Low-level laser therapy for osteonecrotic lesions: effects on osteoblasts treated with zoledronic acid

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Low-level laser therapy for osteonecrotic lesions: effects on osteoblasts treated with zoledronic acid


Abstract

Purpose Clinical studies have shown that low-level laser therapy (LLLT) can improve local tissue healing of bisphosphonate-induced osteonecrosis of the jaw. However, the effects of laser irradiation on bisphosphonate-treated osteoblasts have not been completely elucidated.

Methods Human osteoblasts were cultured in plain culture medium (DMEM). After 48 h, plain DMEM was replaced by DMEM with no fetal bovine serum, for a 24-h incubation followed by addition of zoledronic acid (5 μM) for additional 48 h. Cells were subjected to LLLT (InGaAsP; 780±3 nm; 0.025 W) at 0.5, 1.5, 3, 5, and 7 J/cm², three times every 24 h. Cell viability, total protein production, alkaline phosphatase activity (ALP), mineral nodule formation, gene expression of collagen type I and ALP, and cell morphology were evaluated.

Results LLLT at 0.5 J/cm² increased cell viability of cultured osteoblasts. ALP activity and gene expression, in addition to mineral nodule formation and Col-I gene expression, were not increased by LLLT. LLLT applied to ZA-treated cells increased Col-I expression at 0.5, 1.5, and 3 J/cm² but did not improve any other cell activity assessed.

Conclusion LLLT showed limited effects on bisphosphonate-treated osteoblasts.

Keywords Bisphosphonates · Osteonecrosis · Osteoblasts · Low-level laser therapy · Cell culture

Introduction

Bisphosphonate-related osteonecrosis has been considered to have an adverse effect on bisphosphonate treatment [1, 2]. This condition is described in approximately 1 % of patients, but its incidence is directly related to the type of bisphosphonate, treatment period, and administration of the drug [1, 2]. In addition, oral health conditions, such as the presence of inflammatory conditions and trauma, have also been related to the development and maintenance of osteonecrosis [1–3].

Elucidation of the etiopathogenesis and treatment strategies for osteonecrosis has become critical, since its incidence has increased, associated with the fact that, in most cases, osteonecrosis can be very painful and frequently affects patients’ quality of life [1–5].

Increased incidence of bisphosphonate-related osteonecrosis of the jaw has led to studies of the etiopathogenesis protocols [1, 2] and treatment [3] for this adverse effect of bisphosphonate treatment. It is known that the occurrence of osteonecrosis is associated, at least in part, with the type of bisphosphonate used, its administration, frequency, and duration of treatment. Additionally, this pathological condition may be associated with local factors, such as the presence of biofilm, inflammatory process, and direct cytotoxicity of bisphosphonates to oral mucosa cells [2, 4, 5].

Previous studies have shown that bisphosphonates have toxic effects on osteoblast, fibroblast, epithelial, and endothelial cells [5–7]. In addition, many researchers have
demonstrated that bisphosphonates can delay oral healing process as well as decrease bone formation and neovascularization [8, 9].

Standard strategies for the treatment of osteonecrosis of the jaw include local and systemic antibiotic administration and surgical intervention [1, 3]. Recent clinical reports have demonstrated that treatment of osteonecrotic lesions with low-level laser therapy (LLLT) in association with antimicrobial or surgical treatment improves tissue healing and reduces localized pain [10–13].

Positive effects of LLLT on different cell types have been demonstrated in several in vitro and in vivo studies [14, 15]. However, only scarce data have been provided concerning the effects of laser irradiation on oral tissues, especially on bone cells exposed in osteonecrotic jaw lesions.

Elucidation of the effects of LLLT on zoledronic acid-treated cells may validate or improve the safe use of this non-invasive therapy for osteonecrosis of the jaw for the treatment of patients receiving bisphosphonates and presenting osteonecrosis lesions. Therefore, the aim of this study was to evaluate the effects of LLLT on specific parameters of cultured osteoblasts, whether previously exposed to a specific type of bisphosphonate—zoledronic acid.

Materials and methods

Cell culture

An immortalized human osteoblastic cell line was selected for study (Saos-2–HTB-85). Cells were seeded in wells of 24-well plates in complete culture medium (DMEM-Gibco, Grand Island, NY, USA) supplemented with 10 % fetal bovine serum (FBS-Gibco) and maintained in a 5 % CO2atm at 37 °C.

Zoledronic acid treatment

After 48-h incubation, the plain culture medium in contact with cells was replaced by serum-free DMEM. Following an additional 24-h incubation, ZA at 5 μM was added to the serum-free DMEM, which was maintained in contact with osteoblasts for 48 h.

ZA was used in this study because it is a highly potent bisphosphonate that has been frequently associated with osteonecrosis of the jaws. The ZA concentration used was based on a previous study in which the authors showed that 5 μM is the highest concentration of ZA found in the saliva and bone tissue of patients under ZA treatment [6].

LLLT

LLLT was performed with a laser diode prototype widely used in previous studies (LASERTable, InGaAsP; 780±3 nm; 0.025 W), since it provides complete, uniform, and standardized irradiation of the bottoms of the wells to which the seeded cells are attached [16–18]. Before each experiment, delivery energy was measured at the bottoms of the 24-well plates, to confirm LLLT parameters. The cells were irradiated every 24 h for specific times according to the energy doses of 0.5 J/cm2 (40 s), 1.5 J/cm2 (120 s), 3 J/cm2 (240 s), 5 J/cm2 (400 s), and 7 J/cm2 (560 s). Cells were irradiated at a standardized distance of 2 cm, and the area of irradiation was also standardized at 2 cm2.

The LLLT parameters used in this in vitro study were selected according to previous investigations in which the authors irradiated cultured osteoblasts, demonstrating increased cell viability and function [19–21].

Cell viability—MTT assay

Twenty-four hours after the last irradiation, the osteoblasts’ viability was assessed by MTT assay (n=8), which is a well-described and standardized test that provides cell mitochondrial activity. The protocol was performed as previously described [16].

Total protein production

The total protein production by osteoblasts (n=8), whether exposed to ZA and whether subjected to different levels of LLLT, was analyzed by an end-point colorimetric test as described previously [16].

Alkaline phosphatase activity (ALP)

ALP activity is considered a phenotypic marker of osteoblasts, and alterations to this activity can affect osteoblastic mineralization functions. This assay is based on thymolphthaline hydrolysis by ALP and was performed as previously described (n=8) [22].

Mineralization nodule formation—alizarin red stain

Mineralization nodule formation was assessed by alizarin red stain, which also demonstrates the phenotypic capacity of osteoblasts to form a mineralized matrix [18].

For mineral nodule formation, cells were maintained in an osteogenic culture medium composed of DMEM (Gibco) containing 10 % FBS (Gibco), β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), and ascorbic acid (Sigma-Aldrich) (n=8) [22].
Briefly, after ZA treatment and LLLT application, cells were fixed in 70 % cold ethanol for 1 h. Immediately after the samples were rinsed with deionized water, mineral nodules were detected by alizarin red (40 nm; pH 4.2) incubation under shaking for 20 min at room temperature. For quantitative analysis, mineral nodules were dissolved in 10 % cetylpyridinium chloride (Sigma-Aldrich) for 15 min under shaking. Finally, mineral nodule formation was assessed by absorbance at 692 nm (Thermo Plate, Nanshan District, Shenzhen, China).

Gene expression—real-time PCR

Real-time PCR was used to evaluate the gene expression of Col-I and ALP as previously described \((n=4)\) [23]. These genes participate in matrix synthesis and mineralization.

Briefly, messenger RNA (mRNA) isolation was obtained by the Trizol (Invitrogen, Carlsbad, CA, USA) method, followed by complementary DNA (cDNA) synthesis, with a high-capacity cDNA kit (Invitrogen). For PCR analysis, specific primers and probe sets were designed. PCR reactions were prepared with standardized SYBR® Green reagents (Applied Biosystems, Foster City, CA, USA) for ALP analysis or Taqman reagents (Applied Biosystems) for Col-I and RPL13 analysis. These reactions were performed in the Step One Plus Real Time System (Applied Biosystems). Data were analyzed by Step One Plus Software (Applied Biosystems) with relative quantitation of each mRNA, considering constitutive gene (RPL13).

Scanning electronic microscopy (SEM)

Osteoblast morphology, whether exposed to ZA and whether subjected to LLLT, was evaluated by SEM. Cells were seeded on 13-mm sterile glass discs previously placed on the bottoms of wells of 24-well plates. After the osteoblasts were treated as described in Table 1, the cells were fixed with 2.5 % glutaraldehyde (Sigma-Aldrich), post-fixed with 1 % osmium tetroxide, and dehydrated in increasing ethanol concentrations (30, 50, 95, and 100 %). Finally, the cells were subjected to chemical drying with 1,1,1,3,3,3 hexamethyldisilazane (HMDS, Sigma-Aldrich).

The glass discs with cells on them were mounted in metallic stubs and stored in a desiccator for 7 days. The samples were then gold-sputtered, and cell morphology was assessed by SEM (Inspect Scanning Electron Microscope-S50, FEI, Hillsboro, OR, USA).

Statistical analysis

Cell viability, total protein production, mineral nodule formation, and qPCR data were analyzed by Kruskal–Wallis tests

<table>
<thead>
<tr>
<th>ZA</th>
<th>Energy doses (J/cm²)</th>
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<tr>
<td>+</td>
<td>34 (31–60)</td>
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<tr>
<td></td>
<td>39 (33–52)</td>
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<tr>
<td></td>
<td>36 (34–51)</td>
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<td>36 (34–55)</td>
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<tr>
<td></td>
<td>31 (29–57)</td>
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<tr>
<td></td>
<td>33 (30–58)</td>
</tr>
<tr>
<td>B, a</td>
<td></td>
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<tr>
<td>B, a</td>
<td></td>
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<tr>
<td>B, a</td>
<td></td>
</tr>
<tr>
<td>B, a</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>113 (109–115)</td>
</tr>
<tr>
<td>A, bc</td>
<td></td>
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<tr>
<td>A, a</td>
<td>A, bc</td>
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<td>A, ab</td>
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<td>A, bc</td>
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<td>A, ab</td>
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</table>

Values indicate median (25th to 75th percentiles), \(n=8\)

A, a Same upper-case letters in columns and lower-case letters in rows indicate no statistically significant difference (Mann–Whitney, \(p>0.05\))
complemented by Mann–Whitney tests, for groups. Statistical significance was set at 5%. All experimental protocols were performed on three different occasions.

Results

In general, ZA caused an intense decrease in osteoblasts and viability, and, on the other hand, LLLT at 0.5 J/cm² increased viability of these cultured cells. However, osteoblasts exposed to ZA and subjected to laser irradiation presented no improvement in viability (Table 1).

In terms of total protein production, no difference was observed among all groups in which the cells were subjected to LLLT, whether or not they were exposed to ZA (Table 2). As determined for cell viability, osteoblasts treated only with ZA showed a significant decrease in total protein production.

The ALP activity of osteoblasts, whether or not they were treated with ZA, was not influenced by laser irradiation. The LLLT at energy doses of 3 and 7 J/cm² reduced ALP activity only slightly. In contrast, the same laser energy doses significantly decreased this enzyme activity in osteoblasts previously exposed to ZA (Table 3).

None of the laser energy doses evaluated in this study biostimulated mineral nodule formation by osteoblasts. When the cells were previously treated with ZA, the capacity for mineral nodule formation by cultured osteoblasts was significantly reduced (Table 4).

Gene expression of Col-I by osteoblasts subjected to all laser energy doses tested was similar to that of osteoblasts in the control group. However, for ZA-treated cells subjected to LLLT at 0.5, 1.5, and 3 J/cm², a significant increase in Col-I expression was observed (Table 5).

Increased ALP gene expression was observed only when the osteoblasts were subjected to LLLT at 1.5 J/cm². However, laser irradiation did not modulate the ALP gene expression in those cells previously treated with ZA (Table 6). Osteoblasts subjected only to ZA treatment showed significant decreases in Col-I and ALP expression.

The SEM evaluation of osteoblasts treated with ZA showed the occurrence of intense morphological cell alterations characterized by cytoplasm shrinkage as well as disaggregation and disruption of this cellular structure. Similar osteoblast alterations were also observed when the ZA-treated cells were subjected to LLLT (Figs. 1 and 2).

Increased numbers of cells attached to the glass substrate were seen when the osteoblasts were solely laser-irradiated at an energy dose of 5 J/cm². Cells subjected to all LLLT tested in this study exhibited normal morphology, as observed in the control group (Figs. 1 and 2).

<table>
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<th>Table 3</th>
<th>Alkaline phosphatase activity by osteoblasts after ZA treatment followed by LLLT</th>
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<tbody>
<tr>
<td>ZA</td>
<td>Energy doses (J/cm²)</td>
</tr>
<tr>
<td></td>
<td>0</td>
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<tr>
<td>+</td>
<td>35.56</td>
</tr>
<tr>
<td>−</td>
<td>100.15</td>
</tr>
<tr>
<td>B, ab</td>
<td>(93.96–106.53)</td>
</tr>
</tbody>
</table>

Values indicate median (25th to 75th percentiles), n=8
A, a Same upper-case letters in columns and lower-case letters in rows indicate no statistically significant difference (Mann–Whitney, p>0.05)

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<tr>
<th>Table 4</th>
<th>Mineral nodule formation by osteoblasts after ZA and LLLT treatment</th>
</tr>
</thead>
<tbody>
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<td>ZA</td>
<td>Energy doses (J/cm²)</td>
</tr>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>6.26</td>
</tr>
<tr>
<td>B, a</td>
<td>(5.82–6.82)</td>
</tr>
<tr>
<td>−</td>
<td>96.41</td>
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</tbody>
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Values indicate median (25th to 75th percentiles), n=8
A, a Same upper-case letters in columns and lower-case letters in rows indicate no statistically significant difference (Mann–Whitney, p>0.05)
Support Care Cancer (2014) 22:2741–2748

Table 5 Gene expression of Col-I by osteoblasts after ZA and LLLT treatment

<table>
<thead>
<tr>
<th>ZA</th>
<th>Energy doses (J/cm²)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>0.593 (0.547–0.636) B, a</td>
</tr>
<tr>
<td>−</td>
<td>1.000 (0.983–1.016) A, ab</td>
</tr>
</tbody>
</table>

Values indicate median (25th to 75th percentiles), n=4
A, a Same upper-case letters in columns and lower-case letters in rows indicate no statistically significant difference (Mann–Whitney, p>0.05)

Discussion

Several studies have evaluated the effects of ZA on osteoblasts, since this drug has been related to the etiopathogenesis of osteonecrosis as well as to delayed bone healing and remodeling in patients under ZA treatment [22]. In the present study, a highly cytotoxic effect was observed in osteoblasts treated with ZA, characterized by decreased viability, total protein production, mineral nodule formation, ALP activity and morphology, and gene expression of Col-I and ALP.

Several studies have introduced LLLT as an adjuvant therapy for bisphosphonate-induced osteonecrosis associated with antimicrobial or surgical treatment and have shown that this therapy can accelerate healing and lesion remission [10–13]. Low-power laser-irradiated osteoblasts enhance proliferation and protein synthesis, mainly those related to matrix formation and maturation, such as Col-I [20, 25, 26].

It was shown in the present study that specific parameters of LLLT can biostimulate osteoblasts in culture, increasing their viability. However, no effects of laser irradiation were observed on mineral nodule formation, ALP activity, and expression of genes related to matrix production and mineralization. The absence of positive effects of LLLT on ZA-treated cells may be related to the selected parameters of irradiation, since previous studies have demonstrated that different wavelengths, power outputs, or energy doses can promote diverse cellular responses [24]. As reported by other authors, higher energy doses can increase the metabolism of cultured osteoblasts [26, 27].

The data obtained in the present study may also be related to the fact that cultured osteoblasts were not previously subjected to cellular stress, such as nutritional restriction, which has been reported by many workers as a predictive factor for greater biostimulation [25, 27]. The cellular stress protocol was not used in the present study because the osteoblasts had already been subjected to the toxic effects of ZA, which seemed to be sufficiently high to inhibit the biostimulation of the cultured cells.

Regarding cell morphology, no alterations were observed in cultured human osteoblasts subjected to LLLT at all selected parameters, as previously demonstrated for other cell types [16]. SEM evaluation, which is an important tool for the morphologic evaluation of cells, can determine the cytotoxic effects of several treatments [28, 29] such as observed for ZA [18, 23]. Despite the fact that LLLT did not biostimulate the cultured osteoblasts, cell morphology was maintained during the study, thus determining the cells’ resistance threshold.

The positive effects of LLLT on areas of osteonecrosis could be related to cellular biostimulation, as recently described by our group (eu laser phys) However, so far, only one study has been carried out to assess the effects of LLLT on ZA-treated osteoblasts [30] The authors demonstrated that LLLT applied to cultured human osteoblasts (SaOS-2) subjected to ZA treatment for 24 and 48 h showed increased ALP

Table 6 Gene expression of ALP by osteoblasts after ZA and LLLT treatment

<table>
<thead>
<tr>
<th>ZA</th>
<th>Energy doses (J/cm²)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>0.344 (0.295–0.404) B, a</td>
</tr>
<tr>
<td>−</td>
<td>1.045 (0.905–1.097) A, ab</td>
</tr>
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</table>

Values indicate median (25th to 75th percentiles), n=4
A, a Same upper-case letters in columns and lower-case letters in rows indicate no statistically significant difference (Mann–Whitney, p>0.05)
Fig. 1 Morphology of cultured human osteoblasts (Saos-2) subjected to LLLT at different energy doses (a) Control group. b 0.5 J/cm². c 1.5 J/cm². d 3 J/cm². e 5 J/cm². f 7 J/cm². No significant difference was observed between LLLT-treated cells and the control group. All images show adherent and confluent cells. SEM, original magnification ×200

Fig. 2 Morphology of cultured human osteoblasts (Saos-2) subjected to ZA treatment and LLLT at different energy doses (a ZA. b ZA+0.5 J/cm². c ZA+1.5 J/cm². d ZA+3 J/cm². e ZA+5 J/cm². f ZA+7 J/cm²). For all ZA-treated groups, a significant decrease in adherent cell numbers was observed. For group E, where LLLT was applied, an increase in adherent cells can be observed, compared with the ZA-treated group (a). SEM, original magnification ×200
activity and numbers of viable osteoblasts [30] Despite the similarity of methodology used by the authors compared with that used in the present study, the data obtained in both investigations cannot be compared, because the LLLT specifications were not reported,[30] and because, as already mentioned, current studies have demonstrated that different laser parameters cause various cell responses [15]. Therefore, in addition to several available studies about the effects of LLLT on different cell types, the lack of standardization of laser irradiation protocols for in vitro and in vivo investigations does not allow for adequate comparison among all data obtained.

Considering previous results [18, 30] and based on analysis of the data obtained in the present study, one may suggest that LLLT at specific parameters can promote biostimulation of cultured cells whether or not they are treated with ZA. However, further studies are needed to elucidate the effects of LLLT on ZA-treated cells and to improve and standardize the use of this kind of therapy as an adjuvant for the treatment of osteonecrosis.

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Conflict of interest The authors declare no conflict of interest.

References


