Influence of Beam Shape on *in-vitro* Cellular Transformations in Human Skin Fibroblasts

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ABSTRACT

A variety of strategies have been utilised for prevention and treatment of chronic wounds such as leg ulcers, diabetic foot ulcers and pressure sores[1]. Low Level Laser Therapy (LLLT) has been reported to be an invaluable tool in the enhancement of wound healing through stimulating cell proliferation, accelerating collagen synthesis and increasing ATP synthesis in mitochondria to name but a few[2]. This study focused on an in-vitro analysis of the cellular responses induced by treatment with three different laser beam profiles namely, the Gaussian (G), Super Gaussian (SG) and Truncated Gaussian (TG), on normal wounded irradiated (WI) and wounded non-irradiated (WNI) human skin fibroblast cells (WS1), to test their influence in wound healing at 632.8 nm using a helium neon (HeNe) laser. For each beam profile, measurements were made using average energy densities over the sample ranging from 0.2 to 1 J, with single exposures on normal wounded cells. The cells were subjected to different post irradiation incubation periods, ranging from 0 to 24 hours to evaluate the duration (time) dependent effects resulting from laser irradiation. The promoted cellular alterations were measured by increase in cell viability, cell proliferation and cytotoxicity. The results obtained showed that treatment with the G compared to the SG and TG beams resulted in a marked increase in cell viability and proliferation. The data also showed that when cells undergo laser irradiation some cellular processes are driven by the peak energy density rather than the energy of the laser beam. We show that there exist threshold values for damage, and suggest optimal operating regimes for laser based wound healing.

Key words: Beam shape, cell viability, cell proliferation, cytotoxicity, Low level laser therapy, fibroblasts and in-vitro.

1. INTRODUCTION

LLLT has been employed for its photostimulatory effect on slow-healing ulcers and wounds such as burns, chronic leg ulcers, pressure sores and diabetic foot ulcers[3]. A number of monochromatic radiation sources such as gas state lasers (argon-ion at 488 nm, HeNe at 633 nm), solid state lasers (neodymium: yttrium-aluminum-garnet at 1064 – 1320 nm) and laser diodes (gallium-aluminum-arsenide at 980 nm, gallium-arsenide at 904 nm) have been identified as producing a beneficial biological effect known as "photostimulation" or "biostimulation"; a process reported to generally depend on wavelength and dose of laser irradiation[1]. Lasers of various wavelengths and light emitting diodes (LED) have a wide application in the wound healing research. Al-Watban *et al.* (2003) for instance investigated the effect of polychromatic LEDs in burn healing of non-diabetic and streptozotocin-induced diabetic rats[4]. Previous reports also stated diode lasers at 980 nm show a beneficial effect on wound healing in diabetic mice[5]. Eells *et al.* (2004) reported that laser biostimulation produces its primary effect during the cell proliferation phase of the wound healing process[2]. Literature also report that the biostimulation process has a non-destructive effect on tissue at cellular level and may enhance wound healing[1, 2]. Although many researchers involved in LLLT studies tend to focus on investigating wavelength and dose dependent effects of low energy lasers, they seldom mention the laser beam shape used to treat biological samples. This leads to confusion as to how these fluence values are defined, and how they are achieved.

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For example most authors report laser dosage in terms of J/cm^2 or J/m^2 without specifying the manner in which these fluences were administered to the sample as far as light energy distribution or beam shape is concerned. This makes it impossible to repeat the findings, and difficult to compare one report with another. The present study was performed to determine photobiological effects arising from using a HeNe laser of different beam shapes. This was performed by evaluating changes in cellular processes including cell viability, proliferation and cytotoxicity resulting from laser treatment through investigating how using different beam shapes of various doses would affect wound healing of human skin fibroblasts *in-vitro*.

2. METHODOLOGY

Only in-vitro experiments were performed in this work. A monolayer of human skin fibroblasts which is approximately a few microns in depth was used in this study; the wounds could therefore only be measured according to their diameter and length across the culture plates. Wound healing as a result of irradiation with the three laser beam shapes was evaluated by analysing cellular changes in adenosine triphosphate (ATP), alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) levels.

2.1 Cell culture

Human skin fibroblasts of adherent cell line WS1 (CRL1502) were obtained from the American Type Culture Collection (ATCC). These cells were grown in Eagle's minimal essential medium with 2 mM L-glutamine that is modified to contain 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1 % fungizone and 1 % penicillin-streptomycin and supplemented with 10 % fetal bovine serum (complete medium). The cell cultures were incubated at 37^{o}C with 5 % CO_2 and 85 % humidity (optimum growth condition) in 75 cm^2 flasks. Sub-culturing was done twice a week using a 0.25 % (w/v) trypsin – 0.03 % ethylenediaminetetraacetic acid (EDTA) solution in Hank's balanced salt solution (HBSS) [6]. For experiments, only cells between passages 10 – 35 were used. Before laser treatment approximately 6 x 10^5 cells in 3 ml complete medium (culture supernatant) were seeded from 75 cm^2 flasks into sterile 3.5 cm diameter culture plates and incubated overnight in optimum growth condition. These cells reached 98 – 100 % confluence and were wounded and irradiated.

2.2 Wound formation

All experiments performed were divided into a control (WNI) and a test (WI) condition. Control plates were excluded from the laser irradiations but treated the same as the test plates in every other way. For the simulated wound environment, confluent monolayers were first scratched with a sterile 1ml pipette of 2 mm diameter. After wounding the plates were incubated in optimum growth condition in 800 µl of culture supernatant for 30 minutes before they were irradiated. All scratches were irregular and the "wound" size ranged from 1 – 2 mm in diameter over the length of the culture plate[6].

2.3 Beam shaping and beam shapes

Three beam shapes were used in the experiments reported in this work: a Gaussian beam, a Truncated Gaussian beam, and a Super-Gaussian beam. The use of these beam shapes allows us to probe whether the laser beam interaction with the cells is driven by peak fluence, average fluence or total energy delivered. A brief overview of the beam shapes used is given next.

2.3.1 Gaussian beams

A Gaussian beam has an intensity function given by

$$I_g(r) = I_g \exp(-2(r/w_g)^2), \quad (1)$$

where w_g is the beam radius ($1/e^2$ value) and I_g is the peak intensity. Analytical expressions exist to propagate this type of field through any ABCD matrix system[7].

Gaussian beams have the property that their peak intensity is double their average intensity, and that the beam size w_g is already the second moment radius of this field. In this application the output beam from the HeNe laser was very nearly TEM_{00}, and therefore already a Gaussian shape.

2.3.2 Truncated Gaussian beams

A truncated Gaussian beam has an intensity function given by

$$I_t(r) = \begin{cases} I_t \exp(-2(r/w_t)^2), & |r| \le r_0 \\ 0, & |r| > r_0 \end{cases}, \tag{2}$$

where w_t is the Gaussian beam radius and I_t is the peak intensity. In the limit that $w_t >> r_0$, the beam will approximate a flat-top distribution, and the average intensity will be identical to the peak intensity. This beam is generated by amplitude filtering, and is therefore inherently lossy. However, it is a useful approximation to a flat-top beam.

2.3.3 Super Gaussian beams

A Super-Gaussian beam has an intensity function given by

$$I_s(r) = I_s \exp(-2(r/w_s)^{2p}), \tag{3}$$

where w_s is the beam radius (but not the second moment radius) and I_s is the peak intensity. Analytical expressions exist to propagate this type of field through any ABCD matrix system[8], but they are in general still numerically intensive. In the limit of large p, the Super-Gaussian "edges" become steeper, the peak intensity is equal to the average intensity, and the beam becomes a perfect flat-top. Due to energy conservation, the relationship between the peak values of the Gaussian and Super-Gaussian fields can be written as

$$I_s = \frac{2^{1/p} p w_g^2}{2 w_s^2 \Gamma(1/p)} I_g, \tag{4}$$

The lower peak intensity can be compensated for by decreasing the beam size relative to the Gaussian beam, but this has implications on the propagation of the field thereafter. Several methods exist to create a flat-top beam, including refractive[9] and diffractive methods[10]. In this application a Gaussian beam was focused into a multimode fibre, realizing a nearly uniform output. This output was then imaged to recreate the desired beam shape and size at the sample plane. This method has the advantage of easy use, but the severe limitation of poor energy transmission due to inefficient fibre coupling.

2.4 Laser parameters

A HeNe laser at 632.8 nm adjusted to transmit the above mentioned beam shapes (one shape at a time) was used. Beam radius's of w_g = 1.17 cm, w_t = 2.22 cm and w_s = 1.75 cm were used. A plot of the beam shapes across the diameter of the culture plate is shown in figure 1. Cells were irradiated in 800 µl culture supernatant at room temperature in a dark room. The time of exposure for the different average energy density values (0.2 – 1 J) applied was calculated using the following formula,

$$t = \frac{\phi A}{P}, \tag{5}$$

where t is the time in minutes, Φ is the energy per unit area (specified) in J/cm^2, A is the area (measured – used that of the culture plate) in cm^2 and P is the laser power output (measured using a Coherent labmaster power meter and the Coherent LM-3 HTD detector) in mW. The data for the peak and average intensities used in the experiments are shown in table 1.

Table 1: Data for the actual laser beam shapes used in the experiments.

	Peak Intensity (mW/cm^2)	**Average Intensity (mW/cm^2)**	**Beam Radius (cm)**
Gaussian	9.8	4.9	1.2
Super-Gaussian	0.8	0.6	1.8
Truncated Gaussian	4.3	2.7	2.2

2.5 Cellular transformations

For every assay tested, experiments were done in duplicates and repeated six times. Cells from the WNI and WI plates at a final concentration of approximately 1 x 10^5/ 100 µl were used to perform the ATP cell viability assay at 540 nm using luminescence. A volume of approximately 800 µl of culture supernatant was collected from each plate after laser irradiation for performing the LHD (100 µl) as well as the ALP (100 µl) assays.

2.5.1 Cell viability assay

The CellTiter-Glo luminescent cell viability assay, which is a homogeneous method of determining the number of viable cells in culture based on the quantitation of ATP present, was employed in this study. ATP signals the presence of metabolically active cells in culture. 100 µl of reconstituted CellTiter-Glo reagent was added to an equal volume of cell suspension (after irradiation) in a luminometer tube. The tube contents were mixed on an orbital shaker for 2 minutes to induce cell lysis. These samples were thereafter incubated for a further 10 minutes at room temperature to stabilise the luminescent signal. Luminescence was recorded using a Junior EG & G Berthold Luminometer[11].

2.5.2 Cell proliferation assay

ALP is a membrane bound enzyme released during inflammation, remodeling and cell proliferation. This enzyme has been used as a marker for wound healing and its activity was measured by the colorimetric assay using ρ-nitrophenyl phosphate (ρ-NPP) as a substrate. Following irradiation, 100 µl of culture supernatant was pre-incubated with an equal volume of 0.5 M N-methyl-D-glucamine buffer, pH 10.5, 0.5 mM magnesium acetate, 110 mM sodium chloride and 0.22 % Triton X-100 for 30 minutes at 37^{o}C in a 96 well plate. 20 mM ρ-NPP was added and the reaction was incubated at 37^{o}C for 30 minutes. The amount of ρ-nitrophenol liberated was measured at 405 nm[12].

2.5.3 Cell cytotoxicity assay

The CytoTox 96®, a non-radioactive cytotoxicity assay, was utilised to measure LDH, a stable cytosolic enzyme that is released upon cell lysis. After laser treatment 100 µl of culture supernatant was mixed with an equal volume of reconstituted substrate mix in a 96 well plate. The plate was covered with tin foil and incubated at room temperature for 30 minutes, protected from light. 100 µl of the stop solution was added and the absorbance read at 490 nm[13].

3. RESULTS

After the desired beam shapes had been generated, the distributions were measured, and are shown in figure 1. Figure 2 depicts a section of the 3.5 cm culture plate containing wounded cells. Results presented in figures 3 – 5 were plotted as: (a) function of energy (J) and (b) – (c) peak energy density (mJ/cm^2).

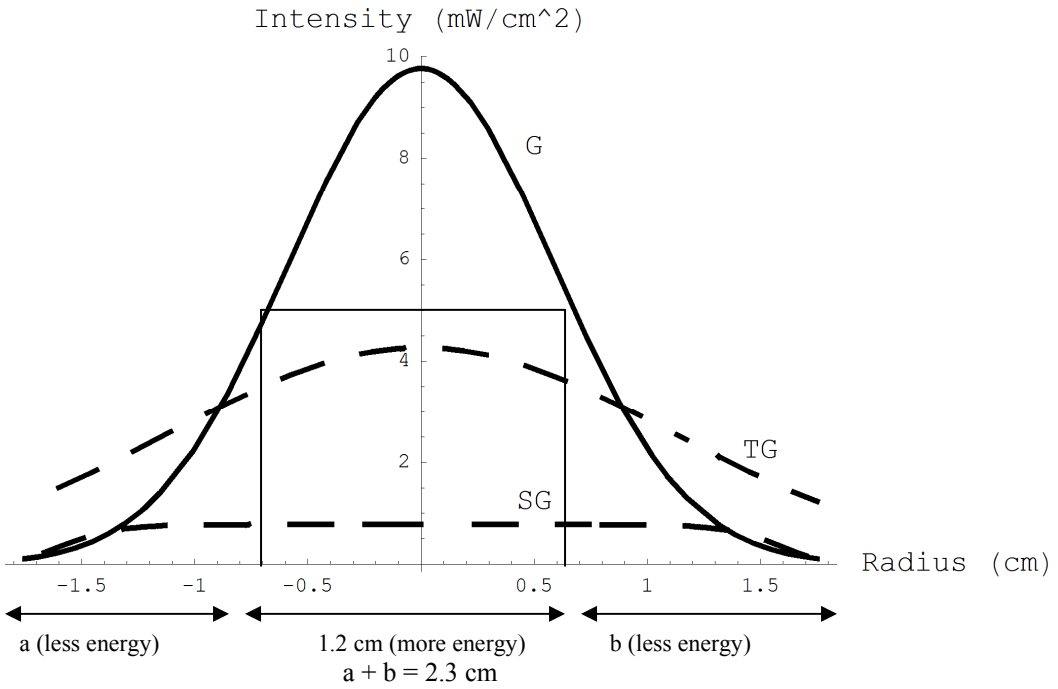

Figure 1: The laser beams used in the experiment are shown with real intensity distributions. The Super-Gaussian has a low peak value due to the lower energy efficiency and the fact that it has a larger beam spread.

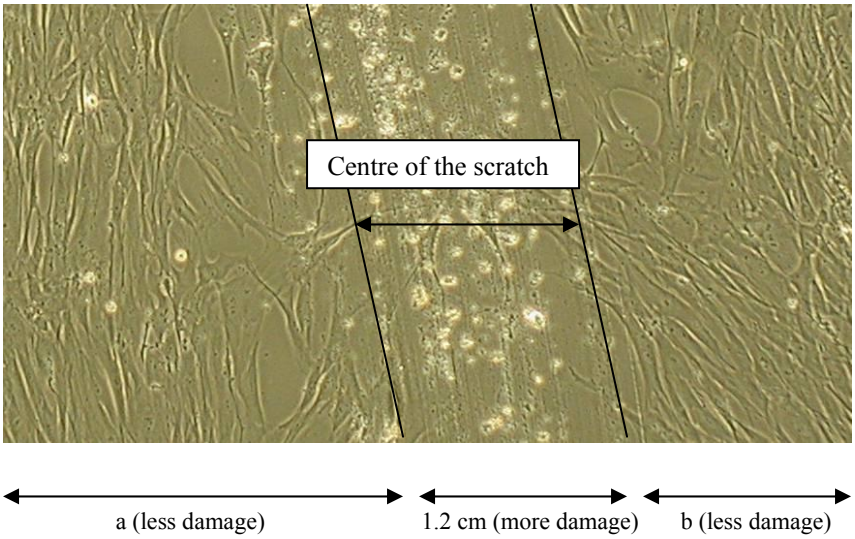

Figure 2: The monolayer of human skin fibroblasts used for experiments. During laser irradiation the center of the culture plate, at roughly 1.2 cm with the scratch (wound) was exposed to more beam peak energy density (around the 5 mJ/cm^2 value – figure 1) compared to the sides a and b.

Following laser irradiation the cells in culture supernatant were incubated at optimum growing conditions. 24 hours later culture supernatant was harvested and cells trypsinised as mentioned before for sample preparation. Then changes in cellular parameters were measured as shown in Figures 3 –5 below.

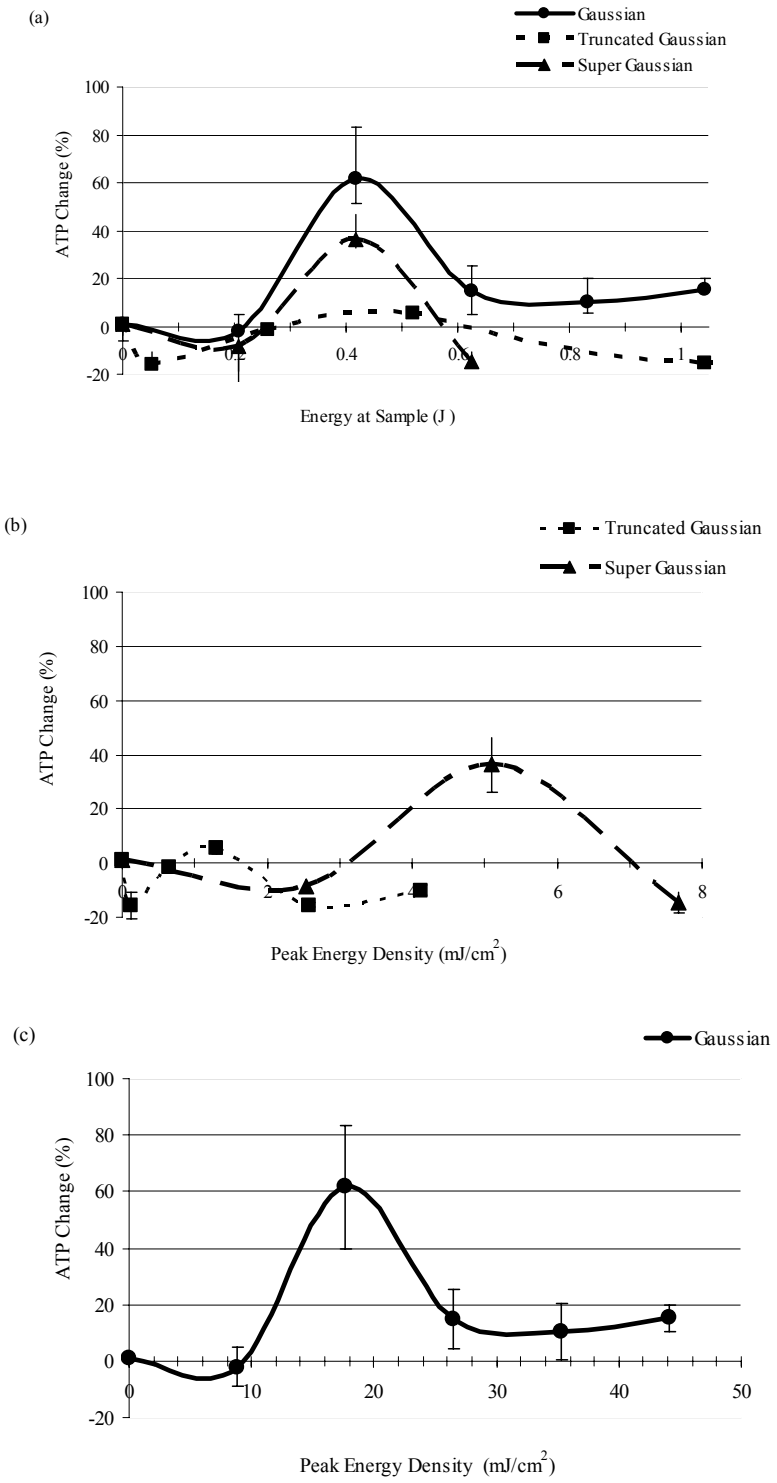

Figure 3: Wounded fibroblasts treated with the three laser beam shapes at doses $0.2 - 1$ J, depict a stimulatory effect i.e. an increase in ATP around 0.4 J for both the G and SG beams with the TG beam showing a lower response at this point. Graph 3a shows the G beam curve is higher than the SG curve at this point. In both graphs 3b and 3c, ATP peak energy density curves for the TG, SG and G laser beams showing disagreeing data to that presented in diagram 3a.

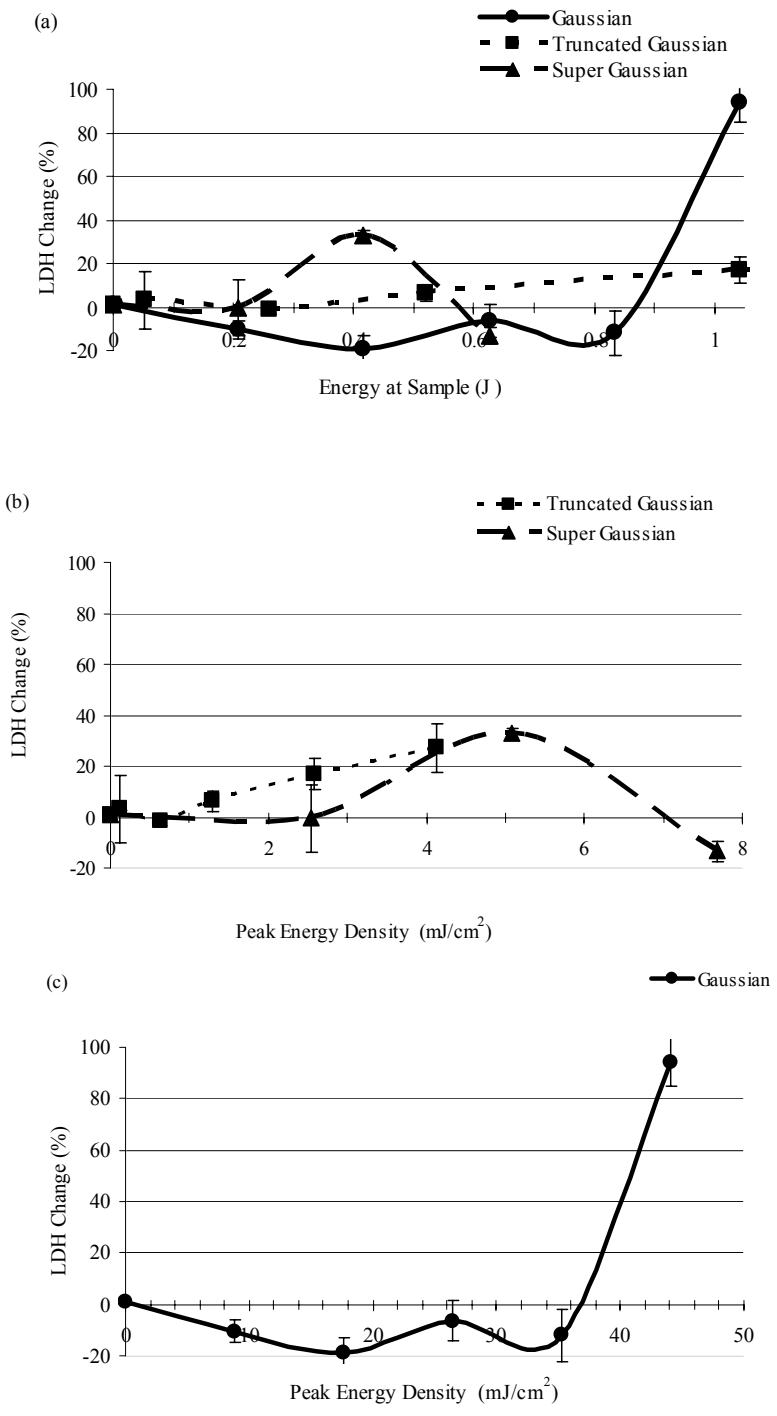

Figure 4: The LDH energy curve (4a) showing data that disagreed with data presented in the peak energy density curves (4b) and (4c). Figure 4b shows that high levels of LDH could be measured when the wounded fibroblasts were irradiated with the TG and SG beams at 5 mJ/cm^2. Graph 4c shows that when the samples were irradiated with the G beam at peak energy densities greater than 35 mJ/cm^2 increase in cell damage was observed.

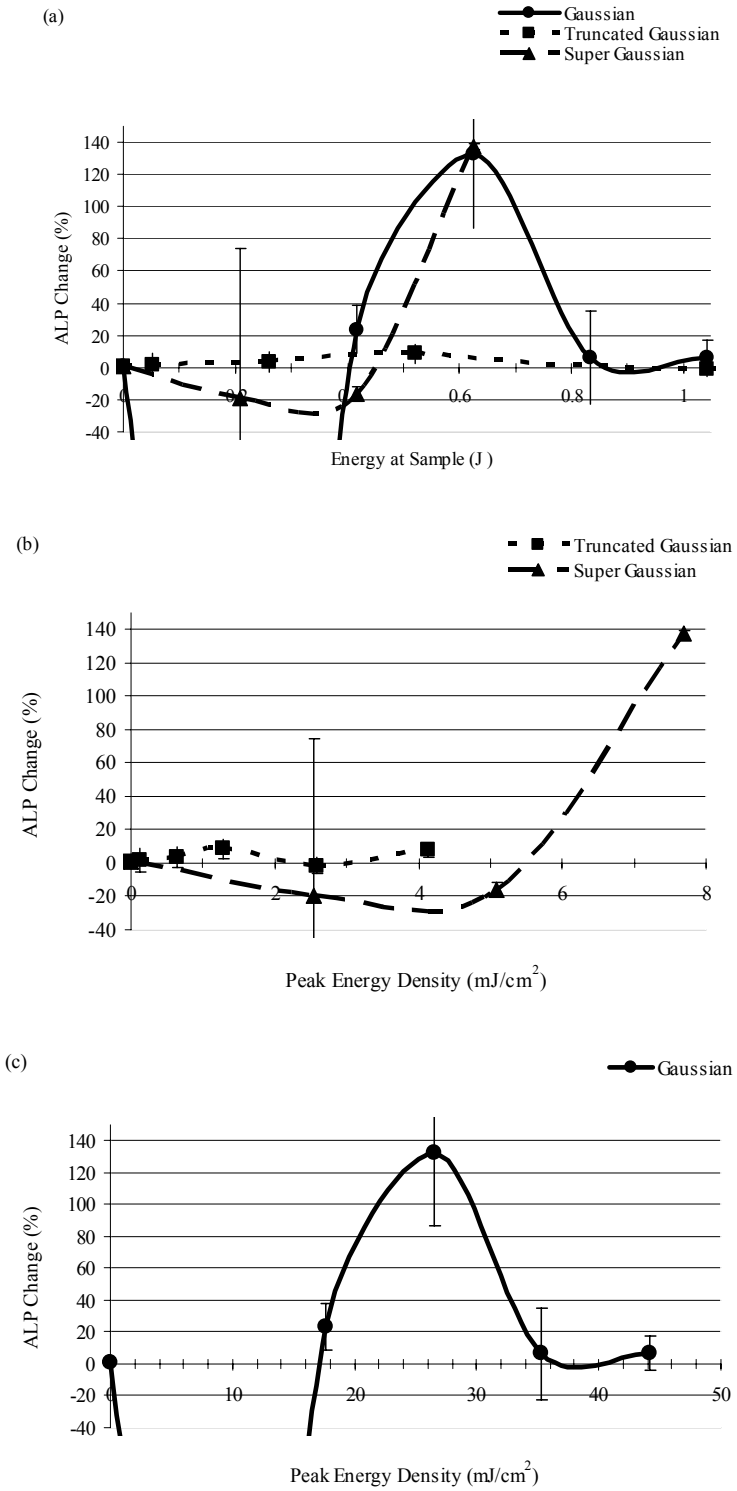

Figure 5: ALP a membrane bound enzyme released in inflammation, remodeling and cell proliferation was utilised as an indicator for wound healing. Both the G and SG beam curves show an increased secretion of this enzyme around 0.6 J, while the TG beam remained unaltered. In figures 5b and 5c ALP peak energy density curves for TG, SG and G showing disagreeing data to that presented in figure 5a.

4. DISCUSSION

In this study we investigated how using different laser beam shapes of the same wavelength would influence wound healing *in-vitro* by measuring changes in cellular responses such as the increase or even decrease in cell viability, proliferation and cytotoxicity as a result of laser irradiation. Our results supported findings stating that laser irradiation of mammalian cells cultivated *in-vitro* can enhance their proliferation[14]. All beam shapes showed a marked increase in ATP (G = 62 %, SG = 36 % and TG = 6 %) around the 0.4 – 0.5 J energy value (figure 3a). Although the calculated amount of total laser energy delivered to the sample at these points was 0.4 and 0.5 J respectively, about 93 % of it was observed to be transmitted through the sample (data not shown). At greater than 0.5 J, ATP levels either remained stable or decreased. Although there was an increase in ALP levels at the 0.5 – 0.6 J values when the samples were treated with the Gaussian and Super Gaussian beams, a very low (9 % increase) response was detected when the cells were irradiated with the Truncated Gaussian beam (figure 5a). Literature reports that photobiological responses promoted by laser irradiation can sometimes take days before their actual detection is possible[15]. For the Truncated Gaussian beam, assays were performed 3 hours and not 24 hours post laser irradiation as was the case with the other two beam shapes. We postulate that this may be reason for the smaller increase in ATP and ALP levels when treating with the Truncated Gaussian beam. In figures 5a to 5c the Gaussian and Super Gaussian beam data shows large error bars and negative values, particularly the around the 0.2 J value, we postulate that this trend occurred as a result of performing background subtractions of culture media when running this assay. More experiments will be done to confirm these results. Higher ATP as well as ALP levels observed when the cells were irradiated with energy doses ranging between 0.4 – 0.6 J corresponds with the finding that Low Level Laser Therapy at low fluences enhances wound healing through stimulating cell proliferation, accelerating collagen synthesis and increasing ATP synthesis in mitochondria[2]. For the ATP and ALP results, the depicted laser peak energy density (mJ/cm^2) data showed no agreement with the laser energy (J) data (figures 3 and 5). There existed no overlap on any of the beam profile curves in the peak energy density data sets, but the graphs presenting the energy data showed a complementary trend of curves between the Gaussian, Super Gaussian and to a lesser extend Truncated Gaussian beam shapes. We interpreted these results as meaning that both the cell viability and cell proliferation processes are energy rather than peak energy density driven. Conversely our LDH data showed that cell damage was promoted by an increase in peak energy density (mJ/cm^2) rather than that in energy (J). The curves presented in figure 4a showed no agreement in their respective trends. At 4.1 mJ/cm^2 (Truncated Gaussian) and 5.1 mJ/cm^2 (Super Gaussian) peak energy density both the Truncated and Super Gaussian beams showed an increase in LDH, there even exists an overlap in these curves at these values (figure 4b). For the Gaussian beam, LDH levels increased when the cells were irradiated at peak energy densities higher than 35 mJ/cm^2 (figure 4c). This effect is illustrated in figures 1 and 2, where at approximately 1.2 cm area of the culture plate the cells got exposed to very high amounts (mostly for the Gaussian beam) of irradiation energy and more damage occurred to the cells. Even though the Truncated and Super Gaussian beams were complementary these results did not agree with the Gaussian beam data, we do not have enough Gaussian beam data at 2 – 4 mJ/cm^2 to comment on its trend at these points. These results suggest that when cells undergo laser irradiation some cellular processes are driven by the peak energy density (mJ/cm^2) rather than the energy (J) of the laser beam. Although treatment with all three beam shapes showed complimentary ALP and ATP results, much higher responses were detected when the cells were irradiated with the Gaussian beam, compared to the other two beam shapes. This may be as a result of its higher peak and average intensities compared to the others (table 1).

5. CONCLUSION

Our conclusion thus far is that laser treatment with a Gaussian beam shape resulted in a marked increase in cell viability and proliferation of normal human skin fibroblasts (cells); this may lead to the promotion of *in-vitro* wound healing. We also observed that for some cellular transformation processes that occurred (increase in ALP and ATP) changes were related to the amount of energy delivered to sample at that point, but for others (increase in LDH) changes depended on peak energy density values of the different beams. This means that the way energy is delivered (beam shape) must be included in any discussion of changes in these cellular processes. However, further experiments need to be completed for us to give full comment on these findings. Future work includes the Bessel beam experiments that will be published elsewhere on completion.

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