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Water-retentive and Anti-inflammatory Properties of Organic and Inorganic Substances from Korean Sea Mud

Jung-Hyun Kim[a], Jeongmi Lee[c], Hyang-Bok Lee[a], Jeong Hyun Shin[b] and Eun-Ki Kim[a,*]

[a]*Department of Biological Engineering*, *Inha University*, *Incheon*, *Korea*

[b]*Department of dermatology*, *Inha University Hospital*, *Incheon*, *Korea*

[c]*College of Pharmacy*, *Sungkyunkwan University*, *Suwon*, *Gyeonggi-do*, *Korea*

ekkim@inha.ac.kr

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Sea mud has been popularly used as an effective base in cosmetic preparations although its biologically-active materials and mechanisms on skin have not yet been fully determined. We isolated humic substances as the major organic substance of the sea mud from a tidal flat in Korea, and investigated their water-retentive properties. Among the three isolated humic substances, humic acid (HA) showed the highest water retentive property (~ 50 % mass increase from water uptake). Based on the observations that mud pack therapy has been traditionally used to soothe UV-irradiated skin, we examined the anti-inflammatory property of the sea mud on UVB-irradiated human keratinocytes (HaCaT cells) by measuring PGE_2 levels produced by keratinocytes in the presence of either the total water or methanol extracts of the mud. The water extract showed higher inhibition of PGE_2 production from HaCaT cells (30 % inhibition) than the methanol extract at 200 ppm (μg/g). We further fractionated the water extract to determine the major components responsible for its anti-inflammatory effect. It was found that the minerals in the mud inhibited PGE_2 production by 83 % at 200 ppm, which is comparable with the inhibitory effect of 1 μM indomethacin. No mud extract showed cytotoxicity at the tested concentrations. The mineral compositions of the mineral extract were determined by ICP-MS, revealing that the sea mud consisted of more than 19 different mineral components, rich in Na^+, Mg^{2+}, and Zn^{2+}. These results imply that the anti-inflammatory effect of the sea mud is largely due to the minerals in the mud. Our research suggests the potential use of the organic and inorganic substances from the sea mud in various skin products as safe biological substances for skin protective purposes.

Keywords: humic substances, humic acid, water retention, mud, anti-inflammation.

Mud has been traditionally used in balneotherapeutic treatment as found in mud pack therapy to treat osteoarthritis and skeletal-muscle diseases [1,2]. Its mechanism of therapeutic action is generally attributed to thermal effects, although several studies have attempted to elucidate other possible modes of actions for its anti-rheumatic and anti-inflammatory properties [3a,3b]. In addition to its therapeutic usage, mud is also used in many skin care products these days. For example, Dead Sea mud has been extensively used as a therapeutic base in mud pack therapy [4a,4b], and as a cosmetic base in various skin preparations [4c].

Mud is composed of diverse amounts of various organic and inorganic substances. Humic substances, which are formed by decomposition of organic matter, are found in many natural materials, such as soil, peat, sea sediment, and lakes. Sea mud also serves as a large reservoir for humic substances. They are sub-classified as humin (insoluble at any pH), fulvic acid (FA, soluble at any pH) and humic acid (HA, soluble at high pH) according to their aqueous solubility [4d]. They have been reported to possess diverse biological activities including antiviral, anti-inflammatory [5a], immunomodulatory [5b], and antiallelochemical [5c] properties. However, no studies have been undertaken on the humic substances from cosmotherapeutic aspects, and the activities of humic substances originating from sea mud have never been evaluated, although it is one of the preferred natural materials added in skin products.

Tidal flats in the western coastal wetland in Korea are one of the five largest tidal flats in the world, providing amphibious habitats for numerous plant and animal organisms. In addition to their ecological importance, these tidal flats play an important role in generating profits for several cities in that mud from the tidal flats is used as an active material in mud pack preparations. Although the mud has been reported to be rich in a

variety of minerals that are beneficial to skin, the cosmotherapeutic effects of mud have never been evaluated on human skin.

In this study, crude sea mud was obtained from a tidal flat from the west coast in Boryeong, Korea to characterize the skin-protective properties of the mud. Boryeong has been hosting an internationally renowned mud festival since 1997 and its mud has been commercially developed as cosmetic products such as mud soaps and packs. We isolated humic substances from the Boryeong mud and measured their water retentive properties to evaluate the potential of each substance as an active ingredient in skin products.

Mud packs have been traditionally used to soothe irritated skin after exposure to UV irradiation. Acute exposure to ultraviolet (UV) leads to cutaneous inflammation that is characterized by erythema and edema, as well as DNA damage and immune suppression [6a]. Lipid mediators, such as prostaglandins (PGs), are released by skin upon UV exposure. In particular, the production of PGE_2 by keratinocytes, which are the major target of sun light, is known to be highly induced on UVB (280 – 320 nm) irradiation [6b.6c]. We evaluated the anti-inflammatory effects of the mud extracts upon UVB irradiated keratinocytes by measuring PGE_2 level.

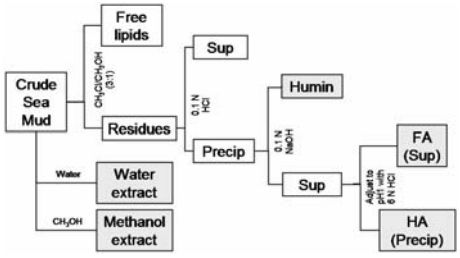

Figure 1: Isolation steps of humic substances from crude sea mud. (FA, fulvic acid; HA, humic acid).

As illustrated in Figure 1, three major humic substances, humic acid (HA), fulvic acid (FA), and humin were prepared from the crude Boryeong sea mud based on their aqueous solubility at different pHs. Yellow ocher, which has been a material popularly used in diverse cosmetic preparations in Korea, was employed as a reference material to compare the water retention capacities of tested materials. Water retentive properties were measured as the relative increased mass of materials over 24 hours (Figure 2). Overall, yellow ocher showed the lowest water retention capacity among the tested substances, and the value for humin was also low and close to that of yellow ocher. HA exhibited the highest capacity, up to ~ 50 % mass increase at 9 hours and retained superior capacity close to 40 % at 24 hours, whereas the water-retention of FA stayed below 20 %, slightly higher than those of humin

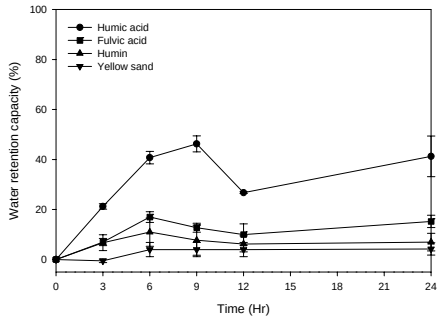

Figure 2: Comparison of water retention capacities of humic acid, fulvic acid, humin, and Korean Yellow sand.

and yellow ocher, during the entire test period. This result suggests the potential application of HA isolated from the sea mud as a natural moisturizing base for skin products.

Anti-inflammatory effects of various types of mud extracts were evaluated using HaCaT cells, which are a human keratinocyte cell line. HaCaT cells were irradiated with UVB and then cultured with either whole methanol or whole water extracts. The level of PGE_2 released from the irradiated cells was measured as an indicator of acute inflammation induced by UVB by enzyme-linked immunosorbent assay (ELISA). Indomethacin, a cyclooxygenase (COX) inhibitor, which is known to inhibit acute skin inflammation induced by UV radiation, was used as a positive control, showing 95% inhibition of PGE_2 production (Figure 3). The whole water extract significantly reduced the PGE_2 level by 30 % when compared with the negative control in which water was added ($p < 0.05$). The whole methanol extract showed 14% inhibitory effect, although this was not statistically significant.

Sea mud is rich in a variety of minerals, which are often claimed to be responsible for the skin-protective effect of the mud without scientific evaluation. Therefore, the whole water extract was further fractionated into the water and mineral extracts, which were then studied to investigate their anti-inflammatory effect again. As displayed in Table 1, both the mineral and water mud extracts inhibited PGE_2 production in a dose-dependent manner within the tested range from 25 ppm (μg/g) to 200 ppm. The inhibitory effect of the mineral extract was higher by 2- to 7-fold than the water extract and the PGE_2 level in the presence of 200 ppm of the mineral extract was almost comparable with that of 1 μM indomethacin. Cytotoxicity of the extracts was evaluated along the concentrations at which the anti-inflammatory effect was measured by assessing the cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Despite the high anti-inhibitory effects, the mineral extract showed no cytotoxicity at any tested concentration (Figure 4).

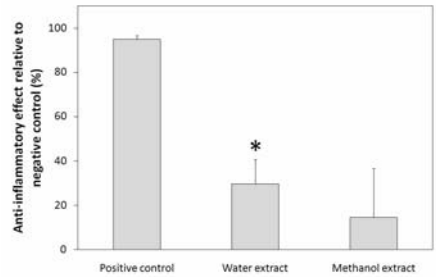

Figure 3: Anti-inflammatory effect of the tested substances, measured by reduced PGE_2 production levels from HaCaT cells in comparison with the negative control (no treatment) after UV irradiation. The positive control was treated with 1 μM indomethacin, and 100 ppm (μg/g) of either methanol or water mud extract was used for comparison. * $p < 0.05$ (n =2).

Table 1: Anti-inflammatory effect of mud water and mineral extracts as PGE_2 level relative to the negative control (%). [a] μg/g.

Concentration	Total water extract	Mineral water extract
25 ppm[a]	7.3	41.1
50 ppm	10.3	65.9
100 ppm	27.9	70.8
200 ppm	43.0	82.9

The water extract resulted in a slightly decreased cell viability at 1 and 100 ppm. Indomethacin exhibited no cytotoxicity at 1 μM (data not shown). Our results suggest that minerals in the mud extract are largely responsible for its anti-inflammatory effect, and that the mud mineral extract could be developed as a safe anti-inflammatory skin-protective agent.

The compositions of the mineral extract were analyzed by inductively coupled plasma mass spectrometry (ICP-MS). The mineral extract was found to contain 19 minerals (Table 1). Seventeen were found in ppb and 2 in ppm concentration ranges. Although copper, zinc, and arsenic were found, which are reported as toxic metals in muds, other toxic metals such as Cr and Pb, which were found in the Dead Sea muds and Dead Sea mud products [6d], were not found in the extract. It was previously reported that magnesium ions (Mg^{2+}) in the Dead Sea mud exhibited an anti-inflammatory effect by inhibiting the antigen-presenting capacity of Langerhans cells [7]. Zinc ions (Zn^{2+}) also showed a therapeutic effect in the wound healing process in mouse skin [8]. Magnesium was found to be the second most abundant ion in the Boryeong mud and a fair amount of zinc was contained as well. Our results support the skin protective roles of minerals in the mud and a study of the individual mineral would reveal the compounds responsible for the anti-inflammatory effect of mud.

In conclusion, we isolated organic and inorganic substances from the sea mud and studied their skin protective properties. Humic acid, which was shown to be the most water-retentive among the tested humic substances, could be used as a natural moisturizing component in skin products. Minerals, the inorganic substances in the mud, were found to be the major components with the anti-inflammatory property against

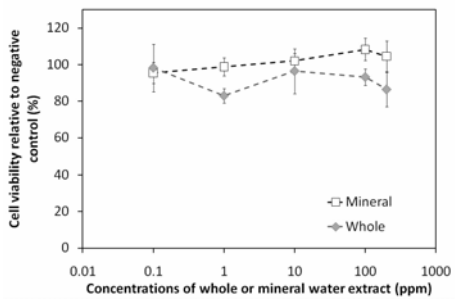

Figure 4: Cytotoxicity of whole and mineral water mud extracts. Relative HaCaT cell viability treated with mud extracts was measured in comparison with the negative control (no treatment) (n = 5).

UVB irradiation. Therefore, the mineral mud extract could be used as a safe natural component in skin preparations aimed to either protect or heal irritated skin from sun exposure.

Experimental

Materials: Crude sea mud was obtained from a tidal flat in the western coastal wetland (Boryeong, Chungnam, South Korea). The sample was taken from 150 cm below the surface to acquire mud of light gray color. Mud was air dried in a dry-oven and then lightly ground in a mortar.

Preparations of humic substances, whole water and methanol extracts, and mineral water extract from sea mud: Humic substances were purified according to the protocols recommended by the International Humic Substance Society and Essington [9]. Free lipids were removed from the sea mud by addition of 200 mL CH_3Cl:CH_3OH (3:1) to 20 g of powdered mud, followed by incubation for 24 h. Residues were washed in 0.1M HCl (10 mL/g sample) and extracted with 1 L 0.1M NaOH under N_2, with intermittent shaking for at least 4 h. The alkaline suspension was then allowed to settle overnight and the humin-containing fraction was collected in the precipitate after centrifugation. The supernatant was acidified with 6M HCl with constant stirring, allowed to settled overnight, and centrifuged to separate the humic acid-containing fraction (precipitates) from the fulvic acid-containing fraction (supernatant). Whole water and methanol mud extracts were prepared by extraction with distilled water and 100% methanol, respectively at room temperature for 24 h (3 mL/g sample), followed by filtration and lyophilization. Organic materials were removed from the whole water extract in a muffle furnace at 550ºC for 1 h, resulting in mineral water extract.

Water retention assay: Saturated NaCl solution is known to maintain 75.0 ~ 76.5% relative humidity, which was used to measure water retentive property, as previously [10a]. Therefore, dried samples including humic substances and yellow ocher were placed in the presence of the saturated NaCl solution in air-tight containers over a period and the weight changes due to

the absorbed water were measured to evaluate their water retention capacity.

Cell culture and UV irradiation: The anti-inflammatory effect of mud extracts was estimated using human skin keratinocyte cell line HaCaT. Cells were grown in RPMI1640 medium (Sigma-Aldrich, USA) containing 10% FBS (fetal bovine serum) and antibiotics (penicillin, 100 U mL; streptomycin, 100 mg mL) at 37°C in a humidified incubator under 5% CO_2. 3.0×10^5 cells were sub-cultured in 6-well plates for 24 h, followed by washing in phosphate buffered saline (PBS), twice. Then UVB irradiation was performed with a bank of 4 FS40 fluorescent lamps (Philips) that emit wavelengths between 280 nm and 320 nm with a peak at 313 nm. While cells were covered in 1 mL PBS, UVB-rays were delivered with a dose of 30mJ/cm^2. After UV treatment, cells were maintained in serum free DMEM supplemented with test substances for 24 h.

PGE_2 measurement: The concentration of PGE_2 secreted in the culture medium was measured 24 h after UV irradiation. Enzyme-linked immunosorbent assay (ELISA) was carried out using a competitive enzyme immunoassay kit (R&D systems, Minneapolis, MN) according to the manufacturer's protocol. The anti-inflammatory property of the tested extracts was evaluated in comparison with indomethacin as a positive control.

Cytotoxicity assessment: Cytotoxicity of mud extracts was determined using MTT assay [10b]. Test substances were incubated with HaCaT cells for 48 h before reading the absorbance of formazan at 540 nm.

ICP mass spectrometry: Inductively coupled plasma mass spectrometry (ICP-MS), which was used to determine the compositions of inorganic components in the mineral water extract, was carried out by Korea Basic Science Institute (Seoul, Korea). Given values are the averages of three measurements.

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