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The Neuroprotective Effects of Progesterone on Experimental Diabetic Neuropathy in Rats

[1,2]H.R. Sameni, [1]M. Panahi, [3]A. Sarkaki, [1]G.H. Saki and [4]M. Makvandi
[1]Department of Anatomical Sciences, Faculty of Medical Sciences,
Ahwaz Jondishapur University of Medical Sciences, Iran
[2]Department of Anatomical Sciences, Semnan University of Medical Sciences, Semnan, Iran
[3]Department of Physiology, Physiological Research Center,
[4]Department of Virology, Ahwaz Jondishapur University of Medical Sciences, Ahwaz, Iran

Abstract: This study was conducted to investigate the neuroprotective effects of progesterone (PROG) on electrophysiological and histomorphometrical alternation in STZ-induced diabetic neuropathy starting from 4 weeks after the diabetic induction. Thirty adult male Sprague-Dawley rats were randomly divided into 3 groups (with 10 rats in each), control (nondiabetic), untreated diabetic and diabetic PROG-treated. Diabetes was induced in adult male rats by a single dose injection of streptozotocin (STZ, 55 mg kg^{-1}, i.p.). In the PROG-treated group, 4 weeks after induce of diabetes; rats were treated with PROG (8 mg kg^{-1}, i.p., every two days) for 6 weeks. Diabetic rats showed a significant reduction in motor nerve conduction velocity (MNCV), mean myelinated fibers (MFs) diameter, axon diameter and myelin sheath thickness in the sciatic nerve after 6 weeks. In the untreated diabetic group endoneurial edema was observed in sciatic nerve and the numbers of MFs with infolding into the axoplasm, irregularity of fibers, myelin sheath with unclear boundaries and alteration in myelin compaction were also increased. Long-term treatment with PROG increased MNCV significantly and prevented all these abnormalities in treated diabetic rats. Our findings indicated that PROG as a therapeutic approach can protect neurophysiologic and histomorphologic alterations induced by peripheral diabetic neuropathy.

Key words: Progesterone, sciatic nerve, diabetic neuropathy, nerve conduction velocity, histomorphometry

INTRODUCTION

Diabetes is a global health problem and its prevalence is said to increase to 366 million worldwide by the year 2025 (Wild *et al*., 2004). Persistent hyperglycemia in diabetic patients despite appropriate therapeutic measures leads to several complications including retinopathy, nephropathy and neuropathy (Kumar *et al*., 2007; Saini *et al*., 2007). Diabetic Neuropathy (DN) is the most common and troublesome complication of diabetes mellitus for which there is no available treatment apart from therapy that normalizes glucose metabolism. Therefore, DN is leading to the greatest cause of morbidity and mortality and resulting in a huge economic burden for diabetes care in developed nations. DN occurs in more than 50-60% of diabetic patients and involves a spectrum of functional and structural changes in peripheral nerves and is the leading cause of non-traumatic lower limb amputation and autonomic failure in diabetic patients (Kumar *et al*., 2007; Saini *et al*., 2007; Andriambeloson *et al*., 2006; Sima, 2003; Yagihashi *et al*., 2007; Rajbhandari and Piya, 2005).

Although diabetic peripheral neuropathy is a multifactorial disorder, it is conditioned by hyperglycemia, deficiencies of insulin and c-peptide. It is characterized by a complex pathogenetic network of interrelated metabolic, neurotrophic and vascular defects (Kumar *et al*., 2007; Saini *et al*., 2007; Sima, 2003; Rajbhandari and Piya, 2005; Greene *et al*., 1999). The most common early disorders of nerve functions include an acute decrease in nerve blood flow and slowing in Nerve Conduction Velocity (NCV), followed by axonal degeneration of both the motor and sensory nerve fibers, paranodal demyelination and loss of myelinated fibers (Andriambeloson *et al*., 2006; Sima, 2003; Calcutt *et al*., 2006).

Earlier studies have demonstrated that there is a relationship between nerve structural lesion and diminish of NCV in chronic diabetic animals (Gebri *et al*., 1999). Experimental DN shares a number of features with human diabetic neuropathy and diabetic polyneuropathy such as the structural, functional and biochemical alterations (Yagihashi, 1997; Biessels *et al*., 1999). Previous investigations have reported that the reduction of NCV, together with the decrease in Na^+, K^+- ATPase activity, is the hallmark of DN (Gebri *et al*., 1999; Berry, 1997).

Corresponding Author: Panahi Marzieh, Department of Anatomical Sciences, Faculty of Medical Sciences, Ahwaz Jondishapur University of Medical Sciences, Postal Code 61335-15794, P.O. Box 189, Ahwaz, Iran Tel: +98-9166166551 Fax: +98-6113332036

Current treatment of DN relies on the control of glycemia, oxidative stress and neural and vascular risk factors including, insulin administration or pancreatic islet transplantation, aldos reductase inhibition, aminoguanidine treatment and neurotrophic growth factor administration, but does not prevent its occurrence or progression totally (Andriambeloson *et al*., 2006; Tesfaye *et al*., 2005; Vincent *et al*., 2004). The effects of steroid hormones, such as progesterone on the reproductive and endocrine systems are well known, moreover recent studies have shown that these hormones also exert a broad spectrum of neuroprotective effects both in the central and peripheral nervous system (Azcoitia *et al*., 2003; Stein *et al*., 2008; Schumacher *et al*., 2007; Leonelli *et al*., 2006). Recently it has been reported that progesterone (PROG) biosynthesis is up-regulated in the spinal cord and peripheral nerves of rats with STZ-induced diabetes (Saredi *et al*., 2005). Furthermore recent data obtained in various laboratories have shown indicated that peripheral nerves represent an important target for the effects of neuroactive steroids (Melcangia *et al*., 2005; Koenig *et al*., 2000).

All of the mentioned findings suggest that neuroactive steroids, such as PROG may represent potential therapeutic tools for the treatment of diabetic neuropathy. In this study we have investigated the neuroprotective effects of PROG on electrophysiological and neuroanatomical alteration in STZ-induced diabetic neuropathy starting from 4 weeks after the diabetic induction.

MATERIALS AND METHODS

Animals, induction of diabetes and drug treatment: This study was conducted on September 2007 to May 2008 in Department of Histology and Physiological Research Center of AJUMS In Iran. Thirty adult male Sprague-Dawley rats obtained from the animal house center of Ahwaz Jondishapur University of Medical Sciences (AJUMS). They were housed in plastic cages (three in each) with controlled temperature (20±2°C), humidity (60-65%) and 12 h light dark cycle (Lighted 7:00 am-19:00 pm). All animals were acclimatized for a minimum period of 2 weeks prior to the beginning of the study. All experiments were carried out in accordance with institutional guidelines. Animals were randomly divided into 3 groups (10 rats in each) including, control group (CO, nondiabetic), diabetes mellitus group (DM, untreated) and diabetic progesterone-treated group (DM+PROG). Diabetes in rats were induced by a single dose intrapritonealy injection of freshly prepared streptozotocin (STZ, 55 mg kg^{-1}, Sigma, USA) in 0.09 M citrate buffer (pH 4.8) (Kumar *et al*., 2007; Saini *et al*., 2007; Andriambeloson *et al*., 2006). The drug was dissolved in citrate buffer. All STZ treated animals received 0.45% NaCl solution after injection time throughout experiment period and 5% fructose solution only in 3rd day of STZ injection. Hyperglycemia was confirmed 48 h after STZ injection by measuring tail vein blood glucose levels using a blood glucose monitoring system (EasyGluco, Infopia Co., Ltd. Korea). Only animals with mean plasma glucose levels above 300 mg dL^{-1} were accepted as being diabetic. Glycemia was also confirmed at the end of the study. Diabetic and control animals were age-matched and maintained for 4 weeks. Four weeks after the diabetic induction in the DM+PROG group, they have treated with progesterone (8 mg kg^{-1} once every two days, i.p., sigma, USA) dissolved in 200 μL sesame oil for 6 weeks. Animals in CO and DM groups received vehicle alone (once every two days, i.p. injection). All animals were provided with standard diet and water. At the end of experiment, all rats were sacrificed under deep anesthetize and their sciatic nerves were taken for morphometrical analysis.

Electrophysiological assessment: The animals were anesthetized with 50/20 mg kg^{-1} ketamine/xylazine i.p. injection to prevent discomfort and then Motor Nerve Conduction Velocity (MNCV) in the right sciatic nerve was measured. During the study, animal's body temperature was maintained at 37°C to ease animal stress from anesthetic. Sciatic-tibial motor MNCV was measured by stimulating distally at the sciatic notch and distally at the knee via bipolar needle electrodes with 10 V, single stimulus (Andriambeloson *et al*., 2006; Kumar *et al*., 2005; Saini *et al*., 2004). Recording needle electrodes connected to bio-potential coupler were placed on paw to detect motor response. The motor response was captured using Dual Bioamplifier, Powerlab/4SP (ADInstruments.Com, Australia). The recording was a typical biphasic response with an initial M-wave, which is a direct motor response due to stimulation of motor fibers. The sciatic-tibial motor MNCV is calculated using two points of stimulation along the nerve and measuring the resultant latency. Latency is measured from initial onset to maximum negative peak. MNCV was calculated by following formula, MNCV = (distance between sciatic and tibial nerve stimulation points)/(sciatic M wave latency-tibial M wave latency) (Kumar *et al*., 2007; Saini *et al*., 2007).

Tissue processing and morphometric analysis: Twenty-four hours after the end of treatment, sciatic nerves specimens between the sciatic notch and the knee were

fixed *in situ* under ketamin/xylazin anesthesia with 4% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 for 20 min. Rapidly after the primary fixation, the nerves were removed, cut into 1-2 mm length segments and fixed for 24 h with 2.5% glutaraldehyde buffered in 0.1 M phosphate buffer. Tissue samples were washed in phosphate buffer, post fixed for 2 h in 1% buffered osmium tetroxide and dehydrated in graded concentrations of acetone and embedded in Epon 812. Transverse semi-thin sections (0.75 μm) were stained with 1% toluidine blue and examined by light microscopy. From each sciatic nerve transverse fascicular area 10 fields, randomly selected at a x1000 magnification, to cover a 0.01 mm^2 area of the nerve. Sections were observed using a light microscope and morphometric analysis was performed with using a computerized image analysis system (Motic Images China Group Co., Ltd.). In each section,, myelinated fibers (MFs) diameter, axon diameter, myelin sheath thickness and g-ratio (axon diameter/fiber diameter) were calculated. At least 200 MFs were measured per animal. Data from fibers with evident degeneration signs, or longitudinal sections of fibers, were not collected.

Statistical analysis: The quantitative data obtained by the experiments have been analyzed by using SPSS version 15.0. Data from experiments with more than two independent variables have been analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc test. All data were expressed as the mean±SEM and the differences were considered to be significant when $p<0.05$.

RESULTS

All diabetic rats showed marked impairment of growth. As shown in Table 1, 48 h post streptozotocin administration; diabetic rats had high blood glucose and no gain of body weight at the end of 10 weeks. Body weight of 10th week diabetic rats was significantly ($p<0.05$) lower than normal control rats. As shown in Table 1, treatment with PROG (6 week), did not significantly affect the blood glucose levels and body weights in diabetic rats.

Electrophysiology: Before the PROG treatment (At the end of 4th week), MNCV was significantly reduced in diabetic groups compared with the values for the control group ($p<0.001$). The diabetic rats treated with PROG showed an improvement in MNCV. The differences in MNCV between untreated diabetic rats and PROG-treated diabetic ones were statistically significant ($p<0.01$) (Table 2).

Morphology and histomorphometry: Light microscopic observations of transverse semi-thin sections of the sciatic nerves in the control group, showed myelinated nerve fibers are in normal morphology and structure. In accordance with earlier studies, a few myelin abnormalities, including myelin infoldings and MFs with irregular shapes were also observed (Fig. 1A). Untreated diabetic rats revealed some abnormalities, including endoneurial edema with dissociation of nerve fibers, degeneration, irregularity and unclear boundary in myelin sheaths. Increase number of MFs with infolding (myelin invagination into the axoplasm) and abnormal-shaped MFs were also observed in the untreated diabetic group (Fig. 1B). Six weeks treatment with PROG significantly prevented all these abnormalities in diabetic rats and myelinated nerve fibers almost are similar control group (Fig. 1C). In the untreated diabetic rats a decrease in the number of large MFs with a reciprocal increase in small MFs as compared with control rats was seen (Fig. 1A, B). But the proportion of MFs with myelin abnormalities was significantly reduced after the treatment with PROG (Fig. 1C).

The mean MFs and axon diameter (μm) were 8.99±0.29 and 6.56±0.23 in the control, 7.12±0.26 and 5.24±0.19 in untreated diabetic and 8.76±0.31 and 6.24±0.19 in PROG-treated diabetic rats, respectively, each

Table 1: Body weight and blood glucose levels of CO, DM and DM+PROG groups

	Body weight (g)		Blood glucose (mg dL^{-1})
Animal groups	Before STZ injection	End of experiment	Before sacrifice
CO (n = 10)	255.1±3.8	384.9±5.9	121±24.185
DM (n = 10)	245.7±2.8	270.7±3.8[a]	544±53.508[c]
DM+PROG (n = 10)	241.9±3.9	282.1±5.3[a]	502±71.632[c]

Data are represented as Mean±SEM, n is the number of animals. [a]$p<0.05$ vs. control, [c]$p<0.001$ vs. control

Table 2: Effect of progesterone on MNCV and histomorphometric parameters (MFs diameter, Axon diameter, Myelin thickness and g ratio) of sciatic nerves

Animal	MNCV (m sec^{-1})	MFs diameter (μm)	Axon diameter (μm)	Myelin thickness (μm)	g ratio
CO	50.32±0.36	8.99±0.29	6.56±0.23	1.25±0.05	0.69±0.014
DM	37.93±0.45[c]	7.12±0.27[c]	5.24±0.19[c]	0.96±0.045[c]	0.74±0.006[c]
DM+PROG	44.96±0.52[b]	8.76±0.31[b]	6.24±0.19[a]	1.18±0.046[b]	0.70±0.009[b]

Data are represented as Mean±SEM, [a]$p<0.05$ vs. DM, [b]$p<0.01$ vs. DM and [c]$p<0.001$ vs. CO and DM+PROG

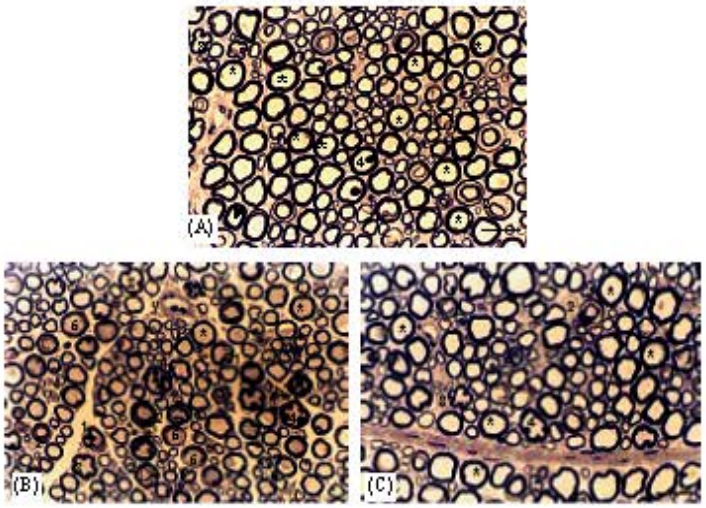

Fig. 1: Light micrographs of transverse semi-thin section of the sciatic nerve. (A) In the control group, myelinated nerve fibers are in normal morphology and structure (*). (B) In untreated diabetic group, nerves revealed certain abnormalities, including: degeneration (1), myelin abnormality and alteration in myelin compaction (2), irregular fiber shape (3), myelin infoldings (4) and unclear boundaries of myelin sheath (5). (C) In the PROG-treated diabetic group, the proportion of axons with myelin abnormality, infolding and irregular shape was significantly reduced. Toluidine blue staining (x400, v = vessel, Scale bar = 20 μm)

diameter was significantly decreased in the untreated diabetic rats compared with the other two groups ($p<0.001$) (Table 2). The mean myelin sheath thickness was significantly reduced in the untreated diabetic rats compared with controls and g ratio was increased in untreated diabetic animals than in control group ($p<0.001$) (Table 2). Treatment with PROG for 6 weeks significantly reversed each diameter reduction in the diabetic rats; however the MFs and axon diameters were displaced toward larger sizes compared with those of the untreated diabetic rats (Table 2).

DISCUSSION

Peripheral neuropathy is one of the many complications of both type 1 and type 2 diabetes mellitus, associated with retinopathy, nephropathy, coronary heart disease, peripheral vascular disease, stroke, pain, skin ulcers, amputation, muscle weakness and extra outcomes of macrovascular and microvascular complications (Sima, 2003; Yagihashi *et al*., 2007; Rajbhandari and Piya, 2005; Little *et al*., 2007). Finally this complications overall impair the patient's quality of life. Therefore, treatment of diabetic neuropathy is a major goal, but despite multiple attempts, no satisfactory management is yet available. Previous studies have shown that neuroactive steroids are potential neuroprotective agents (Azcoitia *et al*., 2003; Saredi *et al*., 2005; Garcia *et al*., 2005; Veiga *et al*., 2006), but using them as therapeutic tools to treat diabetic neuropathy has not received enough attention. Recent studies have reported that steroids such as, dihydroepiandrosterone, progesterone and dihydroprogesterone may prevent vascular and neuronal dysfunction and morphological myelin alterations in the sciatic nerve of diabetic rats (Veiga *et al*., 2006; Yorek *et al*., 2002).

In the present study, we have evaluated the neuroprotective effects of PROG on the development of diabetes-related neuropathy and measured histomorphometric parameters (MFs diameter, Axon diameter, Myelin thickness and g ratio) of sciatic nerves specifically. We found that PROG administration (8 mg kg^{-1}) improved electrophysiological (MNCV measurement) and histopathological signs in STZ-induced diabetic neuropathy.

Development of diabetic neuropathy in STZ-induced diabetic rats was evident from the decrease in sciatic motor nerve conduction velocity. We observed 23% deficit in MNCV in 10 weeks diabetic rats as compared to nondiabetic rats. These results are in accordance with other reports (Kumar *et al*., 2007; Saini *et al*., 2004; Coppey *et al*., 2002; Cotter and Cameron, 2003; Callaghan *et al*., 2005). Several reports indicate that peripheral diabetic neuropathy is a hypoxic neuropathy.

Moreover previous studies have shown that free radicals induce oxidative stress under diabetic conditions to hyperglycemia (Kumar *et al*., 2007; Saini *et al*., 2007; Niedowicz and Daleke, 2005; Pop-Busui *et al*., 2006). Oxidative stress causes vascular impairment leading to decrease in nerve blood flow resulting in endoneurial hypoxia and impaired neural function which may cause MNCV slowing (Saini *et al*., 2007; Yorek *et al*., 2004). On the other hand according to earlier investigations, large MFs preeminently determine the compound MNCV (Gonzalez *et al*., 2005). In our study a reduced number of large MFs with a reciprocal increase in the number of small MFs were also seen in the untreated diabetic rats. Therefore the decrease in MNCV may be due to a decrease in large MFs or the arrested developmental process of MFs. Moreover, the decrease in MNCV could result from alterations in Na^+, K^+-ATPase activity, the membrane environment and histological damage that particularly cause the loss of myelinated large fibers (Gebri *et al*., 1999). Treatment of STZ-induced diabetic rats with PROG improved MNCV that could be due to preserved fibers and axons diameter.

Recent observations have indicated that, increasing endoneurial edema in diabetic rats could result from altered sodium cell gradient related to impairment of Na^+, K^+-ATPase activity (Gebri *et al*., 1999). In diabetic models, the decrease in Na^+, K^+-ATPase activity could be due to metabolic abnormality and histological damage (Gebri *et al*., 1999). It seems that treatment with PROG for 6 weeks prevents histological nerve damage and that it is possible due to improving Na^+, K^+-ATPase activity. In contrast, in PROG-treated rats, amount of endonural edema was decreased.

In earlier studies, our study has also indicated myelin abnormalities in normal peripheral nerves and includes myelin infoldings and MFs with irregular shapes that probably reflect a basal level of nerve fiber damage due to stretch or other mechanical loads (Azcoitia *et al*., 2003; Yorek *et al*., 2002; Gonzalez *et al*., 2005).

This finding in accordance with earlier studies indicates that treatment with STZ increases morphological alteration in the myelinated fibers of the sciatic nerve. The most abundant myelin abnormality observed in our study was myelin infolding in the axoplasm and fiber irregular shapes. Myelin infolding is associated with alterations in myelin proteins such as glycoprotein zero (P0), peripheral myelin protein 22 (PMP 22) and Myelin-Associated Glycoprotein (MAG). Its frequency is increased in aging and different peripheral neuropathies including peripheral diabetic neuropathy (Azcoitia *et al*., 2003; Yorek *et al*., 2002). In the present study some of the significant effects of treatment with PROG is the reduction in the frequency of axons with myelin abnormalities and loss of the proportion of fibers with irregular shapes, induced by STZ treatment.

In agreement with earlier findings, our present results indicate that a significant decrease in the mean diameter of MFs and myelin thickness was also detected in STZ-induced diabetic rats (Azcoitia *et al*., 2003). This may be the results of the massive increase of small size MFs that observed in diabetic sciatic nerves. Treatment of STZ rats with PROG is able to counteract the decrease in mean MFs diameter and myelin thickness in the sciatic nerve. Our study also showed that PROG restored the decrease in number of large MFs in untreated diabetic rats.

These findings confirm that neuroactive steroids such as PROG are able to reduce histomorphological changes associated with STZ-induced diabetes in the sciatic nerve. Therefore neuroactive steroids may also be considered as a therapeutic approach to maintain peripheral nerve myelin integrity during neurodegenerative events (Azcoitia *et al*., 2003; Yorek *et al*., 2002; Schumacher *et al*., 2004).

In conclusion, our findings indicate that PROG may counteract structural alterations in the myelin of rat sciatic nerve fibers induced by experimental diabetes. Although further studies should determine the functional implications and mechanism of this protective effect of neuroactive steroids, our findings suggest that this compound may be considered as a potential therapeutic approach for peripheral diabetic neuropathy.

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