Original Article

Edible bird's nest extract downregulates epidermal apoptosis and helps reduce damage by ultraviolet radiation in skin of hairless mice

Shinya Masuda,[1] Yuko Makioka-Itaya,[2] Tetsuo Ijichi,[2] and Takamitsu Tsukahara[1,*]

[1]Kyoto Institute of Nutrition & Pathology, 7-2 Furuikedani Tachikawa, Ujitawara, Kyoto 610-0231, Japan
[2]Life Science Division, Combi Corporation, Nishibori, Sakura-ku, Saitama 338-0832, Japan

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The purpose of the present study was to examine whether daily intake of edible bird's nest extract reduced ultraviolet-induced damage to skin. Twenty-one female HR-1/Hos mice were divided into control (C, *n* = 7), low-dose (2 mg/kg body weight/day of edible bird's nest extract) (L, *n* = 7), and high-dose (20 mg/kg body weight/day of edible bird's nest extract) (H, *n* = 7) groups. With their left back skin covered with aluminum sheet to prevent exposure, mice were radiated with either ultraviolet A (20 J/cm^2) or ultraviolet B (40 mJ/cm^2) in an alternate manner once daily for 10 weeks. They were gavaged either a solution of saline or edible bird's nest extract every day. The moisture content of the ultraviolet-exposed right back skin was significantly higher in H than in C or L. Histochemical analysis showed that the number of apoptotic epidermal cells on the ultraviolet-exposed skin was significantly lower in L and H than in C. In H, the mRNA expression of superoxide dismutase 2 was significantly higher on ultraviolet-exposed skin than on unexposed skin. Our data suggested that edible bird's nest extract enhanced superoxide dismutase 2 expression and downregulated apoptosis in their epidermis, which likely helped reduce skin damage.

***Key Words*: apoptosis, edible bird's nest extract, skin damage, superoxide dismutase, ultraviolet**

Edible bird's nest (EBN) is a well-known Chinese dish. EBN contains regurgitated saliva of male swiftlets, and thus it is rich in mucinous glycoproteins such as chondroitin glycosaminoglycans and sialylglycoconjugates.[1,2] Consecutive supplementation with EBN is believed to confer beneficial effects such as healthy skin and hence it is often used for medical purposes. Previous studies showed that EBN exhibited epidermal growth factor (EGF)-like activity.[3] Furthermore, digested EBN has been reported to inhibit melanogenesis *in vitro*.[4] We also reported that supplementation with EBN extract (EBNE) maintained dermal thickness of ovariectomized rats.[5] However, to date, there is little information of the effect of EBN supplementation on molecular and cellular processes, which could help improve the health of the host's skin.

Exposure of skin to ultraviolet (UVA and UVB) induces oxidative stress which can lead to serious damage of the epidermis. Both UVA (320–400 nm in wavelength) and UVB (290–320 nm) can penetrate the epidermis and induce DNA damage and apoptosis of epidermal cells.[6–8] Repeated X-ray radiation also increased radicals derived from melanin in the hair and tail skin of mice.[9] Exposure of urban particulate matter to epidermal keratinocytes induced oxidative stress and inflammation, and the supplementation of glycogen enzymatically synthesized from plant starch suppressed the inflammation by decreasing the accumulation of reactive oxygen species (ROS),[10] which suggest the significance of alimentary intervention for the improvement of skin dysfunction. Past work reported that EBNE could reduce production of ROS and improve cell viability in neuroblastoma cells,[11] and that it could downregulate the expression of apoptosis marker genes in rat brain.[12] Superoxide dismutases SOD1 and SOD2, also known as Cu/Zn-SOD and Mn-SOD, respectively, are ubiquitous enzymes that efficiently catalyze dismutation of superoxide anions.[13] In other words, superoxide anions generated by UV irradiation can be dismutated by these SODs to hydrogen peroxide, which is then detoxified by other antioxidant enzymes.[14] Furthermore, a previous study showed that the activity of SOD2 increased *in vitro* after UVA radiation, in time- and dose-dependent manners.[15] Based on this previous evidence, we theorized that supplementation with EBNE could also exhibit the additive effect of SOD activity, which in turn could help reduce oxidative stress and apoptosis of epidermal cells caused by UV radiation and hence, skin damage.

In the present study, we examined the antioxidative effect of EBNE orally supplemented on a daily basis, on the UV-damaged skin of hairless mice. Furthermore, to examine whether EBNE had an additive effect to UV radiation, we evaluated the expression levels of SOD in UV-exposed and unexposed epidermis obtained from the EBNE-treated hairless mice.

Materials and Methods

Animal experiments. All experimental procedures were approved by the Ethics Committee for Experimental Animal Use and Care of Kyoto Institute of Nutrition & Pathology (approval number: 10026CM). The experiment procedure for the UV-radiated mouse model was similar to that of a previous study,[16] with some modifications. Briefly, 21 5-week-old female HR-1/Hos mice were purchased from a commercial breeder (Japan SLC, Shizuoka, Japan). Mice were randomly and equally divided (*n* = 7) into control (C), low-dose (2 mg/kg body weight (BW)/day of EBNE) (L), and high-dose (20 mg/kg BW/day of EBNE) (H) groups. Enzymatically-digested EBNE (Collocalia; Combi Corporation, Tokyo, Japan) was dissolved in a saline solution, and approximately 0.1 ml of this solution was gavaged to mice with a commercial disposable feeding needle, on a daily basis (Fuchigami, Kyoto, Japan).

Mouse groups were housed in an environment-controlled (23–25°C; 40–60% humidity; 12:12 h light–dark cycle) room and kept in separate plastic cages. Throughout the study, mice were given semi-purified chow (AIN-93G, Research Diets Inc., NJ) as the basal diet and tap water *ad libitum*. Supplementation with

*To whom correspondence should be addressed.
E-mail: tsukahara@kyoto-inp.co.jp

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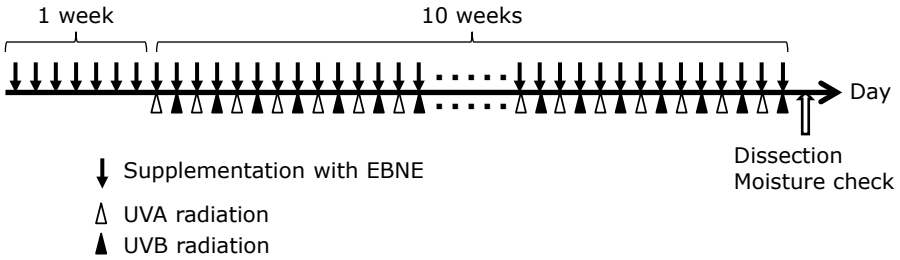

Fig. 1. Schematic presentation of the present experiment with edible bird's nest extract (EBNE) supplementation and ultraviolet ray (UV) radiation. Oral supplementation with EBNE was conducted daily (arrows). After one week, radiation with either UVA (white arrowhead) or UVB (black arrowhead) was conducted in an alternate manner once daily for 10 weeks. Oral supplementation with EBNE was continued during the UV radiation period.

Table 1. Primers and probes used in the present study

Gene name	Primers 5'-3'	GenBank accession number
Superoxide dismutase 1 (Cu/Zn-SOD) (*Sod1*)	F ctctcaggagagcattccatcat	NM_011434.1
	R cagggaatgtttactgcgca	
	P ccgtacaatggtggtccatgagaaacaa	
Superoxide dismutase 2 (Mn-SOD) (*Sod2*)	F gaacttcagtgcaggctgaaga	NM_013671.3
	R aacgccaccgaggagaagt	
	P tgtaacatctcccttggccagagcctc	
Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*)	F ggtgtcttcaccaccatgga	NM_008084.2
	R cagaaggggcggagatgat	
	P aaggccggggcccacttgaa	

Primers and probes were designed and synthesized by Bioreseach Technologies Japan (Tokyo, Japan).

EBNE started as soon as the mouse groups were divided, and a week after, mice were subjected to UV radiation for 10 weeks. Mice were exposed to either UVA (20 J/cm^2) for 50 min or UVB (40 mJ/cm^2) for 30 s in an alternate manner once daily, with the skin of their left back covered with an aluminum sheet (1 cm × 3 cm) to block UV radiation. In a preliminary work, we radiated UVA and UVB for 10 weeks to hairless mice given EBNE, but no skin damage was observed (unpublished data). Therefore, in the present study, to cause severe oxidative stress in the skin, mice were exposed to UVB for 30 min, not 30 s, on one day in week 8.

Prior to dissection of mice, the moisture content of skin in the right back (UV-exposed side) was measured using a commercial moisture meter (Moisture-checker MY-808S; Scalar, Tokyo, Japan). For dissection, all mice were first deeply anesthetized with an intraperitoneal injection of sodium pentobarbital (Somnopentyl; Kyoritsu, Tokyo, Japan). Samples were then collected from skin in both the right and left back sides of each mouse. Portions of the skin samples were immediately soaked in RNA-*later* solution (Thermo Fisher Scientific, MA) and kept at 4°C overnight, after which they were stored at −80°C until the mRNA expression analysis was conducted. The remains of the skin samples were fixed with 10% neutralized formaldehyde solution for histological staining. The procedure of the animal experiments is schematically shown in Fig. 1.

Histological staining. The fixed skin samples were embedded into paraffin wax and cut into 3-μm thick sections. To detect DNA fragmentation, the skin sections were then stained with terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL; *In situ* Apoptosis Detection Kit, Takara Bio, Shiga, Japan). One-cm digital images of the TUNEL-stained skin sections were captured using a light microscope equipped with a digital camera (DP25, Olympus). Afterwards, the number of TUNEL-positive nuclei in the epidermis of mice were counted.

Gene expression analysis. The methods for the RNA extraction and cDNA synthesis were the same as those previously described.[17] Real-time polymerase chain reaction (PCR) was conducted using a Rotor-Gene 6200 (Qiagen, Tokyo, Japan). Primers and TaqMan probes used in this study are listed in Table 1. Optimal primers and probes were obtained from Bioresearch Technologies Japan (Tokyo, Japan). The methods employed for PCR analysis were the same as those previously described.[17,18]

Statistical analysis. To estimate significant differences in TUNEL-positive cell numbers and the relative gene expression between groups, two-way analysis of variance (ANOVA; factors UV radiation and EBNE supplementation) and Tukey-Kramer post hoc comparisons were used. One-way ANOVA followed by Tukey-Kramer post hoc comparisons were used to evaluate significant differences in the moisture content between groups. Grubb's test was used to eliminate outliers. Data were considered statistically significant if $p<0.05$. Values are shown as the means ± SE.

Results

Moisture of the skin after UV radiation. The skin moisture contents in the skin of different mouse groups at week 10 are shown as percentages in Fig. 2. The skin moisture content was found to be significantly higher in H than in C ($p<0.01$) or L ($p<0.05$).

DNA damage in epidermis. Figure 3 shows the number of TUNEL-positive cells in the epidermis collected from left back skin (−UV) and right back skin (+UV). According to the results of the 2-way ANOVA, an interaction effect was observed. Therefore, we conducted multiple comparison tests to detect the differences between six groups (+UV_C, +UV_L, +UV_H, −UV_C, −UV_L, −UV_H). In C, the number of TUNEL-positive cells was found to be significantly ($p<0.01$) higher in the epidermis of +UV skin compared with that of −UV skin. In addition, the

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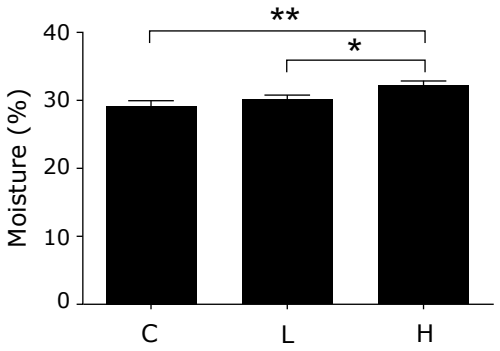

Fig. 2. Moisture content in UV-exposed (right back) skin after 10 weeks of UV radiation. Values are shown as the means ± SE ($n = 7$). *p<0.05, **p<0.01.

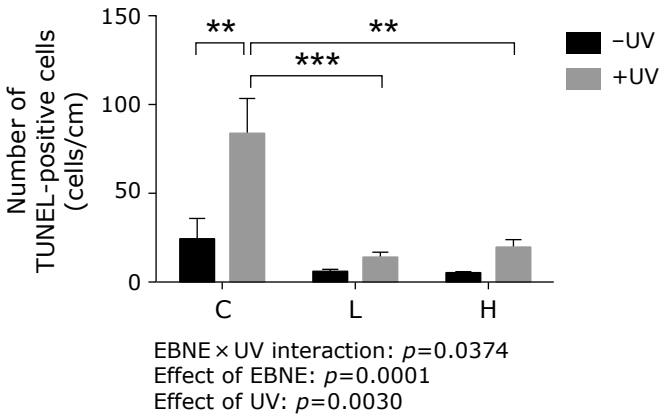

Fig. 3. The number of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL)-positive cells observed in 1 cm of epidermis from UV-exposed (right back, +UV) and unexposed (left back, −UV) samples. Values are shown as the means ± SE ($n = 6$–7). **p<0.01, ***p<0.001.

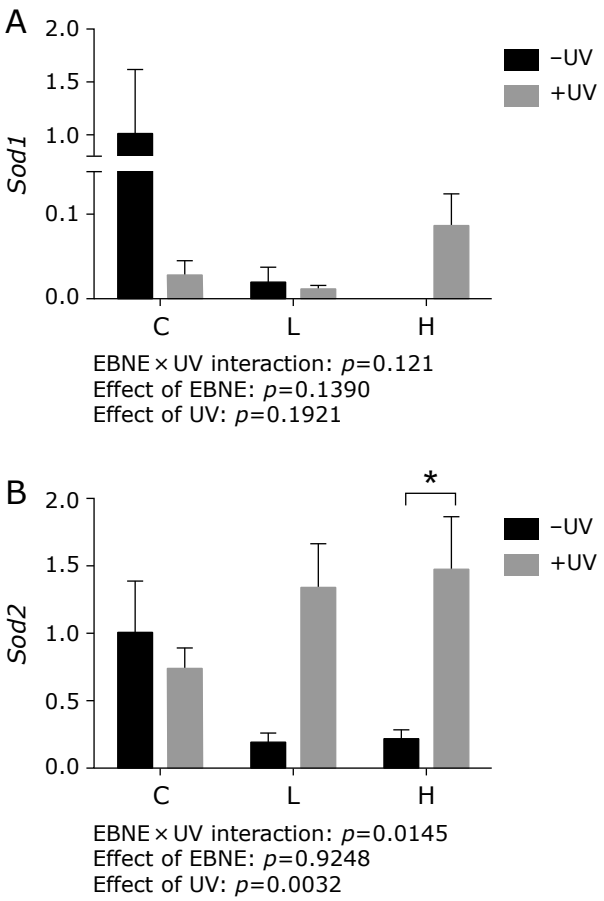

Fig. 4. Expression of (A) superoxide dismutase 1 (*Sod1*) and (B) superoxide dismutase 2 (*Sod2*) in UV-exposed (right back, +UV) and unexposed (left back, −UV) skin samples. Values are the means ± SE ($n = 6$–7). *p<0.05.

number of TUNEL-positive cells was significantly lower in +UV skin of L (p<0.001) and H (p<0.01) than in that of C.

Gene expressions of antioxidant enzymes. Figure 4 shows the expression of *Sod1* and *Sod2* in +UV and −UV skins. The gene expression of *Sod1* did not significantly differ after UV radiation and EBNE supplementation (Fig. 4A). Nonetheless, the 2-way ANOVA of *Sod2* showed an interaction effect. Therefore, we conducted multiple comparison tests to detect differences between the aforementioned six groups again. The expression of *Sod2* was significantly (p<0.05) higher in +UV skin of H than it was in −UV skin of the same mice (Fig. 4B).

Discussion

The results from the present study showed that although exposure to UV for 10 weeks stimulated apoptosis in the epidermis of mice, daily oral supplementation with EBNE helped reduce skin damage induced by this UV radiation.

Similar to the results from the present study, other recent studies conducted elsewhere also showed the protective effects of EBN against oxidative stress. For example, Murugan *et al.*[19] showed that EBN normalized ROS production and oxidative stress in mouse models of hyperglycemic aorta and endothelial cells. This effect was also observed in hippocampal samples of a rat model of lipopolysaccharide-induced neuroinflammation.[20] Moreover, EBN supplementation was reported to enhance antioxidant activity in blood and mRNA expression of antioxidant genes in the liver of obese mice fed a high-fat diet.[21,22] The gene expression related to neurodegeneration and apoptosis in the hippocampus and frontal cortex of ovariectomized rats also improved after EBN supplementation.[12] EBN supplementation was also found to help reduce apoptosis and ROS production in the human neuroblastoma cells of a Parkinson's disease model.[11] We believe that, however, the present study is the first work to show positive effects of EBN supplementation on apoptosis reduction in the skin caused by oxidative stress.

In the present study, an interaction between EBNE and UV exposure was observed in the expression of *Sod2*; however, it was not the case with *Sod1* expression (Fig. 4). While the reason for this discrepancy in the expression of SODs remains unclear, one possible explanation would be that the radiation dosage of UVA was much higher than that of UVB. The expression of *Sod1* is reportedly elevated by UVB radiation.[13] In contrast, UVA radiation increases SOD2 activity *in vitro*,[15] which seems to imply that UVA radiation stimulate the gene transcription of *Sod2* but not *Sod1*. Thus, it could be possible that in the present work, UVA and UVB differentially regulated the gene transcription of cytoplasmic *Sod1* and mitochondrial *Sod2*. We suggest that different irradiation patterns be studied to elucidate the underlying molecular mechanisms in UV-damaged skin following supplementation with EBN.

The moisture content after 10 weeks of UV radiation was higher in H than in the other mouse groups. Furthermore, our results showed that EBNE reduced epidermal apoptosis. These results suggest that daily supplementation with EBNE helped

maintain the protective epidermal barrier function. A reduced capacity of skin to retain moisture can lead to dry skin, which is associated with impairment of the epidermal barrier function.[23,24]

Although the present work did not examine the mechanisms by which oral supplementation with EBNE would exert positive effects on epidermal cells, *in vitro* supplementation with EBN to cultured cells was shown elsewhere to promote cell viability and to exhibit EGF-like activity.[3,11,25] Therefore, it is possible that certain components in EBN act directly on EGF receptors found on the surface of epidermal cells and help increase cell viability. Further investigation is needed to identify the key components in EBNE that confer anti-apoptotic effects to epidermis.

In the present work, the dose of EBNE for H was 20 mg/kg BW/day. This dose would correspond to about 1–2 g/day for humans, which would be a reasonable daily oral intake. Therefore, our results highlighted the significance of EBNE supplementation for a possible preventive and/or therapeutic use to treat damaged skin of both animals and human.

Author Contributions

YM-I, TI and TT designed the experiments. YM-I and TI supplied the materials. TT conducted the experiments. SM carried out the statistical analysis and wrote the draft of the manuscript. TT was responsible for the overall direction of the project and for editing the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest

YM-I and TI are employed by Combi Corporation. SM and TT are employed by Kyoto Institute of Nutrition & Pathology, which has received research funding from Combi Corporation.

References

1 Nakagawa H, Hama Y, Sumi T, *et al*. Occurrence of a nonsulfated chondroitin proteoglycan in the dried saliva of Collocalia swiftlets (edible bird's-nest). *Glycobiology* 2007; **17**: 157–164.
2 Pozsgay V, Jennings H, Kasper DL. 4,8-anhydro-*N*-acetylneuraminic acid. Isolation from edible bird's nest and structure determination. *Eur J Biochem* 1987; **162**: 445–450.
3 Kong YC, Keung WM, Yip TT, Ko KM, Tsao SW, Ng MH. Evidence that epidermal growth factor is present in swiftlet's (Collocalia) nest. *Comp Biochem Physiol B* 1987; **87**: 221–226.
4 Wong ZCF, Chan GKL, Wu KQY, *et al*. Complete digestion of edible bird's nest releases free N-acetylneuraminic acid and small peptides: an efficient method to improve functional properties. *Food Funct* 2018; **9**: 5139–5149.
5 Matsukawa N, Matsumoto M, Bukawa W, *et al*. Improvement of bone strength and dermal thickness due to dietary edible bird's nest extract in ovariectomized rats. *Biosci Biotechnol Biochem* 2011; **75**: 590–592.
6 Bruls WA, Slaper H, van der Leun JC, Berrens L. Transmission of human epidermis and stratum corneum as a function of thickness in the ultraviolet and visible wavelengths. *Photochem Photobiol* 1984; **40**: 485–494.
7 Khan AQ, Travers JB, Kemp MG. Roles of UVA radiation and DNA damage responses in melanoma pathogenesis. *Environ Mol Mutagen* 2018; **59**: 438–460.
8 Timares L, Katiyar SK, Elmets CA. DNA damage, apoptosis and langerhans cells--Activators of UV-induced immune tolerance. *Photochem Photobiol* 2008; **84**: 422–436.
9 Matsumoto KI, Ueno M, Nakanishi I, Indo HP, Majima HJ. Effects of low-dose X-ray irradiation on melanin-derived radicals in mouse hair and skin. *J Clin Biochem Nutr* 2020; **67**: 174–178.
10 Kitakaze T, Yoshioka Y, Furuyashiki T, Ashida H. Enzymatically synthesized glycogen protects inflammation induced by urban particulate matter in normal human epidermal keratinocytes. *J Clin Biochem Nutr* 2020; **67**: 29–35.
11 Yew MY, Koh RY, Chye SM, Othman I, Ng KY. Edible bird's nest ameliorates oxidative stress-induced apoptosis in SH-SY5Y human neuroblastoma cells. *BMC Complement Altern Med* 2014; **14**: 391.
12 Zhiping H, Imam MU, Ismail M, *et al*. Effects of edible bird's nest on hippocampal and cortical neurodegeneration in ovariectomized rats. *Food Funct* 2015; **6**: 1701–1711.
13 Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med* 2002; **33**: 337–349.
14 Kim YS, Gupta Vallur P, Phaëton R, Mythreye K, Hempel N. Insights into the Dichotomous Regulation of SOD2 in Cancer. *Antioxidants (Basel)* 2017; **6**: 86.
15 Poswig A, Wenk J, Brenneisen P, *et al*. Adaptive antioxidant response of manganese-superoxide dismutase following repetitive UVA irradiation. *J Invest Dermatol* 1999; **112**: 13–18.
16 Kon T. Upregulation in antioxidative level of the skin induced by vitamin E supplementation. *Frontiers in Anti-aging of the Skin*, Tokyo, Japan: NTS Inc., 2006.
17 Toyoda A, Iio W, Goto T, Koike H, Tsukahara T. Differential expression of genes encoding neurotrophic factors and their receptors along the septal-temporal axis of the rat hippocampus. *Anim Sci J* 2014; **85**: 986–993.
18 Tsukahara T, Kawase T, Yoshida H, Bukawa W, Kan T, Toyoda A. Preliminary investigation of the effect of oral supplementation of *Lactobacillus plantarum* strain SNK12 on mRNA levels of neurotrophic factors and GABA receptors in the hippocampus of mice under stress-free and sub-chronic mild social defeat-stressing conditions. *Biosci Biotechnol Biochem* 2019; **83**: 2345–2354.
19 Murugan DD, Md Zain Z, Choy KW, *et al*. Edible bird's nest protects against hyperglycemia-induced oxidative stress and endothelial dysfunction. *Front Pharmacol* 2020; **10**: 1624.
20 Careena S, Sani D, Tan SN, *et al*. Effect of edible bird's nest extract on lipopolysaccharide-induced impairment of learning and memory in Wistar rats. *Evid Based Complement Alternat Med* 2018; **2018**: 9318789.
21 Albishtue AA, Yimer N, Zakaria MZA, *et al*. Edible bird's nest impact on rats' uterine histomorphology, expressions of genes of growth factors and proliferating cell nuclear antigen, and oxidative stress level. *Vet World* 2018; **11**: 71–79.
22 Yida Z, Imam MU, Ismail M, *et al*. Edible bird's nest attenuates high fat diet-induced oxidative stress and inflammation via regulation of hepatic antioxidant and inflammatory genes. *BMC Complement Altern Med* 2015; **15**: 310.
23 Asserin J, Lati E, Shioya T, Prawitt J. The effect of oral collagen peptide supplementation on skin moisture and the dermal collagen network: evidence from an ex vivo model and randomized, placebo-controlled clinical trials. *J Cosmet Dermatol* 2015; **14**: 291–301.
24 Verdier-Sévrain S, Bonté F. Skin hydration: a review on its molecular mechanisms. *J Cosmet Dermatol* 2007; **6**: 75–82.
25 Zainal Abidin F, Hui CK, Luan NS, Mohd Ramli ES, Hun LT, Abd Ghafar N. Effects of edible bird's nest (EBN) on cultured rabbit corneal keratocytes. *BMC Complement Altern Med* 2011; **11**: 94.

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