

BASIC RESEARCH

Exercise alleviates hypoalgesia and increases the level of calcitonin gene-related peptide in the dorsal horn of the spinal cord of diabetic rats

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OBJECTIVE: The aim of this study was to evaluate the effects of treadmill training on nociceptive sensitivity and immunoreactivity to calcitonin gene-related peptide in the dorsal horn of the spinal cord of diabetic rats.

METHODS: Male Wistar rats were divided into three groups: control, diabetic and trained diabetic. Treadmill training was performed for 8 weeks. The blood glucose concentrations and body weight were evaluated 48 h after diabetes induction and every 30 days thereafter. The nociceptive sensitivity was evaluated using the tail-flick apparatus. The animals were then transcardially perfused, and the spinal cords were post-fixed, cryoprotected and sectioned in a cryostat. Immunohistochemistry for calcitonin gene-related peptide analysis was performed on the dorsal horn of the spinal cord.

RESULTS: The nociceptive sensitivity analysis revealed that, compared with the control and trained diabetic animals, the latency to tail deflection on the apparatus was longer for the diabetic animals. Optical densitometry demonstrated decreased calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord in diabetic animals, which was reversed by treadmill training.

CONCLUSION: We concluded that treadmill training can alleviate nociceptive hypoalgesia and reverse decreased calcitonin gene-related peptide immunoreactivity in the dorsal horn of the spinal cord of diabetic animals without pharmacological treatment.

KEYWORDS: Hypoalgesia; Tail-Flick Test; Calcitonin Gene-Related Peptide Content; Dorsal Horn of Spinal Cord; Diabetic Neuropathy.

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INTRODUCTION

One of the most common complications of diabetes mellitus is chronic sensorimotor distal symmetric polyneuropathy, with sequelae including painless foot ulceration and occasionally, lower limb amputations (1). Sensory neurons, located in the dorsal root ganglia, are also affected by chronic hyperglycemia, resulting in a decrease in the production (2-4) of the most abundant peptide involved in processing

nociceptive information, the calcitonin gene-related peptide (CGRP) (5,6). Consequently, there is an alteration in sensory behavior, contributing to sensory loss and its consequences.

Recently, several studies have demonstrated the beneficial effects of physical exercise in patients (7) and rats with diabetes (8-11). Physical exercise has been indicated for people with diabetes, yet little is known about the effects of exercise on the processing of nociceptive information; thus, the aim of this study was to investigate the effects of treadmill training on nociceptive sensitivity and the CGRP levels in the dorsal horn of the spinal cord of male diabetic rats.

METHODS

Animals

Thirty male Wistar rats (12 weeks old) from a local breeding colony (ICBS, UFRGS) were housed under standard

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No potential conflict of interest was reported.

Table 1 - Body weight and blood glucose in the studied groups.

Group	Before induction	48 h after induction	30 days		60 days		90 days	
	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)	Weight (g)	Glycemia (mg/dL)
C	298 ± 5	86 ± 5	351 ± 4	90 ± 2	384 ± 3	89 ± 2	406 ± 3	94 ± 3
D	307 ± 16	389 ± 21*	269 ± 10*	525 ± 25*	256 ± 11*	534 ± 7*	254 ± 11*	527 ± 26*
TD	297 ± 9	352 ± 20*	281 ± 11*	438 ± 46*	270 ± 8*	525 ± 22*	290 ± 17*	535 ± 27*

laboratory conditions with food and water available *ad libitum* and a 12:12 h light/dark cycle. All efforts were made to minimize the number of animals studied and their suffering. The animals were cared for in accordance with Arouca Brazilian law (11794/2008) and the recommendations of the Brazilian Society for Neurosciences, the Review Committee of the School of Veterinary Surgery, University of Buenos Aires, and the International Brain Research Organization. Moreover, the study methods were in compliance with the National Institute of Health's Guidelines for the Care and Use of Laboratory Animals (publication no. 85-23, revised 1985), and the Ethical Committee of the UFRGS approved the study under protocol number 2008-062.

Experimental design

The rats were divided into three groups: non-diabetic rats (C), diabetic rats (D), and diabetic rats subjected to treadmill training (TD) for eight weeks. For the nociceptive analysis using the tail-flick test, ten animals were used per group. For the immunohistochemistry studies, six animals were randomly selected per group.

Diabetes induction

After an overnight fasting period (6 h), the rats from the D and TD groups received a single intravenous injection of streptozotocin (STZ; 50 mg/kg of body weight; Sigma Chemicals Co., St Louis, MO, USA) diluted in 10 mM of citrate buffer (pH 4.5), whereas the C group received only the citrate buffer (9,17). Blood collected from the rats' tails was used to measure the blood glucose concentrations using test strips (Performa, Roche, USA). Diabetes was defined as fasting glucose >300 mg/dL in the tail vein blood 48 h after the STZ injection (12). The body weights were measured before diabetes induction and every 30 days thereafter, and the blood glucose concentrations were measured 48 h after the induction of diabetes and every 30 days thereafter.

Maximal exercise test and treadmill training

During the 4th week after diabetes induction, all of the animals underwent adaptation to a treadmill that was originally designed for human use (Runner, São Paulo, SP, Brazil) and was modified for use by rats by walking for 10 min at 5 m/min for four days. On the 5th day, the rats were submitted to a maximal exercise test (MET) consisting of a graded exercise on the treadmill with speed increments of 5 m/min every 3 min, starting at 5 m/min and continuing up to the maximal intensity attained by each rat. The exercise was stopped when each animal remained for more than 50% of the time without run or walk. (13,14). The values obtained in the MET were used to plan the treadmill training program, which began during the 5th week after diabetes induction. To correct the exercise intensity, a second MET was performed during the 5th training week.

Exercise was performed on a treadmill twice per day with an interval of 4 h between each session, five days per week. The training intensity was increased gradually according to the MET results. During the first week, the running sessions lasted 10 min, with increases in duration each week, reaching 60 min in the 7th week, which was maintained until the 8th week. Each training session consisted of a warm-up period, a main period and a cool-off period. During the warm-up period, the rats ran 15% of the session time at 30% of the maximum velocity determined by the MET; during the main period, the rats ran 70% of the session time at 60% of the maximum velocity; and during the cool-off period, the rats ran 15% of the session time at 30% of the maximum MET values (10).

Nociceptive sensitivity analysis

Nociceptive sensitivity was assessed using the tail-flick apparatus (Insight, Ribeirão Preto, SP, Brazil). The rats were wrapped in a towel and placed on the apparatus. A light source positioned below the tail was focused on a point 2–3 cm rostral to the tip of the tail (15). The deflection of the tail activated a photocell and automatically terminated the test. The animals were exposed to the tail-flick apparatus every week to familiarize them with the procedure, as the novelty of the apparatus can itself induce antinociception (16). The tail-flick latency represented the period of time from the beginning of the test to the tail deflection. The final data are presented as a percentage of the control group, which was assigned a value of 100%, and the values are expressed as the means ± standard error of the mean (SEM).

Immunohistochemical procedure

One day after the nociception analysis, rats were anesthetized with sodium thiopental (i.p.; 50 mg/kg; Cristalia, Brazil). Heparin (1000 IU; Cristalia, Itapira, SP, Brazil) was injected into the left cardiac ventricle, and the animals were then transcardially perfused through the left ventricle using a peristaltic pump (Control Company, Friendswood, TX, USA, 20 mL/min) with 400 mL of 0.9% saline solution followed by 400 mL of a fixative solution of 4% paraformaldehyde (Synth, Diadema, SP, Brazil) in 0.1 M phosphate buffer, pH 7.4 (PB). The lumbar spinal cords were removed, post-fixed in the same solution at room temperature for 4 h and cryoprotected by immersion in 15% and 30% sucrose (Synth, Diadema, SP, Brazil) solutions in PB at 4°C until they sank. After these procedures, the spinal cords were quickly frozen in isopentane (Merck, Darmstadt, Germany) cooled in liquid nitrogen and kept in a freezer (-70°C) for further analyses.

Transverse sections (35 µm) from the lumbar spinal cords were obtained using a cryostat (CM1850, Leica, Wetzlar, Germany) at -20°C and collected in PB saline (PBS), pH 7.4. The free-floating sections were pre-treated with 3% hydrogen peroxide for 30 min, carefully washed, treated with 2%

bovine serum albumin (Inlab, São Paulo, SP, Brazil) in PBS containing 0.4% Triton X-100 (PBS-Tx; Sigma Chemical Co, St Louis, MO, USA) for 30 min and incubated for 48 h with polyclonal CGRP-antibody (1:2250; courtesy of Dr. Rodrigo, Cajal Institute, Spain) and gentle stirring at 4 °C. The primary antibody was then removed, and the sections were washed in PBS-Tx for 30 min. The sections were then immersed in a secondary antibody (goat anti-rabbit IgG; Sigma Chemical Co., St Louis, MO, USA) diluted 1:50 in PBS-Tx for 2 h at room temperature with gentle stirring. After washing with PBS-Tx three times for 15 min, a soluble complex of horseradish peroxidase and rabbit anti-horseradish peroxidase (Sigma Chemical Co., St Louis, MO, USA) diluted 1:500 was applied for 1 h 30 min at room temperature. The reaction was revealed in a medium containing 0.06% 3,3'-diaminobenzidine (DAB, Sigma Chemical Co., St Louis, MO, USA) dissolved in PBS for 10 min, and 1 µL of 3% H₂O₂/mL was then added to the DAB medium for an additional 10 min. Finally, the sections were rinsed in PBS, mounted on glass slides, dehydrated in ethanol, cleared with xylene and covered with Entellan (Merck, Darmstadt, Germany) and coverslips. Control sections were prepared by omitting the primary antibody and replacing it with PBS.

Optical Densitometry

Semi-quantitative densitometric analysis was used to measure the intensity of the CGRP immunoreaction using a Nikon Optiphot-2 microscope (100x, Tokyo, Japan) coupled to a Micrometrics camera (Accu Scope, Commack, NY, USA) and Image Pro Plus Software 6.0 (Media Cybernetics, Rockville, MD, USA). The digitized images obtained from the dorsal horn of the spinal cords were converted to an 8-bit gray scale (0–255 gray levels). All of the lighting conditions and magnifications were held constant. The picture elements (pixels) employed to measure the optical density were obtained from squares measuring 1160 µm² (area of interest; AOI) overlaid on the gray scale image. Both the left and right sides of each spinal cord were used. For each rat, 10 measurements were taken from the dorsal horn of the spinal cord. The results for the spinal cords are presented as the total mean value from the three studied regions.

The background staining subtraction and correction were performed in accordance with our previously published protocol (17). The optical density (OD) was calculated using the following formula:

$$OD(x,y) = -\log[(INT(x,y) - BL)/(INC - BL)]$$

where "OD(x,y)" is the optical density at pixel(x,y); "INT(x,y)" is the intensity at pixel(x,y); "BL", or black, is the intensity generated when no light penetrates the material; and "INC" is the intensity of the incidental light.

Statistical analysis

The blood glucose and body weight data were analyzed using repeated measures analysis of variance (ANOVA), and the differences between the groups were assessed using the Bonferroni *post hoc* test. The data obtained from the nociceptive test and the optical densitometry of CGRP-ir were analyzed using a one-way ANOVA and the Bonferroni *post hoc* test. Statistical significance was set at $p < 0.05$. The data were run on the Statistica 6.0 software package (StatSoft,

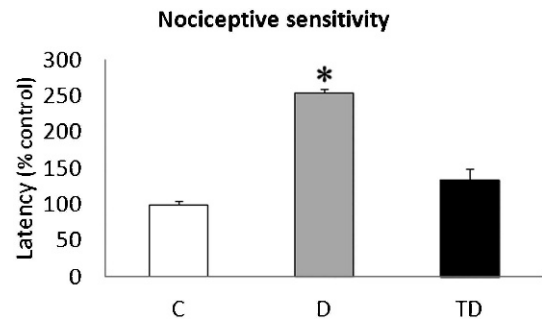


Figure 1 - Nociceptive sensitivity: the latency to tail deflection on the tail-flick apparatus for the C, D and TD groups. ANOVA followed by Bonferroni's *post hoc* test. *corresponds to $p < 0.001$ compared with the C and TD groups.

Inc., Tulsa, OK, USA). All of the data are represented as the means \pm standard error of mean (SEM).

RESULTS

Body weight and blood glucose concentrations

Before diabetes induction, there were no differences in the body weight between the C (298 ± 5 g), D (307 ± 16 g) and TD (297 ± 9 g) groups ($p > 0.05$). However, 30, 60, and 90 days after diabetes induction, the rats from the D (269 ± 10 g; 256 ± 11 g; 254 ± 11 g, respectively) and TD (281 ± 11 g; 270 ± 8 g; 290 ± 17 g, respectively) groups presented lower body weights than the C group (351 ± 4 g; 384 ± 3 g; 406 ± 3 g, respectively; $p < 0.001$; Table 1).

Two days after diabetes induction, the blood glucose concentrations were higher in the D (389 ± 21 mg/dL) and TD (352 ± 20 mg/dL) groups than in the C (86 ± 5 mg/dL) group ($p < 0.001$). The blood glucose concentrations were still higher at 30, 60 and 90 days in the D (525 ± 25 mg/dL; 534 ± 7 mg/dL; 527 ± 26 mg/dL, respectively) and TD (438 ± 46 mg/dL; 525 ± 22 mg/dL; 535 ± 27 mg/dL, respectively) groups after diabetes induction compared with the C group (90 ± 2 mg/dL; 89 ± 2 mg/dL; 94 ± 3 mg/dL, respectively; $p < 0.001$). There were no differences between the D and TD groups at any time (Table 1).

Nociceptive sensitivity

The latency to the tail deflection of the C group was expressed as 100%. Group D required 154.7% more time to deflect the tail than the C group ($p < 0.001$) and 120% more time to deflect the tail than the TD group ($p < 0.001$). The TD

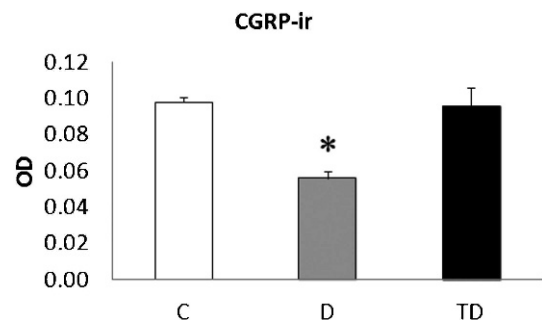


Figure 2 - CGRP immunoreactivity (CGRP-ir) in the dorsal horn of the spinal cord from the C, D and TD animals. ANOVA with Bonferroni's *post hoc* test. * corresponds to $p < 0.05$ compared with the C and TD groups.

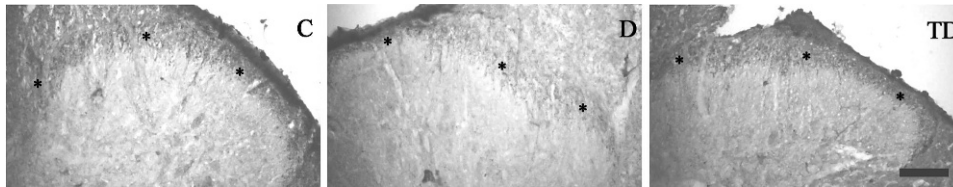


Figure 3 - Digitized images of transverse sections of spinal cord in the C, D and TD animals revealing the CGRP-ir in the dorsal horn of the spinal cord. Note the decreased CGRP-ir in the D group. In the image from the TD animal, note that the CGRP-ir is similar to that of the C animal. The scale bar indicates 100 μ m.

group took only 34.7% more time to deflect the tail than the C group ($p > 0.05$; Figure 1).

Optical densitometry of CGRP-ir

The OD analysis of the dorsal horn of the spinal cord revealed that CGRP-ir was lower in the D (0.056 ± 0.004) group than in the C (0.098 ± 0.003 ; $p < 0.001$) and TD (0.096 ± 0.01 ; $p < 0.05$) groups. There was no difference between the C and TD groups ($p > 0.05$; Figure 2). Representative images are presented in Figure 3.

DISCUSSION

In the present study, we have demonstrated that treadmill training alone, with no pharmacological intervention, can prevent the nociceptive loss caused by the hyperglycemic state in rats with STZ-induced diabetes. This beneficial effect was associated with changes in the CGRP-ir in the dorsal horn of the spinal cord.

As expected, diabetic rats display lower body weights and higher blood glucose levels than non-diabetic rats (8,10). Treadmill training did not alter the body weight or blood glucose levels in rats (8,10,18) or in type 1 diabetic patients (19). STZ induces an insulin-deficient state similar to type 1 diabetes in humans, which is not typically improved by exercise interventions (20), in contrast to type 2 diabetes, which is an insulin-resistant state (21).

In the analysis of nociceptive sensitivity, measured using the tail-flick test, the diabetic rats demonstrated an increase in the latency to deflect the tail from the apparatus, which indicates an STZ-induced loss of sensitivity. These data are in accordance with those of previous studies, which found deficits in motor and sensory nerve conduction (22,23) and thermal (23,24) and nociceptive hypoalgesia (24) in animals. Our findings are also in agreement with clinical data indicating that diabetes in humans can cause nociceptive hypoalgesia (25-27), which is an important determinant of lower limb ulcers and amputations.

However, diabetic rats that were subjected to treadmill training displayed a latency to deflect the tail from the apparatus similar to that of the non-diabetic animals, indicating that training is able to prevent alterations in the sensitivity behavior of diabetic animals. These data have not previously been reported in the literature. We also examined the effects of training on the CGRP-ir in the dorsal horn of the spinal cord, and the data reveal that physical exercise can maintain the content of this peptide in the spinal cord at levels similar to those of control animals.

Diabetes is well known to cause a reduction in the content of CGRP in peripheral nerves (2,3,28), the neurons of the dorsal root ganglia and the dorsal horn of the spinal cord (29). Although physical training has been considered in the treatment of diabetic patients for some time, it was not

known, until now, whether training was able to prevent alterations in nociceptive signaling and CGRP content in the spinal cord. The benefits of treadmill training in these parameters could arise from the effects of nerve growth factor (NGF) and neurotrophin-3 (NT-3). These neurotrophic factors promote neuronal survival and differentiation by activating phosphatidylinositol 3-kinase signaling (PI3-K). Moreover, there are various studies demonstrating that treadmill training increases the level of NGF in the sensory neurons (30) and in the hippocampus (31,32) of NT-3 in the spinal cord (33) and of NGF and NT-3 in the soleus muscle (34,35).

In conclusion, treadmill training is able to alleviate the nociceptive hypoalgesia caused by diabetes and this improvement is related to the content of CGRP in the dorsal horn of the spinal cord.

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AUTHOR CONTRIBUTIONS

do Nascimento PS is the primary author of this manuscript, who organized the research protocol and participated in the research and article preparation. Lovatel GA, Ilha J and Xavier LL participated in the research protocol. Schaan BD participated in the article preparation. Achaval M is responsible for the research and participated in the article preparation.

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