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Effects of Low-Dose Light-Emitting-Diode Therapy in Combination with Water Bath for Atopic Dermatitis in NC/Nga Mice

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ABSTRACT

Background

Light emitting diode (LED) phototherapy and water bath therapy have beneficial effect on atopic dermatitis (AD)-like skin disease. However, not all current treatments work well and alternative therapies are need. The contribution of combination therapy with low-dose 850 nm LED and water bath was investigated on dermatophagoides farina (Df)-induced dermatitis in NC/Nga mice.

Methods

Low-dose LED (10, 15, and 20 J/cm²) irradiation, water bath (36±1°C) were administered separately and together to the Df-induced NC/Nga mice in acrylic jar once a day for 2 weeks.

Results

Combined therapy with low-dose LED therapy and water bath therapy significantly ameliorated the development of AD-like skin lesions. These effects were correlated with the suppression of total IgE, NO, histamine, and Th2-mediated immune responses. Furthermore, combination therapy significantly reduced the infiltration of inflammatory cells and the induction of thymic stromal lymphopoietin (TSLP) in the skin lesions. The beneficial

therapeutic effects of this combination therapy might regulate by the inhibition of various immunological responses including Th2-mediated immune responses, inflammatory mediators such as IgE, histamine, and NO, as well as inflammatory cells.

Conclusions

The combination therapy of LED and water bath might be used as an efficacious, safe, and steroid-free alternative therapeutic strategy for the treatment of AD.

Key words: Light-emitting-diode (LED) phototherapy, Water bath therapy, Combination therapy, Atopic dermatitis (AD), NC/Nga mice

Atopic dermatitis (AD) is a chronic eczematous and pruritic disorder in which the skin is inflammatory, dry, and itchy (1,2). Although the pathogenesis of AD is not well understood, it has been reported that immunological abnormalities, various environmental factors, and skin barrier dysfunction contribute to the development of AD (3,4). The overproduction of immunoglobulin E (IgE), nitric oxide (NO), type-2 helper T cell (Th2) cytokines, and Th2-mediated chemokines are thought to play important roles in the development of AD (5-7). In addition, AD is associated with increased inflammatory cells such as eosinophils and mast cells, and increased substances such as histamine (8,9). Up to the present, the supportive and basic treatments of AD have been studies using such drugs based on topical and system

steroid, and oral immunosuppressants (10,11). However, patients with AD with their drugs in long-term may induce various side effects (12). Therefore, there is need for a new design that has more effective therapeutic strategy for the treatment for AD.

Combination therapy in treating AD provided additive or synergistic effect as well as reduces the range and the side effect of each drug. We and others have shown that combination therapy of topical or oral immunosuppressants plus other immunomodulating agents was more effective than monotherapy in decreasing clinical symptoms severity scores or reducing drug dosage both *in vivo* and *in vitro* experiments (13-15). **Furthermore, combination therapy of phototherapy plus other topical or systemic agents offered enhanced therapeutic effect in AD (16). We recently reported that combination therapy of 850 nm light emitting diode (LED) phototherapy plus low-dose FK-506 in to NC/Nga AD mouse model resulted in a significant improvement (17). Thus, LED phototherapy exerts immunomodulatory effects on allergic diseases such as AD through mechanisms, including inhibition of inflammatory molecules and reduction of inflammatory immune cells, as well as inhibition of Th2-related cytokines. Additionally, bath therapy, including natural mineral water and green tea, has a beneficial effect on skin disease such as AD (18-20). Bathing improves hydration of the skin (21). Moreover, the effects**

of water reported a significant improvement of the skin lesions in AD patients (22). They speculated that bath therapy might regulate the function of impaired skin barrier by improving the skin hydration.

The present study investigated the therapeutic efficacies and regulatory mechanism of low-dose 850 nm LED phototherapy and water bath therapy in combination on the development of Df-induced dermatitis in NC/Nga mice.

MATERIALS AND METHODS

Animals

Eight-week-old male NC/Nga mice were purchased from Charles River Japan (Atsugi, Japan). The mice were adapted for one week before the start of experiments. They were maintained in uncontrolled conventional Laboratory Animal Facilities at the Dongguk University School of Medicine. The animal care and use committee of the research institute at Dongguk University Hospital approved all described studies.

Induction of AD in the NC/Nga mice

Df body ointment (Biostir Inc., Kobe, Japan) was used to induce AD-like skin symptoms in NC/Nga mice as described previously (17). Brief, the hair on the backs of the NC/Nga mice was shaved using an electric shaver, followed by treatment with a skin-hair-remover (Niclean, Ildong, Korea). Barrier disruption was achieved by 4% sodium dodecyl sulfate treatment on the shaved dorsal skin and both surfaces of each ear three hours before Df body ointment (100 mg/mouse) application. These procedures were repeated twice a week for four weeks.

LED irradiation and water bath treatment on Df-induced NC/Nga mice

The mice with a clinical score of 8 were used for treatment with bath alone or in combination with LED (Photonic Planar Integration Inc., Gwangju, Korea) irradiation. The dorsal skin of the mice was irradiated to a LED light with 10, 15, 20, 40, and 80 J/cm² at 850 nm wavelengths. During of bath treatment was 10 min, with a water temperature of 36±1°C. Combination therapy of LED irradiation and water bath are performed simultaneously in acrylic jar once a day for 2 weeks. Each group consisted of five mice.

Scoring of skin lesions

The extent of: (1) erythema/hemorrhage, (2) scarring/dryness, (3) edema, (4) excoriation/erosion was scored as 0 (none), 1 (mild), 2 (moderate), and 3 (severe). The total skin score was defined as the sum of the individual scores (17).

Assay of serum parameters

Mice serum was collected at 48 h after the final administration. The concentration of total immunoglobulin E (IgE) in mouse serum was measured using ELISA kit (Yamasa, Tokyo, Japan). The levels of nitric oxide (NO) and histamine in the serum also were determined by the commercially available total NO detection kit (R&D System, Minneapolis, Minnesota, USA) and histamine EIA kit (Oxford biomedical research, Inc., MI, USA), respectively. The ELISA was performed in accordance with the manufacturer's instructions.

Assay of cytokines, chemokines production

The single-cell suspensions were prepared from the spleen 48 h after the final administration. ELISA on all prepared supernatant was performed at the end of the study using commercially available interleukin (IL)-4, IL-5, IL-13, interferon (IFN)- γ , CCL17/TARC (thymus and activation-regulated chemokine), and eotaxin ELISA kit (R&D System, Minneapolis, Minnesota, USA). For measuring thymic stromal lymphopoietin (TSLP) level, the dorsal skin

of mice were removed and stored at -80°C . Later, the skins were homogenized using the Bullet BlenderTM Blue (Next Advance, Averill Park, NY) at 4°C . The concentrations of TSLP were determined using the ELISA kit (R&D System, Minneapolis, Minnesota, USA).

Histological analyses

Paraformaldehyde-fixed and paraffin-embedded back skin samples from the mice were sliced and then stained with toluidine blue and Congo red to count the mast cells and eosinophils, respectively. The cell density was expressed as the number of cells per five high-power fields (X 400) for each section.

Statistical analysis

The statistical analysis of the experimental data was performed using the Student's t-test. The results are expressed as the mean $\pm$ S.D. A p-value less than 0.05 were considered significant.

RESULTS

Effect of 850 nm LED phototherapy on development of Df-induced AD-like skin symptoms

To investigate the effect of 850 nm LED phototherapy on Df-induced NC/Nga mice, the dorsal skin of the mice was irradiated to a LED light with 10, 20, 40, and 80 J/cm^2 at 850 nm

wavelengths once a day for 2 weeks. The symptoms severity scores were reduced significantly by the irradiation with high-dose (40 and 80 J/cm²) of 850 nm LED (Fig. 1). However, low-dose (10 and 20 J/cm²) of 850 nm LED was not significantly different from that in the control group (Df alone) (Fig. 1). Therefore, we used low-dose (10, 15, and 20 J/cm²) of 850 nm LED in the following experiments.

Inhibitory effect of combination therapy with low-dose 850 nm LED phototherapy and water bath therapy on development of Df-induced AD-like skin symptoms

We investigated the therapeutic potential of combination therapy on the development of Df-induced AD-like skin lesions in the NC/Nga mice. The combination of low-dose 850 nm LED phototherapy and water bath therapy resulted in significantly suppressed the development of AD-like skin lesions compared with that of the group of low-dose 850 nm LED phototherapy alone or water bath therapy alone (Fig. 2A). There was no significant difference between the low-dose 850 nm LED phototherapy alone or water bath therapy alone and the control group (Df alone) (Fig. 2A). Representative clinical features of NC/Nga mice are shown in Fig. 2B.

Inhibitory effect of combination therapy on Df-induced serum IgE, NO, and histamine levels in NC/Nga mice

We analyzed the levels of total IgE, NO, and histamine in the serum of Df-induced NC/Nga mice to characterize the allergic responses after treatment with water bath alone or in combination with low-dose LED phototherapy. As shown in Fig. 3A, B, and C, the total serum IgE, NO, and histamine levels in the combination groups of 850 nm (15 and 20 J/cm²) LED phototherapy and water bath therapy were lower than that in the 850 nm (15 and 20 J/cm²) LED phototherapy alone or water bath therapy alone. In contrast, the released amounts of IgE and histamine were less, and no significant differences were found between the other combination groups and the 850 nm (10 J/cm²) LED phototherapy alone or the water bath therapy alone. However, low-dose 850 nm LED phototherapy alone or water bath therapy alone was not significantly different from that in the control group (Df alone).

Changes of combined therapy on immunologic responses

To clarify the improved mechanism of Df-induced AD-like skin symptoms by combined therapy, we analyzed the Th2-mediated cytokine and chemokines production from cultured splenocytes and skins, which prepared from Df-induced NC/Nga. As shown in Table 1, the decrease in the production of Th2 cytokines such as IL-4, IL-5, IL-13 and Th2 chemokines such as TARC, eotaxin in the combination groups of 850 nm (20 J/cm²) LED phototherapy

and water bath therapy was significant as compared with that in the 850 nm (20 J/cm²) LED phototherapy alone or water bath therapy alone group and the control group (Df alone). Furthermore, this combined therapy significantly decreased the production of TSLP in the skin tissue of Df-induced NC/Nga mice.

Improvement of inflammatory cell infiltration by combined therapy on Df-induced NC/Nga mice

To investigate the inhibitory effects of combined therapy on histological changes in the skin, tissue sections were stained with toluidine blue or Congo red. As shown in Fig. 4A and 4B, the combined therapy of 850 nm (20 J/cm²) LED phototherapy and water bath therapy significantly reduced the number of infiltrated cells, both mast cells and eosinophils as compared with 850 nm (20 J/cm²) LED phototherapy alone or water bath therapy alone and the control group (Df alone).

DISCUSSION

Multiple medication therapy, including topical and systemic steroids such as corticosteroid, and calcineurin inhibitors such as FK-506 and cyclosporine A (CsA), has been the mainstay of treatment for immune-mediated diseases such as AD (23,24). However, long-term use of these drugs may be limited by potential harmful effects. Several studies for

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treatment of patients with AD using combination therapy have been performed (25,26). Thus, combination therapy may provide an alternative treatment for AD, with improvement of therapeutic efficacy, reducing the amount of drug. Recently, we previously demonstrated that combination therapy of two immunosuppressive agents was had a greater therapeutic impact than monotherapy to ameliorate dermatitis in NC/Nga mice model (27). LED is one of the phototherapy which was recently approved for the management of allergic diseases such as AD (28). Thus, LED is known to exert multiple biological effects on the skin diseases. Our previous results showed that treatment of 850 nm LED to NC/Nga mice inhibited the development of AD-like skin lesions and regulated production of Th2-mediated cytokines (29). Additionally, it is well known that bath therapy of natural agents such as mineral water, and green tea has beneficial effects and safety for the treatment of AD (18-20). In this study, we investigated the therapeutic effect and inhibitory mechanism of combination therapy of low-dose 850 nm LED phototherapy plus water bath therapy in Df-induced AD-like skin lesions in NC/Nga mice.

We have shown here that single-modality treatment of high-dose 850 nm LED (40 and 80 J/cm²) to NC/Nga inhibited the development of AD-like skin symptoms, but little effects in NC/Nga mice treated with low-dose 850 nm LED (10 and 20 J/cm²). To further investigate the beneficial effects of low-dose 850 nm LED, low-dose 850 nm LED was performed

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combination therapy with water bath. We found that this combination therapy has a therapeutic effect on Df-induced AD-like skin lesions in NC/Nga mice. Many reports have shown that clinical features of AD was associated with overproduction of total IgE, NO, and histamine levels. The IgE overproduction might be due to Th2 cytokine produced by mast cells, CD4⁺ T cells, and eosinophil-predominant inflammation at the affected skin lesion (30). The histamine level is one of the most important inflammatory mediators in AD because high histamine levels mediate the critical typical symptoms of AD (31). Furthermore, NO may regulate the IgE-mediated function of mast cells including histamine induce from mast cell (32). We and others have reported that Df-induced AD-like skin lesion in NC/Nga mice a significantly increased the production of serum IgE, NO, and histamine (17,33). These results suggest that combination therapy of low-dose 850 nm LED phototherapy and water bath therapy might improve the clinical symptoms of Df-induced NC/Nga mice through the inhibitory effect of immunological pathways including IgE, NO, and histamine.

Inflammatory mediators such as Th2 cytokine and Th2 chemokine are thought to be central to the pathogenesis of AD (7). Th2 cytokine have been considered as the major source for IL-4, IL-5, and IL-13, and regulated IgE synthesis. Moreover, the Th2-specific cytokines and IgE have been reported to be highly produced in the AD-like skin lesions in NC/Nga mice (34). Eotaxin is induced during cutaneous eosinophilia and further increased

chemoattractive activity for eosinophils in the presence of IL-5, whereas TARC produced by Th2 cells and overexpressed by epidermis in AD lesions (35). Therefore, Th2 chemokine, such as eotaxin and TARC, may be an important mediator that contribute and exacerbate AD symptoms. Additionally, thymic stromal lymphopoietin (TSLP) was shown to be significantly increased in lesional AD skin and its expression was associated with Th2 immune response, TARC chemokine, and IgE (31,36). The present study of immune responses clearly shows that Th2 cytokine and Th2 chemokines, and TSLP in combination therapy with low-dose 850 nm LED phototherapy and water bath therapy were significantly lower than in the single modality treatment with either alone and the control mice (Df alone). These results imply that inhibition of Th2-mediated cytokines and chemokines, TSLP might be one of the mechanisms responsible for the inhibitory effect of combination therapy of low-dose 850 nm LED phototherapy and water bath therapy on development of Df-induced AD-like skin lesions in NC/Nga mice.

The aggravation of AD-like skin lesions is dependent on the production of the infiltration of inflammatory cell including eosinophils and mast cells (37). Moreover, the eosinophils and mast cells have been reported to be highly expressed in the Df-induced NC/Nga mice (38). These finding suggest the involvement of IgE-associated Th2 cells in the development of Df-induced AD-like skin lesions in NC/Nga mice. Therefore, recruitment of these inflammatory

cells is considered to play a major role for the management of AD. We found that combination therapy of low-dose 850 nm LED phototherapy and water bath therapy improved the severity of the dermatitis with reduction of inflammatory cellular infiltrate, such as mast cells and eosinophils. These results suggest that inhibition of Df-induced AD-like skin lesions in NC/Nga mice might be mediated, at least in part, by its inhibitory effect on these inflammatory cells. **Collectively, the inhibitory effects of this combined therapy might inhibit the production of IgE, NO, and histamine, probably by reducing the production of Th2-mediated cytokines and chemokines, infiltration of inflammatory cells, as well as the improving of skin hydration.**

CONCLUSION

The combination of LED phototherapy and water bath therapy might be used as an efficacious, safe, and steroid-free alternative therapeutic strategy for the treatment of AD with no severe side effects.

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Table legends

Table 1. Effects of combination therapy on the level of Th2 cytokines and Th2 chemokines in Df-induced NC/Nga mice. IL-4, IL-5, IL-13, eotaxin, TARC, and TSLP were measured on day 7 after the final administration. Data are mean $\pm$ S.D. of five mice. * $p < 0.05$ as compared with untreated control (Df alone) mice (Student's t test).

FIGURE LEGENDS

Fig. 1. Effect of 850 nm LED phototherapy on the Df-induced AD-like skin lesions in NC/Nga mice. 850 nm LED was irradiated to Df-induced NC/Nga mice once a day for 2 weeks at various doses (10, 20, 40, and 80 J/cm²). The clinical skin score is the sum of individual scores judged by the symptoms of erythema/hemorrhage, scarring/dryness, edema,

excoriation/erosion. Each data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's t -test compared with all other groups.

Fig. 2. Therapeutic effect of combination therapy on the Df-induced AD-like skin lesions

in NC/Nga mice. (A) After the removal of hair, Df body ointment (100 mg/mouse) was swabbed onto the dorsal skin and both surfaces of each ear twice a week for 4 weeks. The clinical skin score is the sum of individual scores judged by the symptoms of erythema/hemorrhage, scarring/dryness, edema, excoriation/erosion. Df-induced AD-like skin lesions in NC/Nga mice were untreated (Df alone), or treated with 850 nm LED phototherapy (10, 15, and 20 J/cm²), water bath (10 min in 36 $\pm$ 1°C), or 850 nm LED phototherapy plus water bath once a day for 2 weeks. Each data represents the mean $\pm$ S.D. of 5 mice.

* Statistically significant at $P < 0.05$ using a Student's t -test compared with all other groups.

(B) Representative clinical features of Df-induced NC/Nga mice in the untreated (Df alone), or treated with 850 nm LED phototherapy (10, 15, and 20 J/cm²), water bath (10 min in 36 $\pm$ 1°C), or 850 nm LED phototherapy plus water bath.

Fig. 3. Serum level of IgE, NO, and histamine by combination therapy on the Df-induced AD-like skin lesions in NC/Nga mice. The concentration of IgE (A), NO (B), and histamine (C) in the serum was measured at on day 2 after the final administration. Each data

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represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's t -test compared with all other groups.

Fig. 4. Histological analysis of the skin lesions. Skin samples from the back of the mice were studied at on day 2 after the final administration. The sections were then stained with toluidine blue and Congo red for mast cells and eosinophils, respectively; **(A)** representative histological features in the Df-induced NC/Nga mice from the untreated (Df alone), or treated with 850 nm LED phototherapy (20 J/cm^2), water bath (10 min in $36 \pm 1^\circ\text{C}$), or 850 nm LED phototherapy plus water bath; **(B)** the cell density was expressed as the number of mast cells and eosinophils in five high-power fields (X400 for count) for each section. The data represents the mean $\pm$ S.D. of 5 mice. * Statistically significant at $P < 0.05$ using a Student's t -test compared with all other groups.

Table 1

Groups	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	TSLP (pg/ml)	Eotaxin (pg/ml)	TARC (pg/ml)
Control (Df alone)	152.4±12.1	160.4±24.9	312.4±21.8	60.1±10.1	138.4±21.4	169.1±19.3
Water bath	141.8±19.4	151.8±32.1	298.1±20.6	56.4±9.5	124.4±31.1	159.4±16.1
850 nm LED (20J/cm2)	138.4±15.1	156.1±32.4	284.1±36.9	53.4±12.1	128.1±26.7	143.5±32.1
Water bath + LED (20J/cm2)	95.7±23.1 [*]	102.4±21.5 [*]	201.4±26.7 [*]	34.2±9.1 [*]	84.7±29.4 [*]	93.4±29.6 [*]

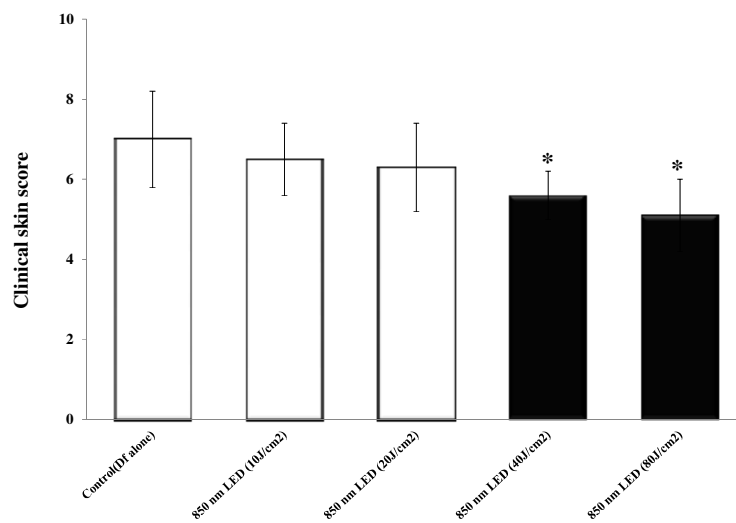
Fig.1

Fig. 2A

Groups	Dermatitis score (Day 0)	Dermatitis score (Day 14)
Control (Df alone)	8.0±0.0	7.3±1.2
Water bath	8.0±0.0	6.7±0.9
850 nm LED (10J/cm ²)	8.0±0.0	6.8±1.1
850 nm LED (15J/cm ²)	8.0±0.0	6.5±0.7
850 nm LED (20J/cm ²)	8.0±0.0	6.5±0.5
Water bath+ 850 nm LED (10J/cm ²)	8.0±0.0	5.9±0.9 [*]
Water bath+ 850 nm LED (15J/cm ²)	8.0±0.0	5.1±0.6 [*]
Water bath+ 850 nm LED (20J/cm ²)	8.0±0.0	4.3±0.9 [*]

Fig. 2(B)

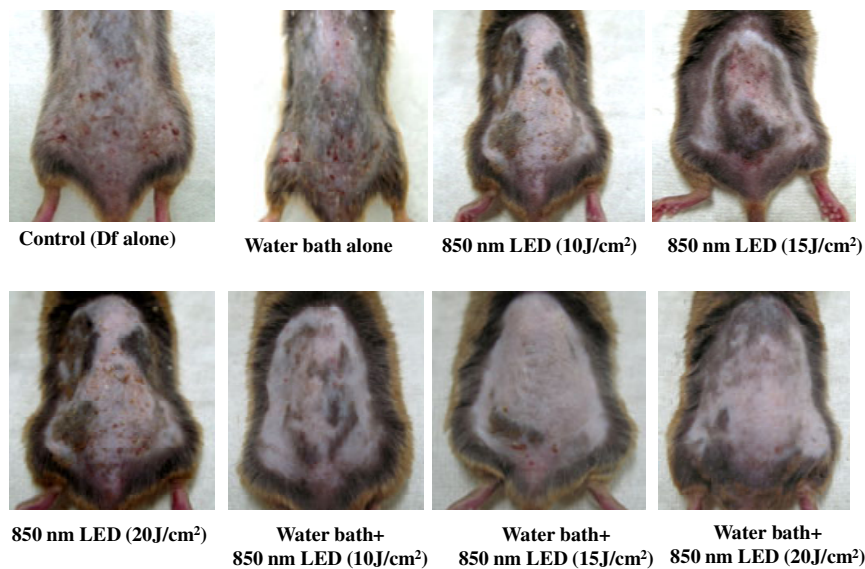


Fig. 3 (A)

IgE

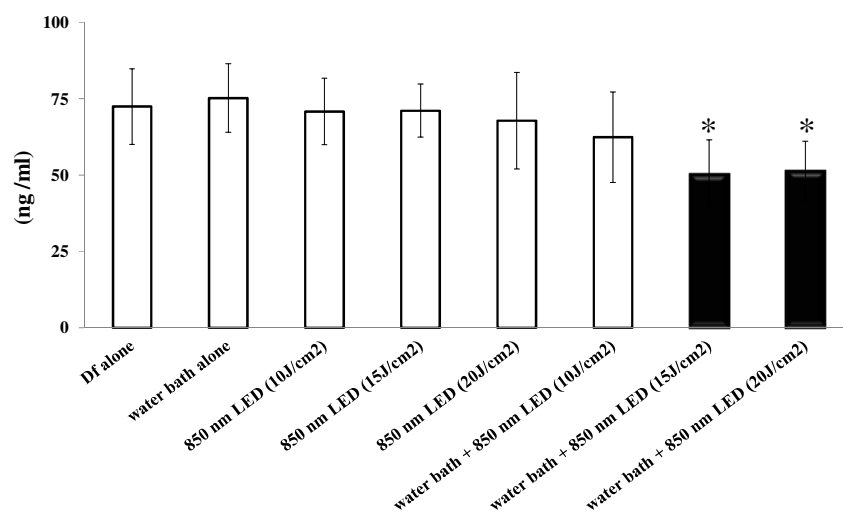


Fig. 3 (B)

NO

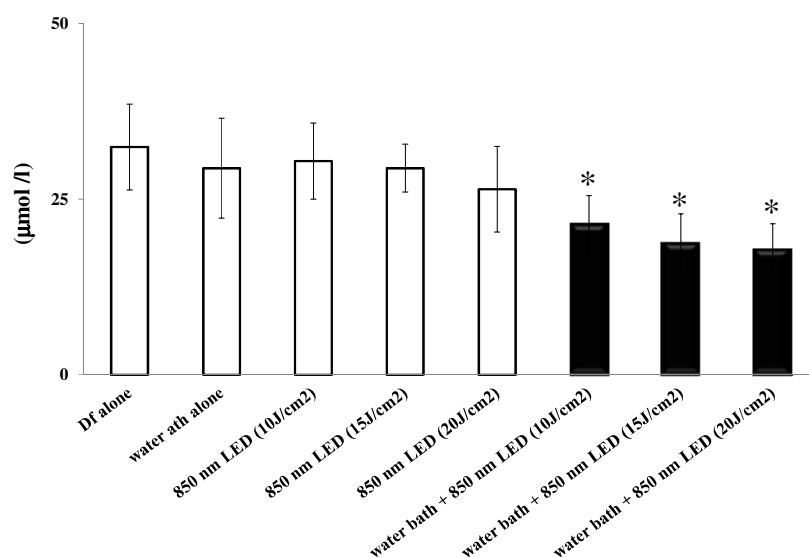


Fig. 3 (C)

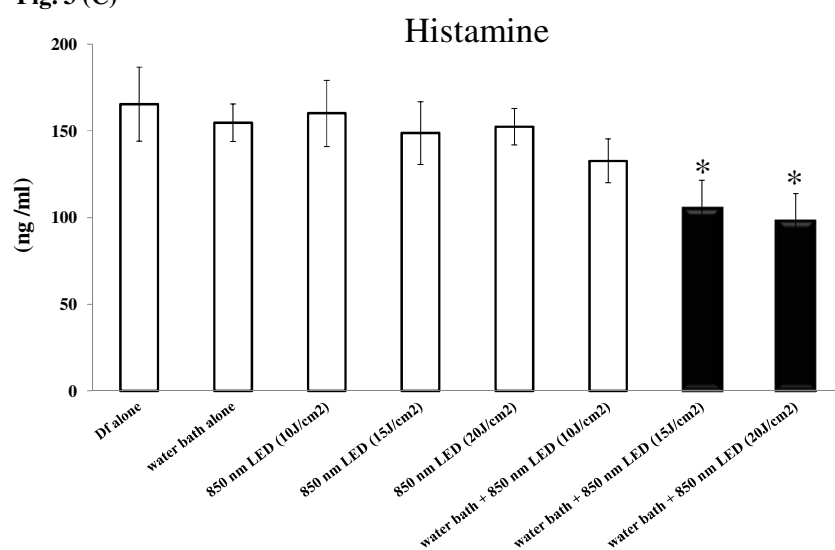


Fig. 4 (A)

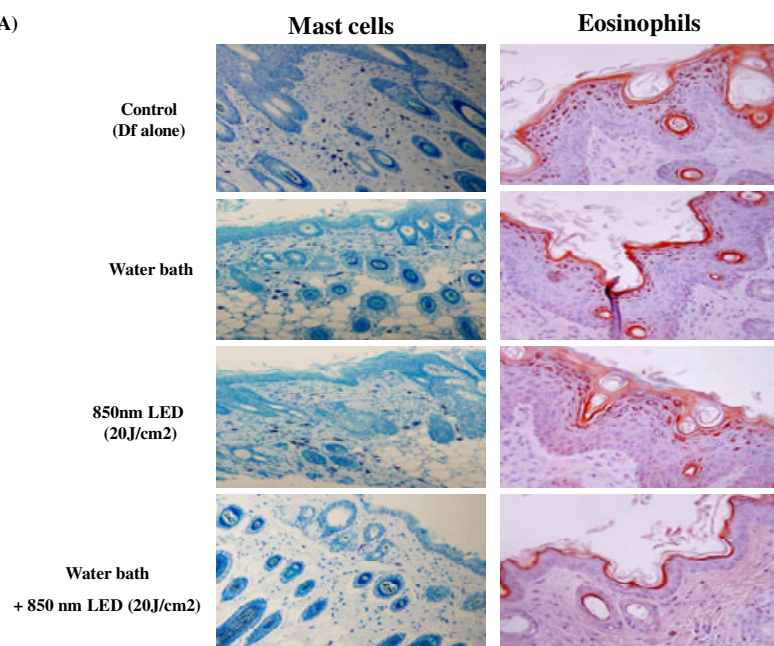


Fig. 4(B)

