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In Vitro Exposure of Wounded Diabetic Fibroblast Cells to a Helium-Neon Laser at 5 and 16 J/cm^2

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ABSTRACT

Objective: **The aim of the present investigation was to assess morphological, cellular, and molecular effects of exposing wounded diabetic fibroblast cells to He-Ne (632.8 nm) laser irradiation at two different doses.** ***Background Data:*** **An alternative treatment modality for diabetic wound healing includes low-level laser therapy (LLLT). Although it's used in many countries and for many medical conditions, too many health care workers are unaware of this therapy, and there is still controversy surrounding its effectiveness.** ***Methods:*** **Normal human skin fibroblast cells (WS1) were used to simulate a wounded diabetic model. The effect of LLLT (632.8 nm, 5 and 16 J/cm^2 once a day on two non-consecutive days) was determined by analysis of cell morphology, cytotoxicity, apoptosis, and DNA damage.** ***Results:*** **Cells exposed to 5 J/cm^2 showed a higher rate of migration than cells exposed to 16 J/cm^2, and there was complete wound closure by day 4. Exposure of WS1 cells to 5 J/cm^2 on two non-consecutive days did not induce additional cytotoxicity or genetic damage, whereas exposure to 16 J/cm^2 did. There was a significant increase in apoptosis in exposed cells as compared to unexposed cells.** ***Conclusion:*** **Based on cellular morphology, exposure to 5 J/cm^2 was stimulatory to cellular migration, whereas exposure to 16 J/cm^2 was inhibitory. Exposure to 16 J/cm^2 induced genetic damage on WS1 cells when exposed to a He-Ne laser *in vitro*, whereas exposure to 5 J/cm^2 did not induce any additional damage.**

INTRODUCTION

THE UNIQUE PROPERTIES of laser light—monochromaticity, coherence, compressibility, and collimation—has allowed for its use in a wide range of medical disciplines, and it has become a new treatment modality in a wide range of disorders, with more applications under investigation.[1] Some uses include acceleration of wound healing; enhanced remodeling and repair of bone; restoration of normal neural function following injury; pain attenuation; and modulation of the immune system.[2] A fair amount of research in laser therapy and cellular biology has been conducted with the desire to obtain reproducible, reliable, scientific results. A wide variety of cell types, including microorganisms, has and can be used as a model and depends on experimental objectives, and various end points have been looked at: proliferation, migration, collagen synthesis, and autocrine production of growth factors.[3] Once a model has been chosen, irradiation parameters (fluence, intensity, and wavelength) need to be determined, and it is these parameters that can lead to negative results. There is controversy surrounding some of the published results as laser light can cause inhibitory or stimulatory effects, which depend upon the choice of parameters.[4]

Diabetes mellitus (DM) is a chronic metabolic disorder caused by the inherited and/or acquired deficiency in the production of insulin or by the ineffectiveness of the insulin produced. Such a deficiency results in increased concentrations of glucose in the blood, which in turn damage many of the body's systems, in particular the blood vessels and nerves. Approximately 150 million people worldwide have DM, a figure that is likely to double by the year 2025 due to population growth, aging, unhealthy diets, obesity, and sedentary lifestyles.[5] The principal complications in DM arise from diseases in the blood vessels (macro- and microangiopathy). Fat and muscle atrophy, combined with sensory loss, due to diabetic neuropathy can result in pressure sores on the feet,

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which may necessitate amputation at a later date. Approximately 15% of diabetic patients will develop foot ulcers, and 6% of these will require hospitalization for the treatment of such ulcers.[6] Amputation is one of the most costly complications of diabetes and places an added economic burden on society due to a loss of earnings—claiming approximately 8% of health budgets in developed countries.[7]

Patients with DM require complex continuous medical attention to prevent acute complications and reduce the risk of long-term complications.[8] Foot ulceration is a major cause of disability in diabetic patients, and every effort and treatment available should be accessible to these patients to prevent or delay adverse outcomes and lower the incidence of amputation. There is a variation in the treatment of foot wounds in diabetic patients, and some of the established treatments include offloading, debridement, dressings, infection management, vascular reconstruction, amputation, and adjunctive medical therapies (e.g., normalization of blood glucose and treatment of edema).[8] Low-level laser therapy (LLLT) has been used in a variety of animal and human models in the treatment of diabetic wounds. It has been shown that laser therapy increases wound closure, improves wound epithelialization, cellular content, granulation tissue, and collagen deposition in diabetic mice.[9] There is a rapid decrease in the inflammatory reaction, cleansing, and acceleration of regeneration processes in infected wounds in diabetic patients.[10,11] LLLT has been shown to induce wound healing in conditions of reduced microcirculation,[12,13] enhance wound tensile strength,[14] accelerate collagen production,[15,16] and enhance angiogenesis.[17]

METHODS

Cell culture

All materials and cells used for cell culture were supplied through Adcock S.A. unless otherwise stated. Human skin fibroblast (HSF) cells, WS1 (ATCC CRL 1502), were grown to confluence in complete Eagle Earle's minimum essential media (EMEM, 12-136F) containing 10% fetal bovine serum (FBS, 14-501AI), 2 mM L-glutamine (17-605F), 0.1 mM non-essential amino acid (NEAA, 13-114E), 1 mM sodium pyruvate (13-115E), and 1% Pen-strep fungizone (17-74SE), at 37°C in 5% carbon dioxide and 85% humidity. A diabetic wound model was based on the published work of Hamuro et al. (2002),[18] who examined the effect of elevated extracellular glucose, thus simulating diabetes,[18–23] on cellular migration. A diabetic cell model was achieved by culturing the cells in complete media containing an additional 17 mMol/L D-glucose.[19] To determine the effects of the laser, cells were detached from the culture vessels by trypsinization (1 ml/25 cm^2, 0.25% trypsin [17-160E]/0.03% EDTA), and approximately 6×10^5 cells 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, and a central scratch, using a sterile pipette, was introduced to simulate a wound.[24]

Laser set-up and irradiation

Cells were exposed once per day on days 1 and 4. Cells were exposed to a He-Ne laser (Spectraphysics; model 127, wavelength 632.8 nm), with an output power of 3 mW/cm^2. The laser beam was expanded and clipped to give a truncated Gaussian beam with a spot size of 3.3 cm in diameter. Output power was measured before each exposure using a Newport Optical Meter (model 80) and averaged approximately 33 mW. A dose of either 5 or 16 J/cm^2 was used, corresponding to an exposure time of 26 min 33 sec and 84 min 23 sec, respectively. Unexposed wounded normal and unexposed wounded diabetic induced cells were treated in the same manner and were used as controls. Cells were exposed in 2000 μL of culture media; the culture media was not replaced as it was used in some of the tests. Each experiment was repeated in triplicate and each test in duplicate.

Morphology, cellular, and genetic parameters

The effect of the laser was determined by looking at cellular morphology, viability, cytotoxicity, apoptosis, and genetic integrity. Cellular morphology was determined by inverted microscopy (Olympus S.A. CKX41); the formation of spindle-shaped cells in wounded cultures was evaluated, characterizing normal processes involved in wound healing.[24] Colony formation, haptotaxis, chemotaxis, and the number of cells in the central scratch were also evaluated. After laser exposure, the culture media was removed and kept for further testing. Cells were removed by trypsonization and re-suspended to a final concentration of approximately 1×10^5/100 μL using the Trypan blue exclusion test. An equal volume of cells was mixed with 0.4% Trypan blue (Sigma S.A. T8154), in Hanks balanced salt solution (Adcock S.A. 10-543F), and the number of viable and non-viable cells was counted using a light microscope (Olympus S.A. BX41).

Cytotoxicity was measured using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega S.A. G4000). This assay quantitatively measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Maximum LDH values for diabetic induced and normal HSF cells was obtained from samples incubated at −80°C; this value was then used to determine the percentage of cytotoxicity. An equal volume of culture media and substrate was incubated for 30 min in the dark at room temperature. Stop solution was added (100 μl), absorbance was read (Bio-Rad S.A. Benchmark Plus Microplate Spectrophotometer A_{490nm}), and percentage of cytotoxicity was determined. Background absorbance from phenol red in the culture media was corrected by including and subtracting a culture medium background control from the results.

Apoptosis was determined by measuring caspase-3 and -7 using the Caspase-Glo™ 3/7 assay (Promega, S.A. TB323). Addition of reagent results in cellular lysis followed by substrate cleavage by caspase-3 and -7 and a luminescent signal is generated by luciferase. Negative controls consisted of reagent and culture media without cells. A positive control was included by inducing apoptosis in 1×10^6 cells/mL using 0.5 μg/mL Actinomycin D. An equal volume of reagent was added to cells; the contents were mixed and incubated at room temperature for 3 h. Luminescence was read using the Junior EG&G Berthold Luminometer. Luminescence is proportional to the amount of caspase activity present.

Genetic integrity was determined by measuring DNA damage using the Comet assay, which was performed according to

Collins.[25] Approximately 2×10^4 cells was embedded in 1% low melting point agarose (Sigma S.A. A-9414) and lyzed in lysis solution (2.5 mM NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100) for 1 h at 4°C. Damaged DNA underwent alkaline unwinding in electrophoresis solution (0.3 M NaOH, 1 mM EDTA). The relaxed coils were pulled out of the nucleoid "head" towards the anode, forming the "tail" of a comet-like image during electrophoresis at 25 V at 4°C for 30 min. Gels were neutralized (0.4 M Tris, pH 7.5) stained with 20 μL of a 1 μg/mL 4′6-diamidine-2-phenylindol dihydrochloride (DAPI; Scientific Group S.A. 32804803), and viewed on a fluorescent microscope, (Olympus S.A. BX41/BX51). One hundred comets per gel were visually analyzed at random, and cells were 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).

Statistical analysis

Unexposed control cells were included in each experiment, the average of which is represented in each graph. Statistical analysis was done using the one-tailed Student t = test using SigmaPlot Version 8.0 and was considered statistically significant at $p < 0.05$ (plotted using positive and negative standard error bars).

RESULTS

Cellular morphology

Number and intensity of colony formation, haptotaxis, chemotaxis, and number of cells in the central scratch were evaluated to determine fibroblast activity. There was no difference in cell structure between diabetic induced and normal

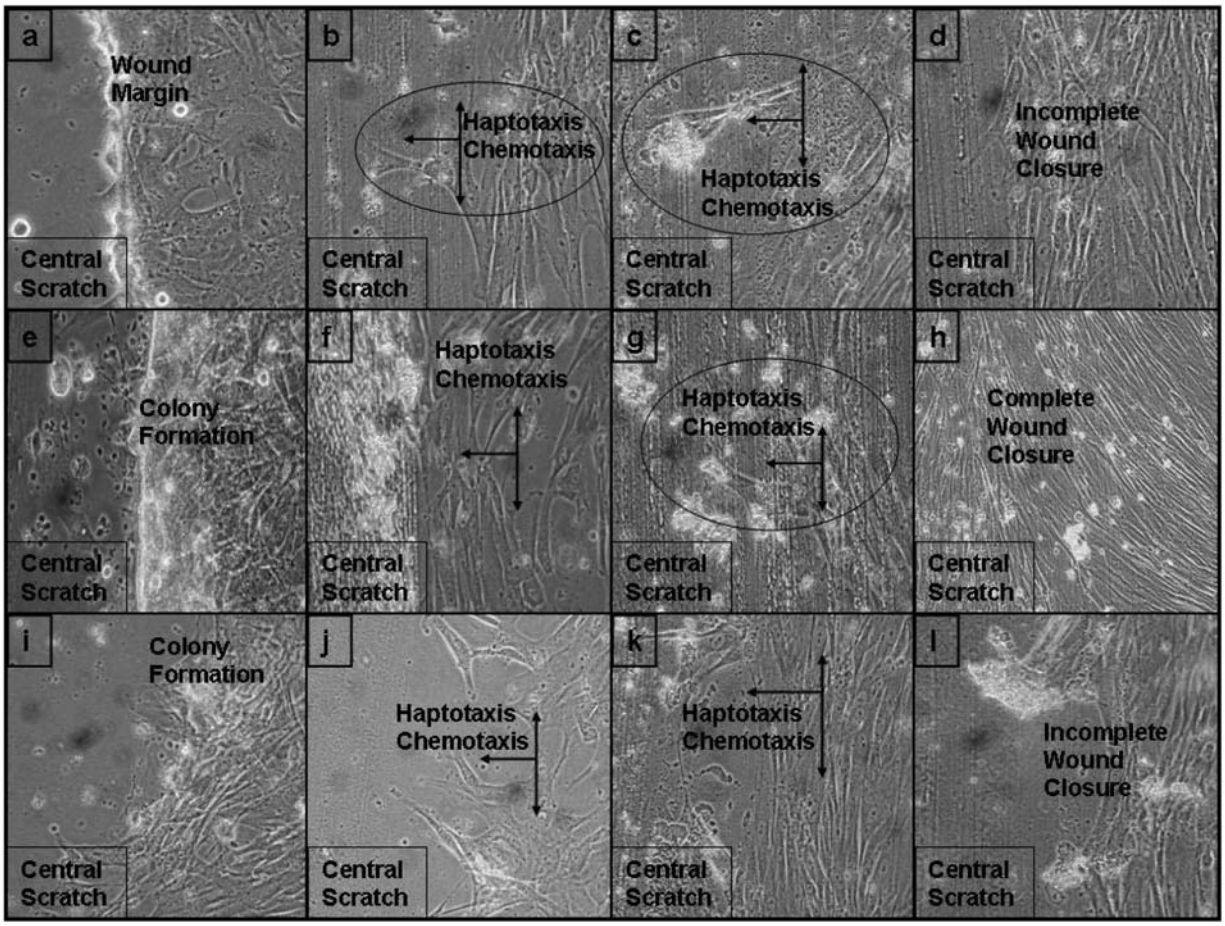

FIG. 1. Diabetic induced, wounded WS1 cells (40× magnification). Unexposed diabetic wounded cells. **(a)** Day 1: A wound margin and the central scratch can be clearly seen to the left of the picture. **(b)** Day 2: There is evidence of haptotaxis and chemotaxis; the cells are changing their direction of growth and are migrating towards the central scratch. **(c)** Day 3: There is still evidence of haptotaxis and chemotaxis, with cells in the center of the central scratch. **(d)** Day 4: The central scratch can still be clearly seen and there is incomplete wound closure. Diabetic wounded cells exposed once a day on days 1 and 4 to 5 J/cm^2. **(e)** Day 1: After laser irradiation, there is colony formation; cells begin to accumulate and group at the wound margin. **(f)** Day 2: There is a much more rapid rate of chemotaxis and haptotaxis; a lot more cells can be seen in the central scratch. **(g)** Day 3: Cells can be seen in the center of the central scratch. **(h)** Day 4: After laser irradiation complete wound closure is noted. Diabetic wounded cells exposed once a day on days 1 and 4 to 16 J/cm^2. **(i)** Day 1: After laser irradiation, there is some colony formation. **(j)** Day 2: There is chemotaxis and haptotaxis; cells are migrating towards the central scratch. **(k)** Day 3: Cells can be seen in the center of the central scratch. **(l)** Day 4: After laser irradiation incomplete wound closure is noted.

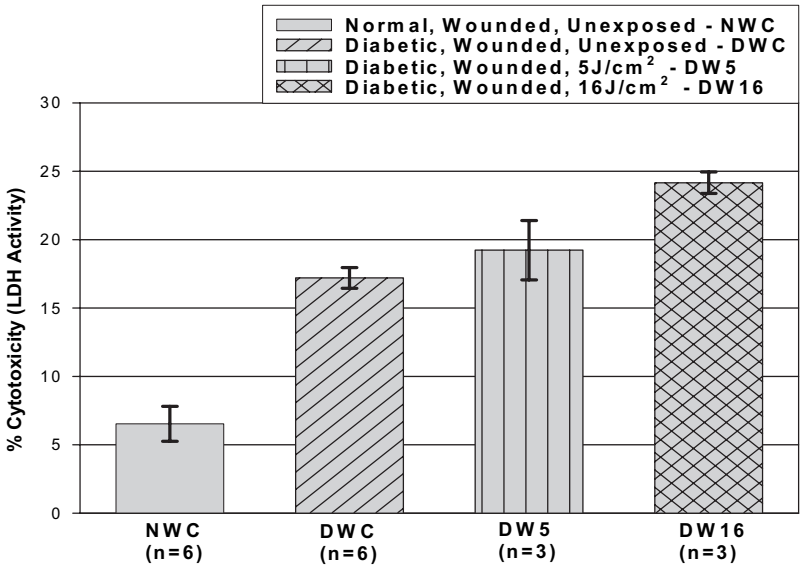

FIG. 2. The release of LDH (A_{490nm}) from frozen WS1 cells (representing maximum LDH) was used to determine percentage cytotoxicity in WS1 cells exposed once a day on days 1 and 4 to either 5 or 16 J/cm^2, mean ± SEM of triplicate. There is a statistical increase when WS1 cells are incubated in 17 mMol/L D-glucose and exposed to He-Ne laser irradiation at a dose of 5 and 16 J/cm^2. There is a significant difference between NWC and DWC ($p < 0.0001$), NWC and DW5 ($p = 0.0010$), NWC and DW16 ($p < 0.0001$), and DWC and DW16 ($p = 0.0010$).

WS1 cells, and no marked morphological changes were observed following LLLT. Corresponding with work conducted by Loevschall and Arenholtd-Bindslev,[26] cells remained long and slender in shape.

Unexposed, normal and diabetic induced, wounded WS1 cells showed signs of haptotaxis and chemotaxis after overnight incubation at 37°C (Fig. 1a–d). Spindle-shaped cells were seen migrating towards the central scratch. By day 3, fibroblast cells could be seen in the center of the central scratch, and on day 4, there were areas of "incomplete wound closure." Cells from either end of the wound margin did not make contact.

Wounded, diabetic induced cells exposed once a day to 5 J/cm^2 on days 1 and 4 remained intact and showed complete wound closure by day 4 (Fig. 1e–h). After irradiation on day 1, there was colony formation, with a dense cluster of cells along the wound margin. After overnight incubation, there was a much quicker rate of chemotaxis and haptotaxis, cells showed structural changes sooner, and cells in the central scratch were more numerous compared to unexposed cells. By day 3, spindle-shaped cells were seen in the center of the central scratch, and there was complete wound closure on day 4. Cells from either end of the wound margin made contact.

However, when exposed to 16 J/cm^2, there was incomplete wound closure by day 4, when wounded, diabetic induced cells were exposed once a day to 16 J/cm^2 on days 1 and 4, (Fig. 1i–l). After laser irradiation on day 1, there was colony formation, with cells beginning to cluster along the wound margin. By day 2, there was evidence of chemotaxis and haptotaxis, with spindle-shaped cells beginning to migrate towards the central scratch. On day 3, there was still evidence of haptotaxis and chemotaxis, and on day 4, after laser irradiation, there was incomplete wound closure.

Cytotoxicity

Percent cytotoxicity as a measure of LDH released into the culture media by lyzed cells was determined spectrophotometrically (A_{490nm}). Simulating diabetes in WS1 cells by growing them in 17 mMol D-glucose increased the amount of released LDH (Fig. 2). There was a significant increase in percentage of cytotoxicity in diabetic wounded unexposed cells as compared to normal wounded unexposed cells ($p < 0.0001$). Exposure of diabetic wounded WS1 cells to 5 J/cm^2 on days 1 and 4 did not induce additional damage on cells when compared to diabetic wounded unexposed cells ($p = 0.5476$), although there was a significant increase when compared to normal wounded unexposed cells ($p = 0.0010$). On the other hand, exposure of cells on days 1 and 4 at a dose of 16 J/cm^2 significantly increased the amount of cellular lysis and damage compared to diabetic wounded unexposed cells ($p = 0.0010$); however, the increase compared to cells exposed to 5 J/cm^2 was not significant ($p = 0.1084$). There was also a significant difference between normal wounded unexposed cells and diabetic wounded cells exposed to 16 J/cm^2 ($p < 0.0001$).

Apoptosis

Apoptosis or programmed cell death was significantly increased in cells exposed to either 5 or 16 J/cm^2 (Fig. 3). When cells were exposed to 5 J/cm^2, the increase in caspase-3 and -7 activity was increased as compared to normal wounded unexposed and diabetic wounded unexposed cells ($p = 0.0002$ and $p = 0.0001$, respectively). The increase in cells exposed to 16 J/cm^2 was also significant when compared to normal wounded unexposed and diabetic wounded unexposed cells ($p = 0.0003$ and $p = 0.0002$, respectively). There was no change between 5 and

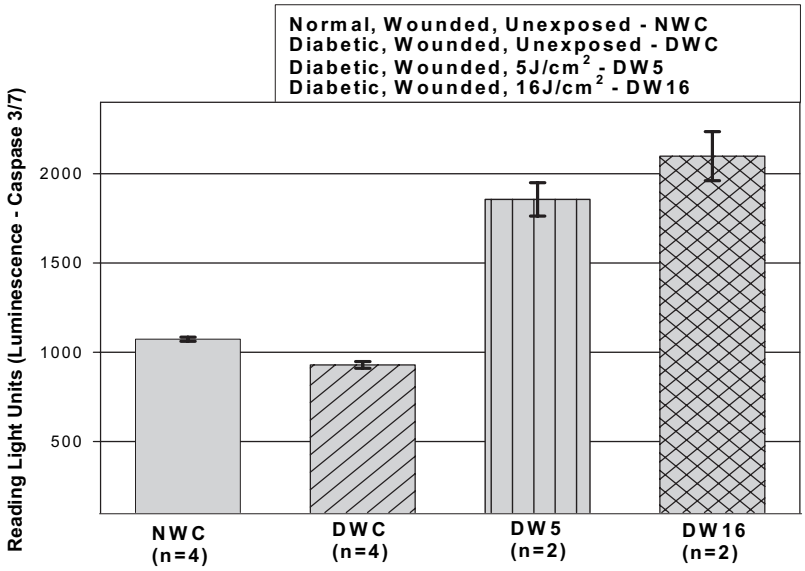

FIG. 3. Apoptosis was determined by measuring caspase-3 and -7 activity in WS1 cells exposed once a day on days 1 and 4 to either 5 or 16 J/cm^2, mean ± SEM of duplicate. There is a statistical increase in apoptosis when cells are exposed to 5 and 16 J/cm^2 as compared to NWC ($p = 0.0002$ and $p = 0.0003$, respectively) and DWC ($p = 0.0001$ and $p = 0.0002$, respectively).

16 J/cm^2. Diabetic wounded unexposed cells had less apoptosis than normal wounded unexposed cells ($p = 0.0006$).

Genetic integrity

The amount of DNA damage was assessed by the Comet assay, performed according to Collins.[25] One hundred comets were scored according to the class they fell into: class 0–4. The class number multiplied by the number of comets in each class gave an arbitrary unit of 0–400. The higher the arbitrary unit, the more DNA damage present. There was no change in diabetic wounded unexposed cells as compared to normal wounded unexposed cells; thus, growing WS1 cells in a hyperglycemic environment for up to 5 days did not induce additional DNA damage (Fig. 4). When WS1 cells were exposed to 5 J/cm^2 once a day on days 1 and 4, there was no change as compared to diabetic wounded unexposed cells and normal wounded unexposed cells. Thus, such an exposure regime *in vitro* did not induce additional DNA damage on cells. However, when WS1 cells were exposed to 16 J/cm^2 once a day on days 1 and 4, there was a significant increase as compared to normal wounded unexposed cells ($p = 0.0007$) and diabetic

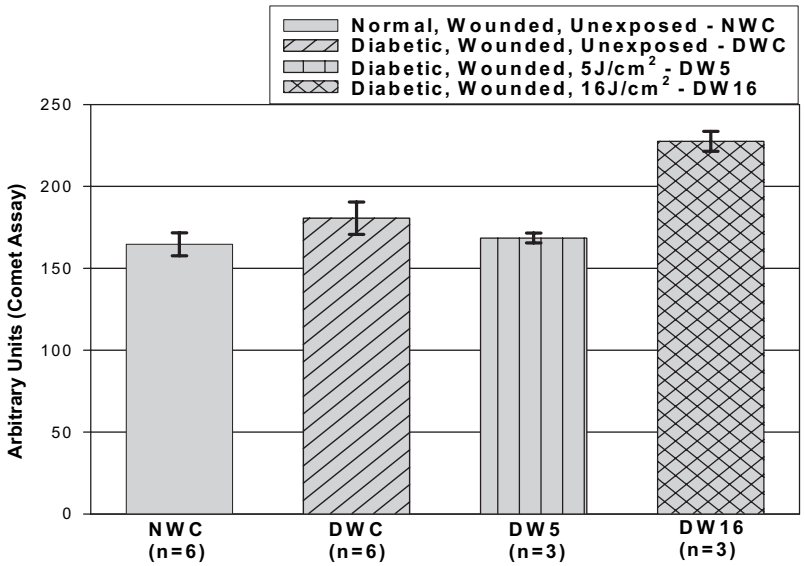

FIG. 4. Genetic integrity was determined by the Comet assay. Comets were scored manually and represented as arbitrary units. WS1 cells were exposed once a day on days 1 and 4 to either 5 or 16 J/cm^2, mean ± SEM of triplicate. There is a statistical increase in DNA damage in cells exposed to 16 J/cm^2. There is a statistical difference between NWC and DW16 ($p = 0.0007$), DWC and DW16 ($p = 0.0165$), and between DW5 and DW16 ($p = 0.0010$).

wounded unexposed cells ($p = 0.0165$), as well as when compared to cells exposed to 5 J/cm^2 ($p = 0.0010$).

DISCUSSION

From the cellular morphology, it can be seen that exposure to 5 J/cm^2 increases cell migration and wound closure in diabetic induced wounded WS1 cells. After a single exposure, there is evidence of colony formation as cells begin to group and accumulate at the wound margin. There is also a greater rate of chemotaxis and haptotaxis and complete wound closure by day 4 as compared to unexposed cells. Exposure to 16 J/cm^2 once a day on days 1 and 4 inhibits cellular migration, cells do not make contact in the central scratch, and there is incomplete wound closure.

Simulating diabetes in WS1 cells by growing them in 17 mMol D-glucose does not have an effect on cellular migration or genetic integrity; it does however increase cellular lysis as measured by LDH release. When percentage viability is calculated using the Trypan blue exclusion test, there is no change in cell viability in wounded diabetic induced cells as compared to normal wounded unexposed cells; this contradicts with results obtained from LDH release. Trypan blue is a stain that enters cells through a damaged membrane; thus, viable cells remain unstained, and non-viable cells stain blue. However, this test does not take into account cells that have lyzed and will be seen as fragments under the microscope, and is thus not as sensitive as the other methods.

In vitro exposure of diabetic wounded WS1 cells to 5 J/cm^2 on days 1 and 4 using a He-Ne laser (33 mW) does not induce additional damage to cells. There is no adverse effect on cellular viability (as determined by Trypan blue), cytotoxicity, and DNA damage. On the other hand, exposure to 16 J/cm^2 is damaging; there is an increase in cytotoxicity and DNA damage. Laser exposure using a He-Ne laser induces apoptosis in cells exposed to both 5 and 16 J/cm^2.

CONCLUSION

Growing WS1 cells in additional 17 mM D-glucose to simulate diabetes does not inhibit cellular migration; this corresponds with work done by McDermott et al.,[19] on SV40 transformed human corneal epithelial cells grown in 17 mM D-glucose. Diabetic wounded WS1 cells irradiated with a He-Ne laser (632.8 nm) to a dose of 5 J/cm^2 show structural and cellular resilience comparable to that of normal and diabetic wounded unexposed skin fibroblast cells. These results may be indicative of the cellular value of LLLT *in vitro*.

Normal and diabetic induced wounded fibroblast cells respond better when incubated overnight; cells show a higher rate of migration and proliferation; and cells are able to respond, adapt, and recover. Human skin fibroblast cells exposed to 5 J/cm^2 *in vitro* to a He-Ne laser respond better than unexposed control cells; and there is a higher rate of migration across the central scratch, with complete wound closure by day 4. Cells exposed to 16 J/cm^2 once a day on 2 non-consecutive days show little migration from the wound margin; there is no migration across the central scratch, indicating an inhibitory effect of irradiation at such a dose. Irradiating diabetic induced WS1 cells to a He-Ne laser to lower fluences (5 J/cm^2) is stimulatory on cellular migration, an important factor in wound healing, whereas higher fluences (16 J/cm^2) are inhibitory.

In a similar study conducted by Hawkins and Abrahamse,[27] there was a statistical decrease in percentage of viability after a single exposure to 5 J/cm^2. Our results show that cells exposed to 5 J/cm^2 on 2 non-consecutive days show similar values as unexposed cells. The increase in cytotoxicity as compared to normal wounded unexposed cells is most likely due to growth in a hyperglycemic media, as there is no difference between cells exposed to either 5 or 16 J/cm^2 and diabetic wounded unexposed cells. Thus, LLLT on 2 non-consecutive days to a He-Ne laser at a dose of 5 or 16 J/cm^2 does not induce additional cellular lysis; it does however induce apoptosis.

A dose of 5 J/cm^2 does not inflict genetic damage when exposed on non-consecutive days. Hawkins and Abrahamse[27] found a significant increase when WS1 cells are exposed once to 5 J/cm^2. This initial damage appears to be repairable, and it is likely that there may be an adaptive response, and cells are able to respond and adapt to the second exposure. More work on DNA damage and repair is warranted.

On the other hand, when cells are exposed on non-consecutive days to 16 J/cm^2, there is a significant difference between unexposed cells and cells exposed to 5 J/cm^2; the damage inflicted on cells is not repaired. A dose 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 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 dose is above the threshold limit.

People with diabetes are 10–15 times more likely to have lower limb amputations than non-diabetic individuals,[28] and these account for 50% of all non-traumatic amputations.[12] Due to the costs involved in the treatment of diabetic wounds, particularly foot ulcers and amputations, and the economic burden placed on patients, caregivers, and society, this research fulfills an ever increasing need to develop a reliable, safe, and cost-effective treatment modality, not only for diabetic wound healing, but also for wound healing in other disease conditions. Despite all efforts in the prevention of lower limb amputation in diabetic patients in the past decade, the incidence continues to rise; thus, appropriate techniques for wound care that can reduce amputation rates are essential.[29] LLLT might be of benefit in selected indications if the existing preliminary data can be confirmed by further prospective and well-controlled studies.[30]

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REFERENCES

1. Council on Scientific Affairs. (1986). Lasers in medicine and surgery. JAMA 256, 900–907.
2. Cabrero, M.V., Failde, J.M.G., and Mayordomo, O.M. (1985). Laser therapy as a regenerator for healing wound tissues. Presented at the International Congress on Laser in Medicine and Surgery.
3. Karu, T. (1998). Basics of the action of momochromatic visible and near infrared radiation in cells, in: *The Science of Low-Power Laser Therapy*. Amsterdam: Gordon and Breach Science, pp. 1–21.
4. Pinheiro, A.L.B., Nascimento, S.C., Vierira, R., et al. (2002). Effects of low-level laser therapy on malignant cells: *in vitro* study. J. Clin. Laser Med. Surg. 20, 23–26.
5. World Health Organization. (2002). Diabetes mellitus. Fact sheet no. 138. Available at: www.who.int/inf-fs/en/fact138.html. Accessed January 10, 2007.
6. Carrington, A.L., Abbott, C.A., Griffiths, J., et al. (2001). A foot care program for diabetic unilateral lower limb amputees. Diabetes Care 24, 216–221.
7. World Health Organization. (1998). Global burden of diabetes. Press release WHO/63. Available at: www.who.int/inf-pr-1998/en/pr98–63.html. Accessed January 10, 2007.
8. American Diabetes Association. (2004). Standards of medical care in diabetes. Diabetes Care 27, S1–S39.
9. Yu, W., Naim, J.O., and Lanzafame, R.J. (1997). Effects of photostimulation on wound healing in diabetic mice. Lasers Surg. Med. 20, 56–63.
10. Khurshudian, A.G. (1989). Use of helium-cadmium lasers in the complex treatment of suppurative diseases in patients with diabetes mellitus. Khirurgiia (Mosk.) 6, 38–42.
11. Kuliev, R.A., and Babaev, R.F. (1991). Therapeutic action of laser irradiation and immunomodulators in purulent injuries of the soft tissues in diabetic patients. Probl. Endokrinol. (Mosk.) 37, 31–32.
12. Schindl, A., Schindl, M., Schon, H., et al. (1998). Low-intensity laser irradiation improves skin circulation in patients with diabetic microangiopathy. Diabetes Care 21, 580–584.
13. Schindl, A., Heinze, G., Schindl, M., et al. (2002). Systemic effects of low-intensity laser irradiation on skin microcirculation in patients with diabetic microangiopathy. Microvasc. Res. 64, 240–246.
14. Stadler, I., Lanzafame, R.J., Evans, R., et al. (2001). 830-nm irradiation increases the wound tensile strength in a diabetic murine model. Lasers Surg. Med. 28, 220–226.
15. Reddy, G.K., Stehno-Bittel, L., and Enwemeka, C.S. (2001). Laser photostimulation accelerates wound healing in diabetic rats, Wound Repair Regen. 9, 248–255.
16. Reddy, G.K. (2003). Comparison of the photostimulatory effects of visible He-Ne and infrared Ga-As lasers on healing impaired diabetic rat wounds. Lasers Surg. Med. 33, 344–351.
17. Garavello, I., Baranauskas, V., and Da Cruz-Hofling, M.A. (2004). The effects of low-level laser irradiation on angiogenesis in injured rat tibiae. Histol. Histopathol. 19, 43–48.
18. Hamuro, M., Polan, J., Natarajan, M., et al. (2002). High glucose induced nuclear factor kappa B mediated inhibition of endothelial cell migration. Atherosclerosis 162, 277–287.
19. McDermott, A.M., Kern, T.S., and Murphy, C.J. (1998). The effect of elevated extracellular glucose on migration, adhesion and proliferation of SV40 transformed human corneal epithelial cells. Curr. Eye Res. 17, 924–932.
20. Coughlan, M.T., Oliva, K., Georgiou, H.M., et al. (2001). Glucose-induced release of tumour necrosis factor–alpha from human placental and adipose tissues in gestational diabetes mellitus. Diabet. Med. 18, 921–927.
21. Morohoshi, M., Fujisawa, K., Uchimura, I., et al. (1995). The effect of glucose and advanced glycosylation end products on IL-6 production by human monocytes. Ann. N.Y. Acad. Sci. 17, 562–570.
22. Morohoshi, M., Fujisawa, K., Uchimura, I., et al. (1996). Glucose-dependent interleukin 6 and tumour necrosis factor production by human peripheral blood monocytes *in vitro*. Diabetes 45, 954–959.
23. Pampfer, S., Vanderheyden, I., and De Hertogh, R. (1997). Increased synthesis of tumour necrosis factor–alpha in uterine explants from pregnant diabetic rats and in primary cultures of uterine cells in high glucose. Diabetes 46, 1214–1224.
24. Rigau, J., Sun, C., Trelles, M.A., et al. (1995). Effects of the 633-nm laser on the behavior and morphology of primary fibroblasts in culture, in: *Effects of Low-Power Light on Biological Systems*. T. Karu and A. Young (eds.). Barcelona; Progress in Biomedical Optics, pp. 38–42.
25. Collins, A.R. (2000). 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.). Chichester, UK: John Wiley and Sons, pp. 83–94.
26. Loevschall, H., and Arenholtd-Bindslev, D. (1994). Effect of low-level diode laser irradiation of human oral mucosa fibroblasts *in vitro*. Lasers Surg. Med. 14, 347–354.
27. Hawkins, D., and Abrahamse, H. (2005). Biological effects of helium-neon laser irradiation on normal and wounded human skin fibroblasts. Photomed. Laser Surg. 23, 251–259.
28. Bloomgarden, Z.T. (2001). American Diabetes Association 60th scientific sessions, 2000. Diabetes Care 24, 946–951.
29. American Diabetes Association. (1999). Consensus development conference on diabetic foot wound care. Diabetes Care 22, 1354–1360.
30. Schindl, A., Schindl, M., Pernerstorfer-Schon, H., et al. (2000). Low-intensity laser therapy: a review. J. Invest. Med. 48, 312–326.

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