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Note

Short-term extracellular glucose exposure alters neurite outgrowth and intracellular reactive oxygen species without altering viability in neuronal cells

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Hyperglycemia is the main trigger for diabetic neuropathic pain and can cause degeneration of the sensory neurons. Here, we explored the effects of high glucose on the cell viability, oxidative stress (intracellular reactive oxygen species (ROS) generation, glutathione (GSH) and neurite outgrowth of primary cultured sensory neurons. Dorsal root ganglion (DRG) neurons isolated from the neonatal rat and mouse neuroblastoma (neuro-2a) cells were exposed to high glucose concentrations. Short-term exposure of high glucose leads to a decrease in the neurite outgrowth as compared to the normal glucose level in the DRG neurons and the neuro-2a cells. However, exposure to high glucose levels did not alter the cell viability. Furthermore, exposure to high glucose levels leads to an increase in the ROS and a decrease in the GSH as compared to the normal glucose levels in the DRG neurons and the neuro-2a cells. These results suggest that short-term exposure of the DRG neurons and the neuro-2a cells to extracellular glucose damages the morphology, thus, hampers the extension of neurite outgrowth without altering the cell viability. The neuronal injury may be induced by high levels of glucose, mediated through the ROS and GSH.

Keywords: Diabetes, DRG neurons, High glucose, H_2DCF-DA, Hyperglycemia, MTT assay, Neuro-2a cells, Neuroblastoma, Neuronal injury, ROS

High glucose results in nerve hypoxia/ischemia, associated with deficits in nerve blood flow and sensory nerve conduction velocity especially in the dorsal root ganglion (DRG) neurons[1]. Glucose uptake in neurons depends on the glucose concentration around the cell[1]. Excessive glucose induces morphological, neurochemical and physiological changes in the sensory neurons leading to pain[1]. Hyperglycaemic injury to sensory nerves induces anatomical modifications such as afferent terminal sprouting, inhibitory interneuron loss etc. to afferent and central neurons[1]. Diabetes is commonly associated with a symmetrical sensory poly-neuropathy. The primary sensory neurons of the peripheral nervous system are located within the DRG. Cultured rat DRG neurons extend neurites that have morphological characteristics similar to those of human sensory axons. When exposed to a variety of toxic insults, cultured DRG undergoes morphological and biochemical changes which closely mimic toxin-mediated damage in adult animals. Interestingly, the serum levels of neurotoxins which produce human neuropathy are similar to those which produce axonal degeneration *in vitro* in DRG[2-4].

Hyperglycemia plays a critical role in the sensory neuronal degeneration. Glucose concentration is one of the factors known to affect the neuronal growth and regeneration. The DRG neurons and the associated cell bodies are particularly vulnerable to the metabolic effects of hyperglycemia, as the DRG is located outside the spinal cord, and thus is not protected by the blood–nerve barrier. It has already been reported that sensory neurons of streptozotocin (STZ)-induced diabetic rats undergo apoptotic cell death *in vivo* and *in vitro*[5]. Further, diabetic rat DRG neurons when treated with nerve growth factor *in vitro*, exhibited significant apoptosis and impaired neurite outgrowth[6,7]. However, the exact underlying mechanism for the extracellular glucose exposure on the neuronal morphology still needs to be understood. Therefore, in the present study, we examined the effects of high glucose insult to the DRG neurons and the mouse neuroblastoma (neuro-2a) cells *in vitro* where acute high glucose exposure influences, directly or indirectly the neurite outgrowth, cell viability and oxidative stress.

Materials and Methods

Primary culture of the DRG neurons

Male Sprague-Dawley rat pups (aged 4-6 days) procured from the Central Animal Facility, National Institute of Pharmaceutical Education and Research (NIPER) were used in this study. All the animal procedure was approved by the Institutional Animal Ethics Committee. The DRG neurons were isolated

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by a method described elsewhere for rat DRG with a minor modification[8,9]. The DRGs (L4-L6) were removed aseptically and were trypsinized (2 mg/mL). The neurons were isolated by gentle trituration with a fire-polished Pasteur pipette at a gradually reducing diameter. The dissociated cells were plated in cover slips pre-coated with poly-D-lysine (PDL) and incubated in Dulbecco's modified Eagle's culture medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic medium at 37°C under 95% relative humidity and 5% CO_2. A high glucose media was prepared by adding additional glucose of the desired amount to the plating media so as to render the final glucose concentration of 10, 15, 30, 45 and 60 mM. After 12 h of plating, the plating medium was replaced with the high glucose medium for 24 h. All chemicals and the kits were purchased in culture grade from Sigma Aldrich, St Louis, MO, USA unless and otherwise mentioned. DMEM, the antibiotic–antimycotic solution and the fetal bovine serum (FBS) were purchased from Gibco BRL, USA.

Neuro-2a culture

The neuro-2a cells were obtained from the National Centre for Cell Science, Pune. The cells were maintained in DMEM supplemented with 10% FBS and 1% antibiotics in 5% CO_2 at 37°C. The medium was replaced after every 2nd day. For differentiation, the cells were switched to serum-starvation medium (0.1 % FBS). The cells were cultured in either low (5.5 mM) or high glucose DMEM medium (10, 15, 30, 45 and 60 mM). For neurite outgrowth induction, the cells were switched to serum-starvation medium.

Characterization of DRG neurons

The surface morphology of the primary cultured DRG neurons (20 ± 4 μm) were examined using scanning electron microscope (SEM) representing the most suitable model to carry out nociception related mechanistic study. The samples were rinsed quickly one time in PBS and were pre-fixed in glutaraldehyde. The post-fixation was performed with a 1% osmium tetroxide solution for 90 min. After they were dehydrated in graded ethanol, the samples were treated with dehydrated alcohol/acetone solution and were examined under SEM. The photographs were used to study the surface morphology of the neurons with the aid of 3D interactive surface plot plug-in, ImageJ.

MTT assay

The DRG neurons and the neuro-2a cells were plated (1×10^5 cells/well) in a 96-well microtiter plate. After 12 h, the culture media were then removed and 200 μL of high glucose media of a specified concentration were added to each well and the plates were further incubated at 37°C under 5% CO_2. At the end of the incubation time, the media was removed. The cell viability under acute high glucose condition was assessed by adding 200 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, 0.5 mg/mL) to each well and then further incubated for 3 h at 37°C. The plates were then flicked and the formazan resulting from the reduction of MTT by the mitochondrial enzymes was extracted using dimethyl sulphoxide. The amount of formazan was determined colorimetrically at 550 nm using a spectrophotometer.

Neurite outgrowth assay

Neurite outgrowth assay was done on the DRG neurons according to the manufacturer instruction (Millipore Neurite outgrowth Assay kit, Chemicon's NS220). The cells were allowed to grow for 24 h and then exposed to normal (5.5 mM) or high (30 mM) glucose medium. The neurite outgrowth was detected by immunofluorescence. The media was aspirated and the cells were washed with PBS, fixed in ice-cold 95% ethanol for 15 min at −20°C twice. Then, the cells were permeabilized in 0.2% Triton X 100 in PBS for 15 min, washed and incubated with goat serum for 30 min at approximately 25°C. The cells were then treated with Alexa-fluor antiactin antibody at a dilution of 1:200 at 4°C for 12 h. The cells were then mounted with mounting media and were observed under a fluorescent microscope (Leica, Solms, Germany) and the images acquired using a charged coupled device camera (Leica DC 300F, Germany) and were analysed by using Image-J software.

Oxidative stress

Intracellular ROS

Neurons (DRG/Neuro-2a cells) were plated into 24-well microtiter plate and exposed to normal glucose, high glucose for 24 h. They were then washed with PBS and were incubated in the dark for 30 min in PBS containing 10 μM of 2',7'-dichloro dihydrofluorescein diacetate (H_2DCFDA). The ROS were estimated by microscopy[9,10].

Glutathione estimation

The neuro-2a cells and the DRG neurons were harvested in cell lysis buffer. The cell lysate was used for measuring the reduced glutathione (GSH) content. An aliquot (0.1 mL) of the lysate was mixed with 5%

sulphosalicylic acid (0.5 mL) and kept in ice for 20 min. The resulting solution was centrifuged for 5 min at 4°C, and 100 µL of the supernatant were mixed with 100 µL of 5,5'-dithiobis-(2-nitrobenzoic acid) in 0.1 M phosphate buffer, (pH 8.0). The mixture was incubated for 10 min at 37°C followed by the measurement of absorbance at 412 nm spectrophotometrically. A standard curve was generated using reduced glutathione as standard. The protein estimation was performed according to Lowry method[11]. The results are expressed as percentage of µM/mg of protein compared to normal control.

Statistical analysis

Data are expressed in percentage of control as mean ± SEM. All statistical analysis was performed using Jandel Sigma Stat 2.0 statistical software. Student's t-test was used for comparison of two groups. P <0.05 was considered as statistically significant.

Results and Discussion

Morphology of DRG Neuron by SEM

Sensory neuronal cell bodies were round or oval in shape in the DRG culture. The SEM analysis showed that the DRG neuronal cell bodies sent axons variable in caliber with small varicosities and sharp terminals with axons crossing over or terminating on the surface neuron (Fig. 1). The configurations of the terminal axons observed under SEM were variable. Some axons would widen on cell surface with various diameter and many appeared no different from the immediate proximal configuration. Normal neurons exhibited enlarged axonal endings. The DRG neurons demonstrated an evidence of dense neurite outgrowth (Fig. 1).

Effect of high glucose exposure on cell viability

No statistically significant difference was observed in cell viability of the DRG neurons and the neuro-2a cells after exposure to extracellular glucose (10, 15, 30, 45 and 60 mM) as compared to the control (Fig. 2). This comparative study provides evidence that neuronal cell like the DRG neurons and the neuro-2a cells are resistant to short-term acute high glucose exposure under *in vitro* condition.

Effect of high glucose on neurite outgrowth

The DRG neurons treated with normal and high glucose concentration were selected on the basis that these concentration did not alter the cell viability. High glucose exposure has significantly reduced the neurite outgrowth density in the DRG neurons as compared to the control. In a similar way, when the neuro-2a cells were exposed to normal and high glucose conditions, a significant reduction in the neurite outgrowth was observed as compared to the control (Fig. 3). This comparative study provides evidence that the neurite outgrowth of the DRG neurons and the neuro-2a cells is reduced on acute high glucose exposure under *in vitro* condition.

Effect of high glucose reactive oxygen species generation (ROS)

The 24 h incubation in high glucose conditions significantly increased the level of ROS as seen from

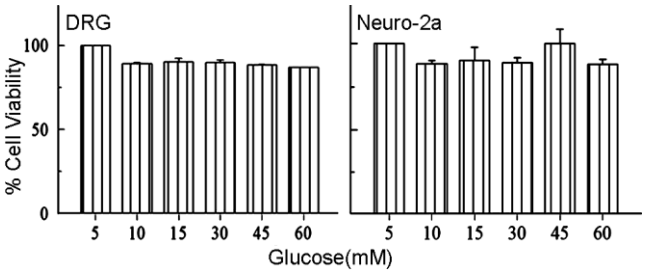

Fig. 2 — Effect of high glucose cell viability in the dorsal root ganglion (DRG) and the neuro-2a cells. [The DRG neurons were incubated in conditions of normal glucose (5.5 mM) and high glucose (10mM -60 mM). Data are expressed in Mean ± SEM. The bar graph showing the percentage cell viability of DRG neurons (left) and neuro2a cells (right) upon exposure to different glucose concentrations after 24 h]

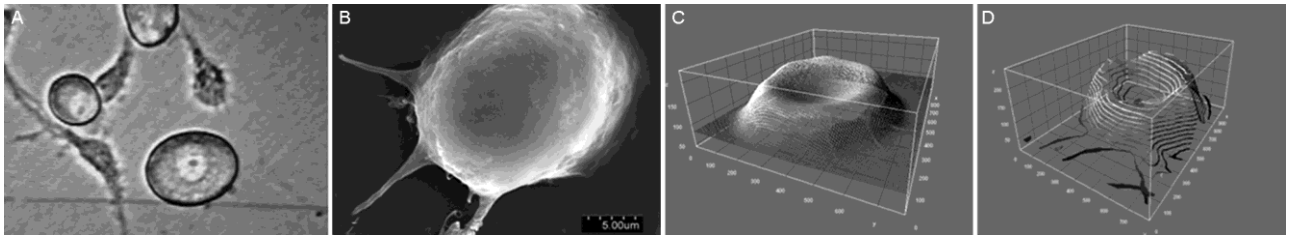

Fig. 1 — Freshly isolated rat dorsal root ganglion (DRG) neurons in normal glucose culture. (A) Depicts bright field photomicrograph; (B) depicts the SEM image of fixed DRG neuron; (C) represent mesh filled 3D surface plot; and (D) represents the isoline 3D surface plot of the DRG neurons. [This particular graph was drawn by using a 3D interactive surface plot plug-in, ImageJ software (NIH) applied on SEM image of the DRG neurons]

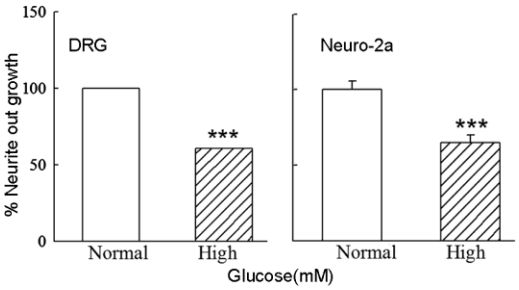

Fig. 3 — Effect of high glucose on the neurite outgrowth in the dorsal root ganglion (DRG) and the neuro-2a cells. [Neurons were incubated in conditions of normal glucose (5.5 mM) and high glucose (30 mM). In the presence of high glucose the neurite outgrowth was significantly reduced in comparison to the control. High glucose also reduced the neurite outgrowth in the neuro-2a cells. The cells were incubated in conditions of normal glucose (5.5 mM) and high glucose (30 mM). ****P* <0.001 *vs*. normal glucose]

the increased intensity of green fluorescence in the DRG and the neuro-2a cells as compared to the normal glucose (Fig. 4).

Effect of high glucose on intracellular GSH

Intracellular GSH was significantly reduced after exposure to the high glucose condition in comparison to the control in the DRG and the neuro-2a cells (Fig. 5).

The present study demonstrates that short-term acute high glucose exposure to DRG neurons and neuro-2a cells hampers the extension of neurite outgrowth without altering the cell viability. Exposure to higher concentrations of glucose also leads to increased ROS generation as well as a decreased cellular GSH level.

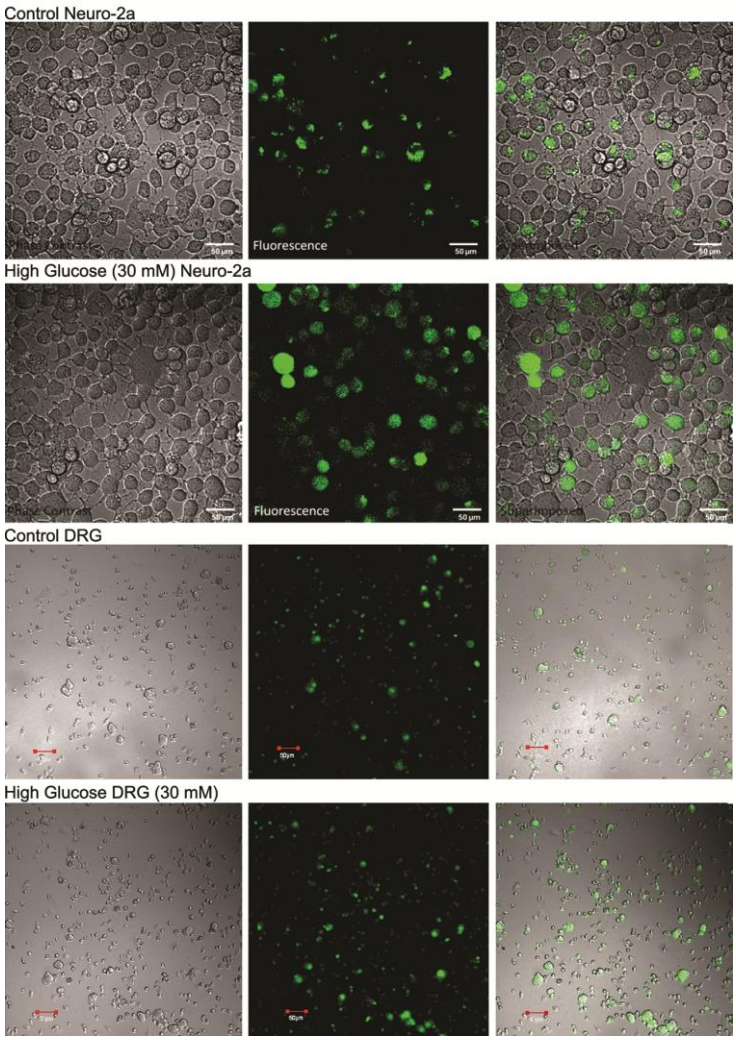

Fig. 4 — Fluorescent images of the neuro-2a cells and the dorsal root ganglion (DRG) neurons were presented before and after exposure with glucose concentrations (5.5 mM and 30 mM). [Effect of high glucose on the intracellular ROS generation in the neuro-2a and the DRG neurons by staining with H2DCFDA and observed at an excitation-wavelength of 495 nm and an emission wavelength of 529 nm. Increased intensity of green fluorescence was observed at high glucose concentrations (30 mM) compared to the control (5.5 mM). The photographs were taken at 40X and 10X for the neuro-2a and the DRG neurons, respectively. Scale bar = 50 µm]

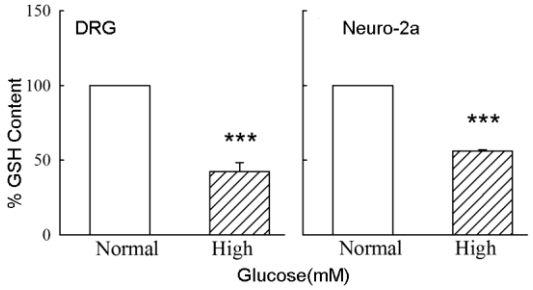

Fig. 5 — Effect of high glucose on the intracellular glutathione (GSH) content in the dorsal root ganglion (DRG) neurons and the neuro-2a cells. [The content of the intracellular GSH was measured in the DRG and the neuro-2a cells by the GSH assay. The results are expressed as percentage of GSH content in high glucose (30 mM) group compared to the control (5.5 mM). ****P* <0.001 Vs Normal Glucose (5.5 mM)]

In pain, the high glucose level promotes neuronal injury through ROS production and the mitochondrial damage[12]. Gene knockout studies have shown that subpopulations of the developing DRG neurons depend on specific neurotrophins for their survival: nerve growth factor for small, peptidergic nociceptive; neurotrophin-3 for large proprioceptive and brain-derived neurotrophic factor for mechanoreceptors and other types of DRG neurons[13,14]. The morphology of axons and dendrites of neurons depends on the dynamics of cytoskeleton which is regulated by diffusing factors from the environment, cell surface receptors, extracellular matrix and intracellular signals. Dissociated DRG neurons maintained in culture begin extending neurites within a day, and within a week the neurite outgrowth become extensive. The cellular changes requiring gene activation such as increased protein and membrane synthesis and cytoskeletal expansion occur during the neurite outgrowth. Ca^{2+} is important as a modulator of cellular events. Many biochemical reactions essential for growth cone motility and neurite outgrowth are sensitive to internal calcium concentration, which is normally very low in the DRG neurons, as in other neuronal cells. An increase in the calcium ion concentration in growth cones might alter the rate of microtubule assembly or disassembly, which is a major component of neurite cytoskeleton and is essential for axoplasmic transport[15]. High glucose exposure to DRG neurons causes reduction in neurite outgrowth probably via decreased intracellular calcium level in the growth cones which reorganizes actin microfilaments and arrests microspikes of neurites[16]. Although the mechanisms underlying the interaction of calcium with the cytoskeleton of the growth cone and axonal terminal remain poorly understood[17,18], it appears to be less important as far as the role of calcium in regulating neurite growth cone motility in sensory neurons is concerned. In cultured mammalian neurons, intracellular free calcium concentration is elevated in neurons showing actively extending neurites as compared to the quiescent neurons. Moreover, the concentration of calcium is higher in the growth cone within the active neurites than in the proximal neurite or soma[19,20]. When the DRG neurons were incubated in high glucose conditions, the amount of neurites and the neurite outgrowth decreased sharply as compared to those incubated under normal glucose condition. In a similar way, when the neuro-2a cells were incubated with normal glucose and high glucose conditions, we observed dramatically decreased neurite outgrowth in the high glucose neuro-2a cells as compared to the normal glucose cells. These evidences suggest that short term exposure of extra-cellular glucose alters the growth of neurites in the DRG neurons and the neuro-2a cells *in vitro*[21].

Diabetes is a complex metabolic disorder characterized by hyperglycemia and biochemical alterations of glucose and lipid metabolism. This abnormal metabolism leads to an increased generation of ROS. Our previously published data suggest that ROS generation increases after exposure to higher concentrations of glucose in a time-dependent manner[9]. In addition, we report that cell viability of the DRG neurons and the neuro-2a cells were unaffected by short-term high glucose exposure. Mitochondrial DNA is susceptible to oxidative damage and these dysfunctional mitochondria have increased ROS leakage. Its various impacts on the neurons like mitochondrial dysfunction, decreased glucose metabolism deactivation of certain key enzymes, increased ROS generation have been widely observed in hyperglycemic treated cell cultures as reported in this study. The ROS generation, intracellular calcium regulation and the release of pro-apoptotic factors lead to changes in the neuronal morphology which further leads to fatal cell damage. We did not observe any change in the cell viability for either the DRG neurons or the neuro-2a cells under different high glucose exposure. This again proved that high glucose medium mainly alters the neurite outgrowth in cultured rat DRG neurons but does not affect cell viability.

Glutathione, a tripeptide present in all the cells, is an important antioxidant[22]. Reduced glutathione

normally plays the role of an intracellular radical scavenger and is the substrate of many xenobiotic elimination reactions. A marked decreased level of reduced glutathione is reported in the plasma of diabetic patients. Results of our study are in agreement with earlier studies[22]. GSH systems may have the ability to manage oxidative stress with adaptational changes in the enzymes regulating the GSH metabolism. There is a negative correlation between GSH and HbA1c in the diabetic patients, which confirms the link between hyperglycemia and the GSH depletion. Indeed, in hyperglycemia conditions, glucose is preferentially used in polyol pathway that consumes NADPH necessary for the GSH regeneration by the GSH-Red enzyme. Therefore, hyperglycemia is indirectly the cause of GSH depletion. As GSH is an important antioxidant molecule, its depletion leads to an increase in the oxidative stress. Free radicals are generated as by-products of normal cellular metabolism; however, several conditions are known to disturb the balance between the ROS production and the cellular defense mechanisms. This imbalance can result in cell dysfunction and destruction resulting in tissue injury. The increase in the level of ROS in hyperglycemia could be due to their increased production and/or decreased destruction by nonenzymic and enzymic catalase (CAT), glutathione peroxidase (GSH-Px) antioxidants.

Conclusion

In this study, we have demonstrated that exposure to short-term high glucose concentrations can induce alterations in the neurite outgrowth of sensory neurons. Further, it provides evidence that extracellular high glucose-induced increase in the intracellular ROS generation and decreased GSH level without altering the cell viability is consistence with the animal models of diabetic neuropathy. In summary, oxidative stress is a critical target for therapeutic intervention in diabetic neuropathy[23].

Acknowledgement

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Author contribution

JNS and SSS designed and critically analyzed the data and wrote the manuscript; GN did experiments related to the neuro-2a culture, NKM did the characterization of DRG neurons with SEM images while SK and DS conducted the culture of DRG neurons and related assays. All authors contributed to the interpretation of data and drafting of the manuscript for the intellectual content.

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