Effect of Laser Irradiation on Mitochondrial Responses of Stressed Keratinocytes

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Summary

Photobiomodulation activates the mitochondrial respiratory chain components which initiates signalling cascades that promotes cellular proliferation and cytoprotection. Human keratinocyte cell cultures were modified to induce changes in mitochondrial membrane potential so that the effect of laser irradiation could be investigated. Control conditions included normal keratinocytes and cells treated with p-(tri-fluoromethoxy) phenyl-hydrazone (FCCP), 2-deoxy-D-glucose (2-DOG) and oligomycin. Experimental conditions were used to induce sub-cytotoxic stress namely; keratinocytes grown in media without bovine pituitary extract (BPE), UVC irradiation and tBHP (tert-hydroperoxide). Results confirm that laser irradiation stimulates the mitochondrial respiratory chain to bring about an increase in ATP production, which ultimately results in photobiomodulation to restore homeostasis of stressed cells.

Introduction

The mechanism of low level laser therapy at the cellular level is based on the increase of oxidative metabolism of mitochondria, which is caused by electronic excitation of components of the respiratory chain. Lasers improve the flow of electrons through the respiratory chain to increase the mitochondrial production of ATP without increasing the production of free radicals. Cell stress results in mitochondrial inhibition which reduces ATP energy output and increases free radical production or reactive oxygen species (ROS) to accelerate common signs of normal ageing [1,2].

Previous reports have shown that cellular functions could be influenced by visible light (400 – 700nm); however the mechanisms by which visible light induces its biological effect remain to be established [3]. Low level laser
irradiation has been found to modulate various biological processes in cell cultures and animal models [4,5]. Recent evidence indicates that cellular proliferation could be triggered by the interaction of helium-neon laser (He-Ne laser, 632.8nm) with the mitochondrial photoacceptor - cytochrome c oxidase [3]. Hu et al. reported that He-Ne irradiation immediately induced an increase in mitochondrial membrane potential (ΔΨ<sub>mt</sub>), ATP, and cAMP via enhanced cytochrome c oxidase activity to elicit photostimulatory effects in mitochondrial processes which ultimately leads to cell proliferation [3]. Mitochondria might be a special target of visible laser light since they contain most of the cellular chromophores [6,7]. The absorption of light by a functioning photoacceptor causes the short term activation of the respiratory chain and oxidation of the NADH pool which leads to changes in the redox state (into a more oxidized state) [1,2,4,8] which stimulates membrane permeability activities and enhances membrane transport mechanisms.

The activation of the electron transport chain results in an increase in proton motive force (pmf, Δμ<sub>H+</sub>), consumption of oxygen and potential of mitochondrial membrane (ΔΨ<sub>mt</sub>) as well as extrasynthesis of ATP and alkalinization of the cytoplasm [9]. It is known that even small changes in ATP level can alter cellular metabolism significantly. There are cellular signalling pathways inside the cell between the photoacceptor and the nucleus and also between the photoacceptor and the cell membrane. In this way the primary reactions occurring under irradiation in the respiratory chain control DNA and RNA synthesis in the nucleus [9]. The photosignal transduction and amplification chain controls cell proliferation. The regulatory role of redox homeostasis, the photoacceptor control over the level of intracellular ATP and the cellular redox state regulate the cellular signalling cascades. Modulation of cellular redox state affects gene expression via transcription factors.

Laser enhanced DNA and RNA synthesis depends on the availability of energy and also on the mechanisms responsible for the absorption of energy and the transduction of photonic energy into chemical energy [3]. The magnitude of the cellular response depends on the cellular redox potential and the physiological status of the cells at the moment of irradiation. Karu (1998) stated that laser light stimulates cells that are growing poorly at the moment of irradiation [1]. It was for this reason that the models were selected to induce homogenous sub-cytotoxic cell stress so that the effect of laser irradiation could be observed and a possible mechanism of action further elucidated. The study aimed to determine if laser therapy would still have an effect if the proposed pathway was blocked or inactivated. The study aimed to determine if certain in vitro models of cell stress could inhibit or prevent the beneficial or therapeutic effect of laser irradiation.

**Materials and Methods**

Human keratinocyte cell cultures designated CCD-1102 KERTr (ATCC CRL-2310) were grown in keratinocyte-serum free media (SFM, Invitrogen 17005075) containing 35ng/ml recombinant epidermal growth factor (rEGF) and 0.05mg/ml BPE. Upon reaching 60-75% confluency, the cells were trypsinized
and 5 X 10^5 cells (in 3ml culture medium) were seeded in 3.4cm diameter culture plates and incubated overnight to allow the cells to attach. Cell cultures were modified to induce changes in mitochondrial membrane potential or to induce sub-lethal or sub-cytotoxic stress (Table 1).

Once the agent was introduced and the cells incubated (Table 1) the cells were washed with warmed phosphate buffered saline for two washes before the culture medium was replaced and the cells returned to the incubator (37°C, 5% CO₂, 80% humidity) for 30 min prior to laser irradiation.

A 648nm diode laser with a power output of 30mW, power density of 1.65mW/cm² and spot size of 3.4cm (area 9.1cm²) was used to irradiate culture dishes with a dose of 1.5J/cm². Studies have shown that 1.5J/cm² results in a significant increase in migration [14] and proliferation of keratinocytes [15].

Table 1. Summary of cell stress conditions induced in human keratinocyte cell cultures.

<table>
<thead>
<tr>
<th>Cell stress</th>
<th>Action</th>
<th>Details</th>
<th>Ref</th>
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<tbody>
<tr>
<td>FCCP</td>
<td>an uncoupler of oxidative phosphorylation</td>
<td>Sigma C2920 10mg</td>
<td>10</td>
</tr>
<tr>
<td>2-DOG</td>
<td>inhibits glucose transport and deplete cells of ATP</td>
<td>Sigma D3179 250mg</td>
<td>10</td>
</tr>
<tr>
<td>Oligomycin</td>
<td>an F₁,F₀-ATP synthase inhibitor and disrupts mitochondrial membrane potential</td>
<td>Sigma 75352mg 10mg/ml</td>
<td>10</td>
</tr>
<tr>
<td>Oligo</td>
<td>an antioxidant and removal reduces cell growth</td>
<td>Invitrogen 0.05mg/ml</td>
<td>11</td>
</tr>
<tr>
<td>BPE</td>
<td>chemically induces oxidative stress</td>
<td>Sigma B2633 100ml</td>
<td>12</td>
</tr>
<tr>
<td>UVC</td>
<td>Ultraviolet-C irradiation 254nm</td>
<td>Philips TUV 30W G30T8</td>
<td>13</td>
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Studies have shown that 1.5J/cm² results in a significant increase in migration and proliferation of keratinocytes. Cell culture dishes were irradiated from a fiber optic positioned at a distance of 8cm above the cell monolayer with the culture dish lid off at room temperature (21°C) in the dark on a dark surface. Cells were irradiated in SFM medium without phenol red to minimize the loss of laser energy through absorption by colored culture medium. The laser tip (±5 mm) was expanded so that the spot size area was the same area as the culture dish. The entire dish was irradiated with a homogenous beam for approximately 7 min 35 sec duration to deliver 1.5J/cm².

Culture plates were irradiated on day 1 and 2 however on day 2, the culture plates were incubated for 30 min at 37°C before the cells were harvested. Digital photos of cells noted changes in cell morphology before the cells were trypsinized and resuspended at a concentration of 8 x 10⁵ cells/100µl in supplemented SFM culture medium. The cell suspension was used to assess cellular responses such as ATP and mitochondrial membrane potential.
tial while the cell culture medium was used to assess cytotoxicity using the LDH membrane integrity assay (Table 2).

**Results and Discussion**

Based on the cell morphology, ATP luminescence and $\Delta \Psi_{m}$, the effect of laser irradiation on stressed cells was assessed. The WST-1 (cell proliferation) and LDH (cytotoxicity) results were used to complement the mitochondrial response assays and to determine if a mitochondrial response translates into a cellular response.

Three specific responses were noted:

(i) **Stimulatory effect of laser irradiation**: normal irradiated cells showed an increase in ATP ($P=0.026$) and increase in $\Delta \Psi_{m}$ indicating an increase in the number of healthy cells. Normal irradiated cells showed a typical cobblestone appearance with an increase in cell number after 24h (Figure 1). Results indicate that laser irradiation stimulates cells that are semi-confluent since keratinocytes were seeded at 5 X $10^5$ cells to avoid the negative effect of contact inhibition. Irradiated cells incubated without BPE showed an increase in ATP and decrease in cytotoxicity (Figure 2A). Removal of BPE results in a decrease in cell growth and an increase in oxidative stress so laser irradiation stimulated cell proliferation (+21.01%). Exposure to 254nm UVC induces apoptosis and DNA lesions. UVC irradiated cells showed the typical keratinocyte morphology without evidence of apoptosis. Laser irradiation resulted in an increase in ATP which translated into an increase in cell proliferation (+18.38%) and decrease in cytotoxicity ($P=0.029$). UVC irradiated cells showed an increase in the number of apoptotic cells when compared to non-irradiated

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**Table 2. Summary of the methods used to assess laser induced cellular responses.**

<table>
<thead>
<tr>
<th>Response</th>
<th>Details</th>
<th>Principle</th>
<th>Detection</th>
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<tbody>
<tr>
<td>Adenosine triphosphate (ATP)</td>
<td>Promega CellTiter Glo luminescent assay (G7570)</td>
<td>Addition of reagent results in cell lysis and generation of luminescent signal proportional to the amount of ATP present which is proportional to the number of cells present in culture.</td>
<td>100μl of reagent and 100μl medium containing cells. Luminescent signal proportional to ATP.</td>
</tr>
<tr>
<td>Mitochondrial membrane potential ($\Delta \Psi_{m}$)</td>
<td>JC-1 mitochondrial membrane potential assay kit (Cayman 10009172)</td>
<td>Changes in $\Delta \Psi_{m}$ are reflected by different forms of JC-1 as either green or red fluorescence. Both forms can be qualified and quantified by fluorescent microscopy, flow cytometry or a fluorescence plate reader.</td>
<td>Healthy cells: JC-1 forms J-aggregates using Ex/Em at 560nm/595nm. Apoptotic or unhealthy cells: JC-1 exists as monomers using Ex/Em at 485nm/535nm.</td>
</tr>
<tr>
<td>LDH</td>
<td>Promega CytoTox 96 non-radioactive cytotoxicity assay (G7890)</td>
<td>The assay measures lactate dehydrogenase (LDH), a stable cytosolic enzyme, released into culture medium upon cell lysis.</td>
<td>50μl of culture medium and 50μl of substrate in 96 well flat bottom plate. Absorbance at 490nm.</td>
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Figure 1. Morphological responses of stressed keratinocytes. FCCP treated cells showed evidence of reversible cell damage consistent with autophagy. 2-DOG and tBHP both showed a significant decrease in cell number with morphological features consistent with apoptosis. Oligomycin showed evidence of reversible damage with large cytoplasmic vacuoles. BPE irradiated cells showed an increase in the growth rate when compared to non-irradiated cells using the WST-1 incorporation assay.

Figure 2. The effect of 1.5J/cm² at 648nm was represented as the percentage change in ATP luminescence and LDH cytotoxicity (A). The JC-1 assay for mitochondrial membrane potential (B) was used to distinguish healthy cells (J-aggregates) from unhealthy cells (monomers). Laser irradiation stimulated the biological function of normal, BPE and UVC irradiated cells with an increase in cell number and metabolic activity which may also exert an anti-apoptotic or cytoprotective effect in tBHP and 2-DOG treated cells (n=6; * P≤0.05).
UVC cells. Laser irradiation was able to stimulate the biological function of normal, BPE and UVC irradiated cells with an increase in cell number and metabolic activity (Figure 2).

(ii) Sub-lethal damage where laser irradiation has a limited effect depending on the compound used: FCCP treated cells showed evidence of autophagy, a reversible process characterized with large autophagic vacuoles, swelling and plasma membrane breakdown. Laser irradiation did not have a stimulatory effect since FCCP inhibits the respiratory chain or oxidative phosphorylation so subsequently no additional ATP was produced. FCCP irradiated cells showed a decrease in ATP ($P=0.022$) and decrease in the number of healthy cells ($P=0.050$) with an increase in the number of unhealthy cells. The decrease in ATP prevents DNA synthesis which limits cell proliferation (-0.2%). FCCP, a mitochondria-specific ionophore, reduced $\Delta \Psi_{mt}$ and ATP production of keratinocyte cells irradiated with 648nm. Oligomycin inhibits ATP synthase and disrupts $\Delta \Psi_{mt}$. Oligomycin treated cells showed evidence of cloudy swelling, a reversible process characterized by large cytoplasmic vacuoles. Oligomycin treated cells showed an increase in the number of healthy cells but also an increase in the number of apoptotic or unhealthy cells ($P=0.048$) when compared to non-irradiated cells. Laser irradiation had no effect on ATP (-0.81%) or cell proliferation (-4.7%). Laser irradiation is known to have a cytoprotective effect however since the compounds inhibit oxidative phosphorylation and ATP synthesis, laser irradiation had a limited effect. Results indicate that mechanism of oligomycin may be similar to that of FCCP which uncouples oxidative phosphorylation however they differ in the degree or extent of the damage.

(iii) Irreversible damage that laser irradiation could not reverse: tBHP and 2-DOG showed evidence of apoptosis. Cell morphology of tBHP and 2-DOG showed changes characteristic of apoptosis including blebbing, loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Figure 1). Cells treated with tBHP and 2-DOG showed a decrease in cell number and ATP. Irradiated tBHP cells showed a significant decrease in ATP ($P=0.006$) and significant increase in cytotoxicity ($P=0.018$) while 2-DOG irradiated cells showed an increase in Caspase 3/7 for apoptosis ($P<0.001$). Both tBHP and 2-DOG irradiated cells showed a decrease in apoptotic cells and increase in healthy cells when compared to the non-irradiated cells (Figure 2B). Laser irradiation had a limited effect since the damage was irreversible and the compounds either inhibited glucose metabolism or depleted the cells of ATP. The results indicate that laser irradiation may have an anti-apoptotic effect since there was a significant increase in $\Delta \Psi_{mt}$ in both tBHP and 2-DOG treated cells.

Conclusion

Mitochondria play a central role in cellular homeostasis, and its homeostatic centre is $\Delta \Psi_{mt}$. Thus, the assessment of $\Delta \Psi_{mt}$ in cells is worth investigating, $\Delta \Psi_{mt}$ may play an important role in the irradiation-induced biologic effect on
human keratinocyte cells. Results confirm reports by Hu et al. [1] that the mechanism of photomodulation involves an increase in $\Delta \Psi_{\text{mt}}$ and ATP content.

Enhancement of cellular metabolism can occur via non-mitochondrial photoacceptors by indirect activation or suppression. The redox regulation mechanism cannot occur solely via the respiratory chain. Other redox chains containing molecules such as NADPH-oxidase, that regulate metabolic pathways, can be photoacceptors for photobiomodulation [1,2]. Susceptibility to irradiation and capability for activation depends on the physiological status of the irradiated cells: the cells, which overall redox potential is shifted to a more reduced state (some pathological conditions), are more sensitive to the irradiation. The control conditions (FCCP, 2-DOG and oligomycin) confirmed that the mechanism of low level laser therapy at the cellular level is based on the increase of oxidative metabolism since the control conditions prevented laser irradiation from having a beneficial or therapeutic effect. The results confirmed reports that the absorption of light by a functioning photoacceptor causes the short term activation of the respiratory chain which stimulates membrane permeability activities and enhances membrane transport mechanisms. Karu stated that the final photobiological response is determined not at the level of primary reactions in the respiratory chain but at the transcription level during cellular signalling cascades [1,2] which may explain why laser irradiation was able to stimulate cell proliferation and metabolic activity in some conditions.

References
potential in yeast cell population by flow cytometry, Microbiology, 147, 3335-3343, 2001.


