

# Quantitative morphological characterization of the tibial nerve in diabetic rats

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RESEARCH

## ABSTRACT

The streptozotocin diabetic rat model has been widely used to study the etiology, pathology and treatment of diabetic neuropathy. There are however, discrepancies among authors with regard to axonal and myelin sheath damage found in the affected peripheral nerves. To quantify the morphological changes produced in the axon and in the myelin sheath of the nerve fibers at the tibial nerve of diabetic rats at 10 and 18 weeks of evolution of diabetes mellitus, diabetes mellitus was induced by the injection of streptozotocin in 12-week-old Wistar rats. A morphometric computerized analysis on myelinated nerve fibers was performed at 10 and 18 weeks of evolution of diabetes mellitus in diabetic animals and in age-paired controls. Morphological changes were quantified by the axonal area, axonal perimeter, form of the axon, as well as by the myelin area, using transversal semithin sections. A reduction of the axonal caliber and the area covered by myelin was observed in diabetic animals compared to age-paired controls. The greatest irregularity observed in nerve fibers of diabetic animals showed the occurrence of greater damage in the axonal area than in the perimeter of axon. This result could indicate a greater effect of diabetes on the cytoskeleton of fiber than on its axolemma. A greater damage of the axon than that of the Schwann cell was evidenced at both time points studied. At 10 weeks, a reduction of a 13.4% for the axonal area and of a 6.7% for the myelin area were found, while 18 weeks after inducing diabetes mellitus a decrease of 16.8% for the axonal area and of 11.2% for the myelin area were observed. The morphological changes of the nerve fibers were greater after 18 weeks of inducing diabetes compared to the 10 weeks of evolution of the disease, thus, an increase in the severity of damage with age was evidenced. Our results indicate the presence of axonal and myelinic mixed neuropathy in the diabetic rat, having mainly an axonal damage.

**Key words:** Axon, Diabetes mellitus, Morphometry, Myelin, Streptozotocin, Tibial nerve

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## RESUMEN

**Caracterización morfológica cuantitativa del nervio tibial en ratas diabéticas.** El modelo de ratas tratadas con estreptozotocina se ha utilizado con mucha frecuencia en el estudio de la etiología, la patología y el tratamiento de la neuropatía diabética. Sin embargo, existen discrepancias entre los autores en cuanto al daño que presentan el axón y la cubierta de mielina de los nervios periféricos cuando existe esta enfermedad. Se decidió cuantificar el daño morfológico que presenta el axón y la cubierta de mielina de la fibra nerviosa en el nervio tibial de ratas diabéticas a las 10 y 18 semanas después de inducida la diabetes mellitas, luego de la pubertad. En ratas Wistar, con 12 semanas de edad, se indujo un cuadro de diabetes mellitas, mediante una inyección de estreptozotocina y se evaluaron las variables: área, perímetro y forma del axón, así como el área de la vaina de mielina de las fibras nerviosas mielínicas, en cortes semifinos transversales. Se evidenció una disminución del área, del perímetro axonal y del área ocupada por la mielina en los animales diabéticos, en comparación con los animales control pareados en edad, así como una mayor irregularidad de las fibras nerviosas en los animales diabéticos. La alteración morfológica en el axón y en la vaina de mielina de la fibra nerviosa de los animales con diabetes, fue superior en la semana 18 con respecto a la semana 10. A las 10<sup>ma</sup> y 18<sup>va</sup> semanas de inducida la diabetes mellitus, se observó una mayor afectación del axón que la de la célula de Schwann. Se constató la presencia de neuropatía mixta axonal y mielínica en las ratas diabéticas, con una afectación principalmente axonal.

**Palabras claves:** axón, diabetes mellitus, estreptozotocina, mielina, morfometría, nervio tibial

## Introduction

Diabetic neuropathy is one of the most common complications of diabetes mellitus (DM). The model of streptozotocin (STZ) diabetic rats has been very frequently used to study the etiology, pathology and treatment of diabetic neuropathy [1-4]. Biochemical, physiological and morphological alterations of the most relevant markers of diabetic neuropathy, have been demonstrated at the neuronal level in those rodents [5-8]. However, there is disagreement among

specialists regarding the contribution of the axon and myelin sheath to the damage of myelinated peripheral nerve fibers in this disease.

According to most specialists, when DM is induced after puberty, the damage of axonal size (diameter, area), is greater than the damage to the myelin sheath (area and diameter of the transversal section) [9]. In contrast, other researchers have shown that the reduction in size of the nerve fiber in diabetic animals

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is predominantly caused by a reduction in the myelin sheath [10, 11]. Other investigations have revealed different findings [12, 13]. Medori and coworkers (1988) reported a proximal increment and a distal decrease in the thickness of the myelin sheath [12]. A certain reduction in the diameter of the axon and an absolute increase in the thickness of myelin, leading to a preservation in the size of the fiber have been also reported [13]. These disagreements could respond to differences in the research protocols used such as: age of DM induction, DM duration and the employed used for morphometric evaluations. The evaluation of any therapeutic intervention in diabetic rats requires the standardization of a model for the disease by every research team. The present study was designed to quantify the morphological damage in the axon and myelin sheath of the tibial nerve of rats with DM induced after puberty, at 10 and 18 weeks of disease evolution.

## Materials and methods

### Experimental groups and induction of diabetes mellitus

Wistar male rats (CENPALAB, Cuba), fed *ad libitum* with food and water were used in all experiments. Two experimental groups with diabetic rats were established (10-weeks DM and 18-weeks DM); two groups of age-matched healthy controls, and a week 0 control group was also included. Diabetes was induced at 12 weeks of age by an intraperitoneal injection of STZ (Sigma) (65 mg/kg body weight) [14]. Seven days after the injection, glucose levels were measured in the blood of the control and treated animals (Reflotron, Boehringer Mannheim). STZ treated animals with glucose levels in the blood above 15 mmol/L, were considered as diabetic [15]. All animals were weighed monthly (Sartorius, Alemania) and water and food consumption was qualitatively monitored through the experiment.

### Morphological study

Animals were anesthetized with chloral hydrate (420 mg/kg, i.p.). Samples were fixed by aortic perfusion: each rat was perfused with 250 mL of saline, followed by 300 mL of the fixing solution (4% paraformaldehyde and 1% glutaraldehyde in phosphate buffer 0.1 mol/L pH 7.4. Tissue samples were taken by the dissection of the right posterior tibial nerve and kept in the same fixing solution for 3 hours at 4 °C. Afterwards, nerve fragments were washed with phosphate buffer, fixed by incubation in 2% OsO<sub>4</sub> for 1 hour at 4 °C, dehydrated in ethanol, treated with propylene oxide and finally embedded in epoxy resin (Durcupán, ACM, Fluka, Switzerland). 1 mm thick semithin transversal sections were sliced with an ultramicrotome (Ultratome IV, LKB, Sweden). Tissue sections were stained with 1% p-phenyldiamine.

### Morphometry

Quantitative microscopic analysis were carried out in an OLYMPOS AH2 microscope (objective 100x), coupled to a video camera and using the DIGIPAT image analysis system (EICISOFT, Cuba). Fields containing the fibers were randomly and systematically

selected. The first field was randomly chosen in a 0 to 6 interval and those separated by 6 fields were selected subsequently [16-18]. A total of 400 fibers was processed for each nerve [19]. The following parameters were measured: axonal area (XA), axonal perimeter (XP), myelin area (MA) and the circularity index (CI) was calculated [ $CI = XA / XP$  (calculated from XP)].

### Statistical analysis

A descriptive data analysis was conducted and the results were expressed as the mean  $\pm$  standard error for every variable evaluated. A variance analysis (ANOVA) ( $\alpha = 0.05$ ), and Duncan's multiple ranges test was applied for group comparison.

## Results

### Clinical observations

The administration of a STZ injection in rats induced a clinical picture of DM, characterized by polydipsia, polyuria, a significant increase in blood glucose levels and a loss of body weight, compared to control animals (table 1). Only two out of 22 rats injected with STZ, were excluded from the study due to glucose levels below the lower limits according to the inclusion criteria (15 mmol/L). Both cases were excluded at the beginning of the experiment. Mortality among diabetic animals during the 18 weeks of the experiment was 40%.

### Morphological study

#### Axonal size

Mean values for the axonal area and perimeter increased with age in either healthy or diabetic animals ( $p < 0.01$ ). However, those values were significantly lower in diabetic rats than in age-matched healthy animals. This finding was consistent at either 10 or 18 weeks after DM induction ( $p < 0.01$ ). These differences were not observed for XP at week 10 of disease evolution (Figure 1). The differences found for XA between healthy and diabetic rats were 13.4% and 16.8% for 10 and 18 weeks DM, respectively.

#### Axonal form

No significant changes related to age were observed in the CI of healthy and diabetic animals. ( $p > 0.05$ ). The

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Tabla 1. Level of glucose in the blood (Glu) and body weight

	DDM Duration (weeks)	Weight (g)		Glu (mmol/L)		
		Initial values	Final values	Initial values	Final values	
	0	289.7	$\pm$ 0.8	7.79	$\pm$ 1.42	-
Healthy rats	10	432.1	$\pm$ 0.7	8.34	$\pm$ 0.56	8.93 $\pm$ 1.28
	n = 15	18	440.5 $\pm$ 0.9	7.32 $\pm$ 1.12	9.80 $\pm$ 0.62	
Diabetic rats	10	220.7	$\pm$ 0.9	20.97	$\pm$ 2.13	26.70 $\pm$ 0.75
	n = 12	18	275.7 $\pm$ 0.6	21.28 $\pm$ 1.89	30.65 $\pm$ 1.22	

Values are expressed as the mean  $\pm$  standard error. n: number of animals.

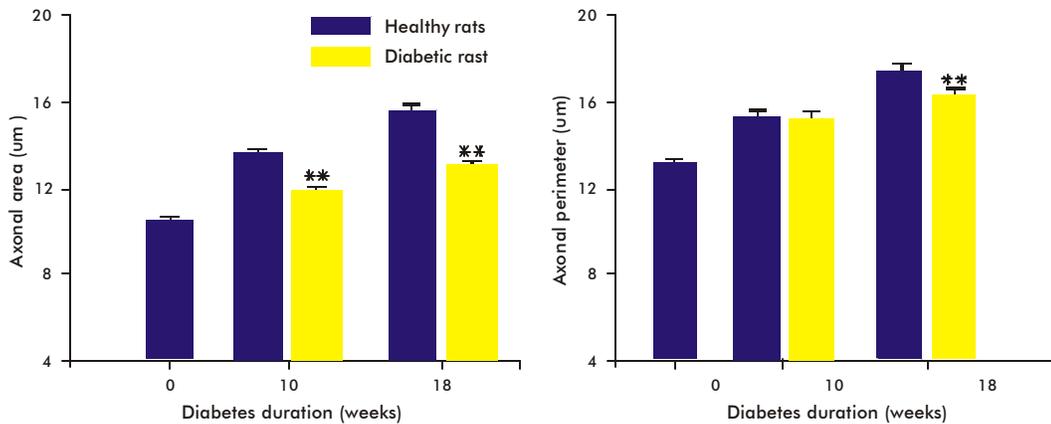


Figure 1. Mean  $\pm$  standard error of axonal area and axonal perimeter of the posterior tibial nervous fiber, in healthy and diabetic animals. \*\* Differences with age paired healthy control animals ( $p < 0.01$ ).

values of this parameter in diabetic rats were significantly lower than those of the age-matched healthy controls and also lower than those in the week zero healthy control group ( $p < 0.01$ ) (Figure 2).

#### Myelin area

Myelin area (MA) increased significantly with age in both healthy and diabetic animals ( $p < 0.01$ ), except between 10 and 18 weeks after DM induction, where no significant increase was observed ( $p = 0.12$ ). The MA was significantly lower in diabetic animals than in the healthy controls of the same age ( $p < 0.01$ ) (Figure 2). Differences between healthy and diabetic rats were 6.7% and 11.2% at weeks 10 and 18 after DM, respectively.

#### Discussion

The process of growth and development in rats extends to a great part of their lives and includes the morphofunctional maturation of peripheral nerves [20, 21]. The instauration of a DM clinical picture in those animals leads to morphological alterations that interfere with the growth process. The morphometric analysis of myelinated peripheral nerve fibers has shown a

reduction in caliber in diabetic animals compared with age matched healthy controls. However, divergent criteria do exist regarding the extent of the contribution of the axon and Schwann cells to these changes. The greatest alterations of axon and myelin sheath are linked to the fast development stage of those structures. This reduction in myelinated peripheral nerve fibers has been considered a maturational deficit secondary to the pathologic insult of diabetes.

In this study, the size of the nerve fiber had a significant increase with age in either healthy or diabetic animals. This was based on an increase in axonal caliber and in myelin sheath area. It is an evidence of the growth and development processes taking place in the peripheral nerves during an important part of postnatal life [22]. Those results agree with studies in healthy rats where the growth of the axonal caliber was observed from week 3 (after study initiation) until 9 month of age [20]. Authors have divergent criteria regarding the behavior of myelin sheath growth in healthy rats. Fraher and coworkers (1990) described a rapid increase in myelin sheath thickness, reaching its maximum values at 3 months of age [20]. Those results do not agree with our findings or those of other

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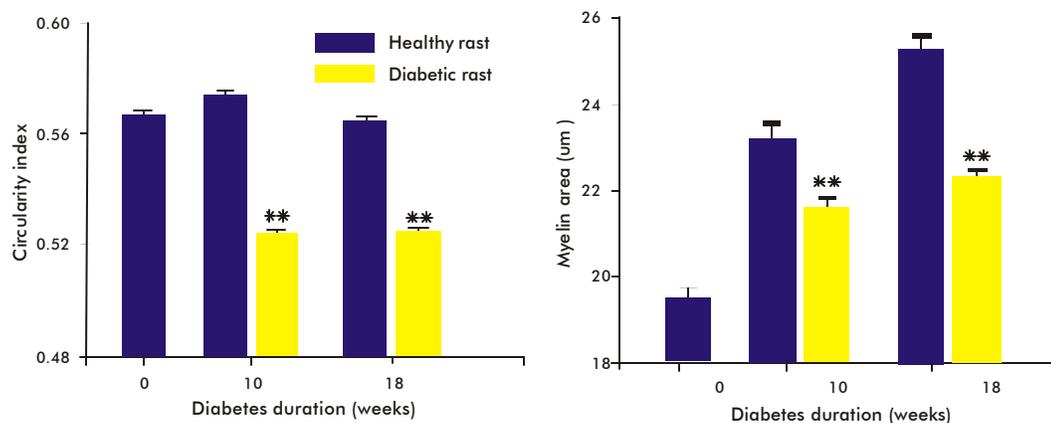


Figure 2. Mean  $\pm$  standard error of circularity index and myelin area of tibial posterior nerve fibers in healthy and diabetic animals. \*\* Differences with age matched healthy control animals ( $p < 0.01$ ).

authors, which indicate a continuous increase in thickness and myelin sheath area 3 months after birth [11, 23]. Moreover, it was shown that experimental DM do not halt peripheral nerves growth, but only slow down this process. This was demonstrated by the observation that the increase in the caliber of fibers (XA and MA) in diabetic animals is less than that observed in the matched controls of the same age. The MA of diabetic animals do not increase significantly between 10 and 18 weeks of DM evolution, although the tendency towards an increase was maintained.

The alteration in axon caliber, expressed by the lower value of XA in diabetic animals compared to age-matched healthy controls, was demonstrated at 10 and 18-weeks DM. The same findings have been reported before by other authors [24-26]. In those studies where DM was induced post-puberty, lower XA values have been found in different nerves from diabetic animals as compared to controls at 12, 20 and 28-weeks DM [24-26]. Likewise, the lack of differences at 2, 4, 6, 8 and 17-weeks of DM in sciatic, tibial, lateral plantar and common peroneal nerves, between diabetic and age-matched control animals had also been reported [11, 23, 26]. The authors ascribed the absence of a difference in XA between healthy and diabetic animals with 17 weeks of evolution, to the late induction of DM (20-21 weeks of age), a period where axonal growth is not a very active process [23]. They thus concluded that at this stage, XA is less vulnerable to damage caused by this disease as compared to XA in studies where DM is induced early, where differences between healthy and diabetic animals have been described from week-12 DM. According to previous data, a certain period of time with DM is required before detecting signs of XA damage, but this time period could be influenced by the age of animals at DM induction.

In regard area, XP represents an indicator of the axon caliber; however, XA represents the axoplasm of the fiber, the perimeter of its axolemma. Most of researchers have reported a reduction of XP in diabetic animals, in compared to the control animals from week-12 after disease induction, but not at 4, 6 and 8 weeks after the induction of diabetes [11, 27]. The results of the present study demonstrate alterations in the axolemma of the fiber at 18-weeks DM but not at 10-weeks DM.

In this experiment, the axonal form did not show variations related to age in either diabetic or control animals, which is in agreement with previous results

[20]. However, a tendency of CI to decrease during development has been reported [28]. The greater irregularity of the nerve fibers in diabetic animals compared to controls, suggest a greater alterations in XA (axoplasm) and XP (axolemma). Several hypotheses have been advanced to explain this irregularity. Experimental data support axonal shrinkage by dehydration, due to tissular hyperosmolarity [24, 29], whereas other authors support growth deficit hypothesis as the main explanation for this greater irregularity [27].

Axonal caliber is basically determined by neurofilaments [30]. It has been described that the initial formation of myelin in the myelinated fibers is determined by the axonal caliber and this influences the thickness and total volume of the myelin sheath. In the present study, significant differences in the MA between diabetic and control animals were found at either 10 or 18-weeks DM, which is similar to previous findings made by others researchers [11, 23, 27].

Several authors hold that when DM is induced during post-puberty, more irregularities in the axonal caliber are found as compared with the function of Schwann cells or the growth of myelin sheath [24, 25, 31]. In contrast, others have observed more alterations in the Schwann cell than in axonal caliber in post-puberty induced DM [11, 23]. The greater alteration of these morphological variables seems to be associated to a stage of faster growth; therefore, the age of DM induction is an important parameter which should be taken into consideration when comparing data provided by different experiments. In this study axonal plus myelinic damage was observed, with an axonal predominance. At 10-weeks after DM, a 13.4% reduction for XA compared with age-matched controls and 6.7% for MA were observed. At week-18 DM, 16.8% of the damage was detected for XA and 11.2% for MA. When severity in neuropathic damage was compared between 10 and 18-weeks DM, a greater alteration of XA and MA parameters was found at the latter time point. On the other hand, regarding myelin, no significant differences were found in diabetic rats between 10 and 18-weeks after DM induction, but these differences were well documented in healthy control animals.

The overall results of this study indicate that diabetic rats displayed a mixed axonal-myelinic neuropathy, with predominance of axonal damage and an increase in the severity of neuropathy with the duration of DM.

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