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Early antioxidant treatment with EGCG prevents diabetic neuropathic pain and oxidative stress damage in nociceptive spinal cord neurons

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Early antioxidant treatment with EGCG prevents diabetic neuropathic pain and oxidative stress damage in nociceptive spinal cord neurons

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Abstract

Aim: To evaluate the effects of a preventive antioxidant treatment of streptozotocin (STZ)-diabetic rats with epigallocatechin gallate (EGCG) in oxidative stress damage and neuronal activation at the spinal cord and in behavioural signs of diabetic neuropathic pain (DNP).

Materials and Methods: Three days after STZ injection, the rats initiated a treatment protocol with an aqueous solution of EGCG (2g/L) in the drinking water during 10 weeks. Mechanical nociception was evaluated before and after EGCG treatment using the paw-pressure and von Frey tests. Spinal cords were immunoreacted against 8-hydroxy-2'-deoxyguanosine (marker of oxidative stress damage; 8-OHdG), and double immunoreacted against 8-OHdG and Fos (marker of neuronal activation).

Results: STZ rats presented increased oxidative stress damage (immunodetection of 8-OHdG) and higher activation of nociceptive spinal neurons (immunodetection of Fos protein), with a contingent of 8-OHdG-positive neurons also expressing Fos than in age-matched controls. In these animals mechanical hyperalgesia (paw-pressure test) and tactile allodynia (von Frey test) were higher than in controls. Treatment with EGCG normalized oxidative stress and neuronal activation to control levels and ameliorated behavioral mechanical nociceptive responses.

Conclusions: The beneficial effects of the preventive antioxidant treatment with EGCG at the spinal cord and behavioral signs of DPN indicate that early detection of diabetes allows the onset of antioxidant treatment that can prevent oxidative stress-induced damage to the somatossensory system.

Key words: diabetes; neuropathic pain; pain; oxidative stress; reactive oxygen species (ROS)

Introduction

Oxidative stress is one of the most prominent features of diabetes and has been implicated in the pathophysiological changes underlying several complications of the disease [1]. Diabetic neuropathy is the most common complication of the disease, affecting approximately 50% of the patients, 10-26% of which complain of pain [2,3]. Diabetic neuropathic pain (DNP) has complex manifestations, such as spontaneous pain, allodynia (increased responses to innocuous stimuli), hyperalgesia (increased responses to innocuous stimuli) and paresthesias (sensation of tingling, tickling, prickling, or burning) [3]. Diabetes-induced oxidative stress accounts for DNP since massive oxidative stress damage has been reported in areas of the somatossensory system involved in the transmission of nociceptive input, namely peripheral nerves and spinal cord [4-6]. Furthermore, antioxidant treatment of diabetic animals reverts oxidative stress damage and behavioural signs of DNP [4]. In the clinical setting, adjuvant treatment of diabetic patients with antioxidants has raised some controversy, with both positive and negative outcomes reported [7].

The spinal cord, the first relay in the transmission of nociceptive input from the periphery to the brain, is affected by diabetes in humans and animal models. Structural and functional abnormalities have been identified at the spinal cord of diabetic patients [8,9]. An increased baseline activity of nociceptive neurons has been reported in the spinal cord of streptozotocin-induced diabetic rats (STZ-diabetic rats). Hyperactivity affected neurons occurring in the superficial laminae of the spinal dorsal horn, which participate in transmission of nociceptive input to the brain [10,11]. Diabetes-induced oxidative stress is likely to account to the neuronal hyperactivity at the spinal cord. In models of traumatic neuropathy, decreasing the

levels of reactive oxygen species (ROS) at the spinal cord reduced behavioural signs of pain and restored normal neuronal activity [12,13]. Due to the high oxidative stress damage in the spinal cord during diabetic neuropathy, it is important to evaluate if preventing the appearance of oxidative stress in the spinal cord of STZ-diabetic rats would also have a beneficial effect in neuronal hyperactivity and DNP.

We recently used an animal model of diabetes, the STZ-diabetic rat, to study the value of an antioxidant treatment in managing DNP. Starting a 2 week treatment of STZ rats with 4 weeks duration of diabetes, with intraperitoneal administrations of α-lipoic acid reversed oxidative stress damage and neuronal hyperactivity at the spinal cord to the level of non-diabetic animals [4]. Although this also improved behavioural signs of DNP accessed by increased sensitivity to noxious mechanical stimuli (mechanical hyperalgesia), a normalization to control levels was not achieved [4]. Since increased responses to innocuous stimuli (mechanical allodynia) were not evaluated and this is a more clinically relevant sign of DNP [9], it remains to ascertain the putative translational application of antioxidants. Epigallocatechin-gallate (EGCG), the major catechin present in green tea, has a similar efficacy when compared with α-lipoic acid in protecting cellular DNA from ROS but has a much higher potency in reducing lipid peroxidation [14,15]. The utility of EGCG in the clinical setting has been demonstrated in several studies, namely in diabetes type 1 by decreasing the risk of its development through several antioxidative and anti-inflammatory actions in a variety of cell types [16,17]. EGCG is an antioxidant particularly indicated for studies of the spinal cord since it crosses the blood-brain barrier and promotes neuronal recovery after spinal cord injury even using a systemic administration route [18,19]. The

mechanisms of action of EGCG are complex and include ROS removal, increase of anti-oxidant enzymes, chelation of transitional metals, modulation of apoptotic pathways and increased expression of neurotrofic factors [17]. Based on the above summarized findings [4], using α-lipoic acid treatment of STZ-diabetic rats after the disease is fully established (4 weeks of diabetes), we hypothesize that using EGCG in STZ-diabetic rats with long term diabetes (10 weeks) and anticipating the onset of treatment to the moment of hyperglycaemia appearance should have a higher effect in behavioural signs of DNP. We further aim to evaluate if oxidative stress damage affects spinal neurons activated by nociceptive stimuli and whether this is could be corrected by the preventive EGCG treatment.

Materials and methods

Animals

Adult male Wistar rats weighing 250-300g at the beginning of the experiments were housed twice per cage in a room with a constant temperature (22±2ºC) and humidity (55±5%) with a 12h light/dark cycle, and received food and water ad libitum. Experiments were performed in accordance with the European Community Council directive 86/609/EEC and the ethical guidelines for the study of pain in conscious animals [20].

Induction of Diabetes

Type 1 diabetes was induced in 16 rats by an intraperitoneal (i.p) injection of STZ (60mg/kg body weight; Sigma-Aldrich, Barcelona, Spain) dissolved in 0.1 M citrate

buffer (pH 4.5). Control animals (n=8) received an i.p injection of the same citrate buffer. Three days later, glucose levels were measured in a blood sample collected from the tail vein using Accu Chek Sensor Comfort (Roche Diagnostics, Berlin, Germany). Only rats with glucose levels above 270 mg/dl were considered diabetic [21]. Glucose levels were also measured at 4 and 10 weeks post-diabetes induction.

Treatment protocol

Three days after the injection of STZ or citrate buffer, a group of STZ-diabetic rats (n=8) initiated the treatment protocol with an aqueous solution of EGCG (2g/L; Holliday & Co, Canada) in the drinking water. The treatment lasted for 10 weeks and these animals formed the “STZ+EGCG group”. Non-diabetic animals (n=8) and the remaining SZT-diabetic rats (n=8) received water without EGCG, forming the “Control group” and the “STZ group”, respectively. Liquid intake was daily measured and the animals were weakly weighted.

Behavioral evaluation

Mechanical hyperalgesia and tactile allodynia were assessed prior and 10 weeks after STZ or vehicle injections using the paw-pressure and the von Frey tests, respectively. Both mechanical hyperalgesia and tactile allodynia are validated behavioural signs in STZ-diabetic rats [21]. All animals were daily handled during the 8 days preceding the behavioural analysis for habituation purposes. The behavioural evaluation was performed before diabetes inductions (week 0), and at the end of treatment (week 10).

In the paw-pressure test, an increasing pressure was applied onto the dorsal surface of the right hindpaw with a cone-shaped plunger using the Randall-Selitto device (Ugo Basile, Italy). The paw withdrawal threshold (PWT) was defined as the force, in grams, that elicited a detectable paw withdrawal. For the von Frey test, the animals were placed in clear acrylic boxes with a metal grid floor and acclimatized for 15 min before testing using an electronic device (Bioseb, France). A linearly increasing force (2,5 g/s) was applied to the plantar surface of the rat hindpaw with a filament (0.5mm). As for paw-pressure test, PWT was settled as the force inducing paw withdrawal. For both tests, the PWT for each animal was calculated as the average of three consecutive measurements with at least a 5 min interval between them.

Sacrifice of the animals

Ten weeks after the onset of treatment, the animals were deeply anesthetized with an i.p. injection of sodium pentobarbital (60 mg/kg body weight) and sacrificed by transcardiac vascular perfusion. Animal sacrifice was performed at least 30 minutes after the last behavioral evaluation. Vascular transcardic perfusion was performed using 200ml of phosphate-buffered saline (PBS) followed by 1000 ml of 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4 [4]. The spinal segments L4-L5 were removed, post-fixed for 2-4h in 4% PFA and cryoprotected overnight in 30% sucrose in 0.1 PBS at 4ºC. Transverse sections, 40 µm thick were obtained using a freezing microtome, collected in 0.1 PBS. One in every 4 sections was processed for immunodetection of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative stress damage of nucleic acid [22]. An additional set of sections was immunoreacted for 8-OHdG combined with

immunoreaction for Fos, a protein synthetized at the spinal cord when the Fos protooncogene is activated by nociceptive stimuliat the spinal cord neurons [23].

Immunohistochemistry against 8-OHdG

The sections used in the immunodetection of 8-OHdG were pretreated with 2 N HCl during 10 min to denature nucleic acids, followed by 5 min immersion in Tris-base 1 M, as described previously [4]. All sections were treated with 1% hydrogen peroxide for 20 min to inhibit endogenous peroxidase activity before incubation for 2h in a blocking solution containing 10% normal swine serum in 0.3% Triton X 25% in PB (PBST). The sections were then incubated during two overnights at 4ºC in mouse anti-8-OHdG (1:1200; Trevigen, USA). After being washed in PBST, the sections were incubated in biotinylated rabbit anti-mouse (1:200; Dako, Denmark) immunoglobulin for 1h. Sections were then washed in PBST, incubated for 1h in the avidin-biotin-complex (Vectastain, Vector Lab, USA) and stained with diaminobenzidine (DAB). Sections were mounted on gelatine-coated slides, air dried and coverslipped with xylol. After observation under a light microscope, the images were acquired using a high resolution digital camera coupled to a computer. Immunolabelling for 8-OHdG was quantified by densitometric analysis of the superficial layers of the spinal dorsal horn (laminae I-III). A bilateral analysis was performed using the ImageJ software (National Institutes of Health, USA). The results are presented in optical units, as described previously [4].

Immunofluorescence against 8-OHdG and Fos

In order to assess oxidative stress damage in spinal neurons activated by nociceptive stimuli, a double immunofluorescence reaction was performed in the additional set of spinal sections. These sections were treated with 1% borohydrate for 20min, before incubation in a blocking solution containing 10% normal horse serum (NHS) in PBST with 0.1 M glycine, for 2h. Sections were then incubated during 2 overnights at 4ºC in a mixture of a mouse anti-8-OHdG (1:500; Trevigen, Gaithersburg, MD, USA) and a rabbit anti-Fos (1:1000; Ab5; Oncogene, Germany) antibody in a 2% NHS solution. After being washed in a 2% NHS in PBST solution, the sections were incubated for 1h at room temperature, in a mixture of two fluorescent antibodies: goat anti-mouse Alexa 594 and goat anti-rabbit Alexa 488, both at 1:500 (Molecular Probes, USA), in a 2% NHS in PBST solution. Sections were examined using an ApoTome microscope (Zeiss, Germany) and the images acquired using a high-resolution camera coupled to a computer. Merged images were obtained using the ApoTome software. The numbers of neurons immunoreactive (IR) for 8-OHdG, for Fos or both were bilaterally counted in the superficial dorsal horn (laminae I-III). The percentages of 8-OHdG-IR neurons that were also Fos-IR were counted. The specificities of primary antibodies against 8-OHdG and Fos were previously demonstrated [4].

Statistical Analysis

SPSS Statistics version 18 (IBM, Armonk, New York, USA) was used for statistical analysis. Means were compared by ANOVA followed by Tukey post hoc test for multiple comparisons. Statistical significance was settled as $p<0.05$. Results are presented as mean ± SEM.

Results

1- Metabolic characterization

All STZ rats developed hyperglycaemia within 3 days post-injection, which was maintained during the time of the experiments, when compared with the non-diabetic age matched controls (Table 1). The STZ group presented a lower body weight and increased liquid intake in comparison to controls (Table 1). The treatment with EGCG did not affect blood glucose concentration, body weight or liquid intake when comparing with STZ-diabetic rats (Table 1). Mean EGCG consumption per day was 494±13.8 mg, indicating a low heterogeneity within the treated group.

2- Effects of EGCG on mechanical nociception

The PWTs in STZ rats were significantly reduced both in the paw-pressure test (Fig. 1A) and von Frey (Fig. 1B) test in comparison with baseline values and the control group. These results confirm the development of mechanical hyperalgesia (Fig. 1A) and tactle allodynia (Fig. 1B) at 10 weeks of diabetes in the STZ-diabetic rat model [11]. The STZ+EGCG group presented higher PWTs both in the paw-pressure (Fig. 1 A) and in the von Frey (Fig. 1 B) tests, when compared with values of untreated STZ rats, indicating an amelioration of mechanical hyperalgesia and tactile allodyinia, respectively. The effects of EGCG in mechanical hyperalgesia appear to be stronger than in tectile allodynia since those differences were more pronounced in the paw-pressure test than in the von Frey test (cf. Figs 1A and 1B). These data indicate that EGCG treatment has a

preventive effect on mechanical nociceptive responses detected in STZ-diabetic rats.

3- Effects of EGCG at 8-OHdG expression at spinal cord

Cells IR for 8-OHdG were identified by a diffuse punctate pattern distributed over the nucleus and cytoplasm which reflects both nuclear and mitochondrial DNA damage [22], as shown in the inset of Fig. 2C.

As shown in Fig. 2, untreated STZ rats presented a significant increase in the expression of 8-OHdG in comparison with the control group (STZ *versus* Control: p=0.013). The treatment with EGCG prevented the increase of 8-OHdG, with statistically significant differences between STZ+EGCG and STZ group (p=0.033). A complete normalization of 8-OHdG expression was obtained after EGCG treatment since 8-OHdG expression in the STZ+EGCG group was similar to the control group (p=0.833).

4- Effects of EGCG at Fos expression at the spinal cord

Fos-IR neurons were recognized by the stained nucleus (Fig 3 B-C). The number of Fos-IR neurons was significantly higher in the STZ group when compared with the control group (STZ *versus* Control: p=0.017) (Fig 3 A). The treatment with EGCG prevented the increased numbers of Fos-IR neurons in the spinal cord of STZ rats, with statistically significant differences between STZ+EGCG and STZ groups (p=0.037). A complete normalization of Fos expression was obtained after EGCG treatment since the number of Fos-IR neurons in the STZ+EGCG group was similar to the control group (STZ+EGCG *versus* Control: p= 0.875).

5- Effects of EGCG at the co-localization of 8-OHdG and Fos

Neurons double-labelled for 8-OHdG and Fos were identified by the diffuse red punctate pattern surrounding the green immunorreaction of the nucleus (Fig. 4B-D).

The percentage of 8-OHdG-IR cells that were also Fos-IR in the STZ group was higher than in the control group (fig 4A). In the STZ, from an average number of 163,33 neurons that were 8-OHdG-IR, 20% were shown to also co-localize for Fos. In the control group, from an average number of 117,79 neurons that were 8-OHdG-IR, only 5% were shown to also co-localize for Fos. The treatment with EGCG prevented the increased co-localization of 8-OHdG and Fos, with statistically significant differences between STZ+EGCG and STZ group ($p=0.037$). In the STZ+EGCG group, from an average number of 96,71 neurons that were 8-OHdG-IR, only 8% were shown to also co-localize for Fos.

Discussion

The present study shows, for the first time, that an antioxidant treatment with EGCG initiated as soon as hyperglycaemia induced-diabetes is detected can prevent the long-term effects of the disease at the spinal cord, namely oxidative stress damage and neuronal hyperactivity. The study also shows that the current EGCG treatment protocol can ameliorate behavioural signs of DNP. The early intervention protocol with an antioxidant is important, inasmuch that clinical trials using antioxidants have given contradictory results, probably because the treatments are usually initiated when the disease is fully established and oxidative stress damage is prominent [1, 7, 24]. In support of this hypothesis, it has been shown that oxidative stress increases gradually during the course of diabetes and

that preventive antioxidant treatments are more effective that curative protocols [25-27].

Treatment of diabetic rats with EGCG normalized oxidative stress damage and neuronal hyperactivity at the spinal cord to the levels of non-diabetic controls. It is likely that these findings are related since EGCG treatment is known to normalize the levels of ROS [28,29]. In traumatic neuropathic pain models, it was shown that excessive levels of ROS at the spinal cord are associated with enhanced activation of receptors involved in excitatory neurotransmission and loss of inhibitory GABAergic transmission [13,30]. It is therefore likely that the increased oxidative stress accounts for the higher neuronal activity detected in the spinal cord of diabetic rats. The normalization of signs of oxidative stress damage and of neuronal hyperactivity by the EGCG treatment was not accompanied by a full reversal of the analyzed behavioural signs of DNP. The magnitudes of EGCG-induced improvement of behavioral signs of mechanical responses were slightly higher in the paw pressure test (mechanical hyperalgesia) than in the von Frey test (tactile allodynia), which refutes our initial hypothesis that tactile allodynia, a more clinically relevant sign of diabetes, is more prone to antioxidant treatment. Curiously, amelioration but not a complete normalization of behavioural responses to mechanical nociception was also detected after EGCG intrathecal delivery in traumatic neuropathic pain models [31,32]. Collectively, the results suggest that both in diabetic and traumatic neuropathic pain models, mechanisms besides the oxidative stress affecting the spinal cord are involved in behavioural pain responses.

In the present study we used a treatment protocol with EGCG in the drinking water to evaluate the effects of this physiological approach. Supporting an action of

EGCG at the central nervous system, it was previously shown that this antioxidant crosses the blood brain barrier and that one of its main targets is the spinal cord [18,33]. We cannot exclude, however that EGCG also acts in other central nervous system areas, since we recently shown that a similar EGCG protocol treatment reverts oxidative stress-induced neurodegenerative changes in pain control centers of the brainstem [34].

By performing a co-localization of a marker of oxidative stress damage (8-OHdG) and a marker of neuronal activation (Fos) our study shows for the first time that at least 20% of the cells affected by oxidative stress at the spinal cord during diabetes are neurons. This conclusion is based on the fact that all Fos-IR cells are neurons [11]. That percentage is underestimated since some spinal cord neurons exhibiting oxidative stress signs probably expressed the Fos protein bellow detection levels or are not activated by nociceptive stimuli. Co-localization of 8-OHdG with pan-neuronal markers, like Neu-N, will allow to enlighten this issue. Another interesting possibility is related to the interplay of oxidative stress and glial cells [35]. It has been demonstrated that the spinal cord of diabetic rats presents increased microglial activation [36]. We will evaluate if the preventive EGCG protocol used in the present study also prevents microglia activation and if the inhibition of microglia with intrathecal delivery of specific microglia inhibitors prevents oxidative stress damage.

It should be noted that the beneficial effects of EGCG in STZ rats are not due to glyceamic normalization as our STZ+EGCG animals remained hyperglyceamic, at the timepoints measured (4 and 10 weeks after diabetes induction). These data are in accordance with the literature that shows that in type 1 diabetes an improvement of the hyperglycaemic condition occurs after EGCG treatment but

the reduction of hyperglycaemia is only achieved using higher EGCG concentrations than those used in the present protocol [37,-39]. Furthermore, a study in type 1 diabetes shows that the efficacy of EGCG in normalizing hyperglyceamia only occurs after longer treatment periods [40]. These conditions were elected in our experimental design since we wanted to evaluate only the effects of correcting the oxidative stress damage without the confounding effect of normalizing glycaemia. The beneficial effects of EGCG antioxidant treatment in amelioration of behavioural signs of diabetic neuropathy match data with other antoxidants, namely α-lipoic acid [4], resveratrol [41] and vitamin E [42]. These results obtained in animal models are, however, yet difficult to translate from bench to bedside since antioxidant treatments are frequently initiated when diabetes is already fully installed. Using protocols of EGCG treatment in early phases of diabetes has better outcomes in other complications of the disease, such as diabetic nephropathy [38], diabetic retinopathy [43], diabetic erectile dysfunction [44], diabetic endothelial dysfunction and atherosclerosis [45] and diabetic cutaneous wound healing [46]. The synergistic effect of insulin treatment with a EGCG protocol initiated in a preventive manner may be useful to manage diabetic neuropathy.

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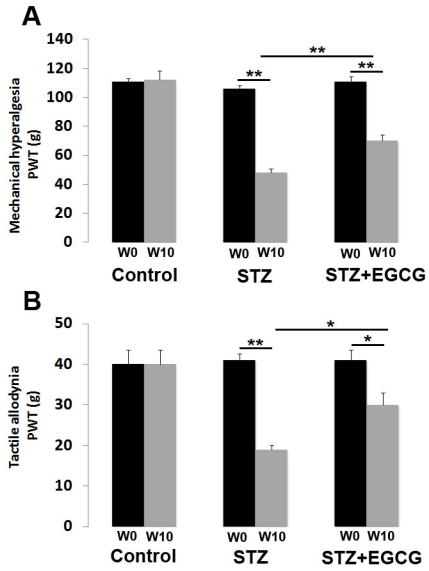
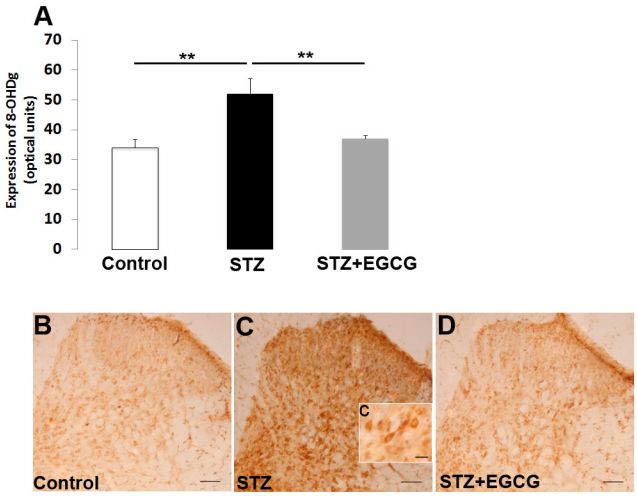
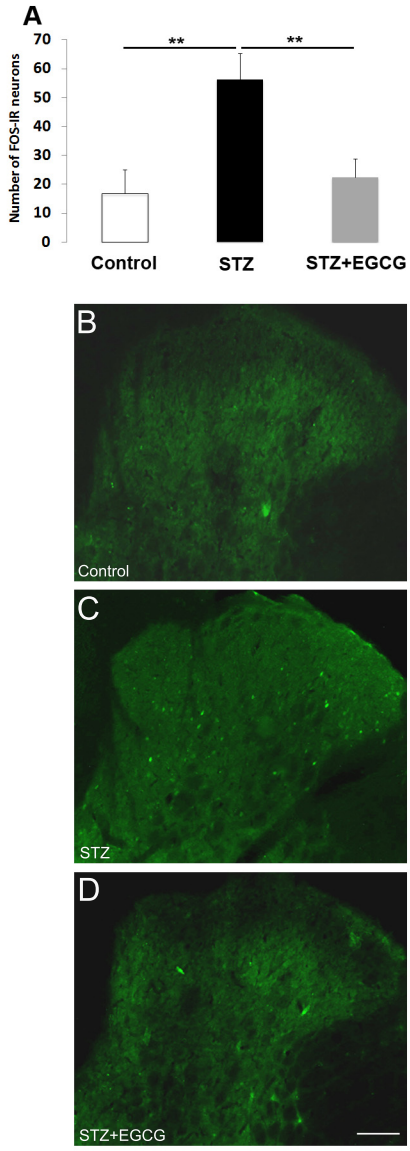
Table 1. Characterization of Experimental Groups

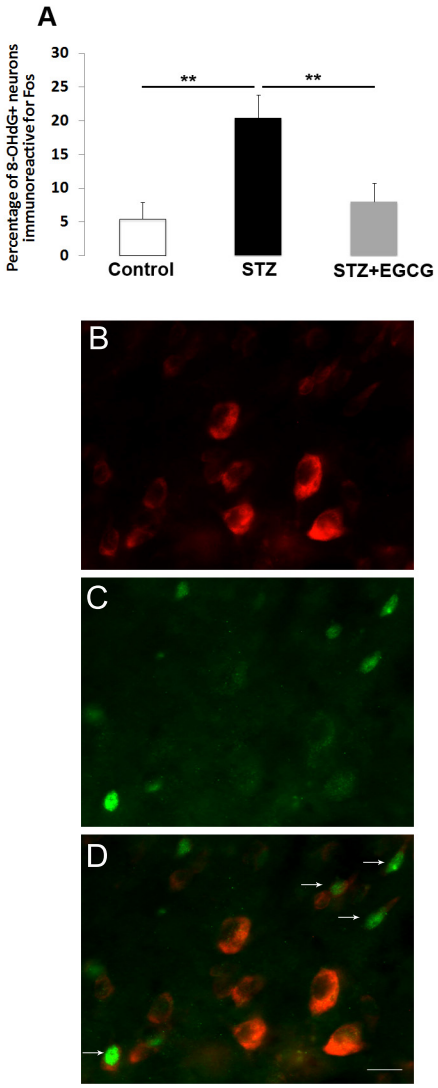
Experimental Groups	Blood glucose concentration (mg/dl)	Body weight (g)	Volume Ingestion (ml/day)
Control	102±21,5	413±4,7	54±13,6
STZ	567±17,9*	291±7,6*	258±27,6*
STZ+EGCG	554±25,7*	288±10,2*	247±31,0*

Mean values ± s.d. EGCG: epigalocathechin-3-gallate; STZ: streptozotocin
*p<0.01 (comparisons with control group)


A
Mechanical hyperalgesia
PWT (g)
140
120
100
80
60
40
20
0
**
**
**
W0 W10
W0 W10
W0 W10
Control
STZ
STZ+EGCG
B
Tactile allodynia
PWT (g)
50
40
30
20
10
0
*
**
*
W0 W10
W0 W10
W0 W10
Control
STZ
STZ+EGCG


A
70
60
50
40
30
20
10
0
Expression of 8-OHDg (optical units)
**
**
Control
STZ
STZ+EGCG
B
C
C
D
Control
STZ
STZ+EGCG


A
Number of FOS-IR neurons
70
60
50
40
30
20
10
0
**
**
Control
STZ
STZ+EGCG
B
Control
C
STZ
D
STZ+EGCG


A
Percentage of 8-OHdG+ neurons immunoreactive for Fos
30
25
20
15
10
5
0
**
**
Control
STZ
STZ+EGCG
B
C
D
STZ+EGCG

Legend of Figures

Fig 1- Effects of EGCG treatment on mechanical hyperalgesia (A) and tactile allodynia using the paw-pressure and the von Frey tests, respectively. Paw withdrawal thresholds (PWT) in control animals, STZ-diabetic rats (STZ group) and STZ-diabetic rats treated with EGCG (STZ+EGCG), before diabetes induction (w 0) and at 10 weeks of disease (w 10). The antioxidant treatment with EGCG ameliorated mechanical hyperalgesia and mechanical allodyinia, but without normalization to control levels. Significance of symbols: * - $p<0.05$; ** - $p<0.01$.

Fig 2- Effects of EGCG treatment on oxidative stress damage at the superficial dorsal horn (laminae I-III) of the spinal cord. (A) Optical density of 8-OHdG, a marker of nucleic-acid oxidative stress damage in control animals, STZ-diabetic rats with 10 weeks of disease (STZ group) and STZ-diabetic rats treated with EGCG (STZ+EGCG group). (B-D) Representative photomicrographs of L4 spinal cord sections after immunoreaction for 8-OHdG in control (B), STZ (C) and STZ+ EGCG (D) groups. The STZ group presents a significant increase in the expression of 8-OHdG when compared with control animals. Treatment with EGCG fully prevented the diabetes-induced oxidative stress damage to control levels. Scale bars: 100μm; scale bar of insert: 25μm. Significance of symbols: * - $p<0.05$; ** - $p<0.01$.

Fig 3- Effects of EGCG treatment on neuronal activity at the superficial dorsal dorn (laminae I-III) of the spinal cord (A) Mean number of Fos-IR neurons per section in

control animals, STZ-diabetic with 10 weeks of disease (STZ group) and STZ-diabetic rats treated with EGCG (STZ+EGCG). B-D are representative immunofluorescence photomicrographs in sections from control (B), STZ (C) and STZ+ EGCG (D) groups. The number of Fos-IR neurons is markedly increased in the STZ group in comparison with the control group and that increase was prevented by the EGCG treatment. Significance of symbols: ** p< 0.01. Scale bar in D: 100μm (all photomicrographs are at the same magnification).

Fig 4- Effects of EGCG treatment on co-localization of 8-OHdG and Fos in the superficial dorsal horn (laminae I-III) of the spinal cord. (A) Percentage of 8-OHdG-IR cells also Fos-IR in control animals, STZ-diabetic with 10 weeks of disease (STZ group) and STZ-diabetic rats treated with EGCG (STZ+8-OHdG group). The percentage of 8-OHdG-IR that are also Fos-IR was higher the STZ group when compared to the control group and that increase was prevented by the treatment with EGCG. B-D are representative immunofluorescence photomicrographs of the same spinal section depicting 8-OHdG immunoreaction (B), Fos-immunostaining (C) or both (D) in a section of a STZ-diabetic rat. Four neurons double-immunostained for 8-OHdG and Fos are marked with arrows in D. Significance of symbols: ** p< 0.01. Scale bar in D: 20μm (all photomicrographs are at the same magnification).

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ANEXOS

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