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Does L-glutamine-supplemented diet extenuate NO-mediated damage on myenteric plexus of Walker 256 tumor-bearing rats?

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ABSTRACT

This study was designed to appraise the relationship between enteric neuropathy and oxidative stress in cancer cachexia under L-glutamine-supplemented diet. Total and nitrergic neuronal populations were investigated in jejunum and ileum in four experimental groups: control (C); control L-glutamine-supplemented diet (CG); Walker-256 tumor (TW); and Walker-256 tumor supplemented with L-glutamine (TWG). In addition, local oxidative stress, neuronal nitric oxide synthase (nNOS) enzyme and nitric oxide (NO) levels were evaluated. Neuronal density and somatic area of the total and nitrergic populations were reduced in TW rats, which was accompanied by high oxidative stress, NO and nNOS levels. L-glutamine supplementation prevented neuronal atrophy, changes in pan neuronal density and nNOS overexpression (ileum), and restored total antioxidant capacity. Nevertheless, the oxidative stress was partially mitigated and no effect was observed on the reduction of nitrergic population and NO levels. L-glutamine-supplemented diet extenuates NO-mediated damage on the myenteric plexus although has a small benefit on oxidative stress.

1. Introduction

Oxidative cellular damage can be induced by high levels of reactive oxygen species (ROS) that surpass the cellular tolerance. Superoxide ($O_2^{\cdot -}$) is one of these ROS produced in the mitochondria under not only physiological but also pathological conditions which can rapidly combine with nitric oxide (NO) to generate peroxynitrite ($ONOO^-$), a reactive nitrogen species (RNS) that may lead to the lipid peroxidation (Fenner et al., 2015; Terra et al., 2012; Yuste, Tarragon, Campuzano, & Ros-Bernal, 2015). In many types of cells, including neurons, ROS levels are controlled by cellular antioxidants, including glutathione (GSH, γ-L-Glutamyl-L-cysteinylglycine), which is the main low-molecular non-enzymatic antioxidant and acts as a cofactor of many enzymes including catalase (CAT) and glutathione peroxidase (GPx). Despite such ingenious cellular mechanisms, ROS and RNS levels can exceed the cellular antioxidant capacity, leading to oxidative and nitrosative stress, a harmful condition that may result in neuronal death and have a crucial role in neurodegenerative diseases (Barreiro, 2016; Fra, Yoboue, & Sitia, 2017; H. R. Freitas et al., 2016; Terra et al., 2012).

The influence of oxidative and nitrosative stress on the pathogenesis of comorbidities related to cancer has been extensively investigated (Argiles, Busquets, Stemmler, & Lopez-Soriano, 2014; Barreiro, 2016; Fenner et al., 2015; Guarnier et al., 2010; Souba et al., 1988). Cachexia is a very serious complication of cancer and results in weight loss

Abbreviations: ABAP, 2,2′-azobis(2-amidinopropane); AUC, areas under the curves; BSA, bovine serum albumin; C, control; CAT, catalase; CG, control supplemented with 2% L-glutamine; CL, chemiluminescence; cm^2, square centimeters; COBEA, Brazilian College of Animal Experimentation; EDTA, ethylenediaminetetraacetic acid; ENS, enteric nervous system; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GIT, gastrointestinal tract; GPx, glutathione-peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, oxygen peroxide; HuC/D, Pan-neuronal marker protein; HuC/D-IR, HuC/D immunoreactivity; IgG, immunoglobulin G; IR, immunoreactivity; K_2HPO_4, potassium hydrogen phosphate; N_2, nitrogen gas; Na_2CO_3, sodium carbonate; NaCl, sodium chloride; nNOS, neuronal nitric oxide synthase; nNOS-IR, nNOS immunoreactivity; NO, nitric oxide; $O_2^{\cdot -}$, superoxide; $ONOO^-$, peroxynitrite; PBS, phosphate-buffered saline; RLU, Relative Light Units; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT, room temperature; SDS-Page, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis; SOD, superoxide dismutase; TBS, tris-buffered saline; T-butyl or t-butyl, tert-butyl-hydroperoxide; T_{ind}, induction time; TRAP, total antioxidant capacity; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; TW, Walker 256 tumor-bearing rats; TWG, Walker 256 tumor-bearing rats supplemented with 2% L-glutamine; UEM, State University of Maringa

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through the loss of skeletal muscle and, eventually, adipose tissue (Argiles et al., 2014; Barreiro, 2016). During the complex development of cancer cachexia, systemic effects are driven by proinflammatory cytokines and oxygen and nitrogen reactive species by depleting antioxidant defenses, particularly GSH, making the tissues vulnerable to oxidative damage (Barreiro, 2016; Guarnier et al., 2010; Pereira et al., 2011). Some intestinal functions such as secretion, immune defense and especially absorption are impaired in cancer cachexia (Samuels et al., 2000). Therefore, for the control of this symptoms it seems to be crucial to preserve the gastrointestinal functions (Godlewski, 2010). Symptoms related to absorptive disorders such as diarrhea, constipation, dysphagia, and abdominal pain are often associated with cancer (DiBaise & Quigley, 1998; Godlewski, 2010). Gastrointestinal disorders frequently exhibit varying degrees of enteric neuronal dysfunction (Godlewski, 2010; Phillips & Powley, 2007). There seems to be a preferential commitment of nitrergic neurons in various neuropathies affecting the enteric nervous system (ENS) (Rivera, Poole, Thacker, & Furness, 2011). Nitrergic neuronal subpopulation (inhibitory motor neurons) of myenteric plexus regulate the smooth muscle relaxation in the gut as well as the intestinal motility, vascular tone, blood flow, mucous secretions, permeability and inflammation in the gastrointestinal tract (GIT) (Kumar, 2008; Phillips & Powley, 2007). In special, NO has a key role in the transmission of the enteric relaxation; however, in high concentrations, NO can induce cell death in neurodegenerative disorders (Kumar, 2008; Rivera et al., 2011). NO is produced in the enteric nervous system from the amino acid L-arginine. This reaction is catalyzed by the neuronal nitric oxide synthase (nNOS) enzyme, which is the main enzyme in several tissues related to NO production, including those from the GIT (Kumar, 2008; Rivera et al., 2011). Therefore, alterations in the nitrergic innervation of the GIT are associated with motility disorders in several pathological conditions, including cancer (DiBaise & Quigley, 1998).

There are some reports that examined the expression pattern of the intestinal nNOS (Fracaro et al., 2016) as well as the NO formation and oxidative stress in the nervous tissue of Walker 256 tumor-bearing rats (Fenner et al., 2015; Freitas, Pompeia, Miyasaka, & Curi, 2001), however, the effects of the regional oxidative stress and cancer cachexia into enteric innervation, notably nitrergic neurons, are still unknown. Is worth to mention that this was the first time that the oxidative stress was evaluated in jejunal samples in cancer experimental model. Additionally, aiming to study the preventive effects of L-glutamine on enteric neuropathy related to cachexia, a supplementation diet was provided to Walker tumor and healthy animals. L-glutamine is broken down into glutamate and used in the synthesis of the antioxidant GSH. Both GSH and L-glutamine have shown antioxidant and neuroprotective properties (Aoyama, Watabe, & Nakaki, 2008; Chen & Herrup, 2012; Lee, Kim, Kim, & Kim, 2015; Pereira et al., 2011).

2. Materials and methods

2.1. Animals and experimental groups

Male Wistar rats (*Rattus norvegicus*) from the Central Animal Facility from the Universidade Estadual de Maringá were kept in individual cages for a period of 14 days in an environment with controlled photoperiod (12-h/12-h dark/light cycle) and temperature (23 ± 2 °C), receiving water and food ad libitum. Thirty-two 57-day-old rats weighing 190–255 g were randomly assigned to four experimental groups (n = 8 per group): control (C), control supplemented with 2% L-glutamine (CG), Walker 256 tumor-bearing rats (TW) and Walker 256 tumor-bearing rats supplemented with 2% L-glutamine (TWG). All the procedures described in this work were approved by the Ethic Committee of Animal Experimentation of the State University of Maringá (UEM), report n° 099/2012, and are in agreement with the ethical principles adopted by the Brazilian College of Animal Experimentation (COBEA).

2.2. Walker 256 tumor cell implantation

A total of 8×10^7 viable Walker 256 carcinosarcoma cells (observed by trypan blue dye exclusion method in a Neubauer chamber) were suspended in a phosphate-buffered saline (PBS) 16.5 mM (pH 7.5) and implanted in animals of groups TW and TWG via subcutaneous injection in the right flank. In the same day and in the same anatomic site, animals from group C and CG received an injection of PBS (pH 7.5) with the same volume. Experimental protocols were performed after 14 days of the tumor inoculation. This experimental period is based on preview studies that observed a 15-day survival time for Walker 256 tumor-bearing rats (Guarnier et al., 2010).

2.3. L-glutamine supplemented diet

Animals from groups CG and TWG received L-glutamine (Deg, São Paulo-SP, Brazil) incorporated to their standard balanced diet. Initially, the standard diet was grinded and reassembled in pellets with 2% L-glutamine (20 g/Kg) (Alves, Alves, Pereira, de Miranda Neto, & Zanoni, 2010; Fracaro et al., 2016; Pereira et al., 2011). The supplementation started at the same day of the tumor cell implementation. Groups C and TW received balanced standard diet from Nuvital® (Nuvilab, Colombo-PR, Brazil) during the entire experimental period.

2.4. Cancer cachexia evaluation

The cachexia of cancer-bearing animals was evaluated at the end of 14 days through weight variation (Iagher et al., 2011) and percentage of total body weight loss – animals are considered cachectic when such weight loss is higher than 10% (Cassolla et al., 2012). Weight variation was calculated by the difference between the final and initial total body weight (minus the tumor mass) for animal in groups TW and TWG (Cassolla et al., 2012; Iagher et al., 2011).

2.5. Tissue preparation for the immunohistochemistry technique

After 14-day of experimental period, animals were fasted for 12 h, weighted and euthanized by intraperitoneal anesthesia (thiopental 40 mg/kg of body weight - Sigma®, St. Louis, MO, USA). The jejunum (exactly 10 cm in midpoint of the small intestine – segment between the pyloric sphincter and the ileocecal junction) and the ileum (first 6 cm from the ileocecal junction towards the proximal end) of each animal were collected, washed with PBS 0.1 M (pH 7.4) and filled with Zamboni's fixative solution by tying up the ends with suture lines. Samples were kept at 4 °C for 18 h then opened along the mesenteric border and successively washed with 80% ethanol to completely remove the fixative. Then, tissues were sequentially dehydrated with an ascendant series of alcohols (95% and 100%), cleared with xylene, rehydrated with a descendent series of alcohols (100%, 90%, 80% and 50%) and kept at 4 °C in PBS solution with 0.08% sodium azide (Costa, Buffa, Furness, & Solcia, 1980). Small slices of fixed jejunum and ileum (around 1 cm^2) were microdissected in a Stemi DV4 binocular stereomicroscope (Zeiss, Jena, Germany) to expose the myenteric plexus, placed between the longitudinal and circular muscular layers. Myenteric whole-mount preparations was obtained by removing the mucosal and the submucosal layers, taking good care to preserve the muscular layer and the myenteric plexus (Zanoni, Tronchini, Moure, & Souza, 2011).

2.6. Immunohistochemistry

To evaluate the enteric neurons by the immunohistochemistry technique, the collected material was processed as previously described (Fracaro et al., 2016). Briefly, the whole-mount preparations of jejunum and ileum were washed three times (10 min each) with PBS (0.1 M, pH 7.4) containing 0.5% Triton X-100 (Sigma®, St. Louis, MO, USA,