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Effects of Quercetin-Supplementation in NADH-Diaphorase Positive Neurons Subpopulations in the Ileum of Rats with Experimental Diabetes Mellitus

Efectos de la Suplementación con Quercetina en Subpoblaciones de Neuronas NADH-diaforasa Positivas en el Ileon de Ratas con Diabetes Mellitus Experimental

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SUMMARY: The effects of quercetin supplementation in NADH-diaphorase positive (NADH-d) neurons of streptozotocin-induced diabetic rats was carried in this study. Fifteen male rats were divided into three groups: normoglycemic (N), diabetic (D) and diabetic supplemented with quercetin (DQ). Whole mount preparations of the muscular layer of the ileum underwent NADH-d histochemistry for evidencing the NADH-d neuronal subpopulation. Quantitative analyzes were performed on 30 random fields, and morphometric analyzes in 100 neuronal bodies and nuclei per animal. The supplementation promoted a 44 % reduction in the neuronal density in D group when compared to N group ($p < 0.001$); a 24.5 % reduction was observed in the DQ group when compared to N ($p < 0.01$). Animals in D group presented an 18.7 % increase in the cell body areas of myenteric neurons when compared to N ($p < 0.001$); DQ group showed a 14.2 % decrease in neuronal areas when compared to D ($p < 0.01$); the nuclear area were similar among the three groups. We conclude that quercetin supplementation was positive for animals with diabetes mellitus.

KEY WORDS: Diabetes mellitus;Enteric neurons; Quercetin; NADH diaphorase.

INTRODUCTION

Diabetes mellitus (DM) lists a group of metabolic diseases and is characterized by presenting a chronic state of hyperglycemia, which leads to increased activity of the polyol pathway, lipid peroxidation, oxidative glycosylation and oxidative stress, with a consequent increased formation of free radicals (Rolo & Palmeira, 2006).

Free radicals can directly affect the sympathetic, parasympathetic, and enteric neural components, resulting in functional loss of multiple systems, such as the gastrointestinal tract. The combination of intracellular signaling disorders (De Giorgio & Camilleri, 2004), morphoquantitative and neurochemical changes of enteric neurons may be linked to relevant clinical problems of neurological manifestations of DM. Therapeutic measures are being concentrated in an attempt to prevent and/or reduce the excess of free radicals generated during the oxidative stress, in order to prevent or lessen the neurological diabetes complications (Shirpoor *et al*., 2007).

Quercetin (3, 5, 7, 3 '-4' - pentahydroxiflavone) stands out among the antioxidants as the main flavonoid present in the human diet. It is found in large quantities in foods such as apples, green tea, pomegranate, broccoli, berries, chard and many others. It exerts biological protective actions in the organism, which include antihypertensive, dysrhythmic, hypocholesterolemic, antihepatotoxicity, anticarcinogenic, antiviral, antiulcerogenic, antithrombotic, anti-ischemic, anti-inflammatory and antiallergic (Formica & Regelson, 1995).

As for the quercetin antioxidant properties, there are reports showing its effectiveness in inhibiting cyclooxygenase and lipoxygenase enzymes involved in the synthesis of eicosanoids from arachidonic acid in its sweeping action of superoxide anions, singlet oxygen and hydroxyl radicals; acting as a chelating agent for transition metal ions such as iron and copper (Formica & Regelson), preventing them to catalyze reactions forming free radicals.

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Increased oxidative stress and reduction in the antioxidative activity observed in experimental and clinical diabetes, have been implicated in the development of neuropathy and in other typical complications of this disease. However, although many studies indicate a nonselective action of the neuropathy, it is known that the enteric neurons and their neurotransmitters, as well as the different intestinal segments, are not affected to the same degree and extent in the presence of diabetes. In this context, there is evidence that the survival of neurons seems to be somehow associated with the ability of these cells to resist to damage from reactive oxygen species (ROS) (Finkel & Holbrook, 2000).

The basis for the proper functioning of the gastrointestinal tract is the normal interrelationship among the various sub-neuronal populations. The morphoquantitative changes in neurons, immunoreactivity, neurotransmitter content and the interaction between the neuronal and glial cells (Korsak *et al*., 2012) are responsible for the appearing of the typical diabetic autonomic neuropathy in the digestive tract, such as diarrhea and constipation, mega colon, slow gastrointestinal motility, gastric stasis and dilation with decreased or increased peristaltic contractions, not to mention the more severe gastric disorders such as gastroparesis, whose symptoms often go undiagnosed, characterized by anorexia, weight loss, nausea and vomiting (Abrahamsson, 1995).

The purpose of this study was to investigate the effects of the quercetin supplementation in NADH-d neuronal subpopulation in the ileum of rats with experimental diabetes, since the pathophysiological conditions present in the diabetes could be generated by the reduction in the levels of antioxidant defense and consequent increase in the oxidative stress, among other factors that may compromise the function of neurons in the myenteric plexus.

MATERIAL AND METHOD

Preparation of Experimental Groups. All the experiment procedures described in this article are consistent with the ethical principles established by the Brazilian College of Animal Experiments (COBEA) and were previously analyzed by the Ethics Committee on Animal Experiments of the State University of Maringá (UEM) (nº053/2009).

Fifteen male Wistar rats (*Rattus norvegicus*) from the Central Animal Facility of the State University of Maringá were used. The animals were divided into three groups, namely: normoglycemic (N), diabetic (D) and diabetic supplemented with quercetin (DQ).

The rats were kept in polypropylene boxes for a period of 120 days, with a 12-hour photoperiod (6:00 - 18:00) and controlled temperature (24 ± 2 °C), receiving standard balanced chow Nuvital® (Nuvilab, Colombo, PR, Brazil) *ad libitum*.

Diabetes mellitus was induced at the age of 90 days in animals from D and DQ groups, after a 14 h fast, by an intravenous injection of streptozotocin (35 mg/kg body weight; Sigma, St Louis, MO, USA) dissolved in citrate buffer, pH 4.5 (10 mM). After the induction of diabetes mellitus, glycemia was determined using the glucose oxidase method (Accu-Chek Active; Roche Diagnostics, Mannheim, Germany) to confirm the establishment of the experimental model.

After streptozotocin injection and diabetes confirmation, the DQ animals received daily water supplemented with quercetin (200 mg/kg body weight). Animals in groups N and D received water without supplementation.

Water intake preliminary evaluation was performed during three consecutive days in order to find the average water consumption per animal in DQ group and allow the calculation of quercetin (200 mg/kg). The animals were also weighed periodically and the group mean was used to maintain the amount of quercetin given during the experiment.

Material collection and processing. After 120 days (210-days-old), the animals were weighed and euthanized following anesthesia with Thiopental® (40 mg/kg I.P.; Abbott Laboratories, Chicago, IL, USA). Blood was collected by cardiac puncture and blood glucose concentration measured using the glucose oxidase method. A laparotomy was performed and ileum were removed.

Histochemistry for disclosure neuronal NADH-diaphorase. The ileum were prepared for the NADH-diaphorase histochemistry (Gabella, 1969) as follows: 5 cm of the ileum of each animal were collected, immediately washed in Krebs, tied at one end and injected with Krebs solution, immersed in Krebs (20 minutes), Triton X-100 (0.3 % for 5 minutes in Krebs, Sigma), washed in Krebs (5 minutes for each solution). The segments were immersed in a solution containing b-NADH (Sigma, St. Louis, MO, USA) and nitro blue tetrazolium (NBT) (Sigma, St. Louis, MO, USA) for 45 minutes. The reaction was interrupted with 10 % formalin solution. They were then dissected under stereomicroscope to obtain membrane preparations. Later, they were dehydrated and mounted between slide and coverslip. The NADH-d reaction product appears in different shades of blue/purple.

Quantitative Analysis. The density of myenteric neurons NADH-d was analyzed using images from the intermediate region of the ileum obtained by sampling. The images were taken by a high resolution camera AxioCam (Zeiss, Jena, Germany) coupled to a light microscope (Olympus BX40), transferred to a computer using the Axioskop Plus (Zeiss, Jena, Germany) software and recorded in a compact disc. Neuronal cell bodies present in 30 images, taken with a 20X objective, were counted for each animal. Data were expressed as neurons/cm^2. Image-Pro Plus 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA) was used to perform the neuronal counting and determination of the images total areas.

Morphometric Analysis. For each animal, 100 nuclei and 100 neuronal cell bodies areas (mm^2) were measured, using same images. Data were expressed in μm^2. The software 4.5.0.29 Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) was used for the morphometric analysis.

Statistical Analysis. The results were analyzed using the Statistica 7.1 and GraphPadPrism 5.1 and written as mean ± standard error of the mean (SEM). Since the morphometric data did not have normal distribution, they were analyzed by randomized block, followed by Tukey test. For other results, the analysis of variance one-way ANOVA, followed by Tukey test were employed. P <0.05 were considered statistically significant.

RESULTS

We observed typical DM signs such as polydipsia and weight loss in animals in D and DQ groups (p <0.05) when compared to N group. In addition, blood glucose was higher (p <0.05) in animals in D and DQ groups when compared to N group (Table I). 120 days after the diabetes induction, the quercetin-supplementation did not affect the fasting plasma glucose in the DQ group when compared to D group (p> 0.05).

Cell bodies of NADH-d neurons were seen in the myenteric ganglia and disposed between the interganglia nerve fibers (Fig. 1).

Myenteric neurons quantitative analysis showed 44 % reduction in the neuronal density in D group (p <0.001) and a 24.5 % reduction in DQ group when compared with N group (p <0.01). There was a 19.5 % preservation in the neuronal density in the supplemented group. Neuronal density in the quercetin-supplemented animals (DQ) was 35 % higher than the D group (p <0.05) (Fig. 2).

The cell bodies areas of NADH-d neurons showed an increase of 18.7 % in the average body cell areas of myenteric neurons in group D when compared to group N (p <0.001). When the ratio between the nucleus and cytoplasm of the neurons of group D is calculated, the nucleus represents 25.7 % of the total cell area, whereas in group N the nucleus represents 31.5 %. On the other hand, the comparison between the nucleus in N and D shows no statistical difference. The neuronal area in the DQ group showed a decrease of 14.2 % when compared to group D (p <0.01). The nucleus of neurons analyzed in the DQ group represents 30.8 % of the cell area,

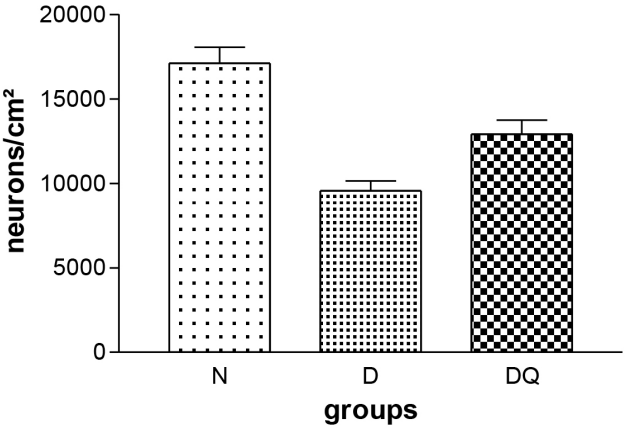

Fig. 2. Density of neurons NADH-d+ (neurons/cm^2) in the animal groups: normoglycemic (N), diabetic (D) and diabetic supplemented with quercetin (DQ). Data expressed as mean ± SEM. n=5 (p <0.05).

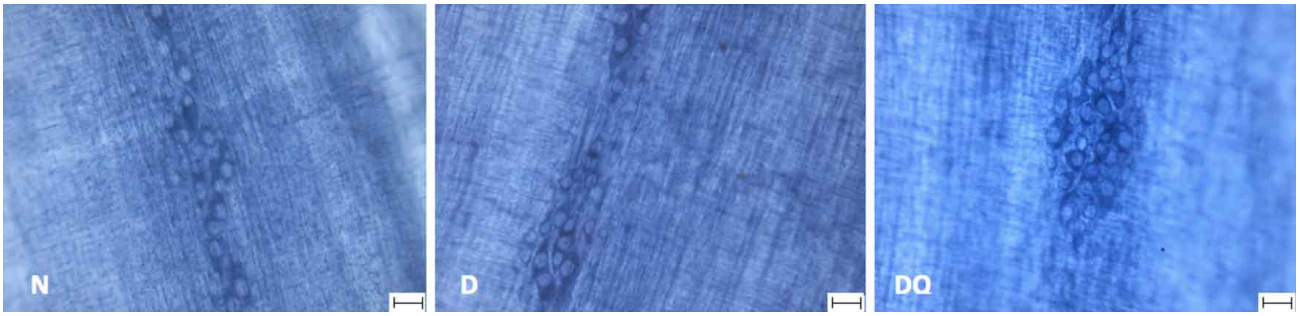

Fig. 1. Micrographs of NADH-d+ myenteric neurons in the ileum of rats in groups: normoglycemic (N), diabetic (D), diabetic supplemented with quercetin (DQ). Calibration bar: 20 mm

close to the result found in group N. The nucleus area and cytoplasm results of animals in groups N and DQ were not statistically different (Table II).

Table I. Parameters evaluated in 210 days old Wistar rats groups: normoglycemic (N), diabetic (D) and diabetic quercetin-supplemented (DQ) (n = 5). Results are expressed as mean ± SEM.

Parameters/Group	N	D	DQ
Initial weight (g)	340.5 ± 8.5[a]	333.0 ± 3.7[a]	367.8 ± 6.3[a]
Final weight (g)	517.4 ± 12.8[a]	301.0 ± 21.4[b]	291.80 ± 8.1[c]
Final Glucose (mg/dL)	137.8 ± 5.4[a]	601.8 ± 40.2[b]	542.0 ± 46.3[b]
Daily water intake (ml/day)	41.82 ± 3.2[a]	113.3 ± 15.6[b]	103.4 ± 21.8[b]

Means followed by different letters in the same line are statistically different according to Tukey test ($p < 0.05$).

Table II. Average body area and neuronal nuclei and the nucleus-cytoplasm ratio of ileum myenteric neurons in rats groups: normoglycemic (N), diabetic (D) and diabetic quercetin-supplemented (DQ) n = 5.

	N	D	DQ
Neuronal body area	166.7 ± 2.1[a]	198.0 ± 3.4[b]	170.3 ± 2.5[a,c]
Nuclei area (μm^2)	52.5 ± 2.0[a]	50.9 ± 1.5[a]	52.5 ± 1.6[a]
Nucleus/cytoplasm	31.5 ± 0.4[a]	25.7 ± 0.8[b]	30.8 ± 1.1[a,c]

Means followed by different letters in the same line are statistically different according to Tukey test ($p < 0.05$).

DISCUSSION

There was no significant difference ($P > 0.05$) in the animals' body weight between groups N, D and DQ at the beginning of our experiment. However, at the end of the experiment we observed that the animals of group N had a weight gain of 52 %, whereas the non-supplemented diabetic rats had an average loss of 6.6 %. Weight loss was even more significant in group DQ (20.67 %). The weight loss in group D may be attributed to increased lipolysis, due to the sharp protein catabolism and dehydration seen in the acute metabolic decompensating stages of the DM. It is evident that quercetin was the causative agent of the weight loss intensification in group DQ, which may have a direct relationship with the anti-lipogenic action of this flavonoid as reported by Shisheva & Schecter (1992).

The quercetin supplementation in DQ did not prevent that the fasting plasma glucose levels were very high, although they are slightly lower, on average, than those seen in non-supplemented diabetics (Table I). Significant hypoglycemic properties have been reported for quercetin in normoglycemic animals (Mahesh & Menom, 2004). Other antioxidant compounds have been evaluated in addition to quercetin and the literature shows conflicting results regarding their effects on fasting glucose levels of diabetic rats. There are reports of hypoglycemic effects by supplementation with vitamin E (Wan Nazaimoon & Khalid, 2002), ascorbic acid (Adeneye *et al*., 2007) a-lipoic acid (Maritim *et al*., 2003), whereas the l-glutamine had no action on glycaemia in a study by Pereira *et al*. (2011).

The enteric nervous system (ENS) plays important roles in the gastrointestinal tract which are governed by the absorption/secretion regulation, motility control and vascular tone. They can be altered by disorders and syndromes arising from changes in the number and morphology of neurons under various pathological conditions, including the DM.

The neuronal density reduction in the myenteric plexus of non-diabetic rats supplemented with quercetin (group D), can be attributed, among other factors, to the reduction of antioxidant defenses and to the concomitant oxidative stress increase, which generates free radicals as observed in endothelial cells (Giuliano *et al*., 1996). Free radicals may react with cellular components causing severe damage, compromising the neuronal functioning both in the central and in the peripheral nervous system.

The reduction in the enteric neurons in diabetic rats and the correlation between these neurons loss to oxidative stress is very well established by literature. Besides developing the neuropathy, the increase in free radicals due to oxidative has been associated with the onset of other chronic complications of diabetes.

When analyzing the data from quercetin-supplemented animals, we observed that the substance was able to partially prevent neuronal degeneration, with results close to those of group N. The lower loss of these neurons in the DQ group when compared to the group D can be attributed to a neuroprotective effect of quercetin supplementation due to its antioxidant properties (Formica & Regelson), which are important to mitigate the damage caused by low levels of other antioxidants, such as vitamins C, E and glutathione (GSH) in plasma and tissues of diabetic animals. It was also found that quercetin increases the activity of superoxide dismutase and reduces the malondialdehyde levels in the aging brain, thus indicating its role in decreasing oxidative stress with consequent improvements in cognition (Spencer, 2009). The quercetin supplementation reduced the loss of HuC/D neurons and enteric glial cells in the duodenum enteric plexus of diabetic rats.

Many studies have assessed the effects of several antioxidants on the enteric neurons of diabetic rats such as: Glutamine as an important precursor of glutathione and, therefore, it could have an action mechanism similar to quercetina (Pereira *et al*., 2011); vitamin C together with vitamin E neutralizes free radicals; vitamin E, which donates electrons to neutralize free radicals, transforms itself into a potent free radical and needs electrons donated by vitamin C to recompose and start acting as an antioxidant while the radical formed by vitamin C is eliminated (Veit & Zanoni, 2012). In general, all the authors who have studied antioxidants found neuroprotective effect evidenced by lower neuronal loss or prevention of morphological changes in the neurons of diabetics.

An increase in the cell body area of NADH-d myenteric neurons in diabetic rats was found in this experiment. The increase in enteric neuronal area in diabetic rats has been attributed to several factors, such as an increase in the synthesis machinery; difficulty of neurotransmitters removal (Arciszewski & Ekblad, 2005) and intracellular edema (Hosking *et al*., 1978).

Similarly to what was observed in this study, supplementation with other antioxidants has helped keep the body cell area of neurons of diabetic rats with almost the same size of normoglycemic animals, as seen in the jejunum with vitamin C-supplementation (De Freitas *et al*., 2008), VIP-ergic neurons in the jejunum of glutamine-supplementation (Alves *et al*., 2010) and vitamin E supplementation in the ileum (Pereira *et al*., 2008).

The maintenance of the cell body area as well as of the nucleus area in the neurons of quercetin-supplemented rats reinforce the evidence of neuroprotection demonstrated by this flavonoid, already evidenced by the lower loss of neuronal density of group D. These data also indicate that the ability to perform autophagy in order to eliminate the undesired cytoplasmic accumulation of compounds or organelles and damaged membrane parts was maintained in the majority of neurons due to the quercetin-supplementation. However, part of the cells did not benefit from this mechanism leading to their elimination (Pietrocola *et al*., 2012). In addition, the areas of the nuclei were not different among the groups and there was no occurrence of minor or pyknotic nuclei which would be common if neurons were going to a condition of apoptosis (Wyllie, 1997).

Summing up, it could be said that the quercetin supplementation in diabetic animals promotes a set of metabolic changes that lead to an increase of weight loss and a slight reduction in blood glucose, whereas the NADH-d neurons in the myenteric plexus of the ileum maintains the nucleus and cytoplasm volume and reduces neuronal loss.

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RESUMEN:Se estudiaron los efectos de la suplementación con quercetina en neuronas NADH-diaforasa positiva (NADH-d) de ratas diabéticas inducidas por estreptozotocina. Quince ratas machos se dividieron en tres grupos: normoglicémico (N), diabéticos (D) y diabéticos suplementados con quercetina (DQ). Las cortes montados de la capa muscular del íleon fueron sometidos a histoquímica de NADH-d para evidenciar la subpoblación neuronal NADH-d. Se realizaron análisis cuantitativos en 30 campos aleatorios y análisis morfométricos en 100 cuerpos y núcleos neuronales, por animal. La suplementación promovió una reducción del 44 % en la densidad neuronal en el grupo D cuando se comparó con el grupo N ($p < 0,001$). Se observó una reducción del 24,5 % en el grupo DQ en comparación con N ($p < 0,01$). Los animales del grupo D presentaron un aumento del 18,7 % en las áreas del cuerpo celular de las neuronas mientéricas cuando se compararon con N ($p < 0,001$). El grupo DQ mostró una disminución de 14,2 % en las áreas neuronales en comparación con D ($p < 0,01$). El área nuclear fue similar entre los tres grupos. Se concluye que la suplementación con quercetina fue positiva para animales con diabetes mellitus.

PALABRAS CLAVE: Diabetes mellitus; Neuronas entéricas; Quercetina; NADH diaforasa.

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