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Research Article

How Should an Increase in Alkaline Phosphatase Activity Be Interpreted?

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Low-level laser therapy, commonly known as LLLT, is the application of low power, monochromatic, and coherent light to injuries and lesions to stimulate healing and give pain relief. There are conflicting reports in the literature regarding the role of ALP. *Objective*: this study aimed to compare the cellular responses of wounded human skin fibroblasts exposed to doses of 0.5 J/cm^2, 2.5 J/cm^2, 5 J/cm^2, or 16 J/cm^2 using LLLT with a Helium-Neon laser (632.8 nm, 18.8 mW power output, 2.07 mW/cm^2 power density, and 3.4 cm diameter spot size or area 9.1 cm^2) to elucidate the role of alkaline phosphatase (ALP) in cell proliferation. *Methods*: cellular responses to laser irradiation were evaluated using ALP enzyme activity, LDH membrane integrity, neutral red for cell proliferation, optical density at 540 nm, and basic fibroblast growth factor (bFGF) expression. *Results*: results suggest that an increase in ALP is negatively correlated with cell growth depending on the concentration of growth factors in the medium. Results also indicate that an increase in ALP may be related to cellular damage. *Conclusion*: since the exact role of ALP is unknown, the ALP enzyme activity assay should be considered in conjunction with other cell proliferation assays such as neutral red, optical density, or more specifically bFGF expression.

1. INTRODUCTION

LLLT has been used to treat many conditions with reports of multiple clinical effects including promotion of healing of both hard and soft tissue lesions. LLLT as a treatment modality remains controversial. The effects of wavelength, beam type, energy output, energy level, energy intensity, and exposure regime of LLLT remain unexplained. Moreover, no specific therapeutic window for dosimetry and mechanism of action has been determined [1].

Many assays require the measurement of alkaline phosphatase (ALP) as an indication of the presence, or for quantitation of specific molecules such as proteins and nucleic acids. ALP is also commonly used as a marker for monitoring the level of antibodies, drugs, enzymes, and other analytes in a wide range of biological tests [2]. ALP is an ectoenzyme anchored to the plasma membrane via a glycosylphosphatidylinositol linkage released in inflammation, remodelling, and cell proliferation and has been used as a marker for wound healing [3]. Little is knownabout functional characteristics of ALP-positive fibroblasts (Figure 1), they are active in protein synthesis and retain the ability to produce type 1 collagen [3, 4].

Experimental wounds of the gingiva exhibit evidence of marked amplification of ALP activity in the outer layers of epithelium adjacent to an incision. The increased enzymatic activity in the region of the healing wound appears to be associated with: (i) the growth and function of outgrowing capillaries, (ii) the activity of fibroblasts in connection with the development of connective tissue fibres, (iii) the activity of macrophages within and around the lesion, and (iv) keratinization proceeding from the margins of the epithelial cicatrix [5].

Abe et al. [6] reported a negative correlation between ALP expression and cell growth, which is accompanied by a change into serum growth factor-independent survival [6]. The upregulation of ALP requires the cessation of proliferation [6]. However, growth arrest alone is unlikely to be sufficient for the elevated expression in wounds and inflammation, because fibroblasts are quiescent (inactive) under normal conditions in vivo whereas they express elevated ALP

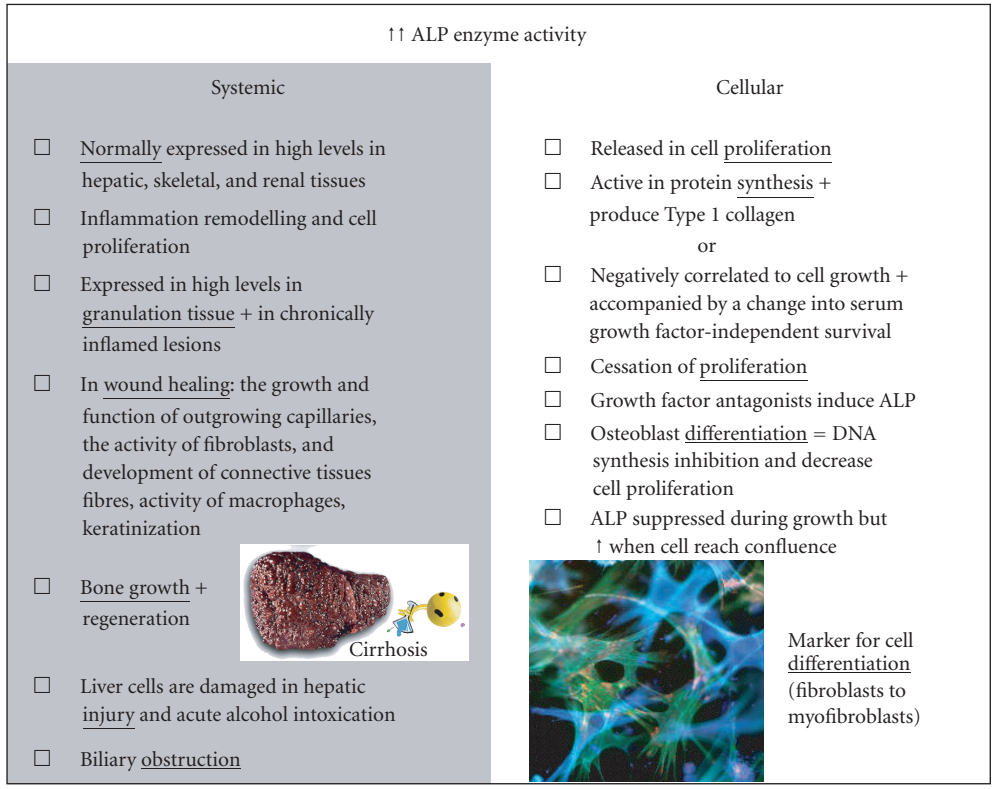

Figure 1: The increase in ALP associated with systemic and cellular changes.

activity in granulation tissue and chronically inflamed lesions [7, 8].

Under pathological conditions such as wound healing and inflammation, morphological and functional features of fibroblasts are modulated by various environmental factors including growth factors, cytokines, and extracellular matrix components [9]. Fibroblasts are responsible for synthesizing collagen, and collagen makes up about 50% of scar tissue. To produce new tissue, fibroblasts proliferate in the wound and migrate with the help of growth factors and glycoprotein that acts as a conduit and binds to both the wound matrix and the fibroblasts. With a reduction in inflammatory stimuli and therefore in the number of inflammatory mediators, the wound milieu is altered to allow new tissue formation. However, fibroblasts only reach the maximum relative number of cells after 5 days post wounding, so any increase in the rate of proliferation would ultimately accelerate the natural healing process of wounds [10]. Fibroblasts undergo phenotypic modulation from an ALP-negative to ALP-positive phenotype as their microenvironment changes during wound healing and chronic inflammation [3].

Under serum deprivation, a fraction of fibroblasts undergo cell death, while others survive and express ALP for at least several weeks [6]. Serum or growth factor deprivation induces ALP expression, which is inversely correlated with cell growth. Growth factor antagonists induce ALP expression when added to cultured cells, while the readdition of serum suppresses the ALP induction [6]. Other studies have reported, in several cell types, that ALP expression represents a marker for cell differentiation [4]. In osteoblasts which express ALP during differentiation, the expression of ALP is upregulated by DNA synthesis inhibition, and is inversely related with proliferation [4].

Several studies have linked the increase in ALP activity with specific biological effects. The anabolic role of androgens in growth and regeneration has implications in periodontal tissues. Studies suggest that androgens and growth factors could affect the activity of the enzyme alkaline phosphatase, being closely linked to anabolic effects on tissues matrices [11]. Williams et al. [12] studied changes in neutrophil precursor populations following an inflammatory stimulus and reported an increase in the number of ALP reactive cells, which was evident first in the myeloblast promyelocyte population at 2 hours [12]. Kanno et al. [13] investigated the biological effects of platelet rich plasma (PRP) on the proliferation and differentiation of 2 human osteoblast-like cell lines and concluded that ALP activity was suppressed during the cell growth phase, but was strongly enhanced when the cells reached confluence [13].

ALP is an enzyme in the blood, intestines, liver, and bone cells. Its chemical structure varies (called isoenzymes) depending on where it is produced which makes it possible to determine where a problem has originated. When bones are growing, when liver cells are damaged, or when biliary obstruction occurs, ALP levels rise considerably [14]. Yue et al. [14] studied the changes of hepatic enzymes after

alcohol intake (80 g ethanol containing beverage) and evaluated the corresponding damage to the human body. The levels of ALP and gamma-glutamyl transpeptidase (gamma-GT) were elevated when the alcoholic concentration reached 0.4 g/L [14].

Stein et al. [15] investigated the effect of LLLT (Helium-Neon [HeNe] laser; 632 nm; 10 mW power output) irradiation on proliferation and differentiation of a human osteoblast cell line. A two-fold enhancement of ALP activity and expression of osteopontin and bone sialoprotein (both osteogenic markers) were much higher in the irradiated cells as compared to nonirradiated osteoblasts. The study concluded that LLLT promotes proliferation and maturation of human osteoblasts in vitro, which may have clinical implications [15].

Khadra et al. [16] investigated the effect of LLLT (Gallium-Aluminium-Arsenide [GaAlAs] diode laser at dosages of 1.5 or 3 J/cm^2) on the attachment, proliferation, differentiation, and production of transforming growth factor-ss (1) (TGF-beta (1)) by human osteoblast-like cells (HOB). Osteocalcin synthesis and TGF-beta (1) production were significantly greater ($P < .05$) on the samples exposed to 3 J/cm^2. However, ALP activity did not differ significantly among the three groups. The results showed increased cellular attachment, proliferation, differentiation and production of TGF-beta (1) indicating that in vitro LLLT can modulate the activity of cells [16].

Guzzardella et al. [17] evaluated whether GaAlAs laser irradiation (780 nm low power laser for 10 consecutive days) can accelerate bone healing. Alkaline phosphatase/total protein (ALP/TP), calcium (Ca), and nitric oxide (NO) were tested on days 7, 14, and 21 to monitor the metabolism of cultured bone. The in vitro results suggested that GaAlAs treatment might play a positive role in bone defect healing [17]. Coombe et al. [18] investigated the effects of LLLT on the human osteosarcoma cell line, SAOS-2. The cells were irradiated as a single or daily dose for up to 10 days with a GaAlAs continuous wave diode laser (830 nm, 90 mW, energy levels of 0.3, 0.5, 1, 2, and 4 joules). No significant early or late effects of laser irradiation on protein expression and ALP activity were found. LLLT was unable to stimulate the osteosarcoma cells utilized for this research at a gross cell population level [18].

The ALP enzyme activity assay certainly has many benefits as changes in cell proliferation can be evaluated over a specific time course. The cell culture medium can be removed throughout the in vitro study without negatively affecting the cells growing in culture. Another benefit is that assay is fairly economical and is relatively simple and easy to perform. The ALP enzyme activity may also have useful applications in monitoring the effects of LLLT on cell proliferation in clinical applications such as wound healing. The ALP enzyme activity obtained from a simple blood sample over a specific time course may show changes in cell proliferation as wounds heal in response to phototherapy.

There are conflicting reports in the literature regarding the role of ALP. This study aimed to compare the cellular responses of wounded human skin fibroblasts exposed to doses of 0.5 J/cm^2, 2.5 J/cm^2, 5 J/cm^2, or 16 J/cm^2 using LLLT with an HeNe (632.8 nm) laser to elucidate the role of ALP in cell proliferation. This study concluded that ALP enzyme activity (in vitro) might be influenced by growth factors in the medium or the amount of cellular damage.

2. MATERIALS AND METHODS

2.1. Cell culture procedure

Human skin fibroblast monolayer cultures (ATCC CRL1502 WS1) were grown in Eagle's minimal essential medium (EMEM) with Earle's balanced salt solution that was supplemented according to previously described methods [19, 20]. Cells were trypsinized using a 0.25% (w/v) trypsin, 0.03% EDTA solution in Hanks balanced salt solution (HBSS), and approximately 6.5×10^5 cells (in 3 mL culture medium) were seeded in 3.4 cm diameter culture plates and incubated overnight to allow the cells to attach [21, 22].

2.2. Laser specifications and exposure regime

Once the fibroblasts had attached, 2 mL of culture medium was removed and a wound was induced before the cells were irradiated. For the simulated wound environment, confluent monolayers were scratched with a sterile pipette of 2 mm diameter and the plates were incubated at 37°C for 30 minutes before they were irradiated [23–25]. Irradiations were performed with an HeNe laser (632.8 nm, 18.8 mW power output, 2.07 mW/cm^2 power density, and 3.4 cm diameter spot size or 9.1 cm^2 area). Wounded unirradiated (0 J/cm^2) cells served as control cells while experimental cells were exposed to 0.5 J/cm^2, 2.5 J/cm^2, 5 J/cm^2, or 16 J/cm^2.

The duration of each exposure for the HeNe laser was calculated at 4 minutes and 15 seconds for 0.5 J/cm^2, 20 minutes and 77, seconds for 2.5 J/cm^2, 40 minutes and 15 seconds for 5 J/cm^2, and 128 minutes and 49 seconds for 16 J/cm^2. The wounded fibroblasts were exposed to combinations of one, two, or three exposures of the specific dose on one day or on two days. Cell culture dishes were positioned under the laser beam and irradiated at room temperature with the culture dish lid off on a dark surface. The cellular response measurements were made between 1 hour and 3 hours after the laser irradiation, to measure the immediate or direct effect of the laser irradiation [26].

2.3. Cellular responses

Following laser irradiation, the fibroblasts were trypsinized from the 3.4 cm culture dishes, and the cell suspension (1×10^5 cells/100 μL) was used to assess changes in cell proliferation (neutral red assay, cell density, and basic fibroblast growth factor (bFGF) expression). The culture medium was used to assess ALP enzyme activity and damage or additional stress caused by the irradiation (LDH membrane integrity).

ALP enzyme assay

Alkaline phosphatase (ALP) assay was performed according to previously published methods [3, 19, 27, 28]. Fifty μL of the culture medium was preincubated with 50 μL of 0.5 M N-methyl-D-glucamine buffer, pH 10.5, 0.5 mM magnesium acetate, 110 mM NaCl, and 0.22% Triton X-100 for 30 minutes at 37°C. Twenty mM *p*-nitrophenyl phosphate (*p*-NPP; Sigma N7653) was added and the reaction was incubated at 37°C for 30 minutes [2, 27, 28]. The amount of *p*-NPP liberated was measured at 405 nm on a Bio-Rad Benchmark Plus Microplate Spectrophotometer.

Neutral red assay

The proliferating activity after irradiation was determined by the neutral red assay (Sigma N2889) based on the ability of living cells to take up the neutral red dye from the medium and retain it in their lysosomes. 5×10^4 cells in complete EMEM were incubated with 10% neutral red (33 μg/mL) for 1 hour at 37°C, fixed with 1% formaldehyde for 30 minutes and solubilized with 1% acetic acid in 50% ethanol for 30 minutes. Absorbance was read at 550 nm [3, 19].

Optical density

Spectroscopy at 540 nm was used to measure cell proliferation or cell density of both irradiated and control samples [29]. The optical density of 5×10^4 cells in 100 μL complete EMEM was measured at 540 nm.

LDH membrane integrity

The CytoTox 96® nonradioactive cytotoxicity assay (Promega G1780) was performed according to previously published methods [19, 20, 30]. Fifty μL of the culture medium was mixed with an equal volume of reconstituted substrate mix. The plate was covered with foil and incubated at room temperature for 30 minutes. Fifty μL of stop solution (1 M acetic acid) was added and the absorbance read at 490 nm [30].

Basic fibroblast growth factor (bFGF)

The indirect enzyme linked immunosorbent assay (ELISA) assay was performed according to previously published methods [31, 32]. Briefly, 100 μl of culture medium was added to 100 μl carbonate-bicarbonate buffer (Sigma C3041) and incubated overnight at 4°C. The following day the coating solution was removed and 200 μl of diluted (5 μg/ml or 1 : 6 500) monoclonal antihuman bFGF (Sigma F6162) primary antibody was added and the plate incubated at room temperature for 2 hours. Each incubation step was followed with three washes of PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). 200 μl of anti-mouse IgG (Fab-specific) peroxidase-conjugated antibody (Santa Cruz sc-2005; 200 μg/0.5 ml) diluted (1 : 4 000) in PBS-T was added as the secondary antibody and incubated for 2 hours at room temperature while 100 μl of TMB substrate reagent (BD Biosciences #555214) was added for colorimetric detection. The orange-yellow color development was stopped after 30 minutes with 1 mol/L H_2SO_4 and the positive wells were read at 450 nm [32, 33].

Statistical analysis

Each experiment was repeated on different populations of normal and wounded fibroblast cells between passage 13–31. Each biological assay was performed in duplicate and the average of the two results was used to obtain a final sample number of $n = 4$. The results were recorded for statistical analysis using SigmaPlot 8.0, and the significant percentage change between the unirradiated control (0 J/cm^2) and the irradiated wounded cells was calculated and graphically represented. The one-way Student *t* test was used to analyze the difference firstly between the unirradiated control (0 J/cm^2) and the irradiated cells and secondly between the different data groups. Statistical significance was accepted at the 0.05 level (95% confidence interval). The ± values or error bars in the figures indicate standard error of the mean (SEM).

3. RESULTS

3.1. Single or double exposure on 1 day or on 2 days

ALP enzyme activity

The ALP enzyme activity was used to assess changes in cell proliferation of wounded fibroblasts following laser irradiation using doses of 0.5 J/cm^2, 5 J/cm^2, or 16 J/cm^2 (Figure 2(a)). Wounded cells exposed to one exposure of 16 J/cm^2 on 1 day showed an increase in the ALP enzyme activity when compared to the wounded unirradiated control and when compared to cells exposed to 0.5 J/cm^2 ($P = .046$). Wounded cells exposed to one exposure of 16 J/cm^2 on 2 days showed a decrease in the ALP enzyme activity when compared to cells exposed to 0.5 J/cm^2 ($P = .035$) and 5 J/cm^2 ($P = .082$). Wounded cells exposed to two exposures of 16 J/cm^2 on 1 day showed an increase in the ALP enzyme activity when compared to the wounded unirradiated control ($P = .045$) and when compared to cells exposed to 5 J/cm^2 ($P = .033$). Wounded cells exposed to two exposures of 16 J/cm^2 on 2 days showed an increase in the ALP enzyme activity when compared to the wounded unirradiated control ($P = .007$) and when compared to cells exposed to 5 J/cm^2 ($P = .0009$). Wounded cells exposed to two exposures of 0.5 J/cm^2 on 1 day and on 2 days showed an increase in the ALP enzyme activity when compared to the wounded cells exposed to two exposures of 5 J/cm^2.

Neutral red assay

The neutral red assay was used to assess proliferating activity after laser irradiation. Wounded cells exposed to one exposure of 0.5 J/cm^2 on 1 day showed an increase in neutral red when compared to the wounded unirradiated control and when compared to cells exposed to 5 J/cm^2 and 16 J/cm^2 (Figure 2(b)). Wounded cells exposed to one exposure of 5 J/cm^2 on 2 days showed an increase in neutral red

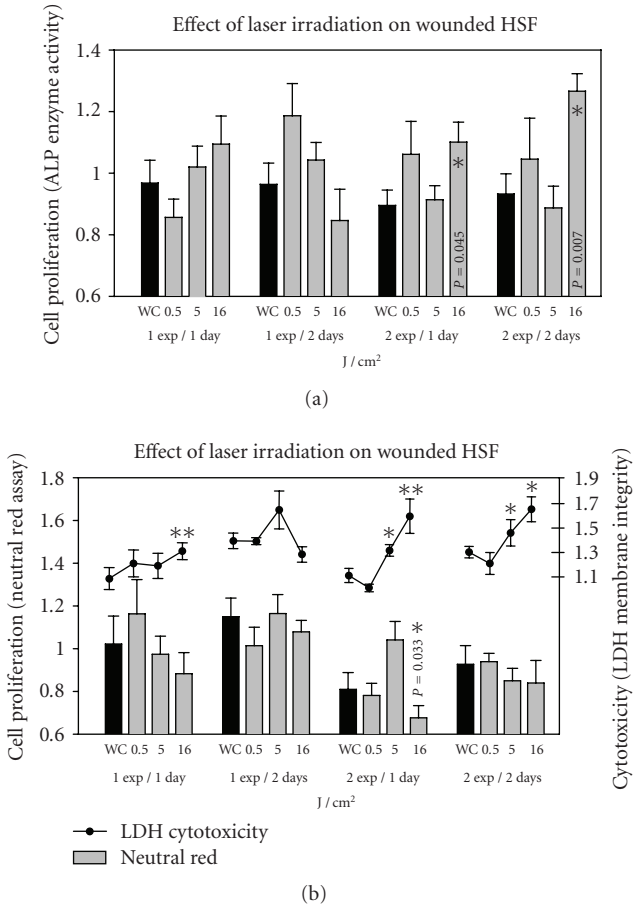

Figure 2: ALP enzyme activity assay was used to measure changes in cell proliferation or cell growth (a). Wounded fibroblasts exposed to two doses of 16 J/cm^2 on 1 day or on 2 days showed an increase in the ALP enzyme activity when compared to wounded unirradiated control cells (WC). The neutral red assay was used to assess proliferating activity (b), and the LDH membrane integrity assay was used to assess cytotoxicity. Wounded cells exposed to two exposures of 16 J/cm^2 on 1 day showed a decrease in cell proliferation and an increase in cytotoxicity, which corresponded to an increase in ALP activity ($n = 4$; $^*P \leq .05$, $^{**}P \leq .001$).

when compared to the wounded unirradiated control and when compared to cells exposed to 0.5 J/cm^2 and 16 J/cm^2. Wounded cells exposed to two exposures of 16 J/cm^2 on 1 day showed a decrease in neutral red when compared to the wounded unirradiated control ($P = .033$) and when compared to cells exposed to 5 J/cm^2 ($P = .0035$). Wounded cells exposed to two exposures of 5 J/cm^2 on 1 day showed an increase in neutral red when compared to the wounded unirradiated control and when compared to cells exposed to 0.5 J/cm^2 ($P = .025$).

Cytotoxicity

The LDH membrane integrity assay was used to assess changes in cytotoxicity following laser irradiation (Figure 2(b)). Wounded cells exposed to one exposure of 16 J/cm^2 on 1 day showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control ($P = .0006$). Wounded cells exposed to one exposure of 5 J/cm^2 on 2 days showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control and when compared to cells exposed to 16 J/cm^2 ($P = .064$). Wounded cells exposed to two exposures of 16 J/cm^2 on 1 day showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control ($P = .009$) and when compared to cells exposed to 0.5 J/cm^2 ($P = .001$) and 5 J/cm^2 ($P = .081$). Wounded cells exposed to two exposures of 5 J/cm^2 on 1 day showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control ($P = .056$) and when compared to cells exposed to 0.5 J/cm^2 ($P = .00008$). Wounded cells exposed to two exposures of 16 J/cm^2 on 2 days showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control ($P = .0001$) and when compared to cells exposed to 0.5 J/cm^2 ($P = .006$) and 5 J/cm^2. Wounded cells exposed to two exposures of 5 J/cm^2 on 2 days showed an increase in LDH cytotoxicity when compared to the wounded unirradiated control ($P = .002$) and when compared to cells exposed to 0.5 J/cm^2 ($P = .095$).

3.2. *Multiple exposures on two consecutive days*

ALP enzyme activity

Wounded fibroblasts showed an increase in ALP enzyme activity after one exposure of 5 J/cm^2 on two consecutive days, which may indicate a phenotypic response related to cell proliferation during wound healing. Wounded fibroblasts showed an increase in the ALP activity after two exposures of 16 J/cm^2 ($P = .006$) and three exposures of 16 J/cm^2 ($P = .001$) when compared to the unirradiated control while the single exposure showed a decrease indicating that multiple exposures of a high dose (16 J/cm^2) may cause additional stress or damage to the cell membrane of the wounded fibroblasts to cause an increase in the release of the ectoenzyme ALP anchored in the plasma membrane. The increase in the ALP activity may indicate that a high dose of 16 J/cm^2 results in the upregulation of ALP expression, which requires the cessation of proliferation [6]. One exposure of 2.5 J/cm^2, two exposures of either 2.5 J/cm^2 or 5 J/cm^2, and three exposures of either 2.5 J/cm^2 or 5 J/cm^2 had an ALP enzyme activity similar to the unirradiated control indicating that the dose and number of exposures did not have a biostimulatory or bioinhibitory effect on the proliferation of wounded fibroblasts (Figure 3(a)).

Basic fibroblast growth factor (bFGF)

bFGF is a potent mitogenic agent for fibroblasts and may play an important role in vivo in cell proliferation and differentiation associated with tissue regeneration and wound healing [31]. Wounded fibroblasts responded with an increase in the release of bFGF after two exposures of 2.5 J/cm^2 on two consecutive days ($P = .012$) while the release of bFGF was similar to the unirradiated control after one exposure, of 5 J/cm^2, two exposure of 5 J/cm^2, and three exposures of

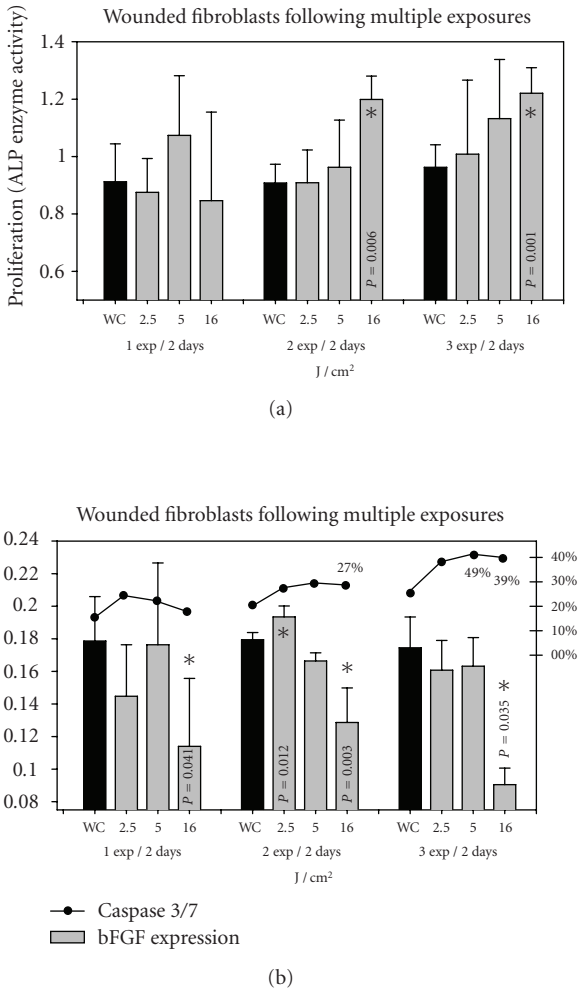

Figure 3: ALP enzyme activity assay was used to measure changes in cell proliferation or cell growth following the irradiation of wounded fibroblasts (a). The ELISA assay for bFGF was used to assess changes in the release of growth factors that regulate cell proliferation during wound repair (b). Wounded cells exposed to 16 J/cm^2 showed an increase in ALP enzyme activity but a decrease in the release of bFGF, which suggests that serum or growth factor deprivation induces ALP expression. ($n = 4$; $^*P \leq .05$).

either 2.5 J/cm^2 or 5 J/cm^2 indicating that the dose and number of exposures did not have a biostimulatory or bioinhibitory effect on the release of bFGF (Figure 3(b)). Wounded fibroblasts responded with a decrease in cell proliferation after one exposure of either 2.5 J/cm^2 or 16 J/cm^2 ($P = .041$), two exposures of 16 J/cm^2 ($P = .003$) and three exposures of 16 J/cm^2 ($P = .035$) indicating a bio-inhibitory effect dependent on the dose (5 J/cm^2 or 16 J/cm^2) and number of exposures. The results also suggest that a single dose of 2.5 J/cm^2 may be too low to stimulate cell proliferation while the cumulative effect of two exposures of 2.5 J/cm^2 is sufficient to exert a biostimulatory effect.

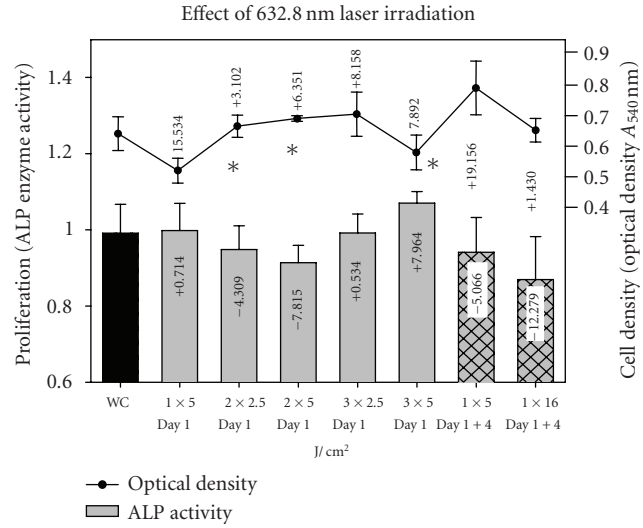

Figure 4: Changes in cell proliferation were determined using ALP enzyme activity and optical density. The ALP enzyme showed that 5 J/cm^2 on day 1 and three exposures of 5 J/cm^2 on day 1 had an increase in ALP activity, which indicates a decrease in cell growth or proliferation. These results correspond to the decrease in cell density at those doses. Literature indicates that tissue destruction, serum deprivation, growth factor deprivation, cell differentiation, and cessation of proliferation are responsible for the increase in ALP activity [6]. Results showed that wounded cells exposed to 5 J/cm^2 on day 1 and day 4 had a decrease in ALP activity but an increase in cell density indicating a stimulatory effect that promotes cell growth or proliferation ($n = 4$; $^*P \leq .05$).

3.3. Multiple exposures on day 1 or on day 1 and 4

ALP enzyme activity

Wounded cells exposed to 5 J/cm^2 on day 1 showed an ALP activity equivalent to the wounded unirradiated control (Figure 4). Wounded cells exposed to two doses of 5 J/cm^2 showed a decrease in ALP activity when compared to the unirradiated control and when compared to three doses of 2.5 J/cm^2 on day 1 indicating an increase in cell growth or proliferation. Wounded cells exposed to three doses of 5 J/cm^2 showed an increase in the ALP activity when compared to the wounded unirradiated control. Wounded cells exposed to two doses of 5 J/cm^2 on day 1, wounded cells exposed to 5 J/cm^2 on day 1 and day 4, and wounded cells exposed to 16 J/cm^2 on day 1 and day 4 show a decrease in ALP activity when compared to the unirradiated control indicating cell growth and proliferation at a higher rate than the control.

The increase in ALP enzyme activity following three exposures of 2.5 J/cm^2 on day 1 or three exposures of 5 J/cm^2 on day 1 corresponds with an increase in caspase 3/7 activity (early marker of apoptosis), decrease in viability, decrease in bFGF, and a decrease in cell density indicating that multiple doses may cause damage, induce early apoptosis, reduce viability, and reduce cell growth or proliferation. An increase in ALP activity may be associated with serum or growth factor

deprivation (bFGF), tissue destruction, reduced cell growth, or cessation of proliferation. Normal or decreased ALP activity may indicate that the cell function has normalized and that the cells are fully functional with sufficient nutrients, serum and growth factors to support cell proliferation or cell growth.

Optical density

Wounded cells exposed to 5 J/cm^2 on day 1 and day 4 showed an increase in cell density when compared to wounded cells exposed to 5 J/cm^2 on day 1 ($P = .012$) clearly indicating the benefit irradiating with a single dose on two nonconsecutive days. Wounded cells exposed to three doses of 5 J/cm^2 on day 1 showed a decrease in cell density when compared to the unirradiated control ($P = .045$) and when compared to wounded cells exposed to two doses of 5 J/cm^2 on day 1 ($P = .044$) indicating that the cumulative dose administered determines whether the total cellular response will be stimulatory or inhibitory. Wounded cells exposed to a single dose of 5 J/cm^2 on day 1 showed a decrease in the cell density, which corresponds to the increase in ALP enzyme activity, which indicates a decrease in cell growth or proliferation. Wounded cells exposed to two exposures of 2.5 J/cm^2 on day 1 and wounded cells exposed to two exposures of 5 J/cm^2 on day 1 showed an increase in cell density which corresponded to a decrease in ALP enzyme activity and an increase in bFGF concentration (Figure 4).

Basic fibroblast growth factor (bFGF)

Wounded cells exposed to two exposures of 5 J/cm^2 on day 1 showed an increase in the release of bFGF when compared to the wounded unirradiated control. Wounded cells exposed to two exposures of 5 J/cm^2 showed an increase in the release of bFGF when compared to three exposures of 2.5 J/cm^2. Wounded cells exposed to three dose of 5 J/cm^2 on day 1 showed a decrease in the release of bFGF when compared to three exposures of 2.5 J/cm^2 on day 1 ($P = .049$). Wounded cells exposed to 5 J/cm^2 on day 1 showed an increase in the release of bFGF when compared to three exposures of 5 J/cm^2 on day 1 ($P = .002$) indicating that the cumulative dose may have an inhibitory effect that decreases the release of growth factor and slows down cell proliferation. Wounded cells exposed to 5 J/cm^2 on day 1 and day 4 showed an increase in the release of bFGF compared to a decrease after 16 J/cm^2 indicating that the laser effect is dependent on the dose (Figure 5).

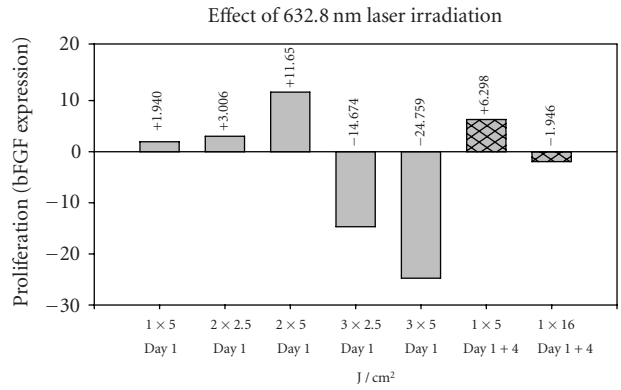

Figure 5: bFGF ELISA was used to determine any changes in the release of the growth factor that regulates cell proliferation during wound repair. Wounded cells exposed to three exposures of 2.5 J/cm^2 or 5 J/cm^2 on day 1 showed a decrease in the release of bFGF when compared to the unirradiated control. Wounded cells exposed to 5 J/cm^2 on day 1 and day 4 and wounded cells exposed to two exposures of 5 J/cm^2 on day 1 showed an increase in the release of bFGF indicating a stimulatory effect that may accelerate cell proliferation and improve the rate of wound healing ($n = 4$; $^*P \leq 0.05$).

4. DISCUSSION

Literature indicates that tissue destruction, serum deprivation, growth factor deprivation, cell differentiation, and cessation of proliferation are responsible for the increase in ALP activity. Results from the single exposure of 16 J/cm^2 on 1 day showed that wounded cells respond with an increase in ALP enzyme activity, decrease in cell proliferation, and increase in cytotoxicity indicating that the increase in ALP activity may be related to changes in cell membrane integrity (Figure 2). Since LDH is a stable cytosolic enzyme that is released upon cell lysis, it is possible that ALP, an ectoenzyme anchored to the plasma membrane, may also be released on cell lysis or membrane damage [19, 34, 35]. Wounded cells exposed to one exposure of 0.5 J/cm^2 on 1 day showed a decrease in ALP activity that correlates to an increase in cell proliferation and increase in cytotoxicity when compared to the unirradiated control cells. Wounded cells exposed to one exposure of 0.5 J/cm^2 or 5 J/cm^2 on 1 day showed a decrease in ALP activity, increase in cell proliferation, and decrease in cytotoxicity when compared to cells exposed to 16 J/cm^2 indicating that the ALP activity is influenced by both membrane integrity and cell proliferation.

Wounded cells exposed to one exposure of 0.5 J/cm^2 on 2 days showed an increase in ALP activity that correlates to a decrease in cell proliferation. The results indicate that the ALP activity is negatively correlated to cell growth, and the up-regulation of ALP requires the cessation of proliferation. Wounded cells exposed to one exposure of 5 J/cm^2 on 2 days showed an increase in ALP activity, increase in cell proliferation, and increase in cytotoxicity when compared to the wounded unirradiated control. Wounded cells exposed to one exposure of 16 J/cm^2 on 2 days showed a decrease in ALP activity, decrease in cell proliferation, and decrease in LDH cytotoxicity supporting evidence that ALP activity is influenced by both membrane integrity and cell proliferation. The results indicate that the level of cytotoxicity has a direct effect on cell proliferation. The results support finding by Hawkins and Abrahamse [34, 35] and Abe et al. [3] which demonstrate that ALP is released during cell proliferation.

Wounded cells exposed to two exposures of 0.5 J/cm^2 on 1 day showed an increase in ALP activity, decrease in cell proliferation, and decrease in LDH cytotoxicity while cells

exposed to two exposures of 5 J/cm^2 on 1 day showed an increase in ALP activity, increase in cell proliferation, and increase in cytotoxicity when compared to the wounded unirradiated control. Wounded cells exposed to two exposures of 16 J/cm^2 on 1 day showed an increase in ALP enzyme activity, decrease in cell proliferation, and increase in cytotoxicity supporting evidence that an increase in ALP activity may be associated with cell membrane damage.

Wounded cells exposed to two exposures of 0.5 J/cm^2 on 2 days showed an increase in ALP activity, increase in cell proliferation, and decrease in LDH cytotoxicity indicating a positive correlation between ALP activity and cell proliferation. Wounded cells exposed to two exposures of 16 J/cm^2 on 2 days showed an increase in ALP activity, decrease in cell proliferation, and increase in LDH cytotoxicity indicating that cell proliferation may be influenced by the degree of damage inflicted on the cells. Wounded cells exposed to two exposures of 5 J/cm^2 on 2 days showed a decrease in ALP activity and decrease in cell proliferation indicating a positive correlation between ALP activity and cell proliferation.

The result for a single exposure of 2.5 J/cm^2, 5 J/cm^2, and 16 J/cm^2 (Figure 3) indicates that ALP is released during proliferation [34, 35] and supports results from Abe et al. [3]. The results indicate that the ALP enzyme activity is directly proportional to the release of bFGF and cell proliferation (neutral red) since all three of the parameters showed the same response: a decrease with a single exposure of 2.5 J/cm^2, an increase with a single exposure of 5 J/cm^2, and a decrease with a single exposure of 16 J/cm^2. However, the results for two or three exposures of 2.5 J/cm^2, 5 J/cm^2, and 16 J/cm^2 indicate a different or contradictory response. The results showed that wounded cells exposed to two or three exposures of 16 J/cm^2 have an increase in the ALP enzyme activity that corresponded to a decrease in the release of bFGF and decrease in cell proliferation (neutral red). Caspase 3/7 activity (early marker of apoptosis) [33] showed a 27% and 39% increase after wounded cells were exposed to two or three exposures of 16 J/cm^2, respectively (Figure 3(b)). The results suggest that two or three exposures of a high dose (16 J/cm^2) may cause additional stress or damage to the cell membrane of the wounded fibroblasts. Wounded cells exposed to three exposures of 5 J/cm^2 showed an increase in ALP enzyme activity, decrease in cell proliferation (neutral red), and 42% increase in caspase 3/7 activity indicating that the degree of cytotoxicity is dependent on both the dose and number of exposures (Figure 3(b)).

Wounded cells exposed to two or three exposures of 16 J/cm^2 have an increase in the ALP enzyme activity and a decrease in the release of bFGF. The results support Abe et al. [6] which suggest that serum or growth factor deprivation induces ALP expression, which is inversely correlated with cell growth [6, 11]. Literature reports that ALP activity is suppressed during the cell growth phase, but is strongly enhanced when cells have reached confluence [13].

The results showed that wounded cells exposed to one exposure of 5 J/cm^2, two exposures of 2.5 J/cm^2, and three exposures of 2.5 J/cm^2 had an increase in ALP enzyme activity, an increase in the release of bFGF, an increase in cell proliferation (neutral red) and, a decrease in caspase 3/7 activity when compared to the other doses. The results indicate that ALP enzyme activity has a positive correlation to cell proliferation and growth factor concentration supporting results from Abe et al. [3]. Results indicate that ALP is influenced by cell proliferation and cell membrane damage. It is difficult to determine which portion of the ALP released is influenced by cell proliferation or growth factor concentration, therefore ALP should not be used independently as a marker of cell proliferation, instead ALP should be used in conjunction with other markers such as neutral red, caspase 3/7, and bFGF concentration to determine the correct effect of phototherapy.

Wounded cells exposed to a single dose of 5 J/cm^2 showed a decrease in ALP enzyme activity, an increase in bFGF concentration, and an increase in optical density when compared to wounded unirradiated control (Figures 4 and 5). Wounded cells exposed to 16 J/cm^2 on day 1 and 4 showed a decrease in ALP enzyme activity, decrease in release of bFGF, and decrease in optical density when compared to wounded cells exposed to a single dose of 5 J/cm^2 on day 1 and day 4. The cytotoxicity for wounded cells exposed to a single dose of 5 J/cm^2 is equivalent to the cytotoxicity for wounded cells exposed to 16 J/cm^2 on day 1 and day 4 indicating that ALP did not accidentally leak out of a damaged cell membrane. The results indicate that a dose of 16 J/cm^2 on day 1 and 4 results in an inhibitory effect (decrease in ALP activity and cell growth) which distinguishes the response from 5 J/cm^2 and demonstrates that higher doses may inhibit cellular responses such as RNA and DNA syntheses, cell mitosis, protein secretion, and cell proliferation.

Wounded cells exposed to two doses of 2.5 J/cm^2 on day 1 showed a decrease in ALP enzyme activity, increase in bFGF, increase in optical density, and decrease in cytotoxicity (LDH membrane integrity) when compared to wounded cells exposed to 5 J/cm^2 on 1 day. Wounded cells exposed to three doses of 5 J/cm^2 on day 1 showed an increase in ALP enzyme activity, a decrease in bFGF, a decrease in optical density, and an increase in cytotoxicity (LDH membrane integrity). Wounded cells exposed to two doses of 5 J/cm^2 on day 1 showed a decrease in ALP enzyme activity, an increase in bFGF, and an increase in optical density but with an increase in cytotoxicity (LDH membrane integrity). The results support Abe et al. [6] which suggest a negative correlation between ALP expression and cell growth. The upregulation of ALP requires the cessation of proliferation [6] and the progression of granulation tissue formation and remodelling, which serves as a marker of successful wound healing. The expression of ALP is upregulated by DNA synthesis inhibition, and is inversely related with proliferation [4].

Wounded cells exposed to three exposures of 2.5 J/cm^2 on day 1 showed an increase in ALP enzyme activity, decrease in bFGF, and decrease in cytotoxicity (LDH membrane integrity) when compared to wounded cells exposed to two exposures of 5 J/cm^2. Wounded cells exposed to three doses of 5 J/cm^2 on day 1 showed an increase in ALP enzyme activity, a decrease in bFGF, and a decrease in optical density. The results support Abe et al. [6] which suggest that serum

or growth factor deprivation induces ALP expression, which is inversely correlated with cell growth. Alpaslan et al. [27] showed an exclusive localization of extracellular ALP activity in the regions of granulation tissue formation. This localization was time related and decreased as healing progressed. Extracellular ALP activity can be used as a simple and reliable histochemical process marker of skin wound healing [27].

5. CONCLUSION

Some reports state that ALP is increased in cell proliferation and wound healing, while other studies indicate that ALP is negatively correlated with cell growth. The results in this study support the latter suggesting that an increase in ALP is negatively correlated with cell growth and is influenced by serum or growth factors in the medium and cytotoxicity. Results from this study indicate that ALP may be related to cellular damage, and the enzyme may be released upon cell lysis. The ALP enzyme activity assay is a useful laboratory assay to measure the responses of cells in culture. However, more research is required to determine the exact role of ALP in cell proliferation since this study indicates that cytotoxicity or cellular damage can influence the level of ALP enzyme activity. Since the exact role of ALP is unknown, the ALP enzyme activity assay should be considered in conjunction with other cell proliferation assays such as neutral red, optical density, or more specifically bFGF.

Results suggest that an increase in ALP may indicate successful wound healing with the cessation of cell proliferation and the progression of granulation tissue formation and remodelling, supporting literature that claims that ALP is released in inflammation, remodelling, and cell proliferation and has been used as a marker for wound healing. Results indicate that ALP can be negatively and positively correlated with cell growth depending on the stage of wound healing and growth factor in the medium. Further studies using 5-Bromo-2′-deoxyuridine (BrdU) antibodies for DNA synthesis will confirm if ALP expression is negatively or positively correlated with cell growth and if ALP is upregulated by DNA synthesis or DNA synthesis inhibition. Further research is required, utilizing different cell models, to more specifically determine the effects of LLLT irradiation and the role of ALP in cell proliferation.

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