Lasers in Medical Science (2019) 34:169–178
https://doi.org/10.1007/s10103-018-2673-8

ORIGINAL ARTICLE

Low-level laser irradiation modulates the proliferation and the osteogenic differentiation of bone marrow mesenchymal stem cells under healthy and inflammatory condition

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Received: 7 September 2017 / Accepted: 12 January 2018 / Published online: 19 November 2018

Abstract

The aim of this in vitro study was to evaluate the effects of low-level laser therapy (LLLT) at different energy intensities on proliferation and osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs) under healthy and inflammatory microenvironments. Human BMSCs and BMSCs from inflammatory conditions (i-BMSCs, BMSCs treated with tumor necrosis factor α; TNF-α) were subject to LLLT (Nd:YAG;1064 nm) at different intensities. We designed one control group (without irradiation) and four testing groups (irradiation at 2, 4, 8, and 16 J/cm^2) for both BMSCs and i-BMSCs. Cell proliferation was measured using colony-forming unit fibroblast assay and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide assay. Osteogenic capacity of cells was determined by alkaline phosphatase (ALP) staining, ALP activity assay, Alizarin Red S staining and the mRNA transcript levels of genes runt-related transcription factor 2 (Runx2), ALP, and osteocalcin. Moreover, the effects of LLLT on secretion of TNF-α in BMSCs and i-BMSCs were measured by enzyme-linked immunosorbent assay. Our results demonstrated LLLT could significantly promote BMSC proliferation and osteogenesis at densities of 2 and 4 J/cm^2. LLLT at density of 8 J/cm^2 could promote the proliferation and osteogenesis of i-BMSCs. However, LLLT at 16 J/cm^2 significantly suppressed the proliferation and osteogenesis of BMSCs both in healthy and in inflammatory microenvironment. Moreover, we also found that the expression of TNF-α was obviously inhibited by LLLT at 4, 8, and 16 J/cm^2, in an inflammatory microenvironment. Considering these findings, LLLT could improve current in vitro methods of differentiating BMSCs under healthy and inflammatory microenvironments prior to transplantation.

Keywords Bone marrow mesenchymal stem cells (BMSCs) · Low-level laser therapy (LLLT) · Inflammation · Proliferation · Osteogenesis

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Introduction

The craniofacial defects, which are caused by trauma, fractures, congenital diseases, and tumor, were considered as the major obstacle for oral and maxillofacial surgeons [1, 2]. Traditional autologous and allogeneic bone grafting often leads to bone resorption and secondary injury. Moreover, some skeletal defects may be too large for autograft transplantation [3]. Recently, tissue engineering based on stem cells provides a new alternative for craniofacial reconstructions. Bone marrow mesenchymal stem cells (BMSCs) are one type of multi-potent stem cells derived from neural crest. Under physiological conditions, BMSCs could migrate to sites of damage and subsequently differentiate into a variety of tissues—bone, cartilage, tendon, muscle, adipose tissue, and neuronal tissue [4–7]. Because of their osteogenic capacity

and tissue origin, BMSCs are considered as an excellent cell source for craniofacial defects repair. Many in vitro and in vivo reports demonstrate that BMSCs associated with scaffolds or not could repair critical-sized cranial defects as seeding cells of bone tissue engineering [8–11]. However, in the process of craniofacial defects repair, we often face inflammatory microenvironment. Many diseases, such as infection after trauma, osteomyelitis of the jaws, and periodontitis, may lead to inflammation in the sites of craniofacial defects. Actually, the multiplex differentiation potential of mesenchymal stem cells (MSCs) depends so much on the microenvironment where they exist. Growing evidences suggest that inflammation could impair the osteogenic capacity of BMSCs [12, 13]. Therefore, how to improve the osteogenesis of BMSCs under healthy and inflammatory condition is one of the key subjects in the field of regenerative medicine and bone repair.

Low-level laser therapy (LLLT) could induce multiple biological effects with a low-powered laser in human tissue [14]. A large number of literatures and articles indicate that LLLT accelerates fracture healing and repairs bone defects [15–18]. In addition to repairing bone tissue damage, LLLT also has the capacity to modulate the biological functions of MSCs. Increased proliferation after LLLT has been reported in BMSCs [19], adipose stem cells [20], and human dental pulp stem cells [21]. Moreover, LLLT also has the capacity to modulate the osteogenic differentiation of the BMSCs. Wu et al. found the alkaline phosphatase (ALP) activity, a marker of early osteoblast differentiation, was significantly increased after LLLT [22].

Although the numerous research papers suggest the bone formation efforts on the BMSCs of the low-energy laser, its functions in the proliferation and osteogenesis of BMSCs under inflammatory condition were still unknown. Herein we evaluate the effects of a neodymium-doped yttrium aluminum garnet laser (Nd:YAG;1064 nm) at different energy intensities on proliferation and osteogenic differentiation of BMSCs under healthy and inflammatory microenvironments. This report is the first to demonstrate the biostimulatory efforts of LLLT on the BMSCs from inflammatory conditions (i-BMSCs).

Materials and methods

Cell isolation and culture

Human BMSCs were cultured as previously described [23]. Briefly, fresh cancellous bone fragments and blood were obtained from five orthognathic patients. Each participant provided written informed consent. The samples were quickly transferred to alpha minimum essential medium (α-MEM; Gibco BRL, Gaithersburg, MD, USA) and washed several times. Then the BMSCs were flushed out from the bone marrow cavity. Next, the resultant medium was centrifuged at 1000 rpm for 5 min. Finally, the deposits (containing cells) were resuspended with α-MEM, containing 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. Zhejing, China), and cultured in six-well plates. Three days later, floating cells and bone chips were removed, and we got the primary BMSCs for continuing culture. After passage, the three passage cells, BMSCs were isolated using immunomagnetic beads with antibodies to detect CD105. Cells from passage 3 to 5 were used in this study. For each experiment, the same passage of BMSCs was used.

To obtain i-BMSCs, we treated BMSCs with tumor necrosis factor α (TNF-α, Pepro-Tech, RockyHill, NJ, USA, 10 ng/ml) and in basic medium for 48 h. Then, the cells were washed with phosphate-buffered saline (PBS) for three times and the culture medium was changed to basic medium without TNF-α.

LLLT treatment

BMSCs and i-BMSCs at passage 3 (1×10^5 cells/well) were cultured in α-MEM (10% FBS) until they reached 80% confluence. Then, cells were scanned by the laser—the laser wavelength and settings of LLLT as previously described [24]. A neodymium-doped yttrium aluminum garnet laser (Nd:YAG;1064 nm; Fidelis Plus III, Fotona; Ljubljana, Slovenia) was applied using a single-probe laser handpiece perpendicular to the surface, scanning the cells. The beam angle was 90°, and the energy intensity applied to cell was adjusted to approximately 0–16 J/cm^2 by applying 0.25 W output power for 20 s. To evaluate the effects of LLLT at different intensities on the proliferation and osteogenic properties of cells, we designed one control group (without irradiation) and four testing groups (irradiation at 2, 4, 8, and 16 J/cm^2) for both BMSCs and i-BMSCs. In each experimental group, we treated cells with LLLT every other day.

Colony-forming unit fibroblast assay

Single-cell suspensions contained 1×10^2 cells which were seeded in six-well plates and cultured for 7 days. The formed colonies were fixed by 4% paraformaldehyde fixation and then were visualized with 0.1% toluidine blue. Colonies were defined as aggregates which contain more than 50 cells and were scored under the microscope (Leica Microsystems,

Heerbrugg, Switzerland). Colony-forming unit fibroblast (CFU-F) assay efficiency was determined by the number of colonies relative to the total number of seeded cells in each plate. This experiment was repeated for three times.

3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide

Cells were cultured in 96-well at a density of 5×10^3 cells/well for 24 h. Then we evaluated the cell proliferation using the MTT assay (Sigma, USA) according to the kit protocol for 7 days and the absorbance was determined at 490 nm. This experiment was repeated for three times.

Osteogenic differentiation

Cells (1×10^5 cells/well) were cultured until they reached 80% of the culture flask. Then, media were changed with osteogenic medium (100 nM dexamethasone, 50 mg/ml ascorbic acid, and 5 mM b-glycerophosphate; Sigma) and cells were cultured for 7 or 21 days.

ALP staining and activity

ALP staining was performed after osteogenic induction for 7 days using a BCIP/NBT ALP Color Development Kit according to the manufacturer's protocol (Beyotime, Shanghai, China).

ALP activity assay was performed after osteogenic induction for 7 days by an ALP kit under the guidance of the manufacturer's instructions (Jiancheng, Nanjing, China). Briefly, the cultured cells were rinsed with PBS and fixed in 4% paraformaldehyde for 30 min. The cell layer was then washed three times with PBS and incubated in alkaline solution for 20 min. Total protein content was determined in the same sample with the bicinchoninic acid (BCA) method using the Pierce protein assay kit (Thermo, USA). ALP activity was normalized to the total protein content and expressed in nanomoles of produced p-nitrophenol per minute per milligram of total protein (nmol/min/mg protein). All the tests were repeated three times.

Alizarin Red staining

After induces by osteogenic medium for 21 days, Alizarin Red staining was performed. Cells were washed with 10% fetal bovine serum PBS twice. Then cells were fixed with 60% isopropanol for 1 min. After that, cells were washed with distilled water for 3 min and 1% Alizarin Red (Sigma) for staining was used.

To quantify Alizarin Red-stained nodules, the stain was solubilized with 0.5 mL 5% SDS in 0.5 N HCl for 30 min at room temperature. Then we put 0.15 mL liquid in to a well of 96-well plate and absorbance value was measured at 405 nm. A standard curve was created by serial dilution of known calcium concentration, and the amount of calcium was calculated by comparison with standard curve. Total protein content was determined in the same sample with the BCA method using the Pierce protein assay kit (Thermo, USA). The amount of calcium was normalized to the total protein content and expressed in micromole of the amount of calcium per milligram of total protein (umol/mg protein). All the tests were repeated three times.

Quantitative real-time polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's standard instructions and converted into cDNA using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). For reverse transcription of mRNA, random-primed cDNA was synthesized from 2 mg of total RNA. Real-time PCR analysis was performed using the SYBR Premix Ex Taq II kit (TaKaRa) and detected on the ABI Prism 7500 HT sequence detection system (Applied Biosystems, Foster City, CA, USA). β-actin was used as endogenous normalization controls for quantitation of mRNAs. The data were analyzed using the $2-\Delta\Delta Ct$ (Livak) relative expression method. All the tests were repeated three times.

Primer pairs were as follows:
Runx2: 5′-CCCGTGGCCTTCAAGGT-3′,
5′-CGTTACCCGCCATGACAGTA-3′;
ALP: 5′-GGACCA TTCCCACGTCTTCAC-3′,
5′-CCTTGTAGCCAGGCCCATTG-3′;
OCN: 5′-CCCAGGCGCTACCTGTATCAA-3′,
5′-GGTCAGCCAACTCGTCACAGTC-3′;
β-actin: 5′-TGGCACCCAGCACAATGAA-3′,
5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′

Cytokine expression assays

In our experiment, we obtained i-BMSCs through treating BMSCs with TNF-α and basic medium for 48 h. After that, the cells were washed with PBS three times and the culture medium was changed to basic medium without TNF-α. Then, we scanned i-BMSCs and BMSCs by the LLLT every other day. After being treated with LLLT for 7 days, we determined TNF-α in the culture supernatant of BMSCs and i-BMSCs using commercially available ELISA kits (R&D Systems, USA) according to the

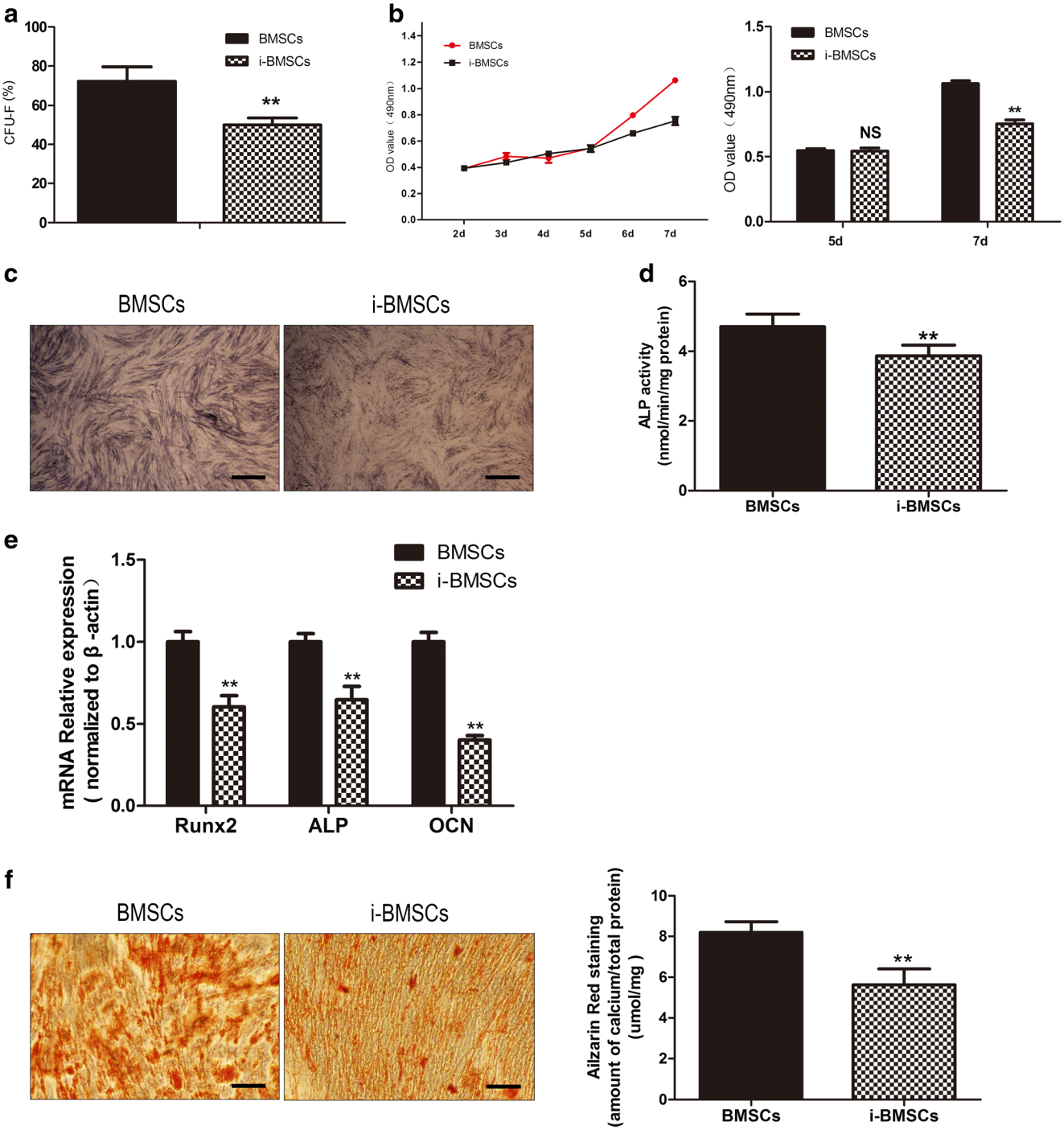

Fig. 1 Effects of TNF-α on the proliferation and osteogenic differentiation of BMSCs. (a) The colony-forming rate (%) of cells was measured after 14 days of culture at low seeding density. (b) The MTT was measured for 7 days to show the effects of TNF-α on the proliferation of BMSCs. (c) Osteogenic differentiation was determined by ALP staining at day 7 after osteogenic differentiation induced. (d) ALP activity was measured by ALP activity assay at day 7 after osteogenic differentiation was induced. (e) The expression levels of the osteogenic genes ALP, Runx2, and OCN were measured by real-time PCR at day 7 after osteogenic differentiation was induced. β-actin was used as endogenous normalization controls for quantitation of mRNAs. (f) Osteogenic differentiation was determined by Alizarin Red S staining at day 21 after osteogenic differentiation was induced. Calcium concentration stained by Alizarin Red S discussed above was examined by calcium level analysis. Data represent mean ± S.D. $*P < 0.05$; $**P < 0.01$; NS, not significant. The scale bar in the micrographs represents 100 μm

manufacturer's protocols. All results of ELISA were normalized to cell number and expressed as pg/10^6 cells. All the tests were repeated three times.

Statistical analyses

All experiments in this study were repeated at least three times. We analyzed data using a χ^2 test and an independent sample t test and presented them by means ± SD. Multiple group tests were analyzed by the Bonferroni correction for post hoc analysis and we considered less than 0.05 as statistically significant.

Results

TNF-α affects proliferation and osteogenic differentiation of BMSCs

After being treated with TNF-α, the proliferation and osteogenic differentiation of BMSCs were measured. Based on colony-forming rate, we found that BMSCs had a higher proliferation capacity than i-BMSCs ($P < 0.05$) (Fig. 1(a)). The MTT showed similar results with CFU-F assay (Fig. 1(b)). Although BMSCs and i-BMSCs exhibited similar proliferation rates within the first 5 days, BMSCs proliferation activity was significantly higher than that of i-BMSCs after 6 days of

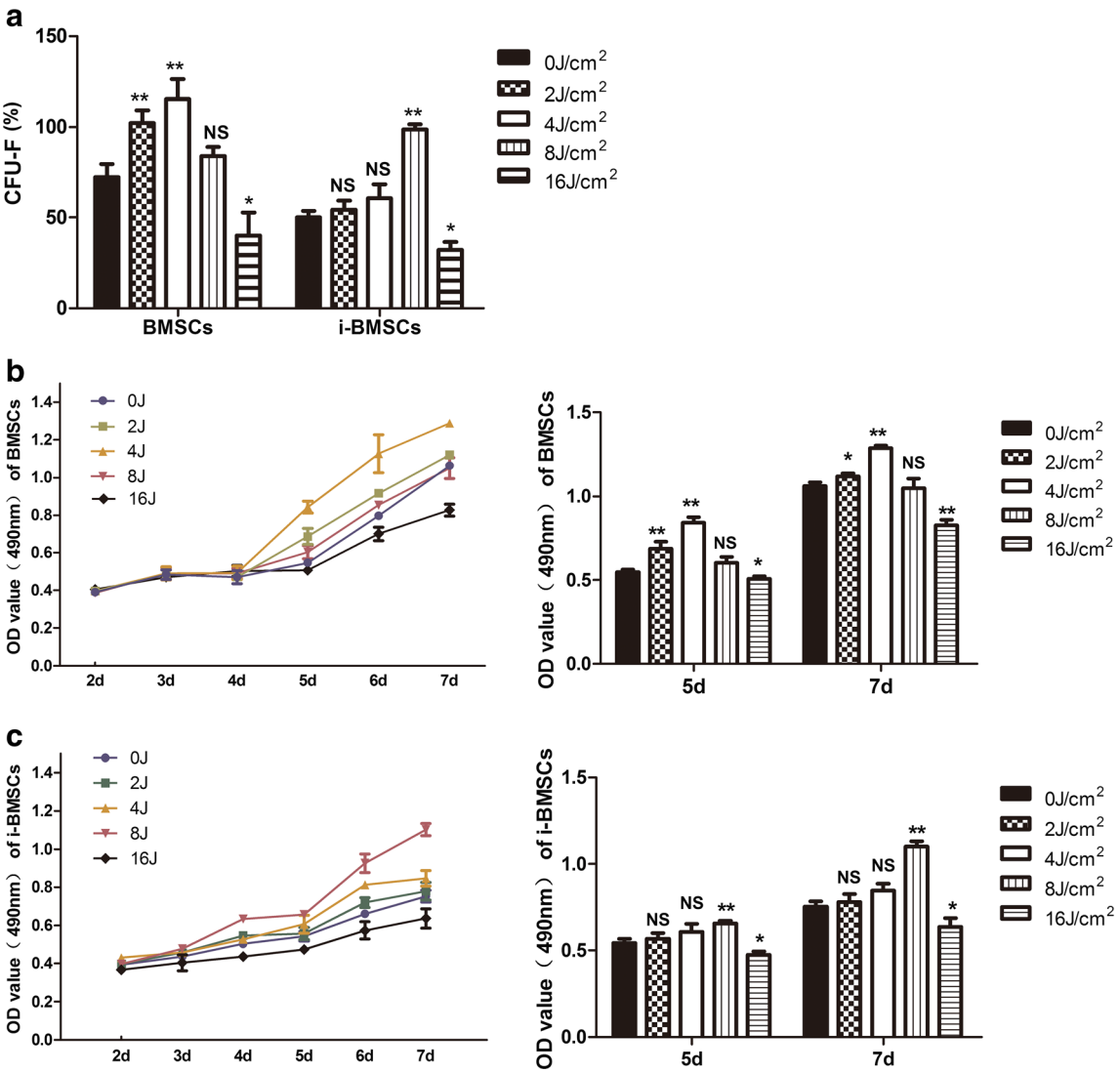

Fig. 2 Effects of LLLT on the proliferation of BMSCs and i-BMSCs. (a) The colony-forming rate (%) of cells was measured after 14 days of culture at low seeding density. (b) The MTT was measured for 7 days to show the effects of LLLT on the proliferation of BMSCs. (c) The MTT was measured for 7 days to show the effects of LLLT on the proliferation of i-BMSCs. Data represent mean ± S.D. *$P < 0.05$; **$P < 0.01$; NS, not significant

incubation ($P < 0.05$). In addition, osteogenic differentiation analysis including ALP staining (Fig. 1(c)), ALP activity assay (Fig. 1(d)), osteogenic genes level (Fig. 1(e)), and Alizarin Red S staining (Fig. 1(f)) showed i-BMSCs lost the osteogenic potential compared with BMSCs ($P < 0.05$).

Effects of LLLT on the proliferation of BMSCs and i-BMSCs

In this study, we measured the proliferation of BMSCs and i-BMSCs using CFU-F assay and MTT. In BMSCs, the abilities to form adherent clonogenic cell clusters were improved after

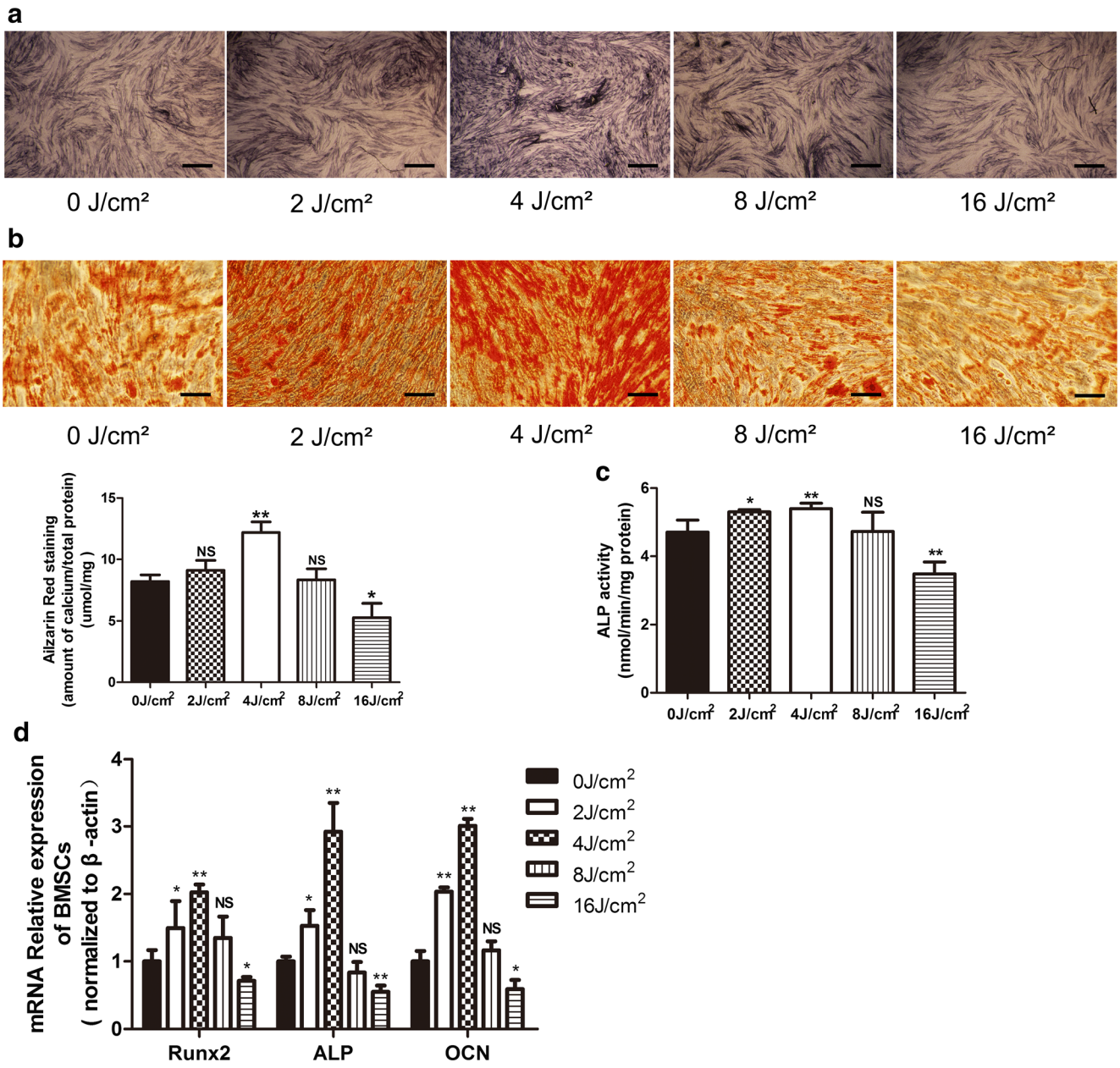

Fig. 3 Effects of LLLT on osteogenic differentiation of BMSCs. (a) Osteogenic differentiation was determined by ALP staining at day 7 after osteogenic differentiation was induced. (b) Osteogenic differentiation was determined by Alizarin Red S staining at day 21 after osteogenic differentiation was induced. Calcium concentration stained by Alizarin Red S discussed above was examined by calcium level analysis. (c) ALP activity was measured by ALP activity assay at day 7 after osteogenic differentiation was induced. (d) The expression levels of the osteogenic genes ALP, Runx2, and OCN were measured by real-time PCR at day 7 after osteogenic differentiation was induced. β-actin was used as endogenous normalization controls for quantitation of mRNAs. Data represent mean ± S.D. *$P < 0.05$; **$P < 0.01$; NS, not significant. The scale bar in the micrographs represents 100 μm

being irradiated at 2 and 4 J/cm^2 ($P < 0.05$). Irradiation at 8 J/cm^2 had no significant effect on the proliferation of BMSCs. After irradiation at 2 and 4 J/cm^2, the MTT activity was increased in BMSCs compared with the control cells at day 5 and day 7($P < 0.05$). The results of CFU-F assay and MTT both showed LLLT even suppressed the BMSCs proliferation at 16 J/cm^2 ($P < 0.05$) (Fig. 2(a, b)).

Compared with the control group, irradiation at 8 J/cm^2 appeared to promote i-BMSCs colony-forming ability and the MTT activity at days 5 and 7 ($P < 0.05$). However, irradiation at 2 and 4 J/cm^2 had no significant effect on the proliferation of BMSCs. Moreover, similar to BMSCs, LLLT at 16 J/cm^2 could apparently inhibit the proliferation of i-BMSCs ($P < 0.05$) (Fig. 2(a, c)).

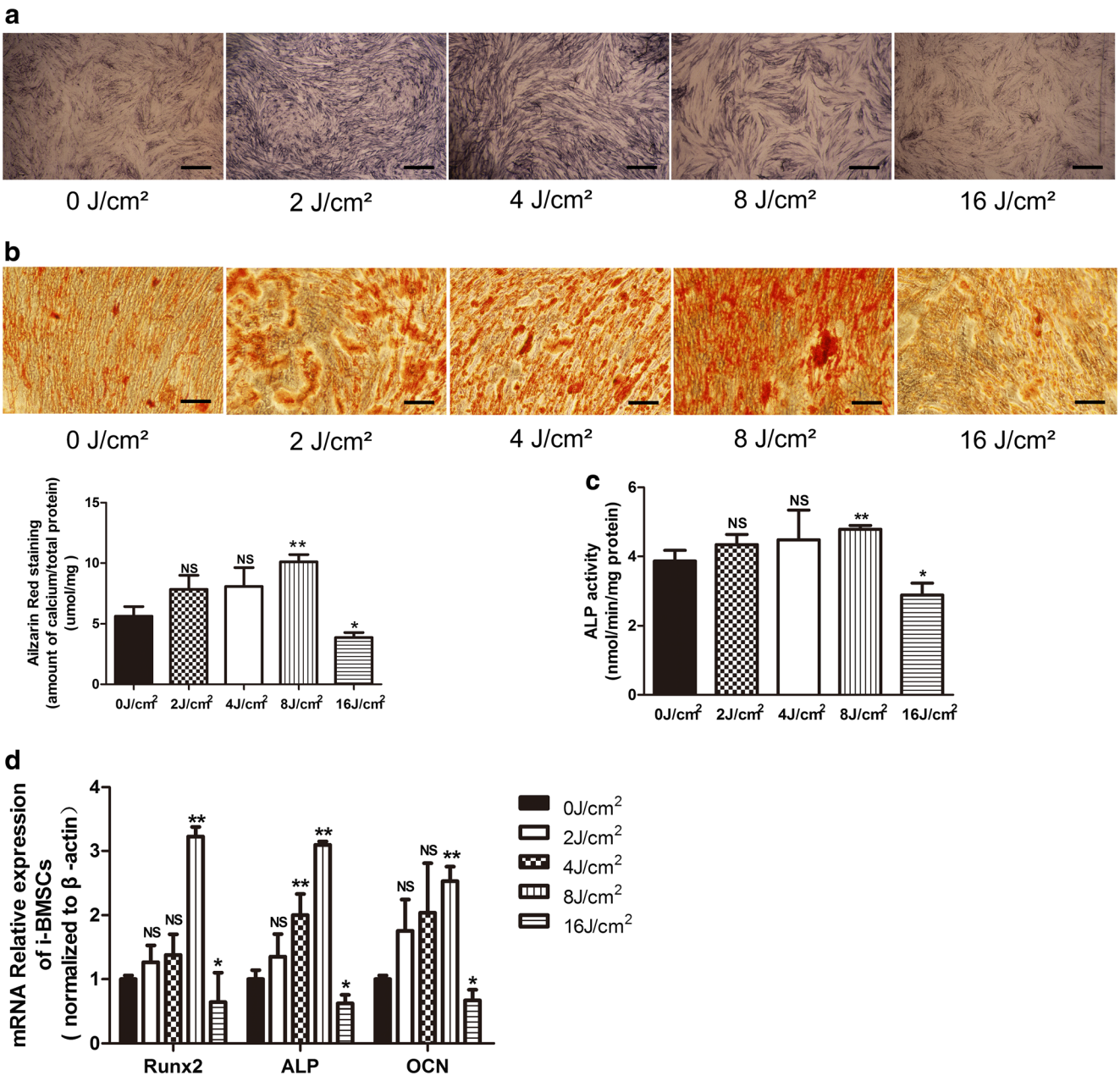

Fig. 4 Effects of LLLT on osteogenic differentiation of i-BMSCs. (a) Osteogenic differentiation was determined by ALP staining at day 7 after osteogenic differentiation induced. (b) Osteogenic differentiation was determined by Alizarin Red S staining at day 21 after osteogenic differentiation was induced. Calcium concentration stained by Alizarin Red S discussed above was examined by calcium level analysis. (c) ALP activity was measured by ALP activity assay at day 7 after osteogenic differentiation was induced. (d) The expression levels of the osteogenic genes ALP, Runx2, and OCN were measured by real-time PCR at day 7 after osteogenic differentiation was induced. β-actin was used as endogenous normalization controls for quantitation of mRNAs. Data represent mean ± S.D. *$P < 0.05$; **$P < 0.01$; NS, not significant. The scale bar in the micrographs represents 100 μm

Effects of LLLT on osteogenic differentiation of BMSCs and i-BMSCs

The effects of LLLT on the osteogenic differentiation of BMSCs and i-BMSCs were analyzed by ALP staining, ALP activity assay, and the mRNA transcript levels of genes Runx2, ALP, and osteocalcin (OCN). Moreover, to characterize the effect of LLLT, we also analyzed mineralized nodule formation by Alizarin Red staining after inducing cell in osteogenic media for 21 days. After being treated with LLLT at 2 and 4 J/cm^2, the ALP levels and the expression of osteogenic genes, including Runx2, ALP, and OCN of BMSCs, apparently increased ($P < 0.05$). However, the mineralized nodules were promoted by irradiation only at 4 J/cm^2 ($P < 0.05$) (Fig. 3(a–d)). In i-BMSCs, the irradiation at 8 J/cm^2 could enhance the osteogenic potential, with the evidence of higher ALP activity, gene expression of ALP, Runx2, and OCN, as well as more mineralized nodules and higher calcium levels ($P < 0.05$) (Fig. 4(a–d)). Among the osteogenic genes in i-BMSCs, the effect of LLLT on Runx2 was most obvious, which showed a significant increase of 3.4-fold ($P < 0.05$). We also found LLLT at 16 J/cm^2 could apparently inhibit the osteogenesis of both BMSCs and i-BMSCs (Fig. 3(a–d) and Fig. 4(a–d)).

Effects of LLLT on the secretion of TNF-α in BMSCs and i-BMSCs

The effects of LLLT on secretion of TNF-α in BMSCs and i-BMSCs were measure by ELISA assay. We although found LLLT cannot significantly affect the secretion of TNF-α of BMSCs, the secretion of TNF-α in i-BMSCs was suppressed by LLLT, and the effects increased as the energy intensity rose within the range between 4 and 16 J/cm^2 (Fig. 5).

Discussion

BMSCs are promising pluripotent stem cells for regeneration, as they have been shown to reconstruct craniomaxillofacial bone injuries in vivo [9–11]. However, the extracellular microenvironment has a direct effect on BMSCs function. Inflammation associated with trauma and postoperative infection can impair the osteogenic capability of BMSCs [12]. In this study, results showed that inflammatory cytokine TNF-α inhibited bone formation of BMSCs as discussed in a previous report (Fig. 1(c–f)) [12]. Liu et al. showed that inflammatory microenvironment positively regulates the proliferation of human periodontal mesenchymal stem cells [23]. However, we found i-BMSCs also exhibit low proliferation ability, which have been revealed by MTT and CFU-F array (Fig. 1(a, b)). Our results are the first demonstration of such a negative effect of inflammation on the proliferation of BMSCs.

The biostimulation of LLLT was firstly described in 1967 by Mester [24]. Fifty years later, thousands of researches demonstrate that LLLT could promote tissue repair, reduce inflammation, and relieve pain in a range of health fields [25]. Recently, the effects of LLLT on the regulation of proliferation and osteogenic differentiation of MSCs have attracted significant attention. In our study, we found LLLT (Nd:YAG;1064 nm) significantly promotes BMSC proliferation at densities 2 and 4 J/cm^2 (Fig. 2(a, b)). These findings are similar to Masoud's study in 2012 [22], which showed that LLLT at densities 2–6 J/cm^2 could significantly enhance BMSC proliferation. Moreover, we also found higher energies of LLLT (8 J/cm^2) could not remarkably increase the proliferation capability. These suggest that LLLT at densities 2–4 J/cm^2 is optimum for the proliferation of BMSCs.

Up to now, only a few researches have been done concerning the effects of LLLT on osteogenic differentiation of BMSCs. Gerbettaz et al. [26] found that LLLT at 4 J/cm^2 could not promote the bone formation ability of BALB/c mice BMSCs. However, another study [22], we mentioned before, demonstrates that the expression level of ALP was increased after treatment of LLLT at 2–4 J/cm^2 in human BMSCs. In this present study, we demonstrated that LLLT at 2–4 J/cm^2 could upregulate the osteogenesis of BMSCs by inducing ALP staining, ALP activity assay, and the osteogenic gene expression (Fig. 3(a, c, d)). In addition, we also noticed that LLLT at

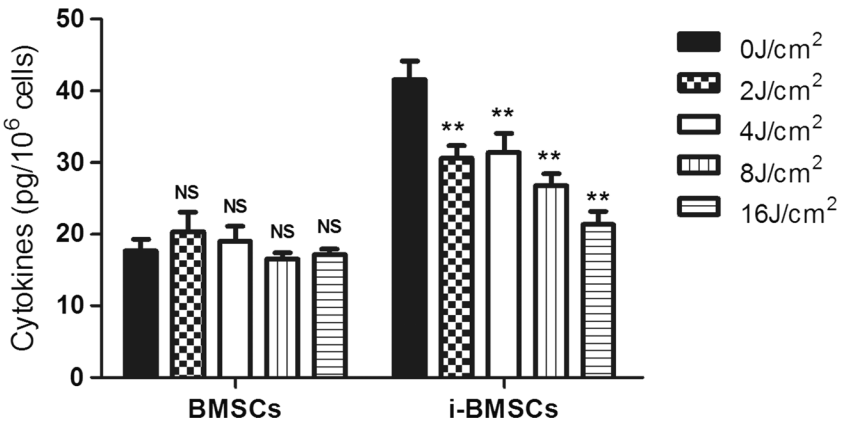

Fig. 5 Effects of LLLT on the secretion of TNF-α and IL-1 in BMSCs and i-BMSCs. The concentration of secreted cytokines TNF-α in the BMSCs and i-BMSCs culture medium were determined by ELISA. Data represent mean ± S.D. *$P < 0.05$; **$P < 0.01$; NS, not significant

2–4 J/cm^2 may enhance the mineralization node formation of BMSCs, but only statistically significant for LLLT at 4 J/cm^2 (Fig. 3(b)). Considering that the formation of mineralization nodes could reflect the mineralization capacity of cells most accurately, the LLLT densities of 4 J/cm^2 were appropriate for osteogenic differentiation of BMSCs.

Considering LLLT could suppress inflammatory reaction through decreasing the expression of cytokine TNF-α (Fig. 5) [27], we study the LLLT effects on the proliferation and osteogenic differentiation of i-BMSCs. Our investigations evidenced that LLLT at density 8 J/cm^2 could promote the proliferation and osteogenesis of i-BMSCs (Fig. 2(a, c) and Fig. 4). This density is higher than the optimum density for the BMSCs proliferation and osteogenesis. All these reveal that BMSCs are more sensitive to LLLT than i-BMSCs. Moreover, after treatment of LLLT at 8 J/cm^2, Runx2 was changed most markedly among the osteogenesis genes Runx2, ALP, and OCN. Runx2 is an osteoblast differentiation factor, which is often expressed in mesenchymal cell types (Fig. 4(d)). Runx2 often promote the level of bone-formatted protein and expressed in terminally differentiated osteoblasts [28]. Interestingly, it has been shown that proinflammatory T cells may inhibit MSC-mediated bone formation via TNF-α-induced downregulation of Runx2 [29]. Therefore, the promotion of bone regeneration by LLLT-induced i-BMSCs may exert an anti-inflammatory effect that reduces levels of TNF-α expression. Our results verified this speculation. In an inflammatory microenvironment, the expression of TNF-α was obviously inhibited by LLLT at 4, 8, and 16 J/cm^2 (Fig. 5).

As other treatments, LLLT is dose dependent, which means the stimulatory effects of LLLT depend on certain energy density. When the LLLT is given in low doses, it may generate a series of stimulatory effects on cells. However when the energy density reached a peak or even stronger, a negative response may achieve [30]. In this study, we noticed LLLT at 16 J/cm^2 significantly suppressed the proliferation and osteogenesis of BMSCs in healthy and inflammatory microenvironment. It might be because this high energy density can damage cells through heating effects which need further investigation to be proven.

Collectively, this study revealed that the effects of LLLT with different energy densities were investigated on BMSCs in healthy and inflammatory microenvironment. The major findings are the following: (1) LLLT at 2–4 J/cm^2 could significantly promote proliferation and osteogenesis of BMSCs. (2) LLLT at 8 J/cm^2 could significantly promote proliferation and osteogenesis of BMSCs in an inflammatory microenvironment. (3) LLLT at 16 J/cm^2 could significantly suppress proliferation and osteogenesis of BMSCs in healthy and inflammatory microenvironment. These findings were the first time to investigate the effects of LLLT on the biological characteristics of i-BMSCs and provide an evidence for clinical application of LLLT. Further research is necessary to examine the mechanism of LLLT effect on stem cell differentiation.

Funding This research was financially supported by grants from the National Natural Science Foundation of China (No. 81701029), the Natural Science Foundation of Gansu Province (No. 1606RJZA185 and 17JR5RA335), and the Autonomous Research Project of State Key Laboratory of Military Stomatology (No. 2016KB02).

Compliance with ethical standards

Ethics statement This article does not contain any studies with human participants performed by any of the authors. Human cancellous bone fragments and blood were collected based on the treatment needs unrelated to this study. Each donor provided written informed consent. All procedures performed in studies were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Conflict of interest The authors declare that they have no conflict of interest.

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