GLUCOSE - INDUCED APOPTOSIS OF SCHWANN CELLS *IN VITRO*

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Jacqueline D. Meyer, B.S.

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DEDICATION

This work is dedicated to all of the members of the of the MCR for their time and talents.

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TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

ABSTRACT

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SUPERVISING PROFESSOR: JOSEPH R. KOKE

The loss of Schwann cells in the peripheral nerves is thought to be responsible for the pathologies associated with diabetic neuropathy. As diabetes causes increased extracellular glucose levels, glycosylation or non-enzymatic glycation of cell surface receptors may interfere with normal signaling between axons and Schwann cells and trigger the apoptotic pathway in Schwann cells. In this study, I tested the hypothesis that high glucose levels emulating hyperglycemia stimulate apoptosis in cultured Schwann cells. Schwann cells were isolated from two-day-old rat pup sciatic nerves, placed in

primary culture, and then incubated in the presence of elevated glucose levels for one to seven days. The glial marker S100 monoclonal antibody identified Schwann cells, and apoptosis was detected using the terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) method. Approximately 50% of the Schwann cells exposed to elevated glucose levels were apoptotic after 24 hours of incubation, and apoptosis of Schwann cells increased with increasing time in culture to 71% on day six in culture. This finding indicates that increased extracellular levels of glucose results in Schwann cell apoptosis *in vitro*.

INTRODUCTION

Diabetic neuropathy is a disabling complication of diabetes mellitus that affects patients with both Type I and Type II diabetes. The link between diabetes and neuropathy could be the high tissue glucose levels that exist in diabetic individuals. Up to fifty percent of all patients with diabetes mellitus experience neuropathies that affect peripheral, autonomic, and cranial nerves (Davis, 1997). Diabetic neuropathy is characterized by degenerative changes of the distal peripheral nerves (Vinik *et al.*, 1992), that result from interrelated metabolic and functional defects (Tomlinson *et al.*, 1994), leading to destruction of unmyelinated and myelinated peripheral nerve fibers (Greene *et al.*, 1999).

Axons manifest metabolic abnormalities associated with hyperglycemia and insulin deficiencies, including increased activation of the polyol pathway, decreased Na^+/K^+ ATPase activity, increased oxidative stress, increased non-enzymatic glycation, and decreased neurotrophism (Sima and Sugimoto, 1999). These metabolic abnormalities may be responsible for the functional abnormalities seen in diabetic neuropathy, which include decreased nerve conduction velocity, decreased blood flow, axonal atrophy and degeneration, segmental demyelination, decreased fiber regeneration, and neuronal apoptosis (Sima and Sugimoto, 1999). The decrease in nerve conduction velocity is thought to be a result of decreased Na^+/K^+ ATPase activity (Greene and

Lattimer, 1984), demyelination, impaired nerve fiber regeneration and fiber loss (Sima and Sugimoto, 1999).

Schwann cells are the myelinating cells of the peripheral nervous system and are derived from the neural crest cells (Mirsky *et al.*, 2002). Schwann cells develop into two separate morphological and functional types: the non-myelinating (terminal) Schwann cells, and the myelinating Schwann cells. These separate types of Schwann cells are determined postnatally by the axonal signals from the nerves with which they are in contact (Son *et al.*, 1996). Schwann cells and neurons have a unique interdependence that is required for survival. Axons secrete survival factors for the Schwann cells, which lay down the basal lamina and provide trophic support for regenerating axons (Son *et al.*, 1996). In Schwann cells from neonates, neuron-derived growth factors termed neuregulins promote differentiation and survival of Schwann cell precursors (Cheng *et al.*, 1998). Neuregulins are a sub-set of a larger family of epidermal growth factors that bind to transmembrane tyrosine kinase receptors (Lemke, 1996). Neuregulins may regulate cell fate, proliferation, and survival in undifferentiated Schwann cells (Lemke, 1996). Neuregulins encompass the glial growth factors (Lemke and Brockes, 1984), acetylcholine receptor-induced activity factor (Falls *et al.*, 1993), neu differentiation factor (Peles *et al.*, 1992), and heregulin (Holmes *et al.*, 1992).

ErbB epidermal growth factor receptors are specific for neuregulins (Reise, 1998). The ErbB family of receptors is made up of ErbB2, ErbB3, and ErbB4 (Lemke, 1996). The ErbB family name originated from the observation that mutated forms of the epidermal growth factor receptors were found in avian erythroblastosis virus B

(Downward *et al.*, 1984). Neuregulin activates erbB receptors, which are tyrosine kinase receptors, through paracrine or juxtacrine signaling (Reise, 1998). ErbB3 and ErbB4 expression changes during neural development, which may suggest that these receptors are coupled to different physiological signaling pathways as well as cellular responses (Reise, 1998). This change in expression around the time of birth, coinciding with the change in Schwann cells' dependency from axonal signals to autocrine signals (Cheng *et al.*, 1998). Neonatal Schwann cells require axonal signals in order to establish a one-to-one Schwann cell: axon ratio; however, in later stages of development, autocrine signals are utilized in order to keep Schwann cells alive during reconstruction of damaged nerves by laying down the basal lamina for axon regrowth (Cheng *et al.*, 1998).

Schwann cell survival is essential for neural regeneration. In diabetic neuropathy Schwann cells dissociate from the axon and do not aid in axonal regeneration. The mechanism for Schwann cell disassociation in diabetic neuropathy has yet to be shown. In this study, I tested the hypothesis that abnormally high glucose levels might be directly responsible for apoptosis of Schwann cells, and I therefore attempted to determine whether elevated glucose concentrations influence apoptosis of Schwann cells *in vitro*.

MATERIALS AND METHODS

Pregnant (e18) female Sprague-Dawley rats were delivered to St. Mary's University in San Antonio, Texas and cared for in St. Mary's University's IACUC approved animal care facility for four days (IACUC approval code # 8-001). Two day-old rat pups were sacrificed by decapitation, followed by sciatic nerve transection. Sciatic nerves were obtained as outlined by Brockes et al., 1979, and stored in 2 ml of HE medium (see Table 1) during dissection.

Primary Cell Culture

All cell cultures were procured under aseptic conditions in a laminar flow hood. Two 350 µl aliquots of 0.3% collagenase were added to sciatic nerves in 2 ml of HE medium, in 45-minute increments, and incubated at 37°C in a shaking water bath for 45 minutes at approximately 60 rpm. The suspension was then transferred into a 15 ml centrifuge tube and centrifuged at 1000 rpm for 5 minutes. The supernatant was discarded, and the cells were resuspended in 1 ml of warmed (37°C) HF medium (see Table 1) per 10 rat pups dissected. The suspension was dispersed by slowly repipetting the pellet, and 1 ml of the suspension was placed into a sterile, glass Petri dish, followed by addition of 9 ml of HF medium to bring the total volume to 10 ml of cell suspension per Petri dish. Cultures were incubated at 37°C in humidified 95% air/5% CO_2 overnight. The following day, HF medium was removed and replaced with 10ml of fresh, warmed (37°C) HF medium, and processed as follows

1. One hundred microliters of 0.115 mM cytosine arabino-furanoside (ARA-C) (in HE medium) was added to each Petri dish;
2. Cultures were then incubated for 48-72 hours in the presence of ARA-C;
3. The HF medium + ARA-C was then removed and replaced by 10 ml of fresh HF medium without ARA-C, and the culture incubated for 5-24 hours.

This cycle (steps 1 - 3) was repeated for 7-10 days until the culture was 98% Schwann cells ("pure culture"), which was determined by microscopic inspection of the cell cultures.

When a pure culture was obtained, HF medium was discarded and replaced with a medium consisting of 1 ml of 2.5% trypsin, 1 ml of 0.4% EDTA, and 8 ml of Saline I (see Table 1). Cells were then mechanically detached by gently swirling the Petri dish while observing the culture using a microscope. The resulting Schwann cell suspension was centrifuged at 1000 x *g* for 10 minutes. The supernatant was discarded, and cells were resuspended in 1 ml of fresh HF medium. Cells were then diluted to a concentration of 15,00-cells/ml in HF as determined by using a hemocytometer. Round, 12.7 mm-diameter, sterile coverslips were first coated with sterile 0.0025% poly-d-lysine, and then placed 1 per well into a 24-well plate (see Fig. 1). Ninety microliters of the Schwann cell suspension was added to each well. The plates were incubated overnight, at conditions stated above, to allow for cell attachment to the coverslips.

Experimental Glucose Treatments

Treatments were set up in a 24-well plate as shown in Figure 1. The experimental media included the control, which was unsupplemented N2 medium (see

Table 1) with a final concentration of 12.5 mM D-glucose; the moderate glucose treatment group, which was N2 medium supplemented with 25 mM D-glucose a final concentration of 37.5 mM D-glucose; and a high glucose treatment, consisting of N2 supplemented with 50 mM D-glucose with a final concentration of 62.5 mM D-glucose. The four columns were designated days 1,3,5, and 6. The six rows were made up of two rows of control groups, two rows of moderate glucose treatment groups, and two rows of high glucose treatment groups. Five hundred microliters of medium was added to each well. Medium was changed daily, and coverslips to which cells were affixed were moved into a new 24 well plate and fixed after one day, three days, five days and six days in culture. Cells were fixed in 4% paraformaldehyde for one hour at room temperature and stored in PBS (phosphate buffered saline (see Table 1) at 4°C until staining and labeling.

In order to control for increased osmotic pressure resulting from increased glucose concentrations, parallel experiments were performed using N2 medium supplemented with 25 mM or 50 mM L-glucose or mannitol.

Immunocytochemistry and Imaging

After fixation and storage at 4°C, the PBS was removed from the coverslips, which were then washed three times by placing 500 μl of PBS in each well, for five minutes at room temperature, then discarding the PBS. After the wash, 200 μl of 10% nonfat powdered milk, reconstituted in PBS was added to each well in order to block non-specific labeling. The coverslips were then washed three times in PBS using the same method stated above. Permeabilization was not necessary for S-100 labeling (preliminary experiments). Following the second wash, 90 μl of S-100 mAb (see Table 2

for antibody sources and dilutions) was applied to the air-dried coverslips. The S-100 mAb was used to identify cytoplasmic glial S-100 calcium-binding protein located in Schwann cells (Barger and Van Eldik, 1992). The anti S-100 mAb remained on the coverslips for one hour at room temperature, in a covered 24 well plate. The coverslips were then washed three times in PBS using the previously stated method. Coverslips were again allowed to air dry. Next, 90 µl of goat anti mouse IgG conjugated to Alexa Fluor 488 (see Table 2 for dilution and source information) was placed onto each coverslip and allowed to incubate for 1 hour at room temperature in a covered 24 well plate. Each coverslip was then washed three times in PBS using the above-stated method. Next, terminal end deoxynucleotidyl transferase – mediated dUTP nick end labeling (TUNEL) was used to identify apoptotic Schwann cells (*In situ* cell death detection kit, TMR red; Roche). The coverslips were washed twice in PBS as stated above, followed by one wash in distilled water to remove PBS. Finally, coverslips were mounted onto slides using 90% glycerol with 0.1 µg / ml of N,N Dimethyl-p-phenyldiamine (in PBS), to avoid fading of the fluorescent probes. Immunocytochemical controls included a positive and negative control for using TUNEL, as stated in the printed protocol (Roche Applied Sciences). The negative control omitted the terminal transferase from the TUNEL assay, and the positive control was incubated in 3000 U/ml (in 50 mM Tris-HCl, pH 7.5) DNase I for ten minutes at room temperature in a covered 24 well plate, to induce DNA strand breaks.

Immunolabeled coverslips were viewed using a Bio Rad MRC 1024 Laser Scanning Confocal Microscope, and images were acquired using Laser Sharp software.

Images were processed using Adobe Photoshop software. Cell counts were performed under epifluorescence illumination on an Olympus microscope, at 600x magnification. The total number of cells seen in each view was counted, and then the cells exhibiting red nuclei from TUNEL were counted. The data were recorded in a Microsoft Excel spreadsheet, and the percentage of TUNEL cells was recorded.

Cell Counts and Statistical Analysis

Cells were counted using epifluroescence microscopy. The total number of cells with S-100 labeling and Schwann cell morphology, in one field at 600X were counted, and TUNEL positive cells were counted in the same field. This was repeated ten times for each coverslip in each treatment. The percentages of Schwann cells that were apoptotic were placed into a Statview spreadsheet, and an ANOVA was run followed by a Fisher's PLSD post hoc test to determine if there was a statistical difference between the total amounts of apoptosis seen between the high glucose and the control treatments, and the difference between the high glucose and mannitol treatments. There were two replicates (n=2) for the mannitol treatment, and four replicates (n=4) for the experimental glucose treatments, which are further detailed in later sections of this paper.

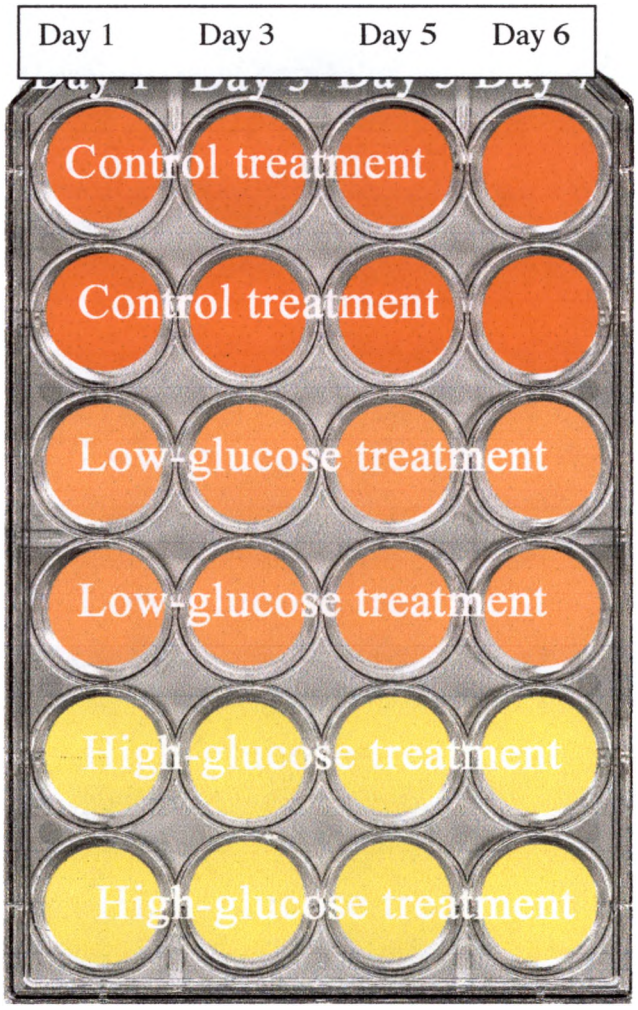
Table 1. Contents of cell culture media, and pH for all media used for Schwann cell culture and experimental protocols.

Media	***Contents***
HE (Brockes et al., 1979)	Dulbecco's Modified Eagles Medium, low glucose (5.6 mM) in 25 mM HEPES buffer, pH 7.3 – 7.4 supplemented with 500 µl of penicillin / streptomycin solution
HF	Dulbecco's Modified Eagles Medium, low glucose (5.6 mM) in 40 mM $NaHCO_3$, pH 7.3 – 7.4, supplemented with 50 ml of heat inactivated fetal bovine serum and 500 µl of penicillin / streptomycin solution
N2	2X Dulbecco's Modified Eagles Medium, high glucose (12.5 mM) in $DDiH_2O$, pH 7.2, supplemented with 10X Ham's F-12 Media, 5 mg/ml insulin (in 1N HCl), 2% Transferrin Stock, 20 nM progesterone, 100 µM putrescine, and 30 nM sodium selenite, 1.4 mM l-glutamine, 0.44 mM $NaHCO_3$, and 1 ml of Gibco Invitrogen 1X Antibiotic-Antimycotic
Saline I	NaCl (138.6 mM) KCl (5.4 mM) Na_2HPO_4 (1.1 mM) KH_2PO_4 (1.1 mM) D-glucose (22.2 mM) 0.5% Phenol Red (15 drops) pH 7.0 – 7.1
Phosphate Buffered Saline (PBS)	NaCl (1.37 mol) KCl (0.03 mol) Na_2PO_4 (0.04 mol) KH_2PO_4 (0.01 mol)

Table 2. Primary and secondary antibodies used for immunofluoresence, dilutions, and catalog numbers.

Antibodies	***Species/ Isotype***	***Dilution***	***Supplier and Catalog Number***
Anti S-100 β subunit mAb	Mouse IgG	1:1000 in PBS	Sigma-Aldrich #S2532
Goat anti-mouse IgG conjugated to Alexa-Fluor 488	Goat IgG	1:1000 in PBS	Molecular Probes #A11017

Figure 1. Contents and set up of 24-well plate. Columns are (from right to left) day 1, day 3, day 5, and day 6. Rows are (top to bottom) N2 without supplemented D-glucose (red), N2 + 25 mM D-glucose (pink) and N2 + 50 mM D-glucose (yellow).


Day 1
Day 3
Day 5
Day 6
Control treatment
Control treatment
Low-glucose treatment
Low-glucose treatment
High-glucose treatment
High-glucose treatment

RESULTS

Based on the previously stated methods, Schwann cell cultures were approximately 99% pure prior to beginning the experimental glucose treatments. All Schwann cells were identified by the presence of S-100 labeling, and cells not labeled were not considered in the analysis. Apoptosis was determined by observation of TUNEL positive nuclei. TUNEL positive controls incubated with DNAse showed labeled red nuclei. Negative controls for labeling did not have any labeling present in the nucleus.

Experimental Glucose Treatments

Thirty-eight percent of the control group Schwann cells fixed on day one were apoptotic (Fig. 2A). Almost 50% of the Schwann cells in the moderate glucose treatment were apoptotic (Fig. 2B), and 45% of the Schwann cells in the high glucose treatment were apoptotic (Fig. 2C). Schwann cells fixed on day one appeared to have shortened processes, and were distributed evenly across the coverslip with small areas of densely packed cells.

On day three, 40% of the Schwann cells in the control group were apoptotic (Fig. 2D), 30% of the Schwann cells in the moderate glucose treatment were apoptotic (Fig. 2E), and 48% of the Schwann cells in the high glucose treatment were apoptotic (Fig. 2F). Cells on day three appeared to have migrated into larger groups of cells with extensions

projecting toward other Schwann cells, with strong S-100 localization around the nuclear membrane, and cellular membrane.

Thirteen percent of the Schwann cells in the control group that were fixed after five days in culture were apoptotic (Fig. 3A). Twenty-nine percent of the Schwann cells in the moderate glucose treatment group that were fixed on day five were apoptotic (Fig. 3B), and 52% of the Schwann cells fixed after five days of incubation in the high glucose treatment were apoptotic (Fig. 3C). After five days in culture, the S-100 labeling became less distinct, and all cells that were not apoptotic were found in large groups of Schwann cells.

After six days in culture, 62% of the Schwann cells in the control group were apoptotic (Fig. 3D), 67% of the Schwann cells in the moderate glucose treatment group were apoptotic (Fig. 3E), and 71% of the cells in the high glucose treatment group were apoptotic (Fig. 3F). S-100 labeling was very faint in most cells, and the lowest amount of labeling was seen in apoptotic Schwann cells (see Figure 3F).

It should also be noted that the Schwann cells seen in large clumps appeared to be somewhat protected from apoptosis than did the single cells not found in clumps, which almost always were apoptotic. Positive controls did not exhibit the same phenomenon.

Statistical Analysis

The percentage of apoptotic Schwann cells from the control groups and the high glucose treatment groups were compared (Figure 4), and the number of Schwann cells that were apoptotic in the mannitol and high glucose treatments groups were compared (Figure 5). A two factor ANOVA and a Fisher's PLSD post hoc test, were run to determine whether a statistical difference existed between the percentage of apoptotic

Schwann cells in D-glucose versus the percentage of apoptotic cells in mannitol, and whether there was a statistically significant difference between the high glucose treatment and the control treatment.

There were apoptotic cells seen throughout the experiment in each treatment. There was a statistically significant difference between the percent of Schwann cells that were apoptotic in the control treatment compared to the high glucose treatment ($P = 0.0216$) as shown in Figure 4. Also, there was a statistically significant difference between the numbers of Schwann cells that were apoptotic in the mannitol treatment versus the high glucose treatment ($P = 0.0230$) as shown in Figure 5.

Controls

The results of the osmotic controls using L-glucose, showed that cultures treated with L-glucose had a similar percentage of apoptotic Schwann cells, as did those treated with D-glucose (Fig 6). A dose dependent increase in nuclear labeling was seen in cells in high L-glucose (supplemented with 50 mM L-glucose) treatments. Because this result was seen, a second osmotic control using mannitol was conducted. The percentage of Schwann cells that were apoptotic in the mannitol treatment was not statistically different from the corresponding percentage in the control treatment ($P=0.4837$).

Figure 2. Confocal micrographs of apoptotic Schwann cells cultured for one day and three days in D-glucose. Apoptotic Schwann cells in control groups after one day in culture (A), in low D-glucose treatment groups after one day in culture (B), in high D-glucose treatment groups after one day in culture (C). Schwann cells in control groups after three days in culture (D), in low D-glucose treatment groups after three days in culture (E), and high D-glucose treatment groups after three days in culture (F). Scale bar represents 10μm. White arrows represent apoptotic nuclei (red labeling).

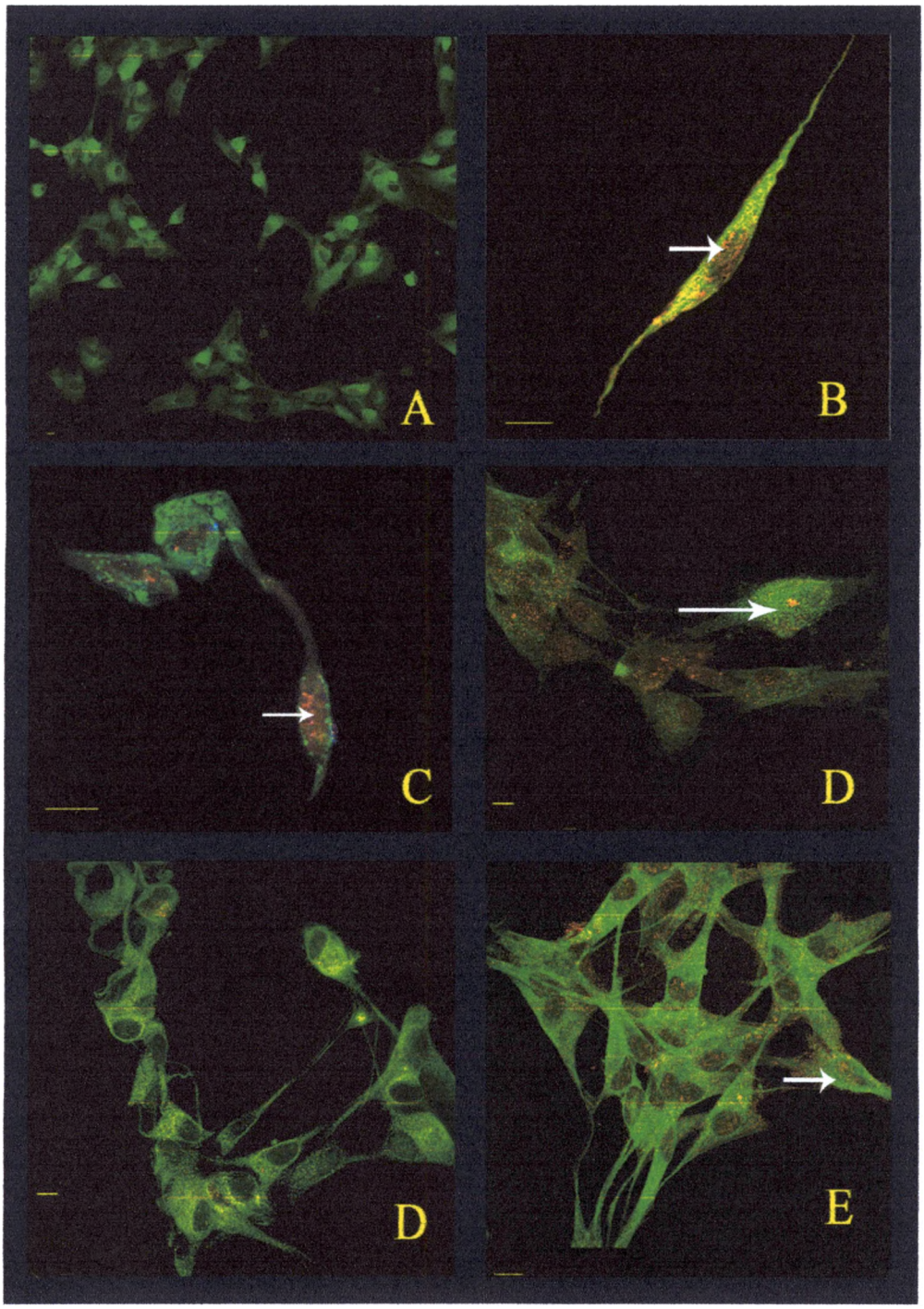
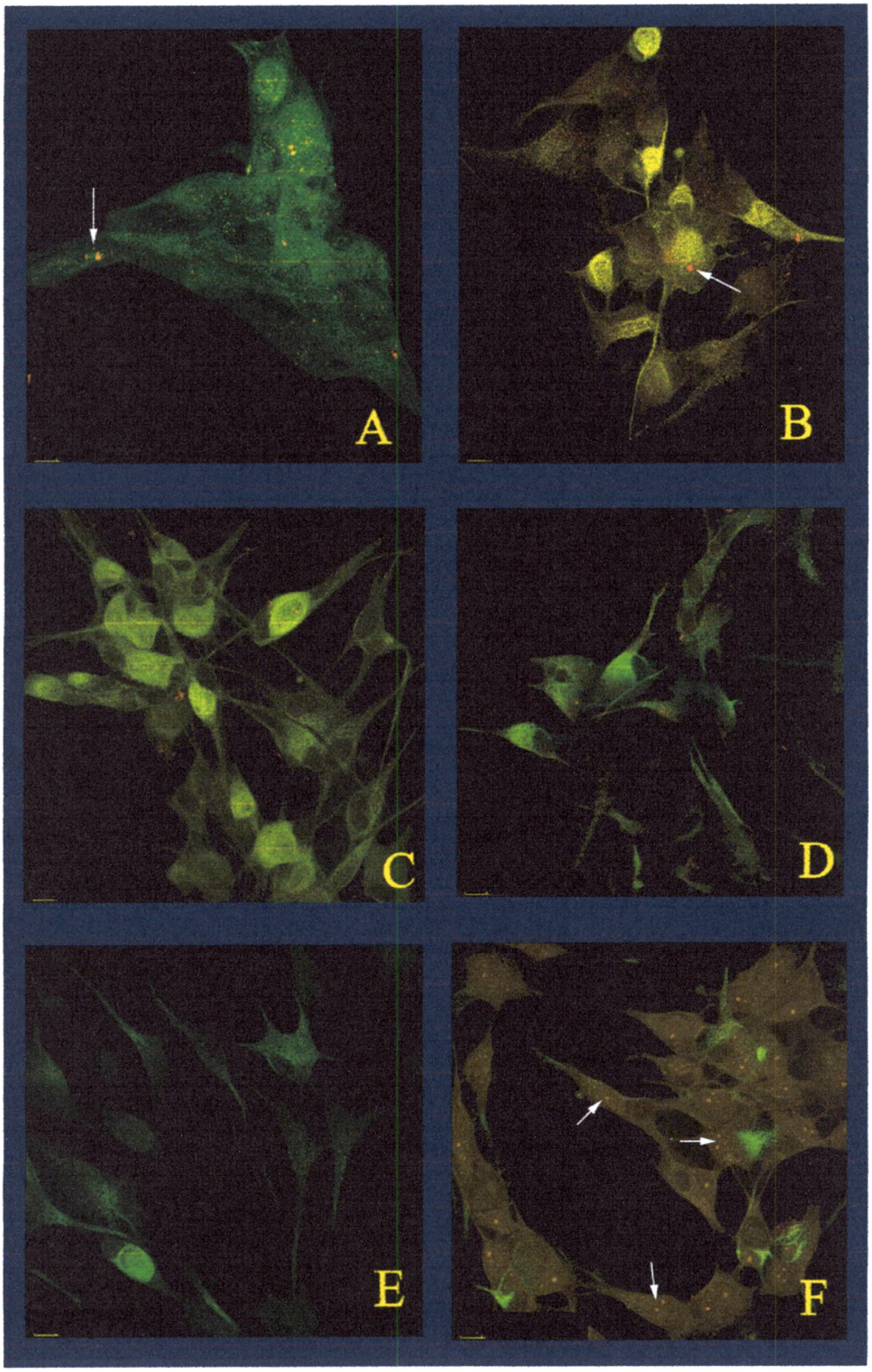

A
B
C
D
D
E

Figure 3. Confocal micrographs of apoptotic Schwann cell control groups after five days in culture (A), low D-glucose treatment groups after five days in culture (B), in high D-glucose treatment groups after five days in culture (C). Schwann cell control groups after six days in culture (D), Schwann cells in low D-glucose treatment groups after six days in culture (E), and Schwann cells in high D-glucose treatment groups after six days in culture (F). Scale bar represents10μm. Red staining indicating apoptotic nuclei is shown with white arrows.


A
B
C
D
E
F

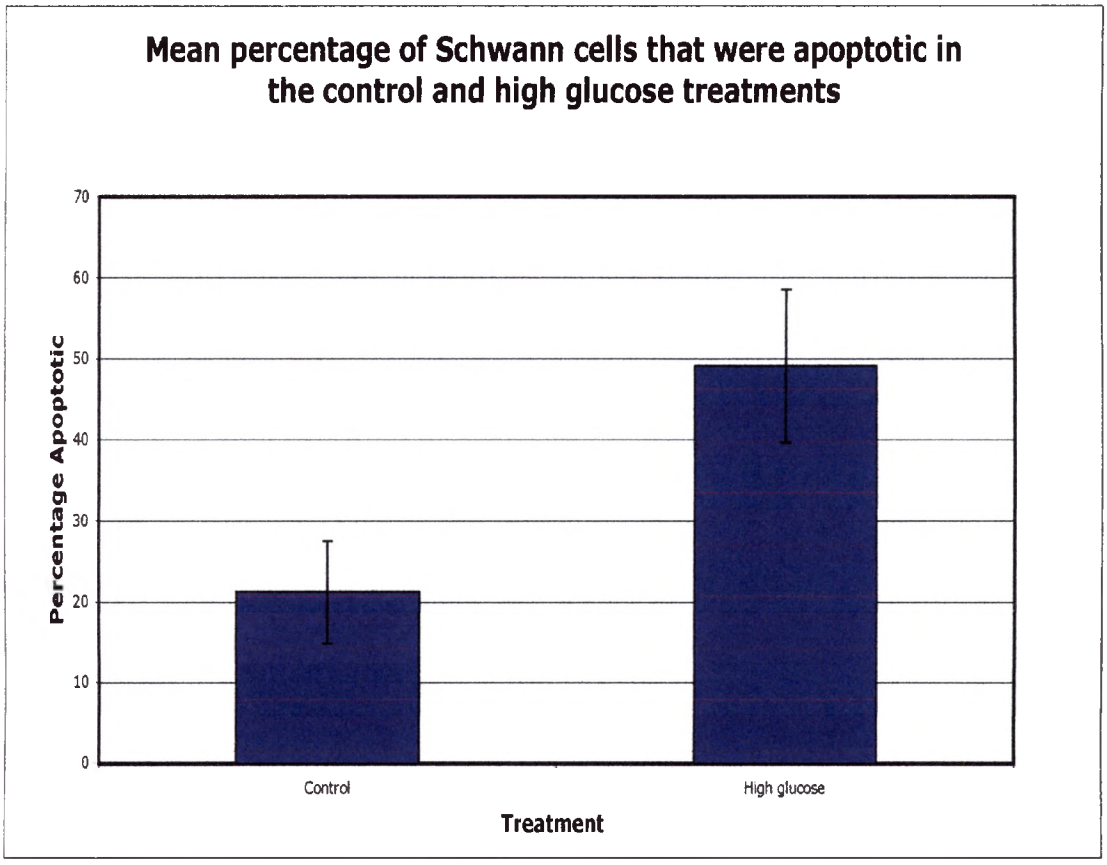


Figure 4. Mean percentage of Schwann cells that were apoptotic in the control and high glucose treatment groups over the duration of the experiment. Bars are representative of the standard error of the mean. The overall percentage of apoptotic cells was based on four replicates (N = 4).

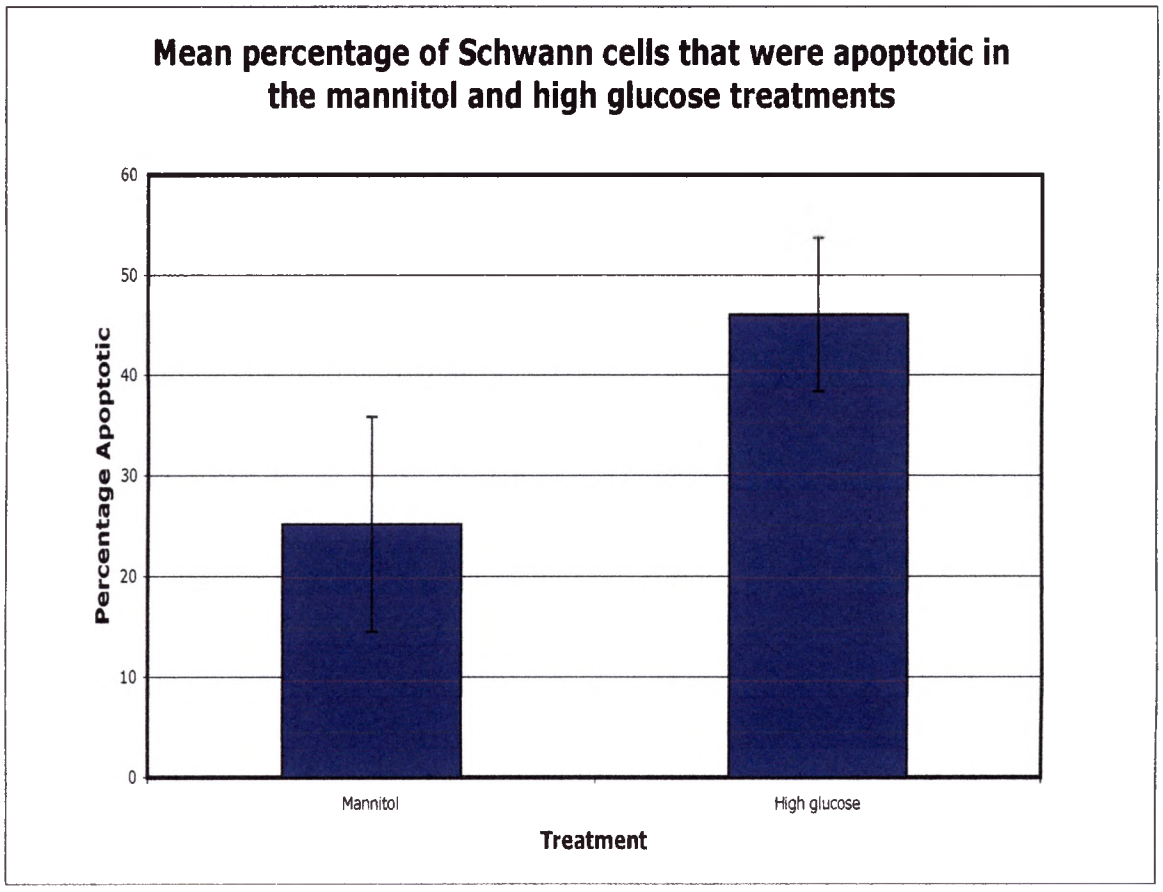


Figure 5. Mean percentage of Schwann cells that were apoptotic in the mannitol and high glucose treatments for the duration of the experiment. Bars are representative of the standard error of the mean. The percentage of Schwann cells that were apoptotic is based on counts from two replicates (N = 2).

DISCUSSION

Experimental Glucose Treatments

Some Schwann cells under the culture conditions used in this study were apoptotic at all time periods, and in all treatments, including the control. In the high glucose treatment, the frequency of apoptosis increased over the duration of the experiment, and was significantly higher than the control, showing that high glucose levels were correlated to apoptosis of Schwann cells *in vitro*.

The use of a serum-free medium was necessary in this study to ensure that there were not exogenous survival factors in the FBS that might rescue Schwann cells from glucose-mediated apoptosis. A baseline level of apoptotic cells were seen in the control treatments, which may have been a result of serum deprivation, which has been shown to cause apoptosis in Schwann cells after one day in serum-free culture media (Syroid et al., 1996). However, the percentage of apoptosis seen in controls for the experiments reported herein were about half of the percentage shown by Syroid *et al.*, indicating that the full effect of serum deprivation may have been deterred by the use of N2, a defined medium. Apoptosis of cells in the control treatments may also have been a result of the concentrations of glucose seen in the control media (12.5 mM), which are still higher than glucose levels seen in humans, which is approximately 5.5 mM D-glucose.

There did not appear to be a statistically significant increase in percentage of apoptotic cells in the moderate glucose treatment, which contained a final concentration

of 37.5 mM D-glucose. One could speculate that Schwann cells in the moderate glucose treatment may have been able to upregulate glucose metabolism, in order to compensate for the increased glucose levels. This could explain why the percentage of Schwann cells that are apoptotic in the moderate glucose treatment group is similar to the percentage of Schwann cells that are apoptotic in the control groups.

The percentage of Schwann cells that were apoptotic in the high glucose treatment group was higher than the frequency of apoptotic Schwann cells incubated in both the control group, and the moderate glucose treatment group at each time period. This result supports the hypothesis that high glucose concentrations may induce apoptosis *in vitro*.

Schwann cells in groups appeared to be somewhat protected from apoptosis by being grouped together, contrary to what was seen in single cells that were not in groups. This observation was also reported by Miersky *et al.* (2002), which shows that Schwann cells at low density were rescued from apoptosis by culture medium from high density Schwann cell cultures. Parkinson *et al.* showed that Schwann cell survival is mediated by autocrine signaling beginning in the neonatal period of development (Parkinson *et al.*, 2002). Autocrine signals, which support long term Schwann cell survival in the absence of neurons, include insulin-like growth factors, platelet derived growth factors, and neurotrophin 3, which act through a mitogen-activated protein kinase pathway (Mirsky *et al.*, 2002). Neuregulin-1 has also been shown to be produced by Schwann cells (Raabe *et al.*, 1996), and may also regulate Schwann cell survival via the autocrine loop. Survival signals secreted by Schwann cells may turn out to have a protective effect against high glucose induced apoptosis, which may explain why more of the Schwann cells treated with high glucose were not apoptotic.

There was not a statistically significant difference between the percentages of apoptotic cells seen in each of the treatment groups, between days. This may have been a result of an early, sustained response of the cells to the high glucose concentrations. If the 50 mM supplemented N2 culture had the greatest effect on the Schwann cells after one day in culture, there would not be another large increase in percentage of apoptosis expected past day one, because the majority of the cells have already been affected. For the statistical comparisons, we compared the control group, mannitol treatment group, and the high D-glucose treatment group, for days one through five. The high glucose treatment and the mannitol treatment groups were compared in order to easily see whether osmotic stress was a key factor in apoptosis of Schwann cells *in vitro*. Schwann cells incubated in the moderate glucose treatment were not included in the statistical comparison, because in previous studies (not published), as in this study, the cells behaved inconsistently in the moderate glucose treatments. In addition, there was not a statistically significant difference between the control group and the moderate glucose treatment group. The majority of the Schwann cells were apoptotic after six days in culture, regardless of the treatment in which they were incubated, therefore, the data from day six was eliminated from statistical analysis. The total number of replicates was low, because several rat pups were required to obtain the number of Schwann cells necessary for each experiment, therefore, each rat pup could not be counted for an individual treatment. Also, the experiment was repeated several times, however there were many early problems with contamination, and cell adhesion to the coverslips, which prevented those data from being included in the results as a replicate.

Mannitol was used as a control for osmotic stress in this study, because it has the same size and molecular weight as glucose. L-glucose was initially used as an osmotic control, because it is not a biologically available form of glucose (Hayashi, 2003). However, when L-glucose was used as a control, the Schwann cells demonstrated similar rates of apoptosis to D-glucose. There were apoptotic cells seen in all time periods and glucose concentrations, with a general increase in the frequency of apoptotic cells with time in the control group, the low L-glucose treatment group and the high L-glucose treatment group. On the basis of the results from the mannitol study, this result was not likely due to osmotic stress but rather may have been a result of non-enzymatic addition of glucose (d or l) to extracellular, or membrane proteins, in a process known as glycation. L-glucose has been shown to form advanced glycation end products (AGEs) in a study that linked AGE formation to neurological impairment and decreased activity of anti-oxidant enzymes *in vivo* (Song *et al.*, 1999). These results further suggest that the mechanism for Schwann cell apoptosis may be due to non-enzymatic glycation of critical signaling proteins, such as neuregulin, or erbB receptors, which are essential to Schwann cell survival (see the next section for further discussion of this hypothesis).

In order to model diabetic neuropathy, it was necessary to utilize high glucose levels to simulate the long-term hyperglycemic conditions seen in individuals with diabetic neuropathy. Normal glucose levels, in a non-diabetic individual can range from approximately 90 mg/dl to 180 mg/dl, depending of the fasting state of the individual. The glucose levels seen in this control are 226 mg/dl, which is slightly elevated above the normal levels, which may account for the apoptosis seen in the control group. The final concentration of glucose in the moderate culture medium (37.5 mM) is equivalent to 675

mg/dl of D-glucose in the blood. The final concentration of glucose in the high glucose culture media was 62.5 mM D-glucose, which is comparable to 1126 mg/dl of blood glucose, which would indicate extreme hyperglycemia.

Mechanisms for Induction of Apoptosis in Schwann Cells

Schwann cell apoptosis seen in high glucose concentrations has been hypothesized to result from the following three interrelated, metabolic disorders seen in diabetic neuropathy: activation of the polyol pathway, oxidative stress and the production of reactive oxygen species (ROS), or nonenzymatic glycation and the formation of AGE products.

The polyol pathway is an alternate glucose metabolism pathway, which is activated when extracellular levels of glucose are high. Glucose is shunted from glycolysis into the polyol pathway, and converted into sorbitol by the enzyme aldose reductase (Greene and Sima, 1993). Sorbitol is unable to cross the cell membrane (Greene *et al.*, 1985), and therefore accumulation of sorbitol may cause osmotic stress in Schwann cells. The second step in the polyol pathway is the formation of fructose from sorbitol. Fructose is a sugar that has been implicated in protein glycation (Takagi *et al.*, 1995). The conversion of sorbitol to fructose is slower than the conversion of glucose to sorbitol, which also increases the intracellular sorbitol levels (Hansen, 2001). The enzyme that converts sorbitol to fructose is sorbitol dehydrogenase, and glycation of sorbitol dehydrogenase decreases the enzyme activity (Hoshi *et al.*, 1996), therefore further elevating sorbitol levels inside the cell. In our study, we show that Schwann cell apoptosis is not influenced by osmotic stress or by activation of the polyol pathway.

Oxidative stress is defined as the production of reactive oxygen species (Sies and Krutmann, 1997), such as superoxides and hydroxyl radicals. Low levels of reactive oxygen species are produced by normal oxidative metabolism and are quenched by endogenous superoxide dismutase and various peroxidases. Recently, it was shown that reactive oxygen species, specifically H_2O_2, lead to apoptosis of PC12 cells, and the addition of neuregulin was able to rescue the cells from apoptosis, (Goldshmit *et al.*, 2001). Goldshmit also showed that neuregulin is unable to rescue these cells when the PI3 kinase pathway is inhibited. This result suggests that the PI3K pathway is involved in reactive oxygen species-mediated apoptosis, and the PI3K pathway is the neuregulin-erbB2/erbB3 (survival signal) pathway. Therefore, we hypothesize that reactive oxygen species may play a role in Schwann cell apoptosis through interaction with the erbB3 receptor, or PI3K pathway proteins.

As suggested in the previous section, protein glycation may be the causative factor for Schwann cell apoptosis in hyperglycemic conditions. In diabetic patients and diabetic animal models, it has been shown that glycation products are present at elevated levels in the peripheral nerve (Vlassara *et al.*, 1981; Vlassara *et al.*, 1983). AGEs have also been shown to accumulate faster in the circulation of diabetic individuals than in non-diabetic individuals (Makita *et al.*, 1991). Transmembrane myelin components such as myelin protein zero (P_o), myelin basic protein and proteolipid proteins have been shown to be glycated in diabetic rat models (Vlassara *et al.*, 1983; Weimbs and Stoffel, 1994).

Previous studies in Dr. Timothy Raabe's lab at St. Mary's University in San Antonio, Texas (Raabe, 2004) have shown that erbB3 receptor expression on Schwann

cells is decreased after three days in hyperglycemic conditions, as shown by both decreased antibody recognition, and by immunoblot analysis. Decreased erbB3 expression has led to the development of severe peripheral neuropathies in other models (Riethmacher *et al.*, 1997). The erbB3 receptor utilizes the PI3K pathway for transmitting survival signals in the Schwann cell. PI3-kinase was found to be decreased in Schwann cells incubated in 50 mM glucose.

Conclusions and Future Studies

Diabetic neuropathy is a multifactoral complication of diabetes mellitus. There are several compounding factors, which make the exact mechanism for Schwann cell apoptosis difficult to elucidate. In this study I have shown (1) that higher than normal glucose levels are correlated significantly with accelerated rates of Schwann cell apoptosis *in vitro*; (2) that apoptosis is not an osmotic effect; and (3) both D-glucose and L-glucose were equally effective in accelerating apoptosis. Non-enzymatic glycation of critical signaling proteins is a possible mechanism for this effect, and possible targets include the erbB3 receptor, and proteins involved in the PI3K pathway. Glycation of these proteins would result in inhibiting Schwann cells from receiving or processing survival signals. Future studies should include studies looking more closely at the effects of both D-glucose and L-glucose, to determine whether AGE formation is occurring in this model, and in intact animal models of diabetic neuropathy.

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VITA

Jacqueline Meyer was born in Austin, Texas, on October 6, 1980, the daughter of Stephen R. Meyer and Pamela A. Meyer. After completing her work at McNeil High School, in Austin, Texas, in 1997, she entered Southwest Texas State University in San Marcos. Jacqueline completed her Bachelor of Science in 2002, and immediately began her graduate work at Texas State University-San Marcos. As a graduate student, Jacqueline taught Functional Biology, Developmental Biology, Anatomy and Physiology, Microbiology, and Cell Physiology. She also presented her research at the Society for Experimental Biology in Washington D.C., in April of 2004.

Permanent Address: 1070 River Rock Rd.

New Braunfels, TX 78130

This thesis was typed by Jacqueline D. Meyer