Probing Cytoskeletal Changes in Neuronal Stem Cells due to Glucose Dysregulation

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2019

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Submitted in partial fulfilment of the requirements

of the degree of

Master of Technology

by

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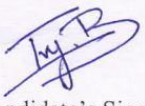
Declaration

I declare that this thesis is a presentation of my original research work. Wherever contributions of others are involved, every effort is made to indicate this clearly, with due reference to the literature, and acknowledgement of collaborative research and discussions.

I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any data/idea/fact/source in my submission. I understand that any violation of the above will be cause for disciplinary action by the institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken despite such requirement.



Candidate's Signature

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DISSERTATION APPROVAL

DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING

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This is to certify that the project report entitled "Probing Cytoskeletal Changes in Neuronal Stem Cells due to Glucose Dysregulation" by Ms Ivy Bhattacharya (Registration No.: 173300019) is based on the work done under my guidance and can be accepted.

Examiners

24/6/19

Supervisor

Layout of the Report

Diabetes mellitus is a disorder in which blood glucose level falls consistently above the normal acceptable range (in humans, this entails blood glucose levels ≥ 11.1 mM). As would be expected, lack of insulin, glucose dysregulation, and subsequent metabolic decompensation has several health implications on almost all the systems of the body.

Neuropathy has been noted to be the most common consequence of longstanding hyper-glycemia. In this project, we focus on the effects that glucose dysregulation has on undifferentiated neuroblastoma cells in vitro. Specifically, we focus on the cytoskeletal changes they undergo due to high and fluctuating glucose concentrations in their immediate surroundings.

We begin with a brief introduction to glucose dysregulation as a consequence of diabetes, and the external manifestations of the same. Next, we define diabetic neuropathy, at the cellular as well as at the systems levels. We discuss existing literature detailing clinical and experimental reports that establish links between glucose fluctuation and neuronal damage on the cellular level, and between impaired glucose tolerance and sensory neuropathy on the systems level. Post that, we limit the scope of this project to studying the cellular manifestations of DN alone. Within this too, we focus solely on the mechanical changes occurring within the cell.

With the scope of the report defined, we move on to the procedure for cell culture and the experimental setups used to study any identifiable changes in (i) morphology, (ii) contractility, (iii) stiffness, and (iv) molecular distribution of active myosin assemblies within the cells, and to relate them to the overarching theme of probing how excessive glucose in the immediate environment affects cellular cytoskeleton in neuronal cells.

Keywords: Diabetic neuropathy, glucose dysregulation, cytoskeleton, cell mechanics, contractility, cell stiffness, myosin

Abbreviations

1. DM: Diabetes Mellitus
2. DN: Diabetic Neuropathy
3. DFU: Diabetic Foot Ulcer
4. IDDM: Insulin Dependent Diabetes Mellitus
5. STZ: Streptozotocin
6. GV: Glycaemic Variation
7. MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
8. ATP: Adenosine Triphosphate
9. SCV: Sensory Conduction Velocity
10. MCV: Motor Conduction Velocity
11. NOS: Nitric Oxide Synthase
12. NO: Nitric Oxide
13. DMEM: Dulbecco's Modified Eagle's Medium
14. FBS: Fetal Bovine Serum
15. PBS: Phosphate Buffered Saline
16. EDTA: Ethylenediaminetetraacetic acid
17. AFM: Atomic Force Microscopy
18. pMLC: phospho-myosin Light Chain

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1. Introduction

Let us first skim through the basics of diabetes mellitus, insulin dysfunction resulting in impaired glucose control and hyper-glycemia, diabetic neuropathy, and the accompanying metabolic disruptions, so as to set the stage for the project and hence, for the rest of the report.

1.1 Neuropathy in Diabetes Mellitus

Diabetes mellitus is a serious, lifelong group of metabolic disorders resulting from defects in insulin secretion, insulin action, or both. Several pathogenic pathways are involved in the development of diabetes: from autoimmune destruction of the pancreatic β cells, resulting in hampered insulin production, to other abnormalities that result in insulin resistance even if production is normal. Due to this deficiency, and/or marked lack of appropriate insulin action, abnormalities in carbohydrate, protein and fat metabolism occur. Diabetes causes many associated metabolic disorders, which accompany changes in insulin action. These may have a direct or indirect impact on the development of complications in patients. However, the most direct effect of insulin dysfunction is on glucose homeostasis in the body.

Peripheral neuropathy, broadly defined as the disease or dysfunction of one or more nerves, is the most common, intractable complication of DM. It involves both somatic sensory and motor nerves, as well as autonomic nerves. The prevalence of DN ranges from 7% within a year post diagnosis to 50% within 25 years post diagnosis. If patients with subclinical levels of neuropathic disturbances are considered, the prevalence might even exceed 90%. Cardiovascular autonomic neuropathy strongly correlates to increased mortality in patients, while the loss of feeling (sensation) in the lower limbs is a high risk for limb amputation due to the development of ulcers, and necessitates extreme cost. [1] Different types of neuropathic syndromes have been observed in people with diabetes; some occur independently, while others occur in combination. Structurally, the pathology of the nerves is essentially the same in both type 1 and type 2 diabetes. However, there seem to be some clinical distinctions in the prevalence of certain kinds of neuropathies. The reason for these distinctions remains unknown.

1.2. Diabetic Neuropathy: Manifestations at the Systems Level

In some cases, patients with poorly controlled diabetes may experience a certain level of discomfort or pain in the lower legs which resolves only after the establishment of euglycemia. In other cases, patients do not develop any major symptoms at all. This kind of neuropathic development, though at the surface, might sound less destructive, is actually, equally, if not more, damaging. Nerve damage at the extremities leads to loss of sensation in those areas. While the palms are less prone to extensive damage, the feet are more likely so, since they bear most of the body-weight, and are also mostly, out of sight. Hence, injuries on the feet that are not picked up

by the nervous system tend to grow worse over time because of unnoticed, repeated insults at the affected area. The body's pain response to damage is an essential part of maintenance and when that is lost, diabetic patients stand the chance of developing severe complications such as diabetic foot ulcers (DFUs), of which peripheral neuropathy is the most significant causal factor. DFUs are the leading cause of non-traumatic lower extremity amputation.

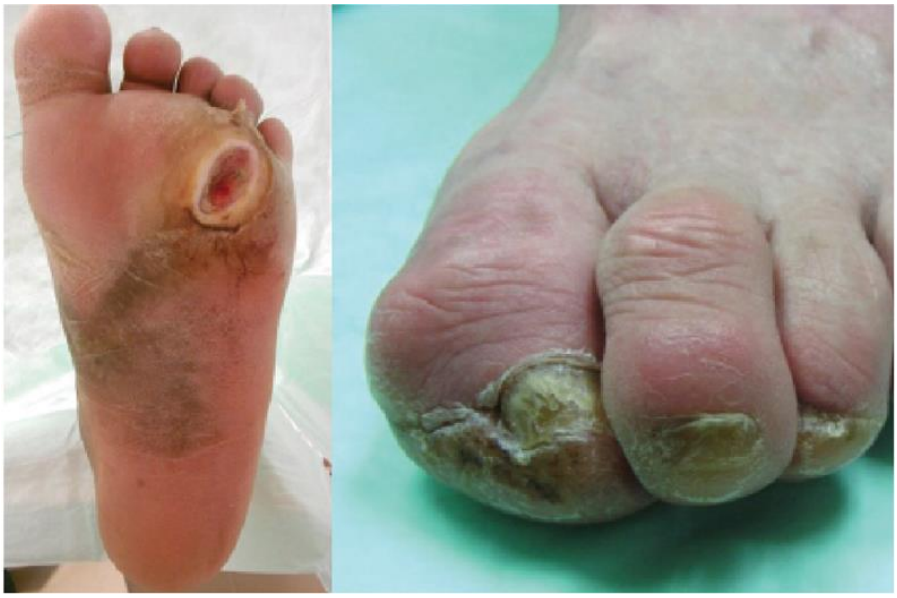


Figure 1: Physiological representations of diabetic foot ulcers

Since small myelinated and unmyelinated nerves get damaged first during the progression of neuropathy, it causes autonomic failure along with thermal and pain detection failure. Autonomic neuropathy, though debilitating if they occur together in the long term, does not have any clear clinical manifestations at its onset. Some markers include gustatory sweating, followed by orthostatic hypotension and diarrhoea. Impotence is relatively common in diabetic men. This is a feature of autonomic DN, though psychogenic and vascular factors do contribute in most cases. Some neuroendocrine responses to hyper-glycemia are blunted in patients with autonomic neuropathy. Most of the effects of autonomic neuropathy are caused by the sympathetic denervation of vascular smooth muscles in specific tissues.

Recent research has clearly established that apart from causing peripheral neuropathy, diabetes also affects the central nervous system in many adverse ways. But for now, this remains outside the scope of this report.

In one of the first studies conducted to find out the relationship between glucose fluctuations and the development of long-term complications in diabetics, it was observed that 'intensive therapy' effectively delays the onset and slows the progression of diabetic retinopathy, nephropathy, and neuropathy in patients with Insulin Dependent Diabetes Mellitus (IDDM). In this study, conventional therapy consisted of one or two daily injections of insulin, including mixed

intermediate and rapid-acting insulins, daily self-monitoring of urine or blood glucose, and education about diet and exercise. Conventional therapy did not usually include daily adjustments in the insulin dosage. The goals of conventional therapy included the absence of symptoms attributable to glycosuria or hyper-glycemia; the absence of ketonuria; the maintenance of normal growth, development, and ideal body weight; and freedom from severe or frequent hypo-glycemia. On the other hand, intensive therapy included the administration of insulin three or more times daily by injection or an external pump. The dosage was adjusted according to the results of self-monitoring of blood glucose performed at least four times per day, dietary intake, and anticipated exercise. The goals of intensive therapy included pre-meal blood glucose concentrations between 3.9 and 6.7 mM, post-meal concentrations of less than 10 mM, a weekly 3-a.m. measurement greater than 3.6 mM, and haemoglobin A_{1c} (glycosylated haemoglobin), measured monthly, within the normal range (less than 6.05 percent). The patients in the intensive-therapy group visited their study centre each month and were contacted even more frequently by telephone to review and adjust their regimens.

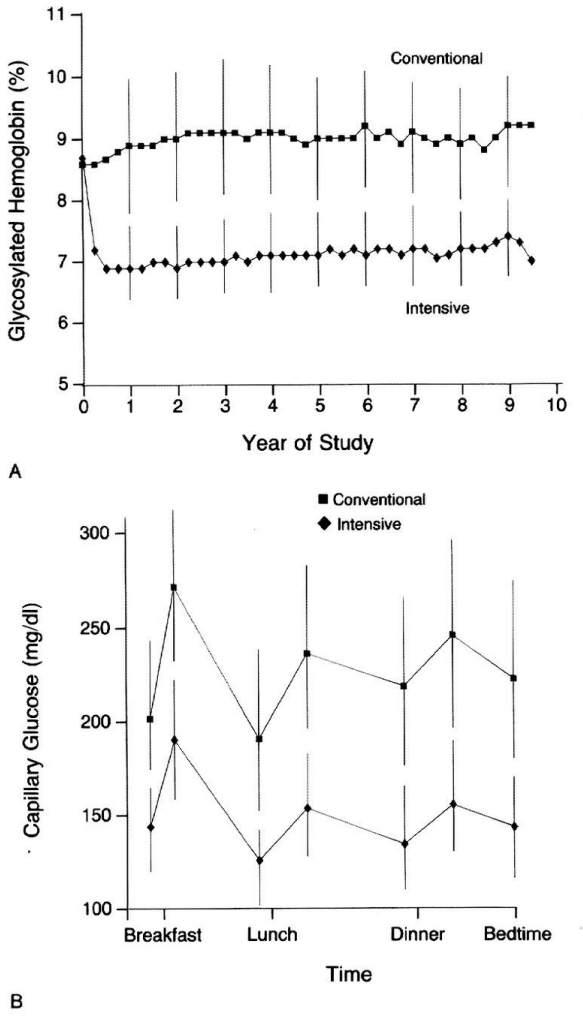


Figure 2. A.: Measurements of (A) Glycosylated Hemoglobin and (B) Blood Glucose in Patients with IDDM Receiving Intensive or Conventional Therapy [Diabetes Control and Complications Trial Research Group et. al, The New England Journal of Medicine, 1993]

Not only are the patients receiving conventional treatment less likely to have a very high concentration of blood glucose, but also lower degrees of glucose concentration fluctuations.

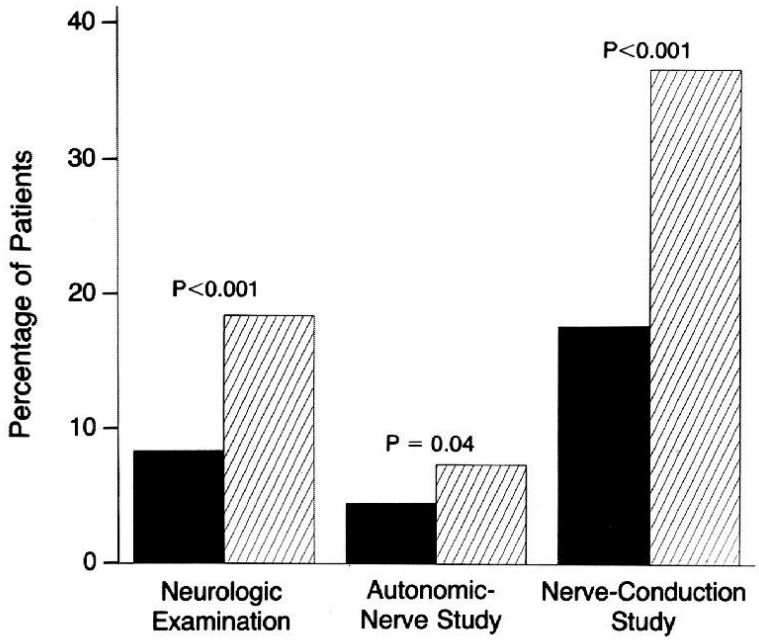


Figure 2.B.: Prevalence of Abnormal Clinical Neurologic Examinations, Abnormal Results of Nerve-Conduction Studies, and Abnormal Autonomic-Nerve Studies at Five Years in Patients Receiving Intensive (Solid Bars) or Conventional (Hatched Bars) Therapy. The analysis included all patients from either cohort who did not have the abnormality in question at base line. [Diabetes Control and Complications Trial Research Group et .al, The New England Journal of Medicine, 1993]

In all the three test conditions, it is clear that the patients that received intensive treatment performed better than patients who received conventional treatment. A neurologic examination is the assessment of sensory neuron and motor responses, especially reflexes, to determine whether the nervous system is impaired. A typical example of such an examination would be the monosynaptic knee-jerk reflex. Autonomic nerve studies evaluate sympathetic and parasympathetic nervous system effects. Examples include recording ECG, EGG, skin temperature and electrodermal activity for evidence of sympathetic/parasympathetic nervous system effects. Nerve conduction velocity (the ability of the nerve to conduct an impulse from one point to another within a reasonable time frame) shows the most rapid decline in hyper-glycaemic patients.

Although the diabetic nerve is known to be hypoxic [3], experiments in rats have shown that hyper-glycaemic, but not normoglycemic hypoxic conditions, give rise to alterations in fast K+ conductance and after-potentials probably due to axonal acidification. [4] This might contribute to the occurrence of pain by the generation of random, ectopic impulses.

Nerve conduction velocity shows a rapid decline in hyper-glycaemic patients. With the correct measures to control for hyper-glycaemia, this returns to normal. One major point of interest here is that the diabetic peripheral nerves show an abnormally strong resistance to ischemic conduction failure. [5] This might be explained by the hypothesis that they switch to anaerobic glycolysis. [6]

Another, relatively recent study shows a clear loss of vertical and total axon density at wound sites in diabetic mice. In human diabetics, it is known that DFU wounds are accompanied with impaired healing, which makes recovery even more difficult, increasing the possibility of an amputation. Here, the authors show that at 7d following a 3mm punch wound, a critical period of healing and reinnervation, both intact skin nearby the wound and skin directly at the wound 6 margins had over 30-50% fewer axons and a larger deficit of ingrowing axons in diabetics, compared with a pre-existing axon deficit of just 10-15% initially. The initial neuropathy is low in this case because the diabetic mice had been injected with STZ just two months prior to the experiments, leaving very little time for the development of secondary diabetic complications. However, the overall findings of this study also suggest striking and unexpected superimposed cutaneous axon loss, beyond that expected of diabetic neuropathy alone, associated with experimental diabetic skin wounding.

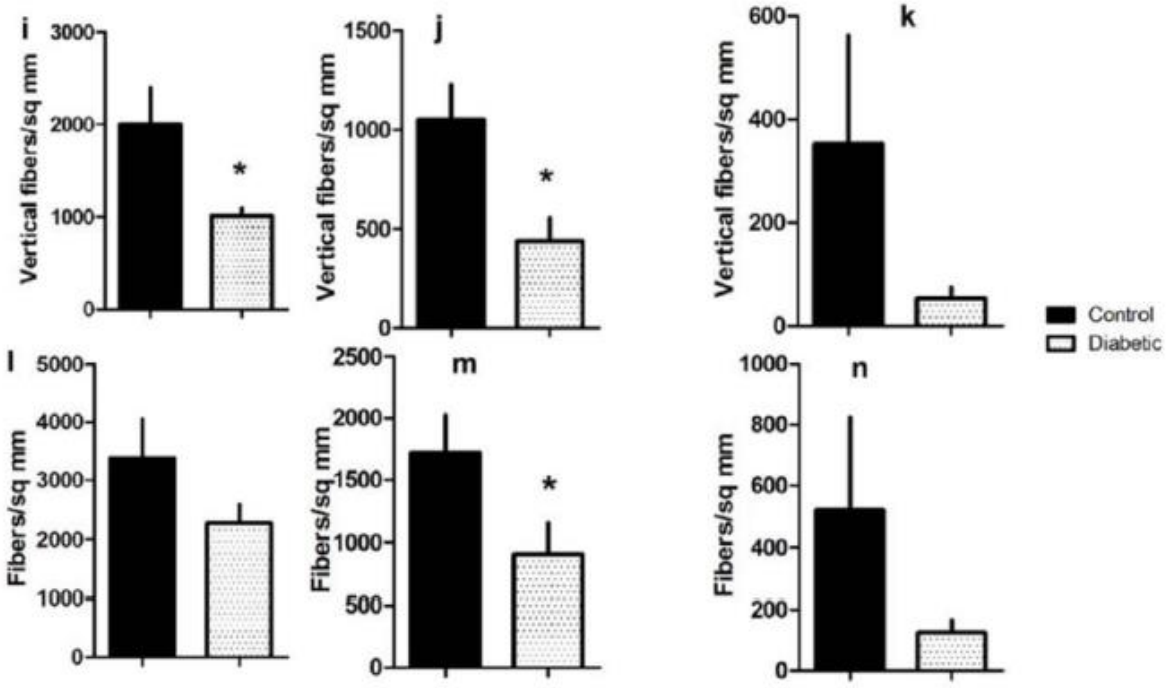


Figure 3: Innervation of three zones associated with dorsal skin wounds shown to be from (i,l) nearby normal skin; (j,m) adjacent wound margin; (k,n) wound zone. Graphs show vertically directed axon profile density (i-k) and total axon profile density (l-n) in diabetics (open bars) and nondiabetics (black bars). [Cheng et. al, PLoS ONE, 2013]

Diabetes is hence associated with a substantial decline in vertically oriented axon profile innervation within nearby normal adjacent skin and wound margins and a decline in total axon profile density in the wound itself. The deficit in axon innervation had a more rapid decline from adjacent skin to core wound tissue in diabetic mice. [7]

1.3. Diabetic Neuropathy: Manifestation at the Cellular Level

1.3.1 Metabolic Changes

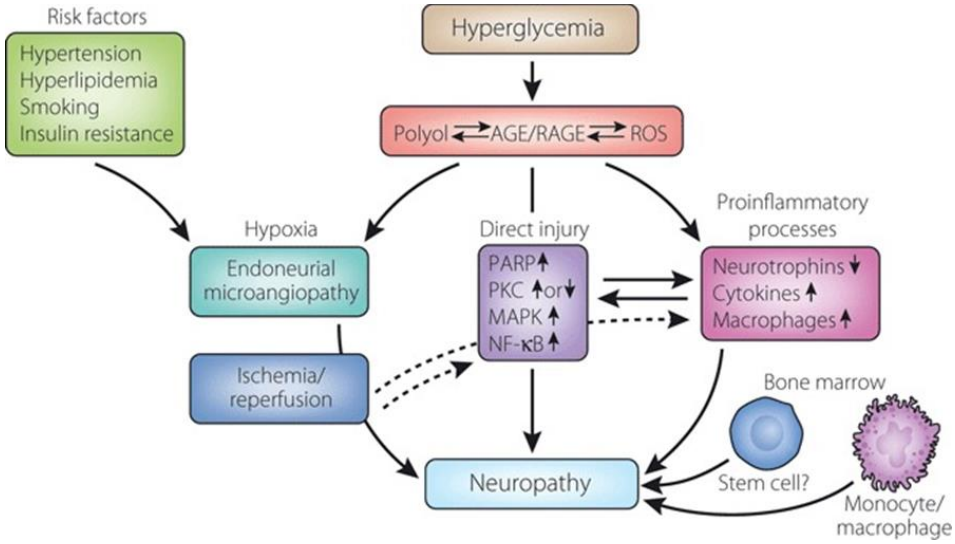


Figure 4: Known metabolic pathogenetic pathways of neuron damage in DN [Yagihashi et. al, Journal of Diabetes Investigation, 2010]

Mechanisms of hyper-glycemia-induced cellular damage were initially reported in the cells of the vascular endothelium. Later, these mechanisms were also observed in peripheral sensory neurons. Glucose uptake is not as rapidly regulated in neurons as it is in endothelial cells. This might explain why neurons are more susceptible to glucose-mediated injury. [8]

In a first-of-its-kind in vitro study on the differentiated SH-SY5Y human neuroblastoma cell line, researchers investigated the effects of constant high and fluctuating glucose concentrations on neuronal cells. They aimed to study the effects of glycaemic variation (GV) upon mitochondrial activity using an in vitro human neuronal model. The metabolic disturbance of GV in neuronal cells was mimicked via exposure of neuroblastoma cells SH-SY5Y to constant glucose or fluctuating (i.e. 6 h cycles) for 24 and 48 h. Mitochondrial dehydrogenase activity was determined via MTT assay. The following figure represents the data.

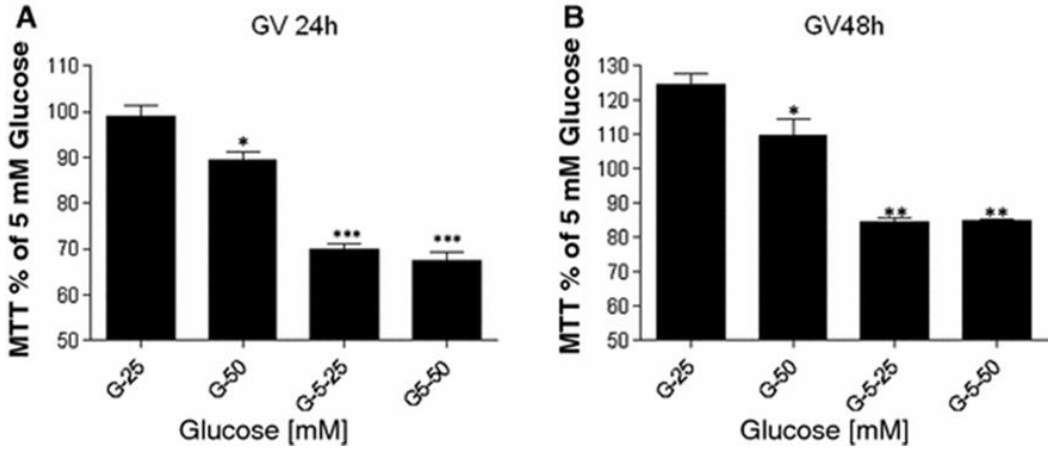


Figure 5: Glucose fluctuation causes mitochondrial dysfunction in differentiated SH-SY5Y neuronal cells [Russo VC et. al, Neurochemical Research, 2012]

Following exposure for 24 and 48 h to constant glucose at 5 or 25 or 50 mM, MTT values are significantly decreased in high 50 mM glucose (*p < 0.05) when compared to the MTT values of 5 mM (normal) glucose. Also, MTT activity at 24 and 48 h is significantly reduced following cyclic glucose fluctuations (G5-25 mM and G5-50 mM). [9]

Let us now look at indirect effects of hyper-glycemia. Intracellular glucose is removed primarily through glycolysis. This process, as we know, generates pyruvate for mitochondrial catabolism to form ATP. Now, excess pyruvate is known to injure neurons through the following known pathways.

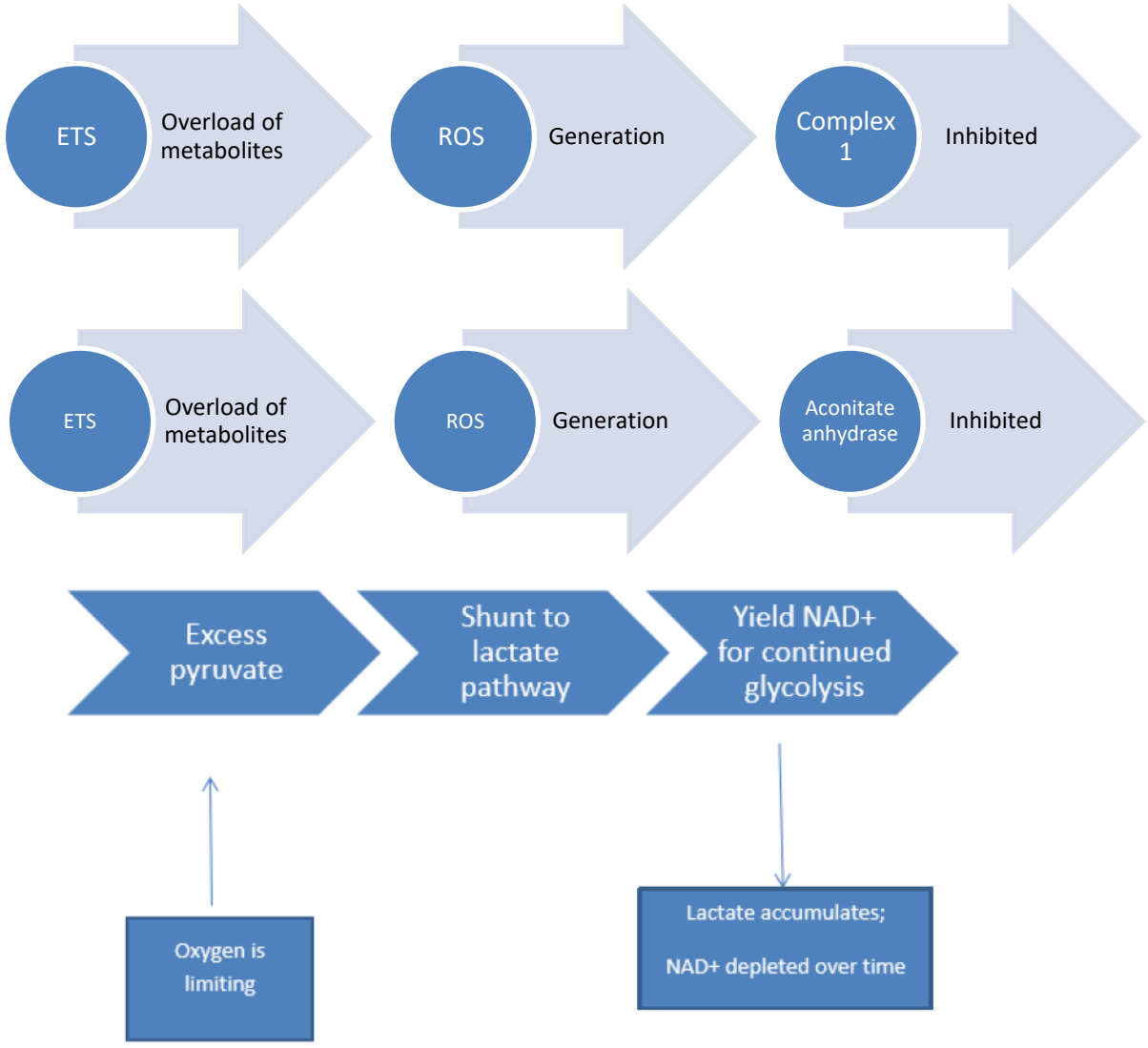


Figure 6: Known metabolic pathways of neuron damage due to excess pyruvate in DN

1.3.2. Mechanical Changes

In a 2004 paper, it was found that DN, characterized in the study by slowing of conduction velocity and axonal atrophy, was accelerated in axons without neurofilaments. The group tested directly the relationship of neurofilaments to diabetic neuropathy by superimposing streptozotocin-generated diabetes on a unique but viable transgenic mouse (described by Eyer and Peterson). These mice expressed a fusion protein in which the carboxyl terminus of the high

molecular weight neurofilament protein (Nf-H) was replaced by beta-galactosidase, in turn blocking normal neurofilament export and rendering axons completely lacking neurofilaments. Despite similar levels of hyperglycaemia, diabetic mice lacking neurofilaments developed progressive slowing of conduction velocity in their motor and sensory fibres between 4 and 8 weeks after the onset of diabetes ($P < 0.05$), unlike diabetic mice with normal neurofilaments, who developed only mild evidence of neuropathy over the same time-frame.

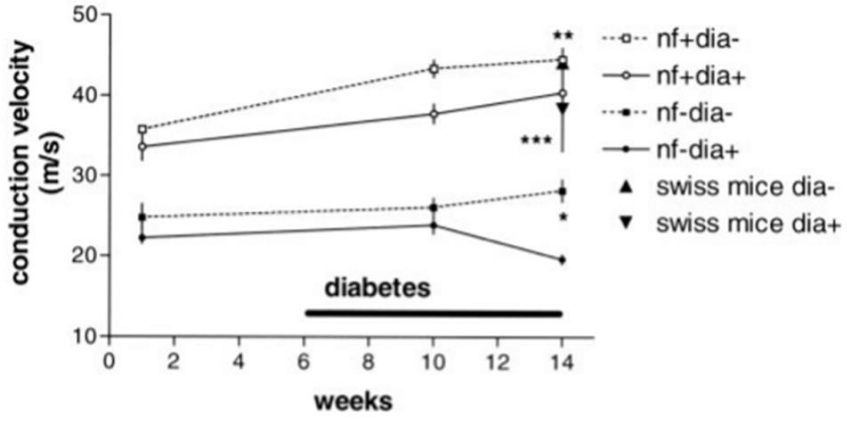


Figure 7: Sensory conduction velocity (SCV) from caudal fibres of mice (Zochodne et. al, Brain, 2004)

At 14 weeks (8 weeks of diabetes) Nf- Dia+ mice had a slower CV than Nf- Dia-. Mice lacking neurofilaments had lower CV than mice with normal neurofilaments. Diabetics with normal neurofilaments and unrelated diabetic Swiss mice had mild CV slowing in both cases.

Diabetic mice without neurofilaments, but not those with neurofilaments, had a progressive decline in the amplitude of the caudal nerve compound action potential too, hence establishing that cytoskeletal and cellular architecture is important during the development of DN. [10]

For a while, although it was known that the neuronal morphology is affected during DN, and that axon dysfunction occurs, the mechanism of this dysfunction remained unknown. A morphometric analysis of sural myelinated axons in diabetic rats and controls showed no difference in the number of sural axons between diabetic and controls (controls, n = 5; diabetic rats, n = 7). But, on analysis of the sizes of these axons, the histogram showed a shift in the calibre of diabetic myelinated axons to smaller size categories. The mean axonal area was also smaller in diabetic rats compared with controls.

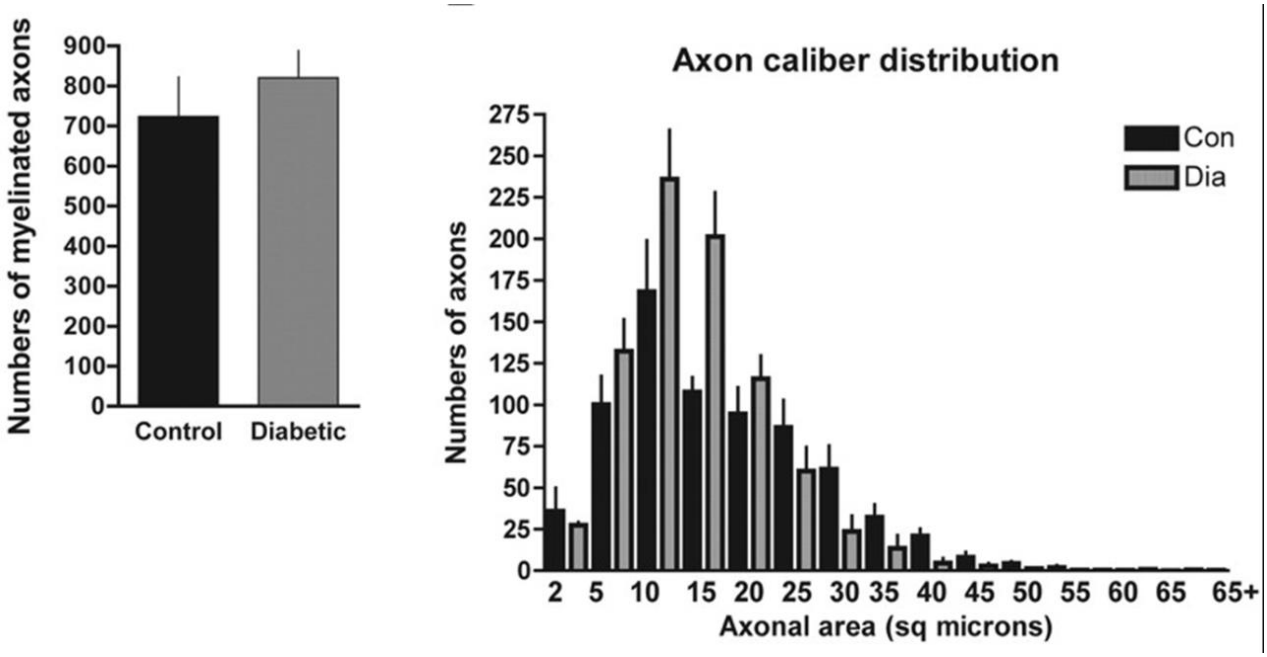


Figure 8: While the difference in axon number remains insignificant in both diabetics and controls, diabetic axons are significantly smaller in size. (Brussee et. al, Diabetes, 2008)

This implies that the primary cause of axon dysfunction in DN is axonal retraction, since the other known ways of axon damage also cause a reduction in axon number. [11]

2. Basic Framework of the Project

A number of clinical as well as in vitro studies in human endothelial cells and human olfactory/neuroepithelial cells have indicated that intermittent high glucose has greater cellular damaging effects than constant high glucose. However, we also know that high glucose alone too has damaging effects on neuronal cells. The cellular and molecular mechanisms involved have also previously been investigated in human neuronal cells. Since glucose is the primary fuel for neurons, and components of the cell cytoskeleton play a primary role in allowing for glucose entry and exit from within the cell, and on cellular biomechanics as well, we choose to focus on exploring the changes in morphology, mechanics and intracellular myosin profiles in response to ambient high glucose conditions and to glucose fluctuations.

2.1. Hypothesis

We hypothesize that changes in extracellular glucose concentration, as in the case of diabetes, cause certain changes in the cytoskeletal structure and intracellular activated myosin profile, leading to higher contractility of neuronal cells, thus causing the axons to retract.

2.2 Objectives

To investigate the hypothesis, we propose the following objectives:

1. To observe the changes in cell morphology when exposed to high or fluctuating glucose concentrations
   Rationale: Major changes in the cytoskeleton will affect the morphology of the cell in the long term.

2. To check for differences in contractility between cells exposed to high or fluctuating glucose concentrations.
   Rationale: The hypothesis proposes increase in cell contractility as a reason for a greater degree of axonal retraction in cells exposed to hyper-glycaemic conditions. Hence, we first explore if this holds using the trypsin deadhesion experiment. [12]
   Curved neurites undergo contraction, straighten up, and retract towards the cell body on deadhesion. Axons show a monotonous decrease in length upon contraction. This decrease in neurite length can reflect its contractile mechanics. [13]

3. To look for changes in cellular stiffness when cells are exposed to high glucose concentrations.

Rationale: There exists an inverse relationship between cell stiffness and de-adhesion response. [12] Earlier studies have indicated that increase in actomyosin contractility (as measured by the trypsin de-adhesion assay in this project) also leads to an increase in cell stiffness. [14]

4. To identify differences in intracellular activated myosin profiles by pMLC staining across constant low or high glucose concentrations.
   Rationale: Neurite straightening and retraction is actively driven primarily by actomyosin contractility. If cellular contractility does indeed increase due to glucose dysregulation, it predicts the existence of a molecular basis for this.

3. Materials and Methods

3.1 Cell culture

The undifferentiated neuronal-type SH-SY5Y human neuroblastoma cell line, derived from metastatic neuroblastoma site in a four-year-old female was used in these experiments. For one experiment, we also used the NIH 3T3 mouse fibroblast cell line, but the culture conditions were consistent across cell lines. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM with L-glutamine and sodium pyruvate, without glucose and sodium bicarbonate (AT186, HiMedia) supplemented with10% manually heat-inactivated foetal bovine serum (HiMedia), 1% penicillin/streptomycin (HiMedia) and the required glucose concentration. Cells were maintained in T-25 (surface area 25 cm^2) cell culture flasks, harvested with 0.5% trypsin-EDTA, and passaged every 2–3 days. For experiments, cells were plated on glass coverslips coated with 5µg/cm^2 rat-tail collagen I (Sigma Aldrich).

3.1.1 Constant glucose conditions

Cells were cultured in DMEM supplemented with 2.5 mM (low), 5 mM (normal), 25 mM (high), or 50 mM (very high) molecular grade D-glucose (HiMedia) with change to fresh media and the same D-glucose concentrations, every 24 h, and repeated for up to 72 h. All experiments with cells maintained at constant glucose concentrations were done at or post the third passage so as to acclimatize the cells to that specific glucose concentration.

3.1.2 Fluctuating glucose

3.1.2.1 Low/Normal to High/Very high

Cells were cultured in 2.5- or 5-mM D-glucose for 6 h (low and normal glucose levels; “low phase”) and then followed by a change of fresh media to elevate both low phase glucose concentrations up to 25 or 50 mM and cultured for another 6 h (high glucose “high phase”). This was then followed by a change to fresh media at 12 h, lowering the glucose levels back to low phase glucose concentration of 2.5 or 5 mM. To minimize cell stress due to media change, fresh treatment media was always incubated at 37 °C and equilibrated with 5 % CO_2 prior to addition to cell cultures. These 6 h cycles with change to fresh media every12 h were repeated for 24 h.

3.1.2.2 High/Very high to Normal/Low

Cells were cultured in 25- or 50-mM D-glucose for 6 h (high and very high glucose levels; “high phase”) and then switched to either 5 mM D-glucose or 2.5 mM D-glucose with

fresh media to lower high phase glucose concentrations to 5 or 2.5 mM and cultured for another 6 h. This was again followed by a change to fresh media at 12 h, elevating the glucose levels back to high glucose concentration of 25 mM or 50 mM. To minimize cell stress due to media change, fresh treatment media was incubated at 37 °C and equilibrated with 5 % CO2 prior to addition to cell cultures. These 6 h cycles with change to fresh media every12 h was repeated for 24 h.

Although diabetic patients might experience fluctuations of blood glucose levels in a range comparable to those selected for our in vitro model, glycaemic fluctuation might occur more frequently than every 6 hourly. However, prior studies have consistently observed that media change at intervals of 2–4 h significantly affected mitochondrial activity of even cells grown in optimal 5 mM glucose. This was not seen when media was changed at intervals of 6 or 12 h, as we have used in these studies. [9]

For this report, we study the morphology and contractility of the cells in both constant as well as fluctuating glucose conditions, but limit ourselves to constant glucose conditions only (2.5 mM, 5 mM, 25 mM, and 50 mM) for cell-stiffness studies and activated myosin profiling.

3.2 Differentiation into neuronal cells

Although differentiated cells have not been used directly in the study, we optimized the differentiation medium for future work on this cell-line. The neuronal-type SH-SY5Y human neuroblastoma cell line was seeded in 24-well plates and cultured for 24 h in 5 mM glucose (normal glucose), complete 10 % FBS, DMEM prior to switch to differentiation treatment with 1 μM, 5 μM , 10 μM , 20 μM, and 40 μM retinoic acid (RA, Sigma) for 48 h to optimize the concentration of retinoic acid required for adequate differentiation. Neuronal differentiation by RA of SH-SY5Y at 48 h (post treatment) was confirmed by change in cell morphology.

Cells show greatest neurite length in conditions of 10 μM RA added to the culture. Hence, for all future work on differentiated cells, we shall use 10 μM RA to induce differentiation.

3.3 Microscopy

Quantification of cell spreading, number of neurites, and neurite length was performed for at least 30 cells per condition across three independent experiments. Cells with at least one neurite were analysed. All images were recorded with an Olympus TX71 microscope at 20 X magnification.

3.4 Trypsin De-adhesion Assay

For neurite de-adhesion experiments, media was removed, and cells were washed once with PBS and then warm trypsin (5 g/L trypsin with 2 g/L EDTA (high activity)) was added. We used a dry-bath to ensure that trypsin and PBS were both maintained at 37ºC throughout the duration of the experiment. Cells were imaged every 10 s at 20X magnification until the neurites retracted from their initial positions, with no further apparent change in length, and after some delay, eventually detached from the substrate along with the cell body.

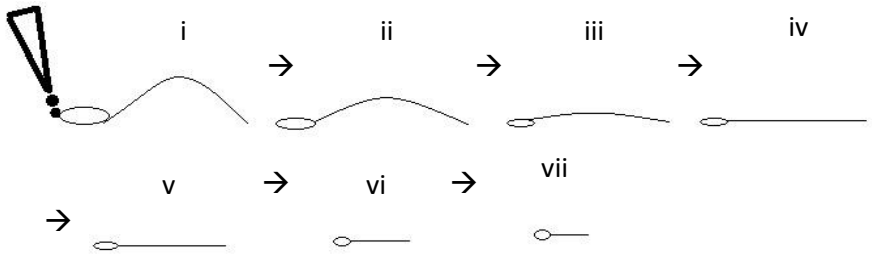


Figure 9: Schematic of neurite on addition of warm trypsin. The initial response is neurite straightening. After the neurite is completely straight, it begins to retract into the cell body

The dynamics of de-adhesion were tracked at maximum for the time span when cells were still attached to the substrate. To quantify de-adhesion, the neurite length was determined by tracing the length of the neurite at different time points using ImageJ (NIH). The time-dependent normalized length was quantified by dividing the current neurite length [L(t)] with the initial neurite length [L(i)]. Thus, the plot of normalized neurite length reduces from a value of 1 (at t = 0, L(t) = L(i)) to a value close to 0, depending on how much the neurite retracts. The normalized length-vs.-time data were then fit to an exponential curve to yield the time constants.

3.5 Atomic Force Microscopy

Cells were cultured in 60 mm tissue-culture petri-dishes for this experiment (wet samples). Culture dishes were mounted onto the stage of an Asylum MFP3D atomic force microscope (Asylum Research) coupled to a Zeiss epifluorescence microscope and indented using a pyramid-tipped probe (Olympus) with nominal spring constant of 30.22 pN/nm. The probe was first calibrated in air using thermal vibration method to determine the exact spring constant. The first 1μm of force-indentation curves for individual cells were fitted with the Hertzian model for a pyramidal tip to obtain estimates of cortical stiffness.

At least 100 cells across two experiments were analysed per condition.

3.6 Immunofluorescence Labelling

For immunostaining, cells were fixed after 24 hrs of culture using 4% PFA in 1x PBS (HiMedia) for 20–25 mins, washed 3 times with 1x PBS solution to remove traces of paraformaldehyde, and permeabilized with 0.1% Triton-X 100 (Sigma) in 1x PBS solution for 5 mins. Cells were blocked with 5% BSA (Sigma) for 1 hour at room temperature (RT) before being incubated with rabbit anti-phospho-Myosin Light Chain (pSer19) primary antibody solution in PBS at the dilution of 1:1000. The following day, cells were washed 3 times with 1x PBS and then incubated with Alexa-Fluor 555 anti-rabbit IgG (Invitrogen) secondary antibody at room temperature (RT) for 2 hrs. Finally, after washing, cells were mounted on glass-slides using mounting medium (Sigma). Cells were imaged at 60x magnification.

4. Results

4.1.High Glucose Induces Reduction in Length of Neurites

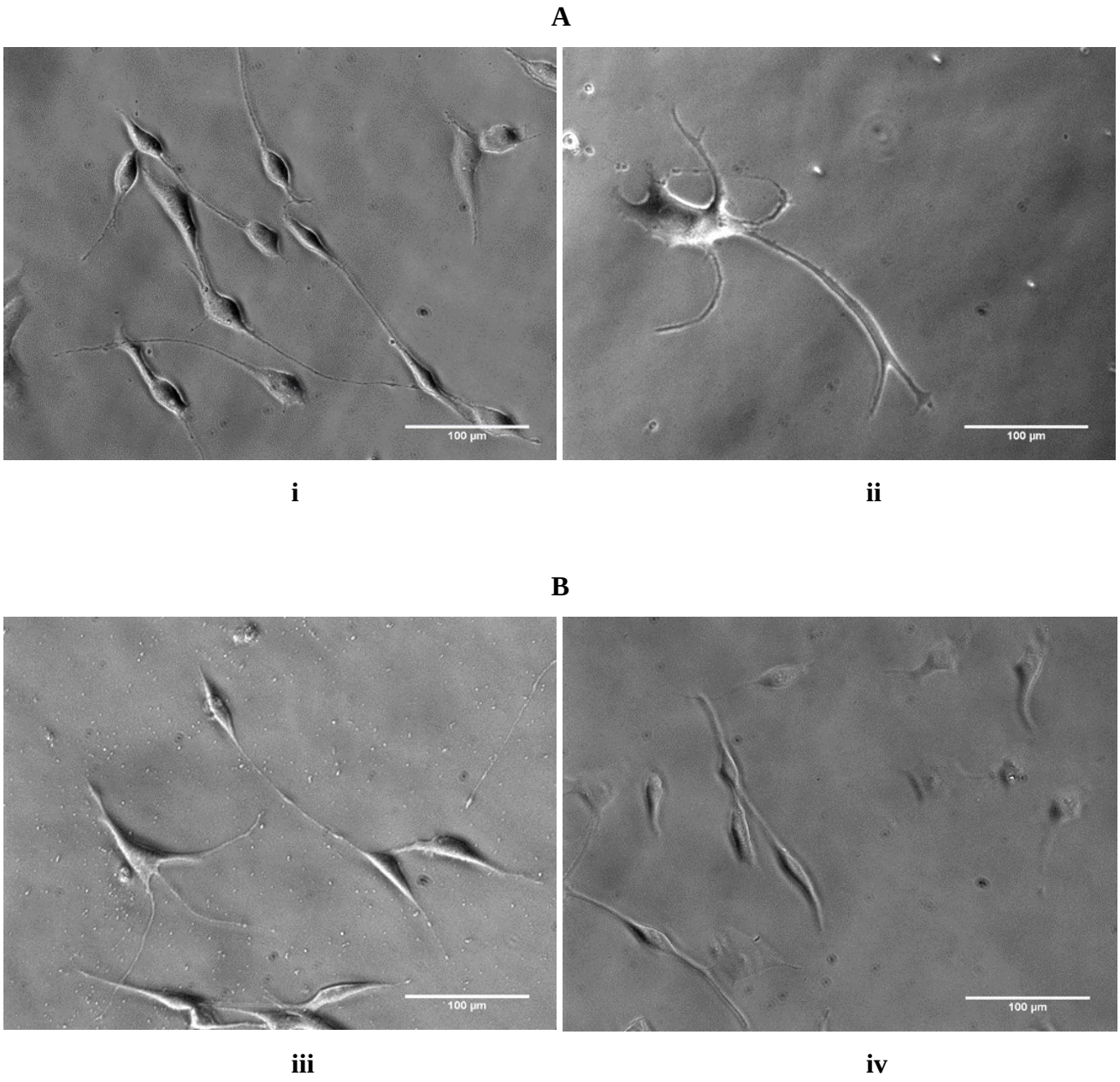
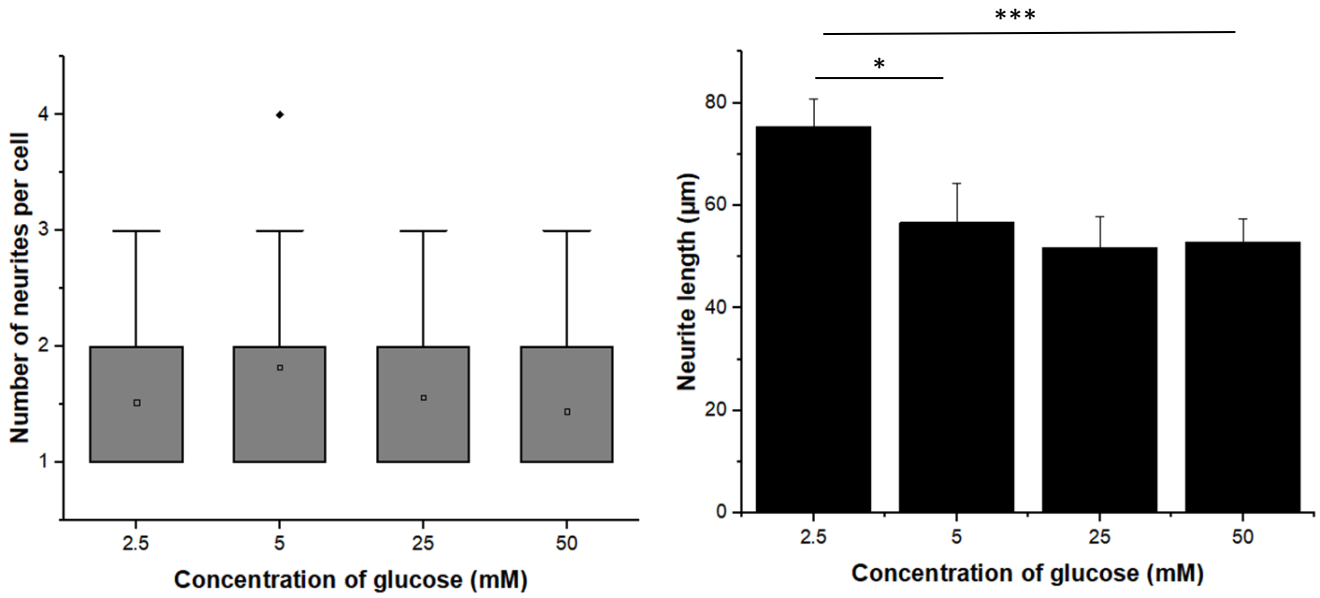


Figure 10: Representative images of SH-SY5Y cells maintained at

A. Low phase D-glucose concentrations of
    i. 2.5 mM
    ii. 5 mM
B. High phase D-glucose concentrations of
    i. 25 mM
    ii. 5o mM



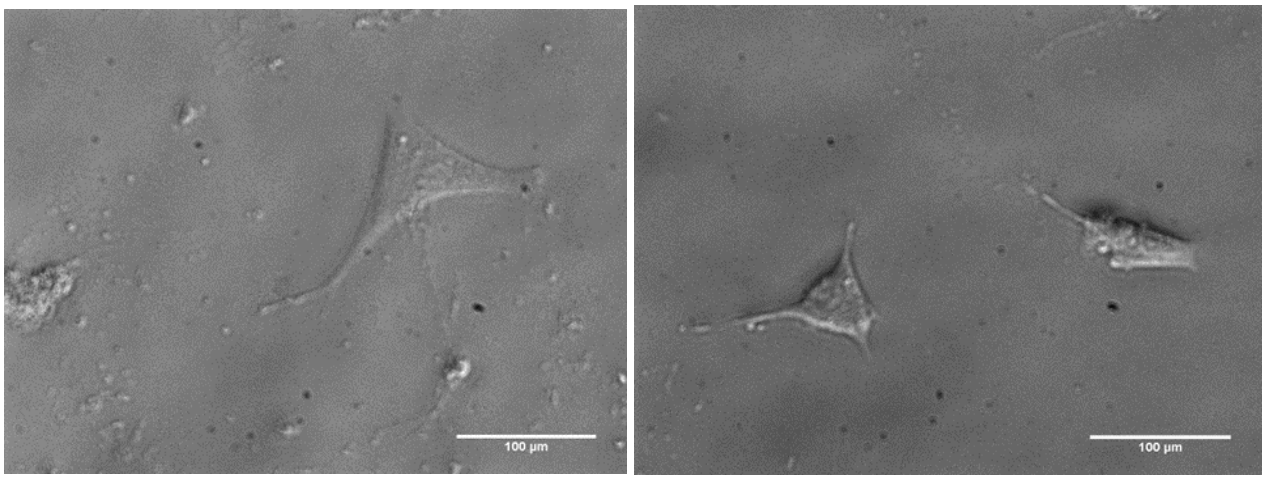
A B

Figure 11. Neurite analysis of 50 cells per condition across 3 independent experiments

A. The number of neurites per cell remains unchanged across the 4 conditions, though there is more variation in neurite numbers at lower concentrations of D-glucose in medium
B. The neurite length is significantly decreased in cells that have been exposed to high D-glucose concentrations. There is no significant difference between cells cultured in concentrations of 2.5 mM and 5 mM

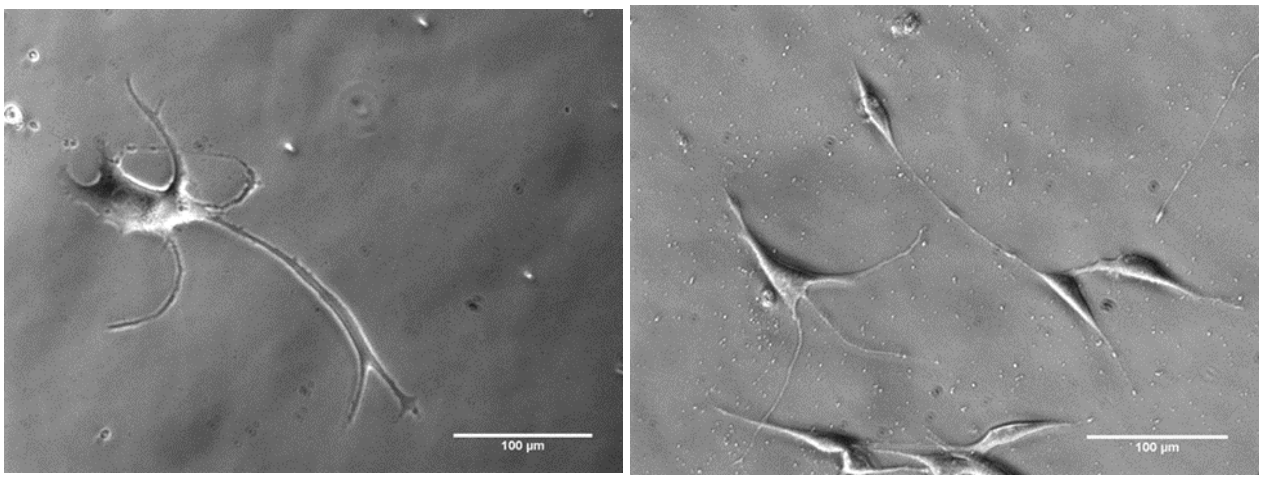
An interesting observation following the neurite morphology experiments was that the cell spread area significantly decreases when cells are exposed to high glucose conditions too. This seems to be the trend not just with the neuroblastoma cell-line, but also with the NIH 3T3 fibroblast cell line.

A



i **ii**

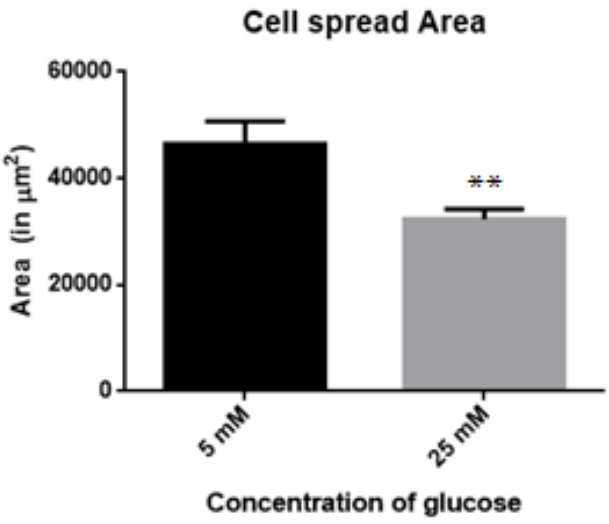
B

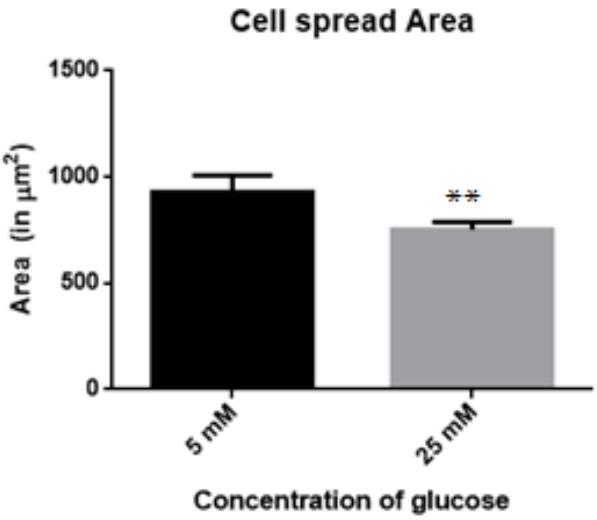


i **ii**

Figure 12: Representative images of A. NIH 3T3 and B. SH-SY5Y cells maintained at D-glucose concentrations of:

i. 5 mM
ii. 25 mM





A. NIH 3T3 B. SH-SY5Y

Figure 13. Decrease in cell spread area following exposure to high glucose. 50 cells were sampled per cell line, over 2 independent experiments. These results are consistent across the two cell-lines.

4.2.High Glucose Does Not Affect Contractility of Neurites

The observations of the trypsin de-adhesion experiments yielded some interesting insights. As mentioned in chapter 3, we focussed on the de-adhesion dynamics of the neurite alone and not the

whole cell. It was observed that first, the curved neurites showed a straightening response to trypsin. After the neurite was completely straightened, it began to retract into the cell body. While most neurites retracted completely into the cell body, others attained a minimum final length, and failed to retract further.

On repeating the same experiment with differentiated cells, we failed to observe any response to trypsin addition. Hence, these results have been excluded from the study, and will be probed into later.

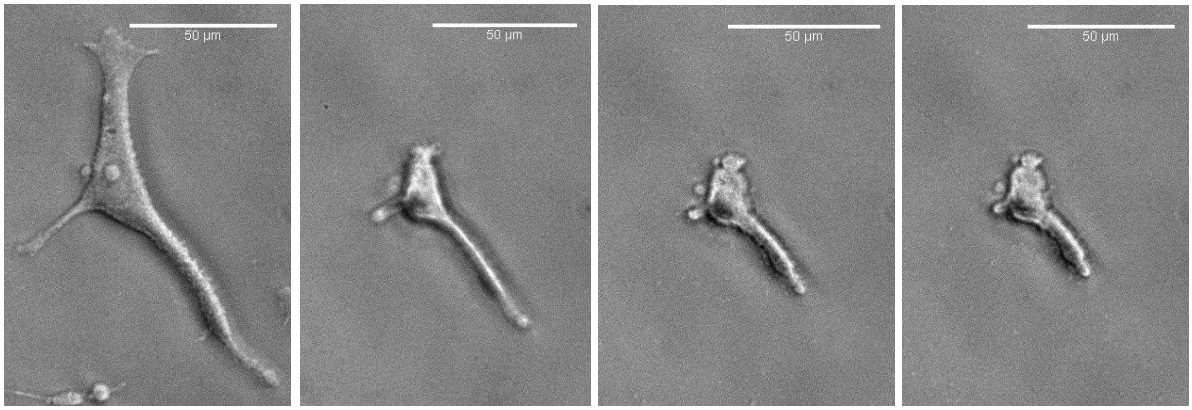


Figure 14: Trypsin-induced straightening and de-adhesion of neurites in 5 mM (normal concentration of) D-glucose

Of the 50 neurites sampled and monitored over 850 seconds, 60.0% (52.9% of cells in low-phase D-glucose, 66.67% of cells in high-phase D-glucose) showed an exponential reduction in length following the addition of warm trypsin. Videos have been included in the supplementary data files.

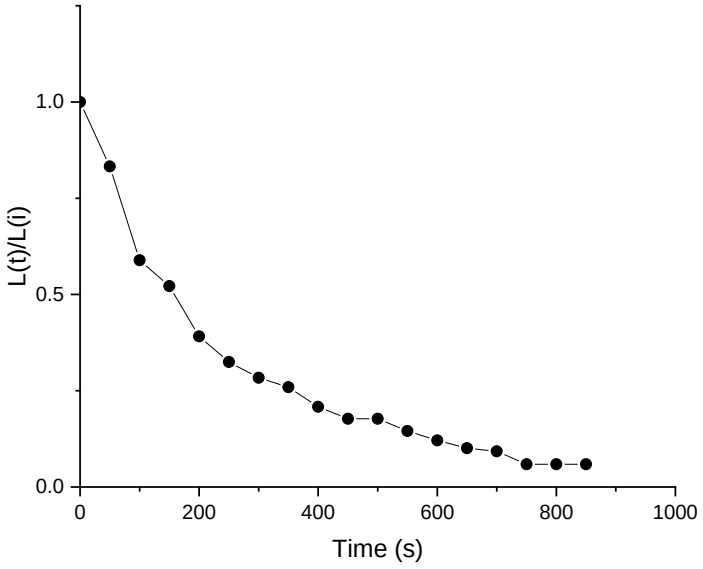


Figure 15: Exponential reduction in length of neurite following trypsin de-adhesion

The other neurites expressed a non-exponential decrease in length profile, most of which were delayed responses to trypsin addition leading to a sigmoidal curve.

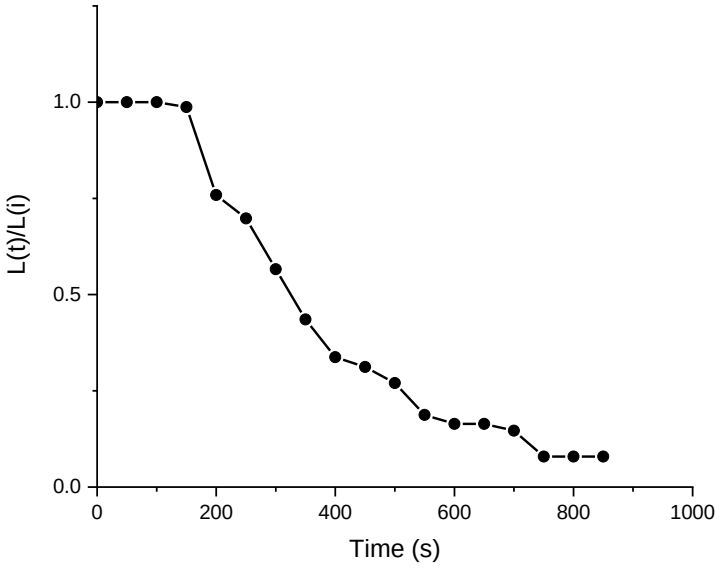


Figure 16: Non-exponential reduction in length of neurite following trypsin de-adhesion

[In Figures 12 & 13, L(t)/L(i) represents normalized neurite length.]

For the purpose of our analysis, we selected the neurites that showed an exponential decay in length, and fitted them into the following curve:

$$1\text{-}C(1\text{-}EXP(-t/\tau))$$

This gave us the values of C and τ for that specific neurite,

where C is indicative of the neurite's contractility
and the time constant τ is indicative of the adhesive forces between the neurite and the matrix

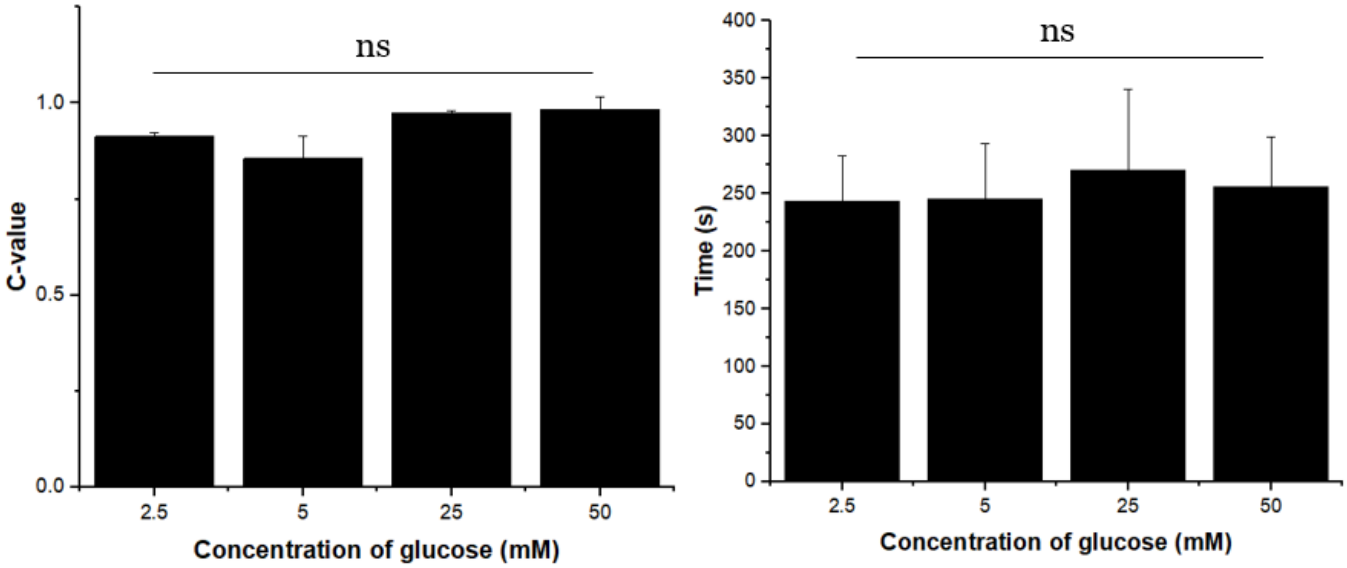


Figure 17: Parameters of contractility (C) and adhesive strength (Ţ) of 30 neurites sampled over 3 independent experiments

We observe no significant change in contractility, so far, from the number of neurites analysed. To state with certainty, whether or not there is a significant difference in the value of Ţ though, the sample size needs to be increased to greater than 30 per condition.

* Post defence, it has been suggested by the evaluators (Prof. Sen, Prof. Manchanda & Prof. Tayalia) that the point of consideration of the beginning of the neurite from the cell body needs to be demarcated more clearly, best identified with a working equation.

4.3.High Glucose Causes Cells to Grow Stiffer

In conjunction with the results of morphology analysis, which show that cells kept in high glucose concentrations have reduced spread area, the results of our Atomic Force Microscopy studies indicate that at higher concentrations of glucose, the SH-SY5Y cells become increasingly stiffer.

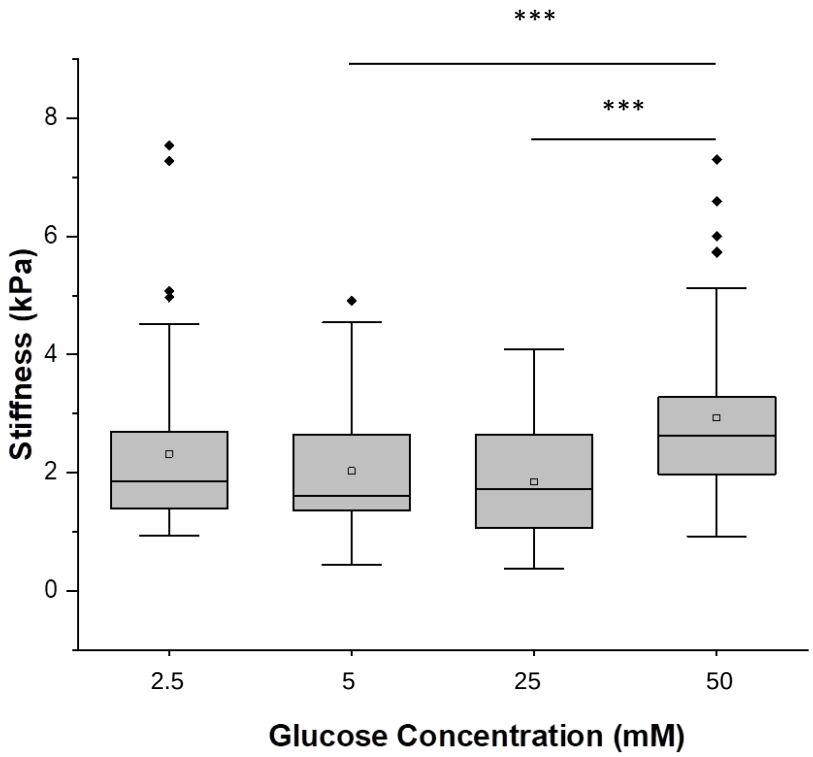
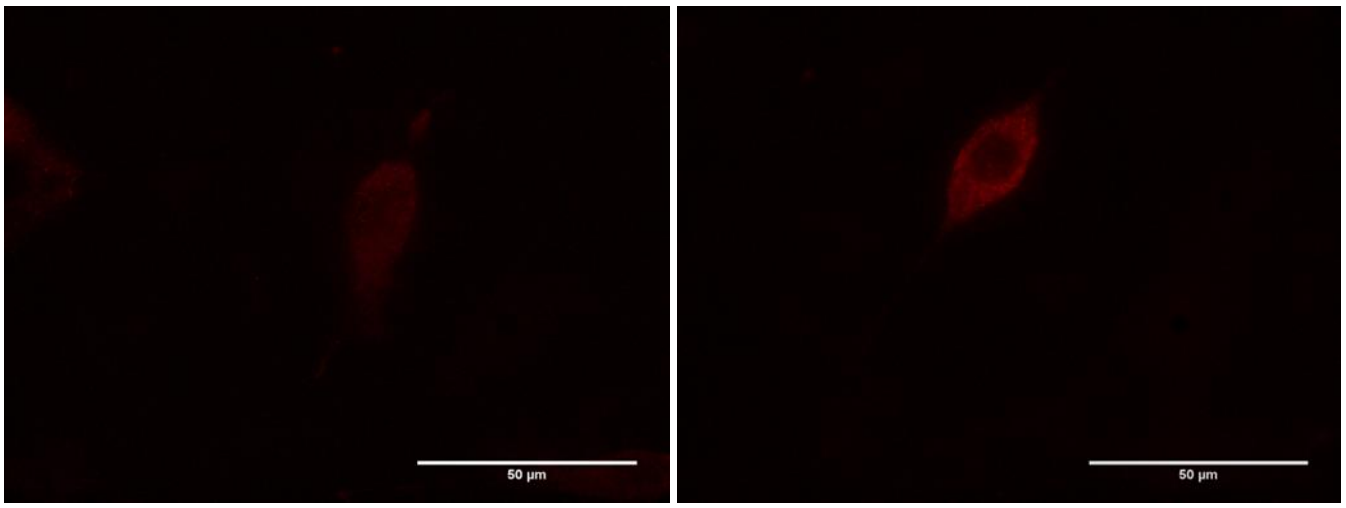


Figure 18: Change in cellular stiffness of undifferentiated SH-SY5Y cells with increase in glucose concentration over of 100 cells per condition, sampled over two independent experiments

4.4.High Glucose Increases Intracellular Levels of pMLC

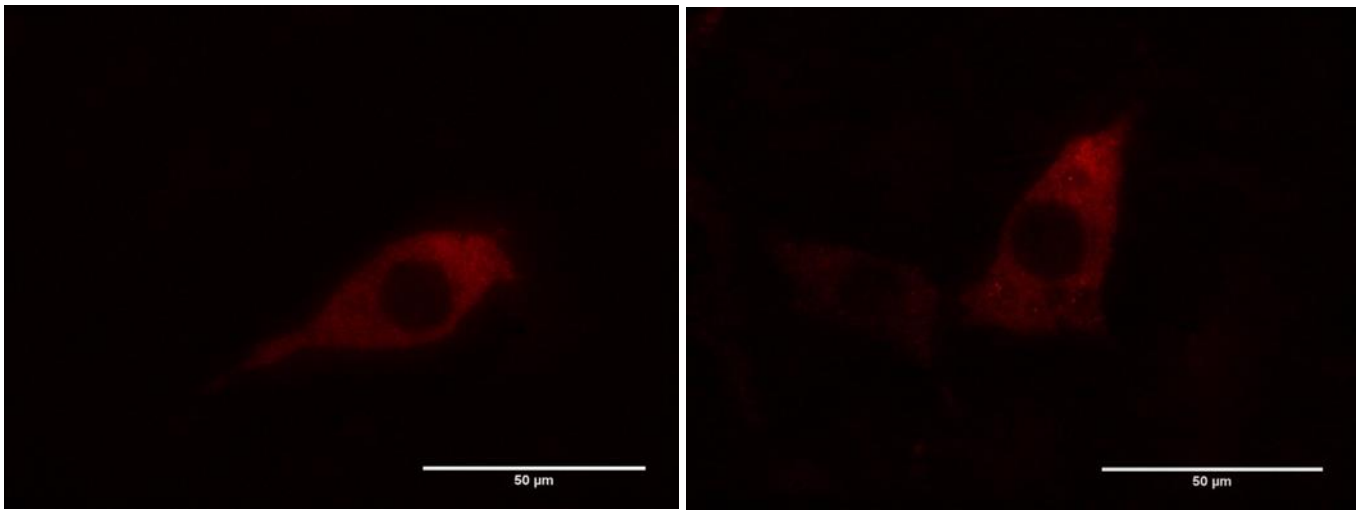
We used staining to compare the intracellular levels of pMLC across the four glucose concentrations. pMLC provides a measure of activated myosin within the cell, which in turn gives an estimate of the molecular basis for enhanced contractility as observed earlier. This experiment was performed only once.

A



i ii

B



i ii

Figure 19: Representative pMLC profiles of SH-SY5Y cells grown in

A. Low phase D-glucose concentrations of
   i. 2.5 mM
   ii. 5 mM
B. High phase D-glucose concentrations of
   i. 25 mM
   ii. 50 mM

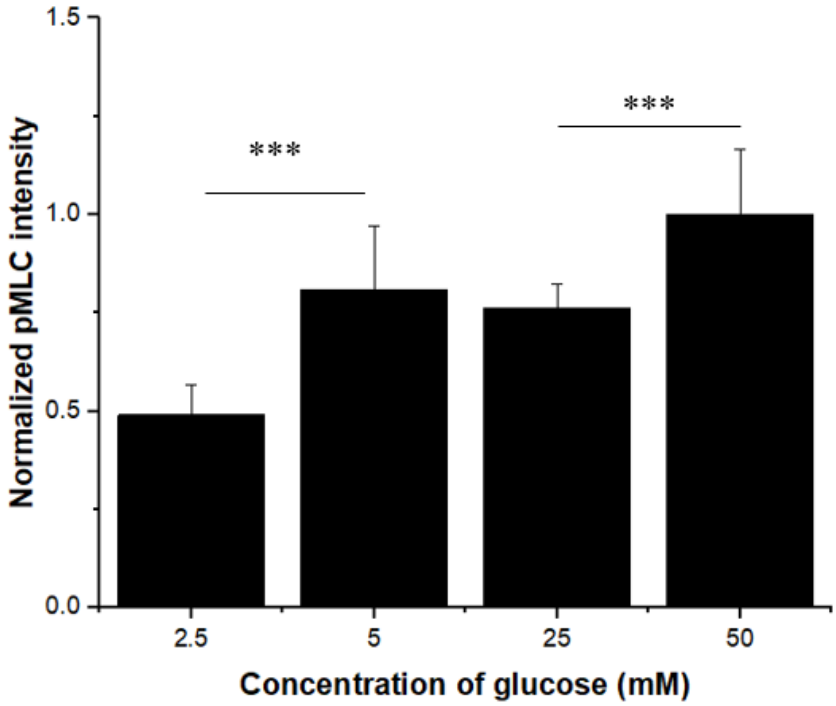


Figure 20: Change in intracellular pMLC profiles of 60 SH-SY5Y cells analysed over 1 experiment

5. Discussion

From our previous report, our experiments show that extracellular glucose concentration affects morphology and contractility of undifferentiated SH-SY5Y cells. In this one, we have solidified the morphology data, although we do not have any conclusive evidence to comment on the contractility. Morphology and trypsin de-adhesion experiments were repeated on differentiated neuronal cells maintained at constant 2.5 mM glucose (low), 5 mM glucose (normal), 25 mM glucose (high) and 50 mM (very high), and the results were noted.

So far, with basic morphology studies, trypsin de-adhesion experiments, AFM, and staining to check for changes in contractility, this in vitro study has shown that sustained high glucose levels have effects on the cytoskeleton in undifferentiated SH-SY5Yneuroblastoma cells. Notably, the studies show a distinct decrease in neurite length as well as spread area in high glucose conditions. The overall stiffness of cells exposed to high glucose conditions shows a clear increase. In the limited sample size of 30 neurites studied per condition, the contractility and adhesion of cells do not show any change over varying glucose concentrations.

With our final objective, we aimed to explore, if cellular contractility does indeed increase due to glucose dysregulation, it predicts the existence of a molecular basis for this. We look at the various components of the cytoskeleton and observe how these are affected due to glucose dysregulation in both undifferentiated as well as differentiated SH-SY5Y cells. For this, we may use staining techniques (to find out the quantity of molecules that help in mechanical transduction of cells) under both normoglycaemic as well as hyperglycaemic conditions and observe any differences between the two conditions (to observe how the cellular mechanics change when one or more of these molecules are disturbed) in either condition. The pMLC profile, indicating the activated myosin levels in the cells, also show an increase across the conditions, although interestingly, there is most difference in the pMLC intensity within the two low phase D-glucose concentration, and the two high-phase D-glucose concentrations, instead of between the high-phase and low-phase conditions expected. The reasons and detailed molecular mechanisms behind these effects are yet to be studied further

We expected to gain some insight into the way extracellular glucose dysregulation affects cell mechanics of neuronal cells, from morphology disturbances to molecular changes in the neuronal cytoskeleton, and so far in the scope of the study, we have been successful.

Constant supply of sustained, normal levels of glucose to the brain is critical for normal neuronal metabolism, with both hypoglycaemia and hyperglycaemia affecting activity, survival and function of neural cells. Our data now supports that even sustained high levels of glucose exposure also triggers some unknown cellular and molecular mechanisms that lead to disturbances in the cellular cytoskeleton. Whilst

an in vivo model would theoretically be more informative in terms of neural survival, the concomitant and compounding direct effects of insulin and counter-regulatory hormones upon neural activity render interpretation of individual molecular processes problematic. Hence, such studies are outside the scope of this project. Thus, within the obvious limitations of an in vitro model system for human neuronal cells, our study suggests that some of the cellular and molecular events leading to neuronal dysfunction, degeneration, or loss during diabetes or other disorders that affect the extracellular glucose concentration could very well be related to changes in the regulation of the cytoskeletal elements. It represents a further, albeit a small step, in understanding the disturbed neural biology leading to the recognized patterns of DN.

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