

# Adaptive Response After 632.8 nm Laser Irradiation Decreases Cellular Damage in Diabetic Wounded Fibroblast Cells

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Running title: *Pre-sensitisation decreases cellular damage in diabetic fibroblast cells*

## ABSTRACT

**Background:** Sensitisation of cells with higher doses of ionising radiation prior to the therapeutic dose induces the so-called adaptive response. This study investigated the effect of laser pre-sensitisation of diabetic wounded fibroblast cells (WS1) with Helium-Neon (He-Ne) laser irradiation.

**Materials and methods:** A He-Ne laser was used to pre-sensitise diabetic wounded WS1 cells with 10 J/cm<sup>2</sup> and then cells received a challenging dose (CD) of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup>. After a repair time of 24 hours, cellular responses were measured.

**Results:** A decrease in DNA damage was seen when cells were pre-sensitised with 10 J/cm<sup>2</sup> before irradiation with a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup> while a decrease in the cytotoxicity of pre-sensitised cells irradiated with a CD of 12 J/cm<sup>2</sup> was also seen.

**Conclusion:** Pre-sensitising with 10 J/cm<sup>2</sup> prior to irradiating with 12 J/cm<sup>2</sup> introduced beneficial effects for cellular repair indicating an adaptive response which may reverse damage caused by higher doses of LLLT.

**KEYWORDS:** LLLT. Pre-sensitisation. He-Ne laser. Cellular damage.

## INTRODUCTION

### Low Level Laser Therapy (LLLT)

Laser light is a monochromatic, coherent, and directional tight beam of light. There are two types of medical lasers, high power or surgical lasers and low level lasers which can be used for bio-stimulation<sup>1</sup>.

Low level lasers were introduced as a therapeutic modality as early as 1968<sup>2</sup>. Several studies of low level laser irradiation in the visible wavelength range (400 – 700 nm) have reported either positive, negative or no effects on cellular functions<sup>3</sup>. The LLLT exposure-response relation has, however, not been clarified, and the basic mechanisms underlying LLLT effects in the therapeutic dose range are still unknown<sup>4</sup>.

Low level lasers aim to bio-stimulate and because of their low power nature the effects are biochemical and not thermal. As such they cannot cause damage to living tissues at a cellular level<sup>5</sup>. While selection of correct wavelength and power level is important, consistent application of the necessary amount of energy is a significant parameter if the best therapeutic effects are to be achieved. The selection of correct parameters could also help to optimise the effects of LLLT.

### Diabetes Mellitus

Diabetes mellitus (DM) is a chronic metabolic disorder caused by inherited or acquired deficiency in production of insulin by the pancreas or by the ineffectiveness of the insulin produced. The high levels of glucose present in the blood damages blood vessels, nerves and other body systems<sup>6</sup>. Approximately 180 million people worldwide have DM and after 15 years, roughly 2% of people become blind as a result of diabetic retinopathy. Patients also develop neuropathy in feet and when combined with reduced blood flow, the chance of foot ulcers and eventual limb amputation increases. Diabetes mellitus is an economic burden on the society<sup>7,8,9</sup>.

### Low Level Laser Therapy For Diabetic Wound Healing

Conventional treatment of diabetic wounds involves many forms of therapies including contact dressings and topical treatments. The different treatments that have been applied do not often give satisfactory results<sup>8</sup>. LLLT has been shown by various studies to be effective in the treatment of diabetic wound healing and the use of lasers as a source of phototherapy looks to be an attractive branch of medicine for the future<sup>10</sup>. Low level laser irradiation has shown to be effective in the treatment of impaired microcirculation, slow-to-heal wounds<sup>5,11,12</sup>, and diabetic retinopathy<sup>13</sup>. Abergel *et al.*, (1987) used a Helium-Neon (He-Ne) laser to irradiate simulated diabetic wounds (*in vitro* and *in vivo*)<sup>14</sup>. They found that laser irradiation was able to significantly enhance healing of diabetic wounds in both experiments. Houreld and Abrahamse (2006) also found a stimulatory effect on diabetic wounded fibroblast cells *in vitro*<sup>15,16</sup>. Similar findings were reported by Kuleiv *et al.*, (1991)<sup>17</sup> and Forney *et al.*, (1999)<sup>18</sup>.

### Adaptive Response

Sensitisation of cells with higher doses of ionising radiation prior to the therapeutic dose induces the so-called adaptive response (AR) where cells develop the ability to activate protective mechanisms that reduce DNA damage<sup>19</sup>.

Pre-sensitisation or AR can be defined as pre-irradiation of the target site using a certain dose of irradiation to pre-condition something before further subjecting it to a CD. Since low level laser has lasting effects that may continue for several days<sup>20</sup>, it is expected that the pre-sensitising dose will have a pre-conditioning effect on the cells, which may influence cellular responses after receiving a CD. When Houreld and Abrahamse (2006) irradiated diabetic wounded fibroblast cells once to a He-Ne laser at a fluence of 5 J/cm<sup>2</sup> there was an increase in ATP, cellular proliferation and cellular damage. However, when irradiated a second time with 2 days between irradiations there was a decrease in cellular damage<sup>15</sup>. They concluded that this was likely due to an adaptive response and that further work on the adaptive response and laser irradiation is warranted as this may play an important role in phototherapy. However, the effects of pre-sensitisation on laser irradiation of wounded diabetic fibroblast cells are still not well known.

## METHODS AND MATERIALS

### Cell Culture

WS1 cells, media and supplements were supplied by The Scientific Group Adcock Ingram unless otherwise specified. Human skin fibroblast cells (WS1, ATCC CRL-1502) were grown in Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt Solution (BSS) modified to contain: 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1.5 g/l sodium bicarbonate, 1% pen-strep and fungizone and supplemented with 10% foetal bovine serum. Cells were incubated at 37 °C in 5% CO<sub>2</sub> and 85% humidity.

The *in vitro* diabetic model was based on the work of McDermott *et al.*, (1998)<sup>21</sup> and Hamuro *et al.*, (2002)<sup>22</sup>, whereby WS1 cells were grown in an additional 17 mM/L D-glucose. To determine the effects of AR of laser irradiation on diabetic wounded WS1 cells, 6x10<sup>5</sup> cells in 3 ml complete EMEM were seeded into 3.3 cm diameter culture plates as determined by the Trypan blue exclusion test. Plates were incubated overnight to allow the cells to attach. A 2 mm diameter wound was induced by a central scratch across a confluent monolayer of cells using a sterile pipette according to Rigau *et al.*, (1996)<sup>23</sup>, then incubated for 30 minutes at 37 °C prior to laser irradiation.

### Irradiation

This study investigated the cellular effects of laser irradiation and pre-sensitisation using a 2 mW/cm<sup>2</sup> He-Ne laser (632.8 nm) with an output of 24 mW. The power output was used to calculate the duration of each exposure namely; 10 J/cm<sup>2</sup> (pre-sensitisation) and a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup>. Hawkins and Abrahamse (2005)<sup>24</sup> and Houreld and Abrahamse (2006)<sup>15</sup> demonstrated that 5 J/cm<sup>2</sup> promoted healing while doses higher than 10 J/cm<sup>2</sup> appeared to produce a significant amount of cellular and molecular damage. Therefore, 5 J/cm<sup>2</sup> was selected with the view that it would equally stimulate healing while 12 J/cm<sup>2</sup> would have a damaging effect.

Cells were divided into two treatment conditions namely; pre-sensitised and non pre-sensitised. Non-irradiated diabetic wounded cells (+W-I+D) were used as control. Diabetic wounded cells received a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup> (+W-I+D) 24 hours post pre-sensitisation followed by a repair

time of 24 hours incubation at 37 °C. Non pre-sensitised diabetic wounded cells only received a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup>. Cellular responses were then assessed. Kreisler *et al.*, (2003)<sup>25</sup> reported that cells showed increased proliferation and viability 24 hours post irradiation so this finding was used as a basis for a 24 hours incubation period in this study.

**Cellular Responses**

The following parameters were measured as indicators of cellular responses on the effects of laser irradiation: *Cell morphology*: Cellular morphology was determined by assessing colony formation (cell regrouping at wound margin), haptotaxis (cell orientation) and chemotaxis (cell migration across the central scratch) using a light microscope<sup>23</sup>.

*Cell viability*: Cell viability was assessed using trypan blue (0.4% w/v) in Hanks BSS in which equal volumes of trypan blue and cell suspension were added and incubated for 5 minutes. Non-viable cells stained blue due to a damaged cell membrane which allowed the dye to penetrate. Viable (clear) and non-viable (blue) cells were counted using an improved Nuebauer haemocytometer counting chamber and the percentage of viable cells was calculated. Mitochondrial activity was also used to assess cell viability by measuring ATP which is found in metabolically active cells. Equal volumes (50 µl) of cells and Glo-reagent (Promega G755A) were mixed. The mixture was vortexed for 2 minutes to induce lysis and incubated at room temperature for 10 minutes. Luminescence was measured in reading light units (RLU) using the Junior EG and G Berthold luminometer.

*Cell and DNA damage*: The LDH (cytotoxicity) and Comet assay was used to assess cellular and DNA damage respectively. Cytotoxicity was measured to determine if laser irradiation caused cell damage or lysis that would cause the release of LDH into the culture media. LDH was measured using the CytoTox 96<sup>®</sup> non-radioactive cytotoxicity assay (Promega G1780). Equal volumes (50 µl) of culture media and reconstituted substrate buffer were mixed and incubated at room temperature for 30 minutes, protected from light. Thereafter, 50 µl of stop solution was added and absorbance read at 490 nm. The Comet assay was performed according to Collins (2000)<sup>26</sup>. A cell suspension (2x10<sup>4</sup> cells/ml) was embedded in 1% low melting point agarose then lysed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100, pH 10) for 1 hour at 4 °C. Damaged DNA underwent alkaline unwinding in electrophoresis solution (0.3 M NaOH and 1 mM EDTA) for 40 minutes at 4 °C. During electrophoresis (300 mA for 30 minutes at 4 °C), relaxed coils were pulled towards the anode forming the tail of a comet like image. Cells were then neutralised (0.4 mM Tris, pH 7.5) and stained with 4'6 diamidine-2-phenylindol dihydrochloride (DAPI). The stained cells were viewed on a fluorescent microscope (Olympus BX41/BX51). One hundred comets per gel were analysed at random and scored according to five recognisable classes ranging from class 0 to 4, with the most damage in class 4.

**Statistical Analysis**

Experiments were repeated six times and assays were performed in duplicate. Results were recorded and graphically represented for statistical analysis using Sigma Plot Version 8.0. Results were considered to be significant if  $P < 0.05$ .

**RESULTS**

*Cell morphology*: There was no difference in structural response between normal non pre-sensitised and normal pre-sensitised cells (micrograph not provided). However, an increase in cell density in irradiated cultures was observed. It was noted that pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (Figure 1D) and the same cells that did not receive a CD (Figure 1B) had similar haptotaxis, chemotaxis, and wound coverage in 48 hours, whereas the same cells non pre-sensitised (Figure 1A) had a decrease in the rate of migration and there was incomplete wound closure in 48 hours. A similar response was observed for non pre-sensitised diabetic wounded cells that received a CD of 12 J/cm<sup>2</sup> (Figure 1E) where there was incomplete wound closure in 48 hours. Similarly, it was observed that non pre-sensitised diabetic wounded cells that were irradiated with 5 J/cm<sup>2</sup> (Figure 1C) had incomplete wound closure in 48 hours. Pre-sensitised diabetic wounded cells that received a CD of 12 J/cm<sup>2</sup> (Figure 1F) showed haptotaxis and chemotaxis in 48 hours.

*Cell viability*: Pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) showed an increase in percent viability although not significant when compared to pre-sensitised diabetic wounded (+W-I+D) cells that did not receive a CD ( $P=0.383$ ), (Figure 2). On the other hand, pre-sensitised cells which received a CD of 12 J/cm<sup>2</sup> (+W+I+D 12 J/cm<sup>2</sup>) showed a decrease in percentage viability when compared to similar cells (+W-I+D) that did not receive a CD. Similarly, diabetic wounded pre-sensitised cells that did not receive a CD (+W-I+D) showed an increase in ATP when compared to the same cells non pre-sensitised. These results correlate with mitochondrial activity in Figure 2; pre-sensitised diabetic wounded cells that did not receive a CD (+W-I+D) showed an increase in ATP when compared to same cells non pre-sensitised. Pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) showed a decrease in ATP when compared to the same cells non

pre-sensitised ( $P=0.747$ ). In contrast, pre-sensitised diabetic wounded cells that received a CD of 12 J/cm<sup>2</sup> (+W+I+D 12 J/cm<sup>2</sup>) showed no difference in ATP when compared to same cells non pre-sensitised ( $P=0.839$ ).

*Cell and DNA damage*: There was no difference in cytotoxicity between pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) and the same cells non pre-sensitised (Figure 3). Similarly, there was no difference in cytotoxicity between pre-sensitised diabetic wounded cells that did not receive a CD (+W-I+D) and the same cells non pre-sensitised. Non pre-sensitised diabetic wounded cells irradiated with 12 J/cm<sup>2</sup> (+W+I+D 12 J/cm<sup>2</sup>) showed an increase in cytotoxicity when compared to pre-sensitised cells that received a CD of 12 J/cm<sup>2</sup> ( $P=0.141$ ). Comet assay results (Figure 3), showed that pre-sensitised diabetic wounded cells that did not receive a CD (+W-I+D) had more DNA damage when compared to same cells non pre-sensitised. But pre-sensitised diabetic wounded cells that did not receive a CD (+W-I+D) had more DNA damage when compared to pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> and 12 J/cm<sup>2</sup> (+W+I+D). Similarly, pre-sensitised diabetic wounded cells that received a CD of 12 J/cm<sup>2</sup> (+W+I+D 12 J/cm<sup>2</sup>) had less DNA damage when compared to similar cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>). However, irradiation with 12 J/cm<sup>2</sup> of non pre-sensitised diabetic wounded cells (+W+I+D 12 J/cm<sup>2</sup>) showed a higher DNA damage than similar cells irradiated with 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) ( $P < 0.001$ ).

**DISCUSSION**

Inducing diabetes in human skin fibroblast cells had no adverse effect on cellular migration *in vitro*. Diabetic wounded cells were still able to migrate across the central scratch in an attempt to close the wound despite being grown in a hyperglycaemic environment. This corresponds with Houreld and Abrahamse (2006)<sup>15,16</sup>. On the other hand, Hamuro *et al.*, (2002)<sup>22</sup> noted that elevated glucose inhibits endothelial migration while McDermott *et al.*, (1998) found that elevated extracellular glucose modulates cell migration, adhesion and proliferation<sup>21</sup>. Numerous reports have been documented where LLLT was found to enhance wound healing<sup>23,24,27</sup>. Pre-sensitised cells that did not receive a CD (+W-I+D) and similar cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) showed complete wound closure, while pre-sensitised and non pre-sensitised cells that were irradiated with 12 J/cm<sup>2</sup> showed incomplete wound closure. This showed that LLLT is beneficial in a dose dependent manner.

The cell viability findings suggest that the pre-sensitised cells that did not receive a CD had greater cell viability and mitochondrial activity than same cells non pre-sensitised. The difference was due to the stimulatory effect of LLLT. Pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D) had greater percentage viability than similar cells that received a CD of 12 J/cm<sup>2</sup> and yet the same cells non pre-sensitised irradiated with 5 J/cm<sup>2</sup> showed more ATP (Figure 2). This can be explained in the light that 10 J/cm<sup>2</sup> has damaging effect and 5 J/cm<sup>2</sup> has stimulatory effect when used as single doses. Therefore when 10 J/cm<sup>2</sup> was used for pre-sensitisation the damage caused led to a repair process making irradiation with 5 J/cm<sup>2</sup> have minimal effect (adaptive response). The increased cell proliferation with 5 J/cm<sup>2</sup> (Data not provided) concurs with the results of Hawkins and Abrahamse (2005)<sup>24</sup> and Houreld and Abrahamse (2006)<sup>15</sup>, who found that there was enhanced cellular proliferation at a similar dose. Irradiating cells with a higher dose than the pre-sensitising dose did not improve cell viability as seen in Figure 2. This might have been because cells adapted to a higher dose than the CD such that the latter led to a decrease in viability or it might have been due to a cumulative effect. Generally, the viability and proliferation of normal fibroblast cells remained as reported in literature<sup>3,28</sup> during the experimental time.

This study found that pre-sensitisation with 10 J/cm<sup>2</sup> was less cytotoxic and beneficial to healing (Figures 1D and 3). Although this was the case, it was observed that 10 J/cm<sup>2</sup> had DNA damaging effect as seen in Figure 3. This corresponds with the findings of Hawkins and Abrahamse (2005)<sup>24</sup>, and Houreld and Abrahamse (2006)<sup>15</sup>, who found that irradiation with doses higher than 10 J/cm<sup>2</sup> had DNA damaging effect. In the literature, it is shown that diabetes contributes to DNA damage by increasing caspase activity<sup>29</sup>. This explains the increase in DNA damage in pre-sensitised diabetic wounded cells that did not receive a CD (+W-I+D) and similar cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>). However, this increase was not found to be significant. There was decreased cell proliferation in non pre-sensitised diabetic wounded cells irradiated with 12 J/cm<sup>2</sup> when compared to the same cells pre-sensitised (Data not provided). This finding correlates with the increased DNA damage in similar cells irradiated with 12 J/cm<sup>2</sup> in Figure 3. Results showed that there was less cellular and DNA damage in pre-sensitised cells that received a CD of 12 J/cm<sup>2</sup> when compared to control cells (+W-I+D) and pre-sensitised cells that received a CD of 5 J/cm<sup>2</sup> ( $P=0.01$ ) (Figure 3). This is attributed to the adaptive response as a higher dose counteracted the effects of pre-sensitisation at a lower dose due to DNA repair after 72 hours.

**CONCLUSION**

Pre-sensitisation has an adaptive response on human skin fibroblast cell culture. When the pre-sensitising dose is higher (10 J/cm<sup>2</sup>) than the CD

(5 J/cm<sup>2</sup>), the effect measured is that of the higher dose; the effect of the lower dose is not noted. Irradiating cells at a higher dose (12 J/cm<sup>2</sup>) than the pre-sensitising dose has beneficial effects. This observation indicates that the damage caused by 10 J/cm<sup>2</sup> is reversible and that the correct dose can stimulate cells to repair and retain normal functional status. It can be concluded that LLLT using a He-Ne laser (632.8 nm) restores cellular function to a varying degree according to the irradiation dose and the repair time used. LLLT has both stimulatory and inhibitory effects as reported in the literature<sup>3</sup>.

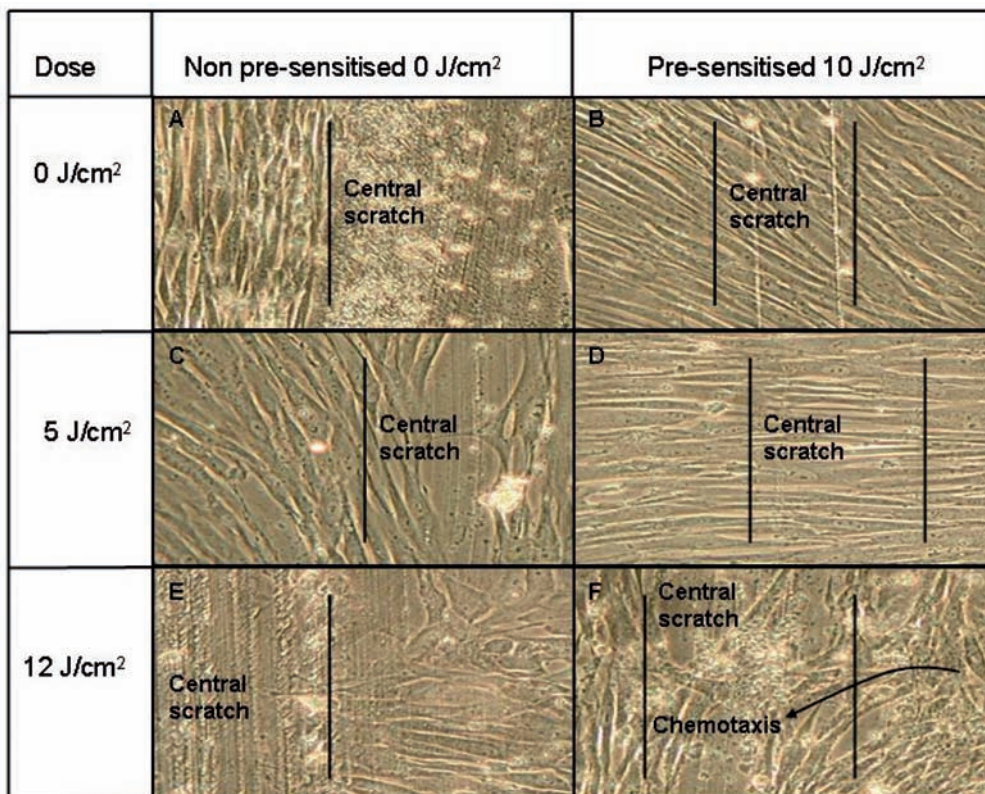
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**FIGURE 1.** Morphology of diabetic wounded WS1 cells; Confluent monolayers of cells were scratched with sterile pipettes to simulate wounds. Non pre-sensitised diabetic cells showed incomplete wound closure in 48 hours (A), whereas there was complete wound closure in 48 hours in cells that were pre-sensitised (B). Although non pre-sensitised cells irradiated with 5 J/cm<sup>2</sup> showed cell chemotaxis, there was incomplete wound closure in 48 hours (C), while pre-sensitised cells that received a CD of 5 J/cm<sup>2</sup> showed complete wound closure in 48 hours (D). Non pre-sensitised cells irradiated with 12 J/cm<sup>2</sup> showed no haptotaxis and chemotaxis across the central scratch in 48 hours (E), whereas pre-sensitised cells that received a CD of 12 J/cm<sup>2</sup> showed haptotaxis and chemotaxis in 48 hours (F). Magnification 200x.

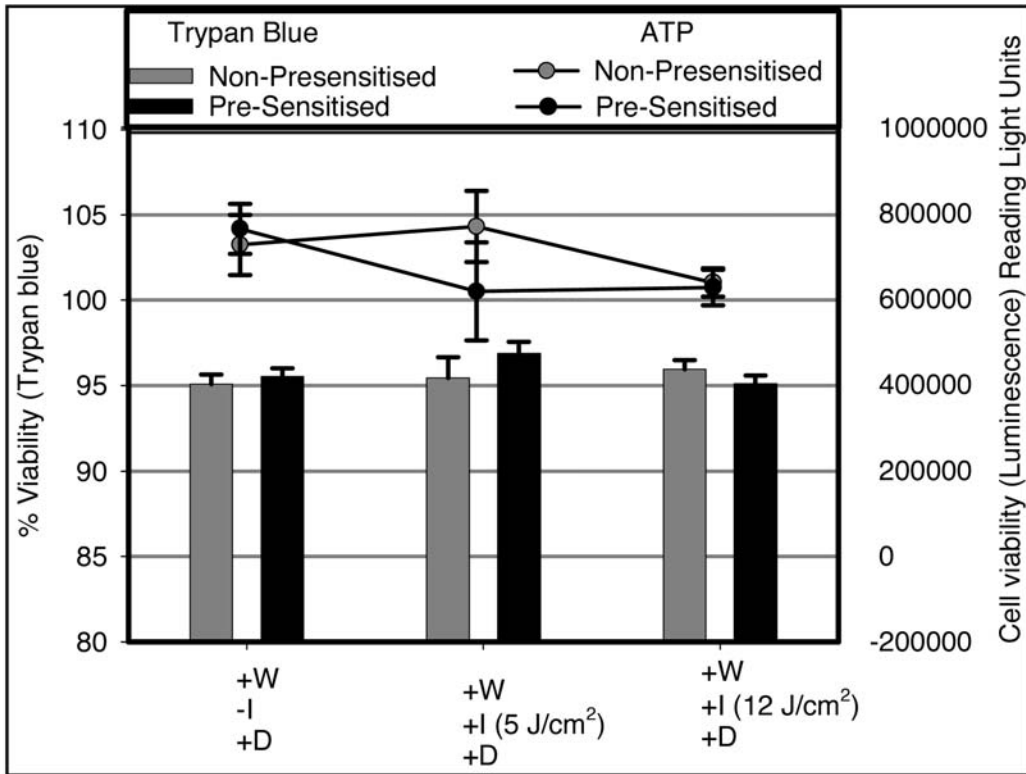


FIGURE 2. The effects of pre-sensitisation (10 J/cm<sup>2</sup>) on cell viability as assessed using trypan blue and ATP luminescent viability assay. Non-irradiated diabetic wounded cells (+W-I+D) were used as controls while pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup> (+W+I+D) were used as experimental group. Pre-sensitised diabetic wounded cells that received a CD of 5 J/cm<sup>2</sup> (+W+I+D 5 J/cm<sup>2</sup>) showed an increase in percentage viability indicating a stimulatory effect, while cells irradiated with 12 J/cm<sup>2</sup> showed a decrease. But non pre-sensitised diabetic wounded cells irradiated with 5 J/cm<sup>2</sup> had increased ATP thus indicating a stimulatory effect.

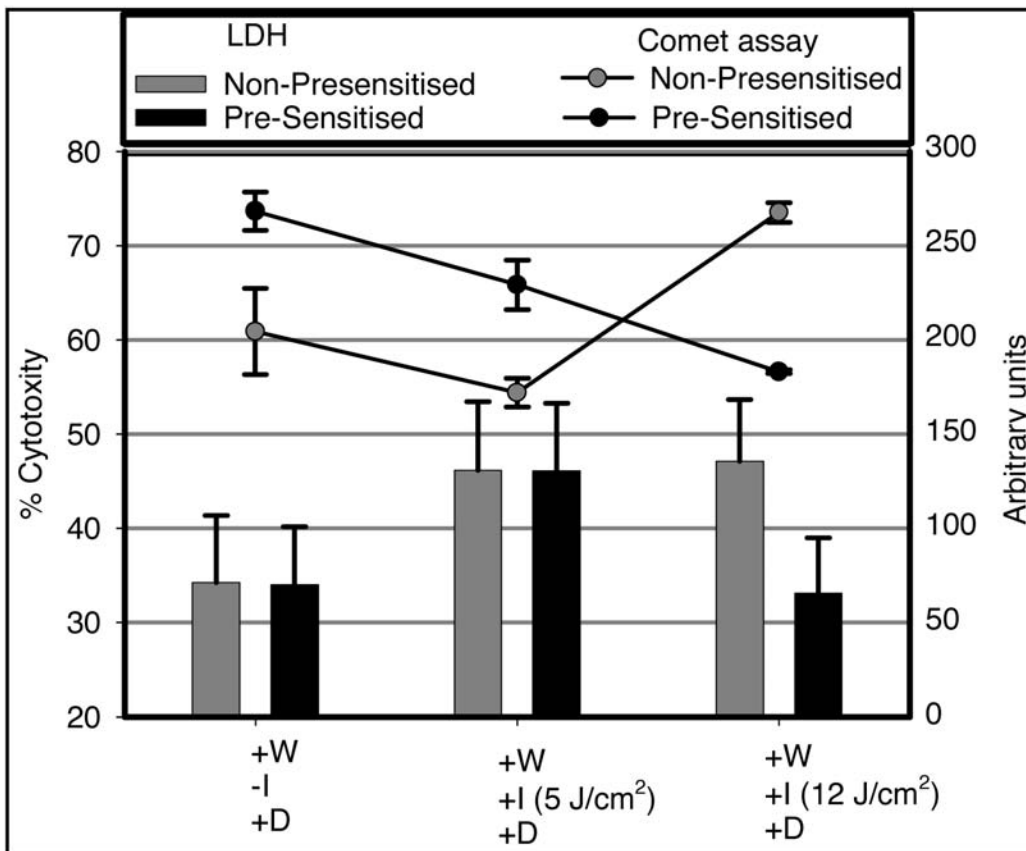


FIGURE 3. The LDH (cytotoxicity) and Comet assay was used to determine membrane integrity and DNA damage following pre-sensitisation with 10 J/cm<sup>2</sup> using a He-Ne laser (632.8 nm). Diabetic wounded cells then received a CD of 5 J/cm<sup>2</sup> or 12 J/cm<sup>2</sup> (+W+I+D). Non-irradiated diabetic wounded cells (+W-I+D) were used as controls. An increase in LDH and arbitrary units indicates an increase in membrane and genetic damage respectively. Pre-sensitised cells that received a CD of 5 J/cm<sup>2</sup> showed more DNA damage when compared to same cells non pre-sensitised ( $P=0.01$ ). Pre-sensitised cells that did not receive a CD showed more DNA damage than similar cells that received a CD of 12 J/cm<sup>2</sup> ( $P=0.01$ ). However, pre-sensitised diabetic wounded cells that received a CD of 12 J/cm<sup>2</sup> (+W+I+D 12 J/cm<sup>2</sup>) showed less cytotoxicity and DNA damage ( $P=0.01$ ) when compared to pre-sensitised cells that received a CD of 5 J/cm<sup>2</sup> indicating that pre-sensitisation is beneficial in a dose dependent manner.