Journal of Biomedical Optics

SPIEDigitalLibrary.org/jbo

Effect of 940 nm low-level laser therapy on osteogenesis *in vitro*

Mohammed Mahmood Jawad
Adam Husein
Ahmad Azlina
Mohammad Khursheed Alam
Rozita Hassan
Rumaizi Shaari

SPIE

Effect of 940 nm low-level laser therapy on osteogenesis *in vitro*

Mohammed Mahmood Jawad,[a,e] Adam Husein,[b] Ahmad Azlina,[c] Mohammad Khursheed Alam,[a] Rozita Hassan,[a] and Rumaizi Shaari[d]
[a]Universiti Sains Malaysia, School of Dental Sciences, Health Campus, Orthodontic Unit, Kota Bharu, Kelantan 16150, Malaysia
[b]Universiti Sains Malaysia, School of Dental Sciences, Health Campus, Conservative Department, Kota Bharu, Kelantan 16150, Malaysia
[c]Universiti Sains Malaysia, School of Dental Sciences, Health Campus, Kota Bharu, Kelantan 16150, Malaysia
[d]Universiti Malaysia Kelantan, Clinical sciences, Faculty of Veterinary Medicine, Kota Bharu, Kelantan 16100, Malaysia
[e]Alnahrain University, Baghdad, Iraq

Abstract. Bone regeneration is essential in medical treatment, such as in surgical bone healing and orthodontics. The aim of this study is to examine the effect of different powers of 940 nm diode low-level laser treatment (LLLT) on osteoblast cells during their proliferation and differentiation stages. A human fetal osteoblast cell line was cultured and treated with LLLT. The cells were divided into experimental groups according to the power delivered and periods of exposure per day for each laser power. The (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) (MTT) assay was used to determine cell proliferation. Both alkaline phosphatase and osteocalcin activity assays were assessed for cell differentiation. All treatment groups showed a significant increase in cell proliferation and differentiation compared to the control group. Regarding the exposure time, the subgroups treated with the LLLT for 6 min showed higher proliferation and differentiation rates for the powers delivered, the 300-mW LLLT group significantly increased the amount of cell proliferation. By contrast, the 100 and 200 mW groups showed significantly greater amounts of cell differentiation. These results suggest that the use of LLLT may play an important role in stimulating osteoblast cells for improved bone formation. [DOI: 10.1117/1.JBO.18.12.128001]

Keywords: low-level laser; osteoblast; proliferation; differentiation; biostimulation; orthodontics.

Paper 130534RR received Jul. 30, 2013; revised manuscript received Nov. 1, 2013; accepted for publication Nov. 5, 2013; published online Dec. 10, 2013.

1 Introduction

In recent decades, various biostimulatory effects of low-level laser therapy (LLLT) have been reported, including wound healing,[1–4] chondral[5] and fibroblast[6,7] proliferation, collagen synthesis,[8] and nerve regeneration.[9] In particular, the acceleration of bone regeneration by laser treatment has been the focus of recent studies[10] because it may hold potential benefits in clinical therapy in orthopedics and dentistry (orthodontics, periodontics, implantology, etc.).

The LLLT indicated that laser irradiation may have a beneficial effect on the wound healing of bone by increasing vascularization,[11] stimulating formation of trabecular osteoid tissue, promoting faster tissue metabolism, and increasing the reaction of bone callus, which will lead to the acceleration of bone regeneration.[12,13] Since bone remodeling in orthodontic treatment comprises modification to the dentoalveolar structure, the effect of LLLT on orthodontics induced interest amongst the researchers.[14–17]

In vitro studies have shown the effects of LLLT on cell cultures, which simulates osteoclastic activity formation of RANK,[16] MMP-9, cathepsin *K*, and $\alpha(v)\,\beta 3$ integrin expression in rat osteoclast precursor cells.[14] Those studies concluded that laser irradiation may induce the differentiation and activation of osteoclasts.[14,16,18,19] Further, other studies demonstrated that laser irradiation stimulates cellular proliferation and differentiation of osteoblast lineage bone nodule-forming cells as well as in bone formation.[20]

Additionally, *in vivo* LLLT stimulation indicated bone regeneration in the midpalatal suture during expansion,[21] increased tooth movement,[16,22] and facilitated the turnover of connective tissues with the acceleration of the bone remodeling process by stimulating osteoblast and osteoclast cell proliferation and function.[15,23] Similar results were observed in human *in vivo* studies.[24,25]

However, there were studies that demonstrated contradictory results suchasc there is no significant acceleration in tooth movement after the LLLT irradiation,[19,23,26,27] the repair was inhibited due to the inhibition of immature collagen expression, and laser delayed the bone formation.[19]

The biostimulation effects of the LLLT on bone repair are directly dependent on the dosage of laser applied.[28] Various parameters that induce changes within cell cultures and lead to an increased healing effect have proven to be effective for several different lasers. Nevertheless, the optimal parameters (wavelength, exposure time, energy density, and power) have yet to be determined.[29] Thus, this study will seek to uncover the optimum laser parameters for the 940-nm LLLT on osteoblast cell proliferation and differentiation.

2 Materials and Methods

2.1 *Cell Culture*

The human fetal osteoblast cell line (hFOB) was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia). The cells were maintained in a growth medium

Address all correspondence to: Mohammed Mahmood Jawad, Universiti Sains Malaysia, School of Dental Sciences, Health Campus, Orthodontic Unit Kubang Kerian, 16150 Kelantan, Malaysia. Tel: +60149398175; Fax: 00609 767 5505; E-mail: dr.mohammedalazzawi@ymail.com

0091-3286/2013/$25.00

consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco, California) with F12 containing 10% (v/v) fetal bovine serum (FBS, Lonza, Maryland) and 1% (v/v) penicillin–streptomycin solution (50-U/ml penicillin and 50 – mg/ml streptomycin; Sigma Chemical, St. Louis, Missouri) at 37°C in a humidified atmosphere of 95% air/5% CO_2; the medium was changed twice weekly.

2.2 *Procedure of LLLT*

The hFOB cells were plated in 96-well plates at a density of 2×10^4 cells/cm^2. After a 24-h incubation for adhesion, the cells were irradiated using a Ga–Al–As diode laser (ezlase, Biolase, California) with a wavelength of 940 nm in a continuous wave mode of operation. The laser beam was delivered by a 0.4-mm-diameter optical fiber defocused at the tip by a concave lens to provide a uniform circle of irradiation that was 7 mm in diameter at the cell layer level; irradiation was performed from 14 mm above the cell layer, as recommended by Aihara et al.[18] The power density of the laser beam was measured with a laser power meter (Thorlabs, New Jersey). The 96-well plates were uniformly irradiated for a period of up to 7 days. The plates were grouped into 3 main experimental groups according to the power delivered: 100, 200, and 300 mW; these laser powers were obtained from a previous preliminary study. Then, each main group was further subdivided to subgroups according to periods of exposure, which either were 3 or 6 min/day for each laser power. The energy densities each subgroup received at 3 or 6 min exposure times, respectively, were as follows: the first group that was exposed to 100 mW received 22.92 or 45.85 J/cm^2; the second group that was exposed to 200 mW received 45.85 or 91.79 J/cm^2; and the third group of 300 mW exposure received 68.78 or 137.57 J/cm^2. When not receiving laser irradiation, the cells were kept in a 5% CO_2 incubator at 37°C. All experimental groups were compared with a control group of cells that did not receive any laser treatment.

2.3 *MTT Assay*

The (3-(4,5-dimethylthiazol-2yl)-2,5 diphenyl tetrazolium bromide) (MTT) is transformed by mitochondrial dehydrogenases of active cells, providing a measurement of cell proliferation and viability.[30] All experimental groups were measured by this test at days 1, 3, and 7. The MTT (Sigma, Missouri M2128) was dissolved at a concentration of 5 mg/ml in sterile phosphate buffered saline, filtered through a 0.22-pm filter to remove any formazan crystals, and stored at 4°C in the dark; The MTT was added to osteoblast cultures in the 96-well plates at a 1:l0 ratio. Following incubation at 37°C for 4 h in 5% CO_2/95% air humidified atmosphere, the supernatants in the wells were removed and replaced with 100 μl of dimethyl sulphoxide per well. The absorbance was measured at the 570-nm wavelength using a microplate reader (Sunrise, TECAN, Switzerland). These values were expressed as the percent viability of the samples versus control cells, which were set equal to 100%.

2.4 *Alkaline Phosphatase Activity Assay*

The Alkaline phosphatase (ALP) is expressed at high levels in osteoblasts; the level of ALP in serum is a systemic indictor of bone formation.[31] The hFOB cells were measured for the ALP activity at days 1, 3, and 7. The procedure was conducted by following the ALP Activity Colorimetric Assay Kit (Biovision, California) manufacturer's protocol. Briefly, the samples were added to the wells of 96-well plates and the volume was brought to 80 μl. Because the colored samples may interfere with optical density (O.D.) readings, sample background controls were maintained by adding the same amount of the sample to separate wells, also bringing the volume to 80 μl. To terminate the ALP activity, 20 μl of stop solution was added to the background sample. Then, 50 μl of 5 mM *p*-nitrophenyl phosphate solution was added to the wells containing the test, background, and control samples. The ALP reaction incubated for 60 min at 25°C in the dark. Twenty microliters of the stop solution was added to each standard and sample reaction, except the background reaction, and after gentle shaking of the plates, a microplate reader (Sunrise, TECAN, Switzerland) was used to measure the O.D. at 405 nm. A standard curve was generated with the assay. The ALP activity was expressed as a relative sample percentage compared with the control, which was set equal to 100%.

2.5 *Osteocalcin Activity Assay*

The Osteocalcin (OST) is a noncollagenous protein found in bone that is secreted solely by osteoblasts and naturally plays a role in the body's metabolic regulation and bone formation. The OST is also implicated in bone mineralization and calcium ion homeostasis.[32] The hFOB cells were measured for OST activity at days 1, 3, and 7. The procedure was conducted by following the manufacturer's protocol for the OST Activity Assay Kit (Invitrogen, California). Briefly, 25 μl of each kit standard, control, or sample was added to the appropriate wells. Then, 100 μl of working Anti-OST-HRP conjugate was added into all wells and incubated for 2 h at room temperature. The solution was aspirated from the wells to discard the liquid, wells were washed 3 times and 100 μl of chromogen solution was added to each well. Then, the plate was incubated for 30 min at room temperature in the dark. To stop the reaction, 100 μl of stop solution was added to each well. A standard curve was generated with the assay. The OST activity was expressed as relative sample percentage compared with the control, which was set equal to 100%.

2.6 *Statistical Analysis*

All experiments were performed in triplicate. Results are expressed as the mean and standard error, and statistical analysis was performed using Kruskal–Wallis and Mann–Whitney tests. A P value ≤ 0.05 was considered statistically significant.

3 Results

3.1 *Cell Proliferation by MTT*

The results showed that there was a significant increase in the cell proliferation rates of all groups compared with the control group in relation to time. The day 7 was the highest among all groups, as shown in Fig. 1.

When comparing all groups at day 7, the subgroups treated with the LLLT for 6 min showed a significantly higher proliferation rate than the subgroups that were treated with the LLLT for 3 min. Additionally, the cell proliferation rate significantly increased when the laser power was increased; the 300-mW laser power had the highest proliferation rate and the 100-mW laser power had the lowest proliferation rate of the groups, as shown in Fig. 2.

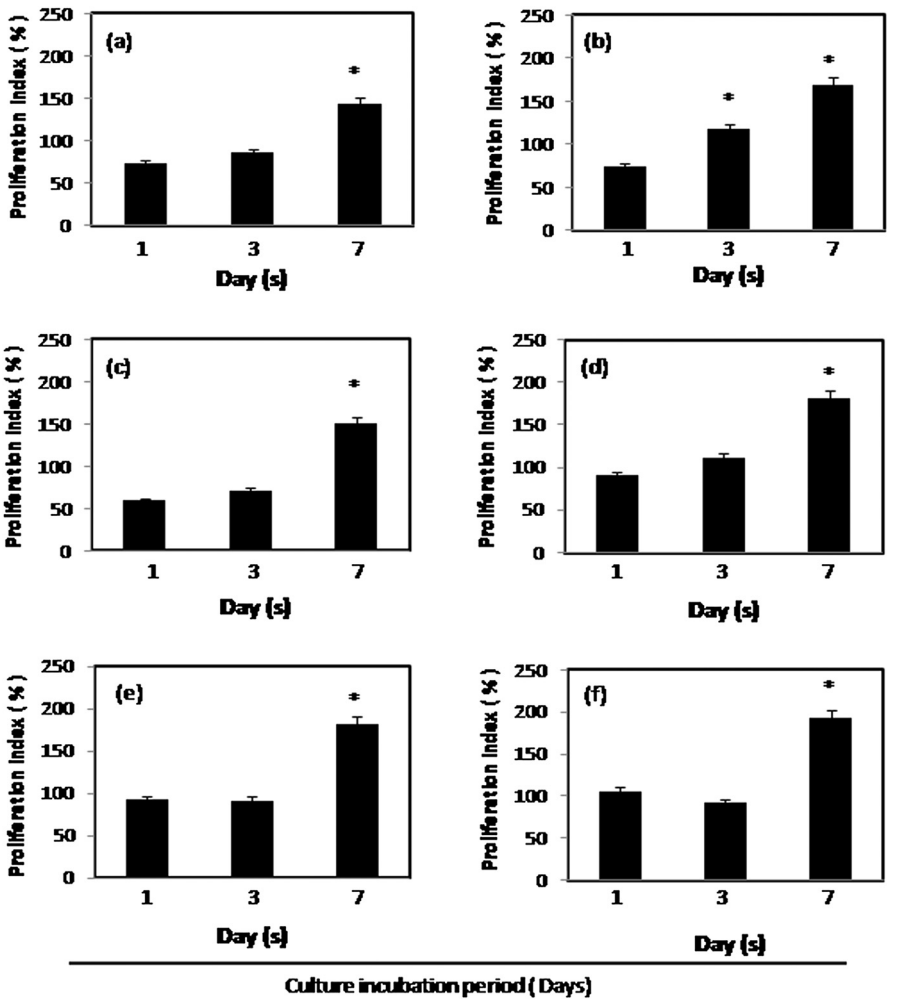

Fig. 1 Effect of laser stimulation on osteoblast cell proliferation. The cells were cultured in the presence and absence of daily laser stimulation. The proliferation percentage of controls, as determined by MTT assay, for different laser powers and times: (a) 100 mW, 3 min; (b) 100 mW, 6 min; (c) 200 mW, 3 min; (d) 200 mW, 6 min; (e) 300 mW, 3 min; and (f) 300 mW, 6 min. The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups on days 1, 3, 7 and control.

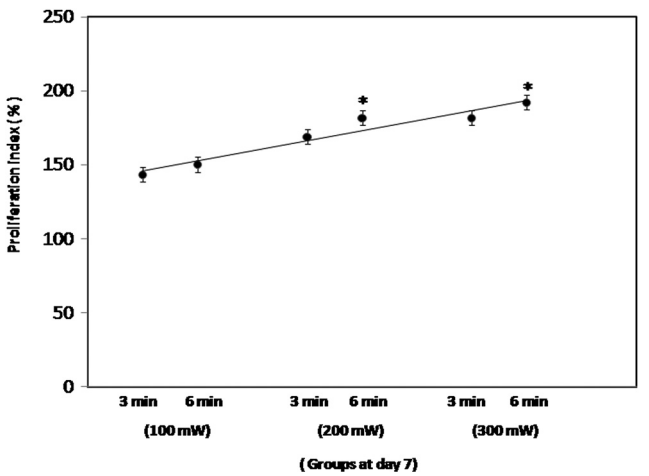

Fig. 2 Effect of laser stimulation on osteoblast cell proliferation at day 7. The cells were cultured in the presence and absence of daily laser stimulation. The proliferation percentage of controls, as determined by MTT assay, for laser treatments at different powers and times: (100 mW, 3 min), (100 mW, 6 min), (200 mW, 3 min), (200 mW, 6 min), (300 mW, 3 min), and (300 mW, 6 min). The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups.

3.2 *Cell Differentiation by ALP and OST Activity Assays*

The results showed that there was an increase in cell differentiation (by ALP and OST assays) in all groups compared with the control group in relation to time. The day 7 was significantly increased in cell differentiation among all groups, as shown in Figs. 3 and 4.

When comparing all groups at day 7, the subgroups treated with the LLLT for 6 min showed a significantly higher differentiation rate (by ALP and OST assays) than the subgroups that were treated with the LLLT for 3 min. Additionally, the cell differentiation rate increased when the laser power was decreased; the ALP activity rate was the more significant at the 100-mW laser power, whereas for the OST activity rate, the 200-mW laser power was the most significant, whereas the 300-mW laser group was not significant among all treatment groups for cell differentiation, as determined by both ALP and OST assays shown in Figs. 5 and 6.

4 Discussion

The primary interest of this study is to understand the bone regeneration which takes place during the LLLT that it may be utilized for orthodontic treatment.[23] In an effort to improve

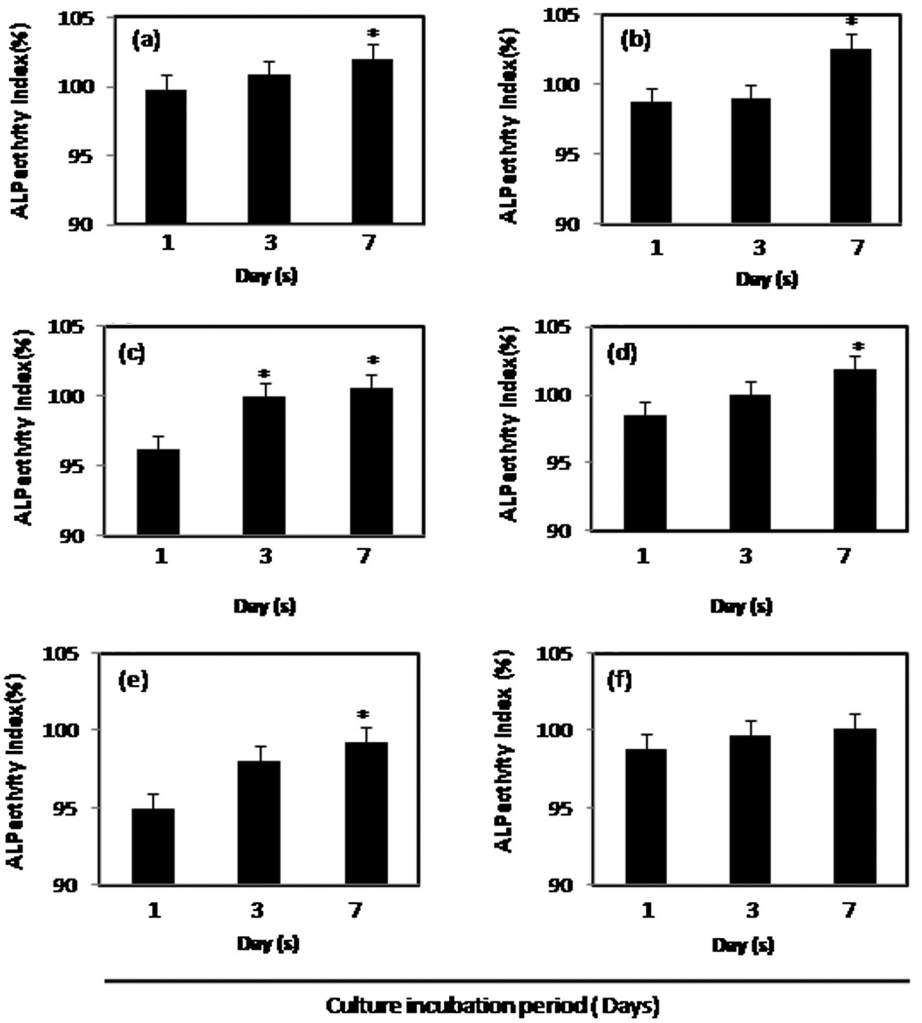

Fig. 3 Effect of laser stimulation on alkaline phosphatase (ALP) activity of osteoblast cells. The cells were cultured in the presence and absence of daily laser stimulation. The ALP percentage of controls for different laser powers and times: (a) 100 mW, 3 min; (b) 100 mW, 6 min; (c) 200 mW, 3 min; (d) 200 mW, 6 min; (e) 300 mW, 3 min; and (f) 300 mW, 6 min. The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups on days 1, 3, 7, and control.

bone regeneration, many biochemical methods involving medicines, such as prostaglandin[33,34] and parathyroid hormone, have been employed. However, these chemicals had an effect on body metabolism and were accompanied by painful procedures. Therefore, their application has been limited.[35]

In this study, we compared the stimulatory effect of different powers of 940-nm LLLT with different exposure times on osteoblasts during cell proliferation. Cell differentiation within the stages of bone formation was also measured by the produced amount of ALP protein from the cells as an early marker for activity and by the produced amount of OST protein which is a marker for the extra cellular calcified matrix formation.[20]

All LLLT treatment groups showed significant increases in cell proliferation and cell differentiation compared to the control group which is similarly described by Ozawa et al.,[20] who reported that the irradiation of LLLT (wavelength of 830 nm) in the early stages of osteoblast-like cells isolated from fetal rat calvariae significantly stimulated cellular proliferation, the ALP activity, and the OST gene expression. Furthermore, Barushka et al.[36] reported that low-energy laser (He–Ne) irradiation after injury positively affected the population of osteoblasts and osteoclasts at the injured site.

Regarding the exposure time, in our study, the subgroups treated with LLLT for 6 min showed higher proliferation and differentiation rates, while the shorter exposure time was not significant, which may be due to the fact that the shorter time of laser exposure delivered lower energy density which is not enough to stimulate the cells. On the other hand, the use of LLLT for a longer time than the range of 6 min was also not significant which may be due to delivering excessive energy density that damages the cells. This was similarly found by Aihara et al.[18] when using the LLLT with osteoclast cells.

For laser power, the 300-mW LLLT group showed the highest cell proliferation rate. By contrast, the 100- and 200-mW groups significantly had the highest cell differentiation rates, whereas the 300 mW-LLLT group had the lowest. For bone healing, the speed and stability of tissue turnover is dependent on cell function, rather than cell proliferation, although an increase in the cell proliferation rate is required. The results suggested that there is an exponential relation between osteoblast cell proliferation and differentiation when the power of LLLT is increased; this result is consistent with Leibur et al., Donahue et al., and others.[37–39] Thus, the use of the lower laser powers of the LLLT is better than the higher one to improve

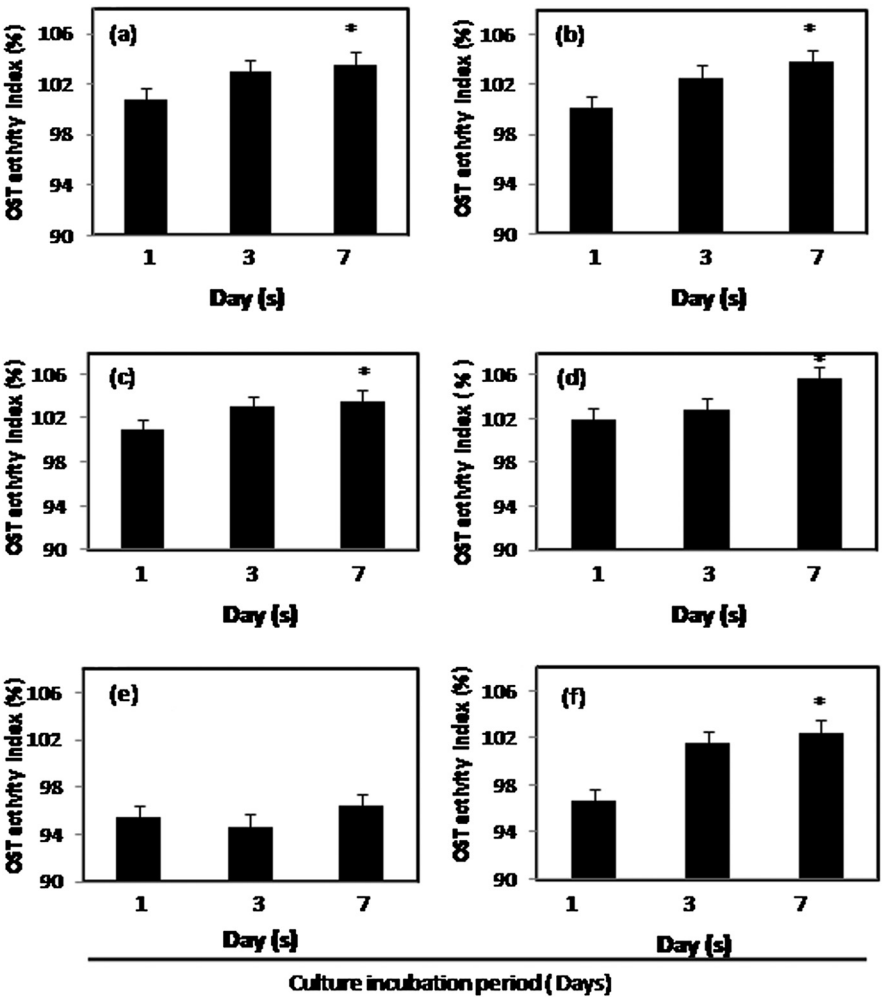

Fig. 4 Effect of laser stimulation on osteocalcin (OST) activity of osteoblast cells. The cells were cultured in the presence and absence of daily laser stimulation. The OST percentage of controls for different laser powers and times: (a) 100 mW, 3 min; (b) 100 mW, 6 min; (c) 200 mW, 3 min; (d) 200 mW, 6 min; (e) 300 mW, 3 min; and (f) 300 mW, 6 min. The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups on days 1, 3, 7 and control.

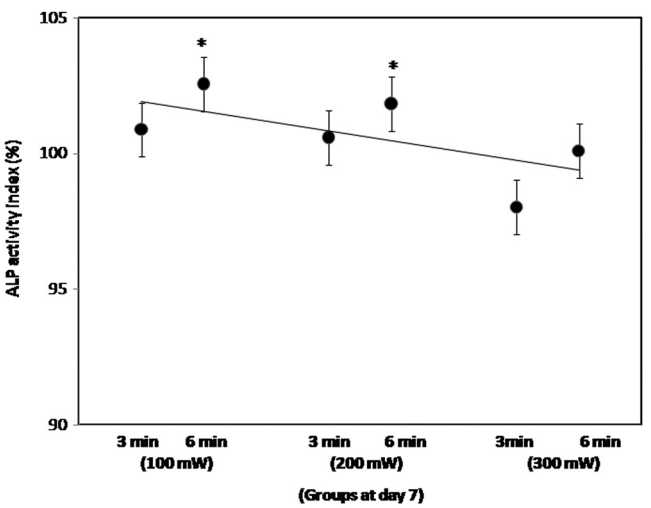

Fig. 5 Effect of laser stimulation on ALP activity of osteoblast cells at day 7. The cells were cultured in the presence and absence of daily laser stimulation. The ALP percentage of controls for different laser powers and times: (100 mW, 3 min), (100 mW, 6 min), (200 mW, 3 min), (200 mW, 6 min), (300 mW, 3 min), and (300 mW, 6 min). The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups and control.

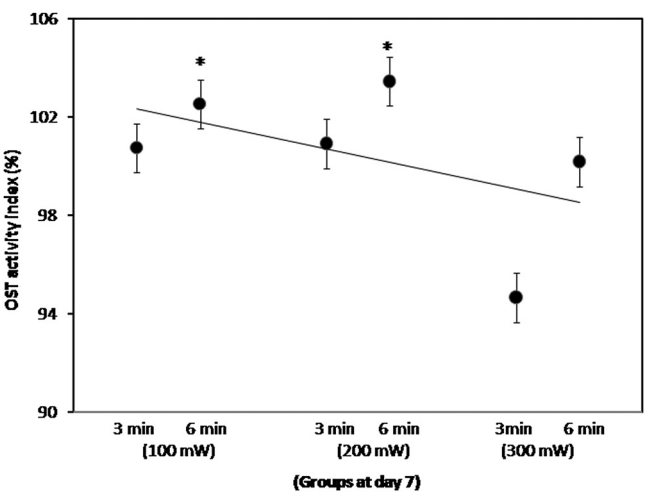

Fig. 6 Effect of laser stimulation on OST activity of osteoblast cells at day 7. The cells were cultured in the presence and absence of daily laser stimulation. The OST percentage of controls for different laser with powers and times: (100 mW, 3 min), (100 mW, 6 min), (200 mW, 3 min), (200 mW, 6 min), (300 mW, 3 min), and (300 mW, 6 min). The data are shown as the mean ± SEM of three separate experiments. * $P \leq 0.05$ between groups and control.

cell differentiation.[20,40] For *in vivo* study, especially in orthodontic movement of teeth, it is recommended to examine these findings with animal models, taking into account our results as a base line to build upon for 940-nm LLLT.

5 Conclusion

On the basis of findings of the present study, the use of LLLT may play an important role in stimulating bone formation by osteoblast cells, particularly when the power does not exceed 200 mW and an exposure time of 6 min is used for the 940-nm LLLT.

Acknowledgments

The authors would like to thank Universiti Sains Malaysia for the financial support (304/PPSG/61312043) and the staff of Craniofacial Biology Laboratory for their assistance.

References

1. S. J. Kim et al., "Effects of low-level laser therapy after Corticision on tooth movement and paradental remodeling," *Lasers Surg. Med.* **41**(7), 524–533 (2009).
2. L. A. D. S. Merli et al., "Effect of low-intensity laser irradiation on the process of bone repair," *Photomed. Laser Ther.* **23**(2), 212–215 (2005).
3. J. Nissan et al., "Effect of low intensity laser irradiation on surgically created bony defects in rats," *J. Oral Rehabil.* **33**(8), 619–924 (2006).
4. D. Ribeiro and M. Matsumoto, "Low-level laser therapy improves bone repair in rats treated with antiâ€ inflammatory drugs," *J. Oral Rehabil.* **35**(12), 925–933 (2008).
5. R. J. Schultz et al., "Effects of varying intensities of laser energy on articular cartilage: a preliminary study," *Lasers Surg. Med.* **5**(6), 577–588 (1985).
6. M. Soudry et al., "Effect of a helium-neon laser on cellular growth: an in vitro study of human gingival fibroblasts," *J. Biol. Buccale* **16**(3), 129–135 (1988).
7. H. H. Van Breugel and P. BÃr, "Power density and exposure time of He-Ne laser irradiation are more important than total energy dose in photoâ € biomodulation of human fibroblasts in vitro," *Lasers Surg. Med.* **12**(5), 528–537 (1992).
8. G. Balboni et al., "Effects of He-Ne/IR laser irradiation on two lines of normal human fibroblasts in vitro," *Ital. J. Anat. Embryol.* **91**(3), 179–188 (1985).
9. J. J. Anders et al., "Low power laser irradiation alters the rate of regeneration of the rat facial nerve," *Lasers Surg. Med.* **13**(1), 72–82 (1993).
10. M. Trelles and E. Mayayo, "Bone fracture consolidates faster with low-power laser," *Lasers Surg. Med.* **7**(1), 36–45 (1987).
11. M. M. Jawad et al., "An overview: laser applications in dentistry," *Int. J. Pharmacol.* **7**(2), 189–197 (2011).
12. S. E. Zahra et al., "The effect of low level laser therapy (LLLT) on bone remodelling after median diastema closure: a one year and half follow-up study," *Orthod. Waves* **68**(3), 116–122 (2009).
13. M. M. Jawad et al., "An overview of laser principle, laser-tissue interaction mechanisms and laser safety precautions for medical laser users," *Int. J. Pharmacol.* **7**(2), 149–160 (2011).
14. M. Yamaguchi et al., "Low-energy laser irradiation facilitates the velocity of tooth movement and the expressions of matrix metalloproteinase-9, cathepsin K, and alpha (v) beta (3) integrin in rats," *Eur. J. Orthod.* **32**(2), 131–139 (2010).
15. B. A. Altan et al., "Metrical and histological investigation of the effects of low-level laser therapy on orthodontic tooth movement," *Lasers Med. Sci.* **27**(1), 131–140 (2010).
16. S. Fujita et al., "Low-energy laser stimulates tooth movement velocity via expression of RANK and RANKL," *Orthod. Craniofacial Res.* **11**(3), 143–155 (2008).
17. M. M. Jawad et al., "Overview of non-invasive factors (low level laser and low intensity pulsed ultrasound) accelerating tooth movement during orthodontic treatment," *Lasers Med. Sci.* (2012).
18. N. Aihara, M. Yamaguchi, and K. Kasai, "Low-energy irradiation stimulates formation of osteoclast-like cells via RANK expression in vitro," *Lasers Med. Sci.* **21**(1), 24–33 (2006).
19. C. Capuccini et al., "Strontium-substituted hydroxyapatite coatings synthesized by pulsed-laser deposition: in vitro osteoblast and osteoclast response," *Acta Biomater.* **4**(6), 1885–1893 (2008).
20. Y. Ozawa et al., "Low-energy laser irradiation stimulates bone nodule formation at early stages of cell culture in rat calvarial cells," *Bone* **22**(4), 347–354 (1998).
21. S. Saito and N. Shimizu, "Stimulatory effects of low-power laser irradiation on bone regeneration in midpalatal suture during expansion in the rat," *Am. J. Orthod. Dentofacial Orthopedics* **111**(5), 525–532 (1997).
22. M. Yamaguchi, "RANK/RANKL/OPG during orthodontic tooth movement," *Orthod. Craniofacial Res.* **12**(2), 113–119 (2009).
23. Y. D. Kim et al., "Low-level laser irradiation facilitates fibronectin and collagen type I turnover during tooth movement in rats," *Lasers Med. Sci.* **25**(1), 25–31 (2010).
24. D. R. Cruz et al., "Effects of low intensity laser therapy on the orthodontic movement velocity of human teeth: a preliminary study," *Lasers Surg. Med.* **35**(2), 117–120 (2004).
25. M. Youssef et al., "The effect of low-level laser therapy during orthodontic movement: a preliminary study," *Lasers Med. Sci.* **23**(1), 27–33 (2008).
26. M. Seifi et al., "Effects of two types of low-level laser wave lengths (850 and 630 nm) on the orthodontic tooth movements in rabbits," *Lasers Med. Sci.* **22**(4), 261–264 (2007).
27. R. C. Rowan, *The effect of two energy density and dose applications of low level laser therapy on orthodontic tooth movement*, Graduate School/Saint Louis University (2010).
28. I. Freitas, V. Baranauskas, and M. Cruz-Hofling, "Laser effects on osteogenesis," *Appl. Surf. Sci.* **154**(5), 548–554 (2000).
29. A. Schindl et al., "Low-intensity laser therapy: a review," *J. Invest. Med.* **48**(5), 312–326 (2000).
30. G. Ciapetti et al., " In vitro evaluation of cell/biomaterial interaction by MTT assay," *Biomaterials* **14**(5), 359–364 (1993).
31. A. Salgado et al., "Preliminary study on the adhesion and proliferation of human osteoblasts on starch-based scaffolds," *Mater. Sci. Eng.: C* **20**(1), 27–33 (2002).
32. N. K. Lee et al., "Endocrine regulation of energy metabolism by the skeleton," *Cell* **130**(3), 456–469 (2007).
33. K. Yamasaki, Y. Shibata, and T. Fukuhara, "The effect of prostaglandins on experimental tooth movement in monkeys (Macaca fuscata)," *J. Dent. Res.* **61**(12), 1444–1446 (1982).
34. K. Yamasaki, "The role of cyclic AMP, calcium, and prostaglandins in the induction of osteoclastic bone resorption associated with experimental tooth movement," *J. Dent. Res.* **62**(8), 877–881 (1983).
35. Y. Kobayashi et al., "Effects of local administration of osteocalcin on experimental tooth movement," *The Angle Orthodontist* **68**(3), 259–266 (1998).
36. O. Barushka, T. Yaakobi, and U. Oron, "Effect of low-energy laser (He–Ne) irradiation on the process of bone repair in the rat tibia," *Bone* **16**(1), 47–55 (1995).
37. E. Leibur and E. Lepp, "Effect of cyclic 3', 5'adenosin-monophosphate on osteogenesis embryonic bone cultivated in tissue culture: an autoradiographic study," *Stomatologija, Baltic Dental Maxillofacial J.* **9**(4), 99 (2007).
38. H. J. Donahue et al., "Differentiation of human fetal osteoblastic cells and gap junctional intercellular communication," *Am. J. Physiol.-Cell Physiol.* **278**(2), C315–C322 (2000).
39. S. Takikawa et al., "Low-intensity pulsed ultrasound initiates bone healing in rat nonunion fracture model," *J. Ultrasound in medicine* **20**(3), 197–205 (2001).
40. T. Ninomiya et al., "Increase of bone volume by a nanosecond pulsed laser irradiation is caused by a decreased osteoclast number and an activated osteoblasts," *Bone* **40**(1), 140–148 (2007).