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Ultrastructural changes in the neuronal superior colliculus in the early stage of streptozotocin-induced diabetes mellitus in rats

Tosawan Upachit, Passara Lanlua and Sirinush Sricharoenvej*

Prannok Road, Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkoknoi, Bangkok 10700, Thailand.

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The superior colliculus (SC) is a visuomotor center involved in the autonomic reflex adjustments of eye movements in response to visual stimuli. Diabetes mellitus (DM) is known to affect some visual pathway structures, but few studies have assessed the effects of diabetes on the SC. The aim of this study was to investigate the ultrastructural changes of SC neurons in the early period of streptozotocin (STZ)-induced diabetes. The ultrastructure was assessed by transmission electron microscopy (TEM). Twenty male Sprague–Dawley rats were divided into two groups (n=10 per group) and were intraperitoneally injected with either STZ (60 mg/kg) in citrate buffer (pH 4.5) to induce DM or with buffer alone as a positive control. The rats were sacrificed 4 weeks after injection and the SC was processed for TEM. Most of the SC neurons in the DM group exhibited either chromatolysis or pyknosis. Chromatolytic neurons had an enlarged nucleus with some chromatin clumping and disruption of the cell membrane. These neurons also exhibited mitochondrial enlargement with rupture of the cristae, distended Golgi complexes and rough endoplasmic reticulum, and numerous secondary lysosomes. By contrast, the pyknotic neurons in the DM group exhibited severe chromatic condensation and dark electron-dense structures in the cytoplasm. The organelles were smaller and had an irregular outline. The neuropil of DM rats had coarse, irregular, swollen dendrites and axons, together with demyelination. In conclusion, this study has provided clear evidence of ultrastructural degeneration in the SC of STZ-induced DM rats. These ultrastructural changes might contribute to the impairments of autonomic eye movement, optokinetic and vestibulo-ocular reflexes, and vision-related learning and memory in patients with DM.

Key words: Superior colliculus, diabetes mellitus, streptozotocin.

INTRODUCTION

The superior colliculus (SC) is an important visuomotor center that controls and adjusts eye movement in response to environmental stimuli, and is influential in automatic perceptual visual function. The SC is the laminar structure located in the midbrain. It consists of alternating neurons and nerve fiber layers, which are

*Corresponding author. E-mail: sirinush.sri@mahidol.ac.th, Tel: +66-2-419-8592. Fax: +66-2-419-8523.

divided into two functional parts: the superficial layer and intermediate and deep layers (May, 2006). The superficial layer receives incoming optic signals from the retinas and the visual cortex. The many variously sized and shaped neurons in this layer receive visual inputs and transmit signals to neurons in the deeper sublayers to integrate the autonomic eye movement reflexes. The intermediate and deep layers of the SC are involved in eye movement, by receiving visual input signals from the superficial layers and afferent projections of several systems related to autonomic eye movement reflexes, including the optokinetic and vestibulo-ocular reflexes (May, 2006).

Diabetes mellitus (DM) is one of the most common chronic metabolic disorders, and affects more than 285 million people worldwide. Prolonged hyperglycemia caused by insulin insufficiency (type 1 DM or insulin-dependent DM) or insulin resistance (type 2 DM or non-insulin-dependent DM) also affect the metabolism of carbohydrate, protein, and lipid (Zhang, 2008). DM is associated with a number of complications, some of which affect components of the nervous system, including visual function. Visual loss, blurred vision, visual defects, and impaired visual acuity are commonly found in patients with DM (Negi and Vernon, 2003). There are also numerous reports of early visual neuronal abnormalities in DM, including histological, physiological, and clinical abnormalities (Antonetti, 2006; Ozawa, 2011). To date, however, few studies have focused on the efferent visual pathways involved in ocular motility and visual reflexes. Prior studies have revealed prolonged reaction times and slower eye movement reflexes, including the loss of eye fixation and gaze shift problem, which are controlled by the SC, in patients with DM (Virtaniemi, 1993; Alessandrini, 1999). Consequently, patients with these visual impairments have difficulties performing daily-life activities, complex task activities, learning, and cognition (Sanders and Gillig, 2009). Therefore, the aim of this study was to assess the ultrastructural changes in the SC in streptozotocin (STZ)-induced diabetic rats using transmission electron microscopy (TEM). So that, the hypothesis of this study was to demonstrate degeneration of neurons and nerve fibers in the SC. These damages will cause the impairment of the eye movement and reflexes in the diabetic patients.

MATERIALS AND METHODS

Twenty male adult Sprague–Dawley rats aged 5 to 8 weeks, weighing 200 to 270 g, were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakhonpathom. The Mahidol University Council's Criteria for Care and Use of Laboratory Animal was adhered to in this study. After acclimatization, the animals were divided into two groups, a DM (n = 10) and a positive control (n = 10) groups.

Rats in the DM group were intraperitoneally injected with a single dose of STZ (60 mg/kg body weight; Across Organics, Janssen Pharmaceuticals, Geel, Belgium) in citrate buffer at pH 4.5. Rats in the positive control group were injected with an equal volume of buffer per kilogram body weight. After a 10 h fast, the urine glucose levels and body weights were monitored daily. Whole-blood glucose levels were measured at 48 and 72 h after injection, and before sacrifice. The animals were sacrificed 4 weeks after injection, corresponding to the early period of DM by halothane inhalation.

The ultrastructural technique was described as in the previous studies (Lanlua, 2012; Sricharoenvej, 2012). After cutting the rib cage to expose the thoracic cavity, 0.1 M phosphate-buffered saline (PBS) was injected into the ascending aorta, and 500 ml of 2.5% glutaraldehyde in 0.1 M PBS was injected to preserve the tissues. Then, the SC was removed and cut into small cubes of about 1 mm^3. These specimens were postfixed in 1% osmium tetroxide in 0.1 M PBS, dehydrated in a graded series of ethanol, cleared in propylene oxide, and soaked in propylene oxide: araldite plastic. The specimens were then embedded in the araldite plastic. The tissue blocks were then sectioned on an ultramicrotome (Leica EM UC6; Leica Microsystems, Vienna, Austria). Semi-thin sections (1–1.5 µm) were stained with toluidine blue and representative areas were observed under a light microscope (Olympus BX41; Olympus, Tokyo, Japan). Next, the embedded specimens containing neurons were serially sectioned (80 to 85 nm thick) using the ultramicrotome and the thin serial sections were stained with 1% uranyl acetate and lead citrate. The neuronal ultrastructure on each SC tissue section was observed and photographed by TEM (JEOL JEM100S; JEOL Ltd., Tokyo, Japan).

Statistic analysis

Quantitative analysis of body weights in each group was expressed as a mean ± a standard deviation (SD). The comparison on the body weights of the positive control and the diabetic groups was performed by using Mann-Whitney U test (SPSS 16.0 software). The value of $p<0.05$ was considered to indicate statistical significance.

RESULTS

At 48 h after injection of STZ in the DM, the mean urine glucose concentration was > 500 mg/dL and the mean whole-blood glucose concentration was > 300 mg/dL, while those in the control were 0 and <300 mg/dL. The body weight was significantly lower in the DM group (256.43 ± 14.07 g) than in the control group (372.33 ± 11.64, $p< 0.05$).

TEM revealed that the ultrastructures of all layers of the SC in the control group were similar in appearance. The normal neurons had large, round, electron lucent nuclei with evenly dispersed fine chromatins and a large dense nucleolus. The nuclear membrane was generally smooth, although some membranes were wrinkled or invaginated. Numerous organelles were concentrically arranged around the nucleus (Figure 1A). By contrast, there were several changes in the neurons in all of the layers of the SC in the DM group. In this group, the neurons in each of the layers exhibited two major degenerative features: chromatolysis or pyknosis. The chromatolytic neurons were enlarged compared with the control neurons. Although most of the nuclear membranes of DM neurons were intact, the chromatin particles were distributed and

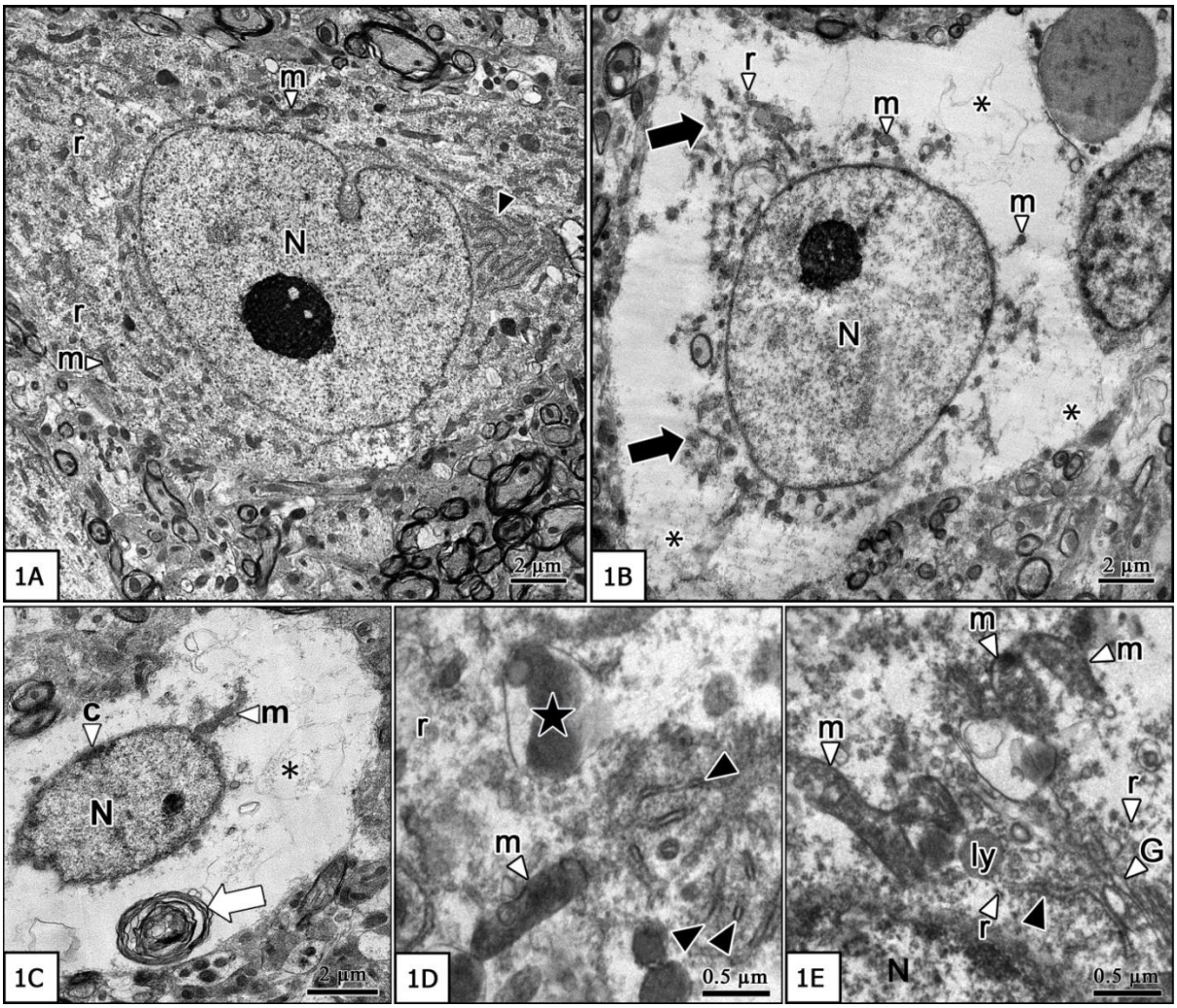

Figure 1. Transmission electron micrographs of SC cross-sections in the control (A) and DM (B–E) groups. Nucleus (N), mitochondria (m), rER (black arrowhead), ribosomes (r), unidentified particles aggregated around the nucleus (black arrows), empty peripheral areas (asterisks), chromatin clumping (C),a large membrane-bound vacuole (a white arrow), Golgi complex (G), primary lysosome (Ly), and a secondary lysosome (a black star).

often formed clumps beneath the nuclear membrane (Figures 1B, C and 2A). Regarding cytoplasmic changes, the SC neurons displayed a loss of organelles, including ribosomes, rough endoplasmic reticulum (rER), and mitochondria in the peripheral area of the perikaryon. The cell organelles also formed clusters around the nucleus (Figures 1B, C and 2A). At higher magnifications, rupture of the rER with short cisternae or small fragments, as well as ribosomal disintegration were also seen. Moreover, enlarged mitochondria with disrupted cristae or ruptured outer membranes were illustrated (Figures 1D and E). Distension of the Golgi complex was also clearly noticed (Figure 1E). Numerous, small secondary lysosomes with dark contents were visible in the cytoplasm (Figure 1D). Large membrane-bound vacuoles, containing a membranous structure, were also observed in the degenerated neurons (Figure 1C).

The second type of neurons in the DM group was pyknotic neurons. These neurons were dark, electron-dense cells with significant abnormalities of the nucleus and cytoplasm. The nuclei and organelles of these neurons were poorly defined, small, and had irregular outlines (Figure 2B). Moreover, the normal neuropils contained several neuronal and glial processes, including dendrites, myelinated axons, and unmyelinated axons (Figure 3A). Both types of axons contained abundant neurofilaments and microtubules, with long and extremely slender mitochondria, but rER and free ribosomes were not observed (Figure 3C). The neurons had enlarged dendrites containing vacuoles with electron-lucent regions (Figures 3B, D and E). The neurofilaments and microtubules were irregularly arranged and the cytoplasmic mitochondria were enlarged (Figures 3D and E). Demyelination was also apparent in the enlarged

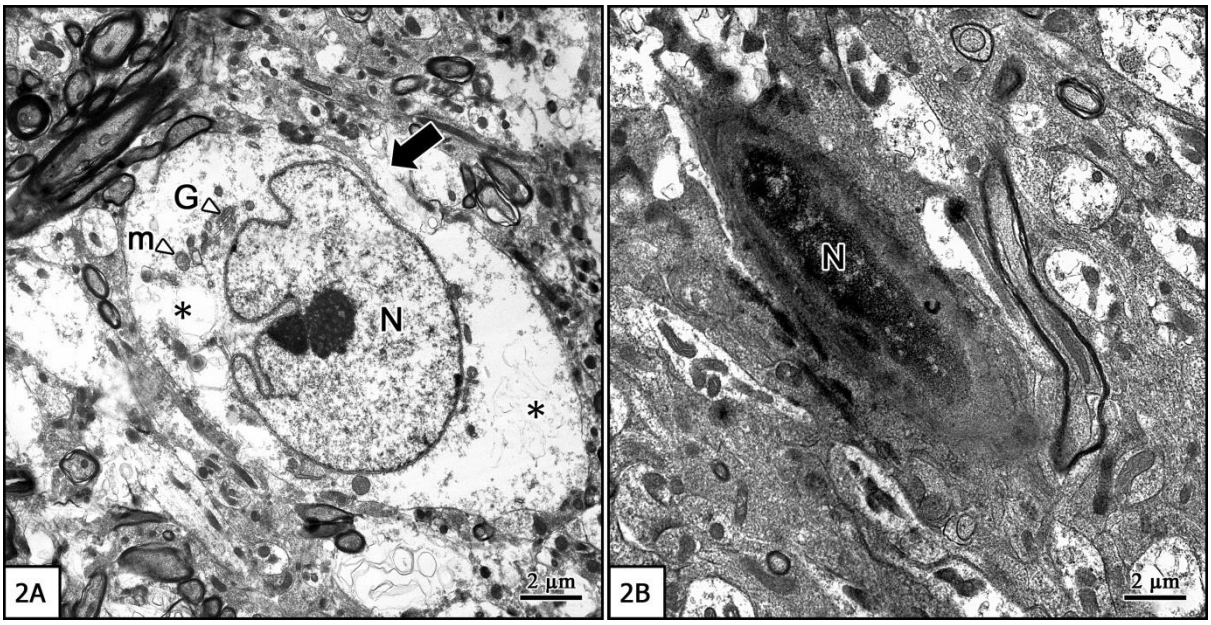

Figure 2. Transmission electron micrographs of chromatolytic neurons (A) and pyknotic neurons (B) in SC cross-sections in the DM group. Nucleus (N), mitochondria (m), Golgi complex (G), clear empty peripheral area (asterisks), and ruptured cell membrane (a black arrow).

neuropils (Figures 3B, D and E). The myelinated axons exhibited localized disarrangement of the myelin sheath, and of neurofilaments and microtubules in the axon cytoplasm (Figures 3D and E).

DISCUSSION

The STZ was used to induce the DM because of the selective destruction of pancreatic beta cells and inhibition of insulin synthesis. Therefore, hyperglycemia occurs due to insulin deficiency (Anderson, 1974; Junod, 1967; Yamamoto, 1981). The most common diabetic complication, which is caused by prolonged hyperglycemia, is microangiopathy. Then, the destruction of vascular wall and reduction of blood supply occur, that affects on the nervous system (Huber, 2006; Li, 1998). The degenerative SC neurons in DM rats in the present study could be classified into two types; chromatolytic and pyknotic neurons. Features of the chromatolytic neurons included enlargement, slight condensation of chromatin in the nucleus, clear cytoplasm with distended cell organelles, and destruction of the neurofilaments. Similar features were observed in the hypothalamic and dorsal motor nuclei of the vagus nerve neurons in previous studies of neurodegeneration (Bestetti and Rossi, 1980; Tay and Wong, 1994). Hyperglycemia in the diabetic state increases the accumulation of glutamate in the extracellular matrix, leading to glutamate excitotoxicity. Glutamate is taken up by neurons via *N*-methyl-D-aspartate (NMDA) and non-*N*-methyl-D-aspartate (non-NMDA) receptors (Portera-cailliau, 1997; Schurr and Payne, 2003). Binding of glutamate to NMDA induces chromatolysis by stimulating cellular intake of calcium ions (Ca^{2+}) that is released from the ER. The accumulation of Ca^{2+} near the cell membrane increases water influx into the neurons (Berridge, 1998) causing cell enlargement with a clear peripheral cytoplasm. Intracellular Ca^{2+}also acts as a second messenger to stimulate protease, lipase, and endonuclease activities (Sundaram, 2012). Intracellular Ca^{2+} also increases the nitric oxide (NO) concentration (Berridge, 1998). Elevated NO and Ca^{2+}concentrations in the mitochondria activate G-proteins, which stimulates the Ras and mitogen-activated protein kinases (MAPK) pathway. MAPK enters the nucleus, where it activates extracellular signal-regulated kinases, FOS, and Ced-3. This signaling pathway promotes the synthesis of proteases, endonucleases and phospholipases, such as calpain and caspase-3, which ultimately degrade the neurofilaments in axons and dendrites (Sundaram, 2012).

Hyperglycemia also increases the generation of reactive oxygen species (ROS), which activate and release cytochrome C from mitochondria to the cytoplasm. Cytochrome C stimulates the expression of caspase-3 (Davi, 2005), which increases the synthesis of endonucleases to cleave DNA, causing chromatin condensation (Huppertz, 1999). High levels of ROS also cause lipid peroxidation of unsaturated fatty acids in the lipid bilayers of cell and organelle membranes. This increases the permeability of the cell's outer membrane, as well as the membranes of the rER, Golgi complex, and

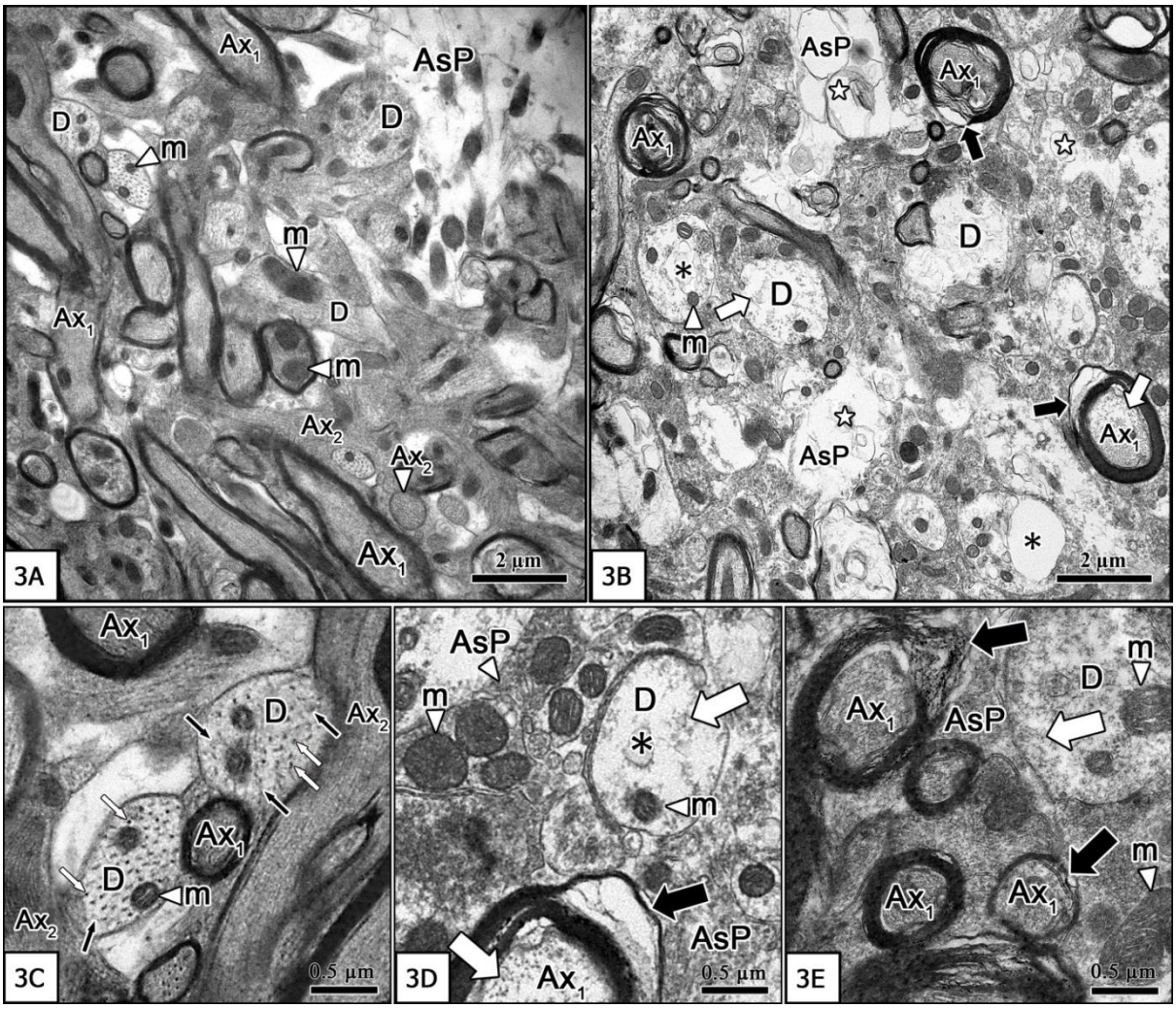

Figure 3. Transmission electron micrographs of SC cross-sections in the control (A,C) and DM (B,D, E) groups. Dendrites (D), myelinated axons (Ax1), unmyelinated axons (Ax2), mitochondria (m), astrocytic process (AsP), vacuolated appearances (asterisks), local disarrangement of myelin sheath (black arrows), degenerations of neurofilaments and microtubules (white arrows), large electron-lucent vacuoles (stars), microtubules (small white arrows), and neurofilaments (small black arrows).

mitochondria. The resulting influx of water causes enlargement of the neurons and cell organelles (Davi, 2005).

Numerous degenerated fragments of organelles in small lysosomes (autophagic membrane-bound vacuoles) in the neurons were observed because of the activity of hydrolytic enzymes. Some secondary lysosomes also contain degenerated membranous organelles, such as mitochondria, which form a concentric pattern known as the myelin figure. These myelin figures have been observed in anoxic-ischemic condition, in cells exposed to potent pro-apoptotic chemicals, and in the diabetic state (Park, 2003; Lanlua, 2012).

Other degenerative features of SC neurons in DM rats were chromatin condensation, cytoplasmic condensation with unidentified cell organelles, and cell shrinkage. These features were consistent with pyknosis or apoptosis, which were observed in previous studies of Park (2003) and Logvinov (2010). As mentioned above, glutamate accumulates in the extracellular matrix. Through an as-yet unknown mechanism, binding of glutamate to non-NMDA receptors increases the mitochondrial Ca^{2+}concentration (Saliñska, 2005). Intracellular Ca^{2+} also upregulates the expression of endonucleases, proteinases, and phospholipases, which leads to degradation of chromatin, rER, Golgi complex, cell membrane, and cytoskeleton in the neurons, as occurs in chromatolysis. These processes result in chromatin condensation and the destruction of cell organelles causes cytoplasmic condensation.

It is also notable that the levels of neurotrophic factors,

such as insulin, insulin-like growth factor-1, neurotrophin-3, and their corresponding receptors are lower in the diabetic state (Lee, 2001; Li, 2005). The changes in these signaling pathways leads to mitochondrial dysfunction, and promote the release of cytochrome C. Cytochrome C stimulates the production of endonucleases, which are responsible for DNA cleavage (Huppertz, 1999). Therefore, cell shrinkage occurs through a variety of processes involving the destruction of the cell membrane, cytoskeleton, and cell organelles.

In conclusion, the results of this study provide clear evidence for significant ultrastructural changes in SC neurons at the early stage of DM in rats. These changes are expected to contribute to the early neurodegenerative changes in the central nervous system in DM. These changes may also contribute to the visual impairments in DM, including autonomic eye movement, optokinetic reflexes, vestibulo-ocular reflexes, and vision-related learning and memory. The present results should provide a foundation for further research to develop therapeutic approaches to prevent or reduce the severity of nervous system disorders affecting vision and related processes that might improve the quality of life of patients with DM.

Conflict of Interest

The authors have not declared any conflict of interest.

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