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Natural reactions of harmed human skin fibroblasts to evaluate the adequacy of in vitro models for cell stress contemplates

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Abstract

The classical central scratch method has been used to successfully induce a wound environment however this model has been criticized. This study aimed to compare the cellular responses of normal fibroblast cells with those of wounded, diabetic wounded, acidic and hypoxic cells to determine if the latter two models are more effective in inducing an injured environment than the central scratch method. The cellular responses that were evaluated included; cell morphology, viability, proliferation, cytotoxicity and DNA damage. The results from this study showed that the biochemical tests were sensitive enough to distinguish changes between normal and wounded, diabetic wounded, acidic and hypoxic cells. Wounded cells showed a decrease in ATP viability, increase in caspase 3/7 activity, increase in proliferation and increase in cytotoxicity and DNA damage when compared to normal cells. This study confirmed that although a central scratch method only wounds 5 - 10% of the surface cells it was sufficient to successfully induce a wound environment *in vitro* . The results suggest that the *in vitro* models may not only be used to study wound healing but also cellular responses related to other pathological conditions such as heart attack, stroke, diabetes and biological or biochemical changes in response to treatment such as laser therapy otherwise known as phototherapy.

Keywords: Acidosis, diabetic, fibroblasts, hypoxia, injury, wounds.

INTRODUCTION

Normal cells in culture, unlike cancer or virus transformed cells show “contact inhibition” of growth and the population density stabilizes at relatively low levels, the precise value varying with the individual cell and the serum. It is this property that provides a suitable environment to study the cellular responses of cells as they react to an insult or injury. The central scratch method has been used with multiple cell types and, as the monolayers heal the wound in a characteristic manner, they have been used to study cell polarization, matrix remodeling, cell migration, and numerous other processes (Cha et al., 1996; Hawkins and Abrahamse, 2005). The injury model simulates *in vivo* mechanical trauma and the processes reflect the behavior of individual cells as well as the properties of the cell sheet as a surrogate tissue. The wounds heal in a stereotypical fashion – cells polarize toward the wound, initiate protrusion, migrate, and close the wound. Progression of these events can be monitored by manually imaging samples fixed at time points over a period of 3 – 24 h or by time-lapse microscopy (Yarrow et al., 2004; Rigau et al., 1995; Wong and Gotleib, 1988).

Other models have been used to simulate an *in vitro* wound environment. The classical scratch method allows wounding of up to 5 - 10% of the surface of a confluent monolayer of cultured cells grown on a culture dish whereas a wounding machine used by Dayem et al. (2003) wounded up to 40% of the surface. Dayem et al. (2003) used a spiral scarificator that develops a large wound over the surface of a confluent monolayer of cul-

tured cells according to methods described by Turchi et al. (2002). Kornyei et al. (2000) used an *in vitro* scratch-wound model to investigate astroglial responses to mechanical injury while Saga et al. (2003), Liang et al. (2004), Farooqui et al. (2004), and Walker et al. (2004) used the cell scratch method to study the proliferative and migratory responses of different wounded cells.

Furthermore, Lau and Yu (2001) focused on the production of four inflammatory cytokines in primary culture and reported that all four cytokines began to increase 1 h post scratch and remained at high levels throughout the experiment.

Diabetes mellitus 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 damages many of the body's systems, in particular the blood vessels and nerves. Houreld and Abrahamse (2007) studied the effects of helium- neon (632.8 nm using 5 and 16 J/cm^2) laser irradiation on wounded diabetic (complete media containing an additional 17 mM/L D-glucose) fibroblast cells by assessing changes in cell morphology, cytotoxicity, apoptosis and DNA damage. The study concluded that laser therapy is a reliable, safe, and cost-effective treatment modality, not only for diabetic wound healing, but also for wound healing in other disease conditions. Hamuro et al. (2002) and Baumgartner-Parzer et al. (1995) also successfully used the diabetic wound model to simulate diabetes and examine the effect of elevated extracellular glucose on cell migration and apoptosis.

Other models that may be considered include a hypoxic, ischemic or acidic conditions, which are created by homogenously insulting the cells by changing the cell culture medium in which the cells are growing. *In vitro* models of hypoxia (oxygen deprivation) are examples where control of oxygen is imperative. However, rapid removal of oxygen can be used to mimic the *in vivo* conditions of heart attack, heart failure, stroke and gangrene. Oxygen is a major player in the maintenance of mitochondrial function, lack of which in the absence of food nutrient like glucose leads to ischemic cell death (Lipton et al., 1999). Consistent exposure to *in vitro* hypoxia, anoxia or ischemia is imperative to allow testing of therapeutic agents and treatments aimed at preventing cell death (Munnus and Arthur, 2002).

Yu et al., (2002) used an *in vitro* ischemic model to study ischemic injury in astrocytes and reported the larg-est decline in the percentage viability of cells during ischemia, which corresponded well to the reduction in ATP and ADP levels in these cultures. Astrocytes remained unaltered after 2 h of ischemia and retained their ability to produce ATP but were moderately or severely damaged after 4 h or after 6 - 8 h, respectively. Studies have showed that short-term intermittent hypoxia for 1 and 4 days appears to exert a protective effect on hearts whereas long-term intermittent hypoxia for 1 and 2 weeks appears to exert deleterious effects (Lee et al., 2006).

Avni et al. (2005) studied the effect of laser irradiation (GaAs, 810 nm) on ischemic reperfusion (I-R) injury in the gastrocnemius muscle of the rat by complete occlu-sion of the blood supply for 3 h. The study found that laser irradiation markedly protects skeletal muscles from degeneration following acute I-R injury. The study also found that the level of antioxidants was elevated following laser irradiation, which may reduce the cytotoxic reactive oxygen superoxides, which together with blood reperfusion aggravates the hypoxia during ischemic injury.

Acidic conditions (low pH) can be found in many pathological conditions including ischemia, diabetes, uremia, wound healing, drug abuse, poison and shear stress (DuBoss, 1998). The pH of the culture medium is one of the most important factors, as growth promoting properties and the selectivity of the culture media are pH dependent. It is important to maintain pH at an optimal level for cells to survive and proliferate. Contact inhibition of growth, growth rate and cell mobility are all strongly influenced by pH of the culture medium (Lie et al., 1972). Miller et al. (1988), Hayter et al. (1992) and Nagira et al. (1995) found that a pH range of 6.8 - 8.2 enhanced human-human hybridoma (HB4C5) cell viability, while ranges out-side demonstrated a decrease in cell viability. Ceccarine and Eagle (1971) found that the rate of growth of skin and lung fibroblasts, whether normal, cancer or virus -transformed were markedly pH dependent as is also the maximum population density attained. Results showed that "contact inhibition" of growth may be determined not only by population density but by artefactual variations in the pH of the medium. Ceccarine and Eagle (1971) reported that the optimum pH for human fibroblasts varies from 6.9 - 7.8. For this study complete EMEM media was adjusted to a pH of 6.7 as it falls outside the optimal growth range for fibroblasts and this pH value has frequently been associated with acidic conditions *in vivo* (DuBoss, 1998; Ceccarine and Eagle, 1971).

This study aimed to evaluate four *in vitro* models that can be used to induce cell stress or injury namely; normal wound (central scratch method), diabetic wound, acidic condition and hypoxic condition. The study aimed to identify an effective *in vitro* model that reproduces an injured or stressed condition so that the biological responses of the cells can be compared to determine if therapy such as laser therapy can be used to stimulate the cells to recover and repair. Phototherapy has been used in laboratories and clinics for years to modulate cell function, resolve inflammation, reduce pain and accelerate healing of soft tissue injuries (Schindl et al., 1999; Ribeiro et al., 2002; Basford, 1995). The effect of phototherapy is especially striking in areas of the body where cells are stressed (hypoxia, decreased oxygen tension or decrea-sed pH) or wounded (Belkin and Schwaltz, 1989) and has been found to biostimulate various biological proces-ses, such as attenuation of ischemic injury in the heart

(Avni et al., 2005).

MATERIALS AND METHODS

Cell culture procedure

Fibroblasts are among the most accessible normal mammalian cell types and are a well-established system for *in vitro* analysis of fibroblast growth, migration and collagen metabolism in wound healing. Human skin fibroblast (WS1) monolayer cultures (ATCC CRL1502) were grown in Eagle's minimal essential medium (EMEM) with Earle's balanced salt solution that was modified to contain 2 mM L-glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 1% fungizone and 1% penicillin-streptomycin and supplemented with 10% (v/v) foetal bovine serum (serum rich medium). The cultures were incubated at $37^{O}C$ with 5% CO_2 and 85% humidity (Ausubel et al., 1994). Cells were trypsinized using a low concentration of 0.25% trypsin, 0.03% EDTA solution in Hanks balanced salt solution (HBSS) for 5 min at $37^{O}C$ to minimize the amount of cellular damage. Approximately 6.5 x 10^5 cells (in 3 mL culture medium containing phenol red) were seeded in 3.4 x 10 cm tissue culture dishes and incubated overnight to allow the cells to attach (Sambrook et al., 1989).

The cells were wounded or stressed on day 1 and the cellular responses were measured on day 4 or day 5 which corresponded to the treatment days selected for laser therapy (Abrahamse et al., 2006; Hawkins and Abrahamse, 2007). Normal cells or un- treated cells (not wounded or injured and not diabetic) were used as control cells.

Central scratch method (*in vitro* wound model)

To perform a wound healing assay, a wound is typically introduced in a cell monolayer according to Cha et al. (1996) using an object such as a pipette tip or syringe needle to create a cell-free zone. For the simulated wound environment, 1 mL of culture medium was removed and the confluent monolayer was scratched with a sterile pipette of 2 mm diameter and each plate was incubated at $37^{O}C$ with 5% CO_2 and 85% humidity until the cellular responses were measured on day 4 or day 5 (Cha et al., 1996; Hawkins and Abrahamse, 2005; Yarrow et al., 2004; Rigau et al., 1995).

Diabetic wounded model

A diabetic wound model was performed according to McDermott et al. (1998) and Hamuro et al. (2002). This was achieved *in vitro* by growing WS1 cells for several passages until required in complete media containing additional D-glucose (17 mM/L). The media had a basal glucose concentration of 5.6 mM/L, thus diabetic induced cells were grown in a total glucose concentration of 22.6 mM/L. The central scratch method was used to simulate a wound and create a cell-free zone (Houreld and Abrahamse, 2007). Each plate was incubated at $37^{O}C$ with 5% CO_2 and 85% humidity until the cellular responses were measured on day 4 or day 5.

Acidosis (low pH) model

Tsubio et al. (1989) adjusted liquid medium by adding hydrochloric acid (HCl) to obtain a pH range of 3 - 7 to verify cell growth therefore the cell culture medium was adjusted by adding 1 N HCl acid (Associated Chemical Enterprises) until the pH was reduced to 6.7. Briefly, 20 µL of HCl was added to 20 mL of complete EMEM (pH 7.4) to obtain a pH of 6.7. The pH was measured using a Thermo Orion pH meter (LABOTEC U.S.A, model 410 A+). After the overnight incubation to allow the cells to attach the complete EMEM was discarded and was replaced with 3 mL of adjusted acidic me-dia (pH 6.7). The cells were incubated at $37^{O}C$ with 5% CO_2 and 85% humidity in the acidic media until day 4 or day 5.

Hypoxia model

In vitro hypoxia was induced by incubating the 3.4 x 10 cm tissue culture dishes in an anaerobic jar (AnaeroPack^{TM} System, Mitsubishi gas Chemical Co. Inc., Japan) with one anaerobic gas pack, which achieves anaerobiosis in 15 min. A methylene blue anaerobiosis indicator was used to monitor oxygen depletion. In this case, cells were being deprived of oxygen, which is important for cellular oxidation. After the overnight incubation to allow the cells to attach, cells were incubated in an anaerobic chamber for 4 h and 15 min. The cells were incubated at $37^{O}C$ with 5% CO_2 and 85% humidity until day 4 or day 5. An incubation of 4 h was selected as further incubation in the anaerobic chamber results in irreversible damage to the cells (Yu et al., 2002).

Biological assays

Changes in normal, wounded, diabetic wounded, hypoxic and acidic fibroblast cell morphology were evaluated by light micro-scopy. Cells were trypsinized from the 3.4 x 10 cm tissue culture dishes and the cell suspension (1 x 10^5 cells/100 µL) was used to assess changes in cell viability (Trypan blue, ATP luminescence and caspase 3/7 activity), cell proliferation (XTT assay) and DNA damage (Comet assay). The culture medium was used to assess cell proliferation markers such as alkaline phosphatase (ALP) enzyme activity and basic fibroblast growth factor (bFGF) while LDH membrane integrity was used to assess cytotoxicity.

Changes in cell morphology: The fibroblast behavior was observed using an inverted microscope. The number and intensity of colony formation, the haptotaxis (direction or orientation) of the edge fibroblasts, the numbers of fibroblasts present in the centre of the scratch and chemotaxis-chemokinesis (migration) were evaluated to determine the activity of wounded and diabetic wounded fibroblasts (Rigau et al., 1995; McDermott et al., 1998). Changes in cell number, lysis of cells and cellular debris were evaluated to determine the extent of damage in hypoxic and acidic cells.

ATP cell viability assay: The assay is based on the quantitation of ATP, which signals the presence of metabolically active or viable cells. An equal volume of reconstituted CellTiter-Glo reagent (Promega G7573) was added to 50 µL of cell suspension. The contents were mixed on an orbital shaker for 2 min and incubated at room temperature for 10 min to stabilize the luminescent signal. Luminescence was recorded in reading light units (RLU) on a Berthold EG & G Junior luminometer (Riss et al., 2002; Crouch, 1993)

Trypan blue: An equal volume of cells was mixed with 0.4% Trypan blue (Sigma T8154) in HBSS and incubated at room temperature for 5-15 min. 10 µL was loaded into a haemo-cytometer-counting chamber and the number of viable (translucent) and non-viable cells (blue) were counted. Percentage viability was calculated as the number of viable cells divided by total cells (viable and non-viable) multiplied by 100 (Ausubel et al., 1994; Sambrook et al., 1989).

Caspase 3/7 activity: The Caspase-Glo^{TM} 3/7 luminescent assay (Promega G8090) measures caspase-3 and –7 activity as an early marker of apoptosis and thus indirectly viability. An equal volume of Caspase-Glo reagent was added to 25 µL cell suspension, contents were mixed for 30 s and incubated at room temperature for 3 h.

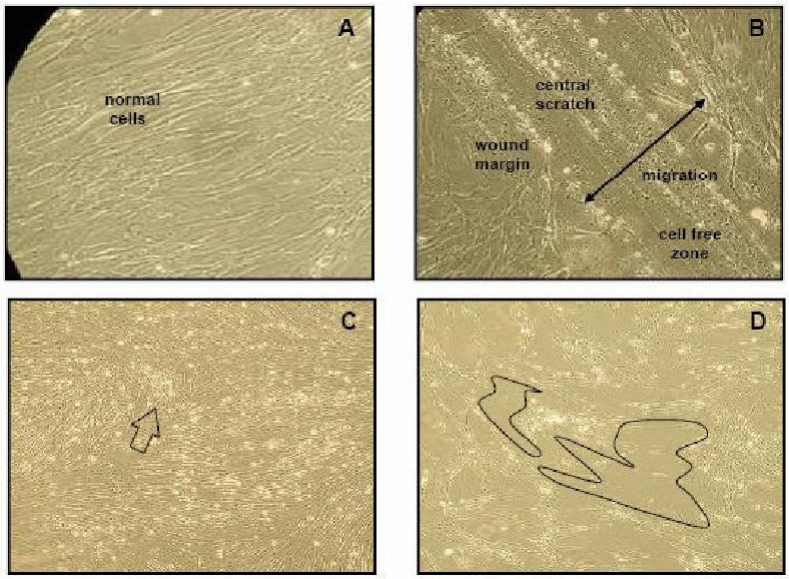

Figure 1. Changes in cell morphology. Normal fibroblasts are slender star-shaped cells that grow in monolayer sheets *in vitro* (A). A wound was introduced in a cell monolayer using the central scratch method to create a cell-free zone. The central scratch method wounded 5-10% of the cultured monolayer cells while the periphery of the culture plate contained un-wounded normal cells. A number of processes including the rate of migration (chemotaxis-chemokinesis), haptotaxis (change in orientation of edge fibroblasts) and number of fibroblasts present in the cell free zone were used to determine the rate of wound closure. Diabetic wounded cells displayed the same morphological pattern as the normal wounded cells (B). Hypoxic cells showed an increase in cell lysis and an increase in the number of cells that had detached from the culture flask (arrow) and were floating in the culture medium (C). The acidic cells showed a decrease in cell number with large spaces (outlined) observed between the cells showing that the cells were not able to grow to confluence (D) . Hypoxic and acidic cells showed evidence of cell lysis, which was supported by a decrease in ATP viability, decrease in XTT for cell proliferation and an increase in LDH cytotoxicity.

Luminescence was recorded in reading light units (RLU) (Nicholson and Thronberry, 1997; Ho et al, 1998).

XTT for cell proliferation: Proliferation was assessed using sodium 3'- (1-(phenylaminocarbonyl)-3, 4- tetrazolium)-bis (4-metho-xy-6-nitro) benzene sulfonic acid hydrate (XTT), (Roche 1465015). Briefly, 2 x 10^4 cells in 80 µL preconditioned media were seeded in a 96 well culture plate (AEC-Amersham S.A. 167008). Cells were incubated at 37^{O}C for 3 h to allow the cells to attach. 50 µL of XTT reagent (0.3 mg/mL) was added and the plate was incubated for 24 h at 37^{O}C. An increase in the number of living cells results in an increase in the overall activity of mitochondrial dehydrogenases. The orange formazan product that was formed was read at 450 nm on a spectrophotometer (BioRad Benchmark Plus Microplate Spec-trophotometer).

ALP enzyme assay: ALP is a membrane bound enzyme that has been used as a marker for wound healing. An increase in ALP activity requires the interruption or cessation of proliferation. An equal volume of culture medium was pre-incubated with 100 µL of 0.5 M N-methyl-D-glucamine buffer, pH 10.5 (0.5 mM magnesium acetate, 110 mM NaCl, and 0.22% Triton X-100) for 30 min at 37^{O}C. 20 mM ρ-nitrophenyl phosphate (ρ-NPP; Sigma N7653) was added and the reaction was incubated at 37^{O}C for 30 min (Abe et al., 2001). The amount of ρ-nitrophenol liberated was measured at 405 nm.

Basic fibroblast growth factor (bFGF): Briefly, 100 µL of culture medium was added to 100 µL carbonate-bicarbonate buffer (Sigma C3041) and incubated overnight at 4^{O}C. The following day the coating solution was removed and 200 µL of diluted (5 µg/mL or 1:6 500) monoclonal anti-human bFGF (Sigma F6162) primary antibody was added and the plate incubated at room temperature for 2 h. Each incubation step was followed with three washes of PBS-T (10 mM phosphate buffer pH 7.4, 150 mM NaCl, 0.05% Tween 20). 200 µL of anti-mouse IgG (Fab specific) peroxidase-conjugated antibody (Santa Cruz sc-2005; 200 µg/0.5 mL) diluted (1:4 000) in PBS-T was used as the secondary antibody while 100 µL of TMB substrate reagent (BD Biosciences 555214) was added for colori-metric detection. The orange-yellow color development was stop-ped after 30 min with 1 mol/L H_2SO_4 and the positive wells were read at 450 nm (Takamiya et al., 2003).

Cytotoxicity (LDH): Cytotoxicity was measured using the CytoTox 96® non-radioactive cytotoxicity assay (Promega G4000) that measures lactate dehydrogenase (LDH), a stable cytosolic enzyme that is released upon cell lysis. Released LDH was measured with a coupled enzymatic assay, which results in the conversion of a tetrazolium salt (INT) into a red formazen product. An equal volume (50 µL) of culture medium and reconstituted substrate was mixed and incubated at room temperature protected from light. After 30 min, 100 µL of stop solution was added, absorbance read (490 nm)

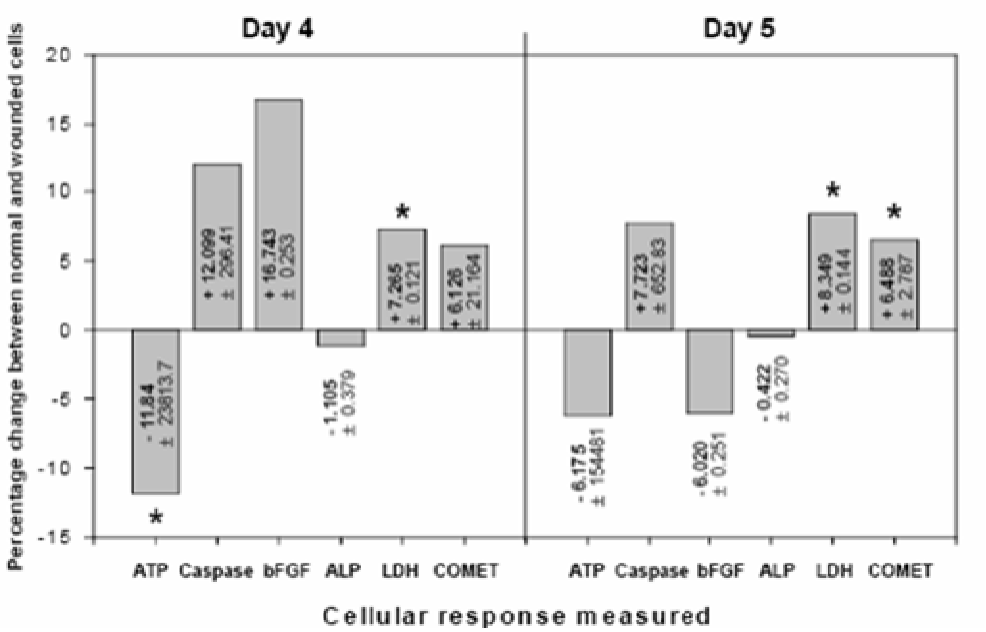

Figure 2. Percentage change between normal and wounded fibroblasts. Wounded cells showed an increase in caspase 3/7 activity and cytotoxicity indicating cellular damage from the central scratch. Wounded cells showed a decrease in viability, an increase in caspase 3/7 activity and an increase in cytotoxicity on day 4 when compared to the normal cells. Wounded cells showed a decrease in viability, a decrease in cell proliferation and an increase in cytotoxicity and DNA damage on day 5 when compared to the normal cells (n=24, * P 0.05).

and % cytotoxicity determined (Moravec, 1994).

Comet assay: The Comet assay was performed according to Collins, 2000 where one hundred comets per gel were visually analyzed at random. 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). Each comet was allocated a value depending on its classification to obtain an overall score ranging from 0 - 400 arbitrary units for each gel. The average arbitrary unit for each slide was calculated with a higher number of arbitrary units indicating more DNA damage.

Statistical analysis

Each experiment was repeated on different populations of fibroblast cells between passages 13 - 31. Each biological assay was performed in duplicate and the average of the two results was used to obtain a final sample number of n = 6-24. The results were recorded using SigmaPlot 8.0 and the significant percentage change between the normal and wounded or injured cells was calculated. The Student-T test was used for analysis and statistical significance was accepted at the 0.05 level (95% confidence interval). The ± value or error bars in the figures indicate standard error of the mean (SEM).

RESULTS

Changes in cell morphology: Monolayer cells were scratched with plastic pipette tips, which removed most of the debris from the wound site (Figure 1B). A total loss of 5 - 10% was calculated by comparing the denuded area to the cell-covered surface as described by Kornyei et al. (2000). The central scratch method wounded 5 - 10% of the cultured monolayer cells while the periphery of the culture plate contained unwounded normal cells however a wound environment was successfully induced, since there were a number of cellular responses that distinguished normal (Figure 1A) from wounded cells (Figure 1B). Wounded cells demonstrated a clear wound margin on either side of the central scratch with some evidence of haptotaxis but with very little migration and few fibroblasts present in the central scratch. Diabetic wounded cells (Figure 1B) displayed the same morphological pattern as the normal wounded cells (Figure 1B) . Hypoxic cells showed an increase in cell lysis and the number of cells that had detached from the culture flask (Figure 1C). The acidic cells showed a decrease in cell number, as the cells were not able to grow to confluence (Figure 1D).

Difference between normal and wounded cells: A number of cellular responses distinguished wounded from normal fibroblasts (Figure 2). The results indicate that the cellular responses measured were sensitive enough to distinguish between normal and wounded cells and also confirmed that cells showed a significant res-ponse to the *in vitro* wound environment that was indu-ced. Wounded cells showed a decrease in ATP cell viabi-lity (P = 0.036) and an increase in LDH cytotoxicity (P = 0.049) on day 4 when compared to normal cells confirm-

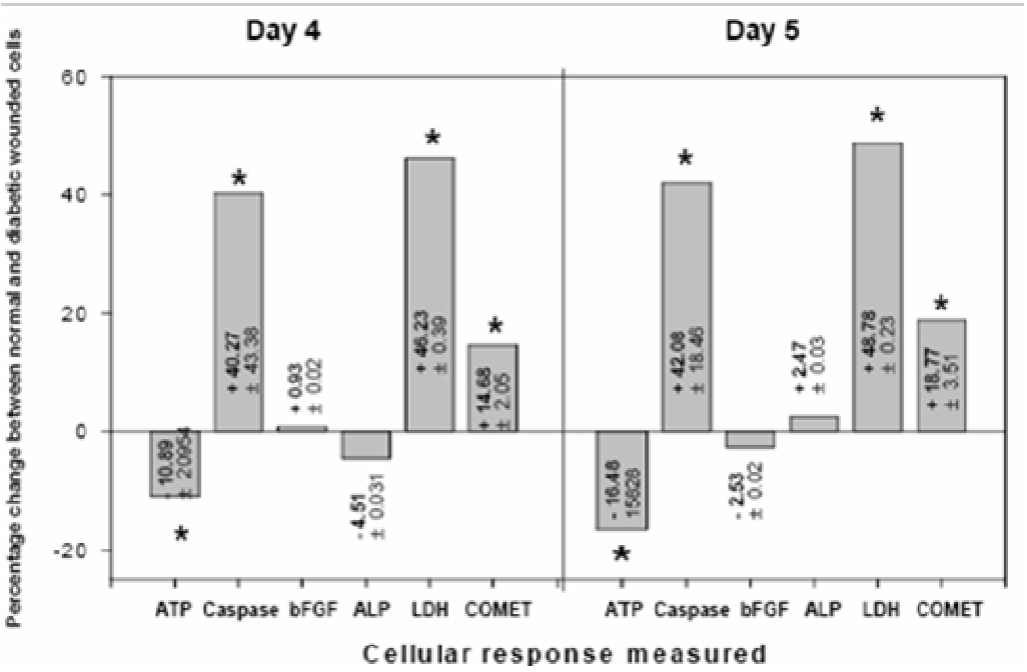

Figure 3. Percentage change between normal and diabetic wounded fibroblasts. There was a significant decrease in the ATP viability after day 4 and day 5 (P<0.05).There was also a significant increase in apoptosis as determined by caspase 3/7 activity, an increase in cytotoxicity as determined by LDH release and an increase in DNA damage as determined by the Comet assay (P<0.0001). There was no change in cellular proliferation as measured by the ALP enzyme activity and bFGF expression (n=24, * P 0.05).

ing that a central scratch method successfully damages the cells and induces a wound environment. Wounded cells showed an increase in cell stress (caspase 3/7 activity), an increase in proliferation rate (increase bFGF and decrease ALP activity) and an increase in DNA damage (Comet assay) on day 4 when compared to the normal control cells. Wounded cells also showed a decrease in ATP viability, an increase in cell stress, an increase in LDH cytotoxicity (P = 0.050) and an increase in DNA damage (P = 0.042) on day 5 when compared to the normal control cells. There was an increase in ATP viability, decrease in caspase 3/7 activity and decrease in cell proliferation when the results from day 5 were compared to day 4 indicating a recovery of cell function however this did not prove to be a statistical significant.

Difference between normal and diabetic wounded cells: Normal unwounded WS1 cells were compared to a diabetic wounded model (Figure 3). When cellular responses were measured on day 4 and day 5, diabetic wounded cells showed a significant decrease in cell viability as was shown by the decrease in ATP luminescence (P = 0.026 and P = 0.009 respectively) and increase in caspase 3/7 luminescence (P<0.0001). There was no significant change in cellular proliferation as determined by ALP and bFGF expression, even after incubation at 37^{O}C until day 5.

Inducing a wound by the central scratch method and incubation in a hyperglycemic environment significantly increased the amount of cellular damage as indicated by the significant increase in LDH and DNA damage on both day 4 and day 5 (P<0.0001). There was an increase in cytotoxicity in diabetic wounded cells after day 5 when compared to day 4. The results show that a diabetic wound model was successfully induced and that differences between normal and diabetic wounded cells could be detected.

Difference between normal and hypoxic cells: When the results of WS1 cells injured by hypoxia were compared with those of normal cells, there was no significant difference in percentage viability on both day 4 and 5 (P = 0.09 and P = 0.11 respectively). There was a significant increase in cell stress as indicated by the LDH cytotoxicity assay in hypoxic cells on day 4 (P = 0.02). There was a significant decrease in ATP viability on day 5 (P = 0.05), while on day 4 the decrease was not significant. The XTT cell proliferation assay showed a decrease in cell proliferation on day 4 and 5; however the response did not prove to be significant. There was a decrease in LDH cytotoxicity on day 5 indicating a recovery or repair of cell function after re-oxygenation (Figure 4).

Difference between normal and acidic cells: The percentage viability (Trypan blue) was significantly reduced in acidic cultures (P = 0.01), while the decrease in ATP

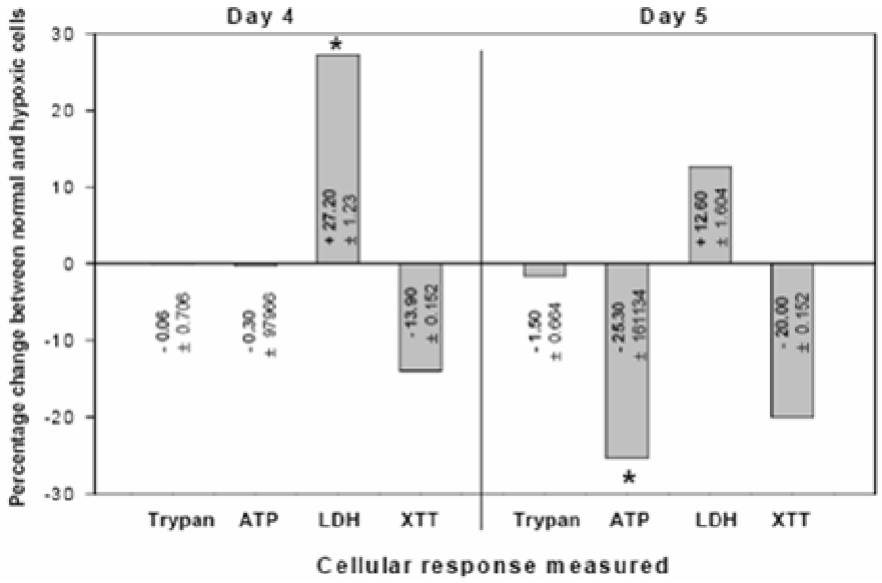

Figure 4. Percentage change between normal and hypoxic fibroblasts. There was a decrease in proliferation (XTT) by 13.9% on day 4 and 20% on day 5. The ATP viability showed little change (-0.3%) on day 4 but decreased to -25.3% on day 5 (P=0.05). Changes in cell membrane integrity as measured by LDH showed an increase of 27.2% (P=0.02) and 12.6% (P=0.16) on days 4 and 5 respectively. (n=6, * P 0.05).

viability was not significant on both days 4 and 5 (P = 0.14 and P = 0.06 respectively). Results showed a statistical increase in LDH cytotoxicity on day 4 (P = 0.03), while there was an improvement on day 5 indicating a recovery or repair response as cells adapted to the environment. The XTT cell proliferation assay showed that there was a statistical decrease in cell proliferation on day 5 (P = 0.05) demonstrating that the acidic condition reduces cell viability, increases cytotoxicity and also impedes or reduces cell proliferation (Figure 5).

The results showed that when the three conditions were compared, wounded and hypoxic (P = 0.05), wounded and acidic (P = 0.001) and acidic and hypoxic (P = 0.001), all three conditions showed differences in the percentage viability as measured by Trypan blue. When ATP viability results were compared, acidic cells showed statistically less viability than wounded cells (P = 0.05) on day 5. When XTT results were compared, acidic cells showed statistically less cell proliferation than wounded cells (P = 0.05) on day 5. Based on the ATP viability and XTT cell proliferation the results indicate that an acidic condition induces more injury or damage than the other two models. Moreover, the decrease in cell viability (percentage viability) and increase in LDH cytotoxicity confirmed that the wounded, hypoxic and acidic models successfully induced cellular injury or damage.

DISCUSSION

The fibroblast responses were sufficient enough to produce measurable differences that could distinguish normal from wounded, diabetic wounded, hypoxic and acidic cells. The results confirmed that the central scratch method successfully induced an *in vitro* wound environment, which was characterized by specific cellular responses (decrease in viability, increase in cytotoxicity and decrease in proliferation). These responses provided information about the cell function (cell viability, cell proliferation and growth factor expression) and the extent of damage (Comet assay, caspase 3/7 activity and LDH cytotoxicity).

A central scratch method was used to simulate a wound environment, however only the cells in the central scratch are wounded while the cells in the periphery of the culture plate remain normal (Figure 1). Kornyei et al. (2000) distinguished three stages in response to scratching: (i) cells surviving the acute damage at wound edge, re-attach to the substrate in 0-30 min following injury, (ii) 1 - 2 h after scratching the cells extend flat, cytoplasmic processes to the denuded area and (iii) migration and enhanced proliferation within and next to the wound site starts a 12 - 24 h post lesion and last until cell density approaches confluence.

Morphologically, although the cells in the periphery of the culture plate are normal, a distinct cell free zone or central scratch can be seen. Changes such as chemotaxis, haptotaxis and number of fibroblasts in the scratch are used to determine if there are changes in cell migration and proliferation (cell density). According to Thomas et al. (1992), cell-cell contacts have diverse effects on migrating cells. Abercrombie and Heaysman, (1996) reported that when a chick heart fibroblast separates from the cohort of cells, it produces multiple lamellapodia along the free leading edge, which seem to compete with

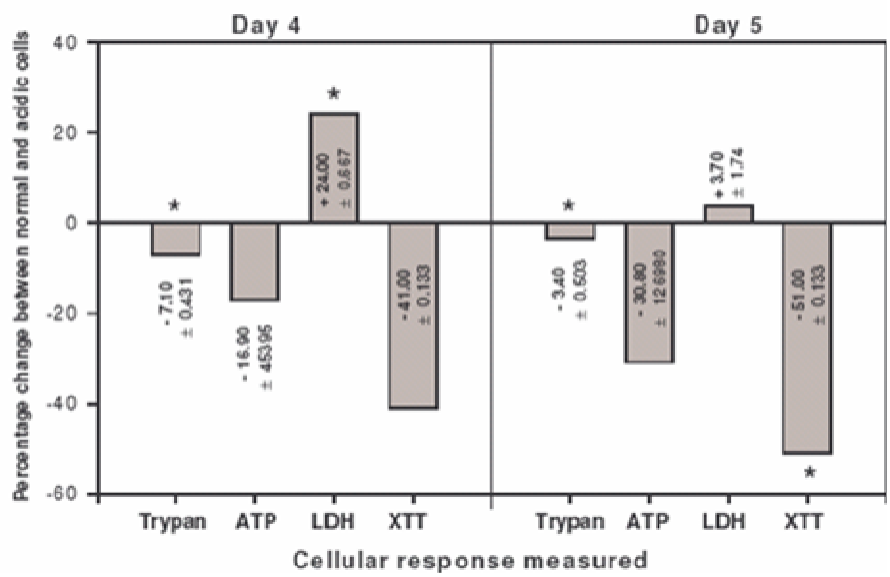

Figure 5. Percentage change between normal and acidic fibroblasts. Acidic cells showed a significant decrease in cell proliferation (P=0.05) on day 5. LDH increased by 24% (P = 0.03) on day 4 which decreased to 3.7% (P=0.45) by day 5. Cell viability using Trypan blue increased from -7.1% (P=0.01) on day 4 to only -3.4% (P=0.01) on day 5 indicating cell recovery or repair (n=6, * P 0.05).

each other for dominance. The result is that the cell makes frequent changes in direction (haptotaxis) and little migratory progress until it rejoins the outward migratory mass of cells. Results suggest that wounded fibroblasts may display a response that is similar to epithelial wound healing, in which cells at the edges of the wound migrate inward to close it (Abercrombie and Heaysman, 1996). It was also reported that while cells that have no contacts with other cells migrated more slowly than those with contacts, the number of contacts did not have a significant effect on the rate of migration.

Wounded cells showed a decrease in ATP viability, increase in LDH cytotoxicity, increase in ALP enzyme activity and increase in DNA damage when compared to the normal cells. Diabetic wounded cells showed a decrease in cell viability and an increase in cytotoxicity and DNA damage. The responses of wounded and diabetic wounded cells confirmed that a central scratch method successfully damaged the cells and simulated a wound environment. The results from this study showed that the parameters were sensitive enough to distinguish changes between normal and wounded cells since wounded cells responded with a decrease in cell viability, increase in cell proliferation, increase cell migration and increase in cell stress (caspase 3/7 activity and LDH cytotoxicity). The results indicate that cellular responses such as the expression of growth factors (bFGF) are stimulated by the physiological status of the cells (wounded). The increase in cell stress (caspase 3/7 activity and LDH cytotoxicity) indicates that the mechanical injury results in an increase in damage. Wounded and wounded diabetic cells showed an increase in caspase 3/7 activity, LDH cytotoxicity and DNA damage when compared to normal cells indicating that the central scratch method results in an increase in cellular damage which could explain why the method is considered as an effective *in vitro* model to reproduce a wounded environment.

Overall, this study proved that the diabetic wounded model and parameters chosen were suitable for basic research investigations. Despite there being no significant difference in cellular proliferation between normal and diabetic wounded cells, diabetic wounded cells were still able to change their direction of growth and migrate into the central scratch therefore cellular migration was not inhibited. This is in contrast with the work of Hamuro et al. (2002) who found an inhibition of cellular migration in human aortic endothelial cells (HAEC) cultured in 10 or 30 mM glucose, while McDermott (1998) found an increase in cellular migration when human corneal epithelial (HCE) cells were grown in 17.5 mM glucose. Diabetic wounded cells showed a significant increase in caspase activity, cytotoxicity and DNA damage compared to normal cells. This was a result of wounding (as shown by the increase in diabetic wounded cells compared to wounded cells) as well as culture in a hyperglycemic environment. A number of studies have found that there is a significant increase in apoptosis (Ho et al., 2000; Baumgartner-Parzer et al., 1995; Susztak et al., 2006; Vincent et al., 2005; Wu et al., 1999), LDH, and DNA damage when cells are grown in conditions of hyperglycemia (Wu et al., 1999; Gonzalez-Correa et al., 2006; Arner et al., 1993). As a result, this increase in stress and damage resulted in a

decrease in cellular viability, possibly due to apoptosis and cellular lysis.

Hypoxic cells showed a decrease in proliferation and increase in LDH cytotoxicity after day 4 and a decrease in ATP viability, increase in LDH cytotoxicity and decrease in proliferation after day 5. One possible explanation for the increase in cellular damage may be formation of free radicals, which are agents for cell injury in a number of different circumstances. Free radicals may be a direct cause of cell damage in hypoxia, chemical injury or radiation damage. Cloudy swelling is an almost universal morphological expression of reversible injury. The cell swells due to the accumulation of water within them (Rippey, 1994). The cytoplasm takes on a granular appearance due to the swelling of the mitochondria and endoplasmic reticulum.

Hypoxic cell injury results in failure of the cell's aerobic respiratory function, that is oxidative phosphorylation with the formation of energy rich ATP, which takes place in the mitochondria. The cell ceases to function and aerobic respiration with the breakdown of glycogen results in the accumulation of lactic acid and phosphates, with a resultant reduction in the pH (increase acidity of the cell). Failure of the sodium pump because of insufficient ATP results in the accumulation of sodium, loss of potassium and net gain of water by the cell, which enlarges. The endoplasmic reticulum becomes swollen. Ribosomes become detached from the rough endoplasmic reticulum and protein synthesis is diminished. The cell membrane may develop blebs and shows increased permeability. The entire cell is thus enlarged and waterlogged.

Acidic cells also displayed similar results of cellular damage, which may indicate a similar mechanism as described above where cell ceases to function and aerobic respiration with the breakdown of glycogen results in the accumulation of lactic acid and phosphates, with a resultant reduction in the pH (increase acidity of the cell). Acidic cells showed a decrease in cell viability, increase in LDH cytotoxicity and decrease in proliferation after day 4 and day 5 indicating that the condition reduces cell function, diminished protein synthesis and reduces ATP viability. LDH results for acidic cells support the results observed for hypoxic cells where there was a significant increase in cellular damage on day 4 but an insignificant change on day 5 indicating that the damage is reversible and that the cells are able to recover and repair.

Results showed that the LDH cytotoxicity was less on day 5 when compared to the results obtained on day 4. This change was also supported by a further decrease in ATP viability and cell proliferation on day 5. The results confirm that the methods successfully induce cellular injury with impaired cell function and a decrease in protein expression however the cytotoxicity and DNA damage results indicate that there is a repair mechanism that has been activated and that the damage is reversible. The caspase 3/7 activity results supports this finding as wounded cells showed a decrease in activity after day 5 indicating a lower rate of apoptosis where repair mechanisms allowed cells to recover and repair. Based on the ATP viability and XTT cell proliferation the results indicate that an acidic condition induces more injury or damage than the wounded or hypoxic models. Although the wounded, diabetic wounded and hypoxic models were able to reduce cell viability, reduce proliferation and increase cytoxicicity, the acidic model appeared to be effective in inducing a homogenous injury but without causing irreversible damage and lysis.

A more homogenous form of mechanical injury could be considered where all the cells in the culture plate are equally wounded and where the insult may simulate a disease or condition that may benefit from a specific treatment. Other forms of mechanical injury to the cells includes: 1.) Cytotoxic drugs or chemotherapeutic drugs such as Decarbonizes (DTIC) that is the first-line chemotherapy for metastatic malignant melanoma. 2.) A change in intracellular pH that may mimic metabolic acidosis that is common with diabetes. 3.) Acetone-induced injury that simulates repeated injury to the stratum corneum that is commonly caused by friction, soaps or organic solvents which results in hyperkeratosis and epidermal thickening (Ajani et al., 2007). 4.) A reduction in the oxygen tension (pO_2) in the range of 0 - 5 mmHg that may mimic hypoxia that is common in chronic inflammation and indolent wou-nds (Abercrombie and Heaysman, 1996) and 5.) An *in vitro* ischemic model where cells are transferred into an anaerobic chamber saturated with $N_2/CO_2/H_2$ mixture (85/5/10) that is common in ischemic heart disease, stro-ke and hypoxia (Yu et al., 2002).

One of the future applications of this work is to develop and use the *in vitro* models to study the effect of phototherapy for wound healing studies. Karu (1998) stated that laser light stimulates cells that are growing poorly at the moment of irradiation. Thus, if a cell is fully functional at the moment of irradiation, there is nothing for laser irradiation to stimulate, and no therapeutic benefit will be observed however if the cells are wounded or injured then laser biostimulation aims to normalize cell function to stimulate healing and repair (Karu, 1998). It was for this reason that this study aimed to identify *in vitro* mod-els that would simulate wounded or injured cells so that biological responses such as viability, proliferation, cytotoxicity could be measured to determine if a specific therapy such as laser therapy is effective in stimulating cells to repair and restore homeostasis. The cell stress models may be used to study the possible implications of phototherapy on pathological conditions such as ischemic heart disease, diabetes, uremia, heart attack, stroke and gangrene.

Further investigation into the pathological processes involved in each model still needs to be performed. Fitsialos et al. (2007) identified 161 new gene markers of epidermal repair after *in vivo* scratch wounding therefore further studies will include the use of more specific and significant biological markers on the mRNA (RT-PCR)

and protein level (ELISA) to identify specific genes involved in the pathophysiology of the skin fibroblast scratch injury, the diabetic scratch injury and also the hypoxic and acidic injuries. Rout et al. (2005) compared expres-sion patterns of genes in fibroblasts isolated from adhe-sion and normal human peritoneum and also examined contributions of TGF- beta1 and hypoxia in the altered expression of specific genes using RT- PCR. The study found that genes that are differentially expressed between normal and adhesion fibroblasts encode molecules involved in cell adhesion, proliferation, differentiation, migration and factors regulating cytokines, transcription, translation and protein/vesicle trafficking. Therefore biochemical markers such as caspase 3/7 activity, bFGF, ATP viability, XTT and LDH cytotoxicity are important biochemical tools to determine the initial cellular responses of cells however these biochemical changes can not give the substantial insight into the pathological processes involved in each condition.

Before *in vivo* experiments, *in vitro* studies with excised human skin – or as a less appropriate alternative, with excised animal skin–have to be performed. The development of human skin models that have the same properties (morphology and functionality) as genuine human skin is of particular significance. Organotypic epithelial "raft" cultures are tissue culture systems that permit the full differentiation of keratinocyte monolayers via culturing of the cells on collagen gels at the air-liquid interface (Andrei et al., 2005) . Shen et al. (2005) used a 3D multilayer model based on the skin physical structure to investigate the transient thermal response of human skin subject to laser heating while other studies have also used a skin equivalent or organotypic model to monitor and observe wound healing processes. Ajani et al. (2007) engineered an *in vitro* model of acetone-induced injury using organotypic epidermal cultures to understand the molecular and cellular responses to the disruption of the permeability barrier. The skin equivalent is a suitable alternative method to animal testing since it represents an advanced alternative to normal cell culture and its natural three-dimensional structure closely reproduces an *in vivo* situation. Future studies may include introducing the cell stress or injury models to the 3D artificial skin constructs to study pathological conditions such as ische-mic heart disease, diabetes, uremia, heart attack, stroke and gangrene.

Conclusion

This study confirmed that although a central scratch method only wounds 5 - 10% of the surface cells it was sufficient to successfully induce an *in vitro* wound environment. The results from this study showed that the biological responses were sensitive enough to show significant differences between normal and injured or stressed conditions. The results from this study showed that a decrease in ATP viability, increase in caspase 3/7 activity, increase in proliferation and increase in cytotoxicity distinguished normal from wounded and diabetic wounded cells. As laser irradiation does not stimulate cells that are fully functional this study demonstrated that the four models; wounded, diabetic wounded, hypoxic and acidic models are effective *in vitro* models that can be used to induce cellular injury or damage. These models can be used for *in vitro* studies to investigate changes in injured cells in response to therapy such as laser therapy. The study also demonstrated that hypoxic and acidic conditions mimic a homogenous stressed or injured environment that may be used as a model to study pathological conditions such as ischemic heart disease, diabetes, ureamia, heart attack, stroke and gangrene.

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