Hindawi Publishing Corporation
Laser Chemistry
Volume 2007, Article ID 80536, 9 pages
doi:10.1155/2007/80536

Research Article

Cellular Damage in Diabetic Wounded Fibroblast Cells following Phototherapy at 632.8, 830, and 1064 nm

Nicolette N. Houreld and Heidi Abrahamse

Laser Research Group, Faculty of Health Sciences, University of Johannesburg, Doornfontein 2028, South Africa

Received 25 September 2007; Revised 12 November 2007; Accepted 15 November 2007

Recommended by Savas Georgiou

Objective. This study aimed to establish if laser irradiation induces cellular and genetic damage. *Background*. Phototherapy has been shown to induce wound healing in diabetic wounds, however little information is known regarding light-induced damage. *Methods*. Diabetic wounded fibroblasts were irradiated with 5 or 16 J/cm^2 at 632.8, 830, and 1064 nm. Damage was assessed by measuring membrane and DNA damages. Cellular migration was determined by microscopy. *Results*. Cells irradiated with 5 J/cm^2 at 632.8 and 830 nm showed a significant decrease in DNA damage while all cells irradiated with a fluence of 16 J/cm^2 showed an increase in membrane and DNA damages. *Conclusion*. This study showed that the comet assay and LDH release were sensitive enough to pick up changes in laser-irradiated cells. This study also showed that cellular and genetic damage inflicted on diabetic wounded cells was dependent on dose and wavelength and that cells are able to recover and respond.

1. INTRODUCTION

Due to its serious complications, diabetes remains an important cause of morbidity and mortality. Principal complications arise from macro- and microangiopathy. The nervous system is also affected, resulting in diabetic neuropathy, leading to sensory loss and subsequent damage to the limbs. Atrophy of fat and muscle tissues, combined with sensory loss, can result in pressure sores on the feet, which, in turn, can lead to lower-limb amputation. This is one of the most costly complications of diabetes, and accounts for 50% of all nontraumatic amputations [1]. Approximately, 15% of people with diabetes will develop foot ulcers, and 6% of these will require hospitalization for the treatment of such ulcers [2]. There is a need to bring researchers together to develop alternative new therapies to improve diabetic wound healing [3]. Phototherapy has been shown to positively effect wound healing in diabetic patients [4].

A large amount of research in photostimulation has been undertaken since the 1970s, and has shown both stimulatory as well as inhibitive effects. For wound healing studies, generally two laser types are used: helium-neon (He-Ne) lasers with a wavelength of 633 nm and semiconductor lasers emitting light in the range of 780 to 950 nm, typically with fluences of 1–5 J/cm^2. The He-Ne laser is the oldest, best documented and most common of all lasers used in photostimulation. This laser emits a red visible light in a continuous wave beam and a mean output power typically between 1 and 10 mW [5]. Despite the low penetration depth, this laser is useful in superficial wound healing since all the targets of wound healing are found in the epidermis and upper dermis [6]. Semiconductor lasers have been found to be more efficient than other more traditional lasers, such as the He-Ne laser. They are smaller, have a higher output power, have a deeper penetration depth and are more cost effective. Neodynium : yttrium-aluminum-garnet (Nd : YAG) lasers produce light in a single crystal of yttrium-aluminium-gallium (YAG) doped with neodymium (Nd) and has a wavelength of 1064 nm, and is in the infrared spectrum. Despite the higher output power, this laser also has biostimulating effects if the dose and power density are within the laser's therapeutic window, however most users of this laser are often unwilling to discuss the biostimulating functions of this laser due to the controversial nature of laser therapy [5]. This laser is commonly used in dermatology and plastic surgery, while the He-Ne and 830 nm diode are typically used for wound healing.

The effect of laser light on target tissue is dependent on the optical, chemical, and mechanical properties of the tissue as well as the characteristics of the incident laser beam

[7]. When a photon hits an object, it may be reflected, transmitted, scattered, or absorbed and the optical properties of a substance are characterized by coefficients for each of these events [7]. When the photon is reflected or transmitted it retains its energy, and when it is absorbed the energy is delivered to the atoms of the absorbing object (photoacceptor or chromophore). This energy may in turn change to heat oscillations (atom/material becomes hot), excite other atoms or molecules (electrons change their energy levels), ionize an atom or molecule (deliver an electron), or break chemical bonds (build new compounds) [8]. At low intensities, the photochemical conversion of the energy absorbed by the photoacceptor is predominant [9]. The effects of phototherapy are photochemical rather than thermal, [5, 10] and the amount of heat generated is insignificant and minimal [11], typically in the range of 0.1 to 0.5°C [12, 13].

A large body of research using cell culture suggests that laser irradiation can nondestructively alter cellular processes [12]. There is evidence that multiple mammalian cells respond to laser therapy. For wound-healing studies, endothelial cells [14], fibroblasts [15, 16], keratinocytes [17], as well as certain classes of macrophages [18] and neutrophils [19] have been used. During wound healing, fibroblasts are critically involved in producing cytokines which initiate the healing process and are key players in the formation of granulation tissue through proliferation, migration, deposition, and remodeling of the extracellular matrix. In addition, fibroblasts differentiate into myofibroblasts which are involved in wound closure and contraction [20]. Pal et al. showed that proliferation of fibroblast cells following He-Ne laser irradiation was dependent on laser exposure parameters (laser intensity, exposure time, and dose) and method of delivery [21]. It was also noted that dose had the greatest influence.

Most studies on DNA damage are performed with ionizing radiation, and little information is known about light-induced DNA damage. In cases of ionizing radiation, DNA damage is usually dramatic, although such studies have revealed strong DNA repair capacity. Even when the overall integrity of a cell's genome is seriously degraded, the damaged DNA can be repaired without directly detectable consequences, however long term mutational damage should not be excluded. Greulich studied laser irradiation (308–450 nm) and light-induced DNA damage and found that at 308 nm a fluence of 0.0001 J/cm^2 was sufficient to produce detectable DNA damage and at 450 nm a fluence of 1 J/cm^2 was required [22]. Damage declined exponentially with wavelength. Kujawa and colleagues found no considerable light-induced DNA damage after irradiating B14 cells to a wavelength of 810 nm at a fluence ranging from 3.75 to 15 J/cm^2 [23]. Hawkins and Abrahamse found a significant increase in DNA damage in normal and normal wounded fibroblast cells irradiated to a He-Ne laser (632.8 nm; 2 mW/cm^2) at a fluence of 5 or 10 J/cm^2 when irradiated once [15], and at a fluence of 10 and 16 J/cm^2 when irradiated on consecutive days [16]. Kao and Sheen treated rat hepatocytes with infrared and low-power laser for 20 minutes and tested for lactate dehydrogenase (LDH) leakage [24]. They found no significant change in laser irradiated cells compared to the control group, while Hawkins and Abrahamse found a significant increase in the release of LDH in irradiated normal and normal-wounded WS1 cells [15, 16]. It is notable that laser effects can be dramatically different depending on the cells being studied.

Due to the seriousness of inadequate wound healing in diabetic patients, phototherapy has been studied as an alternative treatment modality for such healing. There is little information available on the cellular and genetic damage, if any, of such therapies. This study aimed to establish which wavelength (632.8, 830, or 1064 nm) and fluence (5 or 16 J/cm^2) would induce cellular and genetic damage and stimulate wound healing in diabetic wounded fibroblast cells. Diabetic wounded cells irradiated with 5 J/cm^2 at a wavelength of 632.8 and 830 nm showed a significant decrease in oxidative DNA damage and complete wound closure. All cells irradiated with a fluence of 16 J/cm^2 showed an increase in LDH activity and DNA damage and incomplete wound closure.

2. MATERIALS AND METHODS

2.1. Cell culture

Human skin fibroblast cells (WS1, ATCC CRL1502) were grown to confluence in eagles minimum essential medium (BioWhittaker, 12-136F) containing 10% (v/v) fetal bovine serum (Delta Bioproducts, 14-501AI), 2 mM L-glutamine (BioWhittaker, 17-605E), 0.1 mM nonessential amino acids (BioWhittaker, 13-114E), 1 mM sodium pyruvate (BioWhittaker, 13-115E), and 1% (v/v) fungizone (BioWhittaker, 17-745E) at 37°C, 5% carbon dioxide and 85% humidity. An in vitro diabetic wound model was based on Rigau et al., Hamuro et al., and Vinck et al. [25–27]. Briefly, cells were cultured in complete media containing additional 17 mM/L D-glucose and a wound induced by scratching the monolayer of cells with a sterile pipette, exposing a cell free zone with a wound margin on either side. Normal (nondiabetic) control cells were included to determine if the added glucose had an effect on the cells. To determine the effects of the lasers, cells were detached by trypsinization (1 mL/25 cm^2, 0.25% trypsin −0.03% EDTA), and 6×10^5 cells in 3 mL complete culture media 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 wound was induced 30 minutes prior to laser irradiation [25].

2.2. Laser setup and irradiation

Wounded diabetic induced WS1 cells were irradiated in the dark to 3 different lasers, namely, the He-Ne (Spectra-Physics, Model 127), 830 nm diode (World Star Tech TECIRL-70G-830), and Nd : YAG (Coherent) laser. Laser parameters are shown in Table 1. Cells were irradiated with a fluence of 5 or 16 J/cm^2 on days 1 and 4 (72 hours between irradiations). Power output (mW) was measured before each exposure using a newport optical meter (Model 80), and the irradiation time calculated accordingly. Post laser irradiation on day 4, cells were incubated for an hour (37°C) before tests were performed. Normal unwounded unirradiated cells and

Table 1: Laser parameters. (He-Ne[a]-due to expansion of the laser beam output power was lost at the level of the cells, and as a result output power readings were multiplied by 0.871 to convert to the value at the cells; CW[b]-continuous wave emission).

Laser	Wavelength (nm)	Wave emission	Power output (mW)	Power density (mW/cm^2)	Spot size (cm^2)	Duration of irradiation
He-Ne[a]	632.8	CW[b]	23	2.206	9.1	37 min (5 J/cm^2) 2 h (16 J/cm^2)
Diode	830	CW	55	6	9.1	13 min 45 s (5 J/cm^2) 44 min (16 J/cm^2)
Nd : YAG	1064	CW	1000	12.7	78.5	6 min 34 s (5 J/cm^2) 21 min (16 J/cm^2)

diabetic wounded unirradiated cells were used as controls. Cells were detached by trypsinization and resuspended to a final concentration of approximately 1×10^5 cells/100 μL.

2.3. Wound healing (migration)

Each scratch was irregular and the size of the wounds ranged from 1 to 2 mm in diameter [28–30]. Haptotaxis (change in direction of growth), chemotaxis (migration), and the number of cells in the central scratch were examined in wounded cultures by inverted microscopy (Olympus CKX 41) following laser irradiation [25].

2.4. LDH release

Most cytotoxicity assays are based on the fact that the membrane of injured cells becomes permeable and cellular contents are released into the surrounding environment. Lactate dehydrogenase (LDH) is a stable cytosolic enzyme that is released upon cellular membrane damage or lysis.LDH released into the culture media was measured using the CytoTox 96 nonradioactive cytotoxicity assay, (Promega G1780). Maximum LDH values for diabetic induced and normal cells was obtained from samples incubated at −80°C, this value was then used to determine the percentage cytotoxicity. An equal volume of culture media and substrate (50 μL) was incubated for 30 minutes in the dark at room temperature. Stop solution was added (100 μL), absorbance read, (Bio-Rad Benchmark Plus Microplate Spectrophotometer $A_{490\,nm}$), and percentage cytotoxicity determined. Background absorbance from phenol red in the culture media was corrected by including and subtracting a culture medium background control from the results.

2.5. Genetic integrity

Genetic integrity was determined by measuring oxidative DNA damage using the alkaline comet assay, which was performed according to Collins [31]. Briefly, approximately 2×10^4 cells were embedded in 1% low-melting point agarose and lyzed (2.5 mM NaCl, 0.1 M EDTA, 10 mM Tris, pH 10.00, 1% Triton X-100 added before use) for an hour at 4°C. Damaged DNA underwent alkaline unwinding in electrophoresis solution (0.3 M NaOH, 1 mM EDTA) for 40 minutes at 4°C. During electrophoresis (300 mA, 4°C, 30 minutes) the relaxed coils were pulled out of the nucleiod "head" forming the "tail" of a comet-like image. Gels were neutralized by washing 3 times for 5 minutes each in neutralizing buffer (0.4 M Tris, pH 7.50) at 4°C, stained with 4′6-diamidine-2-phenylindol dihydrochloride (DAPI), (Scientific Group 32804803), and viewed on a fluorescent microscope (Olympus BX41/BX51). One hundred comets per gel were visually analyzed at random, and cells scored according to the five recognizable classes of comets, ranging from class 0 (undamaged, no discernible tail) to class 4 (almost all DNA in tail, insignificant head). Each comet was given a value according to the class it fell into (0–4), so that an overall score ranging from 0–400 arbitrary units could be derived for each gel. The higher the arbitrary units, the more DNA damage there is.

2.6. Statistical analysis

Each experiment was performed four times ($n = 4$) on different cell populations, and each test was done in duplicate, the average of which was used. Statistical analysis was done using the one-tailed student's t-test and one-way analysis of variance (ANOVA) using SigmaPlot Version 8.0, and was considered statistically significant when $P < .05$ and plotted using standard deviation bars. Wherever possible, statistical probability was expressed as $P < .05$, $P < .01$, and $P < .001$.

3. RESULTS

Cellular morphology was examined post irradiation for the rate of migration and wound healing ($n = 4$). All wounded cultures irradiated at 632.8 and 830 nm with 5 J/cm^2 showed a higher rate of cellular migration than unirradiated diabetic wounded cells (0 J/cm^2), and cells irradiated with 16 J/cm^2. By day 4, these cells showed complete wound closure, while cells irradiated at 1064 nm with 5 J/cm^2 and unirradiated control cells showed incomplete wound closure, with some open areas between cells (Figure 1). All cells irradiated with 16 J/cm^2 showed incomplete wound closure.

Cellular damage was determined by looking at membrane integrity (LDH), (Figure 2) and DNA damage (comet assay), (Figure 3). The percentage of cytotoxicity was determined from samples incubated at −80°C. In all three wavelengths, diabetic wounded unirradiated cells had a significant increase in cytotoxicity ($P < .001$) and DNA damage ($P < .001$ for 632.8 and 830 nm and $P < .01$ for 1064 nm) compared to normal unirradiated cells. When irradiated at 632.8 nm, diabetic wounded cells showed a significant increase in the percentage cytotoxicity and DNA damage when

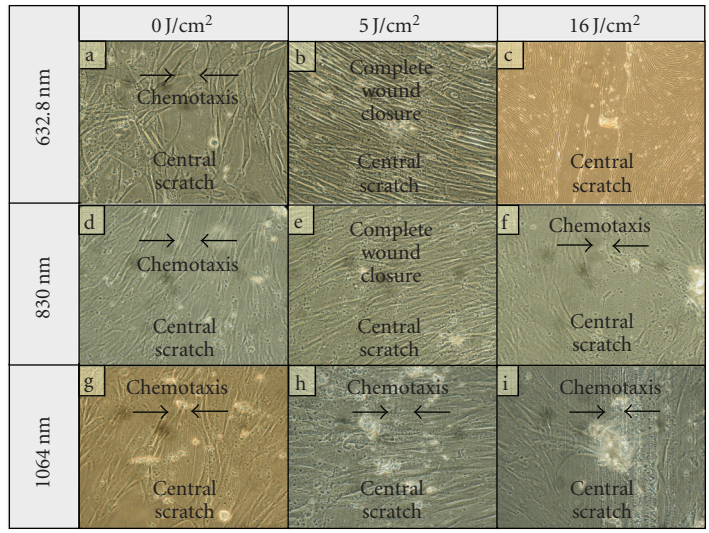

Figure 1: Wound healing was assessed by inverted microscopy in diabetic wounded WS1 cells 1 hour post irradiation on day 4 at 632.8, 830 and 1064 nm with 5 or 16 J/cm^2. All cells irradiated with 16 J/cm^2 showed incomplete wound closure (c, f, i), and there were less cells than the controls (0 J/cm^2), (a, d, g). Cells irradiated at 632.8 and 830 nm with 5 J/cm^2 showed complete wound closure (b, e), while cells irradiated at 1064 nm showed incomplete closure (hours).

irradiated with either 5 J/cm^2 ($P < .05$ and $P < .01$, resp.) or 16 J/cm^2 ($P < .001$) compared to normal unirradiated cells. However, when compared to diabetic wounded unirradiated cells, the increase in LDH released from damaged cells was not significant when irradiated with a fluence of 5 J/cm^2 ($P = .073$), while the increase in cells irradiated with 16 J/cm^2 was significant ($P < .001$). There was a significant decrease in arbitrary units, and hence DNA damage, in diabetic wounded cells irradiated with a fluence of 5 J/cm^2 compared to diabetic wounded unirradiated cells ($P < .05$), while cells irradiated with 16 J/cm^2 showed a significant increase ($P < .01$). Cells irradiated with 16 J/cm^2 showed more cellular damage than cells irradiated with 5 J/cm^2, as shown by the significant increase in the percentage of cytotoxicity and DNA damage ($P < .001$).

When irradiated at 830 nm, there was a significant increase in the percentage of cytotoxicity in diabetic wounded cells irradiated with either 5 or 16 J/cm^2 compared to normal unirradiated cells ($P < .001$), as well as diabetic wounded unirradiated cells ($P < .05$ and $P < .01$, resp.). There was a significant increase in DNA damage in cells irradiated with either 5 or 16 J/cm^2 compared to normal unirradiated cells ($P < .05$ and $P < .01$, resp.). When compared to diabetic wounded unirradiated cells, cells irradiated with a fluence of 5 J/cm^2 showed a significant decrease ($P < .05$), while cells irradiated with 16 J/cm^2 showed no change. Cells irradiated with a fluence of 16 J/cm^2 showed significantly more cellular membrane damage (LDH release) than cells irradiated with 5 J/cm^2 ($P < .05$), while there was no significant difference in DNA damage ($P = .073$). Cells irradiated at 830 nm with 16 J/cm^2 showed less LDH release and DNA damage than the same cells irradiated at a wavelength of 632.8 nm ($P < .01$ and $P = .001$, resp.).

Diabetic wounded cells irradiated with 5 J/cm^2 at 1064 nm showed a significant increase in the percentage of cytotoxicity and DNA damage only compared to normal unirradiated cells ($P < .01$ and $P < .05$, resp.). Cells irradiated with 16 J/cm^2 showed a significant increase inthe percentage of cytotoxicity and DNA damage compared to both normal ($P < .001$) and diabetic wounded unirradiated cells ($P < .001$), as well as cells irradiated with 5 J/cm^2 ($P < .001$ and $P < .01$, resp.). Cells irradiated at 1064 nm with 16 J/cm^2 showed significantly more LDH release compared to the same cells irradiated at a wavelength of either 632.8 or 830 nm ($P < .001$). There was only a significant difference in DNA damage between diabetic wounded cells irradiated with a fluence of 16 J/cm^2 at 1064 and 830 nm ($P < .001$), with more damage seen in cells irradiated at 1064 nm.

4. DISCUSSION AND CONCLUSION

The exact mechanism of action of laser phototherapy is not completely understood. However, it is known that the energy that is incorporated into the chromophore stimulates cellular metabolism [32]. The chromophore is able to transfer the absorbed energy to other molecules and thus cause chemical reactions in the surrounding tissue. Alternatively, the photoacceptor molecule in its activated form can take part in chemical reactions, for example in photodynamic therapy (PDT). Irradiating cells at a certain wavelength may also activate some of their own components, thus altering cellular metabolism, and it is this type of reaction that is believed to form the basis of low-power laser effects [33]. The acceptor molecules' kinetic energy can be increased, thereby activating or deactivating enzymes, which, in turn, are able to alter the physical and/or chemical properties of

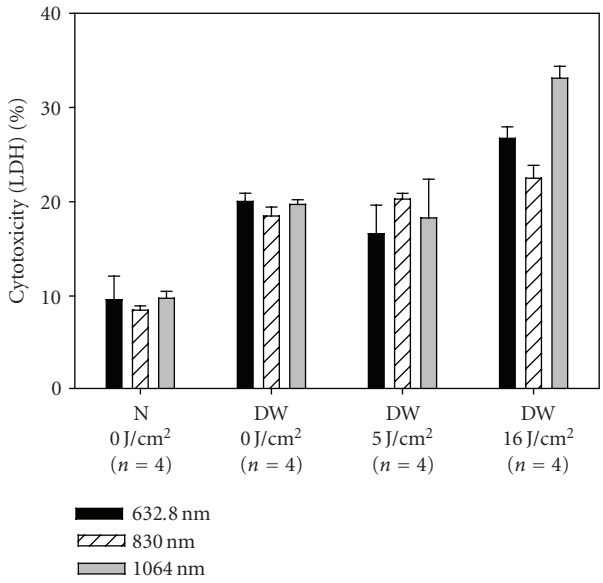

Figure 2: Cellular damage was determined by LDH released from damaged and lysed diabetic wounded (DW) human fibroblast WS1 cells irradiated with 5 or 16 J/cm^2. Unwounded normal (N) and DW unirradiated WS1 cells were used as controls (0 J/cm^2). There was a significant increase in % cytotoxicity in DW cells (0 J/cm^2) compared to N unirradiated cells ($P < .001$). Compared to N cells, all DW cells irradiated with 5 or 16 J/cm^2 at 632.8 nm ($P < .05$ and $P < .001$), 830 nm ($P < .001$ and $P < .01$) or 1064 nm ($P < .01$ and $P < .001$) showed a significant increase in % cytotoxicity. Cells irradiated with 16 J/cm^2 at 632.8, 830, and 1064 nm showed a significant increase in the percentage of cytotoxicity compared to DW cells (0 J/cm^2), ($P < .001$, $P < .01$, and $P < .001$, resp.).

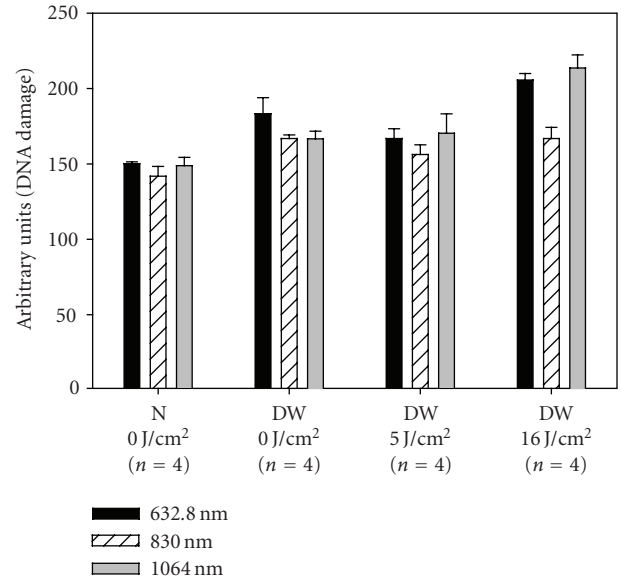

Figure 3: Genetic damage was determined by the alkaline comet assay in diabetic wounded (DW) human fibroblast WS1 cells irradiated with either 5 or 16 J/cm^2. Unwounded normal (N) and DW unirradiated WS1 cells were used as controls (0 J/cm^2). There was a significant increase in DNA damage in DW unirradiated cells compared to N unirradiated cells ($P < .001$). All DW cells irradiated at 632.8, 830, or 1064 nm with 5 J/cm^2 showed a significant increase in DNA damage compared to N unirradiated cells ($P < .01$, $P < .05$, and $P < .05$, resp.), however compared to DW unirradiated cells (0 J/cm^2), cells irradiated at 632.8 or 830 nm showed a significant decrease ($P < .05$). Cells irradiated with 16 J/cm^2 showed more genetic damage than cells irradiated with 5 J/cm^2, with the most damage seen in cells irradiated at a wavelength of 1064.

other macromolecules, such as DNA and RNA [34, 35]. According to Karu, visible and near-infrared (IR) radiations are absorbed by chromophores of the respiratory chain, which alters cellular metabolism [36]. The physical characteristics of the He-Ne and IR lasers permit a high penetration in skin since they are poorly absorbed by water and blood [37], while the laser light from the Nd : YAG laser at a wavelength of 1064 nm is strongly absorbed by water. Wavelengths in the 600 to 700 nm range are chosen for treating superficial tissue, and wavelengths between 780 and 950 nm are chosen for deeper-seated tissues [38]. Although higher wavelengths are generally not used for wound healing, some studies have used such wavelengths [39].

Wounded cells in culture (via the central scratch method) respond to the disruption of cell-cell contact by healing the wound through proliferation and migration [40, 41]. Irradiation at 632.8 and 830 nm with a fluence of 5 J/cm^2 stimulated cellular migration in diabetic wounded fibroblast cells in vitro. These cells showed a higher rate of migration compared to control cells, as well as cells irradiated at 1064 nm. A fluence of 16 J/cm^2 was inhibitory to cellular migration at all three wavelengths. Thus in this model, a fluence of 5 J/cm^2 at a wavelength of 632.8 and 830 nm is stimulatory to migration and promotes wound healing in vitro.

Diabetic wounded unirradiated cells showed a significant increase in LDH and DNA damage compared to normal unirradiated cells. This increase was due to the hyperglycemic environment as a number of studies have found that there is a significant increase in LDH and DNA damage when cells are grown in conditions of hyperglycemia [42–44]. Nakai and colleagues found that high glucose (25 mM/L) enhanced the production of nitric oxide (NO, a free radical synthesized from L-argenine by nitric oxide synthase [NOS, EC 1.14.13.39]) in a human keratinocyte cell line [45], which may be responsible for the increase in damage since in large doses NO is cytostatic which in turn initiate lipid peroxidation, protein damage, or DNA modification [23, 46]. Human dermal fibroblasts produce NO and express both constitutive eNOS and inducible iNOS. iNOS is up regulated during normal wound healing and is expressed in wound macrophages and fibroblasts [47, 48]. NO has been considered to have a biphasic effect in pathological conditions being both beneficial and detrimental depending on the concentration.

Diabetic wounded cells irradiated with a fluence of 5 J/cm^2 at a wavelength of 632.8 or 830 nm showed a significant increase in LDH and DNA damages when compared to normal unirradiated cells. However, when compared to diabetic wounded unirradiated cells, there was a significant decrease

in DNA damage. Cells irradiated at 632.8 nm showed no significant change in LDH, while cells irradiated at 830 nm showed a small significant increase ($P < .05$). The increase in membrane damage seen in these cells is likely due to growing cells in a hyperglycaemic environment and not laser induced, as Hawkins and Abrahamse found no significant difference in LDH between wounded cells irradiated with 5 J/cm^2 using 632.8 or 830 nm in the dark [49].

When diabetic wounded WS1 cells were irradiated with a fluence of 16 J/cm^2 at either 632.8 or 830 nm, the increase in LDH was significant when compared to both normal and diabetic wounded unirradiated cells. LDH is leaked from damaged cellular membranes [50], thus such fluences influence the cellular membrane. Only cells irradiated at 632.8 nm showed a significant increase in DNA damage, cells irradiated at 830 nm had no significant change. Diabetic wounded cells irradiated with either 5 or 16 J/cm^2 at a wavelength of 1064 nm had significantly increased cellular membrane and genetic damage, except in cells irradiated with 5 J/cm^2 compared to diabetic wounded unirradiated cells. This corresponds with Hawkins and Abrahamse who found a significant increase in DNA damage in normal WS1 cells irradiated with a He-Ne laser with 10 or 16 J/cm^2 [16]. Laser irradiation can protect cells from cellular and genetic damage at a fluence of 5 J/cm^2, or induce damage at 16 J/cm^2, and the effects are thus dose dependent. This decrease in damage may be due to an upregulation of enzymes involved in cellular and genetic repair. Zhang and colleagues irradiated human fibroblast cells (HS27) to red-light emitting InGaAIP diodes (Wavelength 628 nm, dose 0.88 J/cm^2) [51]. There was an upregulation of genes related to cell proliferation, antioxidants, and DNA synthesis and repair, to name but a few. Whelan and colleagues irradiated wounded mice with a light emitting diode (wavelength 670 nm, dose 4 J/cm^2) and noticed that genes associated with cell death were downregulated and proliferation and regulatory genes were upregulated [52]. A fluence of 16 J/cm^2 may not sound very high or capable of inducing a significant amount of damage, however, it should be remembered that when exposing cells in vitro the energy absorbed by the cells cannot be dispersed as what occurs in vivo leading to systemic effects. Energy absorbed by the cells in vitro is limited to the culture environment and can thus lead to cellular and molecular damage provided the fluence is above the threshold limit [53].

This study showed that cellular and genetic damage inflicted on diabetic wounded WS1 cells was influenced by hyperglycemia dose and more significantly, wavelength. Despite being subjected to three stressors (hyperglycaema, wound induction, and laser irradiation), diabetic wounded cells irradiated with 632.8 or 830 nm were able to respond in a positive manner and repair damage inflicted on them. A dose of 5 J/cm^2 was stimulatory, cells were protected from additional cellular damage, while 16 J/cm^2 increased cellular damage. A wavelength of 1064 nm showed the most cellular and genetic damage, particularly at a fluence of 16 J/cm^2. In other similar studies, 632.8 and 830 nm with a fluence of 5 J/cm^2 have been shown to positively stimulate cellular viability, proliferation, and wound healing in diabetic wounded cells, while irradiation with 16 J/cm^2 showed the opposite [54]. Irradiation at 1064 nm with either 5 or 16 J/cm^2 showed an increase in apoptosis (caspase 3/7 activity) and a decrease in viability and proliferation. Compared to 632.8 and 830 nm the increase in damage in cells irradiated at 1064 nm with 16 J/cm^2 may have been due to a local heating effect since a wavelength of 1064 nm is strongly absorbed by water. The temperature of the cell culture media was not measured, however if this increase in damage was due to a local heating effect, there would have also been an increase in damage in cells irradiated with 5 J/cm^2. Abergel and colleagues irradiated human skin fibroblasts with an Nd : YAG laser (wavelength 1064 nm) and the temperature of the media was monitored [55]. For comparison, parallel cultures were heated to the same temperature by a tungsten-halogen lamp. There was a significant decrease in collagen production and DNA replication in cultures subjected to the Nd : YAG laser. They concluded that the biochemical alterations in the fibroblast cells could not be explained on the basis of thermal effects [55]. Thus, the increase in damage in diabetic wounded cells irradiated at 1064 nm in this study is probably due to mechanisms other than heating at a dose of 5 and 16 J/cm^2, and further work on this is warranted.

This study showed that not only is it important to choose the correct fluence, but it is also important in choosing the correct wavelength so as not to induce additional cellular and genetic damage. This increase in cellular and oxidative DNA damage may be related to the generation of a large amount of reactive oxygen species (ROS), including NO, and further research is required to determine this relationship. During wound healing there is a balance between the generation of ROS and ROS-scavenging enzymes, or antioxidants. There is an imbalance of ROS in certain disease states, including diabetes. At the same time, literature indicates that there is a small increase in ROS following irradiation which may trigger cell stimulation [56]. It may be that at certain high fluences (such as 16 J/cm^2) and wavelengths (such as 1064 nm) there is a rapid generation of NO which damages cells and genetic material. DNA damage produced by ROS is the most frequently occurring damage, and if unrepaired, it can lead to the production of mutations by insertion of incorrect bases or cell death by blocking DNA replication [57]. There is a direct link between DNA damage and NO, and NO might mediate the formation of both oxidised purines and oxidised pyrimidines and induces the formation of single strand breaks [58–62] (Delaney 1993; Burney 1999; Jaiswal 2000; Li 2005; Davies 2007). NO is unstable and is potentially toxic due to the high reactivity of its unpaired electron. Overproduction of NO can lead to mutagenesis and cell death [59] (Burney 1999). NO induced cytotoxicity is a complex process and involves multiple pathways. This includes inhibition of DNA synthesis, mitochondrial damage, loss of membrane integrity, apoptosis, changes in cell cycle distribution, and the occurrence of DNA strand breaks [63] (Burney 1997). In this study, some of these processes can be seen in cells irradiated at 1064 nm with 16 J/cm^2: there is a decrease in cellular migration and proliferation, loss of membrane integrity as shown by the increase in LDH released into the culture media, and an increase in DNA single strand breaks as determined by the comet assay.

The use of phototherapy for the treatment of diabetic wounds appears to be beneficial; there is an increase in cellular migration and wound closure and a decrease in cellular and genetic damage. Cells were protected from the effects of growing in a hyperglycemic environment. Dube et al. found that pre-irradiation with an He-Ne laser resulted in a dose-dependent decrease in UVA-induced DNA damage [64], while Kholi and Gupta found that He-Ne laser pre-irradiation protected cells from UVC DNA damage [65]. This may be due to the adaptive response, where the first irradiation stimulates cellular repair mechanisms and they are better able to cope with subsequent challenges. Further work of the underlying molecular mechanisms is warranted and could prove this therapy to be a safe alternative treatment modality with no negative side effects. It is necessary to investigate the effects of different wavelengths and doses on different tissue types to properly determine its effect on different pathological conditions.

Damage inflicted in diabetic cells irradiated to 1064 nm was not temperature dependent, and might be due to the generation of ROS above the threshold. The type of DNA damage seen in diabetic wounded cells is oxidative single strand breaks, as this is the type of damage measured by the alkaline comet assay. The decrease in damage in cells irradiated at 632.8 and 830 nm may be due to an upregulation of repair enzymes at low doses, below 5 J/cm^2. Endogenous chromophores in the cell, such as the plasma NADPH oxidase system, absorb visible light and transfer it to nearby oxygen molecules, thus producing ROS. The amount of ROS in cells irradiated with visible light is not enough to become toxic [56], however, direct evidence of ROS creation by red light remains scarce [66]. The molecular actions of laser therapy still remain very unclear and the damaging effects of laser therapy controversial. Further work on repair enzymes and ROS generation is warranted. However, from this study it is evident that laser effects depend greatly on the wavelength, fluence, and type of cells used, and the molecular actions need to be determined at the level of individual cells.

ACKNOWLEDGMENTS

The National Laser Center (NLC), National Research Foundation (NRF) South Africa University of Johannesburg Research Council (URC), and the Medical Research Council (MRC) South Africa supported this research. Lasers were supplied by the NLC.

REFERENCES

[1] A. Schindl, M. Schindl, H. Schon, R. Knobler, L. Havelec, and L. Schindl, "Low-intensity laser irradiation improves skin circulation in patients with diabetic microangiopathy," *Diabetes Care*, vol. 21, no. 4, pp. 580–584, 1998.

[2] A. L. Carrington, C. A. Abbott, J. Griffiths, et al., "A foot care program for diabetic unilateral lower-limb amputees," *Diabetes Care*, vol. 24, no. 2, pp. 216–221, 2001.

[3] S. M. Sweitzer, S. A. Fann, T. K. Borg, J. W. Baynes, and M. J. Yost, "What is the future of diabetic wound care?" *Diabetes Educator*, vol. 32, no. 2, pp. 197–210, 2006.

[4] N. N. Houreld and H. Abrahamse, "Low level laser therapy for diabetic wound healing," *The Diabetic Foot*, vol. 8, no. 4, pp. 182–193, 2005.

[5] J. Tunér and L. Hode, *Laser Therapy. Clinical Practice and Scientific Background*, Prima Books, Grängesberg, Sweden, 2002.

[6] S. J. Gitomer and R. D. Jones, "Laser-produced plasmas in medicine," in *Laser-Tissue Interaction*, S. L. Jacques, Ed., vol. 1202 of *Proceedings of SPIE*, pp. 118–132, Los Angeles, Calif, USA, June 1990.

[7] G. M. Peavy, "Lasers and laser-tissue interaction," *Veterinary Clinics of North America: Small Animal Practice*, vol. 32, no. 3, pp. 517–534, 2002.

[8] P. J. Pöntinen, *Low Level Laser Therapy as a Medical Treatment Modality*, Art Urpo, Tampere, Finland, 1992.

[9] T. I. Karu, *The Science of Low-Power Laser Therapy*, Gordon & Breach Science, Amsterdam, The Netherlands, 1998.

[10] A. Schindl, M. Schindl, H. Pernerstorfer-Schön, and L. Schindl, "Low-intensity laser therapy: a review," *Journal of Investigative Medicine*, vol. 48, no. 5, pp. 312–326, 2000.

[11] H. Loevschall and D. Arenholt-Bindslev, "Effect of low level diode laser irradiation of human oral mucosa fibroblasts in vitro," *Lasers in Surgery and Medicine*, vol. 14, no. 4, pp. 347–354, 1994.

[12] J. R. Basford, "The clinical status of low energy laser therapy in 1989," *Journal of Laser Applications*, vol. 2, no. 1, pp. 57–63, 1990.

[13] A. J. Nemeth, "Lasers and wound healing," *Dermatologic Clinics*, vol. 11, no. 4, pp. 783–789, 1993.

[14] P. Moore, T. D. Ridgway, R. G. Higbee, E. W. Howard, and M. D. Lucroy, "Effect of wavelength on low-intensity laser irradiation-stimulated cell proliferation in vitro," *Lasers in Surgery and Medicine*, vol. 36, no. 1, pp. 8–12, 2005.

[15] D. Hawkins and H. Abrahamse, "Biological effects of helium-neon laser irradiation on normal and wounded human skin fibroblasts," *Photomedicine and Laser Surgery*, vol. 23, no. 3, pp. 251–259, 2005.

[16] D. H. Hawkins and H. Abrahamse, "The role of laser fluence in cell viability, proliferation, and membrane integrity of wounded human skin fibroblasts following helium-neon laser irradiation," *Lasers in Surgery and Medicine*, vol. 38, no. 1, pp. 74–83, 2006.

[17] H. S. Yu, C. L. Wu, C. L. Yu, Y. H. Kao, and M. H. Chiou, "Helium-neon laser irradiation stimulates migration and proliferation in melanocytes and induces repigmentation in segmental-type vitiligo," *Journal of Investigative Dermatology*, vol. 120, no. 1, pp. 56–64, 2003.

[18] S. Young, P. Bolton, M. Dyson, W. Harvey, and C. Diamantopoulos, "Macrophage responsiveness to light therapy," *Lasers in Surgery and Medicine*, vol. 9, no. 5, pp. 497–505, 1989.

[19] Y. Fujimaki, T. Shimoyama, Q. Liu, T. Umeda, S. Nakaji, and K. Sugawara, "Low-level laser irradiation attenuates production of reactive oxygen species by human neutrophils," *Journal of Clinical Laser Medicine and Surgery*, vol. 21, no. 3, pp. 165–170, 2003.

[20] L. S. Wong and M. Martins-Green, "Firsthand cigarette smoke alters fibroblast migration and survival: implications for impaired healing," *Wound Repair and Regeneration*, vol. 12, no. 4, pp. 471–484, 2004.

[21] G. Pal, D. Ashim, M. Kunal, et al., "Effect of low intensity laser interaction with human skin fibroblast cells using fiber-optic nano-probes," *Journal of Photochemistry and Photobiology B*, vol. 86, no. 3, pp. 252–261, 2007.

[22] K. O. Greulich, "Low level laser therapy (LLLT)—does it damage DNA?" to appear in *Laser Partner*, http://www.laserpartner.org/lasp/web/en/2003.

[23] J. Kujawa, I. B. Zavodnik, A. Lapshina, M. Labieniec, and M. Bryszewska, "Cell survival, DNA, and protein damage in B14 cells under low-intensity near-infrared (810 nm) laser irradiation," *Photomedicine and Laser Surgery*, vol. 22, no. 6, pp. 504–508, 2004.

[24] M. J. Kao and L. Y. Sheen, "Effects of infrared and low-power laser irradiation on cell viability, glutathione and glutathione-related enzyme activities in primary rat hepatocytes," *Journal of the Formosan Medical Association*, vol. 102, no. 7, pp. 486–491, 2003.

[25] J. Rigau, C. Sun, M. A. Trelles, and M. Berns, "Effects of the 633nm laser on the behaviour and morphology of primary fibroblasts in culture," in *Effects of Low Power Light on Biological Systems*, T. I. Karu and A. R. Young, Eds., vol. 2630 of *Proceedings of SPIE*, pp. 38–42, Barcelona, Spain, September 1996.

[26] M. Hamuro, J. Polan, M. Natarajan, and S. Mohan, "High glucose induced nuclear factor kappa B mediated inhibition of endothelial cell migration," *Atherosclerosis*, vol. 162, no. 2, pp. 277–287, 2002.

[27] E. M. Vinck, B. J. Cagnie, M. J. Cornelissen, H. A. Declercq, and D. C. Cambier, "Green light emitting diode irradiation enhances fibroblast growth impaired by high glucose level," *Photomedicine and Laser Surgery*, vol. 23, no. 2, pp. 167–171, 2005.

[28] J. C. Yarrow, Z. E. Perlman, N. J. Westwood, and T. J. Mitchison, "A high-throughput cell migration assay using scratch wound healing, a comparison of image-based readout methods," *BMC Biotechnology*, vol. 4, no. 1, p. 21, 2004.

[29] W. Posten, D. A. Wrone, J. S. Dover, K. A. Arndt, S. Silapunt, and M. Alam, "Low-level laser therapy for wound healing: mechanism and efficacy," *Dermatologic Surgery*, vol. 31, no. 3, pp. 334–340, 2005.

[30] D. Cha, P. O'Brien, E. A. O'Toole, D. T. Woodley, and L. G. Hudson, "Enhanced modulation of keratinocyte motility by transforming growth factor-α (TGF-α) relative to epidermal growth factor (EGF)," *Journal of Investigative Dermatology*, vol. 106, no. 4, pp. 590–597, 1996.

[31] A. R. Collins, "Measurement of oxidative DNA damage using the comet assay," in *Measuring in Vivo Oxidative Damage: A Practical Approach*, J. Lunec and H. R. Griffiths, Eds., pp. 83–94, John Wiley & Sons, Chichester, UK, 2000.

[32] A. L. B. Pinheiro, S. C. Nascimento, A. L. Cieira, et al., "Effects of low-level laser therapy on malignant cells: in vitro study," *Journal of Clinical Laser Medicine and Surgery*, vol. 20, no. 1, pp. 23–26, 2002.

[33] T. Karu, "Photobiology of low-power laser effects," *Health Physics*, vol. 56, no. 5, pp. 691–704, 1989.

[34] M. Matic, B. Lazetic, M. Poljacki, V. Duran, and M. Ivkov-Simic, "Low level laser irradiation and its effects on repair processes in the skin," *Medicinski Pregled*, vol. 56, no. 3-4, pp. 137–141, 2003.

[35] S. Takac and S. Stojanovic, "Diagnostic and biostimulating lasers," *Medicinski Pregled*, vol. 51, no. 5-6, pp. 245–249, 1998.

[36] T. Karu, "Primary and secondary mechanisms of action of visible to near-IR radiation on cells," *Journal of Photochemistry and Photobiology B*, vol. 49, no. 1, pp. 1–17, 1999.

[37] C. E. N. de Araújo, M. S. Ribeiro, R. Favaro, D. M. Zezell, and T. M. T. Zorn, "Ultrastructural and autoradiographical analysis show a faster skin repair in He-Ne laser-treated wounds," *Journal of Photochemistry and Photobiology B*, vol. 86, no. 2, pp. 87–96, 2007.

[38] M. R. Hamblin and T. N. Demidova, "Mechanisms of low level light therapy," in *Mechanisms of low level light therapy*, M. R. Hamblin, R. W. Waynant, and J. Anders, Eds., vol. 6140 of *Proceedings of SPIE*, pp. 614001-1–614001-12, Bellingham, Wash, USA, January 2006.

[39] A. Vesnaver and D. A. Dovsak, "Treatment of vascular lesions in the head and neck using Nd:YAG laser," *Journal of Cranio-Maxillofacial Surgery*, vol. 34, no. 1, pp. 17–24, 2006.

[40] B. L. Coomber and A. I. Gotlieb, "In vitro endothelial wound repair. Interaction of cell migration and proliferation," *Arteriosclerosis*, vol. 10, no. 2, pp. 215–222, 1990.

[41] J. M. Zahm, H. Kaplan, A. L. Herard, F. Doriot, D. Pierrot, P. Somelette, and E. Puchelle, "Cell migration and proliferation during in vitro wound repair of the respiratory epithelium," *Cell Motility and the Cytoskeleton*, vol. 37, no. 1, pp. 355–361, 1997.

[42] Q. Wu, J. H. Wang, F. Fennessy, H. P. Redmond, and D. Bouchier-Hayes, "Taurine prevents high-glucose-induced human vascular endothelial cell apoptosis," *American Journal of Physiology*, vol. 277, no. 6, pp. C1229–C1238, 1999.

[43] J. A. Gonzalez-Correa, M. M. Arrebola, A. L. Cansino, et al., "Effects of asprin plus alpha-tocopherol on brain slices damage after hypoxiz-reoxygeneration in rats with type 1-like diabetes mellitus," *Neurosci Letters*, vol. 400, no. 3, pp. 252–257, 2006.

[44] A. Arner, U. Malmqvist, A. Osterman, and B. Uvelius, "Energy turnover and lactate dehydrogenase activity in detrusor smooth muscle from rats with streptozotocin-induced diabetes," *Acta Physiologica Scandinavica*, vol. 147, no. 4, pp. 375–383, 1993.

[45] K. Nakai, S. Fujii, A. Yamamoto, J. Igarashi, Y. Kubota, and H. Kosaka, "Effects of high glucose on NO synthesis in human keratinocyte cell line (HaCaT)," *Journal of Dermatological Science*, vol. 31, no. 3, pp. 211–218, 2003.

[46] Y. G. Kim, "Laser mediated production of reactive oxygen and nitrogen species; implications for therapy," *Free Radical Research*, vol. 36, no. 12, pp. 1243–1250, 2002.

[47] M. B. Witte, F. J. Thornton, D. T. Efron, and A. Barbul, "Enhancement of fibroblast collagen synthesis by nitric oxide," *Nitric Oxide*, vol. 4, no. 6, pp. 572–582, 2000.

[48] M. R. Schäffer, U. Tantry, R. A. van Wesep, and A. Barbul, "Nitric oxide metabolism in wounds," *Journal of Surgical Research*, vol. 71, no. 1, pp. 25–31, 1997.

[49] D. Hawkins and H. Abrahamse, "Changes in cell viability of wounded fibroblasts following laser irradiation in broad-spectrum or infrared light," *Laser Chemistry*, vol. 2007, Article ID 71039, 10 pages, 2007.

[50] R. Moravec, "Total cell quantitation using the CytoTox 96TM non-radioactive cytotoxicity assay," *Promega Notes*, vol. 45, pp. 11–12, 1994.

[51] Y. Zhang, S. Song, C. Fong, C. Tsang, Z. Yang, and M. Yang, "cDNA microarray analysis of gene expression profiles in human fibroblast cells irradiated with red light," *Journal of Investigative Dermatology*, vol. 120, no. 5, pp. 849–857, 2003.

[52] H. T. Whelan, E. V. Buchmann, A. Dhokalia, et al., "Effect of NASA light-emitting diode irradiation on molecular changes for wound healing in diabetic mice," *Journal of Clinical Laser Medicine and Surgery*, vol. 21, no. 2, pp. 67–74, 2003.

[53] N. N. Houreld and H. Abrahamse, "In vitro exposure of wounded diabetic fibroblast cells to a helium-neon laser at 5 and 16 J/cm^2," *Photomedicine and Laser Surgery*, vol. 25, no. 2, pp. 78–84, 2007.

[54] N. N. Houreld and H. Abrahamse, "Laser light influences cellular viability and proliferation in diabetic-wounded fibroblast cells in a dose- and wavelength-dependent manner," *Lasers in Medical Science*, vol. 23, no. 2, pp. 11–18, 2008.

[55] R. P. Abergel, C. A. Meeker, R. M. Dwyer, M. A. Lesavoy, and J. Uitto, "Nonthermal effects of Nd:YAG laser on biological functions of human skin fibroblasts in culture," *Lasers in Surgery and Medicine*, vol. 3, no. 4, pp. 279–284, 1984.

[56] R. Lubart, M. Eichler, R. Lavi, H. Friedman, and A. Shainberg, "Low-energy laser irradiation promotes cellular redox activity," *Photomedicine and Laser Surgery*, vol. 23, no. 1, pp. 3–9, 2005.

[57] Z. Livneh, "DNA damage control by novel DNA polymerases: translesion replication and mutagenesis," *Journal of Biological Chemistry*, vol. 276, no. 28, pp. 25639–25642, 2001.

[58] C. A. Delaney, M. H. Green, J. E. Lowe, and I. C. Green, "Endogenous nitric oxide induced by interleukin-1β in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay," *FEBS Letters*, vol. 333, no. 3, pp. 291–295, 1993.

[59] S. Burney, J. L. Caulfield, J. C. Niles, J. S. Wishnok, and S. R. Tannenbaum, "The chemistry of DNA damage from nitric oxide and peroxynitrite," *Mutation Research*, vol. 424, no. 1-2, pp. 37–49, 1999.

[60] M. Jaiswal, N. F. LaRusso, L. J. Burgart, and G. J. Gores, "Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism," *Cancer Research*, vol. 60, no. 1, pp. 184–190, 2000.

[61] C. Q. Li and G. N. Wogan, "Nitric oxide as a modulator of apoptosis," *Cancer Letters*, vol. 226, no. 1, pp. 1–15, 2005.

[62] C. M. Davies, F. Guilak, J. B. Weinberg, and B. Fermor, "Reactive nitrogen and oxygen species in interleukin-1-mediated DNA damage associated with osteoarthritis," to appear in *Osteoarthritis and Cartilage*.

[63] S. Burney, S. Tamir, A. Gal, and S. R. Tannenbaum, "A mechanistic analysis of nitric oxide-induced cellular toxicity," *Nitric Oxide*, vol. 1, no. 2, pp. 130–144, 1997.

[64] A. Dube, C. Bock, E. Bauer, R. Kohli, P. K. Gupta, and K. O. Greulich, "He-Ne laser irradiation protects B-lymphoblasts from UVA-induced DNA damage," *Radiation and Environmental Biophysics*, vol. 40, no. 1, pp. 77–82, 2001.

[65] R. Kohli and P. K. Gupta, "Irradiance dependence fo the He-Ne laser-induced protection against UVC radiation in *E. coli* strains," *Journal of Photochemistry and Photobiology B*, vol. 69, no. 3, pp. 161–167, 2003.

[66] M. Eichler, R. Lavi, H. Friedmann, A. Shainberg, and R. Lubart, "Red light-induced redox reactions in cells observed with TEMPO," *Photomedicine and Laser Surgery*, vol. 25, no. 3, pp. 170–174, 2007.