Anti-inflammatory activities of light emitting diode irradiation on collagen-induced arthritis in mice (a secondary publication)

Authors: Noboru Kuboyama [1,3], Mitsuhiro Ohta [2,4], Yusuke Sato [1], Yoshimitsu Abiko [3,4]

1: Department of Pharmacology, Nihon University School of Dentistry at Matsudo
2: Department of Oral Diagnosis, Nihon University School of Dentistry at Matsudo
3: Department of Biochemistry and Molecular Biology, Nihon University School of Dentistry at Matsudo,
4: Research Institute of Oral Science, Nihon University School of Dentistry at Matsudo

Background and aims: Rheumatoid arthritis (RA) is an auto-immune disease afflicting multiple joints of the body, where as a result of the increase in inflammatory cytokines and tissue destructive factors such as matrix metalloproteinase (MMP)-3, deterioration of the bones and cartilages of the joints occurs. The present investigation was carried out to study the anti-inflammatory activities of light emitting diode (LED) irradiation on hind paw inflammation in collagen-induced arthritis (CIA) mice models.
Materials and method: RA in the CIA mouse model was induced by immunization of DBA/1J mice with intradermal injections of an emulsion of bovine type II collagen and complete Freund's adjuvant. A total of 20 CIA mice were subdivided into the following groups: control group, CIA group and 2 groups of LED irradiated CIA mice (LED groups) (n=5 per group). The mouse knee joint area in the LED groups (the 570 nm and 940 nm groups) was irradiated with LED energy, three times a week for 500 s per session over 8 weeks at a dose of 5 J/cm^2. The hind paw swelling was assessed by the increase in hind paw thickness. The serum levels of the inflammatory cytokines and arthritic factor MMP-3 were determined with an enzyme-linked immunosorbent assay (ELISA).
Results: In the LED-570 and LED-940 groups at 4weeks after arthritis induction, the swelling inhibition index was 18.1±4.9 and 29.3±4.0 respectively. Interleukin (IL)-1ß, IL-6 and MMP-3 serum levels were significantly lower in the LED-940 group.
Conclusions: LED irradiation, particularly in the near-infrared was effective for inhibition of the inflammatory reactions caused by RA.

Key words: light emitting diode (LED) phototherapy · collagen-induced arthritis mice · hind paw swelling · inflammatory cytokines · MMP-3

Introduction

Rheumatoid arthritis is an auto-immune disease afflicting multiple joints of the body. As the disease progresses, proliferative inflammation of the joint synovia is induced. The increased granulation tissue or pannus produces excessive inflammatory cytokines or tissue destructive factors such as matrix metalloproteinase (MMP)-3, which results in the deterioration of the joints and cartilages [1-3]. It is also reported that inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) play a part in the onset of the disease [4, 5].

As for phototherapy as a modality to treat RA in a rat model, the authors have previously reported the efficacy of linear polarized near-infrared (NIR) light and free-electron laser (FEL) light in achieving an anti-

Addressee for Correspondence:
Noboru Kuboyama
870−1, Sakaecho-Nishi 2, Matsudo, Chiba, 271-8587, Japan
TEL: +81-47-360-9344 FAX: +81-47-360-9344
kuboyama.noboru@nihon-u.ac.jp

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rheumatoid effect by significantly inhibiting the increase of serum inflammatory cytokine and MMP-3 levels and thereby inhibiting the swelling of the hind leg, resorption and erosion of the calcaneus bone in collagen induced arthritis (CIA) rats [6, 7].

Recently, the efficacy of light emitting diodes (LED) irradiation to increase tissue blood perfusion, prevention and amelioration of post-inflammatory hyperpigmentation and atopic dermatitis, enhancement of wound healing and the increase in mitochondrial activity resulting in increased cellular proliferation have all been reported [8-10]. Xavier et al. have reported that LED irradiation (wavelength 880 nm, irradiation time 170 s, total energy density 7.5 J /cm^2, period of the trial of 2 weeks) in a collagen-induced Achilles tendonitis rat model resulted in a decrease in both the emergence of inflammatory cells and the expression of inflammatory cytokines [11]. Others have investigated and compared the effect of low level light therapy (LLLT) with both laser and LED-based sources on zymosan-induced knee joint arthritis in rats. [12] In that study LLLT with the laser source (685 nm and 830 nm) demonstrated efficacy in inhibiting the increase of vascular permeability and hyper algesia whereas the LED source (628 nm) had no effect. In an *in vitro* experimental setting, prostaglandin (PG) E2, cyclooxygenase (COX) and lipoprotein-associated phospholipase (PLA)2 gene expression of human gingival fibroblast culture which usually increases when stimulated with arachidonic acid, was suppressed by treatment with 635 LED irradiation. [13]. In our present study, in order to compare and contrast the anti-inflammatory activity of LEDs with that of NIR and FEL, the treatment parameters of irradiation time (500 s) and total energy density (5 J/cm^2) were maintained. The animals used in the study were CIA mice, a widely acknowledged human RA animal model [14-16].

Materials and methods

The experimental animal

The animals used for this study were 5-week old DBA/1 LacJ male mice (Japan Charles River Inc.) The mice were kept in a climate controlled room at temperature of 23±1°C, humidity 60±10%, diurnal cycle of 12 hours of light and 12 hours of darkness. The mice were fed solid foodstuff (MF, Oriental Yeast Co., LTD.) and filtered tap water *ad libitum*. Following a one week preliminary period, the general well-being of the mice was checked and body weight was measured. Mice weighing 14.6±1.1 g in a healthy condition and with normal growth were used in this study. This study was approved by the Ethics Committee for the Handling of Experimental Animals, Nihon University School of Dentistry at Matsudo. The animals were kept under the rules and stipulations of the Guidelines for Experimental Animals of Nihon University School of Dentistry at Matsudo.

The preparation of the CIA mice

The CIA mice were prepared using 6-week-old DBA/1LacJ male mice. Initial sensitization was performed by administering an emulsion of 0.8 g of type 2 bovine collagen mixed with Freund's Complete Adjuvant at a one-to-one ratio. Ten intradermal injections of 0.05 ml each of this emulsion were administered to the skin on the back. Following the initial sensitization, immunization was performed twice, injecting the same emulsion to the root of the tail and thereby inducing the onset of arthritis **(Figure1 (a), (b))**.

Irradiation parameters for the LED therapy

An LED-based device (Panasonic Co.) was used. The distance between the LED array and the knee joint of the mouse was set at 20 mm. The irradiation area was set to a 15 mm diameter centered on the knee joint for an even field of irradiation. Two wavelengths, 570 nm and 940 nm, were used for the irradiation in separate groups. The exposure time was set at 500 s in continuous wave and the irradiance was set so that the total energy density was 5 J/cm^2. Irradiation at this setting, caused local body temperature elevations less than 0.5 °C as measured with infrared thermography, and was considered too low for any thermal reaction to have taken part in this experiment. The experimental period was set at 8 weeks, and the mice received LED irradiation centered on the knee joint 3 times a week for a total of 24 sessions, under general anesthesia with intra-peritoneal administration of sodium pen to barbitone (35 mg/kg) **(Figure2)**.

Evaluation of arthritis

The swelling of the hind paws of the mice was determined once a week, by measuring the thickness of the hind paws from a lateral view using a stereoscopic microscope. The swelling index and swelling inhibition rate were calculated for both hind paws and the average of these values was used for the evaluation.

Experimental method

Mice with a swelling index of 50% or greater, whose last immunization was 2 weeks prior to the experiment, were divided into 3 groups, with each group

consisting of 5 mice. The groups were the CIA group, the LED-570 group and the LED-940 group. The CIA group received no treatment while the LED-570 and LED-940 groups each received LED irradiation at the parameters given above for both groups. Another group of non-immunized, untreated animals was used as control, for a total of four groups. During the period of the experiment the general conditions of the animals were observed and the body weight and hind paw thickness of all animals were measured once a week.

Inflammatory cytokines and MMP-3 concentrations

Following the 8 weeks of LED irradiation, blood samples were collected through the jugular vein of the mice. Only the serum was used for the assay. The concentrations of serum inflammatory cytokines (TNF-α, IL-1ß, IL-6) were measured using an ELISA kit (R&D Systems), following the manufacturer's instructions. Concentrations of . MMP-3, a rheumatoid factor, were determined using the Assay Kit (Cosmo Bio) by first activating MMP-3 with 4-aminophenylmercuric acetate followed by measuring of fluorescence intensities at an excitation wavelength of 330 nm and a fluorescent wavelength of 395 nm. The data from the measurements were used for calculation of the MMP-3 concentration relative to the control provided with the kit.

Statistical analysis

The results of the experiment were expressed as the mean±standard deviation. The comparison of the results amongst the control group, CIA group,LED-570 group and LED-940 group were analyzed using Tukey's test, where a p-value less than 5% ($p<0.05$) was considered significant.

Results

Bodyweight change

The changes in the bodyweight of the mice are presented in **Figure 3**. At the start of this investigation, the average weight of the mice was 14.6±1.1 g. Following the first collagen immunization, the CIA mice (CIA group, LED-570 group and LED-940 group) all failed to gain as much weight as the control group. At 4 weeks after collagen immunization, there was a significant difference between the CIA mice and the control. The average body weight of the CIA mice was 18.2±3.8 g compared to that of the control mice whose average body weight was 24.9±3.5 g ($p<0.01$). At the end of this experiment, the control mice weighed 30.6±3.2 g, while the CIA group mice weighed 22.7±4.1 g ($p<0.01$), the LED-570 group mice weighed 24.8±4.7 g ($p<0.05$) and the LED-940 group mice weighed 25.6±4.2 g ($p<0.05$). The body weight of all CIA mice was significantly less when compared to the control. However there were no significant differences between the CIA mice groups when compared to each other.

Hind paw swelling of the CIA mice

The sequential changes in the hind paw swelling index of the CIA mice are shown in **Figure 4 (a)**. At the start of this investigation, the average hind paw thickness was 1.57±0.08 mm. At 2 and 4 weeks after the initial collagen immunization, swelling was seen and advanced rapidly; the swelling indices were 37.4±4.6% (2.17±0.27 mm) and 57.6±7.4% (2.50±0.32 mm), respectively. Only those mice whose swelling index was greater than 50% at the start of the LED irradiation were considered to be CIA mice and were included in this experiment. After 2 weeks of LED irradiation, the swelling indices were 56.2±7.3% for the CIA group and 48.6±7.2 % and 44.1±5.5% for the LED-570 group and the LED 940-group respectively.

The swelling inhibition rate is shown in **Figure 4 (b)**. When compared to the CIA group, the inhibition of swelling as a result of LED irradiation at 2 weeks was 13.5±2.1% and 21.6±2.7% for the LED-570 group and LED-940 group respectively, 18.1±4.9% and 29.3±4.0% at 4 weeks, and 17.2±3.7% and 23.8±3.1 % at 8 weeks. The maximum swelling inhibition as a result of LED irradiation was seen at 4weeks. When the difference in the swelling inhibition between the LED-940 and LED-570 mice was compared, LED-940 animals demonstrated a significantly greater inhibition rate compared with the LED-570 group mice.

Figure 5 and **Figure 6** show the gross condition of the hind paws of all groups (control, CIA, LED-570 and LED-940) after 8 weeks. Arthritis was induced in the CIA mice after the second collagen immunization. Multiple joints were affected and were swollen but the most prominent was that of the foot joint of the hind paws. The difference between the LED group and CIA group was easily perceived even through gross observation, where swelling and redness were notably more reduced in the LED group.

The concentration of inflammatory cytokines and MMP-3

The serum concentration of TNF-α, following the 8 week period of LED irradiation of the control group was 1.7±0.3 pg/ml whereas the concentration of the

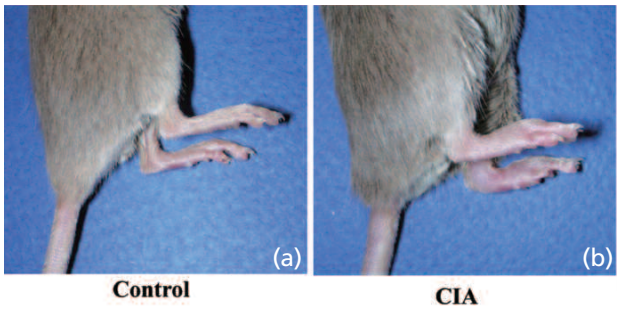

Fig. 1: (a) Observation of hindpaw in normal mice. (b) Observation hindpaw swelling in CIA mouse at 8 weeks after the start of collagen immunization.

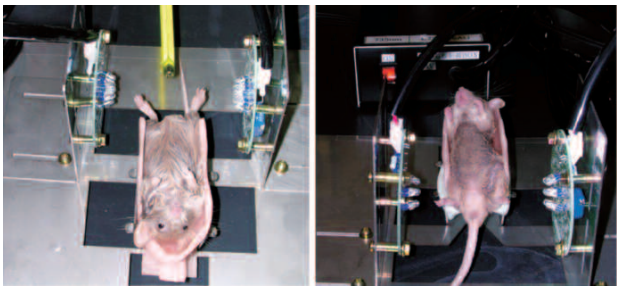

Fig. 2: LED irradiation system image of CIA mouse.

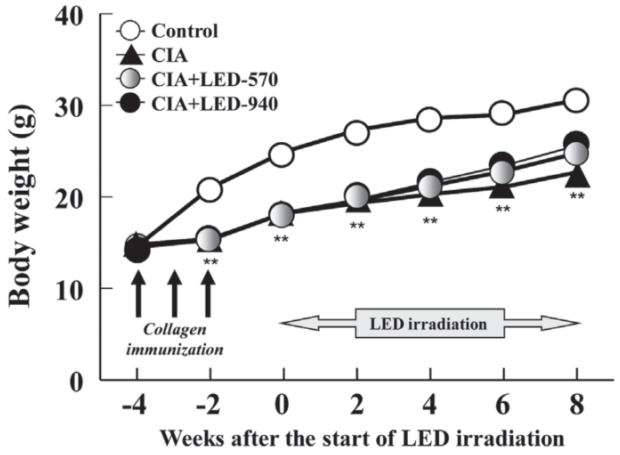

Fig. 3: Changes in body weight on LED irradiation in collageninduced arthritis mice. Data were shown as the mean±S.D. of 5 animals in each group. **: p<0.01, vs. control group.

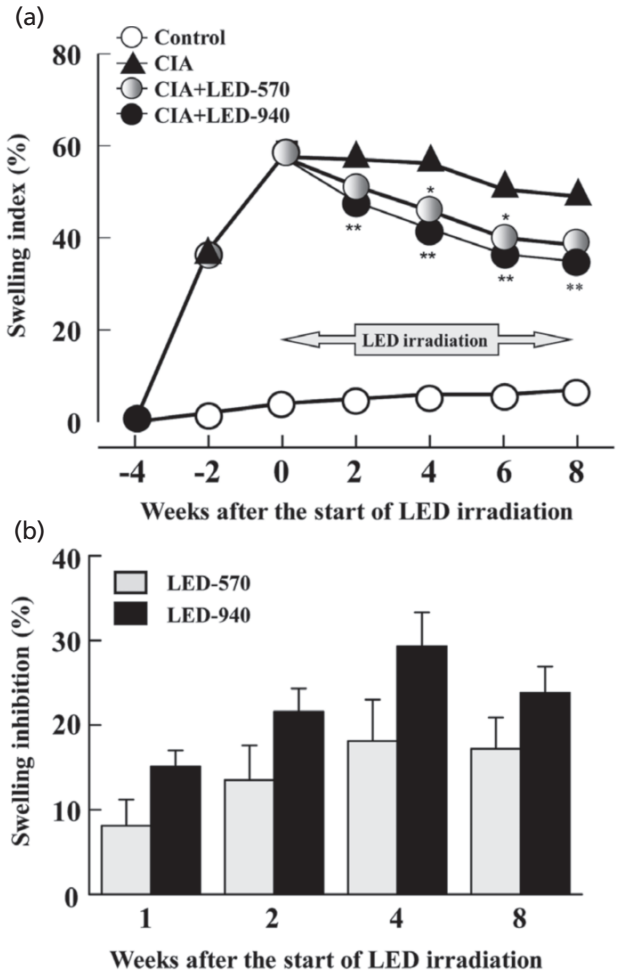

Fig. 4: (a) Effects of LED irradiation on hind paw swelling index in CIA mice. Data were shown as the mean±S.D. of 5 animals in each group. *: p<0.05, **: p<0.01 vs. CIA group.
(b) Effects of LED irradiation on swelling inhibition in CIA mice.Data were shown as the mean±S.D. of 5 animals in each group.

CIA group was 6.8±2.2 pg/ml (p<0.001). The TNF-α concentrations of the LED-570 group and LED-940 group were lower than the CIA group, measuring 5.8±1.9 pg/ml and 4.6±1.3 pg/ml (p<0.05) respectively **(Figure 7 (a))**.

The concentration of IL-1ß was 11.0±1.7 pg/ml for the control group, whereas the concentration of the CIA group was significantly higher, approximately 6 times more, at a concentration of 61.0±13.8 pg/ml. IL-1ß levels of the LED-570 and LED-940 were significantly lower than the CIA group at levels of 52.6±10.6 pg/ml and 41.3±6.5 pg/ml (p<0.05 for both) respectively **(figure 7 (b))**. The concentration of IL-6 in the control group was 5.1±0.8 pg/ml whereas the concentration in the CIA group showed an almost n 8-fold increase at 38.3±8.1 pg/ml (p<0.001). The IL-6 levels of the LED-

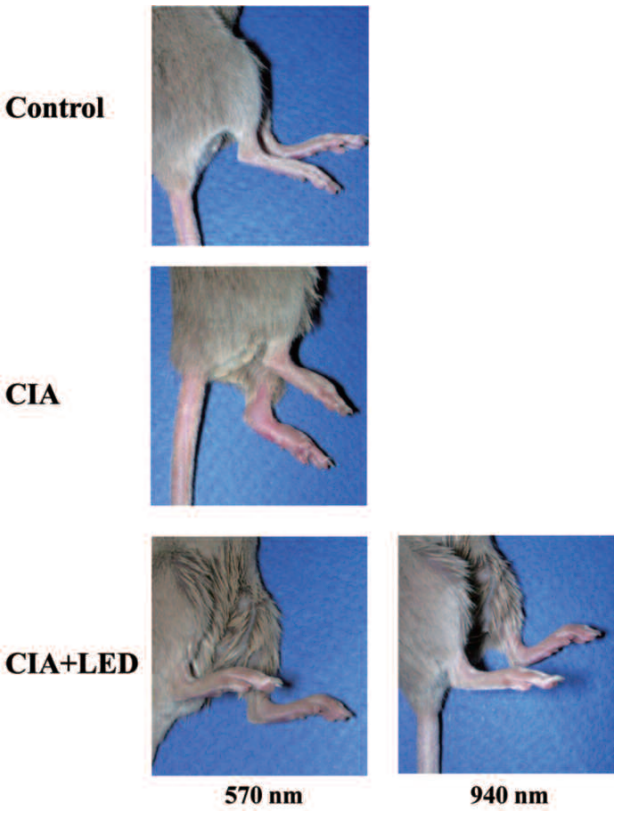

Fig. 5: Photograph of hind paw in CIA mice at 8 weeks after arthritis induction.

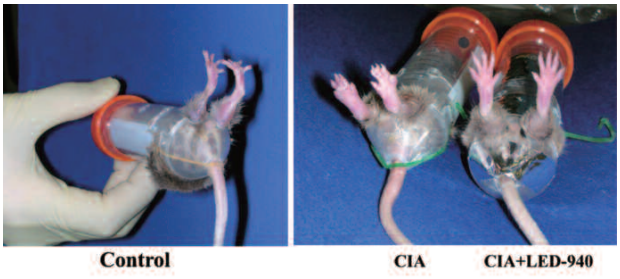

Fig. 6: Photograph of sole print in CIA mice at 8 weeks after arthritis induction.

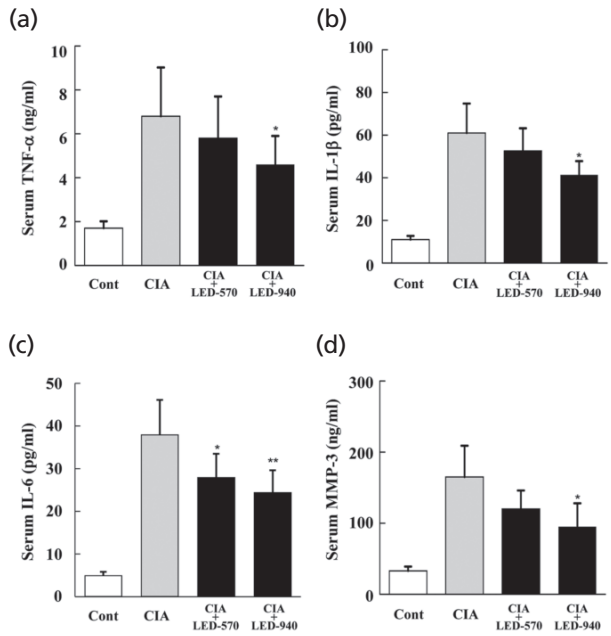

Fig.7: Anti-inflammatory effect of LED irradiation on serum levels of cytokines in CIA mice at 8 weeks after arthritis induction. (a): TNF-α, (b): IL-1ß, (c): IL-6, (d): MMP-3. Data were shown as the mean±S.D. of 5 animals in each group. *: p<0.05, **: p<0.01 vs. CIA group.

570 group and LED-940 group were significantly lower than the CIA group, measuring 28.0±5.5 pg/ml ($p<0.05$) and 24.5±5.1 ($p<0.01$) respectively **(Figure 7 (c)).** The concentration of MMP-3 in the control group was 33.0±6.1 pg/ml whereas the concentration in the CIA group was 165.3±44.7 pg/ml. The levels of MMP-3 in the LED-570 group and LED-940 group were lower than the CIA group, measuring120.2±26.3 pg/ml and 95.1±33.4 pg/ml ($p<0.05$) respectively **(Figure 7 (d))**. LED irradiation at the wavelength of 940 nm significantly lowered or suppressed the increasing concentrations of all inflammatory cytokines (TNF-α, IL-1ß, IL-6) and MP-3. Although a greater decreasing trend was seen in favor of the LED-940 group, comparison between the LED-570 and LED-940 groups showed no statistically significant findings.

Discussion

The aim and goal of the treatment of RA is to first control the pain and inflammatory reactions and secondly to suppress the deterioration of the bone and joints. The mainstream treatment protocols are based on medication where nonsteroidal anti-inflammatory drugs (NSAIDs) are used to alleviate pain and to control the inflammatory reactions, and where steroids and anti-rheumatic drugs are used to control the immunological responses. Recent advances in tactics for the treatment of RA include the targeting of TNF-α. TNF-α plays a central role in the pathophysiology of RA, and biopharmaceutical drugs targeting TNF-α such as the humanized chimera antibodies against TNF-α have appeared. Such drugs directly or indirectly powerfully inhibit the

effect of TNF-α and are now known to be capable of stopping bone and joint destruction completely [17]. However, targeting. TNF-α has its drawbacks in that it can compromise the immune system of the host. Side-effects such as severe infections have been reported. [18] Cases of fulminant hepatitis and related deaths of patients with prior resolved hepatitis B virus (HBV) infections due to the reactivation of the HBV caused by the strong immuno-suppression of anti-TNFα drugs have been reported [19]. Therefore the need for a more safe and effective treatment with less side-effects still exists.

Arthritis in the CIA mouse model is induced by immunization with bovine type 2 collagen of DBA/1 J mice, and the CIA mouse is routinely used as an animal model for human RA. The arthritis of CIA mice closely resembles that of humans in that serum antibodies to type 2 collagen are seen in both and reflect the auto-immune nature of the disease [14, 15, 20], and in that proliferation of synovial cells is observed along with bone and joint destruction and activated osteoclasts. The increase in certain cytokines is also similar. In our current investigation, arthritis of the CIA mice developed following immunization with collagen. The main arthritic site was the foot joint and hind paws of the mice. Signs and symptoms similar to humans such as rubor, tumor and dolor were evident by simple observation.

When the experimental mice were irradiated with LED energy, the swelling inhibition rate was maximum at week 4 of irradiation. The swelling inhibition rates for LED-570 and LED-940 mice were 18.1±4.9% and 29.3±4.0%, respectively, and the swelling inhibition rate in the LED-940 group animals was significantly greater ($p<0.01$) than in the non-treated CIA group. The wavelength of 940 nm is in the near-infrared waveband, compared with the shorter visible yellow wavelength of 570 nm. The longer wavelength is capable of much deeper penetration into living biological tissue, whereas the 570 nm light has blood and melanin as competing chromospheres. It can be proposed that the 940 nm LED energy was transmitted through the epidermis and dermis into the deeper soft tissue, and may have affected the circulation surrounding the joints [21]. Furthermore, the spectral distribution of LED shows that the maximum peak of relative radiant intensity is reached at the wavelength 950nm. The wavelength 940 nm, used in this investigation, may therefore have a high affinity for living tissue and be capable of inducing biological reactions.

In CIA mice, the inflammatory cytokines produced in the pannus are trapped in the tissue and within the joint and cause the inflammatory reaction seen in the hind paws. The results of LED therapy at 940 nm suggest that the LED energy penetrated the tissue and was scattered within the hind leg tissue and joints, enhancing the circulation in these tissues and thereby inhibiting the swelling of the hind legs and paws. The results in ourLED-940 group differ from the results of previous studies using NIR laser and FEL irradiation in that the maximum swelling inhibition associated with NIR laser and FEL was seen at 2 weeks at a inhibition rate of 39.1±5.2% and 31.0±5.8%, whereas in some studies LLLT with LED sources was ineffective.

In RA, the excessive production of inflammatory cytokines by activated macrophages at the site of the inflammation causes the inflammation and induces osteoclast activity [4, 5, 22 ,23]. Therefore, it is important to suppress the production of such cytokines. In the present investigation the production of TNF-α, IL-1ß and IL-6 were suppressed in both the LED-570 and LED-940 groups. This suggests that LED irradiation also acts directly on the macrophages and synovial cells. TNF-α, IL-1ß and IL-6 are osteolytic cytokines in RA. These promote the expression of receptor activator of nuclear factor kappa-B ligand (RANKL) in synovial fibroblasts, the differentiation of osteoclasts and also induce MMP-3 production, all of which play parts in the destruction of the bone and joint [24-27]. In a previous *in vitro* study, the first author has demonstrated that Ga-Al-As diode laser irradiation (Panalas-1000: wavelength 830 nm, total energy density 7.9 J/ cm^2) significantly suppressed the production of PGE_2, IL-1ß and PA in LPS stimulated human gingival fibroblast cell cultures [28-30]. Joint cartilage is composed of mainly type 2 collagen and chondroitin sulfate along with other extra-cellular matrix substances such as hyaluronic acid. In RA, accelerated degradation of the extra-cellular matrix is the cause of cartilage deterioration which leads to the loss of joint function [31].

The destruction of bone and joint cartilage in RA relies on the presence of catabolic enzymes of the extra-cellular matrices such as the MMPs which are produced by synovial cells and joint cartilage cells. - MMP-3 is therefore used as an RA marker, the rise in the concentration of which is in extremely close correlation with joint cartilage destruction [2, 31-33]. In another previous *in vitro* study, Ozawa *et al.* have demonstrated that irradiation of an osteoblast cell line, MC3T3E1, with diode 830 nm diode laser LLLT increased the number of bone nodule formations, the total area of bone formation and the production of collagen and alkaline phosphatase [34]. It is suggested that LED-LLLT achieves its anti-inflammatory and bone resorption suppression

effects through a similar mechanism.

LED phototherapy is gaining attention as the multi-purpose light source of the 21st century. LEDs are now being routinely used for display purposes, traffic lights and domestic lighting, in addition to the rapidly-growing body of literature on their therapeutic indications: further developments of usages are anticipated. LED systems in phototherapy can be grouped into 2 types, those being visible light LEDs (wavelength 400~700 nm, but not including the ultra violet waveband; and near infra-red LEDs (wavelength700-1400 nm. Concerning the safety of LED radiation, there are reports that exposure to blue light LEDs (400~500 nm) causes retinal damage due to the higher photon energy of this waveband than the rest of the visible and near-IR waveband, [35, 36] and that direct viewing of LED light sources emitting over the rest of the visible light spectrum (500-700nm) at close range should be avoided as a common sense safety measure. UV light is known to produce free oxygen radicals at the surface of the epidermal keratinocytes, promoting the production of cytokines such as TNF-α, IL-1ß and IL-6. These cytokines cause damage to dermal cells and extracellular matrix fibers [37, 38]. In the present study visible light LEDs at 570 nm and near infra-red light LEDs at 940 nm were used. These wavelengths were chosen due to the low risk of any skin and ocular damage. The risk of any tissue damage from LEDs is lower than that potentially induced by laser-based therapeutic systems, FEL energy and ultra-violet light since LEDs are per se noncoherent and highly divergent so that the photon intensity of a single LED is much lower than those mentioned previously.

The results of the present investigation showed that LED treatment of a human RA animal model, the CIA mouse, has anti-inflammatory effects where it inhibited and suppressed the swelling of the hind paws, the increase in serum concentrations of inflammatory cytokines and MMP-3. The results suggest that LED irradiation, particularly at the near-infrared wavelength used in the present study, could be effective for the treatment of human RA. Further investigations into elucidating any wavelength dependency of the anti-inflammatory effects may contribute to the further development of low level light therapy as the photomedicine of the 21st Century.

Conclusions

LEDs at the wavelengths of 570 nm and 940 nm were used to treat a human RA animal model, namely the CIA mouse. The chronological changes in hind paw swelling, serum inflammatory cytokines and MMP-3 levels were monitored. From the results the following conclusions were reached: 1. LED irradiation inhibited the swelling of the hind paws. and 2. LED irradiation suppressed the increase in serum inflammatory cytokine levels (TNF-α, IL-1ß, IL-6) and MMP-3 levels. The results of this investigation suggest that LED therapy at appropriate wavelengths could be effective in suppressing the inflammatory reactions associated with RA.

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