THE EFFECTS OF LOW LEVEL LASER THERAPY ON THE PROLIFERATION OF KERATINOCYTES

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Low-level laser therapy (LLLT) is used clinically to stimulate healing, including re-epithelialization of wounds. The aim of this study was to investigate the effects of laser therapy on the proliferation of keratinocytes. The effects of laser emissions at 632.8 nm (continuous wave) and at 904 nm (200 ns pulses at frequencies of 1205 pps and 3125 pps) at energy densities of 0.25–4 J/cm² were investigated.

Phenotypic alterations have been noted in keratinocytes from cultures kept at confluence for several days. Cells from confluent and sub-confluent keratinocyte cultures were used in order to investigate whether these changes could be reversed more quickly, on subculture, following exposure to laser emission. Keratinocytes were grown in either 5% or 1% fetal calf serum (FCS), proliferation being slower in the latter, and the effects of LLLT on them compared.

The results obtained indicate that: (1) LLLT stimulates keratinocyte proliferation; (2) the delay in proliferation observed in keratinocytes taken from confluent cultures may be reduced by laser irradiation; (3) LLLT has a greater effect on cultures grown in the poor conditions of 1% FCS than in the better conditions provided by 5% FCS; and (4) LLLT has biostimulatory properties at energy densities as low as 0.25 J/cm².

KEY WORDS Keratinocytes Laser Proliferation Therapy Wound healing

Introduction

The epidermis is a vital barrier, giving protection from the external environment, reducing dehydration and infection. Open wounds therefore leave the patient vulnerable and can lead to septicaemia.

Treatment of patients with chronic lesions such as non-healing ulcers is expensive, prolonged periods of hospitalization often being required. Pressure sores, for example, were estimated in 1982 to cost the National Health Service in excess of £150 million per year.¹ In addition, prolonged hospitalization may have great financial and social implications for the individual concerned. It is therefore in the interests of the community and the individual patient to achieve rapid healing of wounds. Any treatment which speeds up closure of pressure sores and other chronic skin lesions is potentially of great value, both financially and in reduction of discomfort to patients.

Laser therapy has been shown to increase the rate of re-epithelialization of chronic skin lesions;² indeed, this was considered to be the primary reason for enhanced wound closure in irradiated full-thickness surgical wounds.³ Although the effects of laser therapy on keratinocyte activity have been

observed, they have not been adequately quantified. The aim of this study was to investigate the effects of 632.8 nm coherent continuous wave light and 904 nm coherent light pulsed at 1205 pps and 3125 pps on keratinocyte proliferation *in vitro*.

Although Weiss⁴ described movement as a characteristic of keratinocytes, in normal intact epidermis they are a relatively quiescent population of cells. The epidermis consists of many 'epidermal proliferative units' each with a basal layer of one stem cell and 10 or more 'transient amplifying cells' which together proliferate to provide one cell per day to the overlying column of stratified cells.⁵ In contrast, during re-epithelialization, keratinocytes are highly active; the defect is closed by a combination of epiboly, a form of cell migration, and rapid proliferation of the cells synthesizing the matrix over which they migrate and lysing a path beneath all unviable tissue.

Following injury, there is a lag phase of 12–14 h before keratinocyte migration begins⁶ and of 42–66 h before the onset of proliferation.⁷ This delay is paralleled *in vitro*, freshly isolated keratinocytes and those from confluent cultures being initially unable to migrate across the culture vessel while those from subconfluent cultures can;⁸ this characteristic was named the 'confluent block'. Other features associated with epithelial regeneration are also associated with sub-confluent cultures, for example,

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cells from regenerating epidermis⁹ and sub-confluent cultures¹⁰ exhibit good plasma fibronectin receptor functions whereas those from intact epidermis⁹ and confluent cultures¹⁰ do not; this process is important since during re-epithelialization keratinocytes migrate over a temporary lamina containing fibrin and fibronectin rather than the laminin and type IV collagen of the permanent basal lamina.¹¹ *In vitro* these alterations take several days to occur. Given the differences between confluent and sub-confluent keratinocyte cultures, and given their similarities to cells from intact and regenerating epithelium respectively, it was decided to compare the effects of LLLT on keratinocytes from both sub-confluent cultures and from cultures left at confluence for several days.

Differing reports of the effectiveness of LLLT have been published; in general, those reporting negative results have irradiated fresh surgical wounds^{12,13} whereas those reporting positive results irradiated wounds where poor conditions exist such as burns,² although positive results have also been found by irradiation of surgical wounds.¹⁴ It was decided to irradiate cells cultured in medium containing less than the normal 10% fetal calf serum, growth being retarded in these poorer culture conditions, in an attempt to simulate the poor conditions found in chronic or slowly-healing wounds for three reasons: (1) laser therapy has greater efficacy with such wounds; (2) the potential benefits of stimulating healing in such wounds are greater; and (3) Karu¹⁵ considers cells cultured in poor conditions to have greater potential for stimulation of proliferation than those grown in good conditions.

LLLT has been described as having stimulatory effects at energy densities in the range 1–4 J/cm²,¹⁵ levels below this having no significant effect and greater energy densities being inhibitory. The keratinocytes were therefore irradiated at various energy densities up to, and including, 4 J/cm².

Materials and Methods

Keratinocyte Culture

Transformed guinea pig keratinocytes (obtained from the Eastman Dental School) were grown in 260 ml Nunc culture flasks, the culture medium being 20 ml of DMEM (Gibco) supplemented with 1% penicillin and streptomycin (Gibco) and 10% fetal calf serum (FCS) (Gibco). The cells were cultured in an incubator at 37°C with a saturated atmosphere of 5% CO₂ in air. Sub-culturing was carried out once confluence had been reached, approximately every 3–4 days.

When the cells were fully confluent the medium was decanted and the cells washed with 10% phosphate buffered saline (PBS) (Oxoid) containing

5% penicillin and streptomycin (Gibco). Three ml of 0.25% trypsin with EDTA (Gibco) were added to the flask and left for 30 s after which the trypsin was discarded and the flask left inverted in the incubator for 5 min; any remaining trypsin was thus drained from the cells, preventing further cellular damage. After checking the cells for separation, they were suspended in 3 ml of medium; the suspension was then poured into a centrifuge flask and centrifuged at 1500 rpm for 2 min. The supernatant was discarded and the pellet re-suspended in 3 ml of medium, 1 ml of which was placed in each of three 260 ml Nunc culture flasks, which were then made up with 20 ml of fresh medium. The flasks were replaced in the incubator. Each confluent flask contained approximately 10⁷ cells.

Preparation of Keratinocytes

Difficulty was experienced in growing keratinocytes in medium supplemented with 1% FCS; they were therefore cultured initially in 10% FCS. Prior to irradiation the medium was replaced with fresh medium containing a reduced level of FCS. The level of FCS was reduced from 10% to 5% for cells to be irradiated in medium supplemented with 5% FCS, the level of FCS being further reduced to 3% and then 1% for those cells to be irradiated in medium supplemented with 1% FCS. The cells were left to acclimatize to each new batch of medium for 12 h before the FCS level was reduced further.

Where cells from confluent cultures were to be used, the culture was left at confluence for 48 h prior to changing the medium as stated above. Where cells from sub-confluent cultures were to be used the cells were subcultured one day prior to changing the medium. This ensured that no patches of confluent cells existed within the culture at the time of the experiment.

Irradiation

The laser used was a Space mix-5 mid-laser (Benson Corporation); this has two outputs, a continuous wave He/Ne mode with a wavelength of 632.8 nm and a pulsed infra-red mode of wavelength 904 nm. The 904 nm output has five pulse frequency settings; settings 2 and 4 were used, the pulse frequencies of these being 1205 pps and 3125 pps respectively. The pulse length available was 200 ns. The cells were irradiated in 50 ml culture flasks (Nunc) the centre of which was positioned 10 cm from the front of the laser, the power densities at this point being 6.5 mW/cm² at 632.8 nm, 2.3 mW/cm² at 904 nm (1205 pps), and 5.7 mW/cm² at 904 nm (3125 pps).

The cells were prepared for irradiation in the

following manner. The medium was decanted and the cells rinsed with PBS (Oxoid). Three ml of trypsin with EDTA (Gibco) were added and left for 30 s after which the trypsin was discarded and the flask left inverted in the incubator for 5 min. The cells were suspended in fresh medium and the suspension then poured into a centrifuge tube and centrifuged for 2 minutes at 1500 rpm. The supernatant was discarded and the cells re-suspended in fresh medium supplemented with either 1% or 5% FCS. The cell concentration was determined by taking the average of six haemocytometer cell counts and the suspension was then diluted with fresh medium to give a concentration of 5×10^4 cells per ml.

The periods of irradiation were varied to give doses of 0.25, 0.5, 1, 2 and 4 J/cm². The periods of irradiation are shown in Table 1.

The cells were placed in 50 ml Nunc culture flasks; these were placed upright and the laser beam was directed across the width of the flasks in such a way that the centre of the beam was in the centre of the cell suspension. The flasks were positioned so that the centre of the suspension was at a distance of 10 cm from the end of the laser. Prior to irradiation, each flask was agitated to ensure that all the cells were in suspension; however, considerable settling may have occurred during the longer exposures, it being impossible to agitate the suspensions during irradiation.

After irradiation, the keratinocyte suspensions were plated out into 96-well plates (Nunc), 100 µl from each treatment being pipetted into each well of a row of eight wells giving 5000 cells per well. Six plates were set up for each experiment, the cells in one of the plates being fixed after each subsequent 24 h.

Cell Fixation

After removing the medium, each well of cells was rinsed with 100 µl phosphate buffered saline. The cells were fixed by the addition of 100 µl of methanol to each well. After 4 min, the methanol was removed and the cells allowed to dry in air.

Table 1. Periods of irradiation to which keratinocytes were exposed for each wavelength and pulsing frequency used

Irradiation mode	Period of irradiation (s) to give energy density of J/cm ² :				
	0.25	0.5	1.0	2.0	4.0
632.8 nm	39	77	154	308	616
904 nm	109	217	434	868	1736
(1205 pps)					
904 nm	44	88	175	350	700
(3125 pps)					

Cell Staining

Methylene blue (100 µl) was added to each well and left for 30 min. The methylene blue was then removed, the excess being washed off with three rinses of 100 µl borate buffer. Acid alcohol (100 µl) was added to each well and the plate agitated to ensure complete mixing of the eluted stain and acid alcohol.

The stained plate was then read on an eight channel Multiskan spectrophotometer at a wavelength of 650 nm; the results were statistically analysed using Student's *t*-test.

The above method is a modification of that used by Martin *et al.*¹⁶ who found that macrophage staining was proportional to cell number; Oliver *et al.*¹⁷ recently demonstrated the usefulness and accuracy of this assay method on various cell types. A standard curve was produced for keratinocytes (Figure 1), showing that staining is also proportional to cell number in this cell type.

Results

Due to the large volume of data obtained, a selection of results is presented here which best illustrate our findings.

Keratinocytes from a sub-confluent culture, grown in medium supplemented with 1% FCS and irradiated with 632.8 nm light

By day 3, all energy densities produced significantly increased cell numbers in comparison with the controls (4 J/cm², *p* = 0.05; 0.25, 0.5, 1 and 2 J/

Figure 1. Keratinocyte standard curve

Figure 2. The effect of 632.8 nm laser therapy on keratinocytes from a sub-confluent culture, grown in medium supplemented with 1% fetal calf serum

cm², $p = 0.01$) (Figure 2, Table 2). No treatment produced significant effects at day 4. Energy densities of 0.25, 0.5, 1 and 2 J/cm² producing significantly increased cell numbers again at day 5 ($p = 0.01$).

Keratinocytes from a sub-confluent culture, grown in medium supplemented with 5% FCS and irradiated with 632.8 nm light

All cultures grew at a similar rate, few points being significantly different from either the control or

Figure 3. The effect of 632.8 nm laser therapy on keratinocytes from a sub-confluent culture, grown in medium supplemented with 5% fetal calf serum

other treated cultures (Figure 3, Table 3). However, at days 2 and 3 the culture irradiated at 0.25 J/cm² showed significantly decreased cell numbers compared with the control ($p = 0.01$). By day 6, all energy densities resulted in significantly greater cell numbers than the control (4 J/cm², $p = 0.05$; 0.25, 0.5, 1 and 2 J/cm², $p = 0.01$).

Keratinocytes from a sub-confluent culture, grown in medium supplemented with 5% FCS and irradiated with 904 nm, 1205 pps light

Cell numbers significantly lower than the control were produced in cultures irradiated at 0.5 and 4 J/cm²

Table 2. Absorbance using cells from a sub-confluent culture grown in 1% FCS and irradiated with 632.8 nm light

Day	Data	Control	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.168	0.191	0.189	0.211	0.195	0.191
	S.D.	0.027	0.023	0.026	0.029	0.029	0.035
	p		ns	ns	< 0.01	ns	ns
2	X	0.224	0.248	0.256	0.240	0.261	0.266
	S.D.	0.040	0.024	0.040	0.026	0.026	0.020
	p		ns	ns	ns	< 0.05	ns
3	X	0.239	0.331	0.209	0.0320	0.360	0.265
	S.D.	0.016	0.021	0.021	0.019	0.032	0.021
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.05
4	X	0.362	0.384	0.375	0.362	0.406	0.360
	S.D.	0.064	0.019	0.030	0.039	0.051	0.052
	p		ns	ns	ns	ns	ns
5	X	0.304	0.413	0.428	0.368	0.438	0.332
	S.D.	0.036	0.026	0.042	0.036	0.066	0.020
	p		< 0.01	< 0.01	< 0.01	< 0.01	ns
6	X	0.460	0.438	0.434	0.364	0.448	0.409
	S.D.	0.084	0.033	0.046	0.024	0.088	0.085
	p		ns	ns	ns	ns	ns

Table 3. Absorbance using cells from a sub-confluent culture, grown in 5% FCS and irradiated with 632.8 nm light

Day	Data	Control	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.238	0.216	0.237	0.223	0.237	0.226
	S.D.	0.040	0.024	0.030	0.041	0.023	0.029
	p		ns	ns	ns	ns	ns
2	X	0.420	0.340	0.397	0.387	0.448	0.413
	S.D.	0.055	0.037	0.038	0.044	0.050	0.042
	p		ns	ns	ns	ns	ns
3	X	0.490	0.388	0.458	0.458	0.514	0.472
	S.D.	0.068	0.025	0.025	0.038	0.036	0.054
	p		< 0.01	ns	ns	ns	ns
4	X	0.655	0.637	0.581	0.693	0.732	0.719
	S.D.	0.088	0.077	0.055	0.084	0.072	0.120
	p		ns	ns	ns	ns	ns
5	X	0.931	0.948	0.897	1.065	1.145	1.074
	S.D.	0.119	0.134	0.097	0.149	0.115	0.153
	p		ns	ns	ns	< 0.01	ns
6	X	0.998	1.280	1.300	1.290	1.248	1.153
	S.D.	0.106	0.142	0.088	0.156	0.100	0.115
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.05

Figure 4. The effect of 904 nm (1205 pps) laser therapy on keratinocytes from a sub-confluent culture, grown in medium supplemented with 5% fetal calf serum

cm² at day 3 ($p = 0.05$) and at 0.25 J/cm² on days 3, 4 and 5 ($p = 0.01$) (Figure 4, Table 4). By day 5, energy densities of 1 J/cm² ($p = 0.05$) and 2 J/cm² ($p = 0.01$) produced significantly increased cell numbers. The control curve showed a decreased rate of proliferation between days 5 and 6, but in the irradiated cultures there was no decrease, cultures irradiated at 0.5, 1, 2 and 4 J/cm² producing significantly greater cell numbers than the control ($p = 0.01$).

Table 4. Absorbance using cells from a sub-confluent culture, grown in 5% FCS and irradiated with 904 nm (1205 pps) light

Day	Data	Con- trol	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.238	0.208	0.200	0.226	0.219	0.226
	S.D.	0.040	0.064	0.050	0.026	0.032	0.031
	p		ns	ns	ns	ns	ns
2	X	0.420	0.279	0.352	0.374	0.376	0.387
	S.D.	0.055	0.027	0.035	0.036	0.038	0.031
	p		< 0.01	< 0.01	ns	ns	ns
3	X	0.490	0.361	0.417	0.437	0.443	0.427
	S.D.	0.068	0.036	0.022	0.033	0.033	0.029
	p		< 0.01	< 0.05	ns	ns	< 0.05
4	X	0.655	0.483	0.657	0.683	0.818	0.647
	S.D.	0.088	0.044	0.053	0.051	0.061	0.057
	p		< 0.01	ns	ns	< 0.01	ns
5	X	0.931	0.712	1.004	1.037	1.193	1.108
	S.D.	0.119	0.097	0.074	0.068	0.133	0.108
	p		< 0.01	ns	< 0.05	< 0.01	ns
6	X	0.998	0.970	1.292	1.400	1.422	1.336
	S.D.	0.106	0.123	0.069	0.098	0.140	0.102
	p		ns	< 0.01	< 0.01	< 0.01	< 0.01

Figure 5. The effect of 904 nm (1205 pps) laser therapy on keratinocytes from a sub-confluent culture, grown in medium supplemented with 1% fetal calf serum

Keratinocytes from a sub-confluent culture grown in medium supplemented with 1% FCS and irradiated with 904 nm, 1205 pps light

All energy densities produced significantly increased cell numbers compared with the control (Figure 5,, Table 5) on virtually all days at the $p = 0.01$ level, except at day 6 (0.25 J/cm² and 0.5 J/cm², not significant; 1 J/cm² and 2 J/cm², $p = 0.05$). In addition, significant differences were found between

Table 5. Absorbance using cells from a sub-confluent culture, grown in 1% FCS and irradiated with 904 nm (1205 pps) light

Day	Data	Con- trol	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.168	0.206	0.220	0.235	0.234	0.236
	S.D.	0.027	0.022	0.026	0.026	0.023	0.020
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
2	X	0.224	0.274	0.345	0.381	0.358	0.379
	S.D.	0.040	0.020	0.033	0.029	0.023	0.015
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
3	X	0.238	0.366	0.411	0.465	0.467	0.490
	S.D.	0.016	0.062	0.033	0.039	0.078	0.031
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	X	0.362	0.447	0.598	0.684	0.675	0.656
	S.D.	0.064	0.054	0.058	0.060	0.068	0.016
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
5	X	0.304	0.458	0.482	0.635	0.610	0.651
	S.D.	0.036	0.046	0.023	0.057	0.074	0.058
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
6		0.428	0.470	0.566	0.579	0.644	
	X	0.097	0.072	0.070	0.083	0.096	
	S.D.	0.460	ns	ns	< 0.05	< 0.05	< 0.01
	p	0.084					

cultures irradiated at different energy densities; at day 4, 1, 2 and 4 J/cm² produced greater cell numbers than 0.25 J/cm², ($p = 0.01$), and 1 and 2 J/cm² resulted in greater cell numbers than 0.5 J/cm² ($p = 0.05$). Proliferation was reduced in all cultures after day 4, the cell number declining in cultures treated with energy densities of 0.5, 1 and 2 J/cm².

Keratinocytes from a confluent culture grown in medium supplemented with 1% FCS and irradiated with 904 nm, 1205 pps light

The control culture showed little change in the period studied (Figure 6, Table 6), although there was a slight increase in cell number between days 4 and 5. By two days after plating out, all energy densities produced significantly increased cell numbers compared with the control ($p = 0.01$). The effect of irradiation at 0.25 J/cm² was significantly greater than that of 4 J/cm² by the fourth day ($p = 0.01$). Proliferation of the culture irradiated at 0.5 J/cm² slowed after the fourth day, the rest doing so after day 5, cell number actually declining.

Keratinocytes from a sub-confluent culture grown in medium supplemented with 1% FCS and irradiated with 904 nm, 3125 pps light

Energy densities of 0.5, 1, 2 and 4 J/cm² produced significantly increased proliferation from day 1 until day 5 ($p = 0.01$), 0.25 J/cm² also doing so at days 1 ($p = 0.05$), 3 ($p = 0.01$), and 4 ($p = 0.05$) (Figure 7, Table 7). At day 4, significant differences were observed between the cell numbers of cultures treated at different energy densities (0.5, 1, 2 and

Table 6. Absorbance using cells from a confluent culture, grown in 1% FCS and irradiated with 904 nm (1205 pps) light

Day	Data	Con- trol	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.262	0.311	0.315	0.307	0.311	0.310
	S.D.	0.047	0.039	0.026	0.033	0.040	0.042
	p		< 0.05	< 0.05	< 0.05	< 0.05	ns
2	X	0.170	0.470	0.394	0.427	0.394	0.420
	S.D.	0.024	0.055	0.036	0.036	0.035	0.037
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
3	X	0.180	0.647	0.650	0.662	0.643	0.625
	S.D.	0.022	0.097	0.087	0.088	0.107	0.073
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	X	0.157	0.856	0.825	0.821	0.781	0.759
	S.D.	0.021	0.057	0.064	0.064	0.094	0.038
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
5	X	0.199	1.010	0.863	1.074	1.066	1.021
	S.D.	0.020	0.100	0.116	0.161	0.191	0.098
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
6	X	0.175	0.974	0.919	1.065	1.002	0.918
	S.D.	0.025	0.099	0.091	0.068	0.086	0.080
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Figure 6. The effect of 904 nm (1205 pps) laser therapy on keratinocytes from a confluent culture, grown in medium supplemented with 1% fetal calf serum

Figure 7. The effect of 904 nm (3125 pps) laser therapy on keratinocytes from a sub-confluent culture, grown in medium supplemented with 1% fetal calf serum

4 J/cm² > 0.25 J/cm², $p = 0.01$; 1 J/cm² > 0.5 J/cm² $p = 0.01$; 1 J/cm² > 4 J/cm², $p = 0.01$; 2 J/cm² > 4 J/cm², $p = 0.05$). Cultures exposed to all energy densities, and the control culture showed a decline in cell numbers after four days, the control and cultures treated at 0.25, 0.5 and 1 J/cm² subsequently showing further proliferation.

Keratinocytes from a confluent culture grown in medium supplemented with 1% FCS and irradiated with 904 nm, 3125 pps light

The control showed little sign of proliferative activity (Figure 8, Table 8); the irradiated cultures all showed

Table 7. Absorbance using cells from a sub-confluent culture, grown in 1% FCS and irradiated with 904 nm (3125 pps) light

Day	Data	Con-trol	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.167	0.199	0.210	0.233	0.232	0.231
	S.D.	0.027	0.022	0.030	0.029	0.027	0.027
	p		< 0.05	< 0.01	< 0.01	< 0.01	< 0.01
2	X	0.224	0.249	0.302	0.327	0.343	0.303
	S.D.	0.040	0.026	0.025	0.023	0.024	0.032
	p		ns	< 0.01	< 0.01	< 0.01	< 0.01
3	X	0.239	0.330	0.408	0.520	0.450	0.427
	S.D.	0.016	0.030	0.025	0.035	0.044	0.021
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	X	0.362	0.435	0.527	0.618	0.577	0.515
	S.D.	0.064	0.042	0.042	0.059	0.059	0.020
	p		< 0.05	< 0.01	< 0.01	< 0.01	< 0.01
5	X	0.304	0.323	0.440	0.520	0.562	0.492
	S.D.	0.064	0.028	0.026	0.036	0.023	0.059
	p		ns	< 0.01	< 0.01	< 0.01	< 0.01
6	X	0.460	0.433	0.566	0.566	0.549	0.478
	S.D.	0.084	0.075	0.032	0.032	0.054	0.040
	p		ns	ns	< 0.01	< 0.05	ns

Table 8. Absorbance using cells from a confluent culture, grown in 1% FCS and irradiated with 904 nm (3125 pps) light

Day	Data	Con-trol	Energy density (J/cm ²)				
			0.25	0.5	1	2	4
1	X	0.262	0.313	0.319	0.309	0.284	0.232
	S.D.	0.047	0.045	0.037	0.043	0.040	0.033
	p		< 0.05	< 0.05	ns	ns	ns
2	X	0.170	0.365	0.495	0.443	0.409	0.312
	S.D.	0.024	0.046	0.034	0.029	0.031	0.033
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
3	X	0.180	0.562	0.704	0.739	0.601	0.476
	S.D.	0.022	0.085	0.063	0.063	0.059	0.056
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
4	X	0.157	0.578	0.839	0.837	0.740	0.605
	S.D.	0.021	0.059	0.041	0.049	0.051	0.066
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
5	X	0.199	0.762	1.001	0.791	0.944	0.477
	S.D.	0.020	0.092	0.116	0.103	0.141	0.051
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01
6	X	0.175	0.926	1.023	0.827	0.876	0.560
	S.D.	0.025	0.102	0.062	0.084	0.099	0.144
	p		< 0.01	< 0.01	< 0.01	< 0.01	< 0.01

Figure 8. The effect of 904 nm (3125 pps) laser therapy on keratinocytes from a confluent culture, grown in medium supplemented with 1% fetal calf serum

significantly increased cell numbers ($p = 0.01$) from day 2 onwards. At day 4, just before some of the cell numbers began to plateau, cultures exposed to energy densities of 0.5 and 1 J/cm² produced significantly greater numbers of cells than those exposed to 2 J/cm² ($p = 0.01$) which produced significantly greater cell numbers than energy densities of 0.25 and 4 J/cm² ($p = 0.05$). After day 4 some cultures ceased to increase in cell number, those irradiated at 1 and 4 J/cm² showing decreased cell numbers by day 5.

Discussion

That low level laser therapy using 632.8 nm light has a biostimulatory effect on keratinocytes was clearly demonstrated using cells from a sub-confluent culture grown in 1% FCS (Figure 2). These results, however, were not endorsed when cells grown in 5% FCS were used (Figure 3), few significant results being recorded. These conflicting results may be explained by the difference in culture conditions, cells proliferating slowly due to the poor culture conditions of 1% FCS having a greater potential for stimulation to a higher rate of proliferation than those grown in the relatively better conditions of 5% FCS which are already proliferating at a higher rate.

It has been stated that LLLT has a biostimulatory effect in the energy density range of 1–4 J/cm², greater energy densities inhibiting proliferation;¹⁵ although not inhibitory, the least stimulatory effect in Figure 2 was produced by 4 J/cm², suggesting that this inhibitory effect had been initiated, but was not great enough to block any stimulatory mechanism.

The poor potential for stimulation by LLLT of keratinocytes, grown in 5% FCS as compared with 1% FCS, is again demonstrated by Figures 4 and 5. Cultures irradiated with 904 nm (1205 pps) light produced significantly greater cell numbers at only a few time points when the cells were grown in 5% FCS, some treatments resulting in temporarily reduced cell numbers. In contrast all energy densities produced significantly increased proliferation in cells cultured in 1% FCS. Figure 5 reveals that the

biostimulatory effect of LLLT is dose-dependent. 0.25, 0.5 and 1 J/cm² resulting in progressively greater stimulation of proliferation. The presence of a limiting factor on this stimulation is also demonstrated, the effects of 1, 2 and 4 J/cm² being statistically similar.

Cells taken from confluent cultures exhibited a decreased capacity for proliferation, the control showing very little proliferative activity (Figure 6); in contrast the irradiated cultures all proliferated relatively rapidly. It has been demonstrated that keratinocytes kept at confluence undergo phenotypic alteration¹⁰ producing the confluent block;⁸ the data illustrated in Figure 6 demonstrate that these changes result in a reduced capacity for proliferation and that the capacity for proliferation may be restored by irradiation at 904 nm (1205 pps).

The dose-dependent nature of LLLT biostimulation and the effect of the poor culture medium are confirmed by the data illustrated in Figure 7 using 904 nm (3125 pps) light. At day 4 all treated cultures had greater cell numbers than the control (see Figure 7); significant differences were also observed between the different treatments. A dose-dependent pattern is again clearly demonstrated, stimulation increasing with dose to a maximum at 1–2 J/cm². The results obtained suggest that there may be initiation of inhibitory effects at higher levels resulting in decreased stimulation with 4 J/cm².

The effects of LLLT on the proliferation of keratinocytes from a confluent culture are also demonstrated in Figure 8, irradiation with 904 nm (3125 pps) light resulting in rapid initiation of proliferation.

In producing these results, the laser light may have acted directly on the keratinocytes or alternatively it may have modified the culture medium in such a manner as to stimulate proliferation. There is some evidence that LLLT does act partially by modifying components of culture medium;¹⁸ however, it was found that this effect was very small in comparison with the direct effect on cells. Furthermore, irradiation of the cells in phosphate-buffered saline followed by immediate transfer to fresh medium had an effect of similar magnitude to irradiation of the cells and culture medium.¹⁸

The primary photoacceptors for light are thought to be components of the terminal oxidation redox chain; cytochrome oxidase, for example, is thought to be the photoreceptor for 820 nm light¹⁹ while cytochrome a/a₃ absorbs red light,²⁰ and may be the photoactive molecule upon which the He-Ne light, used here, exerts its effect. Dichromic experiments have revealed the presence of more than one photoacceptor,¹⁵ although whether each photoreceptor is on a different molecule is not known. It is thought that one effect of LLLT is to oxidize the primary photoreceptor, the evidence for this being that methylene blue, an oxidizing agent,

is able to mimic the biostimulatory effects of LLLT and that Na₂S₂O₄, a reducing agent, inhibits these effects.²¹

LLLT has been demonstrated to stimulate cellular respiration; the He-Ne laser for example has been shown to increase the proton gradient across the inner mitochondrial membrane, and to stimulate ATP production.²² This would result in an increase in the energy available for anabolic processes and would therefore allow proliferation to proceed in cells where this had previously been inhibited by low ATP levels. Herbert *et al.*¹⁹ measured ATP levels in lymphocytes before and after irradiation with 820 nm light, and found a low ATP/ADP ratio of 0.9 prior to irradiation which rose to 4.1 when irradiated with an energy density of 1.2 J/cm²; they consider the poor medium with low levels of respiratory substrate to be responsible for the low ATP/ADP ratio. Should medium supplemented with 1% FCS produce a low ATP/ADP ratio in keratinocytes, it may be that stimulation of ATP production was responsible for the results presented here, low ATP levels inhibiting anabolic processes.²³ This mode of action would explain the different results found in keratinocytes grown in 1% FCS and 5% FCS, those grown in 1% FCS proliferating more slowly, possibly due to lower ATP levels as a result of the lower respiratory substrate concentration. They would therefore have greater potential for increased proliferation due to the elevation of ATP levels by the stimulation of terminal oxidation. It has also been suggested that laser therapy might alter the rate of proliferation by the alteration of intracellular H⁺, K⁺ and cyclic AMP levels,¹⁵ all of which are involved in control of mitosis.

The effects of LLLT on ATP production may explain the differing reports of the effectiveness of laser therapy; McCaughan *et al.*¹³ for example, report of no significant effect of 600 nm irradiation at 2 J/cm² on the rate of closure of full thickness excised lesions in guinea pig, and Anneroth *et al.*,¹² found no significant effect when full thickness excised lesions in rats were irradiated at 904 nm. If the cells involved in the repair of these lesions already had high ATP content, further increase may not have been possible. Mester *et al.*,² in contrast, found a significantly increased rate of wound closure in irradiated third degree burns. Cells in the wound irradiated by Mester *et al.*,² will have had poor conditions for growth; burn wounds are ischaemic due to microvascular coagulation and since ischaemia leads to low cellular ATP content²⁴ these cells will have proliferated slowly and will also have had considerable potential for increased proliferation by the stimulation of ATP production.

The results presented here and those of other workers demonstrate that LLLT of various wavelengths has a biostimulatory effect on keratinocytes.

This effect seems to be dependent on the prior poor proliferation of these cells brought about by poor culture conditions. Given these findings, we consider that the effect of LLLT on chronic skin lesions, in which the environmental conditions are poor, merits quantitative investigation.

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