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Transmission Electron Microscopy Studies of the Vestibulocochlear Nerve in Chronic Diabetic Rats

Estudios de Microscopía Electrónica de Transmisión del Nervio Vestibulococlear en Ratas Diabéticas Crónicas

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SUMMARY: It is widely described in the literature that diabetic patients present hearing impairment. Despite the histological alterations of the internal ear structures in these patients as well as in experimental models of diabetes, to the best of our knowledge, an histological evaluation of the vestibulocochlear nerve have not been performed. In the present study, ultrastructural alterations are described and compared between a spinal nerves and a cranial nerve in rats with chronic induced diabetes. Male Wistar rats (n = 12), fed with standard diet from the animal care facility at 42 days of age were used. Induced diabetic animals (n=6) were fasted for 12 hours prior to being injected intraperitoneally with streptozotocin (STZ - 60mg/kg) in a single dose. Control animals (n=6) received (0.01 mol/l citrate buffer, pH 4.5) vehicle alone. Ten weeks after STZ injection the animals were perfused intracardially with Karnovsky solution. Right and left vestibulocochlear nerves were dissected and histologically processed for epoxy resin embedding. Samples were imaged with the transmission electron microscope. Large myelinated fibers with morphological signs of axonal atrophy in the vestibulocochlear nerves were readily observed. These results suggest that chronic STZ-induced diabetes in rats caused alterations in the myelinated fibers and Schwann cells, compatible to the classic diabetes signs and symptoms. Morphological alterations of the vestibulocochlear nerve in diabetes is described for the first time and contributes information for a better understanding of why there are changes in hearing observed in diabetic patients.

KEYWORDS: Experimental diabetes; Vestibulocochlear nerve; Ultrastructure; Myelinated fibers; Axonal atrophy

INTRODUCTION

Among several metabolic diseases, diabetes is the affliction most commonly related with auditory disorders (Maia & Campos, 2005). Studies about the relationship between diabetes and auditory impairment have shown variable results (León-Morales *et al*., 2005). Nevertheless, the sensorineural hearing loss is one the most documented signs of the hearing impairment in diabetic patients (Maia & Campos; Celik *et al*., 1996; Friedman *et al*., 1975; Pessin *et al.,* 2008). Despite the well documented sensorineural hearing loss, controversy remains regarding the etiopathogenesis of the loss (Maia & Campos; Celik *et al.*) as two main theories have been under investigation for many years: angyopathy and neuropathy. Angyopathy in diabetic patients with hearing loss has been characterized by thickening of the basement membrane, particularly on the stria vascularis vessels (Costa, 1967; Jorgensen & Bunch, 1961; Makishima & Tanaka, 1971), as well as associated with endothelial proliferation and narrowing of vessel lumens. In regard to neuropathy, spiral ganglion neurons atrophy and demyelization on the 8th cranial nerve were observed in diabetic patients (Maia & Campos; Makishima & Tanaka). Animal models of diabetes have been widely used in expe-

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rimental studies because they offer promise of new insights into human diabetes (Salgado *et al.*, 2001). Streptozotocin (STZ)–treated rats are an animal model of insulin-dependent diabetes mellitus (IDDM) commonly used to study peripheral nervous system alterations even though the changes do not fully match the alterations observed in clinical conditions. Despite the failure to reproduce the widespread structural abnormalities in peripheral nerves observed in established neuropathy in man, animal studies can be informative as to the changes that develop in the early stages of human diabetes (Rodrigues Filho & Fazan, 2006; Sharma *et al.,* 1985). To investigate the auditory impairment in diabetic animals, brainstem acoustic evoked potentials were studied in STZ-diabetic rats (Rubini *et al.*, 1992) and mice (Hong & Kang, 2008), with similar results. But, to the best of our knowledge, a histological evaluation of the 8th cranial nerve (vestibulocochlear nerve) in STZ-diabetic rats has not been previously undertaken. In this study we have evaluated morphologic changes of the 8th cranial nerve in untreated STZ-diabetic rats and non-diabetic control animals over a 10-week period. The presence of neuropathy was assessed by transmission electron microscopy in the mid segment of the nerves from both right and left sides.

MATERIAL AND METHOD

Experiments were performed on male adult Wistar rats weighing 180-200 g at the beginning of the experiments. Animals were born and raised in a carefully regulated environment maintained at 21 to 23 ºC, 40 to 70 % relative humidity and 12/12 hour light/dark cycle. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ) (60 mg/kg; Sigma Chemical Co., St. Louis, Missouri, USA) on overnight-fasted rats (N = 6). High plasma glucose levels, excessive daily water intake and a large increase in urinary volume confirmed the diabetic state. Control rats (N = 6) received vehicle (0.01 mol/l citrate buffer, pH 4.5) injected intraperitoneally. Fasting blood glucose (mg/dl) was determined with a glucose analyzer (Beckman Instruments, Inc., Brea, California, USA) on the day of the experiment. Rats with fasting glucose levels higher than 150 mg/dl were considered diabetic.

Ten weeks after STZ injection the animals were anesthetized with sodium pentobarbital (Nembutal, 40 mg/kg, i.p.) and initially perfused through the left ventricle first with a phosphate buffered saline 0.05 M solution, pH 7.4 and then with a 2.5 % glutaraldehyde and 4 % paraformaldehyde solution, in 0.1 M cacodylate buffer, pH 7.2. All procedures adhered to The Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication nº 80-23, revised 1978) and the norms of the Ethics Committee for Animal Research of the Federal University of Pernambuco State, Brazil (protocol number 23076.015775/2005-14). Every effort was made to minimize the suffering of the animals as well as the number used. The 8th cranial nerves from both sides were dissected from the spiral ganglion through the point they enter the brain stem. Nerves were placed in the fixative solution for an additional 12 hours. They were then washed in cacodylate buffer, pH 7.2 and their distal segments (close to the brain stem) were excised and processed for epoxy resin embedding (EMbed 812®, Electron Microscopy Sciences, Hatfield, PA, USA) as described elsewhere (Alcântara *et al.*, 2008; Campos *et al.,* 2008). Methods for histological preparation of the nerves were previously described (Fazan *et al.*, 2001; Fazan *et al*., 2002; Fazan *et al.,* 2009; Sato *et al*., 2006). Briefly, before embedding, nerves were oriented to permit semi-thin (0.5 to 1.0 µm thick) transverse sections of the fascicles, which were stained with 1% toluidine blue and observed under the oil immersion lens of an Axiophot II photo-microscope (Carl Zeiss, Jena, Germany). Light microscopy was carried out in order to monitor for high quality of the histological preparation of the nerves. For transmission electron microscopy, thin transverse sections were mounted on 300 mesh copper grids, stained with lead citrate and uranyl acetate, and observed using a JEOL JEM-1230 transmission electron microscope (JEOL-USA, Inc., Peabody, MA, USA), equipped with a digital camera.

RESULTS

Body weight and plasma glucose levels for control and diabetic animals are shown in Fig. 1. Control animals significantly increased body weight from the day of injection to the day of the experiment. Diabetic animals also increased body weight during the 10-week period but there was a significant difference between diabetic compared to controls at the end of the experiment. Diabetic animals gained less weight compared to controls. As expected, plasma glucose levels were significantly increased in STZ-injected animals compared to controls during the 10-week experimental period.

All nerves included in this study showed good preservation of structures and consisted of a single fascicle in both experimental groups. The perineurium was found to be very thin consisting of one layer of flattened connective tissue cells. The endoneurium consisted mainly of longitudinally oriented collagen fibers that occupied much

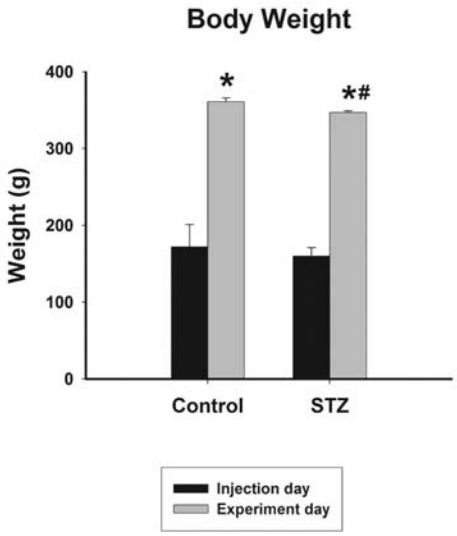
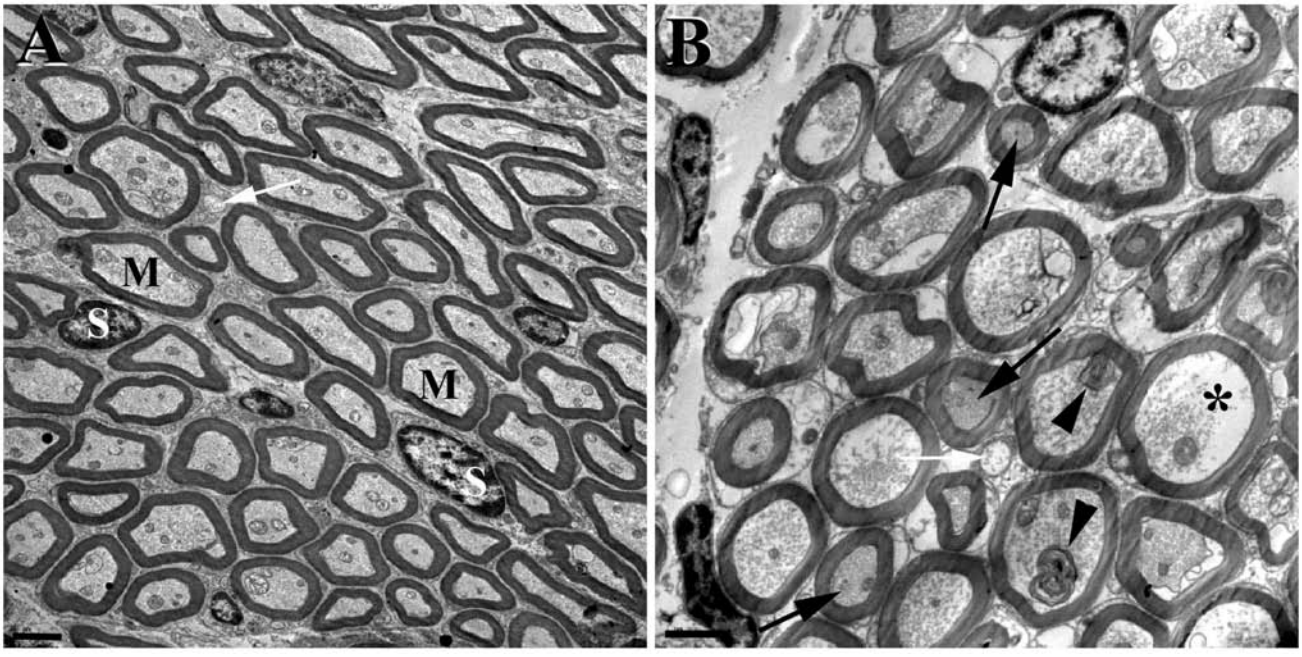


Fig. 1. Upper panel: Body weight on the day of injection (streptozotocin (STZ) or vehicle) (black bars) and 10 weeks later (gray bars). Lower panel: Fasting blood glucose level on the day of injection (STZ or vehicle) (black bars) and 10 weeks later (gray bars). * indicates significant difference compared to the day of injection. # indicates significant difference compared to control group (N = 6 for both groups).

of the space between the myelinated and unmyelinated axons (Fig. 2). Most of the myelinated axons were of large size, intermingled with small caliber myelinated ones. Unmyelinated fibers were rarely seen. No morphological differences were observed between sides in the same group. STZ animals showed large myelinated fibers with clear signs of axonal atrophy (Fig. 2) and swollen Schwann cells. Myelin infolds and balls of myelin were also present (Fig. 2) as were swollen large axons. The unmyelinated fibers were better preserved, despite the presence of some Schwann cells devoid of axons and/or with vacuoles in the cytoplasm. No myelinated or unmyelinated axonal sprouts were evidenced. There was no indication of endoneural capillary damage in the vestibulocochlear nerves of STZ-injected animals. Pericytes were present and mostly normal.

Fig. 2 Electron micrographs of vestibulocochlear nerves from male adult Wistar rats. In the endoneural space of control nerves (A), note the presence of large myelinated fibers (M), Schwann cell nuclei (S) and few unmyelinated fibers (white arrow). The diabetic animals' nerves (B) showed myelinated fibers with clear signs of axonal atrophy (black arrows), myelin infolds (arrowheads) and axoplasmic swelling (*). The white arrowhead points to a normal unmyelinated fiber. Bars = 2 μm.



DISCUSSION

Despite our study being limited to 10 weeks of diabetes, previous studies from our laboratory (Salgado *et al.*; Fazan *et al*., 2006) have demonstrated that 15 days of diabetes is long enough for observing the first morphometric signs of axonal atrophy in this model of diabetes. Axon diameter is thought to be the most important parameter of conduction velocity (Minwegen & Friede, 1984). Although we have not measured nerve conduction velocity in this study, our ultrastructural observations clearly indicate a reduction in the axon size which might contribute to the impaired function of brainstem acoustic evoked potentials observed in rodent models of diabetes (Rubini *et al*., 1992; Hong & Kang, 2008). Our results suggest that there is a vestibulocochlear neuropathy in the STZ-diabetic rat and that this may be an axonopathy, as suggested for other nerves in this diabetes model (Fazan *et al.*, 2006; Bhoyrul *et al*., 1998; Britland *et al.*, 1985; McCalum *et al*., 1986).

The STZ-diabetes model is widely used to investigate the experimental diabetic peripheral neuropathies (Jakobsen, 1979; Jakobsen & Lundbaek, 1976; Sharma *et al*., 1977; Sugimura *et al*., 1980) but few studies have performed a detailed assessment of unmyelinated fibers or capillary morphology in this animal model (Fazan *et al*., 2009). In addition, the ultrastructure of the vestibulocochlear nerve was investigated for the first time. Rodrigues Filho & Fazan demonstrated by light microscopy, axonal atrophy on the phrenic nerves large myelinated fibers. A posterior ultrastructural study of these nerves (Fazan *et al*., 2009) added the evidence of small myelinated fiber neuropathy due to the STZ injection which was clearly associated with severe damage to endoneural vessels in diabetic animals. The present study confirms the axonal atrophy observed by Rodrigues Filho & Fazan but not the blood vessel damage. This could be due to the fact that our STZ-injected animals presented mild diabetes (blood glucose levels between 150 and 250 mg/dl) compared to those in the Fazan *et al.* (2009) study (blood glucose levels above 350 mg/dl) and also that Fazan *et al*. (2009) studied a longer time period of induced diabetes than the present study. This comparison suggests that the blood vessels damage might be related to a higher blood glucose level and the axonal atrophy observed in both investigations is related to the hyperglycemia. Morphological alterations of the vestibulocochlear nerve in experimental diabetes is being described for the first time and such information corroborate to a better understanding of changes in hearing observed in diabetic patients.

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VASCONCELOS, C. C. A.; FAZAN, S. P. V.; MOORE. K. C.; NESSLER, R. A. & VALENÇA, M. M. Estudios de microscopía electrónica de transmisión del nervio vestibulococlear en ratas diabéticas crónicas. ***Int. J. Morphol., 29(1)***:272-277, 2011.

RESUMEN: Se ha descrito ampliamente en la literatura que los pacientes diabéticos presentan discapacidad auditiva. En estos pacientes, a pesar de las alteraciones histológicas de las estructuras del oído interno, así como en modelos experimentales de diabetes, que mejoran nuestro conocimiento, la evaluación histológica del nervio vestibulococlear no ha sido realizada. Se describen y comparan las alteraciones ultraestructurales entre un nervio espinal y uno craneal en ratas con diabetes crónica inducida. Fueron utilizadas 12 ratas Wistar machos, de 42 días de edad, alimentadas con dieta estándar. Los animales diabéticos inducidos (n = 6) se mantuvieron en ayuno por 12 horas antes de ser inyectados por vía intraperitoneal con estreptozotocina (STZ - 60mg/kg) en una sola dosis. Los animales control (n = 6) sólo recibieron inyección de 0.01 mol/l buffer, citrato pH 4,5. Diez semanas después de la inyección de STZ, los animales fueron perfundidos intracardiacamente con solución de Karnovsky. Los nervios vestibulococlear derecho e izquierdo fueron disecados y procesados histológicamente para ser incluidos en resina epoxy. Las muestras fueron estudiadas con microscopio electrónico de transmisión. Fueron observadas fácilmente, grandes fibras mielinizadas con signos morfológicos de atrofia axonal en los nervios vestibulococlear. Estos resultados sugieren que la diabetes crónica inducida por STZ en ratas causó alteraciones en las fibras mielínicas y células del neurilema, compatible, con los signos y síntomas clásicos de la diabetes. Alteraciones morfológicas del nervio vestibulococlear en la diabetes son descritas por primera vez, lo que aporta información para una mejor comprensión de por qué hay cambios en la audición en los pacientes diabéticos.

PALABRAS CLAVE: Diabetes experimental; Nervio vestibulococlear; Fibras mielinizadas; Ultraestructura; Atrofia axonal.

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