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Effects Of Pulsed Electromagnetic Stimulation On Cultured Dermal Fibroblasts From Diabetic And Non-Diabetic Rats

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EFFECTS OF PULSED ELECTROMAGNETIC STIMULATION ON CULTURED DERMAL FIBROBLASTS FROM DIABETIC AND NON-DIABETIC RATS

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BIOMEDICAL ENGINEERING

ABSTRACT

Diabetes is a disease affecting an increasing number people each year. The medical costs of patients with diabetes are substantial due to the many complications associated with the disease. Dermal wounds are one of these complications and are often very difficult to properly heal. Pulsed Electromagnetic Fields (PEMFs) have been introduced as a method by which to speed the healing of dermal wounds. In this study, a device (provided by Biomet/EBI, Parsippany, NJ) for supplying PEMFs in vitro was used to stimulate dermal fibroblasts, one of the key cells in wound healing. These cells were isolated from the genetically diabetic Zucker fa/fa rats and their non-diabetic littermates. Preliminary studies were performed on cells from both diabetic and non-diabetic animals to determine what differences may exist between the two. It was found that dermal fibroblasts from diabetic rats had significantly impaired proliferation compared to cells from non-diabetic rats (Tukey's HSD, $p < 0.05$). Proliferation was also studied under the influence of high glucose at a concentration of 25 mM, and it was found that high glucose impaired the proliferation of dermal fibroblasts from both diabetic and non-diabetic rats (student's t-test, $p < 0.05$). Migration rates of dermal fibroblasts were studied using the

scratch wound assay. It was found that dermal fibroblasts from diabetic rats had significantly impaired scratch wound closure rates compared to cells from non-diabetic rats ($p < 0.05$). For the next set of studies, the effects of stimulation with PEMFs on dermal fibroblasts were determined. It was found that under PEMFs proliferation rate was not significantly changed, with the exception of an increase in proliferation for dermal fibroblasts from one diabetic rat. Additionally, the effects of three different ranges of PEMFs on migration rates of dermal fibroblasts from diabetic and non-diabetic animals were determined using the scratch wound assay. Stimulation with PEMFs significantly altered scratch wound closure rates of dermal fibroblasts, in that, for each range of PEMFs, scratch wound closure of dermal fibroblasts was significantly increased for at least one of the animals ($p < 0.05$). It was concluded that PEMFs produced significant effects on dermal fibroblasts from diabetic and non-diabetic rats and the positive effects may be host and range-dependent.

DEDICATION

I dedicate this work to my family. Thank you to my wonderful husband, Matt, for his love and support. Thank you to my parents, Anita and Mike, for always being there. Thank you to my Grandmother, Martha, who inspired me to pursue this project. You have all helped me to succeed.

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INTRODUCTION

Diabetes Impact

In 2005, it was estimated that 20.8 million people (7% of the population) in the United States (U.S.) have some form of diabetes.[1] The estimated total cost of diabetes in 2002 was $132 billion, which represents 11% of the total U.S. health expenditures. A person with diabetes has to spend approximately 2.4 times more on healthcare than they would if they were not diabetic. This stems from the increased risk of certain medical complications. These complications may include, but are not limited to, heart disease, stroke, blindness, kidney disease, and dermal wounds.[1]

Diabetes is a disease that results in elevated blood glucose levels due to defects in insulin production, insulin action, or both. There are two main types of diabetes: Type 1 and Type 2. The present study will focus on Type 2 diabetes which represents approximately 90% of the diabetic population.[1] Type 2 diabetes, or adult-onset diabetes, usually begins with an insulin-resistance. As the subsequent need for insulin increases, the pancreas is overworked and will eventually lose the ability to produce insulin. Type 2 diabetes is also referred to as non-insulin-dependent diabetes mellitus and is associated with increased age, obesity, and genetics.

Modeling Diabetes

Two models of diabetes were considered for this study. The first was a toxin-induced diabetic model and the second was a genetic model of diabetes. After, preliminary experimentation, the genetic model was chosen for further study. Appendix A gives an overview of the findings for the toxin-induced diabetic animal model.

A genetic model of Type 2 diabetes has been developed in rodents. These animals exhibit many of the characteristics associated with Type 2 diabetes. The Zucker rat was chosen for this study as it is intended to model genetic obesity and Type 2 diabetes. The Zucker rat is a genetically modified animal that possesses a homozygous mutation (fa/fa). The fa allele is an autosomal recessive mutation on chromosome 5. This mutation acts to eliminate leptin receptors in the rat. Leptin is an important protein produced by fat cells in response to increased levels of insulin. Without the leptin receptor, the protein goes unrecognized, thus inducing characteristics associated with Type 2 diabetes. The animals are insulin resistant, hyperinsulinemic, and exhibit obesity at 4 to 5 weeks of age. The lipid body composition is 40-50% by 14 weeks, with lean animals having 20% lipid body composition. {Humans are considered obese if the lipid body composition (a.k.a. body mass index) exceeds 30%, with 18.5% to 24.9% considered normal.} These animals also exhibit hyperphagia; muscle atropy; hyperlipemia (adipocyte hypertrophy and hyperplasia); and hypercholesterolemia. Cleary et. al., 1980 found that Zucker Rats given a lean diet lose body weight, but lipid composition is not changed.[2] Also, Bourgeois et al., 1983 and Goldstein et al., 1985 found that adipoblast cultures from Zucker rats have a slower, more prolonged period of proliferation than cultures from homozygous lean rats.[2]

It should be noted that the Zucker rat does not exhibit hyperglycemia. However, the absence of hyperglycemia in the Zucker rat could model a well-controlled diabetes or at least genetic obesity that is a precursor to Type 2 diabetes. The addition of high glucose media may impair function further in vitro, and will therefore be used to model the poorly controlled diabetic condition.

Dermal Wound Healing

As previously stated, patients with diabetes are a higher risk for medical complications, including dermal wounds. In a non-diabetic patient wound healing will occur in a well documented pattern. First, inflammatory cells, macrophages and neutrophils, will infiltrate the wound to remove any debris and protect from infection. Next, granulation tissue will form as dermal fibroblasts synthesize extra-cellular matrix, comprised mostly of collagen. Lastly, the epidermis will be formed from the migration of keratinocytes over the granulation tissue.

In a diabetic patient the most common dermal wound is the diabetic foot ulcer. These wounds do not follow the normal healing pattern and are often unresponsive to currently available treatments. These wounds may become chronic, taking several months or longer to heal. The progression of acute wounds to chronic wounds is associated with abnormalities present as a result of the patient's diabetic state. These abnormalities include neuropathy, infection, vascular insufficiency, and cellular dysfunction.

Diabetic foot ulcers often develop as a result of neuropathy in the extremities, which is experienced by 30% of people with diabetes.[1] In addition, neuropathy may cause patient to inadvertently apply excessive pressure to the wound, which can result in the cessation of healing or re-injury of a healing wound. Diabetic foot ulcers are also susceptible to polymicrobial infection, which can spread rapidly and lead to significant tissue damage.[3] If the infection is resistant to antibiotics, amputation may be necessary. For this reason, it is very important that diabetic foot ulcers are well cared for with regular dressing changes. These first two issues, excessive pressure and infection, must

be dealt with before treatment of other deficiencies can be effective. Methods such as off-loading, thorough wound cleansing, and frequent dressing changes are necessary as preventive techniques.

Often, the dysfunctions in patient healing cannot be identified specifically because they do not exist at the macroscopic level. Vascular insufficiency and cellular dysfunction are among these. Most diabetic patients experience vascular insufficiency, especially in the extremities, and a decreased angiogenic capability. These conditions prevent the transport of nutrients and oxygen to the wound site, which may lead to ischemia and tissue damage.

Cellular Dysfunction in Diabetic Wounds

Cellular dysfunction within a diabetic foot ulcer has been demonstrated in one of the key cells involved in wound healing, dermal fibroblasts. These cells are responsible for laying down collagen in the repair process and also for cell signaling.[4] An altered or dysfunctional response by dermal fibroblasts is thought to be one of the reasons for a delayed or non-existent healing in diabetic patients. Looking at the most basic, cellular levels of healing may provide a better understanding of the etiology of chronic diabetic wounds.

Several studies have observed differences in collagen metabolism and accumulation in diabetic animals.[5,6] This would be expected if the dermal fibroblasts, the cells responsible for collagen production, were impaired. Further examination has shown that dermal fibroblasts cultured from diabetic patients exhibit decreased collagen synthesis.[7]

Several clinical studies have demonstrated a decreased proliferation rate in patient cultures of diabetic dermal fibroblasts compared to those cultured from non-diabetic patients.[8,9,10,11] Differences have also been seen between diabetic ulcer fibroblasts and diabetic non-lesional fibroblasts.[10] These differences may be attributed in part to a lack of growth factor production and growth factor receptor expression by dermal fibroblasts from diabetic origins.

Studies have been conducted to assess growth factor and growth factor receptor content in different cell types cultured from diabetic ulcers.[12,13] Fibroblasts derived from chronic wounds were found to have a decreased response to growth factors like TGF-β1, platelet-derived growth factor (PDGF), fibroblast growth factor 2 (FGF-2), etc.[3,7,14,15] It would be most beneficial to engineer a method by which to increase both growth factor production and growth factor receptor expression in fibroblasts. In turn, this would help to increase both proliferation and collagen production in dermal fibroblasts.

Diabetic patients often have difficulty controlling their blood glucose levels. Prolonged periods of abnormally high glucose may be experienced until natural insulin takes action or insulin injections are given. High glucose levels, like those seen in diabetic patients, have been shown to contribute to the decreased proliferation of dermal fibroblasts.[16] Previous clinical studies have emphasized good glycemic control as an essential component of successful wound healing.[17]

A decreased migratory capacity has also been observed in fibroblasts cultured from diabetics. Non-lesional fibroblasts from diabetic animals were found to be less responsive to matrix proteins in terms of migration compared to wild-type animals.[4] The impaired state of fibroblasts in diabetics has been well documented, and, therefore,

treatments which correct these dysfunctions are necessary to improve diabetic wound healing.

Electrical Properties of Skin

Endogenous electrical currents may be found throughout the body, including the largest organ of the body, the skin. Human skin is composed of two major layers: epidermis and dermis. Transepithelial potentials (TEPs) have been measured on intact skin as well as across the dermis of wounded areas of skin. The TEPs are the result of actions by sodium channels that allow the diffusion of sodium across cell membranes throughout the skin.[18] Thus, the term "skin battery" was coined to describe the electrical currents present within the skin.[18]

When a wound occurs it can be compared to an electrical leak that allows current to flow freely out of the wound.[19] These natural currents have been measured in vivo, ranging from 140 mV/mm (or 1400 mV/cm) at the wound edge to 0 mV/mm just 3 mm away from the wound on intact skin.[19] A moist environment is required for current to pass through a wound. This is one reason why occlusive wound dressings are essential for proper healing. The dressings hold in moisture that is required to conduct the therapeutic electrical currents. Discovery of these electrical currents induced after wounding has inspired study into the usefulness of exogenous currents applied to wounds and how they may affect healing.

Electrical Stimulation and its Applications

Electrical stimulation (ES) is a treatment method that had been widely examined in orthopedic applications and shows promise for applications to wound healing. Several studies have observed increases in proliferation and migration.[7,8] ES has also been shown to possess certain antibacterial properties, which may prove to be very useful in chronic diabetic wounds, which are prone to infection. Improvements in angiogenesis and tissue oxygenation have also been observed, thus, strengthening the case for use of ES in vivo.

Studies examining the electrical stimulation of fibroblasts have observed their migration toward the cathode.[7] This information can allow manipulation of the cells to promote migration in the most optimal directions to heal a wound. In addition, the bactericidal effects of ES may contribute to better healing, since diabetic wounds have been shown to have decreased inflammatory cell number and action.[20] The mechanisms by which ES aids in cellular processes are numerous and mostly unknown. However, Aaron et al., 2004, hypothesized that electric fields act to change cytoskeletal and plasma membrane structure, enabling the migration of cell surface receptors.[21] ES has been shown to aid in the up-regulation of TGF-β and its receptors in fibroblasts, so this hypothesis may eventually be proven valid.[6]

There are several methods by which cells may be electrically stimulated. The electric current can be applied in the form of or direct current (DC), alternating current (AC), or pulsed current (PC). DC is a continuous, unidirectional flow of charged species, and therefore there is no waveform associated with DC. However, the polarity may be changed to achieve the desired direction of flow. In contrast, the magnitude and direction of AC varies cyclically. The most common waveform for AC is the sine wave, but square

and saw tooth waves are also possible. PC is actually a type of AC and is defined as the brief unidirectional or bi-directional flow of current followed by a relatively longer period of no flow.

Within AC and PC current there are several other parameters to be considered. Figure 1 is a diagram showing these different options for each type of current. The method of stimulation proposed here is in the form of PC, and with PC it is possible to vary many parameters including the waveform phases, symmetry, and charge balance. For this study, biphasic, asymmetrical, unbalanced charge PC was chosen.

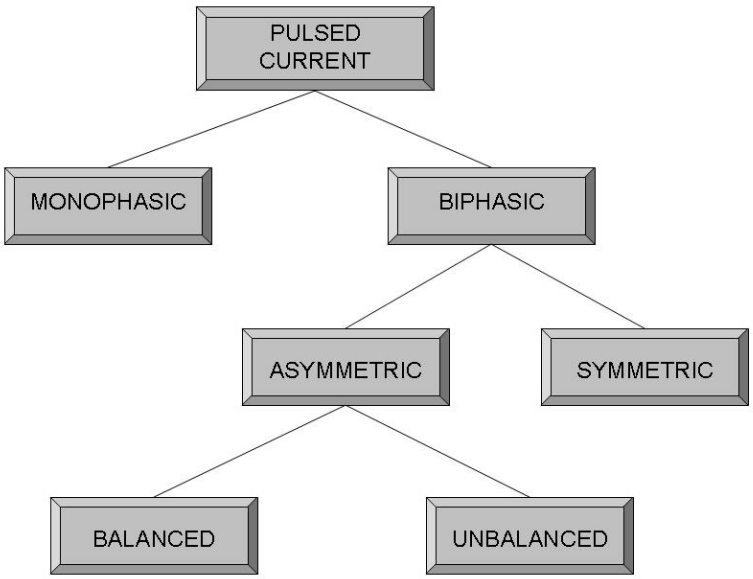

Figure 1: Parameters associated with pulsed current.

There have been some applications of PC to the cells of interest here, fibroblasts. Bourguignon et al., 1987 for example, found that application of a high voltage pulsed galvanic stimulation for a period of 20 minutes at room temperature was able to increase

cellular protein and DNA synthesis in vitro.[20] In addition, they found that these changes may be related to an increase in calcium uptake and increases in insulin receptors on the fibroblast cell surface.[22]

In all of the aforementioned studies, electrodes were used to provide the PC. This method of stimulation is not optimal for patients because it is an invasive form of stimulation where electrodes are often placed in the wound site. This method is not optimal for in vitro studies either due to the electrode products that may be given off in the culture medium, and excessive heat production that may occur. In order to avoid the use of invasive electrodes, pulsed electromagnetic fields (PEMFs) are examined as the method of stimulation for the present study. PEMFs provide a very controlled and practical method for in vitro and future in vivo stimulation. This method allows experiments to be conducted in an incubator using well plates and petri dishes that are commonly used for cell culture assays.

Pulsed Electromagnetic Fields

It is believed that PEMFs do not directly affect the cells that are exposed to them. Instead, the electromagnetic field that is applied induces an electric field across the cells. However, an electric field is only induced when the electromagnetic field is changing. With this principle, steady electric fields can be created by controlling changes in electromagnetic fields. Electromagnetic coils were used in this study to create PEMFs (EBI, Parsippany, NJ). The pulsed current was applied in the pulse-burst form (Figure 2). This form of stimulation provided 20 consecutive pulses during each pulse burst lasting 4.5 milliseconds (ms). The frequency of the burst was approximately 15 Hertz (Hz).

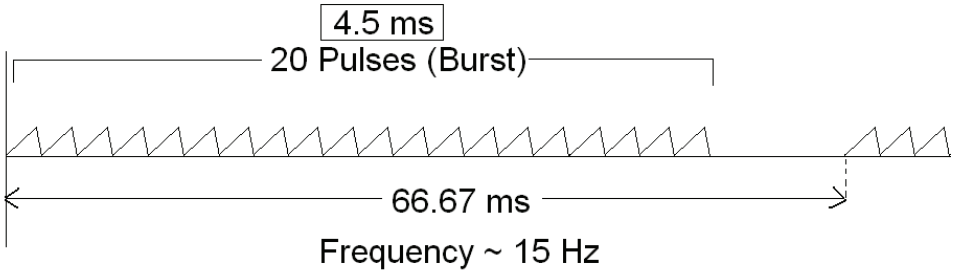

Figure 2: Pulse-burst profile of electromagnetic stimulation.

As previously stated, an electric field is induced when the electromagnetic field is changing. When the electromagnetic field is increasing, a positive electric field is created. Likewise, when the electromagnetic field is decreasing, a negative electric field is created. In this study, the pulse duration was 200 microseconds (μs) and the pulse interval was 25 (μs). It should be noted that during the pulse interval an electric field was also induced, but with opposite polarity since the electromagnetic field was decreasing (Figure 3).

With this setup, a distributed electric field was created within a circular dish. This was achieved by placing the coils on both sides and parallel to the circular dish with cells. This type of stimulation results in a range of fields that increase with increasing distance from the center of the well.

The PEMF strength may be calculated with the following equation

$$E = (r / 2) * (dB/dt) \qquad (mV/cm)$$

where E is the induced electric field, r is the radial distance from the center of the well in centimeters (cm), B is the electromagnetic field in Tesla (T), and t is time in seconds (s).[23] Table 1 shows the ranges of induced electric fields that can be produced by PEMFs in varying size culture dishes.

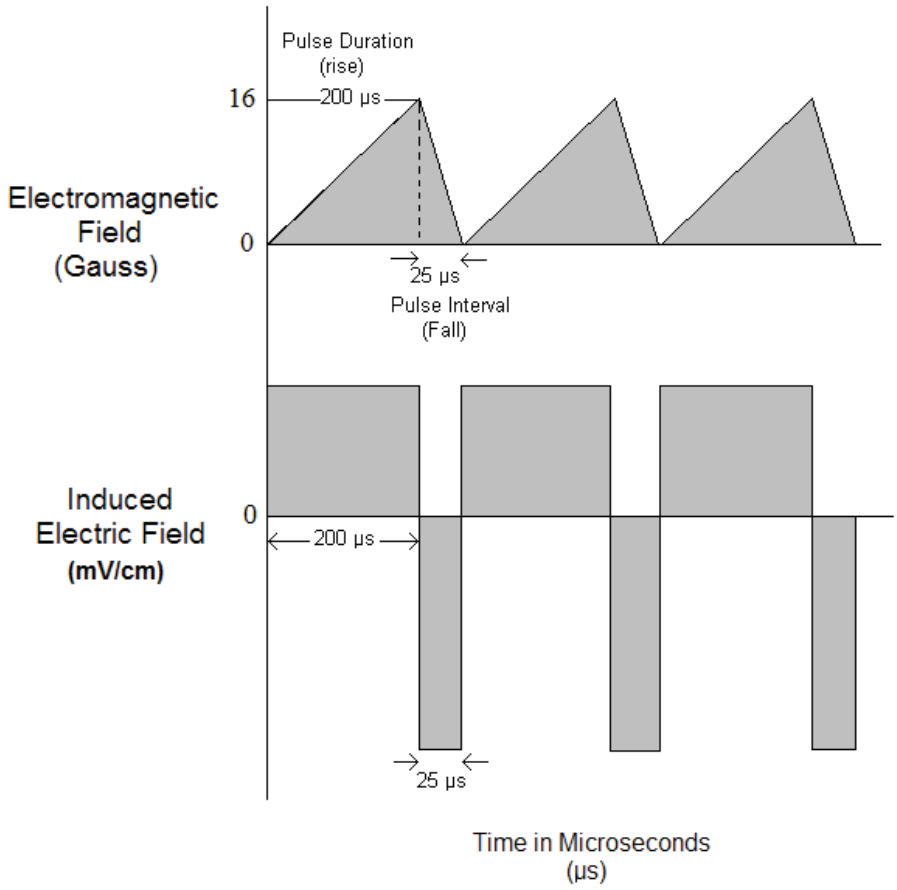

Figure 3: Profile of individual electromagnetic pulses and the resulting electric fields.

Table 1: Ranges of electrical fields induced in various size dishes by PEMFs.

Type of Dish	Culture Area (cm^2)	Radius of Culture Area (cm)	Electric Field Induced Over Pulse Duration (mV/cm)	Electric Field Induced Over Pulse Interval (mV/cm)
96 well Plate	0.32	0.32	0.13	1.02
60 mm Petri	25	2.82	1.12	9.02
100 mm Petri	57	4.26	1.70	13.63
150 mm Petri	148	6.86	2.7	22.0

Studies involving bone cells have found that exposure to PEMFs results in increased levels of growth factor expression.[21, 24] A study examining the effects of PEMFs on several cell types, including fibroblasts, found that while fibroblasts did not respond to the field, they did respond to a conditioned media with a significantly increased proliferation and migration rate.[24] The failure of fibroblasts to respond directly to the PEMF in this study may have been due to the specific pulse strength, duration, and frequency used. Fibroblasts have been shown to respond to pulsed ES at frequencies of 100 Hz.[7] A related study of the effects of PEMFs on neutrophils determined that the cells were in fact responding to the electric field created by the electromagnetic field.[25]

Electrical field gradients are known to be present in embryonic development, directing cell migration.[26] However, the type of electric fields normally present within the body are in the form of high magnitude direct current. The type of stimulation proposed in this study is a very low magnitude pulsed current in the form of PEMFs. Significant effects on cells have been seen under PEMFs, suggesting that pulsed current may be just as beneficial as endogenous direct currents. One study has shown increases in endothelial cell migration under PEMFs.[27] However, little is known about the migration properties of fibroblasts under PEMFs.

Migration studies so far have mainly been done under DC electric fields where a clear polarization is in place with a cathode and anode. There are very few studies examining migration under pulsed fields. Because fibroblasts are key cells in the process of wound healing, it is important to determine how PEMFs may affect migration of these cells. In this study, the scratch wound assay was used to examine migratory properties of fibroblasts. This assay was done by first creating an injury, or a scratch, on a confluent

monolayer of cells, and then taking images of the scratch at various time points. Each image can then be measured to determine how fast cells are migrating to cover the scratch. This assay allowed the comparison of scratch wound closure rates across control and PEMF samples in this study.

GOALS AND HYPOTHESES

The characteristics of dermal fibroblasts from diabetic rats in comparison to non-diabetic rats under standard culture conditions were of interest in this study. It was hypothesized that dermal fibroblasts from diabetic animals would exhibit significantly lower proliferation rates compared to fibroblasts from non-diabetic animals. In addition, it was hypothesized that dermal fibroblasts from diabetic animals would have decreased migratory capabilities compared to dermal fibroblasts from non-diabetic animals.

In most diabetic cases, glycemic control is a problem. For this reason, the rate of proliferation under both high and normal glucose conditions was examined. It was hypothesized that dermal fibroblasts exposed to high glucose concentrations would experience significantly impaired proliferation compared to normal glucose concentrations.

The effects of electric stimulation, in the form of pulsed current, on dermal fibroblasts cultured from diabetic rats was the main point of interest. A specialized setup supplied PEMFs to the cells and migration rates were examined in terms of scratch wound closure. Three separate ranges of PEMFs were examined to determine the optimal range for fibroblasts.

Under PEMFs, fibroblasts were hypothesized to exhibit no significant changes in proliferation rate, but scratch wound closure rates were expected to be significantly improved. Fibroblasts from diabetic animals were hypothesized to show more improvement in scratch wound closure rates than fibroblasts from non-diabetic animals. The largest range of PEMFs were expected to induce the greatest improvement in scratch wound closure.

METHODS

Diabetic Animal Models

An animal model for type 2 diabetes is the genetically diabetic Zucker rat (fa/fa). These rats and their non-diabetic littermates were used for this study. All procedures and care methods were approved by the Institutional Animal Care and Use Committee (IACUC) (Appendix B). Animals were provided food and water ad libitum. Blood glucose levels were checked for a period of 5 days before sacrifice. Animals were humanely sacrificed and dermal tissue was obtained for the establishment of primary dermal fibroblast cultures.

Isolation and Culture of Dermal Fibroblasts from Rats

Following sacrifice, the area of tissue harvest was prepared by shaving and washing with 70% ethanol. Skin was removed from the ventral area and placed in a tube with phosphate buffered saline (PBS) containing 2 times the normal concentration of cell culture antibiotics (4% penicillin/streptomycin). The tubes were placed on ice for transport to cell culture lab. Each piece of skin was then washed with PBS containing 2X antibiotics. Excess fat was removed from the inner layer of tissue. The skin tissues were then minced into fine pieces and placed in 0.25% trypsin-EDTA. The solution was stirred at 37°C for 45 minutes. A quench solution (PBS with 10% horse serum) was then added to the filtrate to stop trypsin action. Next, 70 μm pore cell strainers were used to filter non-digested particles from the solution. Cell solutions were then spun down at 1000 rpm for 10 minutes. After centrifugation, supernatant was discarded and cells were re-suspended in PBS. Cells were then counted to determine yield. Cells from each animal

were split into two groups, normal (5.5mM) and high (17mM) glucose, for subsequent culture. All cells were plated in T25 flasks with Minimum Essential Medium, alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS) and 2% antibiotics to begin culture. Additional glucose in the form of a 45% glucose solution (Sigma, St. Louis, MO) was added to the media of cells to produce the high glucose condition. Cells were grown at 37°C, 5% CO_2, and 95% relative humidity. Successful isolation of dermal fibroblasts was assessed by observing cell morphology. The resulting cells were dermal fibroblasts from normal and diabetic states. Dermal fibroblasts at passages 4-10 were used for all experiments.

Electrical Stimulation Setup

The method of electrical stimulation was pulsed electromagnetic field (PEMF) application through electromagnetic coils (Provided by Biomet/EBI, Parsippany, NJ). The setup consisted of a power unit, incubator, and electromagnetic coils. The power unit sat outside the incubator (Figure 4). The incubators were checked for stray fields using a magnetometer and no such fields were found. The two coils were placed in the incubator with a space in between for cell culture dishes (Figure 5). Stimulation was done in two similar incubators with one serving as the control. The control incubator had the same setup as the experimental incubator, but without activated coils. Diabetic and non-diabetic cells were tested under stimulated and non-stimulated conditions. The time for applied stimulation to fibroblasts was chosen to be 8 hours per day as has been reported to be optimal for fibroblasts.[28]

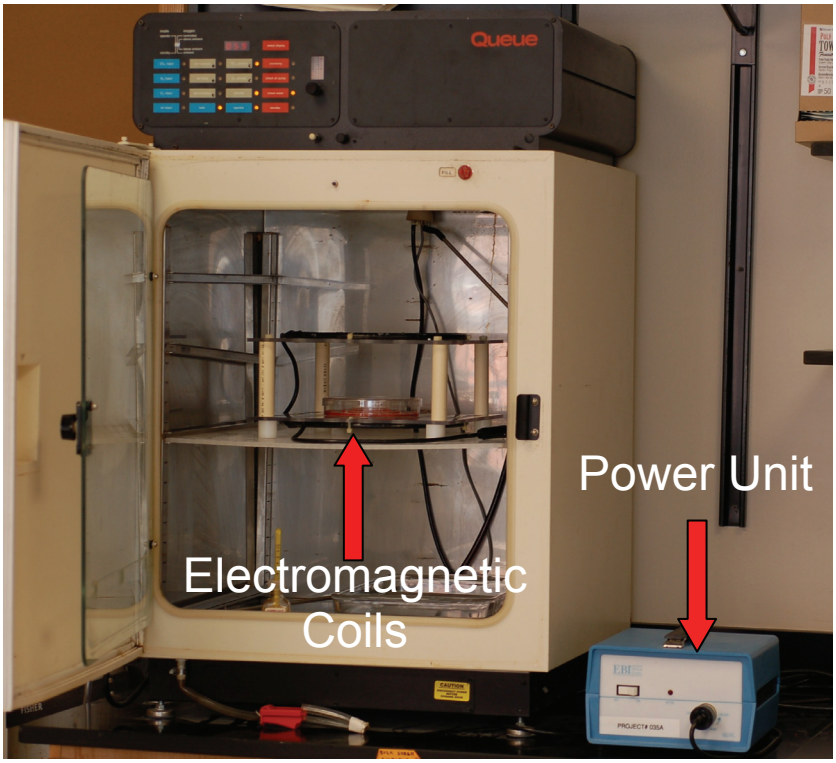

Figure 4: Electrical stimulation setup showing electromagnetic coils and accompanying power unit used to supply PEMFs to the cells.

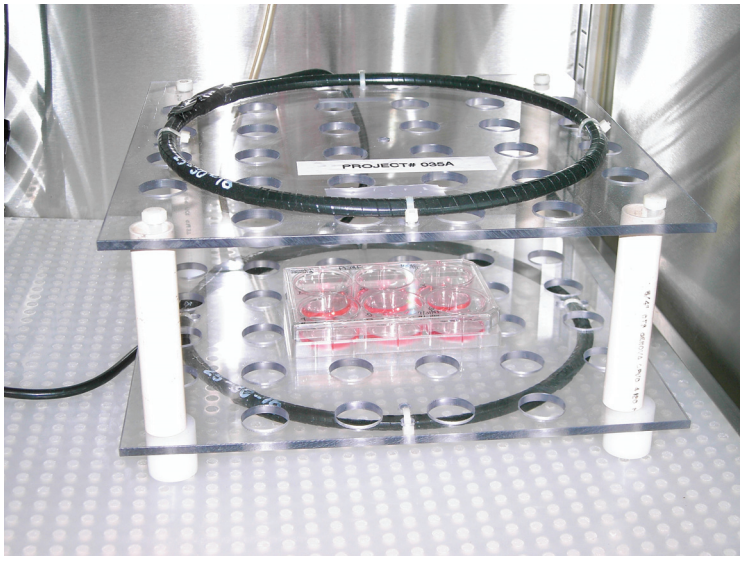

Figure 5: Electromagnetic coils with culture dish placed in the center for in vitro stimulation with PEMFs.

Proliferation Assays

Cell proliferation was quantified using the colorimetric Hexosaminidase assay. This assay has been proven effective in measuring proliferation and has shown higher sensitivity than other commonly used proliferation assays.[29] The Hexosaminidase assay binds an enzyme in the cytoplasm of cells to produce a yellow product at basic pH levels and is quantified using a microplate reader where absorbance is read at 405 nm. Proliferation rates between time points were calculated for comparison between groups.

High Versus Normal Glucose Experiment

Following isolation, cells were plated in either normal glucose medium (4.5 mM) or high glucose medium (17 mM). These levels were chosen because they correspond to normal (100 mg/dl) and high glucose (300 mg/ml) parameters in vivo. Proliferation was quantified using the hexosamindase assay under both conditions for diabetic and non-diabetic fibroblasts. For the proliferation assay, 25 mM glucose levels were added for comparison with current literature.

Tri-target Dish

A special dish was fabricated for use with the scratch wound assay in determining migration properties of dermal fibroblasts (Figure 6). The dish was termed the "tri-target dish" and was fabricated so that three PEMF ranges could be examined separately. The dish prevented diffusion of secreted factors across multiple PEMF ranges so that indirect effects of certain PEMF ranges on each other were excluded. With the new dish, three separate field ranges were isolated for further examination.

The special dish was assembled with three different size petri dishes (150 mm, 100 mm, and 60 mm). The 100 mm dish was affixed concentric with the 150 mm dish, and the 60 mm dish was affixed concentric with the 100 mm dish. The dishes were affixed with small amounts of cyanoacrylate glue and then the edges of the 100 mm and 60 mm dishes were sealed with paraffin wax. The dishes were sterilized with 100% ethanol for 1 hour and then washed with PBS. The components for assembly of the new dish did not create any adverse reaction in the cells, in terms of apoptosis, when observed over a 72 hour time period.

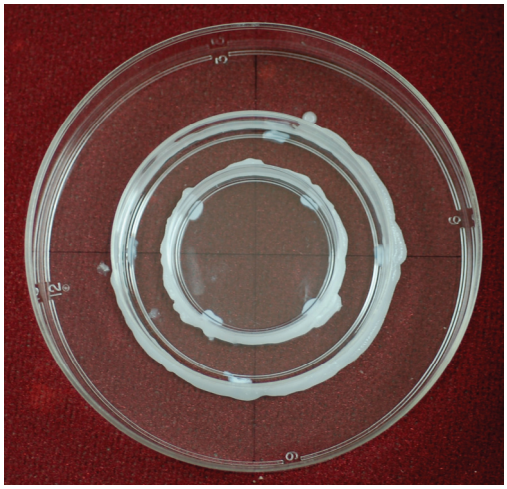

Figure 6: Specially constructed petri dish used for scratch wound assay.

Scratch Wound Assay

The scratch wound assay is a simple method by which to measure cell migration. The assay involves creating a "scratch" on a monolayer of cells and then imaging the artificial wound to measure the migration rate. A scratch wound assay protocol by Liang et al., 2007, was used in this study.[30] For this assay, cells were allowed to reach

confluency on the tri-target dish, and a p200 pipet tip was used to create scratches across each section of the dish. Each plate was washed once with PBS to remove debris, and then fresh medium was added. Scratch wounds were imaged at 10X immediately following scratch creation and at 8 hours, 23 hours, and 28 hours post scratch creation (Nikon Eclipse TE 2000-E Microscope, Nikon Instruments Inc., Melville, NY). Dishes were placed into the specific incubators, control or PEMF, and stimulation was begun immediately.

The aforementioned times were chosen because they correspond to points of interest under the timeframe for PEMF application. The first time point, 8 hours, corresponds to the end of the first round of stimulation and is sufficient time to begin observing cell infiltration and growth over the scratch wound site. The second time point, 23 hours, corresponds to the time just before the second application of PEMF stimulus. This was important in providing a baseline before the second round of stimulation. At 24 hours the second round of stimulation was applied. The last time point of 28 hours was four hours into the second round of stimulation, and was chosen so that the effectiveness of the second round of stimulation could be evaluated.

The PEMF ranges created by tri-target dish may be seen in Figure 7. As previously stated, the dish isolated each range, preventing any effects due to diffusion between ranges. The two scratch wounds were evaluated in four regions of interest (ROI) per PEMF range. The ROIs were captured as pictures and then measured manually (NIS Elements AR 2.30 Software, Nikon Instruments Inc., Melville, NY). An area measurement was taken of the wound area remaining at each time point. One observer

measured all areas. Final evaluation with statistics was done by comparing control samples to those exposed to PEMFs for each range.

There were four groups total, two diabetic and two non-diabetic. Experiments were repeated once for each group, resulting in a total of eight scratch wound pictures for each of the three PEMF ranges. Therefore, the total number of scratch wound pictures measured for all groups was N=32 at each of the three field ranges.

Statistical Analysis

Comparisons were made between dermal fibroblasts from diabetic and non-diabetic rats to determine differences. Normality was warranted for all analyses. Therefore, the student's t-test, the unequal variance t-test, analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) test were used where appropriate. JMP Start Statistical software (SAS Institute Inc., Cary, NC) was used to perform all analyses.

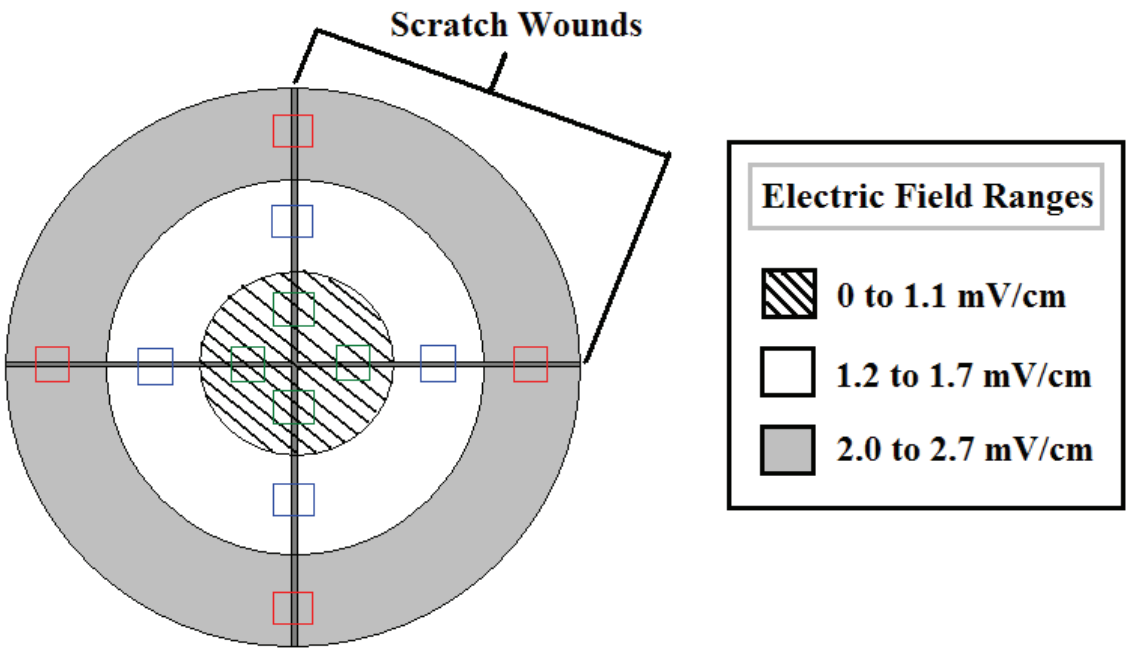

Figure 7: Scratch wound locations on tri-target dish with corresponding electric field ranges. ROIs are shown by square boxes.

RESULTS

Dermal fibroblasts from diabetic animals have impaired proliferation

Proliferation of fibroblasts was measured on days 1, 3, and 6 post-seeding in 96 well plates at a density of 4 x 10^3 cells per well. Fibroblasts from both non-diabetic (wild-type) and diabetic rats were tested for a total of four groups, and there were six replicates per group (n=24). Proliferation rates were calculated for day 1-3 and for day 3-6. For day 1-3, a difference in proliferation rates between the diabetic and non-diabetic groups was observed (ANOVA, $p = 0.002$). However, Tukey's HSD test found that cells from only one of the diabetic rats was different from all other groups ($p < 0.05$) (Figure 8). For day 3-6, differences in proliferation rates were also observed between diabetic and non-diabetic groups (ANOVA, $p < 0.001$). Tukey's HSD test showed that proliferation rates for cells from diabetic rats were different from those of non-diabetic rats ($p < 0.05$) (Figure 9).

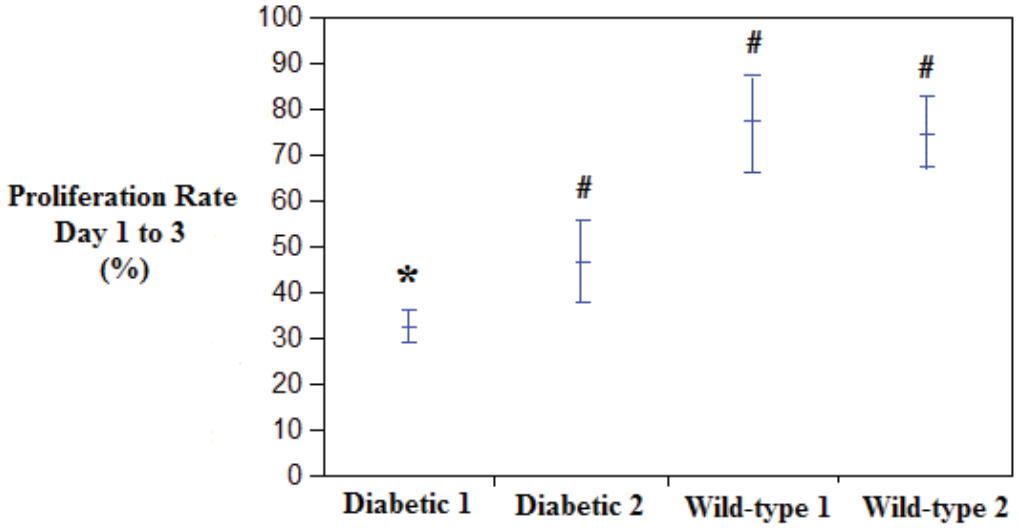

Figure 8: Proliferation rates from day 1 to 3 for dermal fibroblasts.

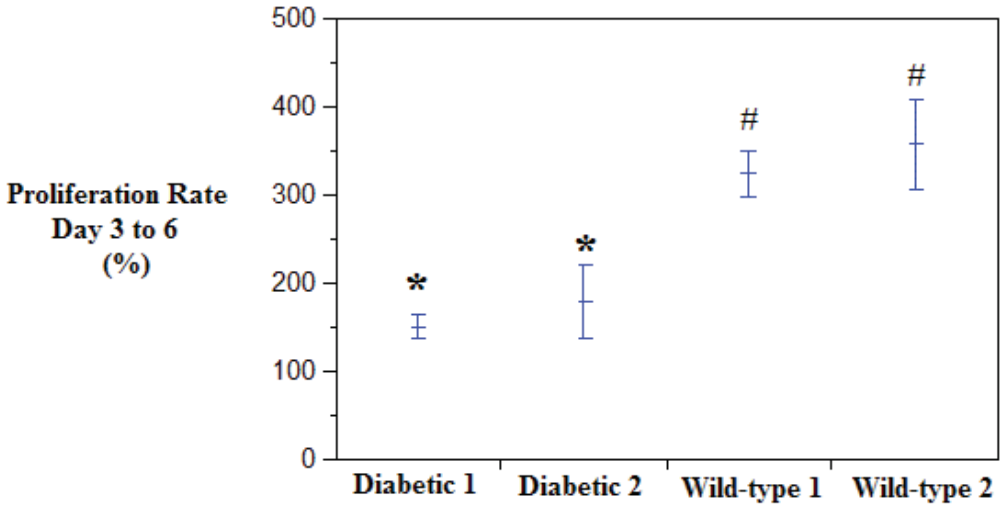

Figure 9: Proliferation rates from day 3 to 6 for dermal fibroblasts.

High Glucose impairs proliferation of dermal fibroblasts

Proliferation of fibroblasts under high glucose conditions (25mM) was measured on days 2, 4, and 6 post-seeding in 96 well plates at a density of 4 x 10^3 cells per well. Fibroblasts from both non-diabetic (wild-type) and diabetic rats were tested for a total of four groups. There were four replicates per group (n=16). Proliferation rates were calculated for day 2-4 and day 4-6. A one-sided t-test (unequal variance t-test when appropriate) was performed for each group to compare normal to high glucose conditions. For day 2-4, proliferation was significantly decreased under high glucose for both non-diabetic animals and one diabetic animal (all $p < 0.03$). One diabetic animal had an insignificant decrease in proliferation rate but still displayed the trend of lower proliferation under high glucose ($p = 0.10$). Table 2 shows the means and standard deviations of proliferation rates from day 2 to 4 for each group. For day 4-6, significant decreases in proliferation were only observed for one of the non-diabetic animals and one of the diabetic animals ($p < 0.05$).

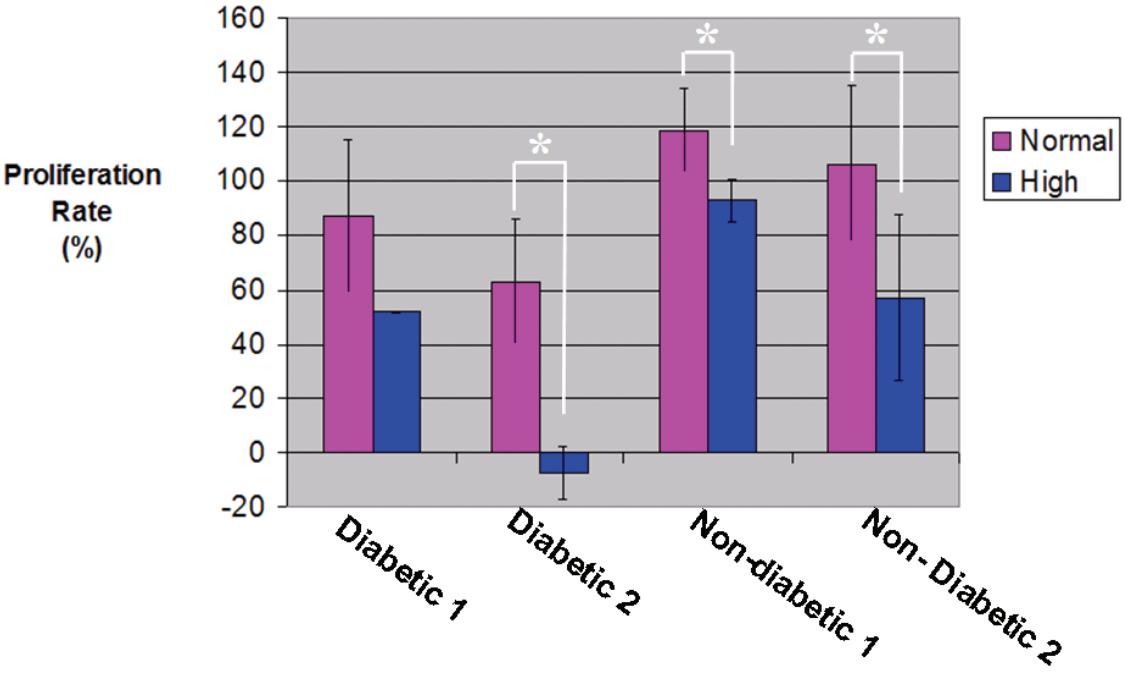

Figure 10: Proliferation rates from day 2 to 4 under high glucose conditions. "Diabetic 2", "Non-diabetic 1", and "Non-diabetic 2" had significantly decreased proliferation under high glucose.

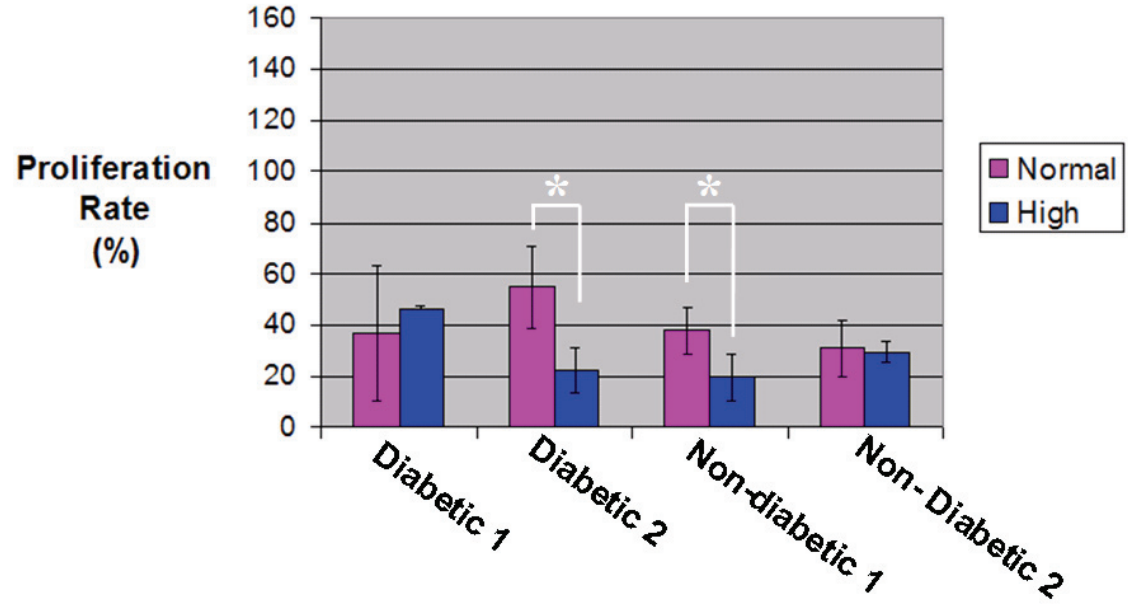

Figure 11: Proliferation rates from day 4 to 6 under high glucose conditions. "Diabetic 2" and "Non-diabetic 1" had significantly decreased proliferation under high glucose.

Proliferation is not affected by PEMFs

Fibroblasts from diabetic and non-diabetic animals were tested in 96 well plates to determine if these small fields affect the proliferation of cells. Cells were seeded at a density of 1 x 10^3 cells per well and tested on days 1, 3, and 6. The fibroblasts from non-diabetic animals showed no difference in proliferation rates from day 1-3 or from day 3-6 between the PEMF stimulated cells and the non-stimulated controls. However, fibroblasts from diabetic animals had a variable response with fibroblasts from one animal showing increased proliferation rates for the stimulated cells for day 3 to 6 ($p = 0.04$).

Impairment in Scratch Wound Closure with Dermal Fibroblasts From Diabetic Rats

Dermal fibroblasts from diabetic and non-diabetic (wild-type) animals were compared in terms of scratch wound closure without the aid of PEMFs. Significant differences in scratch wound closure rates were found at the 8 to 23 hours post-scratch creation time interval (ANOVA, $p < 0.001$). In addition, it was determined that these differences existed between the two groups, diabetic and wild-type, with no significant differences within either group (Tukey's HSD, $p < 0.05$). Dermal fibroblasts from diabetic animals were not able to cover the scratch wounds as quickly as dermal fibroblasts from non-diabetic animals during this time interval (Figure 10).

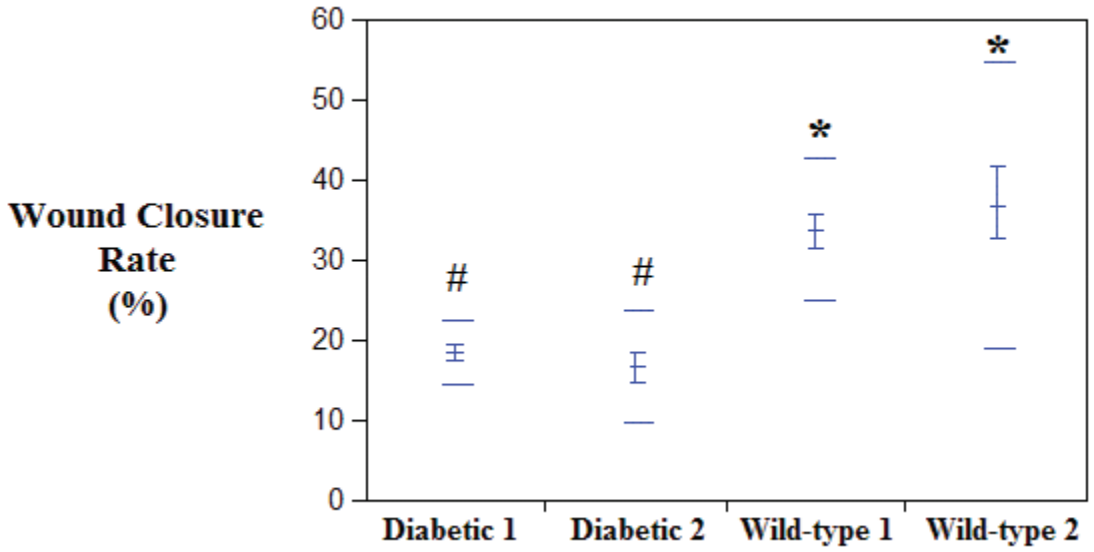

Figure 12: Wound closure rates from 8 to 23 hours post-scratch creation for dermal fibroblasts under control settings.

Effects with Smallest Range of Pulsed Electric Fields

For the smallest observed range of electric fields, 0 to 1.1 mV/cm, both diabetic and non-diabetic dermal fibroblasts showed little change compared to respective controls. Dermal fibroblasts from both diabetic animals showed no change under PEMFs. Dermal fibroblasts from only one non-diabetic animal showed any statistically significant changes between PEMF and control samples (student's t-test, $p = 0.02$). This difference was observed for the time interval from 23 to 28 hours.

Effects with Medium Range of Pulsed Electric Fields

The middle range of electric fields, 1.2 to 1.7 mV/cm, induced minimal effects on dermal fibroblasts. Only one statistically significant difference was observed, and this was for one of the diabetic animals at the 23 to 28 hour time interval (student's t-test, $p = 0.01$).

Effects with Largest Range of Pulsed Electric Fields

For the largest range of electric fields, 2.0 to 2.7 mV/cm, differences were observed for dermal fibroblasts from all but one animal. Dermal fibroblasts from both diabetic animals had differences between PEMF and Control samples for the time interval from 8 to 23 hours (student's t-test, both $p < 0.05$). However, the difference seen for dermal fibroblasts from one of the diabetic animals was actually a decrease in scratch wound closure rate under PEMFs. Dermal fibroblasts from the diabetic animal showing improvement in scratch wound closure rate under PEMFs was also improved at the 23 to 28 hour time interval (student's t-test, $p = 0.01$). Table 3 gives a summary of the scratch wound closure results for dermal fibroblasts from diabetic rats.

Improvement in scratch wound closure rate under PEMFs was seen in dermal fibroblasts from one non-diabetic animal at the 0 to 8 hours time interval (unequal variance t-test, $p < 0.01$). No differences were observed in dermal fibroblasts from the other non-diabetic animal. Table 4 gives a summary of the scratch wound closure results for dermal fibroblasts from non-diabetic rats.

Figure 13 shows representative pictures of dermal fibroblasts from non-diabetic animals closing a scratch wound at 0, 8, and 23 hours. Figure 14 shows representative pictures of dermal fibroblasts from diabetic animals closing a scratch wound at 0, 8, and 23 hours.

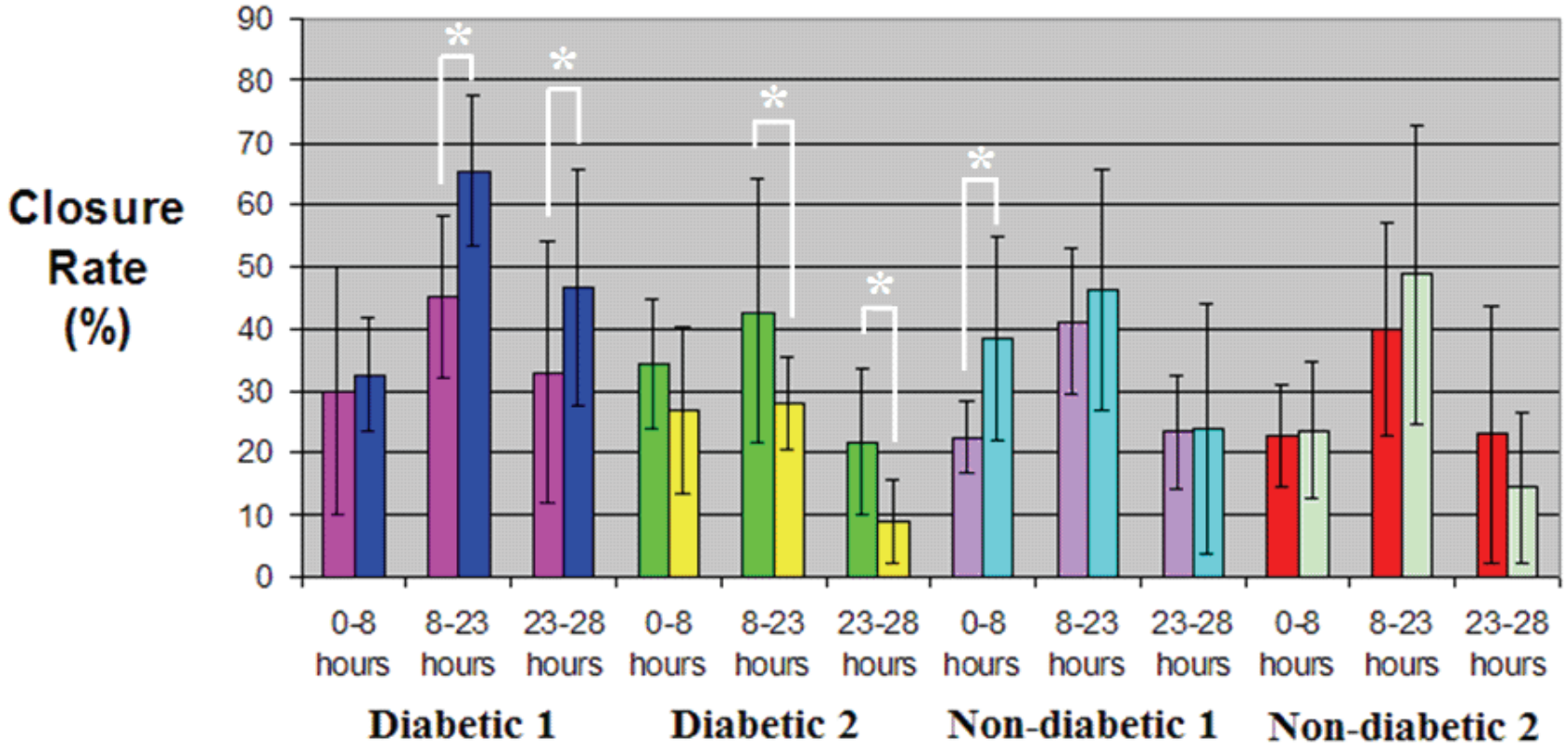

Figure 13: Scratch wound closure rates for each group at each time interval for the largest range of PEMFs. At each time interval, the bar on the left represents the non-stimulated control samples and the bar on the right represents the PEMF stimulated samples. Overall, five significant differences between the control and PEMF samples were found (shown with brackets and asterisks).

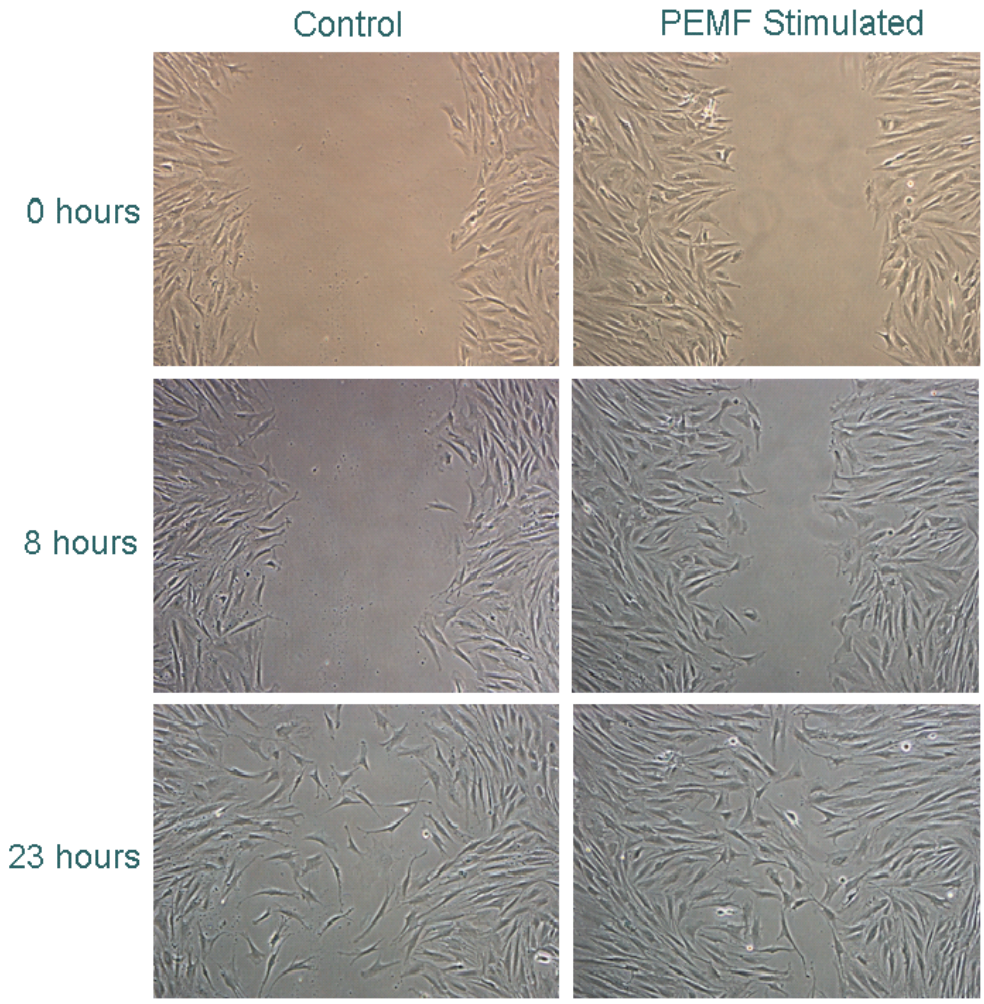

Figure 14: Scratch wounds imaged at 0, 8, and 23 hours post-creation for dermal fibroblasts from non-diabetic rats. Left side shows cells under non-stimulated control settings. Right side shows cells under the largest range of PEMFs (2.0 to 2.7 mV/cm).

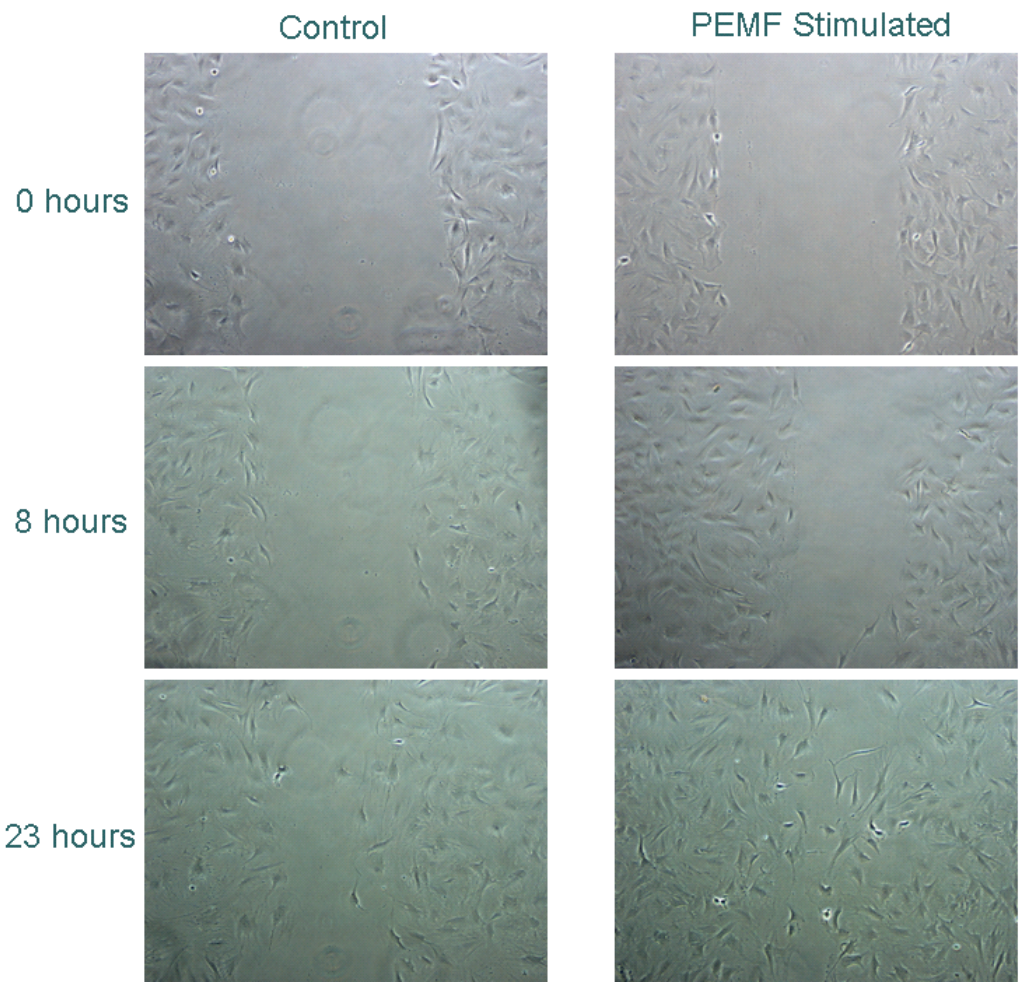

Figure 15: Scratch wounds imaged at 0, 8, and 23 hours post-creation for dermal fibroblasts from diabetic rats. Left side shows cells under non-stimulated control settings. Right side shows cells under the largest range of PEMFs (2.0 to 2.7 mV/cm).

DISCUSSION

It has been widely reported that dermal fibroblasts from diabetic animals and humans are impaired compared to fibroblasts from non-diabetic subjects. In terms of proliferation, dermal fibroblasts cultured from diabetic rats were shown to be impaired in comparison to dermal fibroblasts cultured from non-diabetic rats. The reasons for this impairment are not completely characterized, and, since the diabetic animals used in this study were not hyperglycemic, high glucose conditions cannot be the only factor contributing to this impairment.

Examination of dermal fibroblasts under high glucose conditions revealed impairment in both diabetic and non-diabetic cells, with the exception of a non-significant decrease in proliferation for cells from one diabetic animal.

The fact that differences were not observed on the latter time point was most likely due to contact inhibition that occurs as cells become confluent. The decreases seen in proliferation rates under high glucose are in accordance with the findings of other studies.[16, 17] This confirms the inherent impairment that must be experienced by cells in a diabetic patient with elevated blood glucose levels in addition to all the other factors associated with diabetes.

The effects of low magnitude PEMFs on proliferation were studied and found to be mostly non-significant. This supports the findings of other researchers and suggests that the promotion of healing by these fields must act in other ways.[23] Other studies have reported increases in growth factor production under PEMFs.[21, 23] This may lead to

increases in extracellular matrix production and migration, which are both essential in wound healing.

The scratch wound assay was used to mimic an injury state on a confluent monolayer of cells. Under control settings, it was demonstrated that dermal fibroblasts from diabetic rats are impaired, in terms of scratch wound closure rate, in comparison to dermal fibroblasts from non-diabetic rats. This was expected because a previous study has demonstrated decreased migratory capability of dermal fibroblasts from diabetic subjects.[4]

The scratch wound assay was also used to study the effectiveness of PEMFs to promote healing processes of dermal fibroblasts from diabetic and non-diabetic rats in vitro. The smallest range of PEMFs produced minimal changes in scratch wound closure between PEMF and control samples. However, one significant increase in scratch wound closure was observed in cells from a non-diabetic animal. Dermal fibroblasts from the same animal saw no changes in either of the medium and large ranges of PEMFs. While further experimentation is needed, the optimal range for migration in cells from this animal may be the smallest range.

The medium range of PEMFs was expected to produce minimal changes also, and this was true for dermal fibroblasts from non-diabetic animals. However, an increase in scratch wound closure rate was observed in dermal fibroblasts from one of the diabetic animals. Cells from this same animal saw decreases under the largest range of PEMFs. This suggests that the medium range of PEMFs produces optimal migration in cells from this animal, with the largest range of PEMFs being inhibitory to migration.

It was found that the largest range of PEMFs was most effective in speeding the scratch wound closure rate for dermal fibroblasts from diabetic and non-diabetic animals. Cells from one of the diabetic animals showed increases in scratch wound closure at the latter two time intervals, 8-23 hours and 23-28 hours. Cells from one of the non-diabetic animals showed increases in scratch wound closure rate at the second time interval, 8 to 23 hours. Cells from both the diabetic and non-diabetic animals had no significant improvements in migration under the smallest and medium ranges of PEMFs.

The improvements in scratch wound closure rates under the largest range of PEMFs occurred at the latter two time points. This may be indicative of the increased protein and growth factor production that is suspected to take place during the eight hours of PEMF stimulation and the subsequent actions of these factors in the latter hours after PEMFs are turned off.

Some inhibitory effects were seen under the largest range of PEMFs for dermal fibroblasts from one diabetic animal and no change in dermal fibroblasts from one non-diabetic animal. The inhibitory effects seen may have been due to increased protein and growth factor production that has been shown for other cells types under PEMFs, but this was not measured in this study. The lack of change seen in dermal fibroblasts from the non-diabetic animal may represent the need for a more prolonged study of PEMF stimulation.

Overall, it was found that each range of PEMFs was able to induce positive change in dermal fibroblasts from at least one of the animals. The evidence in this study seems to indicate that the optimal range of PEMFs may be host-dependent, based on the

variability between subjects. However, further study will be needed to confirm this hypothesis.

Applications of electrical stimulation to wound healing are now being brought to the forefront of research. PEMFs were chosen as the method of electrical stimulation for this study due to the non-invasive and practical method of application. It is interesting to note that endogenous currents measured when a wound occurs are much higher in magnitude than the currents induced in this study. However, the evidence supporting the effectiveness of PEMFs for wound healing is mounting.[23, 25, 26] This suggests that perhaps the pulsed form of current has advantages or is at least comparable to direct current in its ability to speed the healing of diabetic wounds.

SUMMARY AND CONCLUSIONS

The initial goals in this study were to examine proliferation rates, under normal and high glucose, plus migration rates of dermal fibroblasts from diabetic and non-diabetic rats. The primary goal was to determine how PEMFs might affect migration capabilities of these cells. This study demonstrated that dermal fibroblasts from diabetic subjects were impaired in several ways. First, dermal fibroblasts from diabetic rats had significantly impaired proliferation compared to dermal fibroblasts from non-diabetic rats. In addition, under high glucose conditions, dermal fibroblasts from both diabetic and non-diabetic cells had significantly impaired proliferation. Lastly, migration properties of dermal fibroblasts from diabetic animals, as examined by scratch wound closure rates, were significantly impaired compared to cells from non-diabetic animals. It was concluded that under the diabetic conditions of the Zucker rat, dermal fibroblasts have a significantly altered state, in terms of proliferation and migration characteristics, in comparison to cells from non-diabetic rats.

Application of PEMFs to dermal fibroblasts produced minimal changes in proliferation. However, dermal fibroblasts from one diabetic animal had significantly increased proliferation rates under PEMFs. From this, it is concluded that dermal fibroblasts from non-diabetic animals were not by considerably affected PEMFs in terms of proliferation. On the contrary, the overall results suggest that impairments in proliferation of dermal fibroblasts from diabetic animals may be improved under PEMFs.

Application of PEMFs to produce local electric fields significantly affected scratch wound closure rates of dermal fibroblasts from both diabetic and non-diabetic animals. The smallest range of electric fields, 0 to 1.1 mV/cm, significantly increased the

scratch wound closure rates in dermal fibroblasts from one non-diabetic animal, while the medium range of electric fields, 1.2 to 1.7 mV/cm, significantly increased scratch wound closure rates in one diabetic animal. Importantly, the largest range of electric fields, 2.0 to 2.7 mV/cm, significantly increased the scratch wound closure rates of dermal fibroblasts from one diabetic and one non-diabetic animal. From these results, it was determined that different magnitudes of PEMFs significantly altered scratch wound closure rates of dermal fibroblasts. It was concluded that different ranges of PEMFs may be important for tailoring treatment to improve the healing of chronic dermal wounds.

FUTURE WORK

The evidence presented in past studies and the present study for the promotion of wound healing with the aid of PEMFs is promising and warrants continuing study. Animal variability was judged to be an influence in this study and, therefore, animal numbers should be increased in future studies. It would be desirable to more closely simulate the dynamic wound environment of a diabetic foot ulcer, for example. More complex co-cultures and the addition of diabetic serum would help to more closely mimic the in vivo situation. Lastly, evaluation of the expression of key growth factors and growth factor receptors within the cells would elicit much more detailed information about the effects of PEMFS.

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APPENDIX A

TOXIN-INDUCED DIABETIC ANIMAL MODEL

Summary of findings for toxin-induced diabetic model

An animal model for Type 1 diabetes can be obtained through the administration of certain toxins, such as streptozotocin (STZ), that act to destroy pancreatic β-cells. This mimics the autoimmune attack on the pancreas that is experienced by subjects with Type 1 diabetes. STZ was used to induce diabetes in the wild-type littermates of the Zucker rats used in the main study.

Two of the wild-type littermates were given an injection of streptozotocin (STZ) at a dose of 65 mg/kg. All other rats, including genetically diabetic, were injected with saline vehicle (control). Vehicle and STZ were administered by intraperitoneal injection. For the diabetic rats, bedding was changed daily and all animals were provided with food and water *ad libitum*. A drop of blood was collected daily after diabetic induction from the tail vein and the glucose level was tested using a glucometer. Blood glucose levels of >300 mg/dl were considered successful diabetes induction. Two days after injection, neither animal was diabetic; therefore, a second injection of STZ was administered. The following day both animals were diabetic with a blood glucose levels >500 mg/dl. After diabetes induction, animals were humanely sacrificed and dermal tissue obtained for the isolation of dermal fibroblasts.

Dermal fibroblasts were isolated using the same procedure stated in the main study. Dermal fibroblasts at passages 4 thru 10 were used for subsequent experimentation. The hexosaminidase assay was used to assess proliferation of the cells. Proliferation rates were compared for dermal fibroblasts from STZ and non-diabetic animal models.

Dermal fibroblasts from STZ animals showed no difference in proliferation rates from day 1-3 compared to dermal fibroblasts from non-diabetic animal (Figure 16) (ANOVA, $p = 0.99$). A large amount of variance was observed for STZ 1.

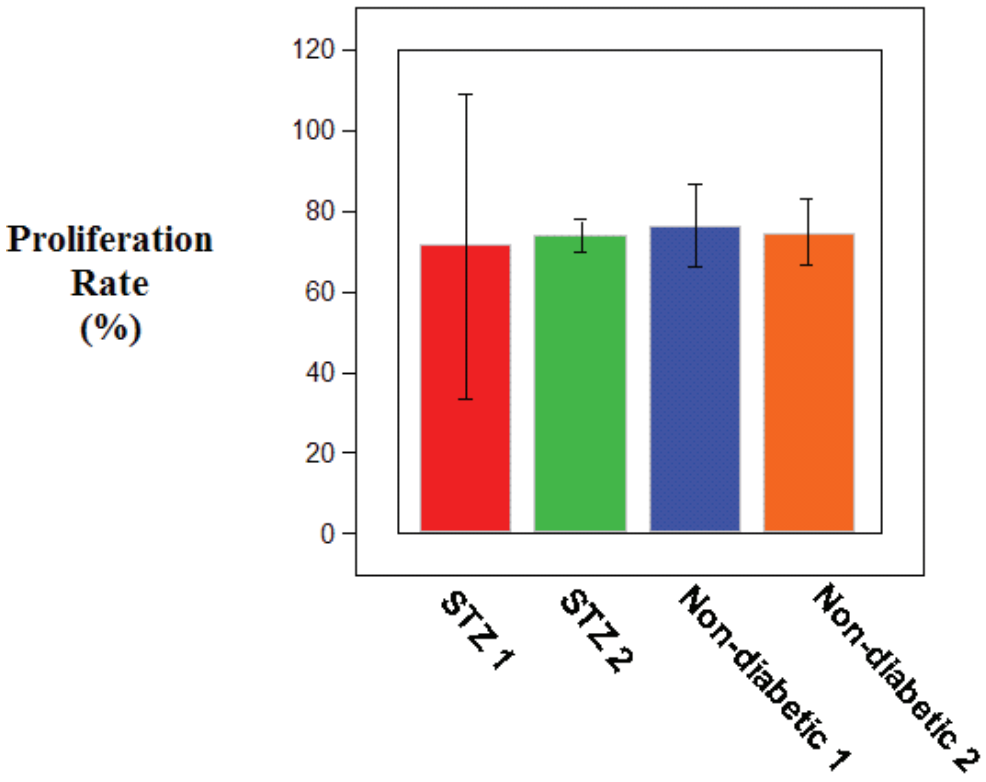

Figure 16: Proliferation rates from day 1 to 3 for dermal fibroblasts from STZ-induced diabetic and non-diabetic rats.

Qualitatively, no morphological differences were observed between dermal fibroblasts from the STZ and non-diabetic animals. The proliferation assay confirmed these similarities between the two different models. One explanation for the lack of difference between the two models may be that the STZ animals were not allowed to survive for a significant period after induction. If the animals were in the diabetic state

for an extended period of time, the differences between dermal fibroblasts from STZ-induced diabetic animals and non-diabetic animals may have been more profound. Also, the lack of endogenously altered genetic makeup within the animals as was present in the Zucker rats may explain why dermal fibroblasts cultured from the STZ-induced animals did not show an altered state. A number of studies have been conducted with toxin-induced diabetic animals. Further examination of the toxin-induced model may provide insight into the degree of its usefulness as a model of diabetes.

APPENDIX B

IACUC APPROVAL FORM

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

Institutional Animal Care and Use Committee (IACUC)

APN 070108022

MEMORANDUM

DATE: January 24, 2007

TO: Donald W. Petersen, Ph.D. Biological Innovations
SBD 616 0007
FAX: 975-6108

FROM: Judith A. Kapp
Judith A. Kapp, Ph.D., Chair
Institutional Animal Care and Use Committee

SUBJECT: **NOTICE OF APPROVAL - Please forward this notice to the appropriate granting agency.**

The following application was reviewed and approved by the University of Alabama at Birmingham Institutional Animal Care and Use COmmittee (IACUC) on January 24, 2007.

Title: Characterization of Diabetic Fibroblasts

Sponsor: Biological Innovations, Inc.

This institution has an Animal Welfare Assurance on file with the Office for Protection from Research Risks (Assurance Number A3255-01) and is registered as a Research Facility with the United States Department of Agriculture. The animal care and use program is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)

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