Synergistic Effects of Chios Gum Mastic Extract and Low Level Laser Therapy on Osteoblast Differentiation

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In the present study, we evaluated the effect of CGM on osteogenic differentiation of cultured osteoblasts, and determined whether combination treatment with LLLT had synergistic effects on osteogenic differentiation.

The results indicated that CGM promoted proliferation, differentiation, and mineralization of osteoblasts at the threshold concentration of 10 μg/ml; whereas, CGM showed cytotoxic properties at concentrations above 100 μg/ml. ALP activity and mineralization were increased at concentrations above 10 μg/ml . CGM in concentrations up to 10 μg/ml also increased the expression of osteoblast-activated factors including type I collagen, BMP-2, RUNX2, and Osterix. The CGM (50 μg/ml) and LLLT (80 mW for 15 sec) combination treatment group showed the highest proliferation levels, ALP activity, and mineralization ratios. The combination treatment also increased the levels of phosphorylated forms of p38, ATF2, PKD, ERK, and JNK. In addition, the osteoblast differentiation factors including type I collagen, BMP-2, RUNX2, and Osterix protein levels were clearly increased in the combination treatment group. These results suggested that the combination treatment of CGM and LLLT has synergistic effects on the differentiation and mineralization of osteoblastic cells.

Key words: Chios gum mastic, Low level laser therapy, Osteoblast differentiation, hFOB 1.19, MC3T3-E1

Introduction

Bone homeostasis is a dynamic process in the human body that balances osteoblastic bone formation and resorption [1, 2]. When the delicate balancing of bone remodeling is disordered in the bone, it can develop into a variety of diseases such as osteoporosis, Paget's disease, rheumatoid arthritis, and tumor formation [3]. Osteoblasts play a key role in the physiological process of bone remodeling. They perform various functions that depend on their differentiation status. Immature osteoblasts regulate the differentiation and activation of osteoclasts, while mature osteoblasts are bone-forming cells that play a crucial role in osteogenesis [4]. During the osteogenic process, many signaling pathways are involved such as mitogen-activated protein kinases (MAPKs), bone morphological protein (BMP), Wnt, and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling [5]. Understanding

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osteoblastic differentiation is very important in improving the treatment of bone diseases.

Chios gum mastic (CGM), the resin produced by *Pistacia lentiscus var Chia* that grows exclusively on the Greek island of Chios, has been known for its aromatic and therapeutic characteristics for thousands of years [6]. It has been used as a dietary supplement and a traditional medicine for stomach and duodenal ulcers in the eastern Mediterranean area [7]. Several studies have demonstrated that CGM has antimicrobial, antifungal, antioxidant, anti-inflammatory, and anticancer properties [8]. Recently, numerous researchers have investigated herbal medicine as a therapeutic agent and supplement in various diseases such as cancer, diabetes, Alzheimer's disease, rheumatism, and metabolic bone diseases. Its use is increasing worldwide [6, 9-12], but there have been no studies on the use of CGM in the osteogenic process or to treat bone diseases until now.

Low-level laser therapy (LLLT) is a well-accepted clinical tool in dentistry and regenerative medicine used to reduce pain and inflammation, prevent tissue death in vivo and in vitro, and promote wound healing, fibroblast proliferation, and nerve regeneration [13, 14]. Recent studies have focused particularly on bone regeneration using laser treatment [15, 16]. LLLT stimulates cellular proliferation and osteoblast differentiation and mineralization in bone formation [14, 17], but its biostimulatory effects are not fully understood. Bone disorders and diseases cause difficulty with bone regeneration. The effects of LLLT co-treated with herbal medicine could be desirable and synergetic in the repair of bone defects.

The present study seeks to determine whether CGM could promote the proliferation of human fetal osteoblastic (hFOB) 1.19 and murine pre-osteoblastic MC3T3-E1 (clonal mouse) cells. In addition, we examined the synergetic effects of LLLT and CGM on the osteoblastic differentiation process.

Materials and Methods

Reagents

CGM was obtained from Mastic Korea (Seoul, Korea). Antibodies against type I collagen, BMP-2, RUNX2, and Osterix were obtained commercially. The Alkaline Phosphatase Assay Kit was purchased from Abcam (Cambridge, MA, USA), and p38, phospho-p38, PKD,

phospho-PKD, ERK, phospho-ERK, JNK, and phospho-JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). The western blotting detection reagent and the SuperSignal West Femto Maximum Sensitivity Substrate that enhanced chemiluminescence was purchased from Pierce (Rockford, IL, USA). Alizarin red S and 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were both obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture

The hFOB 1.19 and MC3T3-E1 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The hFOB 1.19 cells were cultured in DMEM/F-12 1:1 (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS Gold, HyClone). The MC3T3-E1 cells were cultured in an alpha modification of Dulbecco's modified Eagle's medium (α-MEM without ascorbic acid, GIBCO, Grand Island, NY, USA) with 10% FBS at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere. For osteoblast differentiation, the hFOB 1.19 and MC3T3-E1 cells were seeded onto a 24-well or a 100 mm culture dish and allowed to reach confluence, and then they were transferred to α-MEM containing 10% FBS, 10 mM β-glycerophosphate, and 100 µg/ml ascorbic acid incubated at 37°C in 5% CO_2 for 3 or 7 days. The culture medium was replaced every 2 days.

Cell viability and proliferation assay

A cell viability and proliferation assay was performed using an MTT solution (0.5 mg/ml for the final concentration). The hFOB 1.19 and MC3T3-E1 cells (1 x 10^4) were cultured in a 96-well plate and incubated for 24 h. The cells were treated with CGM at various concentrations (1-100 µM) and exposed to LLLT (80 mW for 15 sec). After treatment, 100 µl of MTT solution was added, and the cells were incubated in the dark for an additional 4 h to induce the production of formazan crystals at 37°C. Then, the supernatants were discarded. The medium was aspirated, and formazan crystals were dissolved in DMSO. Cell viability and proliferation were monitored on an ELISA reader (Sunrise Remote Control, Tecan, Austria) at a 570 nm excitation emission wavelength.

Alkaline phosphatase (ALP) activity assay

For the ALP activity assay, 2 x 10^5 cells were plated

in 6-well plates after 24 h. Both types of cells were treated with CGM and LLLT and incubated in osteogenic media at 37°C in 5% CO_2. Following treatment, the cells were incubated with 10 mM of a Tris-HCl (pH 7.6) buffer containing 0.1% Triton-X on ice. Then, 50 μg of cellular fraction proteins were incubated with p-nitrophenol for 30 min at 37°C in the dark. The reaction was quenched by adding sodium hydroxide. The p-nitrophenol levels and the ALP enzyme activity was measured by monitoring the ELISA reader (Sunrise Remote Control) at a 410 nm excitation emission wavelength. The ALP activity of each sample was normalized by protein concentration.

Mineralization analysis

Osteoblast mineralization was measured by the quantification of alizarin red S staining. The hFOB 1.19 and MC3T3-E1 cells were plated onto 24-well plates and treated with CGM and LLLT, and then they were cultured in an osteogenic media for 7 days. The media was changed every 2 days. Both cells were fixed with ice-cold 70% ethanol and stained with alizarin red S to detect calcification. For quantification, cells were stained with ethylpyridium chloride and transferred to a 96-well plate, and the absorbance was measured at 540 nm using a microplate reader.

Western blot assay

Both types of cells were seeded at 2×10^6 in 100 mm culture dishes with the medium and cultured for 1 day. At the end of each treatment of CGM and LLLT, the cell lysates were prepared. Protein concentration determined with a protein assay kit (Bio-Rad, Budapest, Hungary). Proteins were loaded onto a 10 % SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham GE Healthcare, Little Chalfont, UK), where they reacted with each antibody. Then, they were blotted with an HRP-conjugated secondary antibody (1:5000). Immunoblotting using antibodies was performed with a Super Signal West Femto-enhanced chemiluminescent substrate and detected by AlphaImager HP (Alpha Innotech, Santa Clara, CA, USA). Ponceau S staining confirmed equivalent protein loading.

Statistical analysis

Statistical analysis data were expressed ± S.D from at least three independent experiments. We used one-way analysis of variance to analyze the data for cell viability, ALP activity, and alizarin red S staining. The data were analyzed on GraphPad Prism 5.0 (GraphPad Prism Software, San Diego, CA, USA). P values less than 0.05 were considered statistically significant.

Results

Effects of cytotoxicity and cell proliferation on CGM−treated osteoblasts

At first, we examined the cytotoxic effects and cell proliferation of CGM using an MTT assay on the hFOB 1.19 and MC3T3-E1 cells. Both cells were treated with CGM (10-200 μg/ml) and incubated for 24, 48, and 72 h.
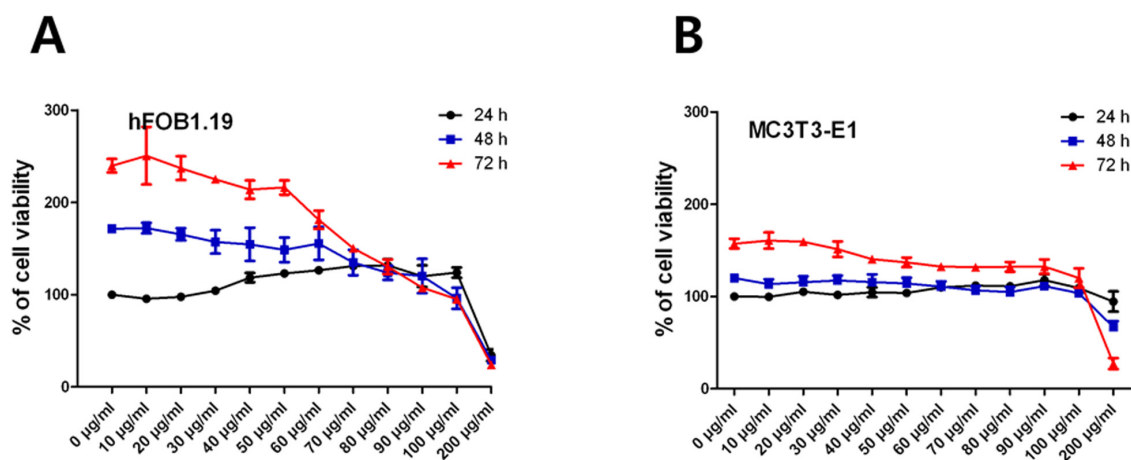

Fig. 1. Effects of Chios gum mastic on the viability and proliferation of osteoblasts. Each value represents the mean of three independent experiments ± S.D (n=6).
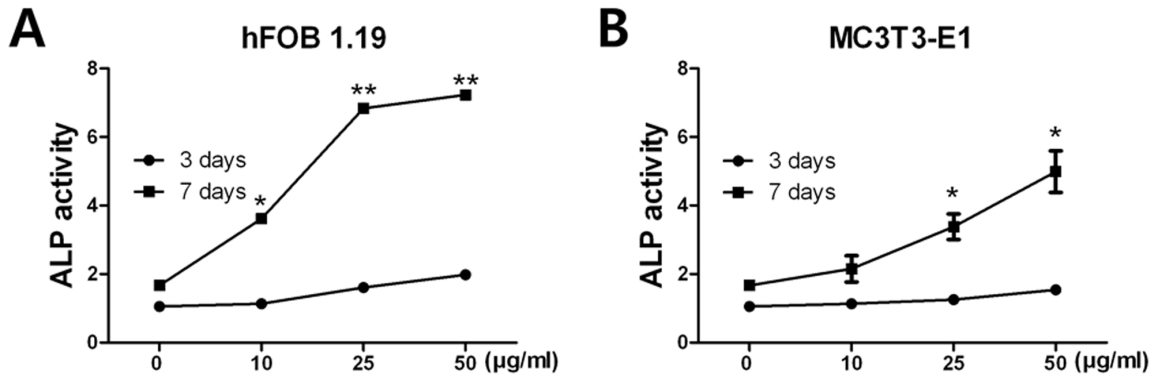
Fig. 2. Alkaline phosphatase activity (ALP) of hFOB 1.19 and MC3T3-E1 cells by Chios gum mastic Osteoblast differentiation marker, ALP activity increased in a time-dependent manner. Each value represents the mean of three independent experiments ± S.D (n=6). *$p<0.05$, ** $p<0.01$, compared with control (non-treated group).
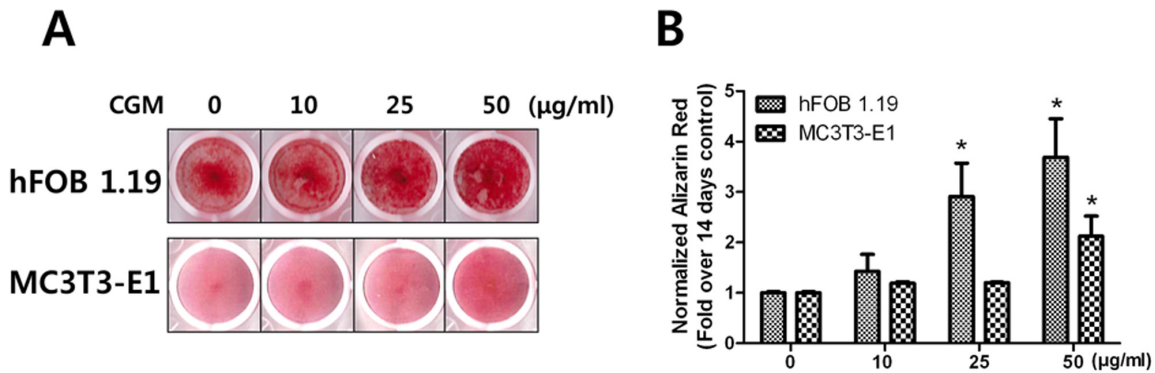

Fig. 3. Effects of Chios gum mastic (CGM) on the osteoblastic mineralization of hFOB 1.19 and MC3T3-E1 cells. Alizarin Red staining was quantified by measuring the absorbance at 570 nm. Data are expressed as the mean ± SD (n = 6). *$p<0.05$ compared with control group (non-treated group).

At 10−50 μg/ml, the CGM did not inhibit cell proliferation on the hFOB 1.19 or the MC3T3-E1 cells; however, at over 100 μg/ml, CGM potently reduced the proliferation of both cell lines dose-dependently. Less than 100 μg/ml of CGM did not have a cytotoxic effect on the hFOB 1.19 or the MC3T3-E1 cells (Fig. 1). Thus, we used a concentration of CGM below 50 μg/ml in subsequent experiments.

Induction of osteoblast differentiation and mineralization by CGM

To find the suitable concentration of CGM on the differentiation and mineralization of osteoblasts, we performed an ALP activity assay and alizarin red S staining on hFOB 1.19 and MC3T3-E1 osteoblastic cells treated with various concentrations of CGM (10-50 μg/ml) and incubated for 3, 7, and 14 days. After CGM treatment, the ALP activities clearly increased in a dose-dependent manner at 7

days on both cell types (Fig. 2). Alizarin red S staining showed the mineralizing matrix of hFOB 1.19 and MC3T3-E1 cells quantified using an ELISA reader. CGM over a concentration of 10 μg/ml induced calcified nodules that stained a deep red color, and it increased in a concentration-dependent manner at 14 days (Fig. 3). In particular, the differentiation and the mineralization activity were higher in the hFOB 1.19 cells than in the MC3T3-E1 cells.

The effects of CGM on osteoblast differentiation further confirmed the osteoblast differentiation makers (type I collagen, BMP-2, RUNX2, and Osterix) by western blot analysis. The CGM treatment were increased expression levels of these proteins in a dose-dependent manner on hFOB 1.19 and MC3T3-E1 cells. Based on the significant increase of ALP activity by CGM, the intensity of alizarin red, and the expression levels of the osteogenic markers, we elucidate that CGM induced osteoblastic differentiation
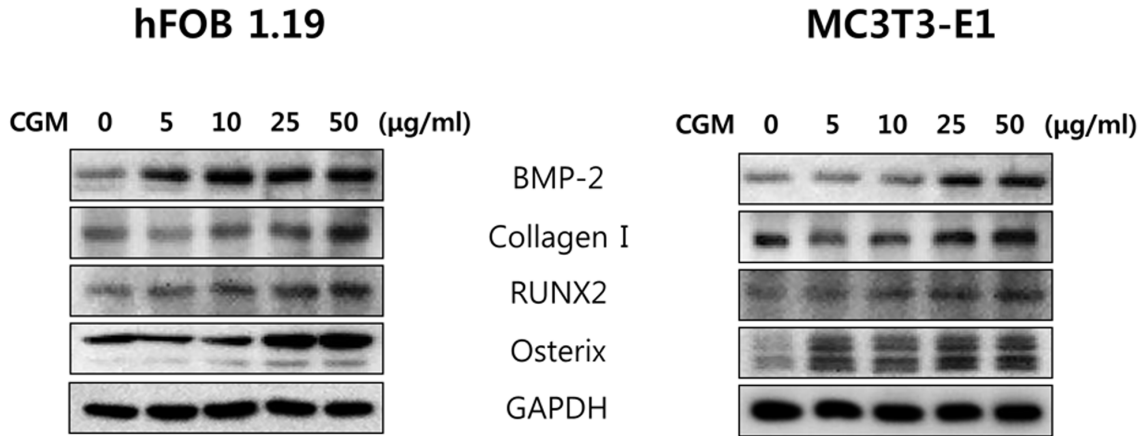
hFOB 1.19

MC3T3-E1

Fig. 4. Chios gum mastic (CGM) activates osteoblast differentiation in related proteins. hFOB 1.19 and MC3T3-E1 cells were treated with various concentrations of CGM. Collagen I, BMP-2, RUNX2 and Osterix were activated by CGM concentration dependently in the both cells.
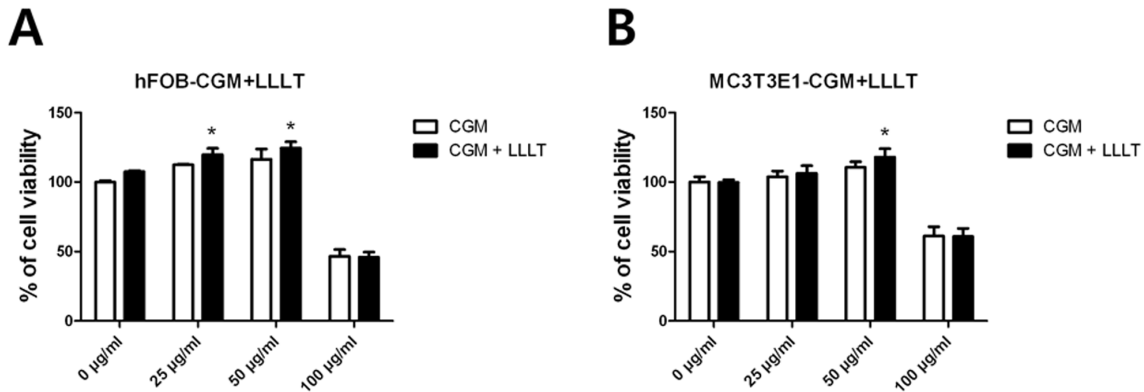

Fig. 5. Proliferation effects of combination treatment of Chios gum mastic (CGM) and low-level laser therapy (LLLT) on osteoblasts. hFOB 1.19 (A) and MC3T3-E1 (B) cells were exposed 80 mW LLLT for 15 sec and then co-treated with 25, 50, 100 μg/ml CGM for 24 h. 50 μg/ml CGM and LLLT combination treatment group was showed highest activity on the both cells. Each value represents the mean triplicated ± S.D (n=6). *$p<0.05$ compared with control group (0 μg/ml CGM).

in the hFOB 1.19 and MC3T3-E1 cells and that it has osteogenic potential.

Synergetic effects of combination treatment of CGM and LLLT on osteoblasts

We next investigated whether the synergistic effects of LLLT occurred on CGM-treated osteoblastic cells. In a previous study, we investigated the suitable power and exposure time of LLLT [18], and we used the same power and exposure time in this study. The hFOB 1.19 and MC3T3-E1 cells were exposed to 80 mW of LLLT for 15 sec, and then they were co-treated with 25, 50, and 100 μg/ml of CGM for 24 h. A combination of 50 μg/ml of CGM and LLLT showed the highest proliferation levels on

both cell types (Fig. 5).

Next, we performed ALP activity and mineralization assays on the hFOB 1.19 and MC3T3-E1 osteoblastic cells. Both cell types were treated with a combination of CGM (50 μg/ml) and LLLT (80 mW for 15 sec) and incubated at 3, 7, and 14 days. The hFOB 1.19 and MC3T3-E1 cells were exposed to 80 mW of LLLT for 15 sec and then co-treated with 50 μg/ml of CGM. After 24 h, the media were changed to fresh osteoblastic differentiation media, and the cells were incubated for 14 days. Alizarin red staining was quantified by measuring absorbance at 570 nm. The CGM and LLLT combination treatment group showed the highest ALP activity at 7 days (Fig. 6) and a deep red color on both cell types at 14
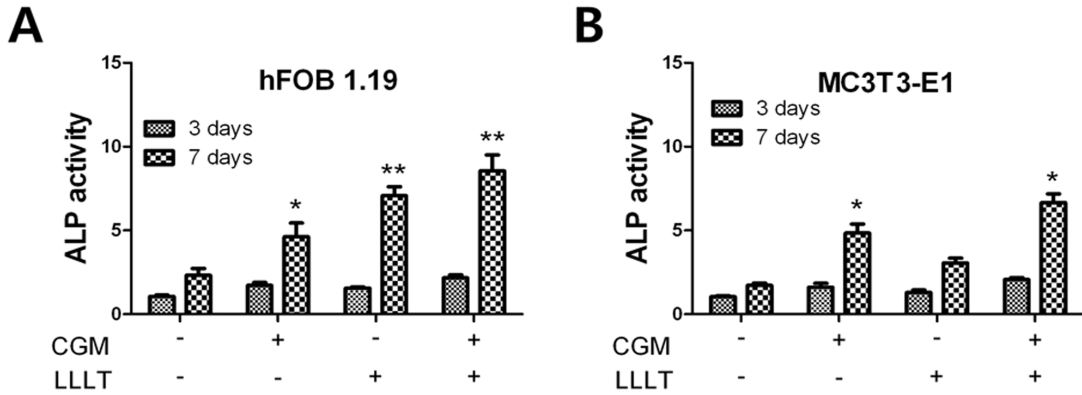
Fig. 6. Effects of ALP activity in Chios gum mastic (CGM) and low-level laser therapy (LLLT) combination treatment. hFOB 1.19 (A) and MC3T3-E1 (B) cells were exposed 80 mW LLLT for 15 sec and then co-treated with 50 µg/ml CGM for 24 h. CGM and LLLT combination treatment group was showed highest activity on the both cells. Each value represents the mean triplicated ± S.D (n=6). *p<0.05, ** p<0.01 compared with control group (0 µg/ml CGM).
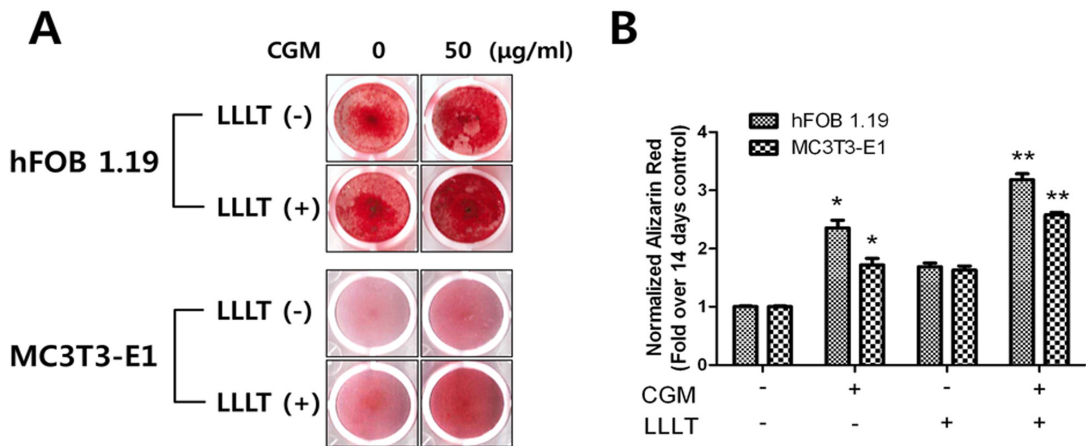

Fig. 7. Mineralization effects of Chios gum mastic (CGM) and low-level laser therapy (LLLT) combination treatment on hFOB 1.19 and MC3T3-E1 cells. hFOB 1.19 and MC3T3-E1 were exposed 80 mW LLLT for 15 sec and then co-treated with 50 µg/ml CGM, After 24 h, medium were changed to fresh osteoblastic differentiation media and incubated for 14 days. It stained with alizarin red S. Alizarin Red staining was quantified by measuring the absorbance at 570 nm. Data are expressed as the mean ± SD (n = 6). *p<0.05 compared with control group non-treated group.
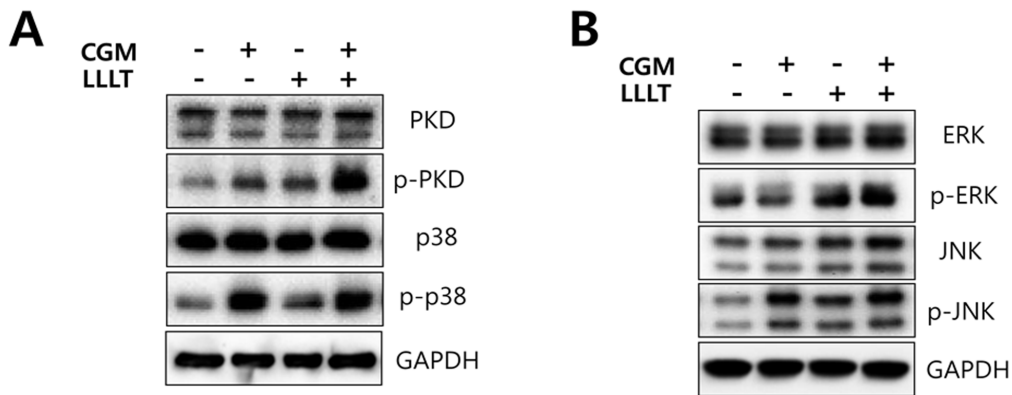

Fig. 8. Involvement in p38 MAPK signaling by Chios gum mastic (CGM) and low-level laser therapy (LLLT) combination treatment on osteoblasts. hFOB 1.19 cells were incubated in CGM in the absence or presence of LLLT for 2 h. Phosphorylation forms of (A) p38, PKD, (B) ERK and JNK were activated by combination treatment group.
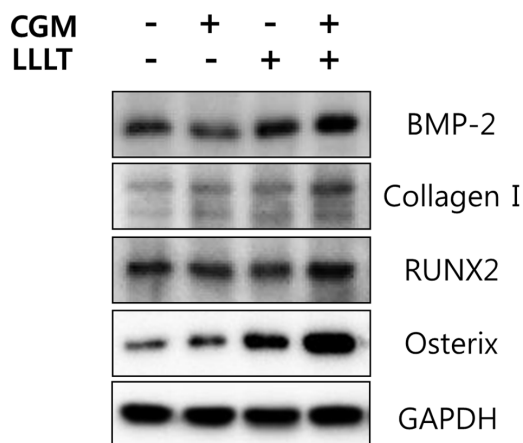
CGM   -   +   -   +
LLLT   -   -   +   +

Fig. 9. Activation of osteoblast differentiation factors by Chios gum mastic (CGM) and low-level laser therapy (LLLT) combination treatment on osteoblasts. hFOB 1.19 cells were incubated in CGM in the absence or presence of LLLT for 2 h. Protein levels of Collagen I, BMP-2, RUNX2 and Osterix were clearly increased in combination treatment group.

days (Fig. 7). Based on the previous results, we selected the hFOB 1.19 cells, which showed higher osteoblast differentiation and mineralization activity.

To find the activation of p38 MAPK signaling and the osteogenic factors by CGM and LLLT combination treatment on osteoblasts, we conducted a western blot analysis. The hFOB 1.19 cells were incubated in CGM in the absence or presence of LLLT for 2 h. The combination treatment group were shown to activate the phosphorylation forms p38, PKD, ERK, and JNK (Fig. 8). In addition, the osteoblast differentiation factors (type I collagen, BMP-2, RUNX2 and Osterix protein levels) clearly increased in the combination treatment group (Fig. 9). These results suggest that p38 signal transduction is an activator for the combination treatment of the CGM and LLLT-induced differentiation and mineralization of osteoblastic cells.

Discussion

In the present study, we prove that CGM induces osteoblast differentiation, and a combination treatment of CGM and LLLT was proven to have synergistic effects on hFOB 1.19 and MC3T3-E1 cells for the first time. Numerous studies indicate that herbal medicine can play an important role in the treatment of various diseases [19]. Over the centuries, herbal medicine, or plant-derived substances,

have been widely used to treat bone disease [20]. CGM has demonstrated numerous and diverse biomedical and pharmacological properties including bacteria eradication, peptic ulcer alleviation, tooth plaque inhibition, cancer prevention, and cardiovascular system protection [21]. Furthermore, other properties are widely known such as antioxidant capacity, hepatoprotective action, and anti-inflammatory effects [22-24]. However, there is no previous research on the biological and physiological properties of CGM in bone homeostasis and disease. Therefore, in the present study, we used hFOB 1.19 and MC3T3-E1 osteoblastic cells to determine the osteogenic potential of CGM.

To find the effects of CGM, we examined the osteoblastic differentiation and mineralization of hFOB 1.19 and MC3T3-E1 cells. The aim of all bone anabolic therapy is to increase the differentiation and mineralization of osteoblasts. [25]. The activity of ALP is known to increase in osteoblasts in early phases of differentiation. ALP is an essential indicator of mineralization capacity, and it is expressed by all osteoblastic cells. ALP activity was reported to be a characteristic of osteogenic cells in cultures, and it was also reported to promote bone matrix mineralization [26]. We observed that CGM stimulated ALP activity and the formation of mineralized nodules at a maximum of 50 μg/ml in the hFOB 1.19 and MC3T3-E1 cells, thereby suggesting a bone anabolic action for CGM in osteoblasts. The CGM treatment were increased dose-dependently bone differentiation markers such as type I collagen, BMP-2, RUNX2, and Osterix in both the osteoblasts.

Various studies reported that LLLT controls pain [27] and anti-inflammatory action [28] and has biostimulatory effects including increasing fibroblast proliferation [29], chondrocyte repair [30], wound healing [31], and collagen production [32]. Recently, researchers have been particularly concerned with promoting bone regeneration using LLLT [17, 33]. LLLT promotes the proliferation and differentiation of human osteoblasts in vitro and in vivo [34-36]. These studies have determined an optimal power of LLLT on bone formation. However, studies of the molecular mechanisms of LLLT are rare. Furthermore, there are no studies on the combined effects of Chinese medicines or plants with LLLT. The present study was designed to examine the synergistic effects of LLLT and CGM, as a combination treatment, on osteoblasts. The CGM (50 μg/ml) and LLLT (80 mW for 15 sec)

combination treatment group showed the highest activity of ALP and mineralization compared to other treatment groups. Overall, the results determine that CGM and LLLT, as a combination treatment, has synergistic effects on the differentiation and mineralization of osteoblasts.

We also investigated whether MAPKs are activated by the CGM and LLLT combination treatment-induced differentiation and mineralization of osteoblasts. MAPKs are a family of serine/threonine protein kinases that play important roles in cell proliferation [37]. JNK, p38, and ERK1/2 are the three main members of MAPK pathways that can be activated by growth factors, deoxyribonucleic acid damage, cytokines, oxidant stresses, and ultraviolet light [38-40]. In addition, MAPK signaling pathways are involved in responses to osteoblastic differentiation [41-43]. It has been reported that activated ERK1/2 is a response to RUNX2, and the activation of p38 regulates the expression of Osterix in osteoblastic differentiation and skeletal development [44, 45]. It has been reported that the protein kinase D (PKD) is involved in the activation of all three MAPKs, BMP-2, and RUNX2 in osteoblastic cell differentiation [46, 47].

In the present study, to evaluate the effects of CGM and LLLT combination treatment on the phosphorylation of p38 molecules, western blotting was performed. As shown in Fig. 8, the CGM and LLLT combination treatment group activated the phosphorylation levels of p38, PKD, JNK, and ERK in hFOB 1.19 cells. Moreover, as shown in Fig. 9, type I collagen, BMP-2, RUNX2, and Osterix markedly increased when hFOB 1.19 cells were combination treated with CGM and LLLT. Overall, these results indicated that CGM and LLLT combination treatment activates and promotes the differentiation of osteoblasts via the p38 MAPK pathway.

Acknowledgements

Conflict of interest

The authors declare that they have no conflicting interest.

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