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Mitochondrial Responses of Normal and Injured Human Skin Fibroblasts Following Low Level Laser Irradiation—An *In Vitro* Study

Innocent L. Zungu, Denise Hawkins Evans and Heidi Abrahamse*

Laser Research Group, Faculty of Health Sciences, University of Johannesburg, Johannesburg, South Africa

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ABSTRACT

Laser irradiation has proved to be very efficient in speeding and improving the quality of healing in pathological conditions of diverse etiologies. However, the mechanisms by which the beneficial effects are attained are not clear. Mitochondria are the primary phototargets during irradiation. The study aimed to establish if laser irradiation had an effect on hypoxic and acidotic cells. The study also aimed to use existing information regarding the possible mechanism of action (established in wounded cells) and apply these principles to acidic and hypoxic irradiated cells to determine whether laser has a stimulatory or inhibitory effect. Cell cultures were modified to simulate conditions of hypoxia (hypoxic gas mixture 95% N_2 and 5% O_2) and acidosis (pH 6.7) whereas the central scratch model was used to simulate a wound. Cells were irradiated with a helium–neon (632.8 nm, 3 mW cm^{-2}) laser using 5 or 16 J cm^{-2} on days 1 and 4. Mitochondrial responses were measured 1 or 24 h after laser irradiation by assessing changes in mitochondrial membrane potential (MMP), cyclic AMP, intracellular Ca^{2+} and adenosine triphosphate (ATP) cell viability. Hypoxia and acidosis significantly reduced MMP when compared with normal nonirradiated control cells. Wounded, hypoxic and acidotic cells irradiated with 5 J cm^{-2} showed an increase in mitochondrial responses when compared with nonirradiated cells while 16 J cm^{-2} showed a significant decrease. The study confirmed that laser irradiation with 5 J cm^{-2} stimulated an increase in intracellular Ca^{2+} which resulted in an increase in MMP, ATP and cAMP, which ultimately results in photobiomodulation to restore homeostasis of injured cells.

INTRODUCTION

Low level laser therapy (LLLT) also known as phototherapy aims to biostimulate and because of its low power nature, the effects are biochemical and not thermal; as such they cannot cause damage to living tissues at the cellular level (1). At low laser fluences of 1–6 J cm^{-2}, cytoplasmic Ca^{2+} levels are increased and mitosis is triggered to enhance cell proliferation whereas at higher fluences of 10–16 J cm^{-2}, the adenosine triphosphate (ATP) cell energy is exhausted due to hyperactivity of Ca^{2+}-ATPase, altering the intracellular pH and osmotic pressure which may cause the cell to explode (2–5).

Mitochondria are sites of oxidative phases of cell respiration. They form chemical reactions which generate ATP, an energy-rich molecule that drives processes responsible for cell growth or proliferation (6–8). LLLT increases ATP production in the mitochondria of the cell. With more energy available, the cell may utilize this fuel to operate more efficiently (9).

It is suggested that the cytochromes and the porphyrins, which are the respiratory chain components in the mitochondria, are the primary photoacceptors in the visible wavelength range (10). When the energy (photon) is absorbed by the cellular membrane photon receptors, a cascade of cellular effects occurs that results in the development of reactive oxygen species (ROS), ATP synthesis, cell membrane permeability changes and nitric oxide release. These effects lead to increased cell proliferation, changes in extracellular matrix synthesis, and local effects on components of the immune, vascular and nervous system. There is an alteration in intracellular pH, which is related to the activation of ATPases. A change in redox state toward oxidation leads to an increase in intracellular Ca^{2+} and stimulation of cell metabolism. High levels of intracellular Ca^{2+} stimulate various biological processes such as RNA and DNA synthesis, cell mitosis and protein secretion. It has been shown that uptake of Ca^{2+} into mammalian cells can be induced by monochromatic red light depending on the dose applied (11). Most of the cellular responses to LLLT are changes by the mitochondrial and membrane activities including mitochondrial membrane potential (MMP) (12). Despite the positive outcome and advocacy of this treatment, there has been a reluctance to accept it due to lack of an understanding of the underlying mechanisms of action (13).

Despite a large amount of research having been undertaken in laser therapy, there are conflicting reports concerning LLLT as a therapeutic modality (14), forcing the therapy to remain unestablished (14–16). Karu states that the magnitude of laser biostimulation depends on the physiological state of the cell at the moment of irradiation (16). This suggests that laser therapy works with a measurable effect in cases of stressed cells and an insignificant effect on normal fully functional regenerating cells.

Injuries can be induced by many ways including hypoxia (oxygen deficiency) and ischemia (blood flow deficiency), extracellular acidosis, chemical agents (drugs and alcohol), physical agents (skin incisions, other traumas and heat),

*Corresponding author email: habrahamse@uj.ac.za (Heidi Abrahamse)

infections, genetic defects (hemoglobin S in sickle cell disease), nutritional defects (vitamin deficiencies and obesity leading to Type II diabetes mellitus) and aging (degeneration and intrinsic cellular senescence) (17–19).

The literature reports that injury reduces MMP which leads to reduced oxidative phosphorylation. When oxidation rate is reduced, ATP production lowers and an alternative source, the glycolytic pathway, is induced. With glycolysis comes accumulation of lactic acid which lowers both extracellular and intracellular pH. Ca^{2+} gets elevated, Na^{+} gets elevated, protein synthesis is significantly reduced, there is nuclear chromatin clumping and increased H_2O leading to cell swelling and cell death (5,12,15,20). When calcium level is highly elevated, it can lead to cell death (21).

However, when cells are injured, the first response is a buildup of repair mechanisms. When the damage is severe and irreparable, the cells are programmed for death by either apoptosis or necrosis as a way of achieving stasis. The stronger and the longer the stimulus, the larger the damage. Response to a given stimulus (injury) depends on the type, status and genetic makeup of the injured cell: skeletal muscles tolerate ischemic conditions for up to 2 h whereas cardiac muscles take up to 20 min (22,23). There are many other underlying conditions which contribute to delayed wound healing (24).

It has been shown that laser irradiation triggers biological changes in a dose-dependent manner (12,25,26). At optimal (stimulatory) dose, laser irradiation causes mitochondrial polarization which improves membrane permeability leading to increased Ca^{2+} flux, pH value, cyclic adenosine monophosphate (cAMP) level, ATP production, anti-apoptotic factors, transcription factors and DNA synthesis. This cascade of cellular events accelerates cell proliferation which promotes healing. When the dose is above the optimum value (inhibitory), cells are further stressed and often results in highly increased calcium levels, depolarized MMP, decreased cAMP level and decreased ATP production. When a low ATP production persists, DNA synthesis reduces significantly, and pro-apoptotic factors increase heralding cell death. In all the different transduction pathways of the cell, mitochondria play a central role in deciding the survival of the cell (5,12,15,25,27).

The focus of this study was to investigate mitochondrial response to phototherapy on normal, wounded, hypoxic and acidotic injuries to human skin fibroblast (HSF) cells to determine whether mitochondria are stimulated by LLLT and gain more insight into how LLLT would benefit the healing process of such injuries.

The central scratch method has been used in many studies to determine the effect of laser irradiation on wounded cells; however, the central scratch method wounds approximately 5–10% of the cultured monolayer cells whereas the periphery of the culture plate contains unwounded normal cells. For this reason the hypoxic and acidotic models were selected to induce homogenous subcytotoxic cell stress so that the effect of laser irradiation could be observed on the entire population of cells.

Cell injuries were performed according to Zungu *et al.* (19) while He–Ne laser irradiation at 632.8 nm was performed twice at 5 or 16 J cm^{-2}. Changes were recorded 1 or 24 h postirradiation in terms of cell morphology, MMP, intracellular calcium (Ca^{2+})i concentration, cAMP, cell viability (ATP), proliferation (XTT) and cell damage (LDH).

MATERIALS AND METHODS

Cell culture. A commercially available HSF cell line (WS1, CRL-1502) was obtained from the American Type Culture Collection. Cells were grown in 75 cm^2 flasks containing minimum essential medium (MEM) (32360-026; Invitrogen, CA) with Earle's balanced salt solution (BSS) modified to contain 2 mM L-glutamine (2530-024; Invitrogen), 1.0 mM sodium pyruvate (11360-070; Invitrogen), 0.1 mM nonessential amino acids (11140-035; Invitrogen), 1% pen-strep fungizone (P06-07100; PAN Biotech GmbH) and 10% fetal bovine serum (10108-157; Invitrogen).

WS1 cells were subcultured twice weekly when 90% confluent, according to standard culture techniques. These adherent cells were removed by trypsinization (1 mL/25 cm) using 0.25% trypsin, 0.03% ethylenediaminetetra-acetic acid in Hanks' balanced salt solution (HBSS) (14170; Invitrogen). Approximately 6.5×10^5 cells (in 3 mL culture medium containing phenol red) were seeded in 3.4 cm diameter tissue culture dishes and incubated overnight at 37°C in 5% CO_2 and 85% humidity to allow the cells to attach.

The cells were injured or stressed on day 1 and incubated for 30 min prior to irradiation. Cellular responses were measured on day 4 (1 h postirradiation) or day 5 (24 h postirradiation) (19,28). The nonirradiated control plates were incubated at 37°C with 5% CO_2 and 85% humidity until the cellular responses were measured on day 4 or day 5 whereas the remaining plates were irradiated on day 1 and day 4 and the cellular responses were either measured on day 4 or on day 5.

Central scratch. A wound was introduced in a cell monolayer according to Rigau *et al.* using a pipette tip to create a cell-free zone (29). For the simulated wound environment, 1 mL of culture medium was removed and the confluent monolayer was scratched with a sterile pipette of 2 mm diameter (3,29,30). Each plate was observed under the microscope to ensure that a reproducible wound of equal length, depth and width was created.

Acidosis. The cell culture medium was adjusted by adding 1 *N* HCl acid (Associated Chemical Enterprises, South Africa) until the pH was reduced to 6.7 (31). Briefly, 20 μL of HCl was added to 20 mL of complete MEM (pH 7.4) to obtain a pH of 6.7. The pH was measured using a Thermo Orion pH meter (model 410 A^{+}; LABOTEC). After overnight incubation to allow the cells to attach, the complete MEM was discarded and was replaced with 3 mL of adjusted acidic media (pH 6.7).

Hypoxia. *In vitro* hypoxia was induced by incubating the 3.4 cm tissue culture dishes in an anaerobic jar (AnaeroPackTM System; Mitsubishi Gas Chemical Co. Inc., Japan). A methylene blue anaerobiosis indicator was used to monitor oxygen depletion. After overnight incubation to allow the cells to attach, cells were incubated in an anaerobic chamber for 4 h and 15 min (as one anaerobic gas pack achieves anaerobiosis in 15 min). An incubation of 4 h was selected as further incubation in the anaerobic chamber results in irreversible damage to the cells (23).

Laser parameters. A helium–neon (Spectra Physics Model 127) with an average power density of 3 mW cm^{-2} and wavelength of 632.8 nm was used to administer a dose of 5 or 16 J cm^{-2} (Table 1).

Biological response. *Morphology*: Morphology was assessed by observing changes in cell orientation (haptotaxis), migration and

Table 1. Laser parameters used to determine the effects of laser irradiation on injured human skin fibroblast cells.

Variable	Parameter
Cell type	Normal and injured
Laser and wavelength	Helium–neon (He–Ne), 632.8 nm
Power density	3 mW cm^{-2}
Wave emission	Continuous (CW)
Dose administered	5 or 16 J cm^{-2}
Average duration	45 min (5 J cm^{-2}) or 150 min (16 J cm^{-2})
Spot size	9.08 cm^2
Repair time	1 and 24 h postirradiation
Number of exposures	2 (days 1 and 4)
Temperature	22 ± 1°C
Irradiation condition	In the dark, dish lid off, against a black surface

wound closure using an inverted light microscope (CKX41; Olympus, PA). Changes in cell morphology were recorded using a digital camera (C-3030 zoom; Olympus Camedia).

Mitochondrial membrane potential: Changes in MMP were assessed using a MitoScreen Kit (5,5′6,6′–tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide, JC-1) (551302; Becton Dickinson and Company, CA) by flow cytometry (BD Facs Calibur) to determine the mitochondrial status of injured cells after laser irradiation. Cells were harvested by trypsinization and a 1 mL cell suspension (1×10^6 cells mL^{-1}) was centrifuged at 400 ***g*** for 5 min at room temperature. The supernatant was removed and discarded. Then 500 μL of freshly made JC-1 working (1:10 dilution) solution was added to each tube and gently resuspended to prevent cells from clumping. Cells were incubated for 15 min at 37°C in a CO_2 incubator and then washed with 2 mL 1× assay buffer and then with 1 mL of assay buffer after centrifugation. The cell pellet was gently resuspended in 500 μL of 1× assay buffer. The samples were then analyzed on a flow cytometer with 488 and 633 nm excitation using forward and side scatter dot plot. Forward scatter detects the size of the cell whereas side scatter detects cell complexities. A two-parameter histogram dot plot is produced where FL1 represents apoptotic cells on the *X*-axis and FL2 represents late apoptotic cells on the *Y*-axis. Fluorescence in both FL-2 and FL-1 indicates cellular polarization. The number of cells represented in each quadrant is expressed as a percentage.

cAMP: Cyclic AMP has been shown to be involved in cell growth and differentiation as well as general metabolism. The determination of cAMP was performed according to manufacturer's protocol (900-067; Assay Designs). Briefly, using unacetylated version of the assay, 100 μL of standards and samples were pipetted into the labeled wells on a 96-well microtiter plate. Cells grown in tissue culture media (1×10^5 cells mL^{-1}) were treated with 0.1 M HCl after first removing the media. The cells were vortexed and then incubated for 10 min at −80°C. If adequate lysis had not occurred, the cells were incubated for a further 10 min. The cells were centrifuged at ≥600 ***g*** at room temperature and the supernatant was used to perform the assay. Fifty microliters of mouse monoclonal antibody to cAMP and 50 μL of cAMP conjugated to alkaline phosphatase were added and the mixture was incubated at room temperature for 2 h on a shaker at 500 rpm with the plate covered and sealed. The plate was then washed three times by adding 400 μL of Tris-buffered saline and finally tapped on a lint-free paper towel to remove any remaining wash solution. Two hundred microliters of the para-nitrophenylphosphate (p-Npp) substrate solution was pipetted to all the wells and incubated at room temperature for 1 h without shaking. After this incubation, 50 μL of trisodium phosphate was added to stop the reaction and the plate was read at optical density 405 nm and background absorbance corrected at 580 nm on a Victor^3 multilabel counter (PerkinElmer).

Intracellular calcium (Ca^{2+})i: Further assessment of mitochondrial activity was made by measuring changes in the level of intracellular Ca^{2+} using QuantiChrom™ Calcium Assay Kit (DICA-500; Bio Assay Systems, CA). Cells were washed twice with PBS (pH 7.4, calcium and magnesium free) and scraped using a cell scraper (S.A. 167008; AEC-Amersham). Cells were then centrifuged at 2000 ***g*** for 5 min and the pellet was resuspended at 1×10^6 cells mL^{-1} in distilled water. Cells were homogenously mixed and sonicated for complete lysis. The lysate was centrifuged at 10 000 ***g*** for 15 min at 4°C. Fifty microliters of the supernatant was transferred into wells of a clear flat bottom 96-well plate and 200 μL of working reagent composed of equal volumes of reagent A (composed of *o*-phthalic acid, polyvinyl pyrrolidone, methylthymol blue and 8-hydroxyquinoline) and reagent B (comprising sodium sulfite and monoethanolamine) was added to each well and the plate tapped lightly to mix. The plate was incubated for 3 min at room temperature and the optical density was read at 612 nm. The absorbance values obtained from an eight-point standard curve were used to calculate calcium concentration (μM). Ca^{2+} was measured in mg mL^{-1} and was converted to μM using the following factor: 1 mg = 250 μM.

ATP luminescence: ATP luminescence was determined with the Cell Titre-Glo luminescent assay (G7571; Promega, WI). Quantification of ATP determines the number of viable cells which reflects the level of metabolically active cells. Equal volumes (50 μL) of cells and reconstituted reagent were mixed for 2 min on an orbital shaker, to induce lysis. The mixture was then incubated at room temperature for 10 min to generate a stable luminescent signal, which was proportional to the amount of ATP present. Luminescence or the amount of ATP (calculated from a standard curve) was measured in reading light units (RLUs) on the Victor^3 multilabel counter (PerkinElmer).

LDH cytotoxicity: The cytotoxicity assay was based on the fact that the membrane of injured cells becomes permeable and cellular contents are released into the surrounding environment. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cellular membrane damage or lysis. LDH released into the culture media was measured using the Cyto-Tox 96 nonradioactive cytotoxicity assay (G1780; Promega). Maximum LDH values were obtained from samples incubated at −80°C; this value was then used to determine the percentage cytotoxicity. An equal volume of culture media and substrate (50 μL) was incubated for 30 min in the dark at room temperature. Stop solution was added (100 μL), absorbance read at 490 nm on a Victor^3 multilabel counter (PerkinElmer) and percentage cytotoxicity determined. Background absorbance from phenol red in the culture media was corrected by subtracting the culture medium background from the results.

Statistical analysis. Each experiment was repeated on different populations of fibroblast cells between passages 13 and 33. The experiments were repeated six times ($n = 6$), and the assays were performed in duplicate. The results were recorded and graphically represented for statistical analysis with Sigma Plot version 8.0. The results were considered to be significant if $P \leq 0.05$ by the Student *t*-test and one-way analysis of variance (ANOVA).

RESULTS

The cellular responses reflect the status of the mitochondria. These changes reflect the responses of stressed cells using the different *in vitro* models of cell stress and indicate how stressed cells respond differently when compared with normal fully functional cells. These changes indicate differences between irradiated and nonirradiated cells.

Effect of laser on cell morphology

Normal cells looked elongated and slender shaped and reached confluence by day 5 of the experimental period (Fig. 1A,B). Irradiation with 16 J cm^{-2} showed reduced cell number as cells were not confluent by day 5 (Fig. 1C). Wounded cells demonstrated a clear wound margin on both sides of the central scratch. After 24 h, cells changed direction and started growing toward the central scratch (haptotaxis) probably in response to the wound in an attempt to close the cell-free zone (chemotaxis). Wounded nonirradiated cells had less cells in the central scratch area and the wound area was not completely covered by day 5 (Fig. 1D). The wounded cells irradiated with 5 J cm^{-2} migrated across the central scratch with more cells in the cell-free zone and the wound area was covered by day 5 (Fig. 1E). Wounded cells irradiated with 16 J cm^{-2} did not cover the wound area in the same period of time (Fig. 1F).

There was no difference in the cell structure of hypoxic fibroblast cells irradiated with 5 J cm^{-2} when compared with nonirradiated hypoxic and normal fibroblast cells (Fig. 1A, G,H), however many of the nonviable cells had detached from the surface of the cell culture plate. The nonirradiated plates showed nonviable cells still attached to the surface of the cell culture plate (Fig. 1G). Nonviable cells had completely detached from culture plates in irradiated (5 J cm^{-2}) samples

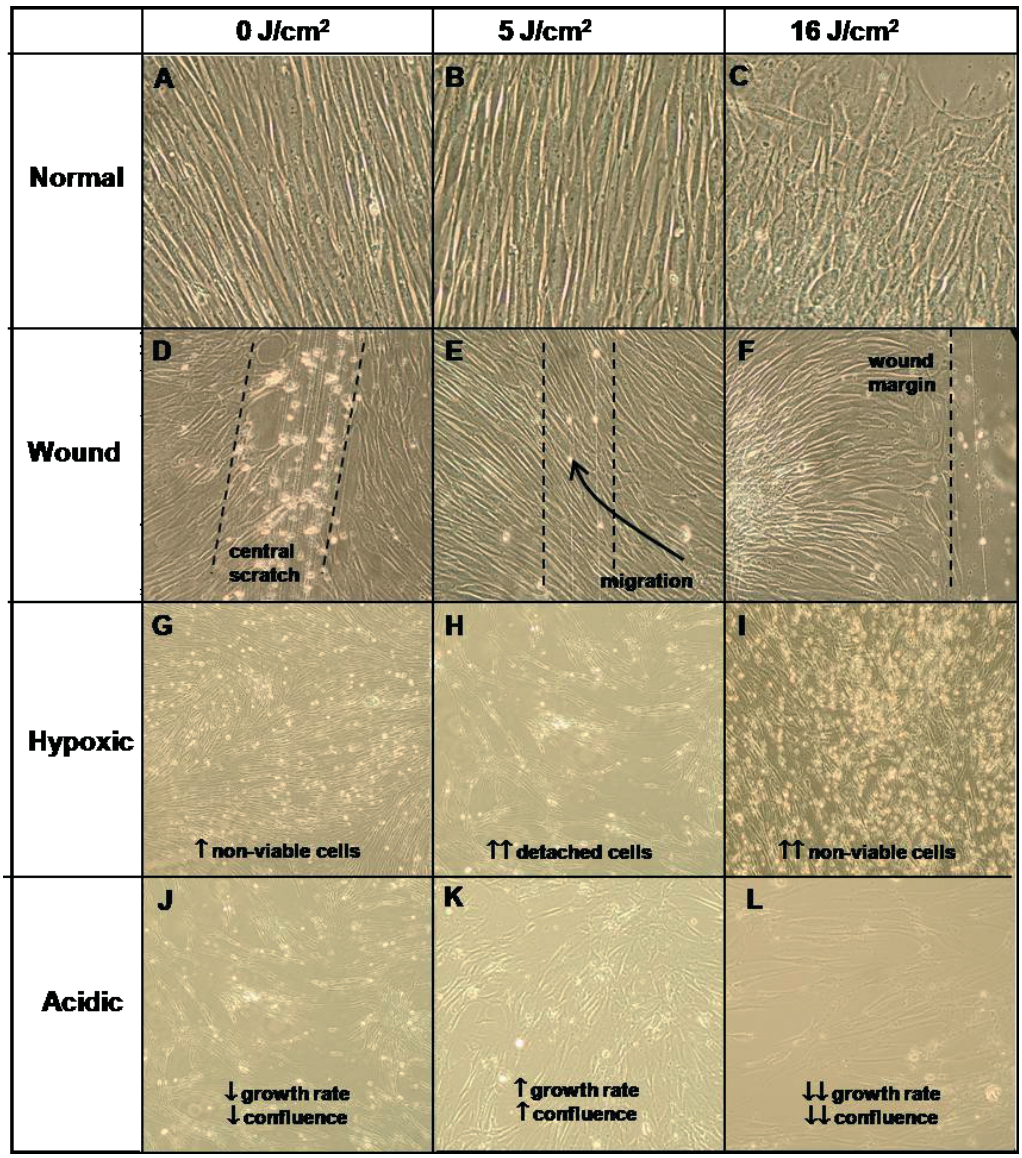

Figure 1. Micrograph of normal, wounded, hypoxic and acidotic WS1 cells irradiated by He–Ne laser (632.8 nm) at 5 or 16 J cm^{-2} taken 24 h postirradiation. Nonirradiated cells or controls (A, D, G, J) showed reduced growth rate. Wounded cells irradiated with 5 J cm^{-2} had wound area covered by day 5 (E). Cells irradiated with 16 J cm^{-2} (C, F, I, L) did not improve their confluence by day 5 and had indication of cell stress and damage and did not cover the wound area by day 5 (F).

and areas of incomplete closure were visible (Fig. 1H). Cells irradiated with 16 J cm^{-2} showed the highest proportion of nonviable cells when compared with normal cells and when compared with wounded nonirradiated or wounded irradiated with 5 J cm^{-2} (Fig. 1I).

Acidotic cells were more confluent after 5 J cm^{-2} irradiation (Fig. 1K) when compared with nonirradiated cells where cells were less confluent (Fig. 1J). A fluence of 16 J cm^{-2} did not improve confluence signifying that the fluence did not promote proliferation. There was a decrease in cell number as indicated by the presence of more areas of incomplete cell closure compared to cells irradiated with 5 J cm^{-2} (Fig. 1L). Cell fragments and debris were also noted in the background and culture media indicating an increase in cell lysis.

Effect of laser irradiation on cellular MMP

As this study aimed to determine whether laser irradiation stimulated the response of mitochondria, only the stimulatory fluence of 5 J cm^{-2} was investigated whereas the inhibitory fluence (16 J cm^{-2}) was excluded (3,26).

Irradiated cells showed an increased percentage of polarized cells (1 h postirradiation) compared with their nonirradiated controls; however, the differences diminished after 24 h (Fig. 2). There was a significant increase in the number of polarized cells in irradiated wounded, hypoxic and acidotic cells when compared with the nonirradiated controls (wounded and wounded irradiated $P = 0.048$; hypoxic and hypoxic irradiated $P < 0.05$; acidotic and acidotic irradiated $P = 0.045$) at 1 h postirradiation. At 24 h postirradiation, the

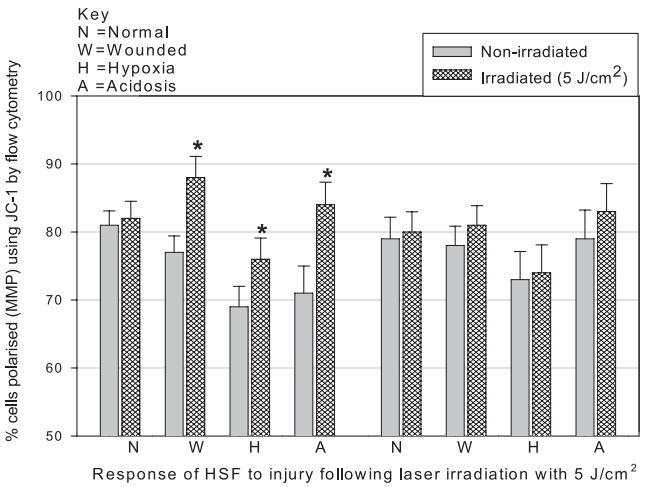

Figure 2. JC-1 was used to determine changes in mitochondrial membrane potential (MMP). There was a significant increase in MMP of cells in wounded, hypoxic and acidotic irradiated cells when compared with their nonirradiated controls ($P < 0.05$) at 1 h postirradiation. However, this response was time dependent and a significant increase in MMP was no longer observed after 24 h postirradiation ($*P \leq 0.05$; $n = 6$).

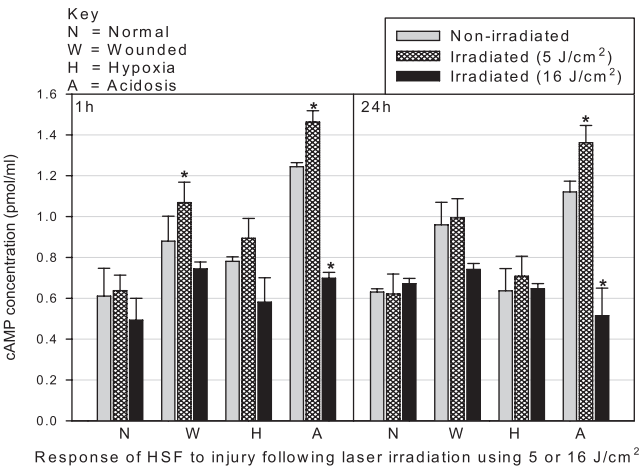

Figure 3. The cAMP assay was used to determine changes in cell mitochondrial activity. Using a fluence of 5 J cm^{-2}, there was a significant increase in cAMP concentration in acidotic condition in both nonirradiated and irradiated cells at 1 and 24 h ($P < 0.001$ and 0.01) and ($P < 0.001$ and 0.001) respectively when compared with normal cells. The concentration was also significantly different between hypoxic and acidotic cells ($P < 0.001$; $P = 0.02$) respectively at both 1 and 24 h postirradiation. When the hypoxic cells were compared with normal cells, the difference was significant at both 1 and 24 h postirradiation ($P < 0.001$; $P = 0.02$). Irradiation with 16 J cm^{-2} had decreased cAMP levels when compared with normal control, their respective controls and cells irradiated with 5 J cm^{-2}. These differences were particularly significant in acidotic conditions (A and AI, $P = 0.01$) at both 1 and 24 h ($*P \leq 0.05$; $n = 6$).

percentage of polarized cells increased in the irradiated wounded, hypoxic and acidotic conditions when compared with their nonirradiated control; however; differences did not prove to be significant.

Effect of laser irradiation on cAMP level

There was no difference in cAMP concentration between irradiated and nonirradiated normal and wounded cells at both 1 and 24 h. There was a significant increase in cAMP concentration in both nonirradiated and irradiated (5 J cm^{-2}) acidotic cells at 1 and 24 h ($P = 0.001$ and 0.001) respectively when compared with normal cells (Fig. 3).

There was a significant increase in irradiated (5 J cm^{-2}) acidotic cells when compared with their nonirradiated control cells ($P = 0.05$). Irradiation with 5 J cm^{-2} elicited an increase in cAMP levels in all the groups at 1 h which diminished 24 h postirradiation. There was a significant increase in cAMP level in wounded cells irradiated with 5 J cm^{-2} when compared with their nonirradiated control cells ($P = 0.05$).

Using a fluence of 16 J cm^{-2}, cAMP level was lower in the stress models than their nonirradiated controls. This decrease was significant in wounded, hypoxic and acidotic cells at 1 h (wounded and wounded irradiated $P = 0.02$; hypoxic and hypoxic irradiated $P = 0.02$; acidotic and acidotic irradiated $P = 0.01$). At 24 h postirradiation, only acidotic cells still showed a significant decrease in cAMP ($P = 0.01$).

Effect of laser irradiation on intracellular Ca^{2+}

Intracellular calcium (Ca^{2+})i was increased in nonirradiated stressed cells compared with normal cells at 1 and 24 h (Fig. 4) supporting evidence that an *in vitro* cell stress environment was successfully simulated. The increase was also noted in stressed irradiated cells using 5 J cm^{-2} when compared with the irradiated normal control cells. There was a statistical increase in irradiated stressed cells compared with their nonirradiated control cells at 1 h (wounded and wounded irradiated $P = 0.04$; hypoxic and hypoxic irradiated $P = 0.045$; acidotic and acidotic irradiated $P = 0.05$). The increase in calcium remained significant 24 h postirradiation (wounded and wounded irradiated $P = 0.035$; hypoxic and hypoxic irradiated $P = 0.043$; acidotic and acidotic irradiated $P = 0.05$).

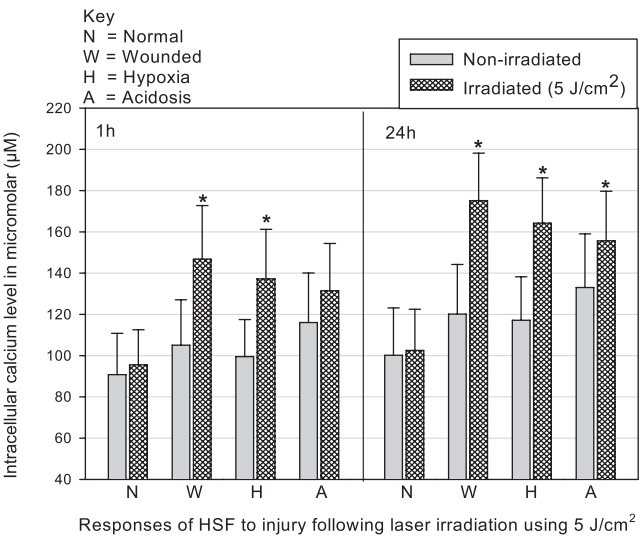

Figure 4. Intracellular calcium level was expressed in μM. Calcium was elevated in all the injury conditions compared with the normal control. A similar trend was seen in irradiated injury conditions when compared with their nonirradiated controls. However, these differences were statistical (WI and W $P = 0.04$; HI and H $P = 0.04$; AI and A $P = 0.05$) at 1 and (WI and W $P = 0.035$; HI and H $P = 0.043$; AI and A $P = 0.05$) 24 h ($*P \leq 0.05$; $n = 6$).

Results confirm that irradiation with 5 J cm^{-2} resulted in an increase in intracellular Ca^{2+}.

Effect of laser irradiation on ATP

In the cells irradiated with 5 J cm^{-2}, the decrease in ATP viability was significant in acidotic cells when compared with normal irradiated cells ($P < 0.05$) at 1 and 24 h postirradiation and similarly the decrease was significant when acidotic cells were compared with wounded and hypoxic cells ($P < 0.05$ and $P = 0.04$, respectively) at 1 h postirradiation (Table 2). This significant decrease in viability was still observed at 24 h postirradiation. There was no difference in ATP between normal and normal irradiated cells.

Irradiated wounded cells showed a significant increase in ATP viability when compared with the nonirradiated wounded cells at both 1 and 24 h postirradiation ($P = 0.02$ and 0.044, respectively). Acidotic cells irradiated with 5 J cm^{-2} showed a significant increase in ATP viability when compared with their nonirradiated control at 24 h postirradiation ($P = 0.05$). There was a significant increase in wounded cells irradiated with 5 J cm^{-2} when compared with wounded cells irradiated with 16 J cm^{-2} ($P = 0.04$) indicating that the physiological state of the cell (wounded, hypoxic or acidotic) and the dose administered (5 or 16 J cm^{-2}) are important factors that influence the cellular responses of the cells. In acidotic cell stress, the increase after irradiation with 5 J cm^{-2} was significant at 1 h ($P = 0.05$) but lost significance at 24 h when compared with their 16 J cm^{-2} irradiated controls ($P = 0.06$).

There was a decrease in ATP luminescence in all cells irradiated with 16 J cm^{-2} both at 1 and 24 h postirradiation. The difference in viability was significant when irradiated acidotic cells were compared with normal control cells ($P = 0.01$). A decrease in ATP viability was also noted when irradiated (16 J cm^{-2}) acidotic cells were compared with their nonirradiated controls ($P = 0.51$) at 1 h and ($P = 0.46$) at 24 h postirradiation indicating that a dose of 16 J cm^{-2} did not stimulate the cells to recover or repair.

Effect of laser irradiation on LDH cytotoxicity

There was an insignificant decrease in cell cytotoxicity when wounded and hypoxic irradiated (5 J cm^{-2}) cells were compared with their nonirradiated controls at 1 h postirradiation (hypoxic and hypoxic irradiated $P = 0.10$; wounded and wounded irradiated $P = 0.18$). The decrease was maintained after 24 h postirradiation. In acidotic cells the decrease was significant at both 1 and 24 h postirradiation (acidotic and acidotic irradiated $P = 0.05$) (Table 2).

Irradiation with 16 J cm^{-2} increased LDH release in all the models at both 1 and 24 h postirradiation. When the cells irradiated with 16 J cm^{-2} were compared with the normal control the differences were significant (normal and wounded irradiated $P = 0.03$; normal and hypoxic irradiated $P = 0.02$; normal and acidotic irradiated $P = 0.004$). At 24 h there was a decrease in LDH cytotoxicity for all the groups compared with LDH values after 1 h. When acidotic cells irradiated with 16 J cm^{-2} were compared with their nonirradiated control cells at 24 h postirradiation, the difference was significant ($P = 0.05$).

DISCUSSION

Though observed under the microscope more than 100 years ago, mitochondria have been recognized as the power plants of cells in the 20th century. They have been identified as the sites of synthesis of many metabolites, for iron/sulfur cluster assembly and other anabolic processes. They are cellular power plants for the production of most of the high-energy rich molecules–ATP.

While the wound area was completely covered following irradiation with 5 J cm^{-2}, the same coverage was not observed with 16 J cm^{-2} by day 5 of the experimental period. This suggests that 16 J cm^{-2} inhibited migration and proliferation which are prerequisites in wound healing. It takes longer for the cell to show morphological changes after losing its biological function (17). This may suggest that 24 h postirradiation with 16 J cm^{-2} may not be enough time to notice morphological changes. However, should the damage be repairable, it may still not show as repair mechanisms start as soon as injury has been incurred (32). Hawkins and Abrahamse found that irradiation with 16 J cm^{-2} caused cell damage and significantly inhibited migration (3,26).

A decrease in cell number was observed in the acidotic model as the pH was below the normal range within which healthy cells are maintained (33). There were however more

Table 2. ATP as measured in reading light units (RLU) and LDH percentage cytotoxicity results.

		ATP (RLU)		% Cytotoxicity	
Type	Dose, J cm^{-2}	1 h	24 h	1 h	24 h
Normal	0	2 532 046 ± 68 730	2645163 ± 75743	22 ± 2.9	18.5 ± 3
	5	2 576 814 ± 70 207*	2655389 ± 122778	19 ± 0.6	20 ± 20
	16	2 413 484 ± 83 721	2555725 ± 47871	24 ± 2.7	21 ± 3.1
Wound	0	2 288 024 ± 67 727	2467180 ± 89771	29 ± 2.3	23.4 ± 3
	5	2 832 888 ± 85 920	2922902 ± 97664*	26 ± 1.6	21 ± 1.5
	16	2 087 108 ± 44 995	2318304 ± 76015	31 ± 2.4	25 ± 3.3
Hypoxic	0	2 074 071 ± 117 134	2548518 ± 71224	28.8 ± 3	23.7 ± 2.7
	5	2 191 064 ± 126 527	2661353 ± 62402	26 ± 1.6	22 ± 2.3
	16	1 796 137 ± 128 202	2262060 ± 78166	31 ± 2.4	27 ± 3.5
Acidotic	0	1 596 137 ± 117 134	1209061 ± 71224	33 ± 3.4	27.7 ± 3.5
	5	1 722 998 ± 100 951	1477862 ± 112618*	24 ± 20*	20 ± 1.3*
	16	1 374 971 ± 129 956	1127902 ± 65680	42 ± 3.3*	34 ± 30*

*$P \leq 0.05$ ($n = 6$).

cells in acidotic irradiated (5 J cm^{-2}) samples. Cells do not proliferate well when pH is low and that an increase in $(pH)_i$ is necessary for mitogenesis (34,35). Irradiation has been shown to facilitate the increase in pH value (36). This could explain the increased confluence in irradiated (5 J cm^{-2}) acidotic monolayers.

In hypoxic cells, there were more nonviable cells in the nonirradiated group; however, after 5 J cm^{-2} these cells detached and viable cells were redirected toward open spaces. Vulnerable cells at the time of hypoxia induction were more susceptible to injury and were not stimulated by laser irradiation to repair which resulted in irreversible damage and reduced viability. During hypoxia, there is reduction in oxidative phosphorylation leading to low or lack of ATP production. According to Yu *et al.* there is a correlation between ATP depletion and cell death under *in vitro* ischemia (23). These cells lose contractility, lipid and fatty acids metabolism membrane integrity leading to cell arrest, apoptosis and necrosis (37). Lactic acid acidosis which slows down cell growth has also been implicated in hypoxia resulting from glycolysis as an alternative source of cell energy (38). Results suggest that irradiation with 5 J cm^{-2} rejuvenates the cells which then start to migrate and proliferate again. After irradiation with 16 J cm^{-2}, there were more nonviable cells. This fluence might have acted as further stressor to cells and more susceptible cells died (26).

It is however important to note that in wound healing *in vivo*, the inflammatory phase has an acidotic microenvironment (36). Whether the same happens *in vitro* in monolayer cell sheets requires further research as it would further support the reasons for slow cellular proliferation in nonirradiated central scratch wounds. Extracellular acidosis reduces intracellular pH which reduces MMP, cAMP, $(Ca^{2+})i$ and ATP. These biochemical changes are influenced by mitochondria and lead to a decrease in the proliferatory and migratory activities of the cells. It was noted that in all three models, nonirradiated cells did not reach confluence by day 5 as was the case after irradiation with 16 J cm^{-2}. The secondary messengers were reduced. There was an increase in MMP, cAMP and $(Ca^{2+})i$ after irradiation with 5 J cm^{-2} which results in an increase in proliferation, migration and cell sheet confluence.

It was noted that irradiation with 5 or 16 J cm^{-2} on normal cells did not elicit any statistically different value when compared with nonirradiated normal cells. Other researchers found that phototherapy did not exert significant effects on cells that were growing optimally (9,12). As phototherapy aims to bring the stressed cells to their normal functional state, it would be energetically inefficient to use phototherapy on cells that are already functioning normally. According to Cai *et al.*, at confluence cells were far less responsive to stimulus and contact inhibition factors play an important role in restricting proliferation when cells reach confluence (39).

Results for wounded cells indicate that 5 J cm^{-2} had a better effect on wounded cells which is in agreement with the findings of Karu, where irradiated wounded (stressed) cells responded with an increase in the rate of chemotaxis, haptotaxis and cell proliferation (40). A decrease in cell damage could be due to the activation of early cell repair mechanisms which are enhanced by 5 J cm^{-2} and elicit an early protective effect (41). In order to interact with the living cell, light must be absorbed by intracellular chromophores. Chromophores such as endogenous porphyrins, mitochondrial and membranal cytochromes and flavoproteins generate ROS which play a significant role in cell activation. Lubart *et al.* demonstrated that various ROS and antioxidants are produced following low energy laser irradiation and suggested that the cellular redox state plays a pivotal role in maintaining cellular activities (41). Results confirm that laser irradiation activates the mitochondrial respiratory chain components which initiate signaling cascades that promote cellular proliferation and cytoprotection. Irradiation is known to downregulate genes involved in apoptosis (42). This may explain the reduced cell damage observed after 5 J cm^{-2} not only in wounded cells but also in hypoxic and acidotic cells irradiated with 5 J cm^{-2}. These results concur with reports that irradiation with 5 J cm^{-2} enhanced cellular viability and reduced cell damage (26) while this study confirms that 5 J cm^{-2} promoted wound closure.

There is evidence that laser irradiation using appropriate laser parameters accelerates Ca^{2+} uptake by mitochondria and cAMP elevation which promotes ATP synthesis leading to stable MMP and cell proliferation (15,18,40). According to Lubart *et al.*, photobiomodulation by laser irradiation involves stimulation of redox activity in the respiratory chain with subsequent effects on intracellular ion concentration including calcium (43). However, the balance of intracellular Ca^{2+} is maintained by MMP whereas on the other hand Ca^{2+} flux regulates MMP. The increase in cAMP level in the irradiated wounded cells was followed by an increase in cell proliferation. This suggests that the mitogenic influence of laser at this dose in fibroblast cells might have been *via* cAMP mediation which further confirms mitochondria stimulation.

Irradiation with 16 J cm^{-2} significantly reduced ATP cell viability and cAMP concentration but increased LDH cytotoxicity. These results are in agreement with reports that laser irradiation using He–Ne laser with 10 and 16 J cm^{-2} significantly reduced HSF cell viability and caused significant cell damage as assessed by LDH (26). This was also confirmed by Tuner and Hode, who stated that higher doses are inhibitory and damage the cells (15). These findings support the notion that the mitochondrial response of wounded cells to laser irradiation is dose dependent—5 J cm^{-2} stimulated whereas 16 J cm^{-2} inhibited.

When the hypoxic cells were irradiated with 5 J cm^{-2}, cell viability improved when compared with the nonirradiated control. This improvement corresponds with the increase in MMP, Ca^{2+} and cAMP levels. Irradiation has been shown to increase electron transfer in purified cytochrome oxidase and upregulates the activity of this complex which increases mitochondrial respiration and ATP synthesis (44). This suggests the link between mitochondrial stimulation (increased MMP), increased ATP synthesis and intracellular Ca^{2+}. According to Johnson and Barton, hypoxia causes mitochondrial ROS production which can lead to disruption of MMP integrity and loss of the ability to generate ATP (45). Cell stress results in mitochondrial inhibition which reduces ATP energy output and increases free radical production or ROS. As the cellular redox state has a key role in maintaining the viability of the cell, changes in ROS may play a significant role in cell activation (41). The findings in this study therefore further prove that the irradiation with He–Ne laser at 5 J cm^{-2} augments recovery pathways enhancing cellular viability and

restoring cellular function following hypoxic injury by stimulating mitochondria.

Results showed that 16 J cm^{-2} did not exert a significant amount of damage on cells injured by hypoxia when compared with damage caused in wounded cells by the same fluence. This could be because hypoxia may have adaptive response to cells which may exert protective effect. Diaz and Wilson categorized ischemic preconditioning into two—"classical" which dissipates within 1–2 h following the stimulus and "late or second window of protection" which reappears from about 24 to 72 h after the initial preconditioning stimulus (46). The duration, frequency, and severity of hypoxic episodes are critical factors that determine whether hypoxia is beneficial or harmful (47). The fate of hypoxic cells is dependent on multiple factors as hypoxia promotes apoptosis and growth inhibition while imposing a strong selective pressure for survival of cells. This selective process promotes genomic instability and the transcriptional activation of genes that enhance survival and growth during tumorigenesis (45,48). Hypoxia induces production of interleukin-6 which stimulates the production of inflammatory proteins (37). Hypoxia also causes localized changes in chromatin structure which facilitates gene-specific regulation and DNA repair (49).

Mechanisms for adaptation to intermittent hypoxia may also provide protection against more severe and/or sustained hypoxia and confer protection against other stresses (47). According to the concept of cross adaptation, development of resistance to one factor confers resistance also to other factors depending on the pattern of gene expression evoked by the primary stress factor (39). This could explain why irradiation with 16 J cm^{-2} did not exert significant damage as was seen in the wounded model. At this point, hypoxia for 4 h might have exerted an adaptive response to the cells. The principle of adaptive response states that when the stressor or presensitisor is stronger than the challenging dose, the challenging dose may not exert its effect (50).

As acidosis is prevalent in many pathological and physiological conditions, the effect on the mitochondria of fibroblasts following irradiation was assessed. Induction of extracellular acidosis in this study can be considered to reflect the conditions of diabetic ketoacidosis, ischemic penumbra and lactic acidosis muscle cramps *in vivo*. Effective control of tissue pH under respective clinical conditions might improve cytotoxic brain edema in ischemic stroke and avoid further acidotic redox stress (51,52).

The results correlate with what others have reported about the effect of extracellular acidic pH on cells. Trevani *et al*. (36) recorded an increase in cytotoxicity which they defined was due to increased ROS production at extracellular acidic condition. This may also explain the increased LDH release which showed increased damage and cytotoxicity. They also noted that extracellular acidic pH (pH_o) causes a drop in intracellular pH (pH_i) and also reduces intercellular adhesion molecule-1 expression on human aortic endothelial cells (36). This could explain the presence of debris and gaps in the morphology of acidic cells (Fig. 1J–L).

Many cellular responses are diminished at lowered extracellular pH, including cytosolic and membrane-associated enzyme activities, iron transport activity, delocalization of protein-bound iron stores leading to oxidative stress, and protein and DNA synthesis (35,52,53). It is very important for the intracellular pH to be in a narrow physiological range for cells to enter the S phase of the cell cycle (54).

In this study, pH was adjusted in MEM-containing bicarbonate to stabilize the pH and avoid pH fluctuations so that the pH remained as close to that of the *in vivo* environment that was being simulated. According to Trevani *et al*., a fall in pH_i might be due to rapid diffusion of CO_2 into the cell originating from the reaction of protons (H^+) with the bicarbonate present in the culture medium (36). This in turn results in overproduction of intracellular protons.

It has been shown that laser irradiation causes an increase in intracellular pH and an increase in intracellular pH has been shown to be a prerequisite for cell proliferation in acidotic conditions (18). In their study, Perez-sala *et al*. (55) blocked the Na/H antiporter and the increase in pH_i was still noted after irradiation, suggesting that irradiation was the cause of the increase. Irradiation with 5 J cm^{-2} might have increased the intracellular pH which promoted Ca^{2+} uptake, MMP, cell viability and proliferation in this study. Intracellular alkalinization has been reported to play a significant role in the suppression of apoptosis and in controlling the rate of cell progression (52,55). Alexandratou *et al*. also noted that laser irradiation triggered the acceleration of Ca^{2+} uptake by human fibroblast cells (18). Other researchers also reported that laser irradiation accelerates the uptake of Ca^{2+} by isolated mitochondria, bull sperm cells, mouse spermatozoa and human lymphocytes (56). It has been shown that uptake of Ca^{2+} into mammalian cells can be induced by monochromatic red light depending on the dose applied (11).

The increase in MMP following irradiation increased at 1 h and decreased at 24 h probably showing that in some cells the decrease was below the basal level. Alexandratou *et al*. found an increase in MMP followed by a gradual decrease to the lower state after irradiation (18). There was no complete collapse of MMP observed in any of the experimental conditions simulated in this study. This further sustains the concept that laser irradiation restores the functionality of the cells to their natural state as laser irradiation aims to normalize or restore homeostasis.

Irradiation with 16 J cm^{-2} did not improve cellular metabolism as viability and proliferation were reduced whereas cell damage or cytotoxicity by LDH was increased. This confirmed that the high irradiation fluence did not support cellular activities that improve viability and proliferation.

The study established that there are measurable changes in hypoxic and acidotic irradiated cells at 1 and 24 h postirradiation. The next step would be to establish a time response to distinguish immediate (< 1 h) and delayed responses (24, 72, 96 h). Immediate changes in MMP and $(Ca^{2+})i$ can be observed using live cell imaging with JC-1 and Fluo 3AM, respectively. Mitochondrial enzyme activity such as complex II (DCIP oxireductase), complex IV (cytochrome *c* oxidase) and succinate phenazine oxireductase (soluble SDH) together with RT-PCR and genetic and enzyme kinetic studies can also be used to determine changes in the expression of enzymes or factors that may regulate cellular changes. A time–response study using assays such as enzyme kinetics, RT-PCR and live cell imaging will provide information which will determine whether the light treatment first alters the level of enzymes and ion transporters or whether laser irradiation immediately

induces an increase in MMP ($\Delta\Psi_{mt}$), ATP and cAMP *via* enhanced cytochrome *c* oxidase activity.

Another important aspect is to ensure that there is cell signaling between the photoacceptors and the nucleus, between the photoacceptor and the nucleus, and also between the photoacceptor and the mitochondria. Studies that assess second messengers (*i.e.* ATP and cAMP or nitric oxide, cGMP and ROS) will be used to determine whether a change in second messengers translates into a change in cellular responses such as activation of protein kinases to change gene transcription and regulate cell behavior. To understand the cellular and molecular responses, it is important to determine whether there is an effect on cellular function such as a change in cell viability, cell proliferation and cytotoxicity.

CONCLUSION

It is well known that mitochondria are very important in apoptosis, aging, calcium homeostasis, cAMP regulation and cell development. Depending on the type of cell and use, mitochondria are functionally heterogenous organelles that are discrete or dynamically networked. Their responses to different stimuli decide whether the cell will survive or commit to apoptosis or necrotic cell death. Mitochondria are believed to be the primary target of photoradiation since they contain cytochrome *c* oxidase or complex IV chromophores. Cytochrome *c* oxidase is a component of the cellular respiratory chain which acts as light receptor or photoacceptor which activates a cascade of cellular respiratory effects upon the absorption of photons.

In conclusion, it has been shown that on the one hand MMP depolarization triggers intracellular Ca^{2+} increases and on the other, intracellular Ca^{2+} changes affect MMP by modulating various ionic currents such as calcium, chloride and sodium calcium exchange currents. It has also been shown that cAMP and Ca^{2+} modulate each other depending on the type of signal and end product to be achieved. It has been shown that most of the cellular responses to LLLT involve changes in the mitochondria and membrane activities including MMP (11,12). Despite the inhibitory effect by 16 J cm^{-2}, irradiation with 5 J cm^{-2} showed positive stimulatory effects through increased levels of cAMP, Ca^{2+} and MMP which promoted cellular viability, repair and proliferation. This study has shown that mitochondria of injured HSF cells are stimulated and activated in response to LLLT using the He–Ne laser in a dose-dependent manner. LLLT influences stressed cells by causing changes in the effect on stressed cells *via* mitochondria.

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