Abstract

**Background** Laser phototherapy promotes cell viability, cell proliferation and migration. This study aimed to determine if laser irradiation could stimulate cellular responses of stressed cells to promote cell survival.

**Materials and Methods** Human keratinocytes were treated with 200 μM hydrogen peroxide (H₂O₂) or 0.4 μg/ml oligomycin to induce autophagy and 5% absolute ethanol (EtOH) or 12 μM tert-butylhydroperoxide (tBHP) to induce apoptosis (control). Cells were irradiated using 1.5 J/cm² with 648 nm and cellular responses were measured after 1 h or after 24 h and 96 h at 37°C.

**Results** Irradiated cells treated with 200 μM H₂O₂ showed an increase in cell proliferation and decrease in intracellular calcium. Irradiated oligomycin treated cells showed a significant increase in intracellular calcium. Irradiated apoptotic (control) cells showed a decrease in ATP viability, an increase in cytotoxicity, decrease in intracellular Ca²⁺ and decrease in cell proliferation.

**Conclusion** Irradiated 200 μM H₂O₂ cells reverted to metabolically active, viable cells capable of proliferating within 96 h of laser irradiation. Changes in intracellular calcium following laser irradiation appear to influence cell survival and proliferation of stressed keratinocytes.

**Keywords** Cell stress, keratinocytes, laser biostimulation, laser phototherapy, Low-Level Laser Therapy (LLLT)

Introduction

Autophagy, a lysosomal process involved in the maintenance of cellular homeostasis, is responsible for the turnover of long-lived proteins and organelles that are either damaged or functionally redundant [8]. This process is important in normal development, differentiation and tissue remodelling but can be induced by a change in environmental conditions such as nutrient deprivation [2-4]. Autophagy has been implicated in several human conditions or pathologies including bacterial and viral infections, ageing, diabetes, cancer, atherosclerosis, neurodegenerative disorders and cardiovascular disease [5]. This catabolic process, also termed Type II programmed cell death, involves self-digestion of intracellular organelles by double-membraned vesicles or vacuoles that encircle the components to be recycled [9]. There is a loss of cell viability with a highly vacuolated morphology however mitochondrial membrane potential remains unaffected. The mitochondria are believed to be the main target for laser therapy. Mitochondria are involved in a range of cellular processes such as generating adenosine triphosphate (ATP), supplying cellular energy, signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth.

Autophagy acts as a pro-survival or pro-death mechanism in different physiological and pathological conditions [5]. It has a cytoprotective role in response to many stresses since the inhibition of autophagy leads to enhanced cell death [6]. It remains to be determined if autophagic death following prolonged stress is due to cellular exhaustion and/or depletion of cellular components or if it is due to the activation of a specific cell death mechanism [8].

Type I programmed cell death or apoptosis is an irreversible, strongly conserved route to death and results in activation of endonucleases, cleavage of DNA into fragments and activation of other proteases (caspases) that lead to cell fragmentation into particles that are then ingested by adjoining cells (phagocytosis), resulting in the removal of dead cells. Defects in apoptosis perturb development, promote tumorigenesis and impair chemotherapy, suggesting that diversion to an alternative cell death pathway such as autophagy or necrosis in these circumstances may be therapeutically beneficial [9]. Apoptosis is cytotoxic while autophagy is cytomodulatory.

It is not clearly established whether the autophagic response can be precisely modulated or regulated to prevent disease or promote health [5]. Manipulation of autophagy may provide a useful way to prevent disease progression or promote cell survival. Since low level lasers do not produce heat but are able to penetrate the interior of cells in a non-destructive manner with highly focused light in the 10-250mW range – stimulating ATP energy production, cell metabolism and membrane permeability to promote healing, reduce pain and stimulate physiological processes – laser therapy may be a safe alternative method to promote cell survival in damaged or stressed cells or preventing conditions such as ageing, diabetes, cancer, atherosclerosis, neurodegenerative disorders and cardiovascular disease where autophagy has been implicated [5].
Low level lasers have been used to promote pain relief, wound healing, immune modulation and to strengthen the regenerative forces of body tissues. Molecularly it is known to stimulate mitochondrial membrane potential (MMP), cytokine secretion and cell proliferation [10]. Laser irradiation has been shown to stimulate the immune system (immuno-corrective) and has anti-bacterial, anti-viral, anti-allergic, anti-toxic, anti-cancer and anti-inflammatory effects [11]. It increases energy and normalizes tissue metabolism, activates ATP-synthesis and energy formation in cells, increases oxidation of energy-carrying molecules and normalizes the parameters of the hormonal, immune and reproductive system. Various mechanisms for the effect have been proposed, including absorption of light by mitochondrial enzymes with localized heating [12], photon absorption by flavins and cytochromes in the mitochondrial respiratory chain affecting electron transport [13], production of singlet oxygen by excitation of endogenous porphyrins [14], and photoactivation of calcium channels resulting in increased intracellular calcium concentration and cellular proliferation [15]. In vitro studies have shown that 648 nm diode laser irradiation (1.5 J/cm²; 3.3 mW/cm²) stimulates cell viability, proliferation and cell signalling of stress induced premature senescent cells indicating that laser irradiation may be beneficial for conditions such as immune senescence, skin ageing, muscle atrophy, premature ageing in patients with advanced heart disease, neurodegenerative disorders and chronic renal failure [16].

Since little is known about the effect of laser irradiation on autophagic cells, this study aimed to determine if laser irradiation with 1.5 J/cm² using 648 nm could stimulate cellular functions of autophagic cells and promote cell survival. Potential benefits of laser therapy on autophagic cells may include: (i) added cell survival, (ii) preventing the onset of apoptosis during nutrient deprivation or (iii) preventing or delaying the onset of several conditions where autophagy has been implicated.

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

<table>
<thead>
<tr>
<th>Action</th>
<th>Details</th>
<th>Ref</th>
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<tbody>
<tr>
<td>Autophagy</td>
<td></td>
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<tr>
<td>Hydrogen peroxide 200 uM H₂O₂</td>
<td>A powerful oxidizer and major contributor of oxidative damage – causes lipid peroxidation of membrane and hydroxylation of proteins and DNA.</td>
<td>18</td>
</tr>
<tr>
<td>Sigma 31642</td>
<td>200 uM H₂O₂ (in EtOH)</td>
<td></td>
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<tr>
<td>500 ml</td>
<td>Add to 3 ml culture medium without rEGF and BPE. Incubate at 37°C for 2 h. Wash with 3 washes of 2 ml warmed PBS. Add fresh medium and replace in incubator (37°C, 5% CO₂, 80% humidity). Everyday for 4 days with 2 days (72 h) recovery. Culture medium was not replaced during the recovery phase.</td>
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<tr>
<td>Oligomycin 0.4 μg/ml Oligo</td>
<td>An F₀-ATP synthase inhibitor and disrupts mitochondrial membrane potential.</td>
<td>19</td>
</tr>
<tr>
<td>Sigma 75352</td>
<td>0.4 ug/ml Oligo (in EtOH)</td>
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<tr>
<td>10 μg/ml</td>
<td>Add to 3 ml culture medium without rEGF and BPE. Wash with 3 washes of 2 ml warmed PBS. Add fresh medium and replace in incubator (37°C, 5% CO₂, 80% humidity). Everyday for 2 days with 30 min recovery.</td>
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<tr>
<td>Apoptosis</td>
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<tr>
<td>Absolute Ethanol 5% EtOH</td>
<td>Induces cellular damage with vacuolization of cells and necrosis.</td>
<td>20</td>
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<tr>
<td>Sigma BCR656</td>
<td>5% EtOH</td>
<td></td>
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<tr>
<td>Cytostatic and Cytotoxic</td>
<td>Add to 3 ml culture medium without rEGF and BPE. Incubate at 37°C for 2 h. Wash with 3 washes of 2 ml warmed PBS. Add fresh medium and replace in incubator (37°C, 5% CO₂, 80% humidity). Everyday for 4 days with 2 days (72 h) recovery. Culture medium was not replaced during the recovery phase.</td>
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<tr>
<td>Tert-hydroperoxide 12 mM tBHP</td>
<td>Chemically induces oxidative stress.</td>
<td>21</td>
</tr>
<tr>
<td>Sigma B2633</td>
<td>12 mM tBHP (in H₂O)</td>
<td></td>
</tr>
<tr>
<td>100 ml</td>
<td>Add to 3 ml culture medium without rEGF and BPE. Wash with 3 washes of 2 ml warmed PBS. Add fresh medium and replace in incubator (37°C, 5% CO₂, 80% humidity). Everyday for 2 days with 30 min recovery.</td>
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Materials and methods

Cell culture

Human keratinocyte cell cultures designated CCD-1102 KERTr (ATCC CRL-2310) were grown in keratinocyte-serum free media (SFM, Invitrogen 17005075) containing 35 ng/ml recombinant epidermal growth factor (rEGF) and 0.05 mg/ml bovine pituitary extract. Upon reaching 60-75% confluency, the cells were trypsinized and 6 X 10^5 cells (in 3 ml culture media) were seeded in 3.4 cm diameter culture plates and incubated overnight to allow the cells to attach.

In vitro models

Oxidative stress has been shown to induce autophagy or apoptotic signalling pathways [17]. The use of chemicals to induce apoptosis or autophagy in in vitro cell culture models has been described elsewhere [16-23]. Several studies have shown that apoptosis can be chemically induced using 33 mM 2-deoxy-D-glucose (2-DOG), 500 μM hydrogen peroxide (H_2O_2), 12 μM tert-butylhydroperoxide (tBHP) and 5% absolute ethanol (EtOH) whereas autophagy can be induced using 15 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), 0.4 μg/ml oligomycin, oridonin, arsenic trioxide (As2O3) and 200 μM H_2O_2 [16-23].

Apoptosis (control) or autophagy was induced by exposing keratinocytes to different chemicals to induce cell stress [16-23] (Table 1). Different incubation times were used to induce autophagy however an apoptotic control was incubated for the same duration. Apoptosis was confirmed by caspase 3/7 activity [16, 23].

648 nm Diode laser irradiation

Digital photographs of the cells were taken everyday to record changes in cell morphology. After the recovery phase cells were irradiated with visible red laser light. A 648 nm diode laser with a power output of 30 mW, power density of 3.3 mW/cm^2 and spot size of 3.4 cm (area 9.1 cm^2) was used to irradiate culture dishes with a dose of 1.5 J/cm^2 (Figure 2). Dosage was
Studies have shown that 1.5 J/cm² results in the dark on a dark surface. Cells were irradiated in monolayer with the culture dish lid off at room temperature and a fiber optic positioned at a distance of 8 cm above the cell area as the culture dish. The entire dish was irradiated with a homogenous beam for approximately 7 min 35 sec duration to energy through absorption by colored culture media. The laser tip [24]. Studies have shown that 1.5 J/cm² results in a significant increase in migration [25] and proliferation of keratinocytes [16, 23, 26]. Cell culture dishes were irradiated from a fiber optic positioned at a distance of 8 cm 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 media without phenol red to minimize the loss of laser energy through absorption by colored culture media. The laser tip 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 1.5 J/cm² (Figure 1).

### Cellular responses

After laser irradiation, cells were incubated at 37°C for 1 h before they were trypsinized and resuspended at a concentration of 8 X 10⁴ cells/100 μl in supplemented SFM media [16, 23]. The cell culture media was removed for the LDH cytotoxicity assay while the cell suspension was used for the ATP luminescent viability assay, WST-1 proliferation assay (24 h and 96 h) and intracellular calcium (Ca²⁺) (Table 2) [27-30]. Changes between the un-irradiated and irradiated samples were graphically presented. Experiments were independently repeated (n=3 to 6).

### Statistical analysis

Differences in cellular functions/parameters (viability, proliferation, intracellular calcium and cytotoxicity) between the irradiated and un-irradiated keratinocytes for each model were estimated using the Student’s t-test or One Way ANOVA for parametric or normally distributed data and the Mann-Whitney Rank Sum Test and Kruskal-Wallis test for non-parametric or non-normally distributed data. Alpha was set at the level of 0.05 (95%). The effect of laser irradiation on a change in viability, proliferation, intracellular calcium or cytotoxicity was estimated for each model (EtOH, H₂O₂, tBHP or oligomycin) compared to normal irradiated keratinocytes. We used normal irradiated human keratinocytes as the reference group – where other variables (i.e. wavelength, dose/fluence, duration of irradiation and irradiation conditions) were kept constant [13, 21, 31]. The change between irradiated and un-irradiated cells for each cellular parameter was calculated and expressed as a percentage (%). Analysis was done using SigmaPlot 8.2 (SYSTAT software) and SAS 9.1 (SAS Institute Inc., Cary, NC).

### Results

**Morphology:** Un-irradiated and irradiated normal keratinocytes (CDD-1102 KERTr) showed a typical cobblestone appearance consistent with normal keratinocyte morphology. Cells treated with 5% EtOH showed a highly vacuolated morphology with evidence of cytoplasmic granules which may be mRNA stress granules or dynamic cytoplasmic foci in which stalled translation initiation complexes accumulate [32]. Cells treated with 5% EtOH showed changes consistent with autophagy however the presence of cytoplasmic stress granules indicates severe dam-

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

<table>
<thead>
<tr>
<th>Action</th>
<th>Incubation</th>
<th>Detection</th>
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<tbody>
<tr>
<td>LDH membrane integrity</td>
<td>Promega CytoTox 96 non-radioactive cytotoxicity assay (G1780)</td>
<td>The assay measures lactate dehydrogenase (LDH), a stable cytosolic enzyme, released into culture medium upon cell lysis.</td>
<td>30 min at room temperature and protected from light.</td>
</tr>
<tr>
<td>WST-1 cell proliferation</td>
<td>Roche WST-1 cell proliferation reagent (11644807 001)</td>
<td>A colorimetric assay for the non-radioactive quantification of cell proliferation, cell viability and cytotoxicity in a 96-well flat bottom plate.</td>
<td>Incubate cell suspension at 37°C and 5% CO₂ for 24 h and 96 h after laser irradiation then add WST-1 reagent. Incubate for 4 h at 37°C and 5% CO₂ with 10 μl WST-1 reagent.</td>
</tr>
<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>Incubate reagent and cells for 2 min at room temperature on an orbital shaker to induce lysis. Incubate for 10 min at room temperature and protected from light. Measure luminescence.</td>
</tr>
<tr>
<td>Intracellular calcium (Ca²⁺)</td>
<td>BioAssay QuantiChrom™ Calcium assay kit (DICA-500)</td>
<td>Phenolsulphonephthalein dye forms a very stable blue coloured complex specifically with free calcium.</td>
<td>Incubate reagent and cells for 3 min at room temperature in 96-well clear bottom plate.</td>
</tr>
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age which suggests apoptosis (cytotoxic) rather than autophagy (cytomodulatory) [16]. Cell morphology of tBHP showed changes characteristic of apoptosis (i.e. cell shrinkage, loss of membrane asymmetry and attachment and controlled disintegration of the cell into apoptotic bodies). Cells treated with 200 μM H2O2 showed a highly vacuolated morphology with double membranes consistent with autophagy. Oligomycin-treated cells showed evidence of cloudy swelling or autophagy, a reversible process characterized by large cytoplasmic vacuoles (Figure 2) [23]. Cells treated with 200 μM H2O2 or 0.4 μg/ml oligomycin showed evidence of autophagy in irradiated cells with a highly vacuolated morphology (arrows) presumed to present extensive recycling of damaged organelles (200 x magnification).

### Autophagy – 200 μM H2O2:
Cells treated with 200 μM H2O2 and irradiated showed an increase in ATP cell viability (p = 0.11), a decrease in LDH cytotoxicity (p = 0.672), a decrease in intracellular calcium (p = 0.024) and an increase in cell proliferation after 96 h (p = 0.014) compared to 200 μM H2O2 un-irradiated cells (Figure 3 and 4), however only intracellular calcium and cell proliferation were statistically significant.

The change between irradiated and un-irradiated 200 μM H2O2 treated cells was calculated and compared to the change between irradiated and un-irradiated normal keratinocytes. Irradiated cells treated with 200 μM H2O2 showed an increase in ATP viability (5% vs. 2%; p = 0.823), a decrease in LDH cytotoxicity (-13% vs. 3%; p = 0.134), a decrease in intracellular calcium (-30% vs. -5%; p = 0.02) and an increase in cell proliferation after 96 h (29% vs. 18%; p = 0.775) compared to irradiated normal keratinocytes, however only a decrease in intracellular calcium was statistically significant.

### Autophagy – 0.4 μg/ml oligomycin:
Cells treated with 0.4 μg/ml oligomycin and irradiated with 1.5 J/cm² did not show an increase in ATP cell viability (p = 0.981), showed a modest decrease in LDH cytotoxicity (p = 0.22), an 11.2% increase in intracellular Ca²⁺ (p = 0.04) and a 4.7% increase in cell proliferation after 96 h compared to 0.4 μg/ml oligomycin un-irradiated cells.

The protein content using the Quanti-IT protein assay (Invitrogen, Paisley UK, Q33211) confirmed the anti-proliferative effect as irradiated cells treated with oligomycin showed a -2.88% decrease (1.39 mg/ml) in protein content or cell volume when compared to un-irradiated cells (1.43 mg/ml). Irradiated cells also showed a decrease in caspase 3/7 activity (-9.33%), a decrease in cytochrome c (-10.07%) and an increase in JC-1 emission fluorescence ratio (p = 0.002).
The protein content using the Quanti-IT protein assay (Invitrogen, Paisley UK, Q33211) confirmed the results as irradiated cells treated with tBHP showed an 11.9% increase (0.963 mg/ml) in protein content or cell volume when compared to un-irradiated cells (0.848 mg/ml) however the NADH CellTiter-Blue® Cell Viability Assay (Promega WI, G8080) showed a significant decrease in fluorescence (indicator of cell number and metabolic activity or viability) in irradiated cells (p = 0.044). The protein content and WST-1 confirms an increase in cell number however the NADH and ATP luminescence confirms a significant decrease in cell viability. Irradiated cells treated with tBHP showed an increase in JC-1 (ΔΨmt) mitochondrial membrane potential (5.4%), a decrease in cytochrome c (-12.5%) and a decrease in caspase 3/7 activity (-11.0%) which indicates an anti-apoptotic effect (results not shown).

The change between irradiated and un-irradiated 12 mM tBHP treated cells was calculated and compared to the change between irradiated and un-irradiated normal keratinocytes. Irradiated cells treated with 12 mM tBHP showed a decrease in ATP viability (-52% vs. 2%; p = 0.04), an increase in LDH cytotoxicity (17% vs. 3%; p = 0.014), a decrease in intracellular calcium (-6% vs. -5%; p = 0.915) and a decrease in cell proliferation after 96 h (-9% vs. 18%; p = 0.902) compared to irradiated normal keratinocytes. Only the ATP cell viability and LDH cytotoxicity showed statistically significant differences between the irradiated 12 mM tBHP treated cells and the un-irradiated cells or the normal keratinocyte cells.

**Discussion**

We demonstrate that in vitro cell stress models using 5% EtOH, tBHP, 200 μM H2O2 or oligomycin induces cellular damage. We demonstrate that laser irradiation with 1.5 J/cm² promotes cell survival of 200 μM H2O2 treated cells by increasing cell proliferation and decreasing intracellular calcium. Oligomycin and H2O2 autophagic cells respond differently to laser irradiation as the in vitro models use different mechanisms of action to induce cell stress which results in different levels of cellular damage. Laser irradiation may contribute to cell survival by...
conserving cellular components until damaged organelles can be recycled or the cellular function of the compromised cells can be restored to maintain homeostasis.

As differentiation of keratinocytes share similarities with programmed cell death, it has been proposed that apoptosis-like mitochondrial impairment triggers keratinocyte differentiation. In particular, it has been shown that the treatment of keratinocytes with inducers of mitochondrial dysfunction (rotenone, staurosporine and protoporphyrin) leads to differentiation-related changes, including flattened morphology, stratification and expression of keratin 10. Reports have suggested that the activation of the apoptotic pathway is necessary for keratinocyte differentiation. A decrease in mitochondrial membrane potential and the release of cytochrome c for transcription factor activation and regulation of gene expression have been reported during in vitro keratinocyte terminal differentiation [32-35]. Studies have reported that a rise in intracellular free calcium is associated with an increase in Ca$^{2+}$ transport across the plasma membrane and is a common mechanism controlling in vitro differentiation in mouse keratinocytes [36, 37]. Both oligomycin and tBHP treated cells showed an increase in mitochondrial membrane potential and decrease in cytochrome c which does not indicate keratinocyte differentiation in these models.

Elevated intracellular Ca$^{2+}$ ([Ca$^{2+}$]) levels trigger growth arrest (anti-proliferative) and induce differentiation of keratinocytes [38]. In H$_2$O$_2$ irradiated cells, the decrease in intracellular Ca$^{2+}$ is consistent with an increase in the growth rate. Results from this study suggest that laser irradiation using 1.5 J/cm$^2$ promotes cell survival since the H$_2$O$_2$ irradiated cells revert to active, viable cells that are capable of actively proliferating within 96 h of laser irradiation. Some studies have suggested that core machinery for autophagy is conserved which plays an important role during proliferation and differentiation [39]. A possible explanation may also be that the over-expression of the anti-apoptotic protein Bcl-2 decreases the endoplasmic reticulum (ER) Ca$^{2+}$ load and protects cells from death. Alternatively buffering of intracellular free calcium can, in some cases, inhibits cell death. The decrease in LDH cytotoxicity in H$_2$O$_2$ irradiated cells supports an anti-apoptotic effect as damage to the cytoplasm and the plasma membrane would result in an increase in the LDH activity. When comparing the change between H$_2$O$_2$ irradiated and normal irradiated cells results suggest that laser irradiation can stimulate H$_2$O$_2$ treated cells so that their cellular function (ATP viability and cell proliferation) is similar or greater (although not statistically significant) than normal irradiated human keratinocytes. The decrease in intracellular calcium is consistent with cell growth and cell survival as increased intracellular calcium concentration can initiate intracellular apoptotic signalling.

Oligomycin itself inhibits ATP synthase, disrupts mitochondrial membrane potential (ΔΨmt) and may be similar to that of p-(tri-fluoromethoxy) phenyl-hydrazone (fCCP) which uncouples oxidative phosphorylation [21]. Studies have reported that oligomycin results in mitochondrial breakdown with concomitant Ca$^{2+}$ influx. Modestly elevating [Ca$^{2+}$], can inhibit apoptosis since [Ca$^{2+}$], mediated multiple signalling cascades (i.e. Ca$^{2+}$/calmodulin-dependent protein kinases kinase, protein kinase B and the phosphorylation of BAD) are critical for cell survival. Results from this study indicate that irradiation with 1.5 J/cm$^2$ results in a modest increase in cell number (<20%) while maintaining membrane integrity with a pro-survival or anti-apoptotic effect. The effect of laser irradiation on ATP viability may be limited in oligomycin treated and irradiated cells as the chemical itself inhibits oxidative phosphorylation and ATP synthesis. Increased [Ca$^{2+}$], in oligomycin treated and irradiated cells may be responsible for an anti-apoptotic or pro-survival effect which protects cells from death and maintains cell numbers. Higher levels of [Ca$^{2+}$], would result in the activation of the apoptotic pathway, trigger growth arrest and induce keratinocyte differentiation. The LDH cytotoxicity, caspase 3/7, cytochrome c and
JC-1 results confirmed an autophagic and not apoptotic cell death pathway. The increase in intracellular calcium is most likely related to the action of oligomycin itself and not due to apoptosis or keratinocyte differentiation. It is well known that laser irradiation protects cells against caspase-mediated apoptosis, most likely by decreasing ROS production, down-regulating pro-apoptotic proteins and activating anti-apoptotic proteins, as well as by increasing energy metabolism.

5% EtOH is both cytotoxic and cytostatic which explains the decrease in cell number observed in the morphology results. The significant increase in LDH cytotoxicity indicates late apoptotic changes since the cytoplasm and the plasma membrane become seriously damaged which would result in an increase in the LDH activity. Cell death can also be induced when Ca²⁺ gains entry from the extracellular medium via the plasma membrane and elevations in intracellular Ca²⁺ may mediate apoptosis. Irradiated 5% EtOH treated cells showed a decrease in intracellular calcium which indicates another mechanism, possibly the lowering of extracellular Ca²⁺, blocking membrane Ca²⁺ channels or by interfering with calcium-dependent secondary messenger systems, which by themselves can trigger apoptosis in vitro [40, 41]. Irradiated 5% EtOH treated cells showed an additional decrease in cell number and increase in cytotoxicity suggesting that laser irradiation may contribute to or precipitate apoptosis in these cells. Laser irradiation may affect apoptotic signalling, enzymes or regulatory proteins which are essential to initiating the apoptotic pathway. These proteins function by either targeting mitochondria functionality or directly transducing the signal via adaptor proteins to the apoptotic mechanisms. Irradiated EtOH treated apoptotic cells showed poor cellular function (significant increase in LDH cytotoxicity, decrease in intracellular calcium and decrease in proliferation) compared to irradiated normal human keratinocytes suggesting that laser irradiation had no beneficial effect on these cells – on the contrary, laser irradiation appears to precipitate apoptosis possibly by interfering with mitochondria functionality however further studies are needed to confirm this.

tBHP uncouples cellular respiration and induces the production of ROS, lipid peroxidation, mitochondrial dysfunction, ATP depletion and impaired active transport. Therefore tBHP itself may be responsible for limiting the effect of laser irradiation on ATP synthesis. Studies have shown that laser therapy attenuates reactive oxygen species (ROS) production [42] or blocks the effect of ROS released [43]. Results from this study suggest that laser irradiation may reduce ROS and exert an anti-apoptotic, protective or pro-survival effect on pro-apoptotic cells (reversible) or repair oxidative stress to reduce apoptotic signals however the significant decrease in viability and small increase in cell number indicates that laser irradiation cannot affect cells that are already committed to apoptosis. Again, laser irradiation may affect apoptotic signalling, enzymes or regulatory proteins which are essential to initiating or stopping the apoptosis pathway. The irradiated tBHP treated cells showed a significant decrease in ATP viability, increase in cytotoxicity and modest decrease in intracellular calcium consistent with a minor increase in cell number (<5%). An increase in [Ca²⁺]; would indicate growth arrest or mediate apoptosis, both at early stages and late steps (post commitment point). Irradiated tBHP treated apoptotic control cells showed poor cellular function (decreased ATP viability, increased LDH cytotoxicity while intracellular calcium and proliferation after 96 h were similar) compared to normal irradiated human keratinocytes.

The switch from a life to a death signal involves the coincidental detection of Ca²⁺ and pro-apoptotic stimuli and depends on the amplitude of the mitochondrial Ca²⁺ signal. Because of the toxicity of Ca²⁺ ions, a low Ca²⁺ concentration must be maintained in the cytoplasm. Cell stress often results in highly elevated calcium levels, depolarised mitochondrial membrane potential, decreased cAMP levels and decreased ATP production. When low ATP production persists, DNA synthesis reduces significantly and pro-apoptotic factors increase heralding cell death [44]. Laser irradiation is believed to stimulate the redox activity in the respiratory chain with subsequent effects on intracellular ion concentration including calcium. Laser irradiation has been shown to cause mitochondrial polarization which improves membrane permeability leading to increased Ca²⁺ flux, pH value, cAMP levels, ATP production, anti-apoptotic factors, transcription factors and DNA synthesis. This cascade of cellular events accelerates cell proliferation [44].

Generation of reactive oxygen species (ROS) through oxidative stress causes cell death or compromises the long-term survival of cells [17]. The role of ROS in the induction of autophagic cell death is not clear however it has been shown that under nutrient starvation, ROS induces autophagy. It has been suggested that ROS could be involved in caspase-independent cell death [45]. ROS has many effects on cells including DNA damage, mitochondrial dysfunction, activation of signalling pathways and activation of transcription factors leading to upregulation of genes [46]. Studies have confirmed that laser phototherapy (gallium-arsenide GaAs, 904 nm, 45 mW average power, 5 J/cm² for 35 s) reduces oxidative stress [47]. Laser phototherapy increases levels of superoxide dismutase (SOD), which is key in the process of clearing ROS, so phototherapy should theoretically help to prevent or even reduce some of the damaging effects of ROS. Studies using visible (634 nm) red laser light have shown that laser irradiation increases nitric oxide (NO) production but decreases intracellular ROS so laser irradiation could reduce the effect of ROS and promote cell survival.

Limitations: These findings should be considered in light of the study limitations. Firstly, cell viability was assessed using the ATP luminescent assay which can under-estimate or over-estimate the metabolic ability of cells to proliferate. However, the assay used is a homogenous method of determining the number of viable cells in culture based on the quantification of ATP, which signals the presence of metabolically active cells. Cells may exhibit delayed death or survival effects that cause a temporary indication of toxicity. Additional assays such as growth curves, clonogenic assays and DNA synthesis (BrdU incorporation) [48] will be explored. Secondly, despite seeding a constant cell number at the beginning of the experiment each chemical (H₂O₂, tBHP, 5% EtOH or oligomycin) affected the cell number differently resulting a different final cell number prior to laser irradiation. To reduce this effect, un-irradiated controls were used for each stress condition while normal keratinocytes were used.
as the reference to estimate the effect of cell type on an increase in viability, proliferation, intracellular calcium and cytotoxicity – changes between the irradiated and un-irradiated cells were calculated and then compared between the models (i.e. normal vs. oligomycin). Thirdly, true assays for apoptosis and autophagy were not reported – although caspase 3/7 results were discussed for oligomycin and IBHP. Future work will include assays to assess apoptosis (by activation of executioner caspases or by observing chromatin condensation and fragmentation using fluorescent probes) and autophagy (by electron microscopy assessing lipidation of the LC3 protein or using other methods recently described 49-51). Lastly, cellular responses were measured within 1 hr, which is sufficient to measure the direct effect of laser irradiation 52, 53 however a longer incubation period (> 24 h) is required to demonstrate cell proliferation (24 and 96 h) and protein expression. Cellular responses were observed after a 1 hr incubation which suggests the mechanism by which laser irradiation with 648 nm reverses autophagy does not require gene transcription however this still needs to be confirmed.

Conclusion

Cells treated with 200 μM H2O2 and irradiated showed changes in intracellular calcium consistent with growth when compared to un-irradiated cells or when compared to irradiated normal keratinocytes. Irradiated 200 μM H2O2 treated cells reverted to metabolically active, viable cells capable of proliferating within 96 h of laser irradiation. Results suggest that laser irradiation at 648 nm stimulated 200 μM H2O2 treated cells so that their cellular function was similar or even greater than that of irradiated normal keratinocytes. The role of intracellular calcium in cell death is controversial – further investigations are warranted to determine the effect of laser irradiation on calcium-dependent secondary messenger systems, calcium channel blockers, apoptotic signalling and regulatory proteins and the combined effect on cell signalling and mitochondrial functionality. Results from this study indicate that visible red laser light can exert different responses in cells as they respond to stress. These include (i) anti-differentiating (ii), stimulating cells to revert to viable actively proliferating cells (iii) promoting cell survival by potentially reducing ROS, and (iv) promoting cell proliferation. Results suggest that laser irradiation may potentially promote a pro-survival or anti-apoptotic effect after cell stress. This points to the potential benefit of irradiating autophagic keratinocytes to prevent disease progression or promote health.

References


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