Nitrosative stress induces proliferation and viability changes in high glucose-exposed rat Schwannoma cells

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Abstract

OBJECTIVES: Schwann cells may be involved in the pathogenesis of several neuropathies, such as those linked to an excess of D-glucose. Indeed, hyperglicemic condition can often result in the production of high reactive/nitrosative oxygen species concentration and possible damage of several cell structures. In the present work attention has been focused on the possible nitrosative effect of hyperglycemia on RT4 Schwannoma cell lines.
METHODS: Cells were cultured for 72hrs in the presence of 180 mM D-glucose. Morphology, growth rate, cell viability, catalase evaluation and Western blot were performed.
RESULTS: In D-glucose-exposed cells, 3-Nitrotyrosine increase and subsequent modifications in cell morphology, growth rate, viability and catalase activity were found.
CONCLUSION: Our findings suggested a possible primary role played by Schwann cells in the hyperglicemic neuropathy pathogenesis, through the excessive production of RNS and a decrease in antioxidant defence systems, bearing out the importance of the "nitrosative hypothesis" in the hyperglicemic-induced nervous system complications.

ORIGINAL ARTICLE

INTRODUCTION

Schwann cells (SCs), the myelin forming cells in the peripheral nervous system, are fundamental for the proper function of peripheral nerves, providing trophic support to axon, especially after nerve damages (Hoke 2006; Chen *et al.* 2007). SCs may play a key role in the pathogenesis of various inflammatory, hereditary and metabolic polyneuropathies (Lehmann & Hoke 2010). Among metabolic neuropathies there are those linked to D-glucose dysfunction. Indeed, peripheral neuropathy is one of the major complications of diabetes mellitus status which can damage Schwann cells directly, and give rise to endoneurial vascular ischemia as well as impaired neurotrophic support (Eckersley *et al.* 2001). The potential etiology of diabetic neuropathy includes, among others, oxidative defects (Brownlee M., 2001; Giacco *et al.* 2010). Oxidative stress occurs in a cellular system when the generation and of reactive oxygen species (ROS) and reactive nitrogen species (RNS) exceeds the antioxidant capacity of that system. If cellular antioxidants such as catalase, superoxide dismutase and glutathione do not remove free radicals, the latter may attack and damage

proteins, lipids and nucleic acids, generating a wide range of pathological conditions (Halliwell 2001; 2006; Murray *et al.* 2008). Glucose oxidation is known to be a considerable source of free radicals mainly when the normally efficient metabolism of glucose is altered with subsequent overloaded metabolic pathways (Maritim *et al.* 2003; Allen *et al.* 2005). Increased oxidative stress is a widely accepted participant in the development and progression of diabetes complications (Ceriello 2000; Maritim *et al.* 2003; Vincent *et al.* 2004). The hyperglycemic condition can be considered a potent initiator of apoptosis through the excessive production of ROS with formation of peroxynitrite and subsequent damage of cellular proteins, membrane lipids, nucleic acids and eventually cell death (Delaney *et al.* 2001). Among others, peroxynitrite can react with either protein tyrosine residues or free tyrosine to produce 3-Nitrotyrosine (Ischiropulos 1998). Accumulation of 3-Nitrotyrosine has been reported in several tissues of diabetic mice, rats and humans (Thuraisingham *et al.* 2000; Pacher *et al.* 2005; Drel *et al.* 2006). Moreover, increased 3-Nitrotyrosine immunoreactivity has been demonstrated in the peripheral nervous system during experimental diabetic conditions (Cheng & Zochodne 2003). This end product, considered a footprint for peroxynitrite mediated damage, can be incorporated in proteins through posttranslational modifications, leading to several pathophysiological consequences (Souza *et al.* 2008).

Since there are many tenable hypothesis on the origin of diabetic complications (advanced glycation and products (AGE) hypothesis, aldose reductase hypothesis and the oxidative stress hypothesis), and considering that for many authors Schwann cells are resistant to oxidative stress injury (Baynes & Thorpe 1999; Vincent *et al.* 2009), the aim of this study was to establish an *in vitro* model using rat Schwannoma cell lines, in order to evaluate the possible importance of nitrosative stress hypothesis in diabetic complications of the peripheral nervous system.

MATERIALS AND METHODS

Undifferentiated RT4-D6P2T rat schwannoma cells (American Type Culture Collection, Rockville, MD, USA) were seeded at a concentration of 5.6×10^5 per ml and grown in phenol-red-free RPMI-1640 medium supplemented with 10% heat-inactivated newborn calf serum, 2 mM L-glutamine, 100 units/ml penicillin G and 100 µg/ml streptomycin sulfate. Cells were incubated for 72 hours at 37 °C in a 5% CO_2, humidified atmosphere in the presence of 180 mM D-glucose (Gluc cells) or in basal conditions (Ctrl cells). Regarding the glucose dose employed, a dose-response curve was performed for the cell line. In rat schwannoma cells the first morphological signs of significant cell suffering (proliferation rate and morphological changes) were appreciable only at 180 mM D-glucose. Until that

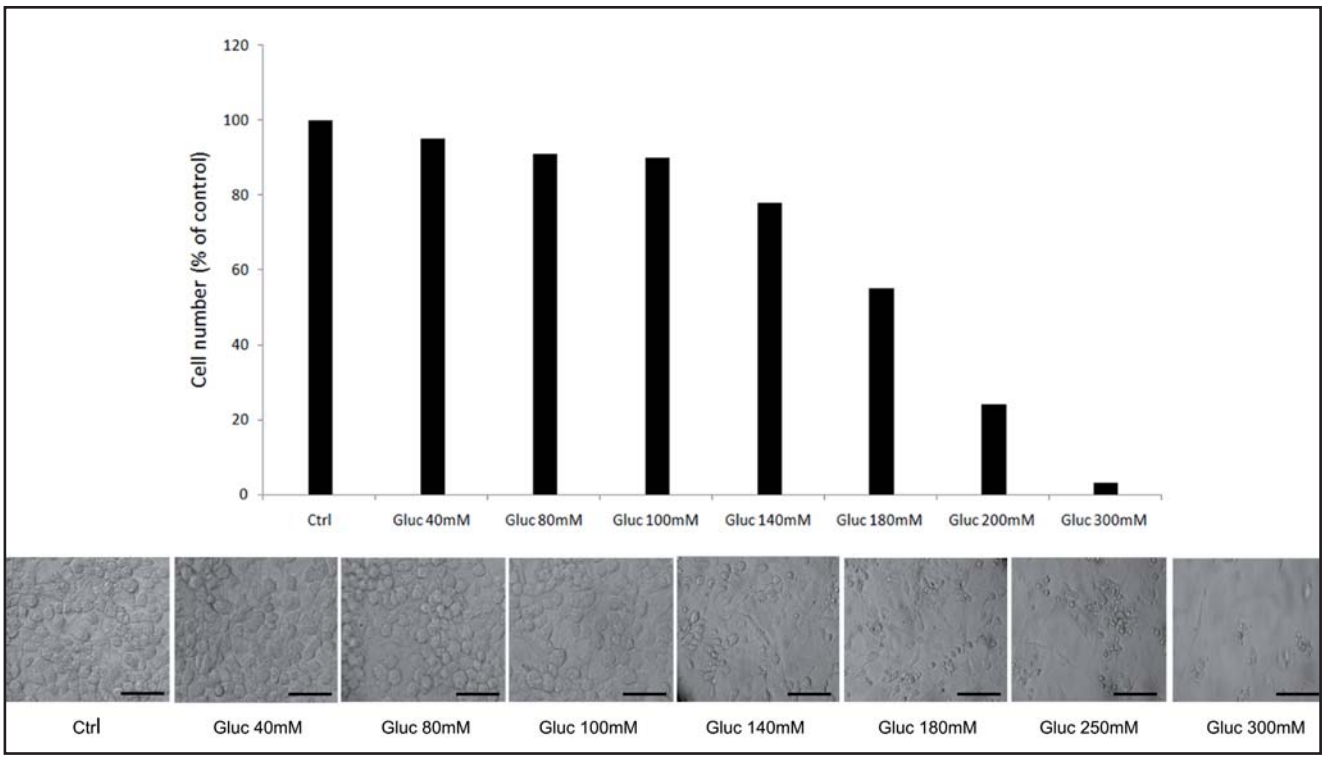

Fig. 1. Dose-response curve. Picture shows the proliferation trend of rat schwannoma cells exposed at different D-glucose concentrations and the related morphological features. The first significant proliferation and morphological changes are detectable at 180mM D-glucose. Each column represents the average of six single experiments. Bar=15µm.

dose there was a normal growth without any significant morphological alteration (Figure 1). Hence, the results and pictures related to RT4-D6P2T cells presented in this paper deal with 180 mM D-glucose.

Cells lysates were collected by using a TO buffer (5 mM TRIS HCl, 2 mM EGTA, 0.1 mM phenyl-methyl-sulfonyl fluoride, pH 8.0) supplemented with protease inhibitors (Complete-mini, Roche, Basel, Switzerland). After protein content determination, samples (60 µg) were loaded and subjected to 10% SDS-PAGE and electrontranferred onto nitrocellulose membranes. Nitrocellulose were incubated overnight at 4 °C with primary antibodies against 3-Nitrotyrosine (polyclonal, 1:500, Sigma, St. Louis, MO, USA) and actin (monoclonal, clone AC-40, 1:1,000, Sigma, St. Louis, MO, USA). Membranes were then incubated with the corresponding anti-mouse or anti-rabbit IgG alkaline phosphatase-conjugated antibody (Sigma, St. Louis, MO, USA) for 1 hr at 37 °C at 1:1,000 or 1:30,000 dilution respectively. Blots were subsequently detected by incubating the membranes with nitro blue tetrazolium/5bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche). Optical density of the bands was evaluated using the freeware Image J freeware. Blot shown are representative of five independent experiments performed.

For immunofluorescence staining cells were seeded on chamber slides (Lab-Tek, Naperville, ILL, USA). Scheduled times, medium characteristics, D-glucose concentrations, etc. were those indicated above. Cells were fixed in methanol at –20 °C and incubated overnight with antibodies against 3-Nitrotyrosine (polyclonal, 1:500, Sigma, St. Louis, MO, USA). Secondary anti-rabbit fluorescein isothiocyanate-conjugated antibody (FITC, AlexaFluor 488, 1:400, Invitrogen, Carlsbad, CA, USA), was used. Evaluation of immunoreactivity level in immunofluorescence was performed by Image J freeware.

For cell proliferation assays, cells were plated in 96-well dishes (1×10^4 cells per well). Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (5 mg/ml, MTT, Cell Titer 96, Promega, Madison, WI, USA). At the end of treatment, the medium was removed and the wells were washed with PBS. The 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed by adding 20µl MTS. The plate was mixed and then incubated for 1hr at 37 °C according to the manufacturer's instruction. MTT [3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] colorimetric assay is to measure reduction of MTT dyes (tetrazolium) into formazan by mitochondrial enzymes in viable cells. Absorbance was measured setting the automatic microtiter reader at 492 nm (Shimadzu UV-1700, Pharma Spec, Japan), in the presence of an appropriate blank (without cells). Relative numbers of live cells were determined based on the optical absorbance of the treated and untreated samples and blank wells using the following formula: Viable cells (%)=(AT–AB)/(AC–AB)×100 where AC is the absorbance of the untreated samples, AT is the absorbance of the treated samples, and AB is the absorbance of the blank. All values are means of triplicate 96-well dishes.

Cell viability was determined by a double-staining procedure using bis-benzimide (Hoechst 33342, Sigma) and propidium iodide (PI, Sigma), and examined by fluorescent microscopy. Cells positively stained with PI were considered to be dead.

Catalase activity was estimated according to the method of Aebi (1984). Catalase degrades hydrogen peroxide which is measured directly by the decrease in the absorbance at 240 nm (Uniskan II, Stanwood Washington, USA). Ice-cold cells were collected and sonicated to rupture cell membranes. The hydrogen peroxide was diluted with PBS, pH 7.0 and its initial absorbance was adjusted between 0.5 and 0.6 of absorbance unit. Catalase activity was expressed as U/ml.

Average values used for analysis are representative of five experiments for each protocol. Data are expressed as mean ± standard error (SEM), and the inter-group analysis was done by Student's t-test or ANOVA. Statistical significance was accepted when $p<0.05$.

RESULTS

Our preliminary work has yielded the following results.

When observed under phase contrast optics after 72hrs of treatment schwannoma, Gluc cells displayed a reduced number and some morphological alterations, such as globular shape, vacuols, in comparison with Ctrl cells (Figure 2). This trend was confirmed by the proliferation assay. Significantly, in D-glucose-exposed cells (Gluc cells) MTS bioreduction revealed a decrease in proliferation rate to approximately 40% of Ctrl cells (Figure 3).

The possible deleterious effect of high-glucose exposure was further studied using Hoechst 33342 and PI double staining. After 72hrs, RT4 Ctrl cells exhibited a high number of Hoechst-positive cells (more than 90%) with homogeneous and compact nuclear morphology. With the addition of 180 mM D-glucose (Gluc cells) a mortality rate of about 60% (PI-positive) was recorded. Because PI is membrane impermeant and generally excluded from viable cells, PI-positive cells were dead cells (Figure 4).

Western blot analysis showed in Gluc cells a higher amount of 3-Nitrotyrosine in comparison with Ctrl cells. Western blot analysis of actin, revealed no differences (Figure 5). The results obtained with Western blot were confirmed by immunofluorescence staining. Indeed Gluc cells were more immunoreactive to 3-Nitrotyrosine than Ctrl cells (Figure 6).

Catalase activity significantly decreased in D-glucose-exposed RT4 cells up to about 40% of Ctrl cells (Figure 7).

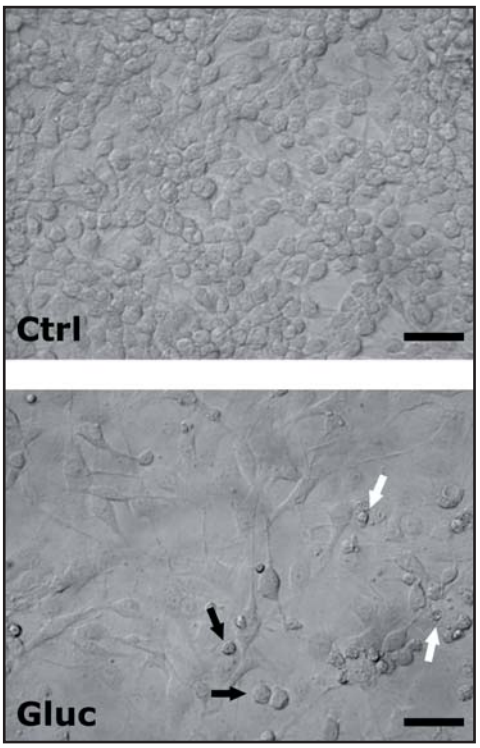

Fig. 2. Phase contrast optics. 180mM D-glucose-exposed cells (Gluc cells) are not confluent and display several morphological alterations such as globular shape (black arrow) and vacuols (white arrow) and, in comparison with Ctrl. Ctrl. Bar =30µm.

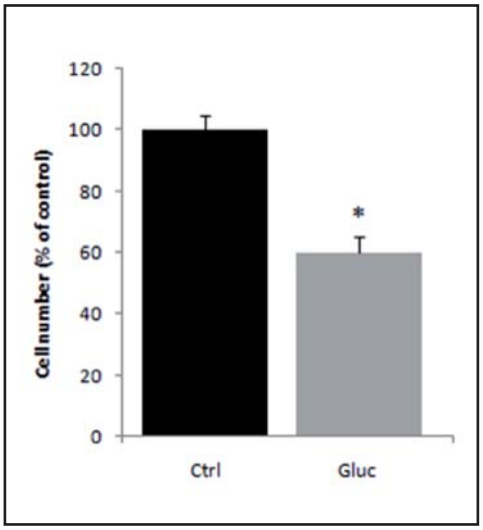

Fig. 3. Proliferation assay. The diagram of proliferation shows a significant inhibition of growth rate in Gluc cells. Data are shown as percentage of maximum value and expressed as mean ± SEM. Each column represents the average of six single experiments. * indicates significant differences from Ctrl ($p<0.05$).

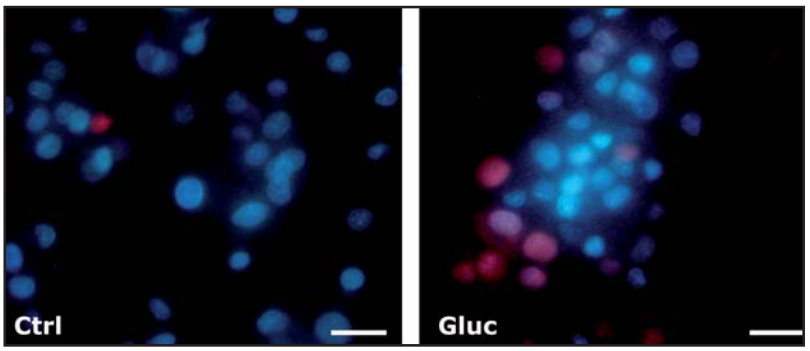

Fig. 4. Viability assay. Hoechst 33342/PI staining reveals a high number of dead cells (red nuclei) in Gluc cells. In contrast, a great deal of living cells (blue nuclei) are present in Ctrl. Bar =15µm.

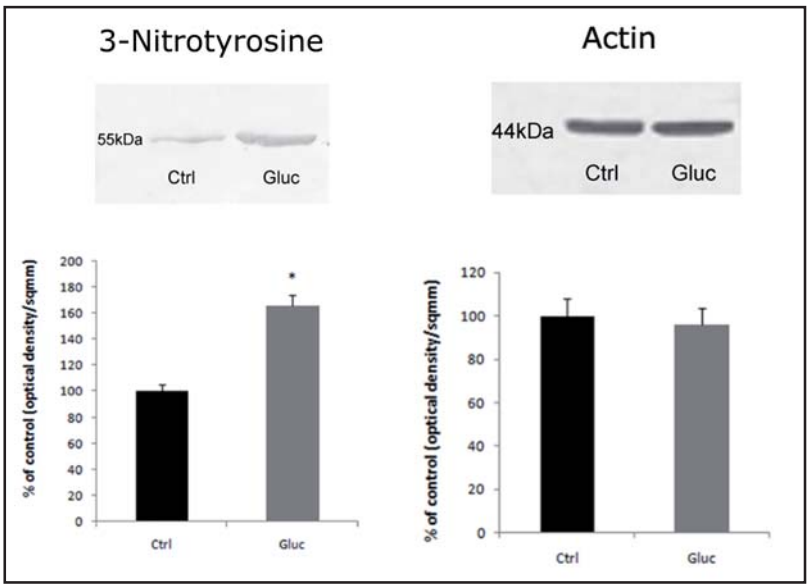

Fig. 5. Western blot analysis. In RT4 rat Schwannoma cells, the amount of 3-Nitrotyrosine is higher in Gluc cells than in Ctrl.. The pattern of actin expression, displays any changes. The diagrams represent the optical density of the bands. * indicates significant differences from Ctrl ($p<0.05$).

DISCUSSION

Hyperglycemic conditions are strongly correlated with increased severity of peripheral neuropathy, through a cascade of systemic changes that involves, in different ways neurons and Schwann cells (Sinnereich *et al.* 2005; Zochodne 2007). Chronic hyperglycemia can exert its detrimental effects, through the increase of oxidative stress (Rosen *et al.* 2001), with a different impact on neuronal and glial cells. Many authors postulated that Schwann cells are considered resistant to oxidative stress injury (Baynes & Thorpe 1999; Vincent *et al.* 2009). Following previous study (Russell *et al.* 1999), we performed an "high glucose experiments", choosing the dose at which the first significant cellular changes were detectable. Based upon the results of our study, we displayed a substantial sensitivity of Schwann cells to oxidative stress. Indeed, Western blot analysis and immunofluorescence staining revealed a higher amount of 3-Nitrotyrosine, in Gluc cells in comparison with Ctrl. 3-Nitrotyrosine is widely considered a fingerprint of oxidative and nitrosative stress, and recent data suggest that this

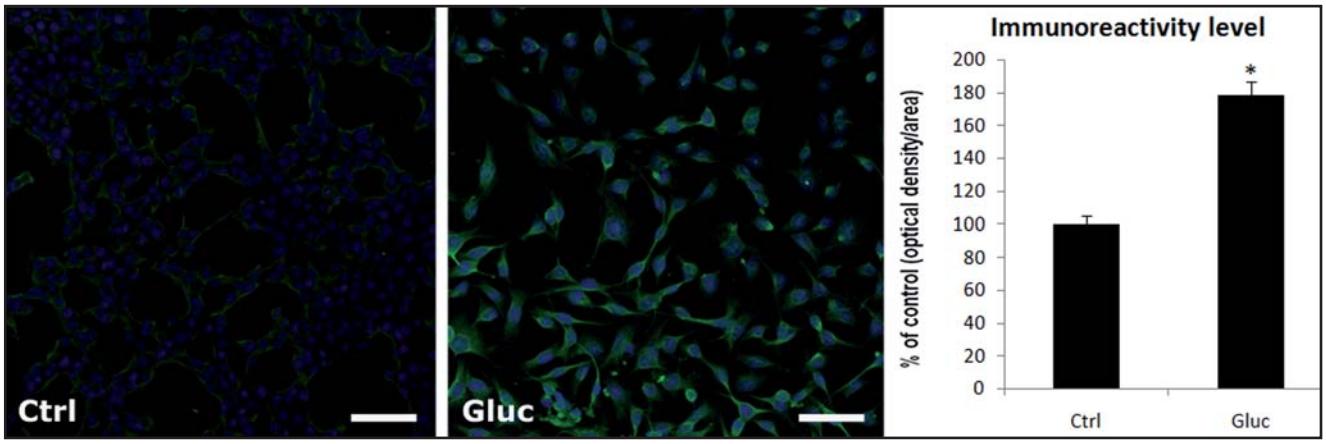

Fig. 6. Immunofluorescence staining. Alexa-Fluor FITC antibody reveal a higher immunoreactivity for 3-Nitrotyrosine in Gluc cells in comparison with Ctrl cells. The diagram quantifies the immunoreactivity level. Bar= 30 µm. * indicates significant differences from Ctrl ($p<0.05$).

modified amino acid could also be a potential neurotoxin (Greenacre & Ischiropulos 2001; Mihm *et al.* 2000; Turko & Murad 2002). It is a general opinion that tyrosine nitration may provoke loss of function of proteins (MacMillan-Crow *et al.* 1996; Hodara *et al.* 2004; Radi 2004), but the molecular basis of 3-Nitrotyrosine deleterious effects is still matter of debate. One of the possible 3-Nitrotyrosine mechanism of action could be its posttranslational incorporation into α-tubulin, blocking the physiological dynamics of microtubules (Eiserich *et al.* 1999; Chang *et al.* 2002; Phung *et al.* 2006). Formation of tyrosine-nitrated α-tubulin may have an effect on microtubule organization, cell division, motility, morphology, cytoplasmic processes elongation (Blanchard-Fillion *et al.* 2006; Gadau *et al.* 2008; 2009). RT-4 schwannoma cells, normally reach the confluent state in 36hrs, and during their growth they emit 2 cytoplasmic processes. The detrimental effect provoked by 3-Nitrotyrosine may explain the morphology alterations (globular shape and absence of cytoplasmic processes), growth rate (reduced number of cells), viability assay (high number of dead cells) observed in Gluc cells. The nitrosative injury induced by high-glucose exposure through the end product 3-Nitrotyrosine could be related to the pattern displayed by one of the most important antioxidants enzyme, catalase, that plays a critical role in protecting cells against the toxic effects of hydrogen peroxide (Dringen *et al.* 2005; Goyal & Basak 2010; Heck *et al.* 2010). The high levels of RNS overload the efficient detoxifying capability of cells leading to an inhibition of catalase antioxidant system. It is well known that low catalase activity levels may promote apoptosis in several types of cells (Kahl *et al.* 2004). This could be in agreement with our result which underlined an appreciable catalase activity reduction in Gluc cells.

Summing up, our result highlighted original data concerning the hyperglicemic nitrosative-induced sensitivity of Schwann cells. Indeed, although some authors have considered Schwann cells as resistant to oxidative stress (Baynes & Thorpe 1999; Vincent *et al.* 2009), we found a considerable increase of 3-Nitrotyrosine, a widely recognized marker of oxidative/nitrosative stress. This could suggest a new insight into the possible crucial role played by Schwann cells in the diabetic neuropathy pathogenesis, through the excessive production of RNS and decreasing of antioxidant defence systems. Moreover, our work may confirm the importance of the "nitrosative hypothesis" in the hyperglicemic-induced nervous system complications.

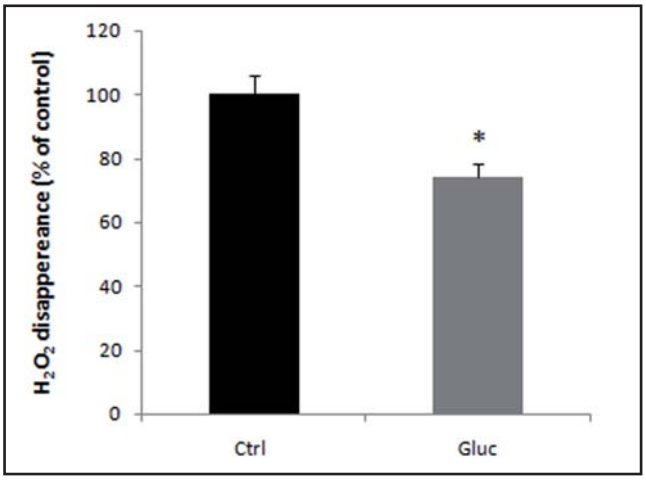

Fig 7. Catalase activity assay. The diagram shows that the impairment of catalase efficiency is more marked in Gluc cells in comparison with Ctrl. * indicates significant differences from controls ($p<0.05$).

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