 4 weeks. Skin conditions were examined before the start of treatment and after 4 weeks of the intervention. The outcomes assessed were facial cheek skin texture, moisture, elasticity, transepidermal water loss (TEWL), the collagen score, epidermal and dermal thicknesses, and ultrasound images of the skin.

All skin parameters were measured in an air-conditioned room at 25 ± 1°C and 50 ± 5% RH. Prior to assessments, subjects washed their faces. After wiping, subjects were acclimated for 30 min. Skin elasticity was measured at the mid-point of the line between the right eye and ear by Triplesense (MORITEX Co., Saitama, Japan). TEWL was assessed using a Tewameter (TM-300, Integral, Japan). Collagen scores and dermal thicknesses were evaluated by DermaLab (Cortex Technology, Hadsund, Denmark). Ultrasound images were analyzed by ImageJ 1.52v using the steps summarized in Fig. 1A. The images obtained of the dermis were divided into a saturated area [hyper-dense extracellular matrix (ECM) area], green area (normal ECM area), and low density shadow area including fat tissue. After subtracting the epidermis image (upper white area) from the pictures, saturated area, green area and shadow area were calculated and output as pixel sizes. Skin surface images were captured using a digital microscope and images of skin texture, particularly shallow wrinkles, were extracted by ImageJ 1.52 V, as shown in Fig. 1B.

**Statistical analysis**

The results of cell-based studies are expressed as the mean ± SE. The significance of differences was examined by a one-way ANOVA, followed by Dunnnett’s test, and *p*<0.05 was considered to be significant. Clinical results are indicated as the mean ± SD. A two-tailed paired *t*-test was used for comparisons between before and after the ingestion of EBN. A probability of *p*<0.05 was considered to be significant.

**RESULTS**

**Keratinocyte and fibroblast proliferation by EBN**

We examined the proliferative effects of EBN on human skin cells (epidermal keratinocytes and dermal fibroblasts). Since keratinocytes play a crucial role in skin barrier function, it is important to evaluate the proliferative
effects of EBN on these cells. Fibroblasts promote ECM and collagen contributes to wound healing and skin elasticity. The results obtained showed that EBN promoted the proliferation of keratinocytes at more than 0.05% and fibroblasts at more than 0.1% (Fig. 2).

**In vitro wound healing effects of EBN**

The effects of EBN on wound healing were examined using the scratch test. As shown in Figure 3A, keratinocyte migration to the scratched cell gap was enhanced by EBN (0.05 and 0.1%). EBN (0.1%) significantly enhanced cell migration, as shown in Figure 3B. These results suggest that EBN promotes wound healing.

![Figure 1](image1.png)

**Figure 1.** Image processing of skin. A) Ultrasound images obtained using DermaLab were divided into RGB images. The saturated area, indicating a hyper-dense ECM, was extracted from red images. Green images show the dermis. Shadows, indicating fat areas, were extracted from blue images. B) Skin surface images obtained using a contrast microscope and converted to a 16-bit image. Shadows in images were enhanced and threshold values were assessed. The shallow wrinkle area was analyzed. These results were shown in Table 1.

![Figure 2](image2.png)

**Figure 2.** Proliferative effects of EBN on keratinocytes and fibroblasts. Each column represents the mean ± SE (n=4). Asterisks denote significant differences from the control at **: \( p < 0.01 \).

![Figure 3](image3.png)

**Figure 3.** *In vitro* wound healing effects of EBN on the keratinocyte layer. A) Cell layer images. B) Area occupied by keratinocytes after the EBN treatment. Each column represents the mean ± SE (n=3-4). The asterisk denotes a significant difference from the EBM untreated group at *: \( p < 0.05 \).
Claudin expression by EBN

TJ connect two closely associated adjacent cells, the membranes of which join together to form a virtually impermeable barrier to fluid. Occludin and claudin are integral plasma membrane proteins located at TJ (20). They form a strong barrier that protects tissue from invading microorganisms, prevents excessive water loss, and selectively transports small solutes through the skin (21) (Fig. 4A). The mRNA expression levels of claudin-1 and -4 were increased by 0.1% EBN (Fig. 4B). The protein expression levels of claudin-1 were increased by EBN from 0.05 to 0.5% and those of claudin-4 by 0.05 and 0.5% (Fig. 4C). Fluorescent

![Image]

**Figure 4.** Effects of EBN on claudin expression in keratinocytes. A) Structure of the epidermal barrier, including tight junctions; B) mRNA expression of claudin-1 and -4. Each column represents the mean ± SE (n=4). Asterisks denote significant differences from the EBN untreated group **: p<0.01; C) Western blotting images and expression ratio of claudin-1 and -4; D) Fluorescent immunostaining images of claudin-4.
images of claudin-4 in EBN-treated cells showed that its expression was localized at the cell membrane (Fig. 4D).

Clinical effects of EBN on skin conditions

Table 1 shows the effects of standardized EBN on epidermal and dermal conditions. TEWL was significantly reduced by EBN, whereas skin moisture was not markedly affected. A microscopic image analysis of the skin surface showed that shallow wrinkle areas and finesse of skin texture were reduced by EBN (Table 1). The following dermal parameters were not markedly affected by EBN: elasticity, the collagen score, and ultrasound image parameters, whereas a significant reduction was observed in dermal thickness.

DISCUSSION

Edible bird’s nest is considered to be good for the skin and its soup is widely consumed as a Chinese delicacy to maintain beautiful skin. As the shape and texture of edible bird’s nest resembles collagen jelly, some people believe that it can supply facial collagen or improve the skin condition such as sagging skin. EBN contains glycoproteins and polysaccharides that have a collagen-like texture when suspended in water. However, it currently remains unclear whether EBN is beneficial for the skin, such as dermal skin elasticity and epidermal barrier function. Based on this simple background, we speculated that EBN may affect epidermal conditions and the structure of the dermis. The in vitro study of EBN revealed its proliferative effects on keratinocytes and fibroblasts. In terms of the cell proliferation effect of EBN except on skin cells, EBN has been previously reported to proliferate retinal cells and uterine cells (9). Thus, its effect is not specific to skin cells but it may act as an EGF-like substances (12). In the present study, EGF was not added to the culture medium of the keratinocyte and fibroblast culture systems. EBN has been reported to exhibit EGF-like activity, but an EGF has not been identified in EBN (11). Although we attempted to detect and measure EGF in EBN using a Western blot analysis with an anti-EGF antibody, we were unsuccessful. Moreover, sialic acid, a major constituent in EBN (5), did not appear to contribute to its cell proliferative effects because sialic acid itself is released from fibroblasts and accumulates in culture medium (22) under normal culture conditions. Thus, the proliferative effects of EBN on fibroblasts appear to be induced by the direct effects of unknown substances in EBN that exhibit EGF-like activity. In contrast, previous findings indicated that sialic acid plays a role in the proliferation of keratinocytes because alpha 2,3-sialic acid, a sialic acid derivative, has been shown to control keratinocyte proliferation and differentiation (23) and sialic acid exists in skin keratinocytes (24). However, sialic acid on the surface of keratinocytes has been suggested to suppress proliferation and induce differentiation (25, 26). Therefore, in the keratinocyte culture system, sialic acid may enhance the differentiation rather than proliferation of keratinocytes.

In addition to its proliferative effects, EBN enhanced the migration of keratinocytes. In the cell scratching mod-

| Table 1. Effect of EBN on epidermal and dermal parameters in cheek skin |
|-----------------------------|-----------------------------|
| Before | After |
| TEWL (g/m²·hr) | 16.8 ± 1.6 | 11.7 ± 1.0** |
| Shallow wrinkle area, indicating skin texture (<1,000 pixel) | 143 ± 12 | 116 ± 19** |
| Thickness of the epidermis (pixel) | 14.2 ± 1.8 | 12.6 ± 1.6 |
| Thickness of the dermis (pixel) | 81.1 ± 10.9 | 69.8 ± 12.4* |
| Moisture (%) | 64.2 ± 1.4 | 54.9 ± 2.0 |
| Elasticity | 18.3 ± 2.0 | 18.6 ± 0.5 |
| Collagen score | 39.0 ± 5.1 | 38.2 ± 4.3 |
| Saturated area (pixel) | 1913 ± 1167 | 1815 ± 1494 |
| Green area (pixel) | 10211 ± 993 | 9907 ± 2802 |
| Shadow area (pixel) | 13650 ± 4153 | 12544 ± 3677 |

Each value represents the mean ± SD (n=5). Asterisks denote significant differences from the value before intervention at *: p<0.05, **: p<0.01.
el of the keratinocyte layer, the occupation of the wound area by keratinocytes has been attributed to migration, not proliferation (27). Keratinocytes play crucial roles in the wound healing process by covering the wounded area as an initial step (28). Thus, the EBN-induced migration of keratinocytes is suggested to be beneficial in the wound healing process. Similar effects have been reported with aloe vera extract (29), which has traditionally been used to treat burned or wounded skin. It promotes the proliferation of keratinocytes (30), and glycoproteins (31) and aloesin (32), a benzopyran derivative, are involved in the effects. Regarding active compounds, since EBN is not a botanical extract, the active component promoting cell migration may be a glycoprotein. Mucin, a glycoprotein, has been identified as a component of EBN (33), and appears to contribute to keratinocyte migration. β-Glucans, fungus- or bacteria-derived β-D-glucose polysaccharides, also promoted the migration of HaCaT cells, a keratinocyte cell line (34). However, β-glucans did not enhance the proliferation of keratinocytes. EBN contains large amounts of polysaccharides, including sialic acid, and enhanced the proliferation of keratinocytes. Therefore, polysaccharides do not appear to be the active component in EBN.

Since the present results showed that EBN enhanced skin cell proliferation and keratinocyte migration, we examined the skin surface protecting effect on TJ (35). In the epidermis, TJ plays a crucial role in the formation and maintenance of epithelial barriers that prevent the invasion of infectious bacteria and harmful particles. In the stratum corneum, the epidermis, ceramide functions as the first skin barrier. TJ in the stratum granulosum acts as the second skin barrier. Therefore, in assessments of the skin barrier function of EBN, it is important to examine the expression of TJ proteins. In the present study, EBN increased the mRNA and protein expression levels of claudin-1 and -4. Both claudins play important roles in the pathology of atopic dermatitis, such as water retention and barrier function (36). Moreover, claudin-1 and -4 increase cell-to-cell adhesion (35). Therefore, EBN strengthens the structure of the epidermis through cell-to-cell binding among keratinocytes. These results demonstrate that EBN enhances epidermal barrier and wound healing by promoting the proliferation and migration of keratinocytes and strengthening TJ.

In terms of the clinical effects of EBN on the dermis and epidermis, we examined keratinocytes in facial skin. EBN ameliorated TEWL, which reduced moisture evaporation from the skin surface without affecting the thickness of the epidermis. These results suggest that EBN affects structures in the stratum corneum, including cornified envelopes and the keratin-filaggrin complex (37). In addition to the above effect, EBN may reduce TEWL by expression of TJ protein including claudins 1 and 4. Moreover, EBN improved shallow wrinkles and skin roughness. In addition to reductions in TEWL, EBN decreased the shallow wrinkle area. Continuous increases in TEWL have been shown to alter the appearance of the skin surface and cause shallow wrinkles and rough skin (38). Thus, EBN was suggested to reduce shallow wrinkles by decreasing TEWL.

Regarding fibroblast parameters, EBN reduced the thickness of the dermis without affecting the density or quality of ECM. Ultrasound images showed that dermal thickness is gradually reduced by aging (39) and the average thickness of the cheek dermis is 1.6 mm, which is thinner than that in the chin area (2.1 mm), at which the texture of skin is harder than that at the cheek (40). On the other hand, dermal thickness was found to be thinner in underweight subjects than in overweight subjects (39). A positive relationship has been reported between dermal thickness and body mass index (41). Thus, although the mechanisms by which EBN reduces dermal thickness currently remain unclear, many factors appear to be involved, such as weight loss, aging, and hormonal changes.

In conclusion, EBN enhanced the proliferation and migration of keratinocytes as well as TJ protein expression. These effects may contribute to reducing TEWL on facial skin, which, in turn, enhances skin surface barrier function and skin texture.

ACKNOWLEDGMENT

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ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Meaning</th>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>EBN</td>
<td>edible bird’s nest extract</td>
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<tr>
<td>D-MEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
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<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<tr>
<td>MTT</td>
<td>3′-[4,5-dimethyl-1,3-thiazol-2-yl]benzenecarboximidamide</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<td>RIPA</td>
<td>radioimmunoprecipitation assay</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>TEWL</td>
<td>trans epidermal water loss</td>
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<td>TJ</td>
<td>tight junction</td>
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CONFLICT OF INTERESTS

The authors declare that they have no known competing financial interests or personal relationships that may have influenced the work reported in this manuscript.

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