



HAL
open science

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

Francesca Comelli, Isabella Bettoni, Mariapia Colleoni, Gabriella Giagnoni,
Barbara Costa

► **To cite this version:**

Francesca Comelli, Isabella Bettoni, Mariapia Colleoni, Gabriella Giagnoni, Barbara Costa. Beneficial effects of a Cannabis sativa extract treatment on diabetes-induced neuropathy and oxidative stress. *Phytotherapy Research*, 2009, 23 (12), pp.1678-n/a. 10.1002/ptr.2806 . hal-00483244

HAL Id: hal-00483244

<https://hal.science/hal-00483244v1>

Submitted on 13 May 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Journal:	<i>Phytotherapy Research</i>
Manuscript ID:	PTR-08-0900
Wiley - Manuscript type:	Full Paper
Date Submitted by the Author:	29-Oct-2008
Complete List of Authors:	Comelli, Francesca; University of Milano-Bicocca, Biotechnology and Bioscience Bettoni, Isabella; University of Milano-Bicocca, Biotechnology and Bioscience Colleoni, Mariapia; University of Milano, Pharmacology Giagnoni, Gabriella; University of Milano-Bicocca, Biotechnology and Bioscience Costa, Barbara; University of Milano-Bicocca, Biothecnology and Bioscience
Keyword:	neuropathic pain, Cannabis sativa, diabetes, cannabinoid, oxidative stress, nerve growth factor



1
2
3
4 **Beneficial effects of a *Cannabis sativa* extract treatment on diabetes-induced**
5
6
7 **neuropathy and oxidative stress**

8
9 Francesca Comelli¹, Isabella Bettoni¹, Mariapia Colleoni², Gabriella Giagnoni¹ and
10
11 Barbara Costa^{1*}
12

13
14 ¹Department of Biotechnology and Bioscience, University of Milano-Bicocca, Piazza
15
16 della Scienza 2, 20126 Milano, Italy
17

18
19 ²Department of Pharmacology, University of Milano, via Vanvitelli 32, 20129 Milano,
20
21 Italy
22

23
24 *Correspondence to: Prof. Barbara Costa

25
26 Department of Biotechnology and Bioscience

27
28 University of Milano-Bicocca

29
30 Piazza della Scienza 2, 20126 Milano, Italy

31
32 Tel:+39-02-64483436; Fax:+39-02-64483450

33
34 e-mail: barbara.costa@unimib.it
35
36

37
38 Short title: Cannabis extract alleviates diabetic neuropathy
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 glycemetic 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 glycemetic 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,

1
2
3
4 cannabinoids are promising analgesics (see Lever and Rice, 2007 for review).
5
6 Cannabinoids are lipophilic compounds originally obtained from *Cannabis sativa* which
7
8 contains more than 60 different cannabinoids. Phytocannabinoids obtained from the
9
10 cannabis plant comprise a range of cannabinoid receptor agonists, partial agonists, and
11
12 antagonists, and many synthetic cannabinoids have also been developed with specific
13
14 receptor affinity and distinct pharmacological profiles. There are two main cannabinoid
15
16 receptors, CB1 and CB2, associated with pain modulation. The first is widely expressed
17
18 in the CNS and peripheral sensory neurons whereas the latter has been found on
19
20 peripheral tissues, including tissues of the immune system and keratinocytes, with
21
22 limited expression in sensory and CNS cells (Pertwee, 2006 for review). Synthetic or
23
24 pure natural cannabinoids have been found useful in neuropathic pain (Bridges *et al.*,
25
26 2001; Fox *et al.*, 2001; Costa *et al.*, 2004b; Costa *et al.*, 2006; Costa *et al.*, 2007) even if
27
28 the most of studies were performed employing the injury of the sciatic nerve as animal
29
30 model of painful neuropathy. To our knowledge there are only two initial studies
31
32 reporting the efficacy of a synthetic CB1 receptor agonist, WIN55,212-2, on tactile
33
34 allodynia in diabetic rats (Dogrul *et al.*, 2004; Ulugol *et al.*, 2004). We have recently
35
36 shown that a controlled cannabis extract, containing multiple cannabinoids, in a defined
37
38 ratio, and other non-cannabinoid fractions (terpens and flavonoids) provided better
39
40 antinociceptive efficacy than the single cannabinoids given alone when tested in the
41
42 chronic constriction injury of the sciatic nerve model of neuropathy (Comelli *et al.*,
43
44 2008). We suggested that other than the pharmacological receptor-mediated effect of
45
46 cannabinoids, the presence of anti-inflammatory and antioxidant compounds in the non-
47
48 cannabinoid fraction of the natural extract might strongly contribute to analgesia
49
50 (Comelli *et al.*, 2008). This background and especially the multiple aetiologies of
51
52
53
54
55
56
57
58
59
60

1
2
3
4 diabetic neuropathy involving oxidative stress, inflammation and nerve damage,
5
6 prompted us to test the same *Cannabis sativa* extract in the rat model of streptozotocin
7
8 (STZ)-induced diabetic neuropathy, in the attempt to propose a new therapy to
9
10 ameliorate this chronic and disabling painful condition after its onset.
11
12

13 14 **MATERIALS AND METHODS**

15 16 **Animals.**

17
18 All experiments performed were in accordance with Italian State and the European
19
20 regulations governing the care and treatment of laboratory animals (Permission n°
21
22 101/2004B) and conformed to the guidelines for the study of pain in awake animals
23
24 established by the International Association for the Study of Pain (Zimmermann, 1983).
25
26 All efforts were made to minimize the number of animals used and their discomfort.
27
28 Male Wistar rats weighing 200-220 g (Harlan, Italy) were housed under controlled
29
30 temperature ($22\pm 1^{\circ}\text{C}$), humidity ($60\pm 10\%$) and light (12h/day) and allowed to
31
32 acclimatise for at least one week before the tests.
33
34
35
36

37 38 **Induction of diabetes.**

39
40 Type 1 diabetes was induced through chemical pancreatectomy by a single
41
42 intraperitoneal injection of STZ (Sigma, Italy) at 60 mg/kg, freshly prepared in citrate
43
44 buffer 0.1 M pH 4.5. Diabetes was verified one week later by measurement of blood
45
46 glucose concentration by a glucometer (Lifescan One Touch Ultra glucose meter,
47
48 Milan, Italy) on a sample of blood obtained from a tail prick. Only rats with a blood
49
50 glucose level above 250 mg/dL were selected for the experiments. Control animals
51
52 received an intraperitoneal (i.p.) injection of citrate buffer. Blood glucose level and rat
53
54 body weight were monitored over the whole period of the experimental study.
55
56
57
58

59 60 **Drugs and treatments.**

1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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

1
2
3
4 middle of the plantar surface of the hind paw. The filament exerted an increasing force
5
6 starting below the threshold of detection and increasing until the animal removed its
7
8 paw. Withdrawal threshold was expressed as threshold level in g.
9

10 11 **Sample preparation.**

12
13 Thirty-five days following STZ, 150 min after the last administration of eCBD or its
14
15 vehicle, pain assessment was recorded and animals were sacrificed. The livers and the
16
17 sciatic nerves were quickly and carefully removed and washed with ice-cold saline
18
19 solution. 2.5 g of liver were homogenized in four volumes of ice-cold 0.15 M KCl and
20
21 centrifuged at 9000 g, at 4°C for 10 min. Supernatants were centrifuged at 100000 g, at
22
23 4°C for 1 h in order to obtain the cytosolic fractions which were ultracentrifuged again
24
25 in presence of HPO₃ (1:4, v:v), used as a protein precipitant. These latter fractions were
26
27 used for reduced (GSH) and oxidized (GSSG) glutathione content assay. Part of the
28
29 liver was stored at -20°C and used for malondialdehyde (MDA) assay. Sciatic nerves
30
31 were immediately stored at -80°C until the neurotrophic factor nerve growth factor
32
33 (NGF) was assayed. Protein concentrations of all sample tissues were assayed by the
34
35 method described by Lowry *et al.* (1951) with bovine serum albumin as standard.
36
37
38
39
40
41

42 **GSH and GSSG assay.**

43
44 The GSH and GSSG content in the hepatic cytosolic fractions was analyzed
45
46 fluorimetrically with 350 nm and 420 nm as excitation and emission wavelengths,
47
48 accordingly to the method of Hissin and Hilf (1976), using ophthalaldehyde (OPT) as
49
50 fluorescent reagent. Briefly, for the GSH measurement, to 0.1 ml of the 100000 g
51
52 supernatant, 1.9 ml of a 0.1 M phosphate-EDTA (5 mM) buffer, pH 8, was added. For
53
54 the GSSG measurement, 0.5 ml of cytosol was incubated for 30 min with 200 µl of 0.04
55
56 M *N*-ethylmaleimide to prevent oxidation of GSH to GSSG. After the incubation, 4.3
57
58
59
60

1
2
3
4 ml of 0.1 N NaOH was added to this mixture. For both GSH and GSSG, the final assay
5
6 mixture (2.0 ml) contained 100 μ l of the diluted cytosol, 1.8 ml of phosphate buffer, and
7
8 100 μ l of the OPT solution (1% in methanol). The GSH and GSSG concentration was
9
10 calculated using a standard curve with known amounts of GSH and GSSG (Sigma
11
12 Aldrich, Milano, Italy), and expressed as μ g/mg protein.
13
14

15 16 **MDA assay.**

17
18 The MDA level, an indicator of free radical generation, was estimated in liver
19
20 homogenate in a ratio 1 g tissue to 9 ml potassium phosphate (50 mM) plus EDTA (0.1
21
22 mM) buffer, pH 7. The lipid peroxide level was determined using the thiobarbituric acid
23
24 test of Ohkawa *et al.* (1979). Briefly, 0.2 ml of homogenate was added to 0.8%
25
26 thiobarbituric acid, 8.1% sodium dodecyl sulfate (SDS) and acetic acid (20%) in
27
28 distilled water. After heating for 60 min in a water bath at 95°C, the mixture was then
29
30 cooled and extracted with a mixture of n-butanol/pyridine (15:1, v:v). The absorbance
31
32 of the reaction product present in the upper organic layer separated by centrifugation
33
34 was measured spectrophotometrically at 532 nm. The MDA content was calculated
35
36 employing 0.156 mM/cm as the extinction coefficient and was expressed as nmol
37
38 MDA/g tissue.
39
40
41
42

43 44 **NGF assay.**

45
46 Sciatic nerves were homogenized in a cold lysis buffer (250 μ l). The homogenates were
47
48 centrifuged at 4500 g at 4°C for 10 min, and the resulting supernatants were then diluted
49
50 5-fold with Dulbecco's phosphate buffer solution. Samples were acidified to pH < 3.0
51
52 by adding 1 N HCl and then neutralized with 1 N NaOH to pH 7.6. NGF protein levels
53
54 were determined by enzyme-linked immunosorbent assay (ELISA) using an ELISA kit,
55
56 according to the manufacturer's instructions (Promega, USA). The absorbance at 450
57
58
59
60

1
2
3
4 nm was recorded on a microplate reader (Multiskan® EX, ThermoLabSystem). NGF
5
6 levels were determined by interpolation with standard curve and normalized to protein
7
8 content in each tissue sample.
9

10 11 **Statistical analysis.**

12
13 All data are expressed as the mean \pm SEM and analyzed using analysis of variance
14
15 (ANOVA) followed by Tukey's post-hoc test for multiple comparison. Student's *t* test
16
17 was used to compare the values from two groups. Differences were considered
18
19 significant at $p < 0.05$.
20
21

22 23 **RESULTS**

24 25 **Effect of STZ injection on pain behaviour.**

26
27 The withdrawal latency and mechanical threshold were tested 7, 14, 21, 28 and 35 days
28
29 after a single administration of STZ (60 mg/kg, i.p.). Before STZ administration, the
30
31 rats withdrew their left and right hind paws from radiant heat with a latency of about 10
32
33 s and sustained a mechanical force of about 38 g. Seven days after the STZ
34
35 administration the pain behaviour of diabetic rats was unmodified in respect to control
36
37 animals (Fig. 1A, B). Diabetic rats showed a significant increase in the paw withdrawal
38
39 latency to thermal stimuli starting from the day 14th after STZ (Fig. 1A). This thermal
40
41 hypoalgesia further increased during the subsequent days of observation reaching the
42
43 maximum between day 28 and 35 (Fig. 1A). Only on day 28th after STZ, diabetic rats
44
45 developed a significant mechanical allodynia demonstrated by the decrease in the
46
47 mechanical force sustained by diabetic rats *versus* non diabetic (Fig. 1B). Mechanical
48
49 allodynia was still present the subsequent week, corresponding to the 35th day after STZ
50
51 injection (Fig. 1B).
52
53
54
55
56
57
58
59
60

1
2
3
4 **Effect of eCBD treatment on diabetes-induced thermal hypoalgesia and**
5
6 **mechanical allodynia.**
7

8
9 Diabetic rats were orally treated with vehicle or with eCBD once a day for seven days,
10 starting the day 28th after STZ, when both thermal hypoalgesia and mechanical
11 allodynia were evident. Thermal hypoalgesia and mechanical allodynia were still
12 present in diabetic rats treated with vehicle, whereas the repeated treatment with eCBD
13 significantly relieved mechanical allodynia and restored the physiological thermal pain
14 perception at the highest dose employed (30 mg/kg) (Fig. 2A, B). The single
15 administration of eCBD at the highest dose evoked only a slight relief of mechanical
16 allodynia at 150 min (30.42±0.611 g for eCBD treated diabetic rats *versus* 26.20±1.20 g
17 for diabetic rats) (data not shown).
18
19
20
21
22
23
24
25
26
27
28
29

30 **Effect of eCBD treatment on diabetes-induced hyperglycemia and body weight**
31 **loss.**
32
33

34
35 To ascertain whether the relief of diabetic neuropathy induced by eCBD was due to a
36 glycaemic control, glucose level was measured after the pharmacological treatment (day
37 35 after STZ) and compared with the basal one (day 28 after STZ) (Fig. 2C). As
38 expected, by the 2nd day after administration of STZ, rats developed hyperglycemia:
39 their blood glucose level (502.2±42.05 mg/dL) was statistically different from that of
40 control animals (135.7±14.31 mg/dL) (data not shown). Hyperglycemia became greater
41 at 28 days after STZ (Fig. 2C). The blood glucose level of rats treated for one week with
42 eCBD (30 mg/kg) did not differ either from that of diabetic rats treated with vehicle or
43 from the level before starting the treatment (Fig. 2C), suggesting that the
44 pharmacological treatment did not affect the hyperglycemia induced by STZ. The body
45 weight of diabetic rats became significantly lower than that of controls during the four
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 weeks after STZ injection; in fact, as shown in Fig. 2D, diabetic rats had lost 20% of
5
6 their original body weight *versus* a physiological increase of about 15% showed by
7
8 control rats, with initial body weights similar in control and diabetic groups (224.6 ± 5.63
9
10 g and 222.4 ± 3.92 g). After the week of treatment, diabetic rats treated with vehicle
11
12 showed a further reduction in their body weight; conversely, the repeated administration
13
14 with eCBD evoked a significant attenuation of the body weight loss of diabetic rats
15
16 (Fig. 2D), showing that the pharmacological treatment led to an amelioration of the
17
18 general health of rats.
19
20
21
22

23 **Effect of eCBD treatment on oxidative stress.**

24
25 The content of GSH and GSSG, as well as the MDA level, were measured as hepatic
26
27 markers of diabetes-induced oxidative stress. As expected, STZ administration induced
28
29 a significant depletion of both GSH and GSSG, with a ratio (GSH/GSSG) that
30
31 decreased more than two times in respect to the physiological value necessary to carry
32
33 out the glutathione-linked defensive activity (Fig. 3A, B, C). Accordingly, in the same
34
35 animals the level of MDA was markedly increased, as sign of free radical generation
36
37 (Fig. 3D). The repeated treatment with the eCBD restored the physiological level of
38
39 GSH, with a smaller effect upon GSSG (Fig. 3A, B). However, as consequence, the
40
41 correct ratio GSH/GSSG was completely restored after eCBD treatment (Fig. 3C). In
42
43 addition, the treatment with eCBD significantly reduced MDA level to the control level
44
45 (Fig. 3D), highlighting the potent antioxidant and anti-radical effect of eCBD.
46
47
48
49
50

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

52
53 NGF concentration was significantly decreased (35%) in the sciatic nerve of diabetic
54
55 rats compared with controls. This downregulation was completely reversed by eCBD
56
57 treatment that brought NGF at a value no more statistically different from that of non
58
59
60

1
2
3
4 diabetic animals (Fig. 4), indicating a positive effect of eCBD on diabetes-induced
5
6 impairment of peripheral nerve support by NGF.
7
8

9 **DISCUSSION**

10
11 Diabetes is one of the most frequent pathologies in developing countries and it is
12
13 estimated that the number of people affected with diabetes worldwide is projected to be
14
15 366 million by year 2030 (Wild *et al.*, 2004). Uncontrolled chronic hyperglycemia in
16
17 diabetic subjects leads to several complications including nephropathy, retinopathy,
18
19 neuropathy. This latter is the most common complication, affecting more than 50% of
20
21 diabetic patients. Evidence suggest that most of the neuropathic pain states, including
22
23 diabetes-induced neuropathy, are not effectively controlled by first-line therapy with
24
25 drugs such as antidepressants, anticonvulsants and opioids. Thus neuropathic pain is
26
27 still considered to be relatively refractory to most of the analgesics. Recent treatment
28
29 strategies have focused on the action of cannabinoids in neuropathic pain and growing
30
31 evidence highlighted the efficacy of many cannabinoid receptor agonists to exert anti-
32
33 hyperalgesic effect in nerve injury-induced neuropathic pain (Bridges *et al.*, 2001; Fox
34
35 *et al.*, 2001; Costa *et al.*, 2004b; Costa *et al.*, 2006; Costa *et al.*, 2007). In contrast,
36
37 much less is known of the effects of cannabinoids in diabetes-induced neuropathic pain
38
39 (Dogrul *et al.*, 2004; Ulugol *et al.*, 2004). In spite of the efficacy reported, for many
40
41 cannabinoids the therapeutic employment is precluded by the concomitant adverse
42
43 effects (hypothermia, sedation, hypomotility) and by the possibility of tolerance and
44
45 dependence development. We recently found that a controlled cannabis extract,
46
47 containing multiple cannabinoids, in a defined ratio, and other non-cannabinoid
48
49 fractions (terpenes and flavonoids) provided better antinociceptive efficacy than the
50
51 single cannabinoid given alone, when tested in a rat model of neuropathic pain elicited
52
53
54
55
56
57
58
59
60

1
2
3
4 by sciatic nerve injury (Comelli *et al.*, 2008). In addition to potentiating the
5 pharmacological efficacy of cannabinoids, the use of controlled extracts could also
6 decrease the adverse effects following *in vivo* administration. For instance, it was
7 established in humans that the psychoactive effects of THC were significantly
8 attenuated when CBD was also present (Dalton *et al.*, 1976). Collectively, the findings
9 from our previous work strongly support the idea that the combination of cannabinoid
10 and non-cannabinoid compounds, as present in eCBD extract, provides significant
11 advantages in the relief of neuropathic pain in terms of efficacy and a lack of central
12 effects (Comelli *et al.*, 2008). In the present work we showed the therapeutic efficacy of
13 the same eCBD in relieving diabetes-induced neuropathic pain. The model employed by
14 us consisting in STZ-induced diabetes, evoked in rats mechanical allodynia and thermal
15 hypoalgesia. Our observation is in agreement with various studies that demonstrate
16 profound mechanical hyperalgesia with thermal hypoalgesia or no changes in thermal
17 withdrawal thresholds in diabetic animals (Malcangio and Tomlinson, 1998; Fox *et al.*,
18 1999). Similarly, human diabetic patients often develop a compromised ability to
19 perceive tactile sensation, particularly in the most distal limbs (Norrzell *et al.*, 2001),
20 and they have longer withdrawal latency than non diabetic patients when exposed to
21 both warming and cooling thermal stimuli (Navarro and Kennedy, 1991). Here we
22 demonstrated that the repeated treatment with eCBD evoked a significant attenuation of
23 mechanical allodynia and restored the physiological thermal nociceptive perception.
24 These effects were elicited by 30 mg/kg of extract, a dose higher than that necessary to
25 relieve nerve injury-induced neuropathic pain (15 mg/kg, as previously demonstrated by
26 us (Comelli *et al.*, 2008)), suggesting that although similar behavioural symptoms are
27 observed both in nerve injury- and STZ-induced neuropathy, it is possible to assume
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 that such symptoms may not share the same aetiology. Of particular relevance for a
5 possible future clinical employment, it is the ability of eCBD to alleviate diabetic
6 neuropathy in a therapeutic regimen and through the oral route of administration.
7
8 Concerning the mechanism of action, multiple hypothesis can be postulated in the light
9 of the miscellaneous of compounds present in the extract and on the basis of the
10 complexity of the mechanisms underlying diabetic neuropathy. The relief of pain
11 induced by eCBD might be ascribed to a receptor-mediated action. In our previous work
12 we demonstrated that eCBD counteracted nerve injury-induced neuropathic pain only
13 through the transient receptor potential vanilloid subfamily member 1 (TRPV1)
14 (Comelli *et al.*, 2008) that is highly expressed on primary sensory neurons where it
15 functions as polymodal nociceptor (see Szallasi *et al.*, 2007, for review); both
16 antagonists, through the blockade of the receptor, and agonists, through the
17 desensitization of the receptor, behave as analgesics. In diabetic subjects small fiber (the
18 unmyelinated C and the thinly myelinated A δ) damage is responsible for the allodynia
19 and hypoalgesia and both type of fibers constitutively express TRPV1. On these bases,
20 it is possible to speculate a TRPV1 receptor involvement in the relief of diabetic
21 neuropathy induced by eCBD, even if this hypothesis awaits further experimental
22 investigation.
23
24
25
26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45

46
47 Hyperglycemia has been reported to result in increased polyol pathway activity,
48 oxidative stress, advanced glycation end product formation, increased activation of
49 protein kinase C, nerve hypoxia/ischemia and impaired NGF support (see Pop-Busui *et*
50 *al.*, 2006, for review) and all these pathways contribute to the development of diabetic
51 neuropathy. Therefore we first tested whether the eCBD-induced relief of diabetic
52 neuropathy can be due to its action upon hyperglycemia. However, the results showed
53
54
55
56
57
58
59
60

1
2
3
4 that the repeated treatment with eCBD did not result in a decrease of blood glucose
5 level, demonstrating that the relief of neuropathy occurred without affecting
6
7 hyperglycemia. One of the important consequences of chronic hyperglycemia is the
8
9 enhanced oxidative stress resulting from imbalance between production and
10
11 neutralization of reactive oxygen species (ROS). Particularly, the diabetes-associated
12
13 free radical injury, i.e. accumulation of lipid peroxidation products, depletion of GSH,
14
15 decrease in GSH/GSSG ratio and down regulation of key antioxidant enzymes, have
16
17 been detected not only in the liver but also in peripheral nerves, dorsal root and
18
19 endothelial cells in different animal model of diabetes (Nagamatsu *et al.*, 1995; Low *et*
20
21 *al.*, 1997; Romero *et al.*, 1999). Accordingly, we observed a decrease in GSH in the
22
23 liver of diabetic rats, probably due to an increased utilization following the diabetes-
24
25 induced oxidative stress. Previous studies have reported that there was an increased
26
27 lipid peroxidation in liver of diabetic rats (Yilmaz *et al.*, 2004) that can be due to
28
29 increased oxidative stress in the cell as a result of depletion of antioxidant scavenger
30
31 systems. Lipid peroxide-mediated tissue damages have been observed in the
32
33 development of type I and type II diabetes (Feillet-Coudray *et al.*, 1999). The repeated
34
35 administration of eCBD increased the GSH content in the liver leading to a restore of
36
37 the defence mechanism and significantly decreased the liver lipid peroxidation. eCBD
38
39 may help to control free radicals as both CBD and THC offered protection to cells
40
41 against oxidative stress by scavenging free radicals. In fact it has been shown that
42
43 compared with other commonly used antioxidants, CBD and THC protected neurons to
44
45 a greater degree than the dietary antioxidants α -tocopherol and ascorbate (Hampson *et*
46
47 *al.* 1998). In addition, CBD has been shown to exert antioxidant effects both *in vitro*
48
49 (Chen and Buck, 2000) and in various preclinical models of neurodegeneration and
50
51
52
53
54
55
56
57
58
59
60

1
2
3
4 inflammatory disorders (Malfait *et al.*, 2000; Costa *et al.*, 2004a) through a receptor-
5
6 independent mechanism. Of interest, many other substances present in the extract
7
8 possess well established antioxidant properties (tocopherol, monoterpenes (Grassmann,
9
10 2005)), which could prominently contribute to the antioxidant effect observed after
11
12 eCBD treatment. In conclusion, the protective antioxidant action of eCBD provides the
13
14 protection against oxidative damage in STZ-induced diabetes that also strongly
15
16 contributes to the neuropathy development. In support of this mechanism of action there
17
18 is the demonstration that several antioxidants such as α -lipoic acid, taurine and β -
19
20 carotene ameliorate nerve function deficit in experimental diabetic neuropathy (Karasu
21
22 *et al.*, 1995).
23
24
25
26
27

28 There is strong support for the hypothesis that reduced levels or activity of NGF play a
29
30 significant role in the pathogenesis of diabetic neuropathy (Pittenger and Vinik, 2003),
31
32 since the integrity of distinct subpopulations of nociceptive sensory neurons are highly
33
34 dependent on neurotrophic support provided by NGF. In addition, the retrograde
35
36 transport of NGF in the sciatic nerve is also decreased in rats after STZ (Hellweg *et al.*,
37
38 1991). The low level of NGF could be due to either reduced production or transport of
39
40 NGF in diabetes or both, possibly as a result of glucose-induced oxidative stress
41
42 (Pittenger and Vinik, 2003). Clinical studies have confirmed that there are changes in
43
44 NGF mRNA levels in the skin of diabetic subjects (Anand *et al.*, 1996) that may be
45
46 pertinent to progressive loss of epidermal C fibers and subsequent thermal hypoalgesia.
47
48 Unfortunately, clinical trials of NGF therapy in diabetic patients have not been
49
50 successful (Apfel *et al.*, 2000), in part because of the limitation in the exogenous NGF
51
52 delivery and tolerability. We believe that a compound able to induce or enhance
53
54 endogenous NGF production could represent an important therapeutic tool to prevent
55
56
57
58
59
60

1
2
3
4 the nerve damage associated to the loss of NGF support. In this sense, we have reported
5
6 here that the NGF content in the sciatic nerve of diabetic rats was restored to normal
7
8 following the repeated treatment with eCBD. It is possible to hypothesize that the
9
10 eCBD-induced prevention of diabetes-induced thermal hypoalgesia as well as the nerve
11
12 degeneration consequently to hyperglycemia might be ascribed to the ability of the
13
14 extract to normalize the NGF support, even if the mechanism underlying such an effect
15
16 is still unknown and deserves further investigations.
17
18

19
20 In conclusion, the repeated treatment with eCBD in diabetic rats resulted in an
21
22 amelioration of allodynia and of impaired thermal perception along with an
23
24 improvement of oxidative damage and NGF support, suggesting the beneficial effects of
25
26 this cannabis extract diabetic neuropathy.
27
28

29 30 **Acknowledgments**

31
32 We are grateful to GW Pharmaceuticals for kindly supplying *Cannabis sativa* extract.
33
34

35
36 Authors state no conflict of interest.
37

38 **REFERENCES**

- 39
40 Anand P, Terenghi G, Warner G, Kopelman P, Williams-Chestnut RE, Sinicropi DV.
41
42 1996. The role of endogenous nerve growth factor in human diabetic neuropathy. *Nat*
43
44 *Med* **2**:703-707.
45
46
47 Apfel SC, Schwartz S, Adornato BT, Freeman R, Biton V, Rendell M, Vinik A,
48
49 Giuliani M, Stevens JC, Barbano R, Dyck PJ. 2000. Efficacy and safety of
50
51 recombinant human nerve growth factor in patients with diabetic polyneuropathy: A
52
53 randomized controlled trial. rhNGF Clinical Investigator Group. *JAMA* **284**:2215-
54
55 2221.
56
57
58
59
60

- 1
2
3
4
5 Bridges D, Ahmand K, Rice AS. 2001. The synthetic cannabinoid WIN55,212-2
6
7 attenuates hyperalgesia and allodynia in a rat model of neuropathic pain. *Br J*
8
9 *Pharmacol* **133**: 586-594.
10
11
12 Chen Y, Buck J. 2000. Cannabinoids protect cells from oxidative cell death: a receptor-
13
14 independent mechanism. *J Pharmacol Exp Ther* **293**:807-812.
15
16
17 Comelli F, Giagnoni G, Bettoni I, Colleoni M, Costa B. 2008. Antihyperalgesic effect
18
19 of a *Cannabis sativa* extract in a rat model of neuropathic pain: mechanisms
20
21 involved. *Phytother Res* **22**: 1017-1024.
22
23
24 Costa B, Colleoni M, Conti S, Parolaro D, Franke C, Trovato AE, Giagnoni G. 2004a.
25
26 Oral anti-inflammatory activity of cannabidiol, a non-psychoactive constituent of
27
28 cannabis, in acute carrageenan-induced inflammation in the rat paw. *Naunyn*
29
30 *Schmiedebergs Arch Pharmacol* **369**:294-299.
31
32
33 Costa B, Colleoni M, Conti S, Trovato AE, Bianchi M, Sotgiu ML, Giagnoni G. 2004b.
34
35 Repeated treatment with the synthetic cannabinoid WIN 55,212-2 reduces both
36
37 hyperalgesia and production of pronociceptive mediators in a rat model of
38
39 neuropathic pain. *Br J Pharmacol* **141**: 4-8.
40
41
42 Costa B, Siniscalco D, Trovato AE, Comelli F, Sotgiu ML, Colleoni M, Maione S,
43
44 Rossi F, Giagnoni G. 2006. AM404, an inhibitor of anandamide uptake, prevents
45
46 pain behaviour and modulates cytokine and apoptotic pathways in a rat model of
47
48 neuropathic pain. *Br J Pharmacol* **148**: 1022-1032.
49
50
51
52 Costa B, Trovato AE, Comelli F, Giagnoni G, Colleoni M. 2007. The non-psychoactive
53
54 cannabis constituent cannabidiol is an orally effective therapeutic agent in rat
55
56 chronic inflammatory and neuropathic pain. *Eur J Pharmacol* **556**: 75-83.
57
58
59
60

- 1
2
3
4 Dalton WS, Martz R, Lemberger L, Rodda BE, Forney RB. 1976. Influence of
5
6 cannabidiol on delta-9-tetrahydrocannabinol effects. *Clin Pharmacol Ther* **19**: 300-
7
8 309.
9
10
11 Dogrul A, Gul H, Yildiz O, Bilgin F, Guzeldemir ME. 2004. Cannabinoids block tactile
12
13 allodynia in diabetic mice without attenuation of its antinociceptive effect. *Neurosci*
14
15 *Lett* **368**:82-86.
16
17
18 Feillet-Coudray C, Rock E, Coudray C, Grzelkowska K, Azais-Braesco V, Dardevet D,
19
20 Mazur A. 1999. Lipid peroxidation and antioxidant status in experimental diabetes.
21
22 *Clin Chim Acta* **284**:31-43.
23
24
25
26 Fox A, Eastwood C, Gentry C, Manning D, Urban L. 1999. Critical evaluation of the
27
28 streptozotocin model of painful diabetic neuropathy in the rat. *Pain* **81**:307-316.
29
30
31 Fox A, Kesingland A, Gentry C, Mcnair K, Patel S, Urban L, James I. 2001. The role of
32
33 central and peripheral cannabinoid receptors in the antihyperalgesic activity of
34
35 cannabinoids in a model of neuropathic pain. *Pain* **92**: 91-100.
36
37
38 Grassmann J. 2005. Terpenoids as plant antioxidants. *Vitam Horm* **72**:505-535.
39
40
41 Hampson AJ, Grimaldi M, Axelrod J, Wink D. 1998. Cannabidiol and (-)Delta9-
42
43 tetrahydrocannabinol are neuroprotective antioxidants. *Proc Natl Acad Sci U S A*
44
45 **95**:8268-8273.
46
47
48 Hargreaves KM, Dubner R, Brown F, Flores C, Joris J. 1988. A new and sensitive
49
50 method for measuring thermal nociception in cutaneous hyperalgesia. *Pain* **32**: 77-
51
52 88.
53
54
55 Hellweg R, Wöhrle M, Hartung HD, Stracke H, Hock C, Federlin K. 1991. Diabetes
56
57 mellitus-associated decrease in nerve growth factor levels is reversed by allogeneic
58
59 pancreatic islet transplantation. *Neurosci Lett* **125**:1-4.
60

- 1
2
3
4 Hissin PJ, Hilf R. 1976. A fluorometric method for determination of oxidized and
5
6 reduced glutathione in tissues. *Anal Biochem* **74**:214-226.
7
8
9 Karasu C, Dewhurst M, Stevens EJ, Tomlinson DR. 1995. Effects of anti-oxidant
10
11 treatment on sciatic nerve dysfunction in streptozotocin-diabetic rats; comparison
12
13 with essential fatty acids. *Diabetologia* **38**:129-134.
14
15
16 Lever IJ, Rice AS. 2007. Cannabinoids and pain. *Handb Exp Pharmacol* **177**: 265-306.
17
18
19 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the
20
21 Folin phenol reagent. *J Biol Chem* **193**: 265-275.
22
23
24 Low PA, Nickander KK, Tritschler HJ. 1997. The roles of oxidative stress and
25
26 antioxidant treatment in experimental diabetic neuropathy. *Diabetes* **46**: S38-S42.
27
28
29 Malcangio M, Tomlinson DR. 1998. A pharmacological analysis of mechanical
30
31 hyperalgesia in in streptozotocin/diabetic rats. *Pain* **76**:151-157.
32
33
34 Malfait AM, Gallily R, Sumariwalla PF, Malik AS, Andreacos E, Mechoulam R,
35
36 Feldmann M. 2000. The nonpsychoactive cannabis constituent cannabidiol is an oral
37
38 anti-arthritic therapeutic in murine collagen-induced arthritis. *Proc Natl Acad Sci U*
39
40 *S A* **97**:9561-9566.
41
42
43 Martin CL, Albers J, Herman WH, Cleary P, Waberski B, Greene DA, Stevens MJ,
44
45 Feldman EL. 2006. Neuropathy among the diabetes control and complications trial
46
47 cohort 8 years after trial completion. *Diabetes Care* **29**: 340-344.
48
49
50 Nagamatsu M, Nickander KK, Schmelzer JD, Raya A, Wittrock DA, Tritschler H, Low
51
52 PA. 1995. Lipoic acid improves nerve blood flow, reduces oxidative stress, and
53
54 improves distal nerve conduction in experimental diabetic neuropathy. *Diabetes*
55
56 *Care* **18**:1160-1167.
57
58
59
60

- 1
2
3
4 Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, Naruse K,
5
6 Kasuya Y, Mizubayashi R, Miwa K, Yasuda Y, Kamiya H, Ienaga K, Sakakibara F,
7
8 Koh N, Hotta N. 1999. A protein kinase C-beta-selective inhibitor ameliorates
9
10 neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes* **48**: 2090-2095.
11
12
13 Navarro X, Kennedy WR. 1991. Evaluation of thermal and pain sensitivity in type I
14
15 diabetic patients. *J Neurol Neurosurg Psychiatry* **54**:60-64.
16
17
18 Norrsell U, Eliasson B, Frizell M, Wallin BG, Wesslau C, Olausson H. 2001. Tactile
19
20 directional sensibility and diabetic neuropathy. *Muscle Nerve* **24**:1496-1502.
21
22
23 Ohkawa H, Ohishi N, Yagi K. 1979. Assay for lipid peroxides in animal tissues by
24
25 thiobarbituric acid reaction. *Anal Biochem* **95**:351-358.
26
27
28 Pertwee RG. 2006. The pharmacology of cannabinoid receptors and their ligands: an
29
30 overview. *Int J Obes (Lond)* **30**: S13-S18.
31
32
33 Pittenger G, Vinik A. 2003. Nerve growth factor and diabetic neuropathy. *Exp*
34
35 *Diabetes Res* **4**:271-285.
36
37
38 Pop-Busui R, Sima A, Stevens M. 2006. Diabetic neuropathy and oxidative stress.
39
40 *Diabetes Metab Res Rev* **22**:257-273.
41
42
43 Romero FJ, Martínez-Blasco A, Bosch-Morell F, Marín N, Trenor C, Romá J. 1999.
44
45 Glycemic control and not protein kinase C inhibition prevents the early decrease of
46
47 glutathione peroxidase activity in peripheral nerve of diabetic mice. *J Peripher Nerv*
48
49 *Syst* **4**:265-269.
50
51
52 Szallasi A, Cortright DN, Blum CA, Eid SR. 2007. The vanilloid receptor TRPV1: 10
53
54 years from channel cloning to antagonist proof-of-concept. *Nat Rev Drug Discov*
55
56 **6**:357-371.
57
58
59
60

- 1
2
3
4 Tomlinson DR, Fernyhough P, Diemel LT. 1997. Role of neurotrophins in diabetic
5 neuropathy and treatment with nerve growth factors. *Diabetes* **46**: S43-S49.
6
7
8
9 Ulugol A, Karadag HC, Ipci Y, Tamer M, Dokmeci I. 2004. The effect of WIN 55,212-
10 2, a cannabinoid agonist, on tactile allodynia in diabetic rats. *Neurosci Lett* **371**:
11 167-170.
12
13
14
15
16 Wild S, Roglic G, Green A, Sicree R, King H. 2004. Global prevalence of diabetes:
17 estimates for the year 2000 and projections for 2030. *Diabetes Care* **27**:1047-1053.
18
19
20
21 Yilmaz HR, Uz E, Yucel N, Altuntas I, Ozcelik N. 2004. Protective effect of caffeic
22 acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in
23 diabetic rat liver. *J Biochem Mol Toxicol* **18**:234-238.
24
25
26
27
28 Zimmermann M. 1983. Ethical guidelines for investigations of experimental pain in
29 conscious animals. *Pain* **16**: 109-110.
30
31
32
33 Zochodne DW. 1999. Diabetic neuropathies: features and mechanisms. *Brain Pathol* **9**:
34 369-391.
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
59
60

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 \pm 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 \pm 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 \pm 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 \pm S.E.M. of 6-8 rats. ** p <0.01 vs non diabetic; ° p <0.05 vs diabetic.