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Beneficial effects of a *Cannabis sativa* extract treatment on diabetes-induced neuropathy and oxidative stress

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Short title: Cannabis extract alleviates diabetic neuropathy

Abstract

Neuropathy is the most common complication of diabetes and it is still considered to be relatively refractory to most of the analgesics. The aim of the present study was to explore the antinociceptive effect of a controlled cannabis extract (eCBD) in attenuating diabetic neuropathic pain. Repeated treatment with cannabis extract significantly relieved mechanical allodynia and restored the physiological thermal pain perception in streptozotocin (STZ)-induced diabetic rats without affecting hyperglycemia. In addition, the results showed that eCBD increased the reduced glutathione (GSH) content in the liver leading to a restore of the defence mechanism and significantly decreased the liver lipid peroxidation suggesting that eCBD provides the protection against oxidative damage in STZ-induced diabetes that also strongly contributes to the neuropathy development. Finally, the nerve growth factor content in the sciatic nerve of diabetic rats was restored to normal following the repeated treatment with eCBD suggesting that the extract was able to prevent the nerve damage caused by the reduced support of this neurotrophin. These findings highlighted the beneficial effects of cannabis extract treatment in attenuating diabetic neuropathic pain, possibly through a strong antioxidant activity and a specific action upon nerve growth factor.

keywords: neuropathic pain; *Cannabis sativa*; diabetes; cannabinoid; oxidative stress; nerve growth factor

INTRODUCTION

Diabetic polyneuropathy, the most common of the peripheral neuropathies, occurs widely in the western countries as a long-term complication of diabetes. Given that diabetes affects approximately 246 million people worldwide, it is estimated that 20-30 million people are affected by symptomatic diabetic neuropathy. The pathogenesis of diabetic neuropathy includes many factors such as metabolic, vascular, autoimmune, neurohormonal growth factor deficiency and oxidative stress (see Zochodne, 1999 for review). Based on these observations, several therapeutic drugs, including antioxidants (Low *et al.*, 1997), selective PKC inhibitors (Nakamura *et al.*, 1999) and neurotrophic factors (Tomlinson *et al.*, 1997) have been used to treat diabetic neuropathy, even if the magnitude of the effects in humans has been smaller than the expected for many of them. In addition, as for other types of painful neuropathies, tricyclic antidepressants, anticonvulsants and opioids are employed in order to obtain pain relief, even if it has been estimated that these therapies led at best to 50% reduction of pain in 50% of patients. Furthermore, the poor efficacy of these drugs is often associated to severe adverse effects. Finally, there is a general consensus that a strict glycemic control may diminish the risk of developing a disabling peripheral neuropathy (Martin *et al.*, 2006), suggesting that the prevention of diabetic neuropathy remains the best strategy. Thus, early diagnosis of diabetic neuropathy, followed by drug therapy combined with glycemic control may be warranted to prevent at least the progression of diabetic neuropathy. However, the future goal in treating diabetic neuropathy should be not only to prevent or delay the painful neuropathic symptoms, but also to completely relief pain and possibly to promote the regeneration of degenerate nerve fibers after the onset of the pathology. Among the emerging substances very effective against neuropathic pain,

cannabinoids are promising analgesics (see Lever and Rice, 2007 for review). Cannabinoids are lipophilic compounds originally obtained from *Cannabis sativa* which contains more than 60 different cannabinoids. Phytocannabinoids obtained from the cannabis plant comprise a range of cannabinoid receptor agonists, partial agonists, and antagonists, and many synthetic cannabinoids have also been developed with specific receptor affinity and distinct pharmacological profiles. There are two main cannabinoid receptors, CB1 and CB2, associated with pain modulation. The first is widely expressed in the CNS and peripheral sensory neurons whereas the latter has been found on peripheral tissues, including tissues of the immune system and keratinocytes, with limited expression in sensory and CNS cells (Pertwee, 2006 for review). Synthetic or pure natural cannabinoids have been found useful in neuropathic pain (Bridges *et al*., 2001; Fox *et al*., 2001; Costa *et al*., 2004b; Costa *et al*., 2006; Costa *et al*., 2007) even if the most of studies were performed employing the injury of the sciatic nerve as animal model of painful neuropathy. To our knowledge there are only two initial studies reporting the efficacy of a synthetic CB1 receptor agonist, WIN55,212-2, on tactile allodynia in diabetic rats (Dogrul *et al*., 2004; Ulugol *et al*., 2004). We have recently shown that a controlled cannabis extract, containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid fractions (terpens and flavonoids) provided better antinociceptive efficacy than the single cannabinoids given alone when tested in the chronic constriction injury of the sciatic nerve model of neuropathy (Comelli *et al.*, 2008). We suggested that other than the pharmacological receptor-mediated effect of cannabinoids, the presence of anti-inflammatory and antioxidant compounds in the non-cannabinoid fraction of the natural extract might strongly contribute to analgesia (Comelli *et al.*, 2008). This background and especially the multiple aetiologies of

diabetic neuropathy involving oxidative stress, inflammation and nerve damage, prompted us to test the same *Cannabis sativa* extract in the rat model of streptozotocin (STZ)-induced diabetic neuropathy, in the attempt to propose a new therapy to ameliorate this chronic and disabling painful condition after its onset.

MATERIALS AND METHODS

Animals.

All experiments performed were in accordance with Italian State and the European regulations governing the care and treatment of laboratory animals (Permission n° 101/2004B) and conformed to the guidelines for the study of pain in awake animals established by the International Association for the Study of Pain (Zimmermann, 1983). All efforts were made to minimize the number of animals used and their discomfort. Male Wistar rats weighing 200-220 g (Harlan, Italy) were housed under controlled temperature (22±1°C), humidity (60±10%) and light (12h/day) and allowed to acclimatise for at least one week before the tests.

Induction of diabetes.

Type 1 diabetes was induced through chemical pancreatectomy by a single intraperitoneal injection of STZ (Sigma, Italy) at 60 mg/kg, freshly prepared in citrate buffer 0.1 M pH 4.5. Diabetes was verified one week later by measurement of blood glucose concentration by a glucometer (Lifescan One Touch Ultra glucose meter, Milan, Italy) on a sample of blood obtained from a tail prick. Only rats with a blood glucose level above 250 mg/dL were selected for the experiments. Control animals received an intraperitoneal (i.p.) injection of citrate buffer. Blood glucose level and rat body weight were monitored over the whole period of the experimental study.

Drugs and treatments.

Cannabis sativa extract with an high cannabidiol (CBD) content (eCBD) is a gift of GW Pharmaceuticals (UK). eCBD contains 64.5% CBD, 4% tetrahydrocannabinol (THC), <4% of other cannabinoids (cannabigerol, cannabichromene, cannabidivarin, cannabidiolic acid) and minor components (terpenes, sterols, triglycerides, alkanes, squalene, tocopherol, carotenoids). The compound was dissolved in a 1:1:18 mixture of ethanol:cremophor:saline.

Diabetic rats, randomly divided in three groups of 6-8 rats each, received orally the drug vehicle or eCBD (15 or 30 mg/kg), once a day for 8 days, starting from the day 28th after the STZ injection. The effect of the acute administration of eCBD was studied in diabetic rats treated with vehicle for 7 days challenged with the drug the day of the behavioural evaluations.

Assessment of thermal hyperalgesia and mechanical allodynia.

Responses to thermal and mechanical stimuli were measured before diabetes induction and subsequently to STZ injection once per week for five weeks. On day 28th after STZ injection, the pharmacological treatment began just after the pain behaviour evaluations and at the end of treatment (on day 35) the evaluations were performed 150 min after the last eCBD administration. Heat hypersensitivity was tested according to the Hargreaves procedure (Hargreaves *et al*., 1988) using the Plantar test (Ugo Basile, Varese, Italy). Briefly, animals were placed in a clear plexiglass box and allowed to acclimatise. A constant intensity radiant heat source was aimed at the midplantar area of the hind paw. The time, in seconds, from initial heat source activation until paw withdrawal was recorded. Mechanical allodynia was assessed using the Dynamic Plantar Aesthesiometer (Ugo Basile, Varese, Italy). Animals were placed in a test cage with a wire mesh floor, and the tip of a von Frey-type filament was applied to the

middle of the plantar surface of the hind paw. The filament exerted an increasing force starting below the threshold of detection and increasing until the animal removed its paw. Withdrawal threshold was expressed as threshold level in g.

Sample preparation.

Thirty-five days following STZ, 150 min after the last administration of eCBD or its vehicle, pain assessment was recorded and animals were sacrificed. The livers and the sciatic nerves were quickly and carefully removed and washed with ice-cold saline solution. 2.5 g of liver were homogenized in four volumes of ice-cold 0.15 M KCl and centrifuged at 9000 g, at 4°C for 10 min. Supernatants were centrifuged at 100000 g, at 4°C for 1 h in order to obtain the cytosolic fractions which were ultracentrifuged again in presence of HPO_3 (1:4, v:v), used as a protein precipitant. These latter fractions were used for reduced (GSH) and oxidized (GSSG) glutathione content assay. Part of the liver was stored at -20°C and used for malondialdehyde (MDA) assay. Sciatic nerves were immediately stored at -80°C until the neurotrophic factor nerve growth factor (NGF) was assayed. Protein concentrations of all sample tissues were assayed by the method described by Lowry *et al.* (1951) with bovine serum albumin as standard.

GSH and GSSG assay.

The GSH and GSSG content in the hepatic cytosolic fractions was analyzed fluorimetrically with 350 nm and 420 nm as excitation and emission wavelengths, accordingly to the method of Hissin and Hilf (1976), using ophthalaldehyde (OPT) as fluorescent reagent. Briefly, for the GSH measurement, to 0.1 ml of the 100000 g supernatant, 1.9 ml of a 0.1 M phosphate-EDTA (5 mM) buffer, pH 8, was added. For the GSSG measurement, 0.5 ml of cytosol was incubated for 30 min with 200 µl of 0.04 M *N*-ethylmaleimide to prevent oxidation of GSH to GSSG. After the incubation, 4.3

ml of 0.1 N NaOH was added to this mixture. For both GSH and GSSG, the final assay mixture (2.0 ml) contained 100 µl of the diluted cytosol, 1.8 ml of phosphate buffer, and 100 µl of the OPT solution (1% in methanol). The GSH and GSSG concentration was calculated using a standard curve with known amounts of GSH and GSSG (Sigma Aldrich, Milano, Italy), and expressed as µg/mg protein.

MDA assay.

The MDA level, an indicator of free radical generation, was estimated in liver homogenate in a ratio 1 g tissue to 9 ml potassium phosphate (50 mM) plus EDTA (0.1 mM) buffer, pH 7. The lipid peroxide level was determined using the thiobarbituric acid test of Ohkawa *et al.* (1979). Briefly, 0.2 ml of homogenate was added to 0.8% thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in distilled water. After heating for 60 min in a water bath at 95°C, the mixture was then cooled and extracted with a mixture of n-butanol/pyridine (15:1, v:v). The absorbance of the reaction product present in the upper organic layer separated by centrifugation was measured spectrophotometrically at 532 nm. The MDA content was calculated employing 0.156 mM/cm as the extinction coefficient and was expressed as nmol MDA/g tissue.

NGF assay.

Sciatic nerves were homogenized in a cold lysis buffer (250 µl). The homogenates were centrifuged at 4500 g at 4°C for 10 min, and the resulting supernatants were then diluted 5-fold with Dulbecco's phosphate buffer solution. Samples were acidified to pH < 3.0 by adding 1 N HCl and then neutralized with 1 N NaOH to pH 7.6. NGF protein levels were determined by enzyme-linked immunosorbent assay (ELISA) using an ELISA kit, according to the manufacturer's instructions (Promega, USA). The absorbance at 450

nm was recorded on a microplate reader (Multiskan® EX, ThermolabSystem). NGF levels were determined by interpolation with standard curve and normalized to protein content in each tissue sample.

Statistical analysis.

All data are expressed as the mean ± SEM and analyzed using analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparison. Student's *t* test was used to compare the values from two groups. Differences were considered significant at $p<0.05$.

RESULTS

Effect of STZ injection on pain behaviour.

The withdrawal latency and mechanical threshold were tested 7, 14, 21, 28 and 35 days after a single administration of STZ (60 mg/kg, i.p.). Before STZ administration, the rats withdrew their left and right hind paws from radiant heat with a latency of about 10 s and sustained a mechanical force of about 38 g. Seven days after the STZ administration the pain behaviour of diabetic rats was unmodified in respect to control animals (Fig. 1A, B). Diabetic rats showed a significant increase in the paw withdrawal latency to thermal stimuli starting from the day 14th after STZ (Fig. 1A). This thermal hypoalgesia further increased during the subsequent days of observation reaching the maximum between day 28 and 35 (Fig. 1A). Only on day 28th after STZ, diabetic rats developed a significant mechanical allodynia demonstrated by the decrease in the mechanical force sustained by diabetic rats *versus* non diabetic (Fig. 1B). Mechanical allodynia was still present the subsequent week, corresponding to the 35th day after STZ injection (Fig. 1B).

Effect of eCBD treatment on diabetes-induced thermal hypoalgesia and mechanical allodynia.

Diabetic rats were orally treated with vehicle or with eCBD once a day for seven days, starting the day 28th after STZ, when both thermal hypoalgesia and mechanical allodynia were evident. Thermal hypoalgesia and mechanical allodynia were still present in diabetic rats treated with vehicle, whereas the repeated treatment with eCBD significantly relieved mechanical allodynia and restored the physiological thermal pain perception at the highest dose employed (30 mg/kg) (Fig. 2A, B). The single administration of eCBD at the highest dose evoked only a slight relief of mechanical allodynia at 150 min (30.42±0.611 g for eCBD treated diabetic rats *versus* 26.20±1.20 g for diabetic rats) (data not shown).

Effect of eCBD treatment on diabetes-induced hyperglycemia and body weight loss.

To ascertain whether the relief of diabetic neuropathy induced by eCBD was due to a glycemic control, glucose level was measured after the pharmacological treatment (day 35 after STZ) and compared with the basal one (day 28 after STZ) (Fig. 2C). As expected, by the 2nd day after administration of STZ, rats developed hyperglycemia: their blood glucose level (502.2±42.05 mg/dL) was statistically different from that of control animals (135.7±14.31 mg/dL) (data not shown). Hyperglycemia became greater at 28 days after STZ (Fig. 2C). The blood glucose level of rats treated for one week with eCBD (30 mg/kg) did not differ either from that of diabetic rats treated with vehicle or from the level before starting the treatment (Fig. 2C), suggesting that the pharmacological treatment did not affect the hyperglicemia induced by STZ. The body weight of diabetic rats became significantly lower than that of controls during the four

weeks after STZ injection; in fact, as shown in Fig. 2D, diabetic rats had lost 20% of their original body weight *versus* a physiological increase of about 15% showed by control rats, with initial body weights similar in control and diabetic groups (224.6±5.63 g and 222.4±3.92 g). After the week of treatment, diabetic rats treated with vehicle showed a further reduction in their body weight; conversely, the repeated administration with eCBD evoked a significant attenuation of the body weight loss of diabetic rats (Fig. 2D), showing that the pharmacological treatment led to an amelioration of the general health of rats.

Effect of eCBD treatment on oxidative stress.

The content of GSH and GSSG, as well as the MDA level, were measured as hepatic markers of diabetes-induced oxidative stress. As expected, STZ administration induced a significant depletion of both GSH and GSSG, with a ratio (GSH/GSSG) that decreased more than two times in respect to the physiological value necessary to carry out the glutathione-linked defensive activity (Fig. 3A, B, C). Accordingly, in the same animals the level of MDA was markedly increased, as sign of free radical generation (Fig. 3D). The repeated treatment with the eCBD restored the physiological level of GSH, with a smaller effect upon GSSG (Fig. 3A, B). However, as consequence, the correct ratio GSH/GSSG was completely restored after eCBD treatment (Fig. 3C). In addition, the treatment with eCBD significantly reduced MDA level to the control level (Fig. 3D), highlighting the potent antioxidant and anti-radical effect of eCBD.

Effect of eCBD repeated treatment on NGF production in the sciatic nerve.

NGF concentration was significantly decreased (35%) in the sciatic nerve of diabetic rats compared with controls. This downregulation was completely reversed by eCBD treatment that brought NGF at a value no more statistically different from that of non

diabetic animals (Fig. 4), indicating a positive effect of eCBD on diabetes-induced impairment of peripheral nerve support by NGF.

DISCUSSION

Diabetes is one of the most frequent pathologies in developing countries and it is estimated that the number of people affected with diabetes worldwide is projected to be 366 million by year 2030 (Wild *et al*., 2004). Uncontrolled chronic hyperglycemia in diabetic subjects leads to several complications including nephropathy, retinopathy, neuropathy. This latter is the most common complication, affecting more than 50% of diabetic patients. Evidence suggest that most of the neuropathic pain states, including diabetes-induced neuropathy, are not effectively controlled by first-line therapy with drugs such as antidepressants, anticonvulsants and opioids. Thus neuropathic pain is still considered to be relatively refractory to most of the analgesics. Recent treatment strategies have focused on the action of cannabinoids in neuropathic pain and growing evidence highlighted the efficacy of many cannabinoid receptor agonists to exert anti-hyperalgesic effect in nerve injury-induced neuropathic pain (Bridges *et al*., 2001; Fox *et al*., 2001; Costa *et al*., 2004b; Costa *et al*., 2006; Costa *et al*., 2007). In contrast, much less is known of the effects of cannabinoids in diabetes-induced neuropathic pain (Dogrul *et al*., 2004; Ulugol *et al*., 2004). In spite of the efficacy reported, for many cannabinoids the therapeutic employment is precluded by the concomitant adverse effects (hypothermia, sedation, hypomotility) and by the possibility of tolerance and dependence development. We recently found that a controlled cannabis extract, containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid fractions (terpenes and flavonoids) provided better antinociceptive efficacy than the single cannabinoid given alone, when tested in a rat model of neuropathic pain elicited

by sciatic nerve injury (Comelli *et al*., 2008). In addition to potentiating the pharmacological efficacy of cannabinoids, the use of controlled extracts could also decrease the adverse effects following *in vivo* administration. For instance, it was established in humans that the psychoactive effects of THC were significantly attenuated when CBD was also present (Dalton *et al.*, 1976). Collectively, the findings from our previous work strongly support the idea that the combination of cannabinoid and non-cannabinoid compounds, as present in eCBD extract, provides significant advantages in the relief of neuropathic pain in terms of efficacy and a lack of central effects (Comelli *et al*., 2008). In the present work we showed the therapeutic efficacy of the same eCBD in relieving diabetes-induced neuropathic pain. The model employed by us consisting in STZ-induced diabetes, evoked in rats mechanical allodynia and thermal hypoalgesia. Our observation is in agreement with various studies that demonstrate profound mechanical hyperalgesia with thermal hypoalgesia or no changes in thermal withdrawal thresholds in diabetic animals (Malcangio and Tomlinson, 1998; Fox *et al*., 1999). Similarly, human diabetic patients often develop a compromised ability to perceive tactile sensation, particularly in the most distal limbs (Norrsell *et al*., 2001), and they have longer withdrawal latency than non diabetic patients when exposed to both warming and cooling thermal stimuli (Navarro and Kennedy, 1991). Here we demonstrated that the repeated treatment with eCBD evoked a significant attenuation of mechanical allodynia and restored the physiological thermal nociceptive perception. These effects were elicited by 30 mg/kg of extract, a dose higher than that necessary to relieve nerve injury-induced neuropathic pain (15 mg/kg, as previously demonstrated by us (Comelli *et al*., 2008)), suggesting that although similar behavioural symptoms are observed both in nerve injury- and STZ-induced neuropathy, it is possible to assume

that such symptoms may not share the same aetiology. Of particular relevance for a possible future clinical employment, it is the ability of eCBD to alleviate diabetic neuropathy in a therapeutic regimen and through the oral route of administration. Concerning the mechanism of action, multiple hypothesis can be postulated in the light of the miscellaneous of compounds present in the extract and on the basis of the complexity of the mechanisms underlying diabetic neuropathy. The relief of pain induced by eCBD might be ascribed to a receptor-mediated action. In our previous work we demonstrated that eCBD counteracted nerve injury-induced neuropathic pain only through the transient receptor potential vanilloid subfamily member 1 (TRPV1) (Comelli *et al*., 2008) that is highly expressed on primary sensory neurons where it functions as polymodal nociceptor (see Szallasi *et al*, 2007, for review); both antagonists, through the blockade of the receptor, and agonists, through the desensitization of the receptor, behave as analgesics. In diabetic subjects small fiber (the unmyelinated C and the thinly myelinated Aδ) damage is responsible for the allodynia and hypoalgesia and both type of fibers constitutively express TRPV1. On these bases, it is possible to speculate a TRPV1 receptor involvement in the relief of diabetic neuropathy induced by eCBD, even if this hypothesis awaits further experimental investigation.

Hyperglycemia has been reported to result in increased polyol pathway activity, oxidative stress, advanced glycation end product formation, increased activation of protein kinase C, nerve hypoxia/ischemia and impaired NGF support (see Pop-Busui *et al.*, 2006, for review) and all these pathways contribute to the development of diabetic neuropathy. Therefore we first tested whether the eCBD-induced relief of diabetic neuropathy can be due to its action upon hyperglycemia. However, the results showed

that the repeated treatment with eCBD did not result in a decrease of blood glucose level, demonstrating that the relief of neuropathy occurred without affecting hyperglycemia. One of the important consequences of chronic hyperglycemia is the enhanced oxidative stress resulting from imbalance between production and neutralization of reactive oxygen species (ROS). Particularly, the diabetes-associated free radical injury, i.e. accumulation of lipid peroxidation products, depletion of GSH, decrease in GSH/GSSG ratio and down regulation of key antioxidant enzymes, have been detected not only in the liver but also in peripheral nerves, dorsal root and endothelial cells in different animal model of diabetes (Nagamatsu *et al*., 1995; Low *et al*., 1997; Romero *et al*., 1999). Accordingly, we observed a decrease in GSH in the liver of diabetic rats, probably due to an increased utilization following the diabetes-induced oxidative stress. Previous studies have reported that there was an increased lipid peroxidation in liver of diabetic rats (Yilmaz *et al*., 2004) that can be due to increased oxidative stress in the cell as a result of depletion of antioxidant scavenger systems. Lipid peroxide-mediated tissue damages have been observed in the development of type I and type II diabetes (Feillet-Coudray *et al*., 1999). The repeated administration of eCBD increased the GSH content in the liver leading to a restore of the defence mechanism and significantly decreased the liver lipid peroxidation. eCBD may help to control free radicals as both CBD and THC offered protection to cells against oxidative stress by scavenging free radicals. In fact it has been shown that compared with other commonly used antioxidants, CBD and THC protected neurons to a greater degree than the dietary antioxidants α-tocopherol and ascorbate (Hampson *et al.* 1998). In addition, CBD has been shown to exert antioxidant effects both *in vitro* (Chen and Buck, 2000) and in various preclinical models of neurodegeneration and

inflammatory disorders (Malfait *et al*., 2000; Costa *et al*., 2004a) through a receptor-independent mechanism. Of interest, many other substances present in the extract possess well established antioxidant properties (tocopherol, monoterpenes (Grassmann, 2005)), which could prominently contribute to the antioxidant effect observed after eCBD treatment. In conclusion, the protective antioxidant action of eCBD provides the protection against oxidative damage in STZ-induced diabetes that also strongly contributes to the neuropathy development. In support of this mechanism of action there is the demonstration that several antioxidants such as α-lipoic acid, taurine and β-carotene ameliorate nerve function deficit in experimental diabetic neuropathy (Karasu *et al*., 1995).

There is strong support for the hypothesis that reduced levels or activity of NGF play a significant role in the pathogenesis of diabetic neuropathy (Pittenger and Vinik, 2003), since the integrity of distinct subpopulations of nociceptive sensory neurons are highly dependent on neurotrophic support provided by NGF. In addition, the retrograde transport of NGF in the sciatic nerve is also decreased in rats after STZ (Hellweg *et al*., 1991). The low level of NGF could be due to either reduced production or transport of NGF in diabetes or both, possibly as a result of glucose-induced oxidative stress (Pittenger and Vinik, 2003). Clinical studies have confirmed that there are changes in NGF mRNA levels in the skin of diabetic subjects (Anand *et al*., 1996) that may be pertinent to progressive loss of epidermal C fibers and subsequent thermal hypoalgesia. Unfortunately, clinical trials of NGF therapy in diabetic patients have not been successful (Apfel *et al*., 2000), in part because of the limitation in the exogenous NGF delivery and tolerability. We believe that a compound able to induce or enhance endogenous NGF production could represent an important therapeutic tool to prevent

the nerve damage associated to the loss of NGF support. In this sense, we have reported here that the NGF content in the sciatic nerve of diabetic rats was restored to normal following the repeated treatment with eCBD. It is possible to hypothesize that the eCBD-induced prevention of diabetes-induced thermal hypoalgesia as well as the nerve degeneration consequently to hyperglycemia might be ascribed to the ability of the extract to normalize the NGF support, even if the mechanism underlying such an effect is still unknown and deserves further investigations.

In conclusion, the repeated treatment with eCBD in diabetic rats resulted in an amelioration of allodynia and of impaired thermal perception along with an improvement of oxidative damage and NGF support, suggesting the beneficial effects of this cannabis extract diabetic neuropathy.

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Figure legends

Fig. 1. Time course of pain threshold values of STZ (60 mg/kg, i.p.)-administered (diabetic) or control (non diabetic) rats, after thermal (A) and mechanical (B) stimuli. Withdrawal latency to heat and mechanical threshold of the paws are expressed as s and g, respectively, and data represent mean ± S.E.M. of 6-8 rats. ***p<0.001, **p<0.01, *p<0.05 vs non diabetic.

Fig. 2. Effect of eCBD (15 and 30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28th after STZ injection, on thermal hypoalgesia (A) and on mechanical allodynia (B). The effective dose of eCBD on pain behaviour (30 mg/kg, p.o.) was also tested on glucose level (C) and on body weight (D) by comparing the values between the beginning (D28) and the end (D35) of the treatment. Data represent mean ± S.E.M. of 6-8 rats.***p<0.001, **p<0.01, *p<0.05 vs non diabetic ; °°°p<0.001 vs diabetic.

Fig. 3. Effect of eCBD (30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28th after STZ injection, on reduced glutathione (GSH) (A) and on oxidized glutathione (GSSG) (B) in the cytosolic fraction of liver. Panel C represents the ratio between reduced and oxidized glutathione. In panel D is shown the effect of the repeated treatment with eCBD on the level of malondialdehyde (MDA) in the liver. Data represent mean ± S.E.M. of 6-8 rats. ***p<0.001, *p<0.05 vs non diabetic; °°p<0.01 vs diabetic.

Fig. 4. Effect of eCBD (30 mg/kg, p.o.) given daily to diabetic rats for one week, from day 28th after STZ injection on nerve growth factor (NGF) production in the sciatic nerve. Data represent mean ± S.E.M. of 6-8 rats. **p<0.01 vs non diabetic; °p<0.05 vs diabetic.