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Effects of metabolic syndrome on the ultrastructure of the femoral nerve in aging rats

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Summary. The aim of the present study was to characterize the morphometry of the femoral nerve in aging rats with metabolic syndrome compared to controls. Systolic blood pressure and fasting plasma glucose were measured, and myelinated and unmyelinated fibers in the femoral nerves were quantitatively assessed under electron microscopy. Aging rats exposed to a regimen of metabolic syndrome developed elevation of plasma glucose concentration, mild hypertension and polyneuropathy characterized by a decrease in myelin fiber area, axon diameter, myelin sheath thickness and myelin fiber loss in the femoral nerve. The histogram of size distribution for myelinated fibers and axons from the aging rats of the control group was bimodal. For aging MS animals, the histogram turned out to be unimodal. The ultrastructure of unmyelinated fibers and of Schwann cells in 18-month-old rats was well preserved. Granules of lipofuscin were seen in unmyelinated fiber axons of 18-month-old rats with MS. The damage percentage of the large myelinated fibers has increased significantly in 18-month-old and 18-month-old (MS) rats in relation to the controls. No significant difference was observed among the groups for the g-ratio. Comparing the three groups, the number of neurotubules and neurofilaments in myelinated fibers of 18-month-old rats with MS was significantly smaller than for the groups of 18-month-old and 14-month-old rats. The overall changes seen in the femoral nerve from aging rats seem minor compared to the changes in the aging rats with MS, suggesting that long-term MS accelerates the progressive modifications in peripheral nerves that develop in old age.

Key words: Diabetes, Peripheral nerve, Morphometry, Aging rats

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Introduction

Overconsumption of fructose, particularly in the form of soft drinks, is increasingly recognized as a public health concern (Brown et al., 2008; Agrawal and Gomez-Pinilla, 2012). A high fructose diet causes numerous pathological changes, including oxidative stress and metabolic syndrome (Ross et al., 2009).

Metabolic syndrome (MS) is a collection of cardiometabolic risk factors that includes obesity, insulin resistance, hypertension, and dyslipidemia. Although there has been significant debate regarding the criteria and concept of the syndrome, this clustering of risk factors is unequivocally linked to an increased risk of developing neural and cardiovascular disease (Kawamoto et al., 2005). Peripheral neuropathy, with clinical manifestations of pain, sensory, autonomic and even motor dysfunctions, is a major common complication of MS (Sumner et al., 2003; Tracy and Dyck, 2008; Tesfaye et al, 2010; Dyck et al 2011; Papanas et al, 2011; Callaghan et al., 2012; Smith, 2012).

Several authors have studied the impact of MS on nerve structure in models with young animals (Dockery

and Sharma, 1990; Ochodnická et al., 1995; Imada et al., 2007) but a follow-up study using an aging animal model has not been performed yet to study the adaptive peripheral nerve changes of MS.

We have designed the present study in male Wistar rats fed high-fructose diets in order to establish the role of metabolic state in the pathogenesis of polyneuropathy in an aging model. The rats fed conventional diets were used as control. In these groups, time courses of body weight, systolic blood pressure (SBP) and fasting plasma glucose, were measured in a parallel manner during four months. Following these experiments, a morphometric study of large and small myelinated fibers in the femoral nerve fibers was assessed under electron microscopy. Morphometric data, such as the nerve area, number of myelinated fibers, diameter of the myelin sheath and of the myelinated axon and density of neurotubules and neurofilaments in rats undergoing MS were studied for a period of four months.

Materials and methods

Animals and treatment

The experiments were performed on male Wistar rats (*Rattus norvegicus*) purchased from the Laboratory Animal Center of São Judas Tadeu University, São Paulo, Brazil. The animals were housed in plastic cages with access to food and water ad libitum and maintained on a 12h light/dark cycle at room temperature (23-26°C). The experimental protocols were approved by the Institutional Animal Care and Use Committee of the university. The present study was carried out in accordance with the National Institute of Health Guidance for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996.

The animals were randomly divided into three groups (n=8) at the age of 14 months. A group (14-month-old) was sacrificed immediately. The others were assigned to either the 18-month-old group or the 18-month-old MS group. Rats in the MS group were fed with a conventional diet and water with fructose (D-fructose, 100g/L) (Suzuki et al., 1997), for 16 weeks, to induce the metabolic syndrome. The consumption of fructose was measured every two days by subtracting the total volume offered from the remaining volume. Body weight and food consumption of each rat per day-and-night were measured regularly. The 14-month-old rats were raised under usual laboratory conditions until the age of 18 months.

Measurement of SBP and glucose

The body weight of each rat was measured at the end of the experiment. The SBP was verified every four weeks by using the tail cuff plethysmography method. Blood was collected for glucose analysis. The animals had their blood glucose measured by glucometer (Accu-Chek Active, Roche). Rats were considered diabetic when the blood glucose levels were greater than 250 mg / dL three days after induction.

Electron microscopic examination of nerve fibers

Under pentobarbital anesthesia (40-50 mg: kg; i.p.), a small piece of the right femoral nerve was excised from the proximal end and fixed in 2.5% glutaraldehyde. This was later osmicated in 1% OsO_4. Semithin cross sections were cut at about 1 mm thickness from the specimen embedded in the Epon-812 resin and stained with toluidine blue after trimming carefully under the light microscope. Ultrathin sections (40-60 nm) were cut perpendicularly to the axis of nerve fibers. They were observed after staining with uranyl acetate and lead citrate, and microphotographs were taken under an electron microscope (JEOL Ltd., Japan). Pathological changes were observed, including myelin lamina rarefaction, focal demyelination or vacuolization.

Morphometric evaluation

The area of each nerve was measured by using an image-processing system (Axio Vision, Zeiss) at x50 magnification. The total numbers of myelinated fibers was counted, and the average diameters of individual axons (one-half of the sum of the measured maximum and minimum diameters) were measured from photographic prints (x1500). The ratio of axon diameter to the total fiber diameter (g- ratio) was also calculated for the three groups.

The density of myelinated fibers was calculated from the area of the nerve and the total number of myelinated fibers. Five electron photomicrographs of myelinated fibers per animal were taken (x50,000). The neurotubules and neurofilaments were counted over a representative unit rectangular sampling area (2.16 μm^2) (Fig. 1).

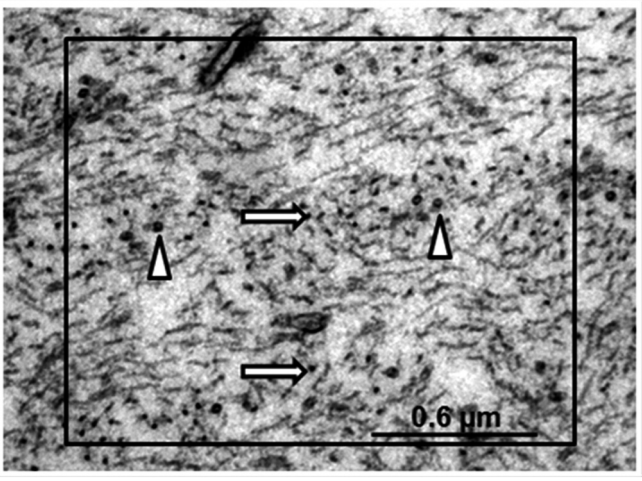

Fig. 1. Unit rectangular sampling area (2.16 μm^2), from myelinated fiber, for counting neurotubules (arrowheads) and neurofilaments (arrows).

Statistical analysis

Values were expressed as media ± SD. The one sample Kolmogorov-Smirnov test was used to check the normality distribution of the variables. Comparisons among groups were made by using a one-way ANOVA combined with a Tukey's *post hoc* test. A value of $p<0.05$ was considered as significantly different.

Results

Effects of MS on body weight, SBP and plasma glucose levels

There was no significant difference between the body weight of the 18-month-old group in the beginning and the end of the experiment: 445 g to 450 g. However, the mean body weights of the 18-month-old MS rats increased significantly from the beginning of the experiment, 448 g, to the end of the experiment, 610.5 g. The 18-month-old rats showed normal and stable SBP during the experiment, while 18-month-old MS rats developed hypertension. The plasma glucose concentration was significantly higher in the 18-month-old MS group than in 18-month-old rats (Fig. 2).

Morphological changes

Photomicrographs of representative semithin sections of femoral nerves obtained from 14-month-old, 18-month-old, and 18-month-old rats with MS appear in Fig. 3.

There were two major morphological changes in the femoral nerve of the 18-month-old MS group compared to the 18-month-old and 14-old-month groups: (1) enlargement of inter-fiber space consequent to the loss of myelinated fibers (Fig. 3C); (2) myelin breakdown or disruption of large myelinated fibers and myelin sheath infolding. Compared with the severe disruptions present in the large myelinated fibers, only mild pathological alterations were observed in the small myelinated fibers and in unmyelinated fibers of the femoral nerve in the 18-month-old MS group of rats, compared to controls (Fig. 4C-F). The axolemma and the Schwann cells covering are well maintained in all unmyelinated fibers of 18-month-old rats (Fig. 4B,E) and 18-month-old rats with MS (Fig. 4C,F). In addition, lipofuscin depositions are also seen in unmyelinated axons of 18-month-old rats with MS (see black arrowheads in fig. 4F) but not in control rats (Fig. 4D).

Morphometry

The quantitative data on the morphological parameters of the femoral nerves are summarized in Table 1. Comparing the three groups, the mean fascicular areas, the densities of myelinated fibers, the mean axon diameters and myelin sheath thickness of 18-month-old rats with MS were significantly smaller than for the groups of 18-month-old and the 14-month-old rats. The corresponding values for 18-month-old rats were significantly smaller than for 14-month-old rats. Aging had no effect on g-ratio at any group.

Histograms showing myelinated axon diameter distribution in 14-month-old rats, 18-month-old rats, and 18-month-old MS rats are displayed in Fig. 5.

Quantitatively, the percentage of damaged large myelinated fibers presented by the groups was the following: 36% of the 14-month-old group, 54% of the 18-month-old group and 67% of the 18-month-old rats with MS ($p<0.05$) (Fig. 6). The quantitative analysis did not display any significant differences in the amount of damaged small myelinated fibers among the studied groups (18.7%, 17.7% and 18.3% for the 14-month-old, 18-month-old and 18-month-old rats with MS, respectively, $p>0.05$).

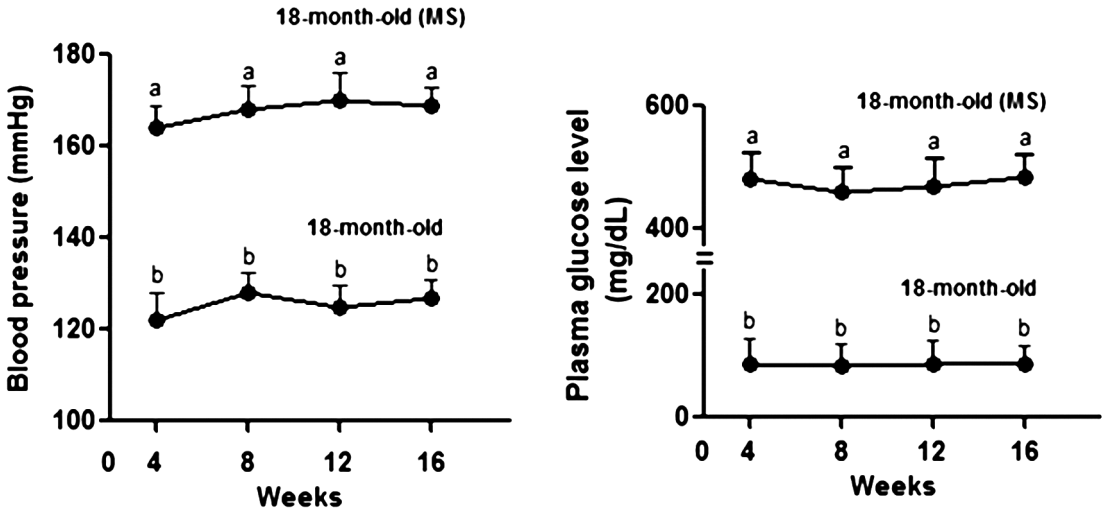

Fig. 2. SBP and plasma glucose levels in 18-month-old and 18-month-old MS groups of rats during the 4-month period of study. Rats in the 18-month-old MS group showed hypertension and high glucose levels in relation to 18-month-old rats. a, bSBP and plasma glucose levels with different superscripts are significantly different ($p<0.05$) using ANOVA.

Although slight damage was observed in the 18-month-old rats with MS compared to the 14-month-old group, when quantified (Fig. 6), the levels of damaged unmyelinated fibers did not differ significantly among the three groups (7.1%, 8.2%, 8.0% for 14-month-old, 18-month-old and 18-month-old rats with MS, respectively, $p>0.05$).

The mean number of neurotubules and neurofilaments per unit area (Fig. 7, Table 2) was smaller in myelinated axons in both the groups of 18-month-old rats and 18-month-old MS rats than in control rats.

Discussion

The present study evaluated the morphological and quantitative effects on the myelinated and unmyelinated fibers of the femoral nerve of aging rats when consuming a high fructose solution during a four-month period. The advantage of using morphometric studies is obtaining unbiased and accurate estimations (Rafati et al., 2013).

Although many experimental models of metabolic syndrome have been used (Portha et al., 1989; Kodama et al., 1993; Naderali et al., 2003; Oliveira et al., 2005; Mota and Rostom de Mello, 2006), in the present study, we chose the model of Busseroles et al. (2002), who utilized a diet rich in fructose.

The weight gain of aging rats fed a high energy diet are in accordance with the results of previous studies by Messier et al. (2007), Stranahan et al. (2008) and Brito et al. (2008), which reported that high fructose diets induced weight gain in rodents. Contrarily, no weight gain was observed by Takatori et al. (2008), Zamami et al. (2008) and Van der Borght et al. (2011). According to Rafati et al. (2013), a probable explanation might be a different route of administration and another reason might be the use of different species of experimental animals.

In accordance with the present results, Catena et al. (2003); Brito et al. (2008) and Takatori et al. (2008) showed that consumption of fructose affects the glucose levels in fasting state. However, Ueno et al. (2000), Axelsen et al. (2010) and Van der Borght et al. (2011) showed that consumption of fructose did not affect glucose levels in fasting state. It is possible that different administration routes and different species of animals

Table 1. Quantitative analysis of structural changes in the nerve area, fiber diameter, number of fibers, axon diameter, myelin sheath thickness and g- ratio in the femoral nerve of rats aged 14-month-old, 18-month-old and 18-month-old (MS).

Group	14-mo-old	18-mo-old	18-mo-old (MS)
Nerve area (mm^2)	0.039±0.004	0.026±0.003*	0.016±0.004**
Fiber diameter (μm)	11.3±0.6	7.8±0.5*	6.5±0.3**
Number of fibers	2800±170.3	2100±140.5*	1800±120**
Axon diameter (μm)	6.1±0.4	4.4±0.3*	3.0±0.2**
Myelin sheath thickness (μm)	2.6±0.3	1.7±0.2*	1.3±0.2**
g ratio	0.54±0.10	0.56±0.11	0.6±0.12

Vallues are means ± SD. *Significantly different from 14-mo-old rats, $p<0.05$. **Significantly different from 18-mo-old rats, $p<0.05$

Table 2. Density (numbers/2.16 μm^2) of neurotubules and neurofilaments in the femoral nerve.

Group	Neurotubules density	Neurofilaments density	p
14-month-old	75±5	35±4	<0.05
18-month-old	45±3*	28±2*	<0.05
18-month-old (MS)	36±2*,**	19±3*,**	<0.05

Values are mean ± SD. *Significantly different from 14-month-old rats, $p<0.05$. **Significantly different from 18-month-old rats, $p<0.05$.

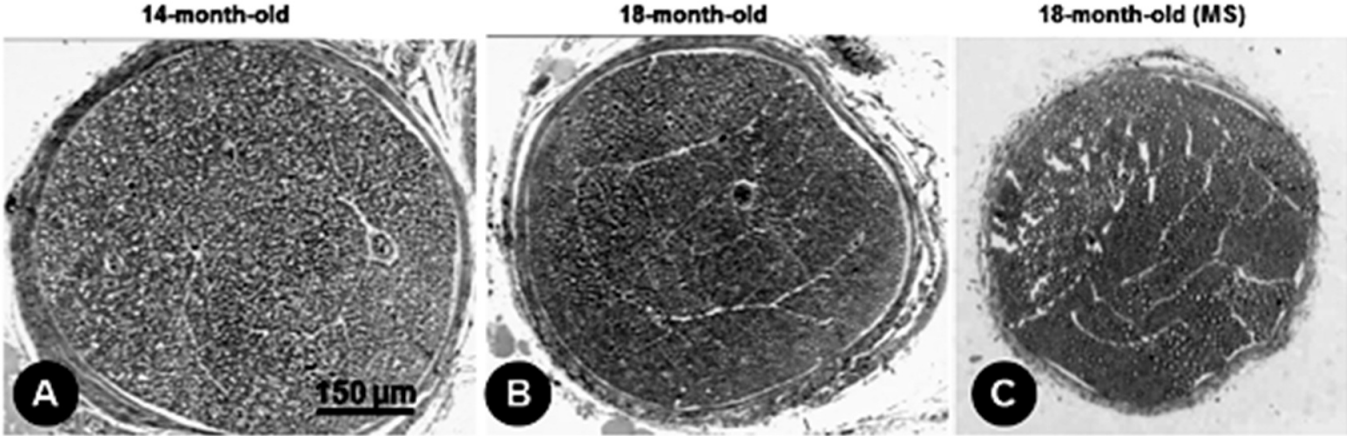

Fig. 3. Photomicrographs of tranverse semithin sections, stained with toluidine blue, obtained from three representative femoral nerves in 14-month-old **(A)**, 18-month-old **(B)**, and 18-month-old rats with MS **(C)**, respectively. Enlargement of inter-fiber space can be seen in C.

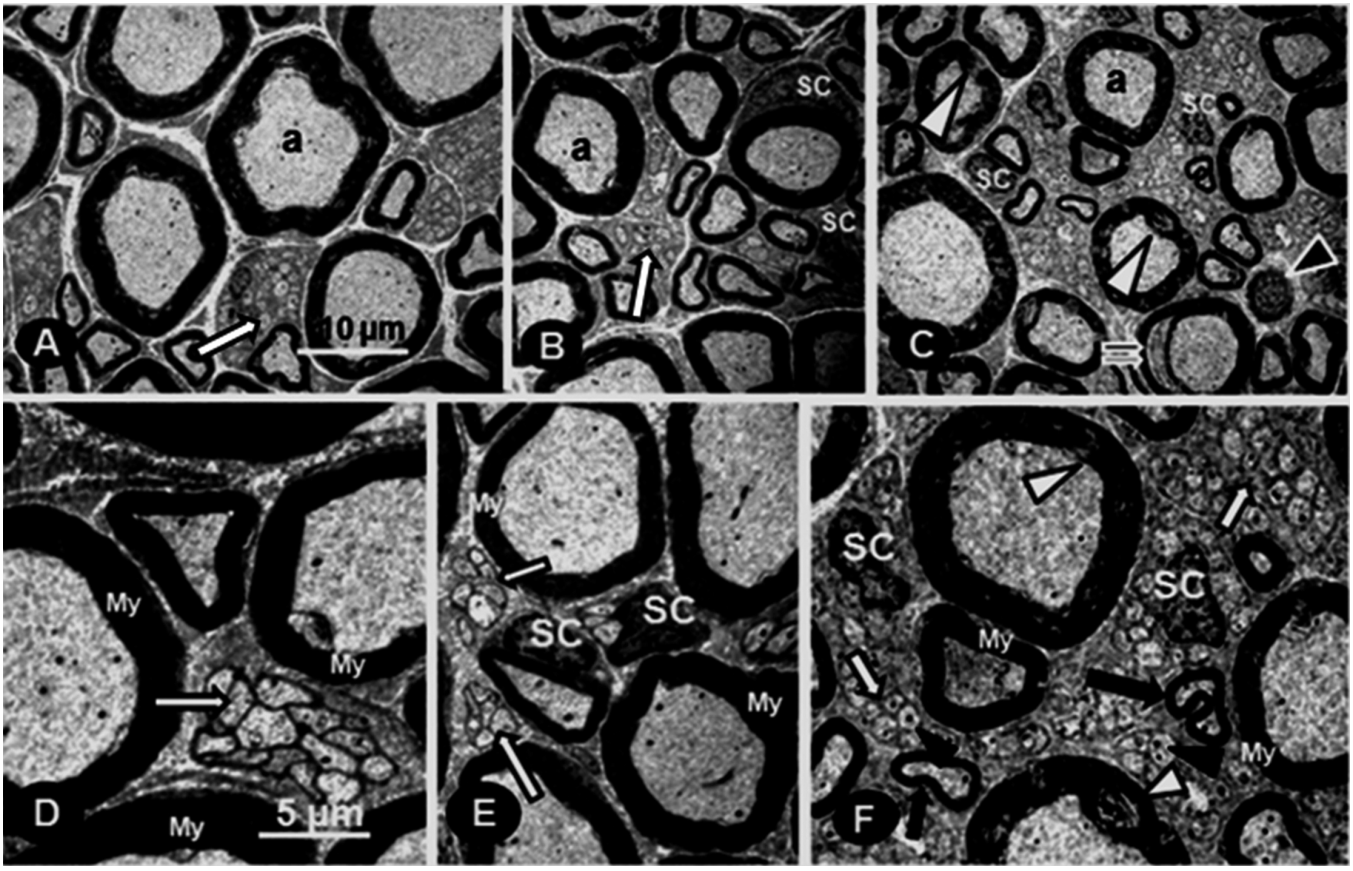

Fig. 4. The effects of aging and aging plus MS on the myelinated and unmyelinated fiber structures in the femoral nerves. Electron microscopic photomicrographs show the cross section of the femoral nerve fibers in the 14-month-old **(A, D)**, 18-month-old **(B, E)**, and 18-month-old rats with MS **(C, F)**. Pathological changes characterized by myelin disruption (double arrows) **(C),** myelin sheath infolding (black arrow) **(F)** and axon degeneration (white arrowhead) **(C, D)** are seen in large myelinated fibers of 18-month-old rats with MS. The ultrastructure of unmyelinated fibers (white arrows) and of Schwann cell (SC) in 18-month-old (B, E) is well preserved in comparison with 14-month-old rats **(A, D)**. Granules of lipofuscin (black arrowheads) are also seen in unmyelinated fiber axons of 18-month-old rats with MS **(F)**. 10 μm is the scale bar for A-C and 5 μm is the scale bar for D-E. a: myelinated axon. My: myelin sheat. Scale bars: A-C, 10 μm; D-E, 5 μm.

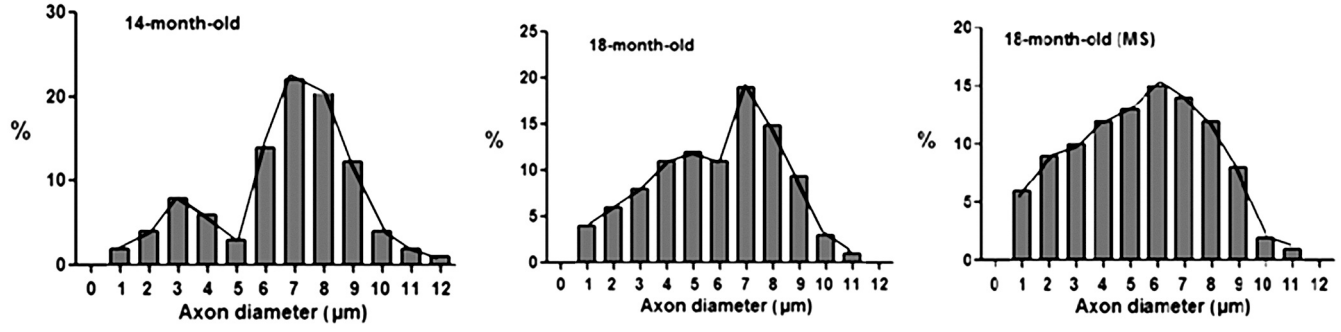

Fig. 5. Histograms showing the frequency distribution of axon diameter of femoral nerve. These histograms include all nerves measured: a total of 120 fibers of seven 14-month-old rats, 120 fibers in eight 18-month-old rats, and 120 fibers in eight 18-month-old MS rats. Note the bi-modal distribution for the 14-month-old rats, and the left-ward shift of the distribution for the 18-month-old and for the 18-month-old (MS) rats.

may explain the controversy observed.

The results of the present study showed a dramatic increase in the levels of SBP due to a high-fructose diet, when compared with the values in rats fed a normal diet, suggesting that metabolic changes associated to obesity are critical factors in the early processes that lead to MS. As hypertension was only observed in rats with MS, a high-fructose diet is likely to be the contributing factor to its generation, an observation previously demonstrated in both humans and animals (Soleimani and Alborzi, 2011). Increased dietary fructose intake in rodents has shown to recapitulate many aspects of metabolic syndrome by causing hypertension, insulin resistance and hyperlipidaemia (Soleimani, 2011). Recent studies demonstrated that increased dietary fructose intake stimulates salt absorption in the small intestine and kidney tubules, resulting in a state of salt overload and thus, causing hypertension (Soleimani and Alborzi, 2011).

Fructose induces systemic hypertension through several mechanisms mainly associated with deleterious effects on target organs (kidney, endothelium, heart) exerted by the byproducts of its metabolism, such as uric acid. The kidney is particularly sensitive to the effects of

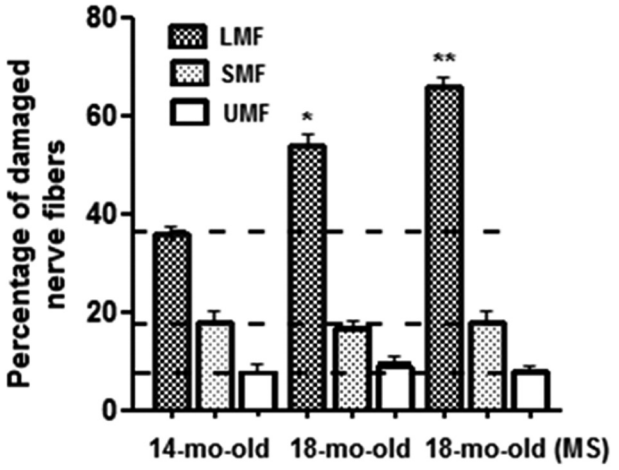

Fig. 6. Percent damage to large myelinated fibers (LMF), small myelinated fibers (SMF) and unmyelinated fibers (UMF) in the femoral nerves of rats from 14-month-old, 18-month-old and 18-month-old MS groups. *Values of LMF from 18-month-old significantly elevated compared with 14-month-old and 18-month-old-(MS) rats ($p<0.05$). **Values of LMF significantly elevated compared with 14-month-old rats ($p < 0.05$).

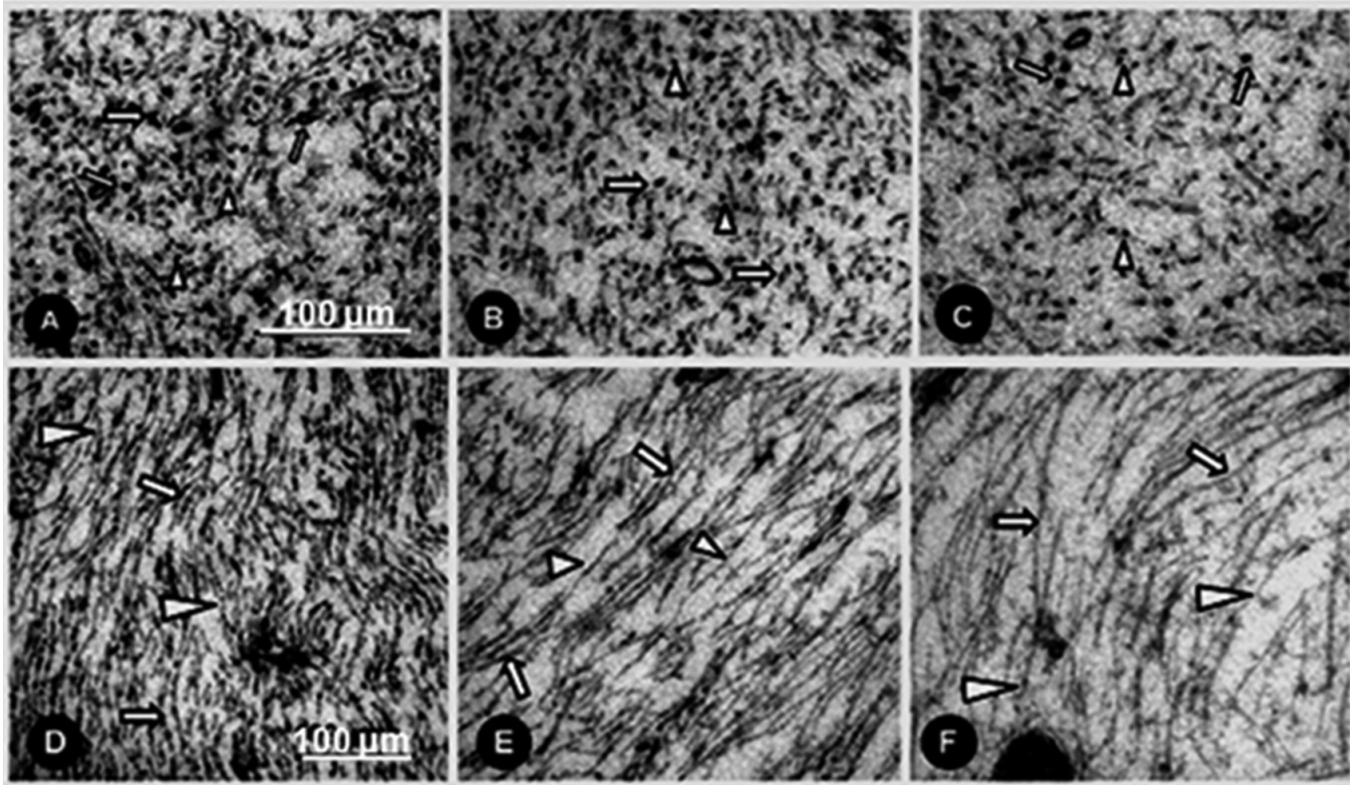

Fig. 7. The effects of aging and aging plus MS on the neurotubules (arrows) and neurofilaments (arrowheads) from myelinated fiber structures in the femoral nerve. Electron microscopic photomicrographs show the cross sections **(A-C)** and longitudinal sections **(D-F)** of the femoral nerve fibers in the 14-month-old **(A, D)**, 18-month-old **(B, E)**, and 18-month-old rats with MS **(C, F)**. The number of neurotubules and neurofilaments in myelinated fibers of 18-month-old rats with MS was significantly smaller than that for the 18-month-old and that for 14-month-old rats.

fructose because high loads of this sugar reach renal tissue. In addition, fructose increases reabsorption of salt and water in the small intestine and kidney. Thus, the combination of salt and fructose has a synergistic effect in the development of hypertension (Madero et al., 2011).

The present study showed that the consumption of a high fructose solution for 16 weeks associated with aging reduced the diameter and the number of myelinated fibers, the diameter of the myelin sheath and of the myelinated axon, and the density of neurotubules and neurofilaments of the femoral nerve in rats. The demyelination and reduction of fibers dimension were comparable to those formerly reported in peripheral nerves, and could represent causal factors in slowing the conduction velocity of action potentials (Dockery and Sharma, 1990; Fahim et al., 2000).

It was shown that rats consuming fructose have an increased amount of apoptotic cells in the central nervous system (Van der Borght et al., 2011). It is possible that by consuming fructose, the neuron metabolism (both somatic and visceral) is impaired and consequently, the number of nerve fibers is reduced. A mechanism that can be responsible for neuronal loss and consequent nerve fiber loss is that fructose intake directly affects the spinal neurons, disrupting their plasma membrane (Agrawal and Gomez-Pinilla, 2012). Another possibility is that high fructose concentrations induce the toxic effects (e.g. hypophosphatemia, hyperuricemia) (Farah et al., 2006) that could be responsible for the neural changes observed in the present study.

The ratio of the inner axonal diameter to the total outer diameter or g-ratio is widely used as a functional and structural index of optimal axonal myelination (Chomiak and Hu, 2009). In the present study, no significant difference was observed among the values of g-ratio for the three groups, which were between 0.5 and 0.6. This result means that aging and aging associated with MS have promoted a uniform influence on the myelin sheath and the axon. Using the same model for the vagus nerve, Alcantara et al. (2008) also showed a g -ratio distribution for most of the fibers between 0.5 and 0.6. The g-ratio of a myelinated axon is optimized to achieve maximal efficiency. This concept is supported by the observations that during the recovery process from demyelinating disease, the axons undergo an initial period of hyper-remyelination and increased diameters, and then, eventually revert to the normal g-ratio (Perrot et al., 2007).

It is well known that the microtubules and neurofilaments are essential for the function of peripheral nerves because they participate in the processes of plasticity, cell signaling and transport (Helfand et al, 2003; Baas and Ahmad, 2013). Furthermore, they determine the caliber of the nerve, the pattern of growth, stabilize the axolemma, and encourage the basic machinery required for both anterograde and retrograde axoplasmic transport (Bernal et al., 2007; Ouyang et al., 2013).

The present study demonstrated a significant decrease in the number of neurotubules and neurofilaments in the femoral nerve of rats submmited to consumption of a high-fructose solution for 16 weeks. Iturriaga (1985) has found a reduced content of microtubules in myelinated axons from sural nerves in diabetic rats. Since the axon-diameter depends on the integrity of neuronal cytoskeletal proteins (Yagihashi, 1995; Shea et al., 2009), we can speculate that as consequence of axonal atrophy, the axonal transport of the nerve growth factor (NGF) and neurotrophin-3 (NT-3) produced in peripheral tissues was impaired in the femoral nerve of 18-month-old MS rats.

Based on the present study results, in which fructose or one of its metabolites might induce neuronal changes in the femoral nerve, we believe our findings are relevant in understanding the effects of MS in humans.

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