Fucus extract: cosmetic treatment for under-eye dark circles

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Synopsis

Background: Dark circles around the eyes are a complex issue with two main possible causes, the accumulation of melanin in the skin around the eyes and the accumulation of heme resulting from blood leakage. The free heme produced in this manner is highly cytotoxic, proinflammatory and pro-oxidative. Aims: To evaluate the effect of Fucus extract on heme oxygenase-1 (HO-1) stimulation activity, and to study its *in vitro* anti-inflammatory, antioxidative, and collagen stimulation activity. Methods: The HO-1 stimulation activity was first evaluated at gene level by reverse transcriptase- polymerase chain reaction targeting specific HO-1 gene, and then followed by Western blot in protein level. The *in vitro* anti-inflammatory effect was measured by quantification of interleukin-8 (IL-8) level. The *in vitro* antioxidative activity was measured. Collagen stimulation activity was quantitatively measured by the amount of deposited collagen I in the extracellular matrix. Results: Fucus extract was identified to have HO-1 stimulation activity at both gene and protein level. By stimulating this enzyme, it promotes the degradation of toxic heme to its protective catabolites (CO, Ferritin, and bilirubin) and reduces the source of dark circles. In addition, Fucus extract showed good anti-inflammatory efficacy. The strong antioxidation property of Fucus extract can reduce eye bags and wrinkles while its collagen boosting activity will potentially reduce fine lines and wrinkles. Conclusion: Fucus extract is a novel product that brings a quadruple approach to the treatment of under-eye dark circles.

INTRODUCTION

DARK CIRCLES AND CAUSES

Dark circles around the eyes are a complex issue with many possible causes. Based on recent research, two factors are clearly defined as major causes. First is the accumulation of melanin in the skin around the eyes. Melanin is produced by melanocytes and is transferred to keratinocytes to protect the nucleus from ultraviolet radiation. Selective accumulation of melanin around eye area will cause a dark circle. Second is the accumulation of heme resulted from blood leakage (1). The dilated blood vessels in the dermis lead to blood leakage into the surrounding areas including epidermis. Degradation of leaked red blood cells leads to the release of heme and other catabolites.

Heme is the prosthetic group of hemoproteins found in red blood cells. It contains an iron ion (Fe^{2+}) held within a heterocyclic organic ring known as a porphyrin

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(Figure 1). Under normal conditions, heme is intact within hemoproteins, but can be released under oxidative stress and became free heme. The free heme produced in this manner is highly cytotoxic, most probably due to the iron atom contained within its porphyrin ring, which can undergo Fenton chemistry to catalyze production of free radicals in an uncontrolled manner (2). This property of free heme can also sensitize a variety of cell types to undergo programmed cell death in response to proinflammatory stimuli. This deleterious effect is thought to play an important role in the pathogenesis of certain inflammatory diseases (3).

When the inflammation occurs around the eyes, it can, in turn, exacerbate further blood leakage. This downward spiral will cause negative effects in the eye area including the formation of dark circles. Therefore, it is critical to scavenge and dispose of free heme as quickly as possible.

Heme is degraded by an essential enzyme called heme oxygenase (HO) to carbon monoxide, iron and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase (Figure 2). Research has shown that carbon monoxide at low concentrations can act as an endogenous mediator of cellular signaling, and has antioxidation, anti-inflammation and antiapoptotic activities (4). The iron produced by the degradation of heme is sequestered in ferritin. Bilirubin, and biliverdin are also very good antioxidants (5,6). Thus HO turns free heme, a very toxic molecule, into several cytoprotective products.
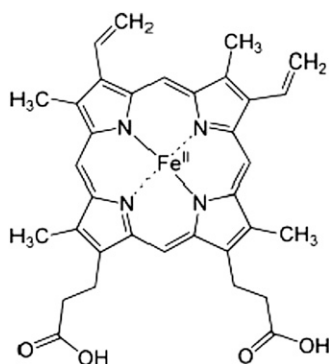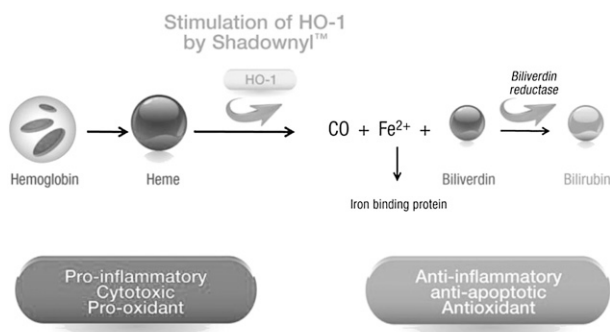

Figure 1. Heme.

Figure 2. Heme catabolites and their properties.

HO occurs as three isozymes: an inducible heme oxygenase-1 (HO-1) and constitutively expressed heme oxygenase-2 (HO-2) and heme oxygenase-3 (HO-3) (7). HO-1 can be induced by variety of stimuli, which indicate that HO-1 plays a vital role in maintaining cellular homeostasis in addition to heme degradation (8).

THE PROTECTIVE ROLES OF HO-1

HO-1 mediated protection of cells and tissues is supported by several animal models of oxidative injury and acute inflammation (9,10). In these models, HO-1 elevation confers potent resistance to stress, cell injury, and lipopolysaccharide-induced death. Blocking of HO activity abrogated cytoprotection, resulting in severe tissue damage. In addition, increased HO-1 expression levels have been clinically demonstrated to resolve of a wide variety of inflammatory conditions (11). HO-1 activity forms a feedback loop by attenuating leukocyte adhesion and migration and by promoting the resolution of inflammatory responses. Overexpression of HO-1 in endothelial cells and fibroblasts protected these cells from TNF-α-mediated apoptosis (12). HO-1 also plays a role in the regulation of angiogenesis, and overexpression of HO-1 has accelerated cutaneous wound healing in mice (13).

THE PROTECTIVE EFFECT OF HO-1 IS ALSO THROUGH ITS PRODUCTS:

(i) CO inhibits proliferation of vascular smooth muscle cells, platelet aggregation, and protects against apoptosis. In addition, CO inhibits proinflammatory genes while augmenting anti-inflammatory cytokine production (14,15).

(ii) HO-1 induction is accompanied by increased ferritin synthesis. A recent study demonstrated that ferritin plays a central role in cellular antioxidant defense and cytoprotection (16). The elevated level of ferritin would result in increased resistance to iron-mediated oxidative stress (17).

(iii) It was shown *in vitro* that at micromolar concentrations, both biliverdin and bilirubin are efficient free-radical scavengers (5). In liposomes, bilirubin suppressed oxidation even more effectively than α-tocopherol or vitamin E, which is regarded as an excellent antioxidant. These results indicate that bilirubin is a very good antioxidant and provides potent protection against oxidative injury and inflammation (18).

HO-1 and its products: CO, ferritin, and bilirubin all working together, play an essential role in antioxidation, anti-inflammation, and cytoprotection.

COLLAGEN AND WRINKLES

Collagen, a group of naturally occurring fibrous proteins, is a major component of the extracellular matrix (ECM) that supports tissues. It is also the main component of fascia and skin (19), providing them with strength and elasticity. With aging, the amount of collagen production in the skin decreases, which results in the formation of fine lines and wrinkles (20). This is especially evident around the eye area.

Collagen is composed of a triple helix of amino acid chains and is produced and assembled by fibroblast cells through a series of steps. By identifying active cosmetic ingredients that can stimulate collagen production, the appearance of fine lines and wrinkles can be minimized.

MATERIALS AND METHODS

REAGENT AND CHEMICALS

Human IL-1β and IL-8 ELISA kit were from RnD systems (RnD system, Minneapolis, MN); Mouse anti-HO-1 antibody was from BD Biosciences (San Jose, CA); Mouse anti-β actin was from Sigma (Sigma, St Louis, MO); Goat anti-mouse HRP antibody from Santa Cruz Biotechnology, Inc. (Dallas, TX); SV 96 total RNA isolation kit from Promega (San Luis Obispo, CA); Probes of HO-1 and GUSB from Life Technologies (Grand Island, NY); 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox, RIPA buffer, and a cocktail of protease inhibitors are from Sigma. All medium including Dulbecco's modified Eagle's medium (DMEM), medium 154, and media supplement are from Invitrogen (Life Technologies). BCA assay kit from Pierce (Thermo Fisher Scientific Inc. Rockford, IL).

CELL CULTURES AND TREATMENT PROTOCOL

Normal human dermal fibroblasts (NHDF) were obtained from Invitrogen (Life Technologies) and were subcultured in DMEM medium supplemented with fetal bovine serum (FBS 10%), penicillin (100 U/ml) and streptomycin (100 µg/ml), and incubated at 37°C in a humidified incubator with 5% CO_2.

Normal human epidermal keratinocyte (NHEK) were also obtained from Invitrogen and were subcultured in medium 154 with supplement kit at 37°C with 5% CO_2.

After confluence, cells were seeded in 6-well or 96-well plates and incubated for two more days before treatment.

Tested products were diluted into respective treatment medium to different concentrations, and then were added to the cells.

RNA ISOLATION AND RT-PCR ON HO-1

Cells were grown to confluence and then were treated with tested product in their respective medium for 24 h at 37°C with 5% CO_2. After treatment, cells were rinsed once and total RNA was extracted from the cells using Promega SV 96 total RNA isolation kit. Two hundred nanograms of total RNA was used for reverse transcription with M-MLV and oligo (dT) primers. Real time PCR was performed with probe sets specific for human HO-1 (Hs01110250_m1). These reactions were run in duplex, with probe sets for GUSB, a housekeeping gene for data normalization. The cycling conditions for the RT-PCR are as following: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 60 s. Data were analyzed with ΔCt in Excel.

WESTERN BLOT

Cells were grown to confluence and then treated with tested product in their respective medium for 3 days for NHEK cell and for 24 h for NHDF cells at 37°C with 5% CO_2. After rinsing once with PBS, cells were processed for Western blotting by lysis in RIPA buffer containing a cocktail of protease inhibitors. The lysate was sonicated for 15 s, centrifuged 10 min at 12000g, and the supernatants were quantified for protein content by BCA assay. HO-1 protein levels were then analyzed by electrophoresing 30 µg cellular protein in a 4%–20% acrylamide gradient gel (Bio-Rad, Hercules, CA), transferring to a nitrocellulose membrane and blotting 90 min with mouse anti-human HO-1 antibodies and mouse anti-β-actin antibodies (to normalize protein loading). After washing, the membrane was blotted with HRP-linked anti-mouse antibodies for 60 min. HO-1 and β-actin bands were resolved and quantified by chemiluminescence on a Kodak Image Station 4000R. Cell treatments, extractions, protein quantifications, and Western blots were performed in triplicate.

DPPH ASSAY

Fucus vesiculosus water extract was diluted to 0.5%, 1%, 2%, and 5% in PBS. Trolox was dissolved in ethanol and diluted to concentrations of 2.5, 5, 10, 20, 30, 40, and 50 µM, which is used to generate a standard curve. DPPH was dissolved in ethanol to make 100 µM solution. In a 96-well plate, 100 µL of sample or standard solutions was added to wells in six replicates, and then 100 µL of DPPH solution was added to triplicate wells, and 100 µL of ethanol without DPPH to the other triplicate wells for background reading. After mixing at room temperature for 20 min, absorbance was read at 510 nm. In order to minimize background interference for colored samples, the background reading is subtracted from the final absorbance of these samples.

IL-8 ASSAY

Cells were pretreated for 4 h with basal medium (Invitrogen, medium 154 with no growth supplement). Then, cells were incubated overnight in treatment media (Invitrogen, medium 154 with 10 ng/ml of IL-β and tested product added) at 37°C with 5% CO_2. The level of IL-8 released into culture medium was measured by ELISA method. In brief, mouse anti-human IL-8 was coated overnight in a 96-well plate. Samples and standard were then added to the plate and incubated for 2 h. After washing, a biotinylated goat anti-human IL-8 was added to the plate and incubated for 2 h. A streptavidin-conjugated horseradish peroxidase and substrate was used to measure the amount of IL-8 content by recording the optical density at 450 nm with correction at 540 nm.

COLLAGEN I ASSAY—DELFIA METHOD

Collagen I was assayed using a method developed internally (Immunoassay method for *in vitro* measurements, patent application filed). After treatment with the products, cells

were disrupted with dedicated lysis solution. This solution allows the disruption of cell membranes without solubilizing the deposited matrix. The deposited collagen I was detected by primary antibody anti-collagen I (Interchim, Montlucon, France) and with the secondary antibody anti-IgG coupled to a DELFIA® Europium (Perkin Elmer, Courtaboeuf, France) probe. Europium-related fluorescence (λ_{exc}, 340 and λ_{em}, 615 nm), proportional to the quantity of deposited collagen was measured.

STATISTICAL ANALYSIS

The statistical analysis (two-tailed) for *in vitro* studies were performed using Student's *t*-test and analysis of variance on the multiple comparisons with a threshold of significance set to 5% ($p < 0.05$).

RESULTS AND DISCUSSION

EFFECT OF FUCUS EXTRACT ON HO-1 STIMULATION *IN VITRO*

Skin responds to environmental assault and endogenous factors by changing the vascular integrity, leading to the blood leakage and heme accumulation in the surrounding tissue space and causing dark circles. In this study, experiments were performed to evaluate the effect of Fucus extract in the HO-1 stimulation activity in cultured cells.

The HO-1 stimulation efficacy of Fucus extract is evidenced first at the gene level by RT-PCR (Figure 3). For initial screening, both fibroblasts and keratinocytes were treated with 5% of the Fucus extract in their respective medium for 24 h at 37°C with 5% CO_2. Total RNA was isolated, cDNA was synthesized and used as template, and a probe set specific to HO-1 gene was used for this reaction. The HO-1 mRNA expression profile was compared to non-treated control, and the results from both cell types are described in Table I.
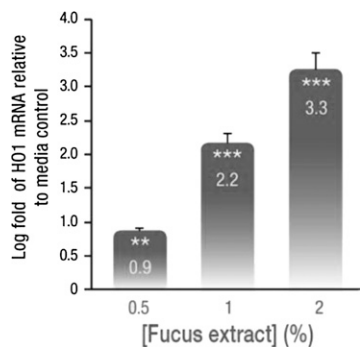

Figure 3. Fucus extract stimulates heme oxygenase-1 gene expression in keratinocytes- (Log base is 2, 1log = 2 fold) Mean ± SD on 3 assays – Paired Student's t- test - **: $p < 0.01$- ***: $p < 0.001$ compared to control.

Table 1
Fucus extract up-regulated HO-1 mRNA expression in both fibroblast and keratinocyte cells

Cell type	NHDF	NHEK
HO-1 $\log_2$ fold change relative to control	1.6	5.8
HO-1 fold change relative to control	3	55

Results were further confirmed by a dose-dependence study in keratinocyte as shown in Figure 3.

At concentrations from 0.5% to 2%, Fucus extract positively stimulated HO-1 gene expression in keratinocyte culture. At 1%, Fucus extract stimulated HO-1 to 2.2 log, which is more than 4-fold of control; at 2%, the stimulation went to about 10-fold of control ($p < 0.001$).

The HO-1 stimulation activity of Fucus extract at protein level analyzed by Western blot is presented in Figure 4 for keratinocyte and in Figure 5 for fibroblast cells.

Fucus extract at 1% and 2% concentrations induced HO-1 protein in keratinocyte significantly in a dose-dependent manner (Figure 4). Quantitative densitometric analysis of protein band intensities showed that at 1%, Fucus extract induced HO-1 protein about 6-fold of control, at 2%, it induced HO-1 protein about 30-fold of control. The p values of paired Student's t-test for both concentrations are less than 0.001 when compared to control, which is statistically significant.

In fibroblast cells (Figure 5), HO-1 protein was induced by Fucus extract at both 1% and 2% concentrations too. When the analyzed protein band intensities were compared to control for a paired Student's t-test, the p values for both concentrations are less than 0.01, which is statistically significant. The induced HO-1 protein content in fibroblast cells reached maximum levels when treated with 1% of Fucus extract.

These results clearly show that Fucus extract is capable of inducing HO-1 in both keratinocyte and fibroblast cells, suggesting Fucus extract could have a beneficial
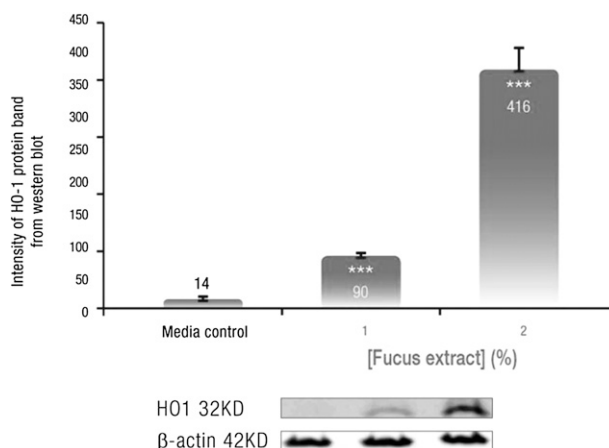

Figure 4. Fucus extract increased HO-1 content in keratinocyte by western blot. Mean ± SD on 3 assays – Paired Student's t test - ***: p < 0.001.
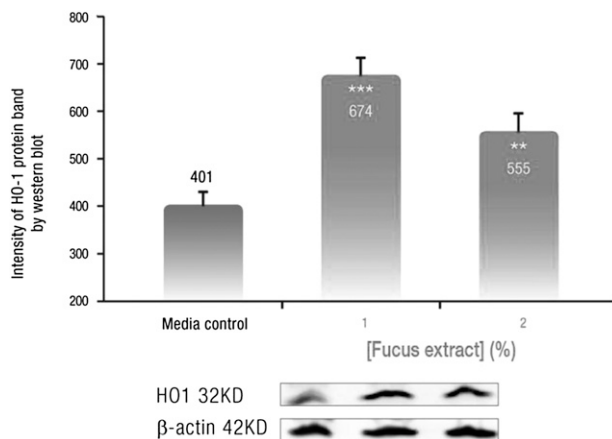
Figure 5. Fucus extract increased HO-1 content in fibroblast by western blot. Mean ± SD on 3 assays – Paired Student's t test -**: p < 0.01 - ***: p < 0.001.

effect in reducing dark circles around the eyes caused by blood leakage and heme accumulation.

EFFECT OF FUCUS EXTRACT ON ANTIOXIDATION BY DPPH ASSAY

Antioxidants help to prevent and repair damage to your body's tissue by slowing or preventing the effect of free radicals, and help to protect your skin from the damaging effects of the sun (21).

DPPH is a common abbreviation for the organic chemical compound 2,2-diphenyl-1-picrylhydrazyl. It is a dark-colored crystalline powder composed of stable free-radical molecules. It is used in a common assay for determining the antioxidant capacity of active cosmetic ingredients.
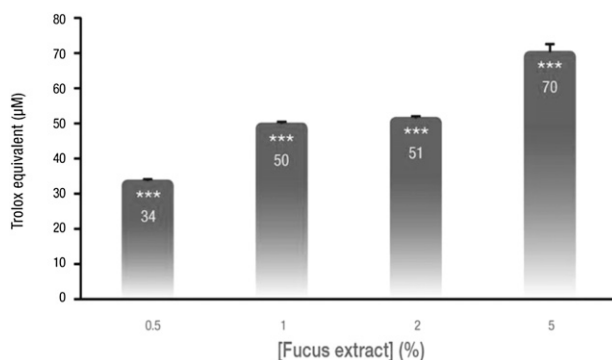

Figure 6. Fucus extract: Anti-oxidation effect Mean + SD of triplicate assays - Paired Student's t test - ***: p < 0.001.

Trolox is a water-soluble form of vitamin E. It is commonly used as a benchmark for antioxidant capacity evaluation, especially for water extracts like Fucus extract.

As analyzed by the DPPH assay, Fucus extract showed dose-dependent antioxidation activity at concentrations from 0.5% to 5%. As shown in Figure 6, 1% Fucus extract is equivalent to about 50 µM Trolox in its antioxidation capacity. When compared to a negative control using a paired Student's t-test, the p values for all concentrations tested are less than 0.001, which is statistically significant.

EFFECT OF FUCUS EXTRACT ON ANTI-INFLAMMATION IN KERATINOCYTES (*IN VITRO* TEST)

Interleukin-8 (IL-8) is a chemokine produced by macrophages and other cell types such as epithelial cells. This chemokine is one of the major mediators of the inflammatory response. Under normal growth conditions, a very low level of IL-8 cytokine is detected in the medium of normal keratinocyte cultures. But when cells were treated with IL-1β, the IL-8 level secreted to media is elevated. The anti-inflammation property of Fucus extract was evaluated by IL-8 ELISA under the treatment of IL-1β (Figure 7). Control is the treatment without Fucus extract added.

As shown in Figure 7, the IL-8 level induced by IL-1β was reduced by the Fucus extract treatment in a dose-dependent manner at concentrations from 0.5% to 2%. At 1%, Fucus extract reduced IL-8 level to about 40% of control, and at 2%, to about 25% of control.

Since IL-8 is often associated with inflammation, reduction of chemokine IL-8 is a positive indicator of anti-inflammation efficacy.

EFFECT OF FUCUS EXTRACT ON COLLAGEN I IN FIBROBLAST CELLS (*IN VITRO* TEST)

In this experiment, we evaluate the capacity for Fucus extract to stimulate collagen I synthesis in cultured human fibroblast cells. The assay shown below measures collagen I deposited in the ECM as opposed to secreted collagen.
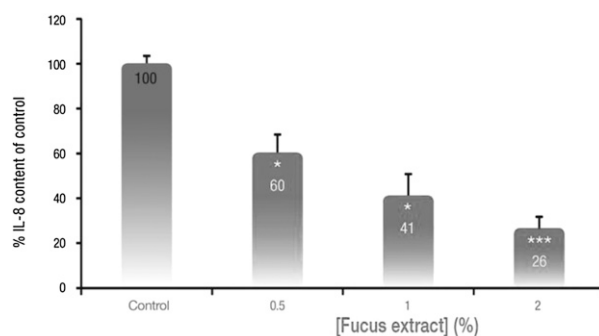

Figure 7. Fucus extract decreases IL-8 content compared to control Mean ± SEM on 3 assays in triplicate - Student's t test - *: p<0.05 - ***: p<0.001.
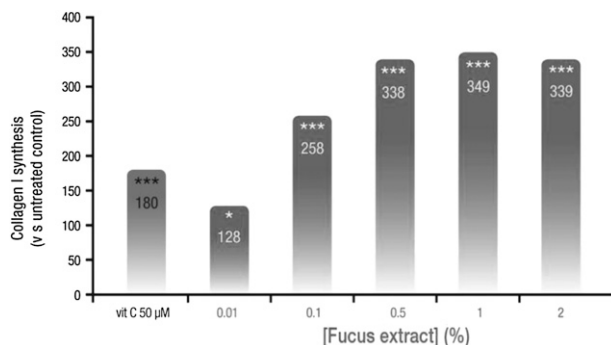
Figure 8. Fucus extract increase the collagen I on fibroblast cells. Mean ± SD on 3 assays in triplicate – Student's t test - *: p<0.05 - ***: p<0.001.

As shown in Figure 8, Fucus extract increased collagen I in the ECM in a dose-dependent way. The increases were significant at all concentrations tested (0.01% to 0.5%). At 0.01%, 0.05%, 0.1%, and 0.5%, Fucus extract enhanced collagen synthesis to 1.3-, 1.6-, 2.5-, and 3.7-fold of control, respectively. Vitamin C, the positive control increased collagen significantly to 1.8-fold of control. At 0.1% and above, Fucus extract increased collagen more than vitamin C.

Vitamin C at 50 µM, a commonly used bench mark in collagen I stimulation assays, was used as a positive control.

CONCLUSION

This study demonstrated that Fucus extract is a novel product with quadruple *in vitro* efficacy. Fucus extract stimulates *in vitro* the production of HO-1 enzyme. In addition, Fucus extract was found to be an anti-inflammatory, antioxidative, and collagen boosting ingredient. Ingredients with such properties are likely to aid in alleviating the appearance of dark circles around the eye area. Additional *in vivo* studies are necessary to confirm our hypothesis that Fucus extract could aid in periorbital rejuvenation.

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