Acta Scientiarum

http://periodicos.uem.br/ojs/
ISSN on-line: 1807-863X
Doi: 10.4025/actascibiolsci.v45i1.61368

Administration with L-glutamine and L-glutathione protects the nitrergic innervation of the penile tissue of diabetic rats

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ABSTRACT. Erectile dysfunction is caused due to neuropathy, resulting from a high oxidative stress, in this way treatment with antioxidants may be promising. Aim of this work was to investigate the effects of the administration of 2% L-glutamine and 1% L-glutathione on the penile tissue of diabetic rats analyzing the nerve fibers that expressing Nitric Oxide Synthase Neuronal (nNOS). Forty-eight male Wistar rats distributed into six groups were used: normoglycemic, diabetic, normoglycemic administered with 2% L-glutamine, normoglycemic administered with 1% L-glutathione, diabetic administered with 2% L-glutamine, and diabetic administered with 1% L-glutathione. After a 120 days experimental period, the animals were euthanized, and the penile tissues were collected and processed for the subsequent immunohistochemical procedure (nNOS) and posterior varicosities morphometry analysis. Diabetic rats administered with L-glutamine and with L-glutathione displayed larger varicosity areas of 14 and 15% compared to the diabetic group ($p < 0.05$). On the other hand, the administration of 2% L-glutamine and 1% L-glutathione in normoglycemic animals promoted a reduction of 3.3% and 2.4% compared to the normoglycemic group ($p < 0.05$). We concluded that both L-glutamine and L-glutathione administrations exerted a protective effect on the penile nitrergic innervation of diabetic rats, which can have a positive impact on the erectile function and that their use in normoglycemic animals should be better investigated.

Keywords: antioxidant; diabetes mellitus; penis; nitric oxide; free radicals.

Received on October 29, 2021.
Accepted on October 13, 2022.

Introduction

Diabetes mellitus (DM) is a disease characterized by hyperglycemia that may cause various complications (Bernstein, Busik, & Henry, 2002), affecting the peripheral nervous system and causing neuropathy (Vinik, 1999), leading to erectile dysfunction development. According to Dhaunsi, Yousif, Makki, Akthar and Benter (2017), erectile dysfunction (i. e. impotence) is one of the problems related to DM, causing impairment in the quality of life of several patients.

To initiate the penile erection, the neurotransmitter Nitric Oxide (NO) has a fundamental function leading to vascular relaxation of the corpus cavernosum smooth-muscle of humans, which occurs due to the conversion of L-arginine to NO, mediated by the enzyme neuronal Nitric Oxide Synthase Neuronal (nNOS) (Toda, Ayajiki, & Okamura, 2005) and endothelial Nitric Oxide Synthase (eNOS) (Tao, C. Tasdemir, S. Tasdemir, Shahabi, & Liu, 2017). The eNOS is typically localized in the vascular and sinusoidal endothelium of the penis, whereas nNOS is mainly distributed in the non-adrenergic and non-cholinergic nitrergic nerve terminals (Tao et al., 2017).

The penile tissue from a diabetic individual presents, along with increased oxidative stress, the uncoupling of eNOS and nNOS, and the upregulation of NADPH oxidase, conceivably producing the molecular basis for chronically reduced endothelial and neuronal NO (Musicki & Burnett, 2017). Studies have shown that NO-mediated corpus cavernosal smooth muscle relaxation was reduced in diabetic men with impotence, demonstrating the importance of this neurotransmitter for erection (Saenz de Tejada, Goldstein, Azadzoi, Krane, & Cohen, 1989; Bemelmans, Meuleman, Doesburg, Notermans, & Debruyne, 1994).

Neuropathy occurs as a consequence of the increased oxidative stress that occurs in DM (Vincent, Russel, Low, & Feldman, 2004). All cellular components are susceptible to lipid peroxidation by reactive

oxygen species, causing changes in the cell membrane structure and permeability, leading to cell death (Ferreira & Matsubara, 1997). Furthermore, NO may react with free radicals due to the formation of peroxynitrite, thereby decreasing its expression and activity and facilitating penile tissue dysfunction (Young, Yu, Baterman, & Brockerman, 2004).

DM is also associated with decreased glutathione levels (Loven et al., 1986). L-glutathione acts as a potent endogenous antioxidant (Curi et al., 2005; Hermes-Uliana et al., 2014; Panizzon et al., 2016) and has L-glutamine as its precursor (Alves, Alves, Pereira, Miranda Neto, & Zanoni, 2010; Pereira et al., 2011). Studies with other antioxidants demonstrated that quercetin promoted a positive effect on penile tissue of diabetic rats due to the improvement of erectile dysfunction inhibiting oxidative stress (Zhang et al., 2011). Furthermore, 2% vitamin E also demonstrated positive results in the nNOS-immunoreactive (-IR) varicosities (Tronchini, Miranda Neto, & Zanoni, 2011) and cinnamon essential oil and cinnamaldehyde also improved the erectile function (Onder, Yilmaz-Oral, Jerkovic, Akdemir, & Gur, 2019).

Therefore, we aimed to evaluate the effects of 1% L-glutathione and 2% L-glutamine administrations on penile tissue of diabetic rats by analyzing the varicosities area of NO-expressing nerve fibers.

Material and methods

Animals

We performed all animal manipulation according to the Brazilian Society of Science in Laboratory animals (SBCAL). The procedures were approved by the Ethics Committee in Animal experimentation of *Universidade Estadual de Maringá* (UEM) under the protocol number 009/2005. Forty-eight male Wistar rats (*Rattus norvegicus*), 90 days old, were used from the Central Animal Facility of the *Universidade Estadual de Maringá*. We distributed the animals into six groups with 8 animals each: normoglycemic (N), diabetic (D), normoglycemic administered with 2% L-glutamine (NG), normoglycemic administered with 1% L-glutathione (NGT), diabetic administered with 2% L-glutamine (DG), and diabetic administered with 1% L-glutathione (DGT). The animals were housed in polypropylene cages measuring 40 cm x 33 cm x 17 cm (length, width, and height) and maintained at a controlled environmental temperature (23 ± 2°C) and lighting regime (12-h 12-h^{-1} dark $light^{-1}$ cycle). Food and water were *ad libitum*.

The DM induction was performed after 14 hours of fasting period using an intravenous injection of streptozotocin (35 mg kg^{-1} body weight; Sigma, St. Louis, USA) dissolved in citrate buffer solution pH 4.5 (10 mM) (Tronchini et al., 2010; Hermes-Uliana et al., 2014; Panizzon et al., 2016). After four days, we obtained a drop of blood from the animal's tail to measure glucose levels by glycoso-dye-oxidoreductase photometric determination Accu-Chek Active glucometer, Roche Diagnostics GmbH, Mannheim, BW, Germany). We used rats with glycemia higher than 200 mg dL^{-1}.

During the experimental period, animals of the N and D groups (non-supplemented groups) were fed with standard balanced Nuvital feed (Nuvilab, Colombo, PR, Brazil). The diets of the NG and DG groups comprised the ground standard diet supplemented with 2% L-glutamine (20 g kg^{-1}) (Deg, São Paulo, SP, Brazil) (Hermes-Uliana et al., 2014; Panizzon et al., 2016). The animals of the NGT e DGT groups received 1% L-glutathione (10 g kg^{-1}) (Deg, São Paulo, SP, Brazil) (Hermes-Uliana et al., 2014; Panizzon et al., 2016).

Collection and processing of the material

At 210 days of age, after 4 hours of food restriction, the animals were weighed and anesthetized with intraperitoneal thiopental (40 mg kg^{-1} body weight) (Abbott Laboratories, Chicago, IL, USA). We collected blood by cardiac puncture for glucose measurement (glucose oxidase method). The entire penis was collected and processed for the nNOS immunohistochemical technique.

Immunolocalization of the nNOS: study of the varicosities in nerve fibers of the penis

The penile tissue was fixed in Zamboni's solution (4% paraformaldehyde and 0.4% picric acid in phosphate buffer) for 18 hours. After that, the tissue was washed repeatedly with 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 12 hours. Afterward, cryoprotection was carried out in a solution of 18% sucrose in 0.1 M PBS for 24 hours. After immersion in the soaking medium for frozen tissue (OCT 4583 compound; Tissue-Tek), the tissue was frozen in liquid nitrogen and stored in a freezer at -80°C. We used a cryostat to make 12 µm semi-serial cuts, placing five slices per animal on microscope slides previously prepared with poly-L-lysine adhesive and stored at -4°C.

We washed the slides three times in PBS containing Triton X-100 (0.5%) and then proceeded to incubate in bovine serum albumin (1% BSA in PBS) for 1 hour. Then the samples were incubated for 48 hours at room temperature in primary rabbit antibody solution against nNOS (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Afterward, we performed three washes in PBS and incubation in an Alexa Fluor 488 anti-rabbit secondary antibody solution (1:400; Molecular Probes, Eugene, Oregon, United States) for two hours at 4°C, followed by three more washes in PBS (0.1 M, pH 7.4). We then prepared the slides with buffered glycerol (9:1) and stored them in the refrigerator. We omitted the primary antibody for the negative control (Tronchini et al., 2010).

Morphometric analysis of the nNOS-IR varicosities on penile tissue nerve fibers

For each animal, we used the software Image-Pro Plus 4.5.0.29 (Media Cybernetics, Silver Spring, MD, USA) to measure the areas (μm^2) of 100 varicosities using images captured by a Moticam® 2500 5.0 Mega Pixel high-resolution camera (Motic China Group Co., Shanghai, China) coupled to an Olympus fluorescence optical microscope® BX40 (Olympus Co., Japan). The images were transferred to a microcomputer through the software Motic Images Plus® 2.0ML (Motic China Group Co., Shanghai, China) and recorded.

Statistical analysis

We subjected the results to statistical analyses using Statistica 7.1 and GraphPad Prism 5.1 software. The morphometric data were analyzed by Block Design followed by Tukey's test. All other results were analyzed with a one-way analysis of variance (ANOVA) followed by Tukey's test. We considered $p < 0.05$ values statistically significant.

Results

The initial weight for all groups was similar ($p > 0.05$; Table 1). Animals of the N, NG, and NGT groups had a significant increase in the final weight compared to the initial weights. The D, DG, and DGT groups had lower body weight than their respective controls and these groups were hyperglycemic compared to the N, NG, and NGT controls, respectively ($p < 0.05$; Table 1).

Table 1. Physiological Parameters (PP) assessed: initial weight (IW/g), final weight (FW/g), final glycemia (FG mg^{-1} dL^{-1}). Groups: normoglycemic (N), diabetic (D), normoglycemic administered with 2% L-glutamine (NG), normoglycemic administered with 1% L-glutathione (NGT), diabetic administered with 2% L-glutamine (DG) and diabetic administered with 1% L-glutathione (DGT). n = 8 rats per group.

PP	N	NG	NGT	D	DG	DGT
IW	340 ± 4.7	313 ± 7.8	335 ± 7.3	313 ± 8.3	312 ± 7.4	317 ± 4.9
FW	496 ± 13.6	429 ± 19.1*	463 ± 16.7	306 ± 11.9*	272 ± 12.1*	287 ± 11.3*
FG	149 ± 3.3	163 ± 4.7	127 ± 7.9	524 ± 33.3*	603 ± 7.78**	571 ± 22.1*

Values expressed as mean expressed as mean ± SE. * $p < 0.05$ when compared to C; ** $p < 0.05$ when compared to D.

Representative photomicrographs of the immunostaining are shown in Figure 1. In this study, we found that the D group nNOS-IR varicosities area suffered an expressive reduction (vs N; $p < 0.05$), whereas the DG and DGT groups presented a larger area of the varicosities than the D group ($p < 0.05$, Figure 2). On the other hand, administration with 2% L-glutamine in normoglycemic animals (NG) promoted reduction of 3.3% ($p < 0.05$) and 1% glutathione (NGT group) a reduction of 2.4% (vs N; $p < 0.05$) (Figure 2A).

Most of the nNOS-IR varicosities varied from 2 to 31 μm^2 (N group), 3 to 19 μm^2 (NG group), 3 to 15 μm^2 (NGT group), 2 to 15 μm^2 (D group), 4 to 17 μm^2 (DG group) and 3 to 16 μm^2 (DGT group) (Figure 2B).

Discussion

We confirmed the establishment of the DM experimental model due to the increased final glycemia and the absence of body weight gain in D, DG, and DGT groups. The 2% L-glutamine and 1% L-glutathione administrations did not reverse the high glycemic levels presented by DG and DGT animals. We observed higher values than the diabetic group. The glycemic levels indicate that the results we found are a consequence of the antioxidant effects from both administrations and not the result of an indirect glycemic improvement. Other studies using the same administration also did not present a reversal in glycemic levels of the diabetic animals supplemented with both substances (Hermes-Uliana et al., 2014; Panizzon et al., 2016).

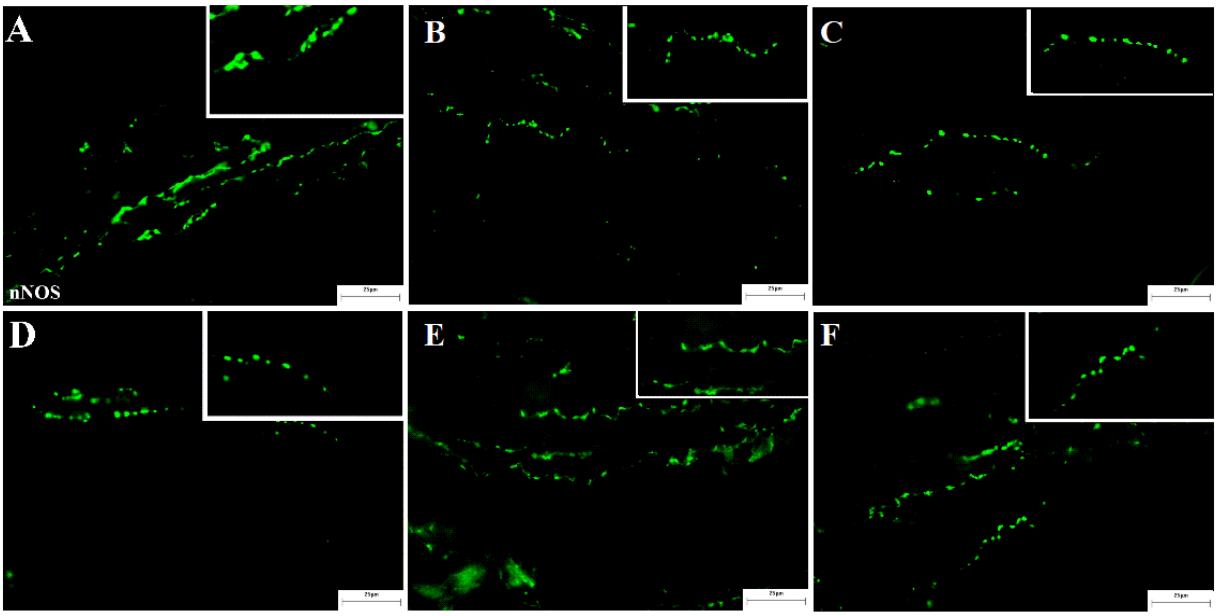

Figure 1. Penile neuronal nitric oxide synthase (nNOS) varicosities in rats from the groups normoglycemic (A), normoglycemic administered with 2% L-glutamine (B), normoglycemic administered with 1% L-glutathione (C), diabetic (D), diabetic administered with 2% L-glutamine (E) and diabetic administered with 1% L-glutathione (F). Calibration bar 25 µm.

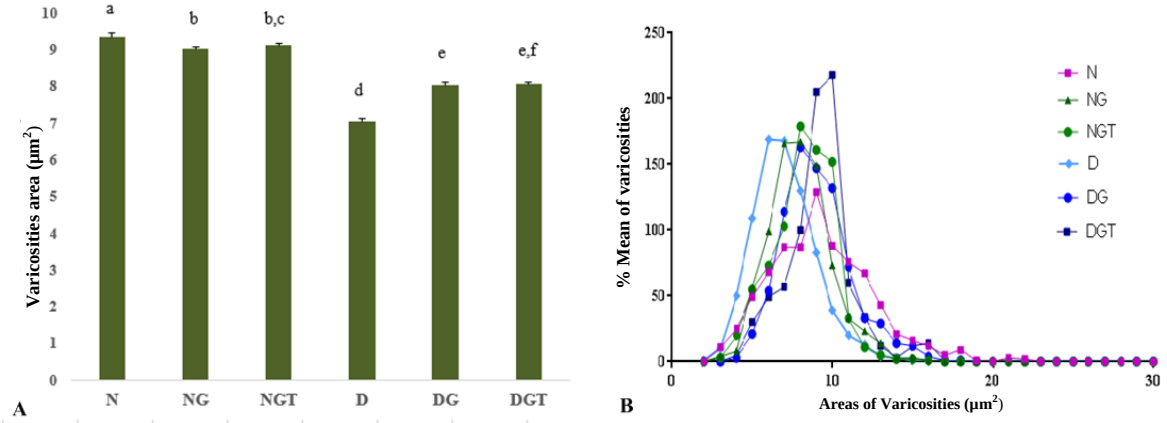

Figure 2. A) Means and standard errors of mean (SEM) in the area of neuronal nitric oxide synthase (nNOS) varicosities in penile tissue. The mean values followed by different letters on the columns are statistically different ($p < 0.05$). B) Distribution of the relative frequencies of the varicosities areas of the nNOS-IR neuron fibers in the penis tissue. Groups: normoglycemic (N), diabetic (D), normoglycemic administered with 2% L-glutamine (NG), normoglycemic administered with 1% L-glutathione (NGT), diabetic administered with 2% L-glutamine (DG) and diabetic administered with 1% L-glutathione (DGT). n = 8 rats per group.

Erectile dysfunction is induced by a combination of neurogenic, vasculogenic, endocrinological, metabolic, use of drugs, and psychological causes. These dysfunctions may be classified as psychogenic, organic, and mixed psychogenic and organic (Defeudis et al., 2015). The penis is innervated by the autonomic nervous system through sympathetic and parasympathetic fibers and non-cholinergic and non-adrenergic fibers (NANC) (Martínez-Salamanca, Martínez-Ballesteros, & Carballido, 2010). Furthermore, NANC fibers convert the amino acid L-arginine to NO through the nNOS enzyme. For this reason, NO strikes muscle cells and activates the Soluble Guanylate Cyclase that converts Guanosine Triphosphate (GTP) to Cyclic Guanosine Monophosphate (cGMP), leading to cavernous smooth muscle relaxation and thereby stimulating the erection. In addition to NO, polypeptide neurotransmitters (e.g., Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase Activator Polypeptide (PACAP)) are found in penile tissue (Toda et al., 2005). Regarding the area of the nNOS-IR varicosities, our study showed that diabetic animals displayed a 25% area reduction compared to normoglycemic rats. Similar results were observed by Tronchini et al. (2010) using the same experimental model.

The nervous tissue dysfunction in diabetics is caused by direct effects of free radicals, which result in cell death due to the lipid peroxidation process induced by changes in cell membrane structure and

permeability (Ferreira & Matsubara, 1997). Oxidative stress may be triggered by several mechanisms that are accentuated during DM, such as the activation of the polyol pathway due to the accumulation of sorbitol and fructose, leading to nerve degeneration (Afzaal, Singh, & Saleem, 2002; Giugliano, Ceriello, & Paolisso, 1996), and oxidative glycosylation that forms accumulate advanced glycation end products (AGEs), promoting the hardening of the endothelial cells (Kikuchi et al., 2003). For these reasons, the increased production of free radicals and the reduction in endogenous antioxidant defense system capacity may be crucial for cell death (Dam et al., 1998). We evaluated oxidative stress in the jejunum of diabetic rats with the same experimental period as the animals used in this study, and an increase of oxidative stress was observed (Hermes-Uliana et al. 2014). Although oxidative stress was not evaluated in the penile tissue of this study, possibly the results would be similar since oxidative stress in diabetics occurs systemically. The decreased nNOS-IR varicosities area in diabetic animals may be related to insulin reduction. Insulin resistance may lead to disturbances in the subcellular signaling pathways required for NO production (Trussell & Legro, 2007).

Our results demonstrate that the DGT group displayed larger nNOS-IR varicosities compared to the D group. In diabetic neuropathy, antioxidants are reduced because of increased lipid peroxidation and high levels of free radicals that inactivate NO (Young et al., 2004; Kuyvenhoven & Meinders, 1999). The positive effect may have occurred due to L-glutathione, the main cellular antioxidant and crucial to the cellular defense against oxidative stress (Cruzat, Rogero, Borges, & Tirapegui, 2007, Hermes-Uliana et al. 2014). Administration of L-glutathione becomes even more important since its levels are decreased in DM (Lu, 2009). This decrease occurs due to the conversion of oxidized glutathione-dependent oxidation of NADPH to reduced glutathione by glutathione reductase. The NADPH is found in low concentrations since the polyol pathway is also dependent on this reducing agent (Vincent et al., 2004).

The DG group also displayed larger nNOS-IR varicosities compared to the D group. L-glutamine is one of the main substances used for the reduction of free radicals since this amino acid is a precursor for the L-glutathione synthesis (Cruzat et al., 2007), in the catabolic process (Curi et al., 2005). L-glutamine also acts in the maintenance of cellular structures and functions, in addition to the modulation of stress proteins activation, which is part of the main signaling pathways for the survival of cells when exposed to stressors, such as reactive oxygen species (Cruzat, Petry, & Tirapegui, 2009). Furthermore, L-glutamine may promote protein synthesis and increase the availability of substrates for various systems involved in the tissue repair process (Cruzat et al. 2007).

We observed that the nNOS-IR varicosities of the NG and NGT groups showed a significant size reduction compared to the N group. Such an effect may have occurred due to an excess of glutamine and glutathione in these animals. Even though L-glutamine is the precursor molecule for the antioxidant L-glutathione, it can generate glutamate, promoting neuronal toxicity (Amores-Sánchez, & Medina, 1999; Matés, Pérez-Gómez, Castro, Asenjo, & Márquez, 2002). Another hypothesis for this decrease in size of nNOS-IR varicosities in the NG group is that dietary administration with L-glutamine may contribute to a reduction in NO formation since glutamine may act on arginine/NO metabolism through the inhibition of the enzyme nitric oxide synthase (Amores-Sánchez & Medina, 1999; Matés et al., 2002).

According to Panizzon et al. (2011), normoglycemic animals produce all the L-glutathione necessary. The 1% administration possibly caused an excess and after its degradation resulted in the production of glutamate and cysteine, which at high levels has cytotoxic potential. In DM, the lack of insulin causes protein depletion, as it is essential for protein synthesis. For this reason, both L-glutathione and L-glutamine administrations were important in the protein synthesis, including the nNOS enzyme.

Conclusion

We concluded that the administration with 1% L-glutathione and 2% L-glutamine increased the sizes of the nNOS-IR varicosities in the diabetic animals. These antioxidants protect the nerve fibers arranged in penile tissue from oxidative stress and thereby improve NO production. However, these results are only valid in animals susceptible to pathological conditions, such as diabetes used in this study, since glutamine and glutathione levels are reduced. Therefore, these supplements should be used with caution in the absence of disease since they may influence the synthesis of NO in penile tissue.

Acknowledgements

The project was funded by a grant from the Fundação Araucária (*Apoio ao Desenvolvimento Científico e Tecnológico do Paraná*, Brazil) and CAPES (*Coordenação de Aperfeiçoamento de Pessoal de Nível Superior*, Brazil).

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