**BRAZILIAN ARCHIVES OF
BIOLOGY AND TECHNOLOGY**

A N   I N T E R N A T I O N A L   J O U R N A L

Combination Vitamin C and Vitamin E Prevents Enteric Diabetic Neuropathy in the Small Intestine in Rats

Jacqueline Nelisis Zanoni[1*] and **Catchia Hermes-Uliana**[2]
[1]*Departamento de Ciências Morfológicas; Universidade Estadual de Maringá; Maringá - PR - Brasil.*
[2]*Universidade Federal de Mato Grosso do Sul; Coxim - MS - Brasil*

ABSTRACT

The present study evaluated the effects of supplementation with a combination of vitamin C and vitamin E on NADH-diaphorase-positive (NADH-d$^+$) and neuronal nitric oxide synthase (nNOS)-immunoreactive myenteric neurons in the duodenum and ileum in diabetic rats. Forty rats were distributed into the following groups: normoglycemic (N), normoglycemic supplemented with vitamin C and vitamin E (NS), diabetic (D), and diabetic supplemented with vitamin C and vitamin E (DS). Vitamin C was added to the drinking water, and vitamin E was incorporated in the diet (1%). After 120 days, the animals were euthanized, and the duodenum and ileum were subjected to NADH-d and nNOS staining. Quantitative and morphometric analyses of myenteric neurons were performed. Diabetes reduced NADH-d$^+$ neurons in the D group. The density of nitrergic neurons was not changed by diabetes or vitamin treatment. Hypertrophy of the cell body area of NADH-d$^+$ and nNOS-immunoreactive neurons was observed in both intestinal segments. Combined supplementation with vitamin C and vitamin E prevented the reduction of the density of NADH-d$^+$ neurons and hypertrophy, demonstratred by both techniques. Supplementation with a combination of vitamin C and vitamin E promoted myenteric neuroprotection in the small intestine in diabetic rats.

Key words: ascorbic acid, α-tocopherol, diabetes mellitus, myenteric plexus, enteric nervous system

INTRODUCTION

Diabetes mellitus is a chronic degenerative disorder with multiple endocrine etiologies that result from genetic and environmental factors (Geirsdottir et al. 2012). The disease requires the continuous management of patients to prevent acute and chronic complications (Zitkus 2014). Among the chronic complications caused by diabetes, neurological manifestations are the most common, affecting both the autonomic and peripheral nervous systems and impairing the quality of life of patients (Chandrasekharan et al. 2011). Our research group found that diabetes mellitus reduced the density of myenteric neurons and enteric neural plasticity in various segments of the digestive system (Takahashi et al. 1997; Zanoni et al. 1997; Fregonesi et al. 2001; Defani et al. 2003; Zanoni et al. 2003; Pereira et al. 2006; Tashima et al. 2007; Zanoni et al. 2007; De Freitas et al. 2008; Roldi et al. 2009; Alves et al. 2010; Pereira et al. 2011; Zanoni et al. 2011; Lopes et al. 2012; Hermes-Uliana et al. 2014). These changes in enteric innervation that arise from diabetes are attributable to persistent hyperglycemia that produces numerous metabolic changes. In an effort to explain the relationship, between the extent and severity of hyperglycemia and the development of diabetic neuropathy, multiple mechanisms have been described. The main factor has been proposed to be oxidative stress, which may result from the increased

---

*Author for correspondence: zanonijn@gmail.com

production of reactive oxygen species precursors and/or the low efficiency of enzyme systems (Vincent et al. 2004; Chandrasekharan et al. 2011; Stavniichuk et al. 2014).

Agents that act on oxidative stress pathways can reduce or prevent diabetic neuropathy. Vitamin C (ascorbic acid) is a water-soluble antioxidant that inhibits aldose reductase, thus preventing sorbitol formation. Vitamin E (α-tocopherol) is a liposoluble antioxidant that plays an important role in the inhibition of lipid peroxidation by reducing oxidative stress. Promising results have been reported with the use of vitamin C (Pereira et al. 2006; Zanoni et al. 2007; Zanoni et al. 2009) and vitamin E (Pereira et al. 2008; Roldi et al. 2009) in the prevention of diabetic neuropathy. The combined use of vitamin C and vitamin E showed positive effects in reducing oxidative stress (Aksoy et al. 2005; El-Hadjela et al. 2013). Vitamin C regenerates oxidized vitamin E, thereby rescuing molecular stability (Zaken et al. 2001). However, the use of a combination of these antioxidant vitamins to prevent changes in the enteric nervous system has not been investigated.

The aim of the present study was to evaluate the effects of supplementation with a combination of vitamin C and vitamin E on NADH-diaphorase-positive (NADH-d$^+$) and neural nitric oxide synthase (nNOS)-immunoreactive myenteric neurons in the duodenum and ileum in rats with experimental diabetes mellitus.

MATERIAL AND METHODS

Animals

All of the procedures described in this study are consistent with the ethical principles adopted by the Brazilian Society of Science in Laboratory Animals (SBCAL) and were approved by the Ethics Committee on Animal Experimentation of the State University of Maringá (protocol no. 054/2006).

Male Wistar rats *(Rattus norvegicus*; n = 40), 13 weeks of age and weighing 300-400 g, were used. The rats were randomly distributed into four groups: normoglycemic (N), normoglycemic supplemented with vitamin C and vitamin E (NS), diabetic (D), and diabetic supplemented with vitamin C and vitamin E (DS). The animals were kept in polypropylene cages with a 12 h/12 h light/dark cycle and controlled temperature (24 ± 2ºC).

Diabetes was induced in the D and DS groups by an intravenous injection of 35 mg/kg streptozotocin (Sigma, St. Louis, MO, USA) dissolved in 10 mM citrate buffer (pH 4.5) after fasting for 14 h. Three days after the induction of diabetes, glucose levels were determined using a glucose oxidase test to confirm establishment of the experimental model. We used animals with > 250 mg/dL glucose.

Vitamin C (Sigma, St. Louis, MO, USA) was added to the drinking water (1 g/L/day), and vitamin E was incorporated in the diet (Nuvital; prepared at a 1% concentration each week) in the NS and DS groups.

Material collection and processing

After 120 days, the animals were euthanized after they were weighed and intraperitoneally anesthetized with 40 mg/kg thiopental (Abbott Laboratories, Chicago, IL, USA). The duodenum and ileum from all of the animals were collected.The duodenum was collected using the Trendz ligament as a reference. Segments from 20 animals (five per experimental group) were collected and processed for NADH-d immunohistochemistry to determine metabolically active neurons, and the other 20 were processed for nNOS immunohistochemistry to determine nitrergic neurons.

Histochemical technique for NADH-d (Gabella 1969)

The segments from each animal were washed, bound at one end, injected with Krebs solution. Then, they were immersed for five min in a Triton X-100 0.3% solution (Sigma, St. Louis, MO, USA), and washed twice in Krebs solution (5 min each). The segments were immersed in a solution that contained β-NADH (Sigma, St. Louis, MO, USA) and nitroblue tetrazolium (NBT; Sigma, St. Louis, MO, USA) for 45 min. The reaction was stopped with buffered formalin solution. The segments were then dissected under a stereomicroscope to obtain preparations of the total muscle layer, dehydrated, diaphanized, and mounted in Permount medium.

Immunohistochemical technique for nNOS (Wrzos et al. 1997)

The segments were washed with 0.1 M phosphate-buffered saline (PBS; pH 7.4), injected with Zamboni fixative (Stefanini et al. 1967), and stored in fixative for 18 h at 4°C. The segments

were then dehydrated in an ascending series of alcohol (80, 95, and 100%), diaphanized in xylene, rehydrated in a descending series of ethanol (100, 95 and 80%) for 30 min in each solution, and stored in PBS. The segments were dissected under a stereomicroscope to remove the mucosa and submucosa, yielding whole-mount muscular layer preparations. The whole mounts were washed twice in PBS with 0.5% Triton (Sigma, St. Louis, MO, USA) and blocked for 1 h in PBS with 2% bovine serum albumin (BSA; Sigma, St. Louis, MO, USA) at room temperature. The segments were then incubated in a primary antibody solution that contained anti-nNOS (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and diluted in PBS, 2% BSA, and 0.1% Triton X-100 at room temperature with shaking for 48 h. After incubation, the whole mounts were washed three times in PBS for 5 min each. They were then incubated in a solution that contained rabbit anti-AlexaFluor 488 secondary antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). They were then washed three times with PBS, mounted in glycerol gel, and stored in a refrigerator. The negative control was performed by omitting the primary antibody.

Quantitative analysis of NADH-d$^+$ and nNOS-immunoreactive myenteric neurons

The quantification of myenteric neurons was performed using a sample from the intermediate region of the intestinal circumference of each animal. The total area that was quantified for each animal was 10.38 mm^2. The results were converted to cm^2.

Morphometric analysis of NADH-d$^+$ and nNOS-immunoreactive myenteric neurons

The measurement of the areas of the cell bodies of NADH-d$^+$ and nNOS-immunoreactive myenteric neurons was performed using images captured with a high-resolution AxioCam camera (Zeiss, Jena, Germany) coupled to an Axioskop Plus light microscope (Zeiss, Jena, Germany). The images were transferred to a computer using AxioVision 4.1 software. Image-Pro Plus software was used to measure neuronal cell bodies in 100 μm^2 areas for each animal per group (i.e., a total area of 500 μm^2 per group) for each of the two techniques of neuronal identification.

Statistical analysis

The data were statistically analyzed using Statistica 7.1 and GraphPad Prism 5.1 software and are expressed as mean ± standard error. The morphometric data were subjected to delineation blocks followed by the Tukey test. The other data were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Quantitative analysis of NADH-d$^+$ myenteric neurons

The number of NADH-d$^+$ neurons was reduced in both the duodenum and ileum in animals in the D group compared with the N group ($p < 0.05$). Supplementation with vitamin C and vitamin E promoted neuroprotection, preserving the number of neurons in the group DS compared with the D group ($p < 0.05$; Fig. 1). No difference was found between the N and NS groups ($p > 0.05$).

### Quantitative analysis of nNOS-immunoreactive myenteric neurons

No changes in the density of nNOS-immunoreactive neurons were observed between any of the groups in either segment ($p > 0.05$; Fig. 2).

Morphometric analysis of NADH-d$^+$ myenteric neurons

An increase was observed in the cell body area in the D group compared with the N group ($p < 0.01$). In the DS group, supplementation with vitamin C and vitamin E prevented the increase in cell body area compared with the D group ($p < 0.01$). No differences were found between the N and NS groups ($p > 0.05$; Table 1).

Morphometric analysis of nNOS-immunoreactive myenteric neurons

Diabetes mellitus increased the cell body area of nNOS-immunoreactive neurons in the D group compared with the N group ($p < 0.01$). Hypertrophy was prevented in the DS group compared with the D group. No difference was found between the N and NS groups ($p > 0.05$; Table 1).
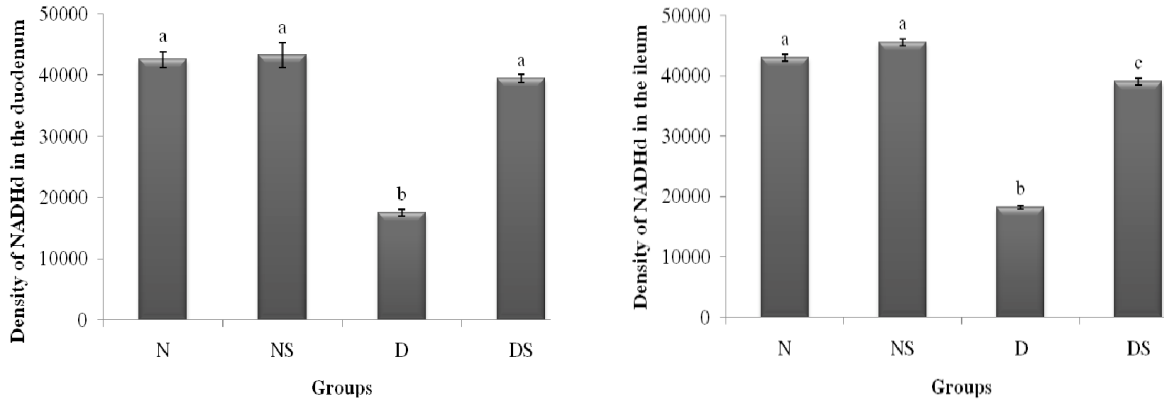
Figure 1 - Density of myenteric neurons NADH-d$^+$ in the duodenum and ileum of groups: normoglycemic (N), normoglycemic supplemented with vitamin C and vitamin E (NS), diabetic (D) and diabetic supplemented with vitamin C and vitamin E (DS). Mean values followed by different letters are statistically different according to Tukey's test (p < 0.05). Results were expressed as mean ± standard error. n = 5 rats per group.
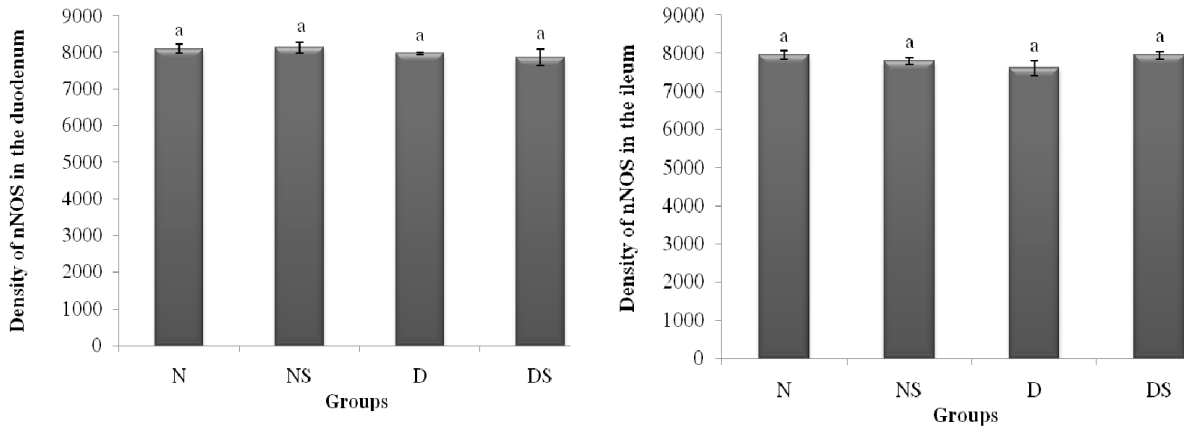
Figure 2 - Density of myenteric neurons nNOS in the duodenum and ileum of groups: normoglycemic (N), normoglycemic supplemented with vitamin C and vitamin E (NS), diabetic (D) and diabetic supplemented with vitamin C and vitamin E (DS). Mean values followed by different letters are statistically different according to Tukey's test (p < 0.05). Results were expressed as mean ± standard error. n = 5 rats per group.

Table 1 - Cell body area (μm^2) of NADH-d$^+$ and nNOS-immunoreactive myenteric neurons in the duodenum and ileum in the following groups: normoglycemic (N), normoglycemic supplemented with vitamin C and vitamin E (NS), diabetic (D), and diabetic supplemented vitamin C and vitamin E (DS).

	Duodenum		Ileum	
	NADH$^+$	nNOS	NADH$^+$	nNOS
N	256.5 ± 2.50[a]	226.3 ± 2.88[a]	258.6 ± 2.51[a]	231.0 ± 2.97[a]
NS	252.8 ± 2.57[a]	213.0 ± 2.42[a]	252.3 ± 2.60[a]	216.2 ± 2.68[a]
D	353.6 ± 3.39[b]	479.4 ± 6.35[b]	351.2 ± 3.35[b]	430.5 ± 7.05[b]
DS	257.4 ± 2.52[a]	227.7 ± 3.32[a]	260.3 ± 2.42[a]	216.2 ± 2.91[a]

Mean values followed by different letters in the same column are significantly different (*p* < 0.05, Tukey's test). The results are expressed as mean ± standard error. *n* = 5 rats per group.

DISCUSSION

The present results showed that 35 mg/kg streptozotocin was sufficient to induce diabetes in the D and DS groups, in which typical signs of diabetic status were observed, thus confirming reliable establishment of the experimental model.

Metabolically active neurons were affected by diabetes, with reductions of the density of NADH-d$^+$ myenteric neurons in the D group of 58.8 and 57.5% in the duodenum and ileum, respectively. The reduction of neuronal density is frequently found in experimental models of diabetes for the specific myenteric subpopulation (Pereira et al. 2006; Zanoni et al. 2007) and total population of neurons (Pereira et al. 2009).

Although the NADH-d technique does identify all neurons, it marks neurons that exhibit an increase in enzymatic activity, thus allowing the assessment of whether an increase or decrease in metabolism occurs. Thus, we can infer that diabetes promoted a reduction of neuron metabolism, leading to the death of a portion of its neuronal population. We observed a reduction of more than half of these neurons, and diabetic neuropathy was observed, similar to studies that used other experimental models of diabetes and evaluated different regions of the gastrointestinal tract, including the stomach (Takahashi et al. 1997; Fregonesi et al. 2001), duodenum (Pereira et al. 2006; Zanoni et al. 2011; Lopes et al. 2012), jejunum (Defani et al. 2003; De Freitas et al. 2008; Alves et al. 2010; Hermes-Uliana et al. 2014), ileum (Zanoni et al. 2003; Shotton and Lincoln 2006; Pereira et al. 2011), cecum (Zanoni et al. 1997; Zanoni et al. 2011), and proximal colon (Tashima et al. 2007; Roldi et al. 2009). The development of neuropathy affects different types of enteric neurons, which are responsible for the control of important functions, such as motility, secretion, blood flow, and growth aspects of the local mucosal immune system (Furness 2012). One of the factors that is responsible for damage to the enteric nervous system is oxidative stress. Hyperglycemia activates the polyol pathway, which increases the production of sorbitol. This increase results in cellular stress that leads to a decrease in intracellular antioxidant defenses. It can also induce an increase in the concentration of advanced glycosylation products, thereby altering cell function. Hyperglycemia can also activate nuclear transcription factors, triggering an increase in the expression of inflammatory mediators. The combination of these mechanisms alters the production of oxidants, causing cellular stress and resulting in structural damage (Calles-Escandon and Cipolla 2001; Vincent and Russell 2004; Brownlee 2005; Voukali et al. 2011).

In addition to the reduction of neuronal density, hypertrophy of the cell body area occurred of NADH-d$^+$ neurons in the duodenum (37.8%) and in the ileum (35.8%), possibly in an attempt to maintain physiological conditions of the neurons that survived. Similar results were reported in previous studies in diabetic rats (Pereira et al. 2011; Lopes et al. 2012; Hermes-Uliana et al. 2014). Adaptive mechanisms of the remaining neurons attempt to compensate for neuronal loss by increasing protein synthesis to maintain their function and target tissue (Zanoni et al. 2002; Hermes-Uliana et al. 2014).

Furthermore, the NADH-d technique marks the most metabolically active neurons (i.e., neurons that have more mitochondria or larger-volume organelles that are normally considered large compared with others; Araújo et al. 2009). Therefore, diabetes may have increased the neuronal activity of surviving neurons, thus promoting hypertrophy.

Nitrergic neurons (nNOS) did not exhibit changes in density with diabetes. Previous reports showed that this neuronal subpopulation may or may not be affected by diabetes mellitus. One factor to consider is the specific intestinal segment that is evaluated. In the duodenum (de Mello et al. 2009) and ileum (Wrzos et al. 1997; Zanoni et al. 2003; Pereira et al. 2008), the density of nNOS neurons was maintained. These results support the hypothesis that inhibitory neurons are more protected (Chandrasekharan and Srinivasan 2007), showing greater resistance of this neuronal subpopulation to death induced by free radicals that are present in high concentrations during the hyperglycemic state that is characteristic of diabetes (Pereira et al. 2008).

Despite the similarity in the nitrergic neuronal density observed in the present study, significant increases in the cell body area of 111.8 and 86.4% were found in the duodenum and ileum, respectively. Hypertrophy can be considered a form of compensation in an attempt to avoid the death of these neurons. Nitric oxide plays a role in the preservation of enteric neurons (Cowen et al. 2000). In concert with vasoactive intestinal peptide (VIP), NO is important for neuronal adaptation, maintenance, and survival (Lin et al. 2004).

Hypertrophy suggests an increase in the expression of the nNOS enzyme (Adegathe et al. 2003; Shotton et al. 2003). Thus, an increase in neurotransmitter levels possibly occurred, which can promote gastrointestinal changes because NO is a major inhibitory neurotransmitter in the intestine (Furness 2012).

An important consideration is that diabetic patients exhibit alterations in intestinal motility, which may result in an increase in intestinal transit time or diarrhea (Rodrigues and Motta 2012). Intestinal transit may be imbalanced in diabetes. The hypertrophy of nitrergic neurons has been shown to be responsible for non-adrenergic and non-cholinergic relaxation and smooth muscle (Takeuchi et al. 1998), which may be related to an increase in intestinal function.

Other studies reported an increase in the cell body area of nitrergic neurons (Fregonesi et al. 2005; Zanoni et al. 2003; Shotton and Lincoln 2006). The increased synthesis of NO by neurons in an attempt to compensate for the reduced availability of NADPH to nNOS in diabetes is a possible explanation for the increase in nitrergic neuron cell bodies (Fregonesi et al. 2005).

The use of antioxidant agents is one way to minimize or even prevent the damage caused by diabetes in the enteric nervous system. The use of two combined antioxidant vitamins in the present study showed promising results. We observed neuroprotection that prevented the reduction of the density of NADH-d$^+$ neurons in both segments of the small intestine. The protection afforded to NADH-d$^+$ neurons by vitamin C in diabetic animals was previously reported (Zanoni et al. 2007). These studies demonstrate the benefits of vitamin C therapy to scavenge free radicals, increase the levels of vitamin E, decrease the levels of lipid peroxidation in plasma, and increase the activity of glutathione peroxidase, with the consequent prevention of nerve dysfunction (Garg and Bansal 2000).

Vitamin C supplementation has been used for the treatment of neurological complications associated with diabetes because it reduces the concentration of sorbitol, inhibits aldose reductase, and reduces capillary fragility and oxidative stress (Will and Byers 1996), in addition to increasing the antioxidant defenses that are needed to combat oxidative events to preserve and protect neurons against lipid peroxidation (Nike et al. 2005).

Vitamin E acts on the lipid peroxidation process to protect polyunsaturated fatty acids from attack by free radicals (Traber 2007). The presence of vitamin E in neuronal cell membranes, particularly in the inner mitochondrial membrane, makes its use in diabetes promising. The combination of these antioxidant effects prevented enteric neuroplasticity, demonstrated by both techniques in both intestinal segments. The present findings suggest a synergistic effect that may prevent the changes caused by diabetes.

The combination of vitamin E with other antioxidants, such as vitamin C, also has beneficial effects on oxidative stress. Aksoy et al. (2005) found that treatment with a combination of these two vitamins 6 weeks after diabetes induction enhanced the antioxidant system and increased glutathione, superoxide dismutase, and glutathione peroxidase in erythrocytes. Vitamin E supplementation alone or combined with vitamin C was shown to modulate apoptosis (Barroso et al. 1997). Supplementation with these vitamins alone also showed promising results with regard to enteral neuroprotection (Pereira et al. 2006; Zanoni et al. 2007; Pereira et al. 2008; Roldi et al. 2009; Zanoni et al. 2009). However, other studies that tested the effects of these vitamins alone found that vitamin C (Pereira et al. 2009) and vitamin E (Tronchini et al. 2012) did not preserve enteric neurons in diabetic rats. These results demonstrate that the combination of these antioxidants may more markedly reduce oxidative stress and thus reduce the deleterious effects of diabetes. Further studies are needed to assess the effects of this combination on the enteric nervous system.

CONCLUSION

Supplementation with a combination of vitamin C and vitamin E promoted myenteric neuroprotection in the small intestine in diabetic rats, which may be promising for the prevention enteric diabetic neuropathy.

REFERENCES

Adeghate E, al-Ramadi B, Saleh AM, Vijayarasathy C, Ponery AS, Arafat K, et al. Increase in neuronal nitric oxide synthase content of the gastroduodenal tract of diabetic rats. *Cell Mol Life Sci*. 2003; 60: 1172-1179.

Aksoy N, Vural H, Sabuncu T, Arslan O, Aksoy S. Beneficial effects of vitamins C and E against oxidative stress in diabetic rats. *Nutr Res*. 2005; 25: 625- 630.

Alves EP, Alves AM, Pereira RV, Miranda Neto MH, Zanoni JN. Immunohistochemical study of vasoactive intestinal peptide (VIP) enteric neurons in diabetic rats supplemented with L-glutamine. *Nutr Neurosci*. 2010; 13: 43-51.

Araújo EJA, Hermes C, Miranda-Neto MH, Almeida EC, Sant'ana DMG. Atrophy of the nitrergic myenteric neurons in the descending colon rats submitted to protein and vitamin deficiency. *Int J Morphol*. 2009; 27: 939-945.

Barroso MP, Diaz CG, Lluch GL, Malagon MM, Crane FL, Navas P. Ascorbate and a-tocopherol prevent apoptosis induced by serum removal independent of Bcl-2. *Arch Biochem Biophys*. 1997; 343: 243-248.

Brownlee M. The pathobiology of diabetic complications a unifying mechanism. *Diabetes*. 2005; 54 (6): 1615-1625.

Calles-Escandon J, Cipolla M. Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev*. 2001; 22 (1): 36-52.

Chandrasekharan B, Srinivasan S. Diabetes and the enteric nervous system. *Neurogastroenterol Motil*. 2007; 19: 951-960.

Chandrasekharan B, Anitha M, Blatt R, Shahnavaz N, Kooby D, Staley C, et al. Colonic motor dysfunction in human diabetes is associated with enteric neuronal loss and increased oxidative stress. *Neurogastroenterol Motil*. 2011; 23: 131-138.

Cowen T, Johnson RJ, Soubeyre V, Santer RM. Restricted diet rescues rat enteric motor neurons from age related cell death. *Gut*. 2000; 47 (5): 653-660.

De Freitas P, Natali MRM, Pereira RVF, Miranda Neto MH, Zanoni JN. Myenteric neurons and intestinal mucosa of diabetic rats after ascorbic acid supplementation. *World J Gastroenterol*. 2008; 14 (42): 6518.

De Mello ST, Miranda Neto MH, Zanoni JN, Furlan MM. Effects of insulin treatment on HuC/HuD, NADH diaphorase, and nNOS-positive myoenteric neurons of the duodenum of adult rats with acute diabetes. *Dig Dis Sci*. 2009; 54: 731-737.

Defani MA, Zanoni JN, Natali MRM, Bazotte RB, Miranda Neto MH. Effect of acetyl-L-carnitine on Vip-ergic neurons in jejunum submucous plexus of diabetic rats. *Arq Neuropsiquiatr*. 2003; 61(4): 962-967.

El-hadjela D, Zine K, Leila B. The beneficial effect of combined administration of vitamins C and E on renal function and selected parameters of antioxidant system in diabetic rats fed zinc-deficient diet. *Afr J Biotechnol*. 2013; 12 (43): 6232-6240.

Fregonesi CE, Molinari SL, Alves AM, Defani MA, Zanoni JN, Bazotte RB, et al. Morphoquantitative aspects of nitrergic myoenteric neurons from the stomach of diabetic rats supplemented with acetyl-L-carnitine. *Anat Histol Embryol*. 2005; 34: 93-7.

Fregonesi CE, Miranda Neto MH, Molinari SL, Zanoni JN. Quantitative study of the myenteric plexus of the stomach of rats with streptozotocin-induced diabetes. *Arq Neuropsiquiatr*. 2001; 59: 50-53.

Furness, JB. The enteric nervous system and neurogastroenterology. *Nat Rev Gastroenterol Hepatol*. 2012; 9 (5): 286-294.

Gabella G. Dettection of nerve cells by histochemical technique. *Experientia*. 1969; 25: 218-219.

Garg MC, Bansal DD. Protective antioxidant effect of vitamins C and E in streptozotocin induced diabetic rats. *Indian J Exp Biol*. 2000; 38: 101-104.

Geirsdottir OG, Arnarson A, Briem K, Ramel A, Jonsson PV, Thorsdottir I. Effect of 12 week resistance exercise program on body composition, muscle strength, physical function, and glucose metabolism in healthy, insulin-resistant, and diabetic elderly Icelanders. *J Gerontol A Biol Sci Med Sci*. 2012; 67 (11): 1259-1265.

Hermes-Uliana C, Panizzon CP, Trevizan AR, Sehaber CC, Ramalho FV, Martins HA, Zanoni JN. Is l-glutathione more effective than l-glutamine in preventing enteric diabetic neuropathy? *Dig Dis Sci*. 2014; 59(5): 937-948.

Lin Z, Sandgren K, Ekblad E. Increased expression of oxide synthase in culture neurons from adult rat colonic submucous ganglia. *Auton Neurosci*. 2004; 114: 29-38.

Lopes CR, Ferreira PE, Zanoni JN, Alves AM, Alves EP, Buttow NC. Neuroprotective effect of quercetin on the duodenum enteric nervous system of streptozotocin-induced diabetic rats. *Dig Dis Sci*. 2012; 57 (12): 3106-3115.

Niki E, Yoshida Y, Saito Y, Noguchi N. Lipid peroxidation: mechanisms, inhibition, and biological effects. *Biochem Biophys Res Commun*. 2005; 338: 668-676.

Pereira MAS, Liberati AP, Souza IDS, Zanoni JN. Density and morphometry of overall myenteric neurons population from the duodenum of diabetic rats (*Rattus norvegicus*) under ascorbic acid supplementation. *Int J Morphol*. 2009; 27 (2): 413-418.

Pereira MAS, Bagatin MC, Zanoni JN. Effects of the ascorbic acid supplementation on NADH-diaphorase myenteric neurons in the duodenum of diabetic rats. *Biocell*. 2006; 30 (2): 295-300.

Pereira RV, Miranda Neto MH, da Silva Souza ID, Zanoni JN. Vitamin E supplementation in rats with experimental diabetes mellitus: analysis of myosin-V and nNOS immunoreactive myenteric neurons from terminal ileum. *J Mol Histol*. 2008; 39: 595-603.

Pereira RV, Tronchini EA, Tashima CM, Alves EP, Lima MM, Zanoni JN. L-glutamine supplementation prevents myenteric neuron loss and has gliatrophic effects in the ileum of diabetic rats. *Dig Dis Sci*. 2011; 56 (12): 3507-3516.

Rodrigues MLC, Motta MEFA. Mechanisms and factors associated with gastrointestinal symptoms in patients with diabetes mellitus. *J Pediatr*. 2012; 88: 17-24.

Roldi LP, Pereira RV, Tronchini EA, Rizo GV, Scoaris CR, Zanoni JN, et al. Vitamin E (α-tocopherol) supplementation in diabetic rats: effects on the proximal colon. *BMC Gastroenterol*. 2009; 9: 88.

Shotton HR, Clarke S, Lincoln J. The effectiveness of treatments of diabetic autonomic neuropathy is not the same supplying different organs. *Diabetes*. 2003; 52: 157-164.

Shotton HR, Lincoln J. Diabetes only affects nitric oxide synthase-containing myenteric neurons that do not contain heme oxygenase 2. *Brain Res*. 2006; 1068: 248-256.

Stavniichuk R, Shevalye H, Lupachyk S, Obrosov A, Groves JT, Obrosova IG, et al. Peroxynitrite and protein nitration in the pathogenesis of diabetic peripheral neuropathy. *Diabetes Metab Res Rev*. 2014; 28.

Stefanini M, De Martino C, Zamboni L. Fixation of ejaculated spermatozoa for electron microscopy. *Nature*. 1967; 4: 216:173.

Takahashi T, Nakamura K, Itoh H, Sima AA, Owyang C. Impaired expression of nitric oxide synthase in the gastric myenteric plexus of spontaneously diabetic rats. *Gastroenterol*. 1997; 113 (5): 1535-1544.

Takeuchi T, Niioka S, Yamaji M, Okishio Y, Ishii T, Nishio H, et al. Decrease in participation of nitric oxide in nonadrenergic noncholinergic relaxation of rat intestine with age. *Jpn J Pharmacol*. 1998; 78 (3): 293-302.

Tashima CM, Tronchini EA, Pereira RV, Bazotte RB, Zanoni JN. Diabetic rats supplemented with L-glutamine: a study of immunoreactive myosin-V myenteric neurons and the proximal colonic mucosa. *Dig Dis Sci*. 2007; 52 (5): 1233-1241.

Traber MG. Vitamin E regulatory mechanisms. *Annu Rev Nutr*. 2007; 27: 347-362.

Tronchini EA, Trevizan AR, Tashima CM, Pereira RV, Zanoni JN. Supplementation with 0.1% and 2% vitamin e in diabetic rats: analysis of myenteric neurons immunostained for myosin-V and nNOS in the jejunum. *Arq Gastroenterol*. 2012; 49 (4): 284-290.

Vincent AM, Russell JW, Low P, Feldman EL. Oxidative stress in the pathogenesis of diabetic neuropathy. *Endocr Rev*. 2004; 25: 612-628.

Voukali E, Shotton HR, Lincoln J. Selective responses of myenteric neurons to oxidative stress and diabetic stimuli. *Neurogastroenterol Motil*. 2011; 23: 964-e411.

Wrzos HF, Cruz A, Polavarapu R, Shearer D, Ouyang A. Nitric oxide shyntase (NOS) expression in the myenteric plexus of streptozotocin-diabetic rats. *Dig Dis Sci*. 1997; 42: 2106-2110.

Will JC, Byers T. Does diabetes mellitus increase the requirement for vitamin C?. *Nutr Rev*. 1996; 54 (7): 193-202.

Zanoni JN, Buttow NC, Bazotte RB, Miranda Neto MH. Evaluation of the population of NADPH-diaphorase-stained and myosin-V myenteric neurons in the ileum of chronically streptozotocin-diabetic rats treated with ascorbic acid. *Auton Neurosci*. 2003; 104: 32-38.

Zanoni JN, Hernandes L, Bazotte RB, Miranda-Neto MH. Terminal ileum submucous plexus: Study of the VIP-ergic neurons of diabetic rats treated with ascorbic acid. *Arq Neuropsiquiatr*. 2002; 60 (1): 28-31.

Zanoni JN, Miranda Neto MH, Bazotte RB, de Souza RR. Morphological and quantitative analysis of the neurons of the myenteric plexus of the cecum of streptozotocin-induced diabetic rats. *Arq Neuropsiquiatr*. 1997; 55 (4): 696-702.

Zanoni JN, Pereira PGC, Pereira MAS. Analysis of myenteric neurons of the cecum of diabetic rats after supplementation with ascorbic acid. *Int J Morphol*. 2009; 27 (2): 387-392.

Zanoni JN, Pereira RVF, Freitas PD. Effect of the ascorbic acid treatment on the NADHd-positive myenteric neurons of diabetic rats proximal colon. *Braz Arch Biol Technol*. 2007; 50 (1): 31-38.

Zanoni JN, Tronchini EA, Moure SA, Souza ID. Effects of L-glutamine supplementation on the myenteric neurons from the duodenum and cecum of diabetic rats. *Arq Gastroenterol*. 2011; 48 (1): 66-71.

Zaken V, Kohen R, Ornoy A. Vitamins C and E improve rat embryonic antioxidant defense mechanism in diabetic culture medium. *Teratology*. 2001; 64 (1): 33-44.

Zitkus BS. Update on the American Diabetes Association Standards of Medical Care. *Nurse Pract*. 2014; 39 (8): 22-32.