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Desired and side effects of the supplementation with L-glutamine and L-glutathione in enteric glia of diabetic rats

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ABSTRACT

Background/Aims: Enteric neuropathy associated with Diabetes Mellitus causes dysfunction in the digestive system, such as: nausea, diarrhea, constipation, vomiting, among others. The aim of this study was to compare the effects of supplementation with 2% L-glutamine and 1% L-glutathione on neurons and enteric glial cells of ileum of diabetic rats.
Methods: Thirty male Wistar rats have been used according to these group distributions: Normoglycemic (N), Normoglycemic supplemented with L-glutamine (NG), Normoglycemic supplemented with L-glutathione (NGO), Diabetic (D), Diabetic supplemented with L-glutamine (DG) and Diabetic supplemented with L-glutathione (DGO). After 120 days, the ileum was processed for immunohistochemistry of HuC/D and S100β. Quantitative and morphometric analysis have been performed.
Results: Diabetic rats presented a decrease in the number of neurons when compared to normoglycemic animals. However, diabetes was not associated with a change in glial density. L-Glutathione prevented the neuronal death in diabetic rats. L-Glutathione increased a glial proliferation in diabetic rats. The neuronal area in diabetic rats increased in relation to the normoglycemics. The diabetic rats supplemented with L-glutamine and L-glutathione showed a smaller neuronal area in comparison to diabetic group. The glial cell area was a decreased in the diabetics. The diabetic rats supplemented with L-glutamine and L-glutathione did not have significant difference in the glial cell body area when compared to diabetic rats.
Conclusion: It is concluded that the usage of L-glutamine and L-glutathione as supplements presents both desired and side effects that are different for the same substance in considering normoglycemic or diabetic animals.

1. Introduction

Diabetes mellitus is associated with disorders in the gastrointestinal tract (GIT), which include symptoms, such as: nausea, swelling, abdominal pain, diarrhea, constipation and delay in the gastric emptying (Bytzer et al., 2001). The pathogenesis of changes caused by diabetes in the GIT is still under investigation and the role of enteric nervous system (ENS) in this pathogenesis increased appreciation (Chandrasekharan and Srinivasan, 2007). The ENS is present throughout the GIT and coordinates motility, blood flow and secretion. It composed of neurons and enteric glial cells (EGCs) (Furness, 2006). The cell bodies of enteric neurons are in ganglia within myenteric and submucosal plexues. EGCs are found in all intestine layers, but are more prevalent within ganglia (Ruhl, 2005). Many studies have been carried out in rat models of dia-

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betes, that have evaluated damage to the ENS, especially structural and functional alterations that reach the enteric neurons in different GIT segments, as: stomach (Fregonesi et al., 2001), duodenum (Takahara et al., 2001), ileum (Pereira et al., 2008; Shotton and Lincoln, 2006; Zanoni et al., 2003), jejunum (Defani et al., 2003; De Freitas et al., 2008), cecum (Zanoni et al., 1997), and proximal colon (Tashima et al., 2007). However, there are fewer studies in relation to ECGs under the same experimental condition. It is expected that both neurons and ECGs can be affected by oxidative stress brought by diabetes.

Studies using supplementation with several antioxidant substances have revealed positive effects on the enteric neurons, for instance, vitamin C (Pereira et al., 2006), vitamin E (Roldi et al., 2009), and *Ginkgo biloba* (da Silva et al., 2011). Alves et al. (2010) and Pereira et al. (2011) investigated the effects of supplementation with 2% L-glutamine in the ENS of diabetic rats and in both works, the supplementation helps protect the ENS. Ueno et al. (2002) used 1% L-glutathione to verify the action on the neuropathy and nephropathy of diabetic rats and they suggests a potential usefulness of supplementation with L-glutatione to reduce diabetic complications.

L-Glutamine, an amino acid (Kuyvenhoven and Meinders, 1999), and L-glutathione, a nonprotein thiol (Ueno et al., 2002), both play and important role in intermediary metabolic pathway regulation (Wu, 2009). l-Glutamine is a substrate in forming L-glutathione (Wu et al., 2004), the main endogenous antioxidant because it controls free radicals homeostasis (Wu et al., 2004; Forman et al., 2009), which also presents a detoxifying role of xenobiotics in the organism (Joseph et al., 1997).

Aiming to extend these previous studies, the present study compares the effect of supplementation with 2% L-glutamine and 1% L-glutathione on enteric neurons and the ECGs in the ileum myenteric plexus of experimental induced-diabetic rats. To our knowledge, these therapies have not been tested simultaneously in the same study previously.

2. Material and methods

2.1. Procedure on animals

All experimental procedures were done according to the ethical principles adopted by the Brazilian Society for Laboratory Animal Science (SBCAL/COBEA) and were approved by the Ethics Committee on Animals Experiments of the Universidade Estadual de Maringá (UEM), under register numbered 2009/2005.

Thirty Wistar rats, all raised from a UEM breeding colony, were used in this study. The animals were randomly distributed into six groups with 5 animals in each one, which are: normoglycemic (N), normoglycemic supplemented with 2% L-glutamine (NG), normoglycemic supplemented with 1% L-glutathione (NGO), diabetic (D), diabetic supplemented with 2% L-glutamine (DG), and diabetic supplemented with 1% L-glutathione (DGO).

At 90 days of age, the animals were housed in polypropylene boxes, they received water and ration *ad libitum* and were kept on environmental conditions controlled by temperature (24 ± 2 °C) and lighting (cycle 12 h light/12 h dark). The non-supplemented animals (groups N and D) got standard balanced rations Nuvital (Nuvilab, Colombo, PR, Brazil). For the NG and DG groups, L-glutamine (DEG, São Paulo, SP, Brazil, code: 30192) was incorporated into the standard ration at concentration of 2% (Alves et al., 2010; Pereira et al., 2011). For the NGO and DGO groups L-glutathione (DEG, São Paulo, SP, Brazil, code: 30324, purity: 97,5%) was incorporated into the standard ration at concentration of 1% (Ueno et al., 2002). Food intake was evaluated every 15 days (g/day).

Animal groups D, DG and DGO were subjected to a fourteen-hour fast, before an intravenous injection of streptozotocin (35 mg/kg body weight; Sigma, St. Louis, MO, USA, code: S0130, purity: ≥98%) dissolved in citrate buffer solution pH 4.5 (10 mM). Diabetes was verified by blood glucose test, four days after induction. A drop of blood was obtained from the tail of the animals to measurement of blood glucose through photometrical determination of glucose by glucose dye oxidoreductase (Accu-Chek Active glucometer, Roche Diagnostics GmbH, Mannheim, BW, Germany). Only animals having blood glucose levels >200 mg/dl were used. The animals used in this study are the same as those used by Hermes-Uliana et al. (2014).

2.2. Material collection and processing

At 210 days of age, animals were weighed and killed under anesthesia with thiopental (40 mg/kg body weight, i.p: Abbott Laboratories, Chicago, IL, USA). Blood was collected by cardiac puncture for measurement of blood glucose (glucose oxidase method). After celiotomy, the distal ileum was removed, washed with phosphate-buffered saline (PBS, 0.1 M, pH 7.4) and carefully filled with Zamboniís fixative solution (Stefanini et al., 1967), fulfilling the space previously taken by content in such way that the segment could not be distended.

For immunohistochemistry, ileum segments were fixed as a tube for 18 h in the same Zamboni fixative solution (Stefanini et al., 1967). At the end of this period, the segment extremities were open so that the content (fixative) could be drained. Then, the segment was opened along the mesenteric border and successively washed with 80% alcohol until total fixative removal. Next, the tissue was dehydrated in alcohols (95% and 100%) followed by diaphonizaed in xylol and then rehydrated in alcohols descendent series (100%, 90%, 80%, 50%). The segment was stored 4 °C in PBS containing sodium azide at (0.04%). The ileum was cut in small segments of approximately 1 cm^2 and then subsequently microdissected to remove the mucosa and submucosa layers.

2.3. Immunohistochemistry

The whole mounts of ileum were washed twice for 10 min in PBS solution containing 0.5% Triton X-100 (Sigma). Then, they were incubated for 1 h in blocking solution (PBS + 0.5% Triton X–100 + 2% bovine serum albumin (BSA; Sigma) + 10% goat serum). After this period, the tissues were incubated for 48 h at room temperature in the blocking solution containing specific primary antibodies against HuC/D (produced in mouse: 1:500; Molecular Probes, Eugene, OR, USA, Cat no. A21271) and S100β (produced in rabbit; 1:200; Sigma, St Louis, MO, USA, Cat no. S2644). The tissues were washed three times in PBS solution + 0.5% Triton X-100 for five minutes and incubated for 2 h in room temperature with the secondary antibodies: Alexa Fluor 488–conjugated Donkey anti-mouse IgG; 1:250 (Molecular Probes, Eugene, OR, USA, cat no.: A21202) and Alexa Fluor 568–conjugated Donkey anti-rabbit IgG; 1:500 (Molecular Probes, Eugene, OR, USA, cat no.: A10042). After this time, the whole mounts were washed again three times for five minutes in PBS solution and mounted on slides with Prolong® Gold Antifade with DAPI (Molecular Probes). As a negative control, primary antibody was omitted.

2.4. Quantitative analysis of immunohistochemistry

All quantification was performed on ganglia located in the intermediate region (60°–120°, 240°–300°; of the intestinal circumference of each animal, considering 0° as the mesenteric insertion). Images captured by AxioCam (Zeiss, Jena, Alemanha) high resolution camera were used. The images were captured using