

BEHAVIORAL CHANGES OF WISTAR RATS WITH EXPERIMENTALLY-INDUCED PAINFUL DIABETIC NEUROPATHY

JOSÉ ARTUR C. D'ALMEIDA*, CARLOS M. DE CASTRO-COSTA**,
CARINA H. FROTA***, JANAIRA F. SEVERO***,
TARCISO D. S. ROCHA***, TRÍCIA F. NOGUEIRA***

ABSTRACT – With the purpose of studying data on spontaneous customary changes in diabetic rats, we induced diabetes in 28 Wistar rats with streptozotocin. The animals were observed for 27 weeks in an attempt to characterize spontaneous customary changes that could suggest signs of chronic pain. Morphine, as a central-acting potent analgesic and its specific antagonist naloxone, were used. Our results evidenced in the animals a clinical syndrome similar to human diabetes. Long-term customary analysis revealed a significant ($p < 0.05$) increase of scratching and resting/sleeping behaviors, but diminished motor, eating and grooming customs. Moreover, the thermal tests revealed hyperalgesia in 43% of the animals, what may corroborate the meaning of scratching as a sign of pain. Pharmacological tests with morphine showed a significant ($p < 0.05$) inhibition of scratch, with concomitant increase of motor and eating activities and diminished rest/sleep capacity. Naloxone antagonized the effects induced by morphine. Such results suggest that these animals exhibit evoked behavior of hyperalgesia and that scratch may possibly be a spontaneous manifestation of chronic pain also in Wistar rats with this experimental model of painful diabetic neuropathy.

KEY WORDS: chronic pain, painful neuropathy, experimental diabetic neuropathy, spontaneous scratching behavior, morphine, naloxone, long-term profile.

Mudança do comportamento de ratos Wistar no modelo experimental de neuropatia diabética dolorosa

RESUMO – Com o objetivo de estudar dados sobre mudança de comportamento em ratos com neuropatia diabética dolorosa, induzimos diabetes em 28 ratos Wistar com estreptozotocina. Os animais foram então observados ao longo de 27 semanas com o propósito de caracterizar mudanças espontâneas em seus hábitos que pudessem sugerir sinais de dor crônica. Morfina como um potente analgésico de ação central e seu antagonista específico naloxona foram utilizados. Nossos resultados evidenciaram nos animais uma síndrome clínica semelhante ao diabetes humano (poliúria, catarata, perda de peso, neuropatia sensitivo-motora de predomínio distal). A análise comportamental revelou aumento dos comportamentos de coçar-se e descansar/dormir, e diminuição das atividades motoras e hábitos de comer e limpar-se. Além disso, os testes térmicos revelaram sinais de hiperalgesia em 43% desses animais, o que corrobora o significado de coçar-se como sinal de dor. Os testes farmacológicos com morfina evidenciaram inibição significativa ($p < 0,05$) no hábito de coçar-se, com aumento recíproco das atividades motoras e de alimentar-se, e diminuição do tempo de descansar/dormir. A naloxona antagonizou os efeitos da morfina. Tais resultados sugerem que esses animais exibiram comportamento evocado de hiperalgesia e que o hábito de coçar-se é possivelmente uma manifestação espontânea de dor crônica nesse modelo de neuropatia diabética dolorosa em ratos Wistar.

PALAVRAS-CHAVE: dor crônica, neuropatia dolorosa, neuropatia diabética experimental, comportamento espontâneo de coçar-se, morfina, naloxona, perfil a longo prazo.

Laboratory of Experimental Neurology (Department of Physiology and Pharmacology), Federal University of Ceará (UFC), Brazil: *MD, MsC; **MD, MsC, PhD; ***Student. Aceite: 22-junho-1999.

Dr. José Artur Costa D'Almeida – Laboratório de Neurologia Experimental, Departamento de Fisiologia e Farmacologia UFC – Rua Cel. Nunes de Melo 1127 – 60430-270 Fortaleza CE – Brasil. Fax: 55 85 2439333.

Among the several types of painful diabetic neuropathies, the sensory-motor symmetric polyneuropathy outstands for its frequency. In spite of the substantial progresses in the understanding of nociception and in the increase of the number of drugs for therapeutic purpose, the results of the treatment of those patients are still frustrating and unsatisfactory¹. This supports the need of developing further studies and research on painful diabetic neuropathy. However, the difficulties derived from the complexity of the human pain, and from the ethical limitations for experimental research in humans, lead to develop and use of animal models of diabetic painful neuropathy.

In a review of the literature, references on behavior quantification of chronic pain in diabetic rats are still lacking. Since the scratching behavior has been shown to be increased in animals with adjuvant arthritis²⁻⁴ and in rats with constrictive sciatic mononeuropathy⁵⁻⁷, we were interested in determining whether that behavior would be also increased in diabetic rats with neuropathy signs, evidencing, this way, a behavioral parameter of chronic pain in this model of experimental painful diabetic neuropathy.

METHOD

Thirty-six Wistar rats were used, ranging from 6 to 7 weeks of age, and weighing between 100 and 125g. The animals were kept in ambient temperature (25°C), with natural light during the day and access to the water and food ad libitum. All experiments, clinical and behavior observations were accomplished between 7:00 a.m. and 7:00 p.m.

Diabetes was induced through intraperitoneal administration of the drug Streptozotocin (Sigma Co., St. Louis, MO), and the glycemia was quantified with the use of ribbons in a glycometer. The animals that presented glycemia below 230mg% were excluded from the research.

Daily clinical exams were carried out to determine muscular atrophy, coloration and aspect of the coat, color of the eyes, presence of parasites, signs of local or widespread infection, vocalization, gait and autotomy signs. Besides these clinical observations, we used a stairway of three steps to evaluate muscular force and dexterity of the animals. To confirm the motor implication, the parameters of the Sciatic Functional Index were used⁸.

In the evaluation of the sensibility, the thermal test consisting in the immersion of each hindpaw in water with temperature of 40°C (non-noxious stimulus) and 46°C (noxious stimulus) was used, as described by Attal et al.⁹.

The experimental group (n= 28) was subdivided into subgroup 1 and subgroup 2. Subgroup 1 (n= 12) included animals of which hindpaw withdrawal latency to the thermal stimulus of 46°C was below 8 sec. (cut-off chosen according to the results of the control groups). Subgroup 2 (n= 16) included animals of which hindpaw withdrawal latency to the thermal stimulus of 46°C was the same or above 8 sec.

For behavioral observations, each animal was put in a wooden box measuring 100x50x50 cm, with a glass front wall to allow the observer to follow all behaviors of the animals. Then, each animal was observed during 30 min., and the behaviors of scratching, grooming, rearing, sniffing, resting/sleeping, eating and freezing were quantified with the help of a specific program in a IBM-PC type computer. The behavior observations began in the 12th week up to the 27th week of diabetes.

From the data of quantification of the scratching behavior in the experimental group, another subgroup, denominated Subgroup 3 (n= 20), was created. Subgroup 3 consisted of the animals that presented persistent increase of the scratching behavior above 12 sec. along the weeks of observation. As control group of these observations, we used 8 normal animals.

With the objective of evaluating the effect of a potent central-acting analgesic on the behaviors presented by the diabetic animals, we used morphine in the doses of 2 and 4 mg/kg, and its antagonist, naloxone, in the dosis of 1 mg/kg. Nine diabetic animals of the subgroup 3 were used as experimental group. The control group of these experiments was the same animals before the treatment with drugs.

We followed the ethical guidelines of the International Association for the Study of Pain – IASP, concerning the use of conscious animals in experimental pain¹⁰.

For the statistical analysis of the results, a computer Sigma Stat® program was used, with the following tests: “t” de Student test for normal population distribution and non-parametric tests (Mann-Whitney Rank Sum test and Wilcoxon test) for independent or related samples, respectively. Results were regarded as being significant when p values were lower than 0.05.

RESULTS

Starting from the 4th week of the disease up to 27th week of observations, we noted that the diabetic animals presented the following clinical alterations: yellowed coloration of the coat, increase of the abdominal volume, opaque crystalline lens and relative decrease of weight in relation to the controls. Spontaneous vocalization was not observed during the whole time of the disease evolution. In none of the animals, autotomy, paresis of a limb or isolated muscular group was observed. Most diabetic animals presented distal muscular atrophy, and some of them presented it more proximally. The parameters of the Sciatic Functional Index revealed distal motor damage ($p < 0.001$).

The thermal test with temperature of 40°C evidenced similar results in the diabetic animals and in the control group along 26 weeks of follow-up. In the thermal test with temperature of 46°C, two different subgroups were formed: The subgroup 1, (43% of the experimental group), presented hyperalgesia (hindpaw withdrawal latency below 8 sec.), and the subgroup 2, (57% of the experimental group) presented hypoalgesia (hindpaw withdrawal latency equal or above 8 sec.).

The subgroups 1 and 2 presented mean values of scratching behavior superior to the control group, with significant increased values ($p < 0.05$) in almost all the study. The analysis of the other behaviors revealed an increase in the time of resting/sleeping and a decrease of the motor (rearing, sniffing), eating and grooming behaviors. The behavioral changes evidenced in the diabetic rat model seemed to be persistent and progressive for at least 27 weeks (Fig 1).

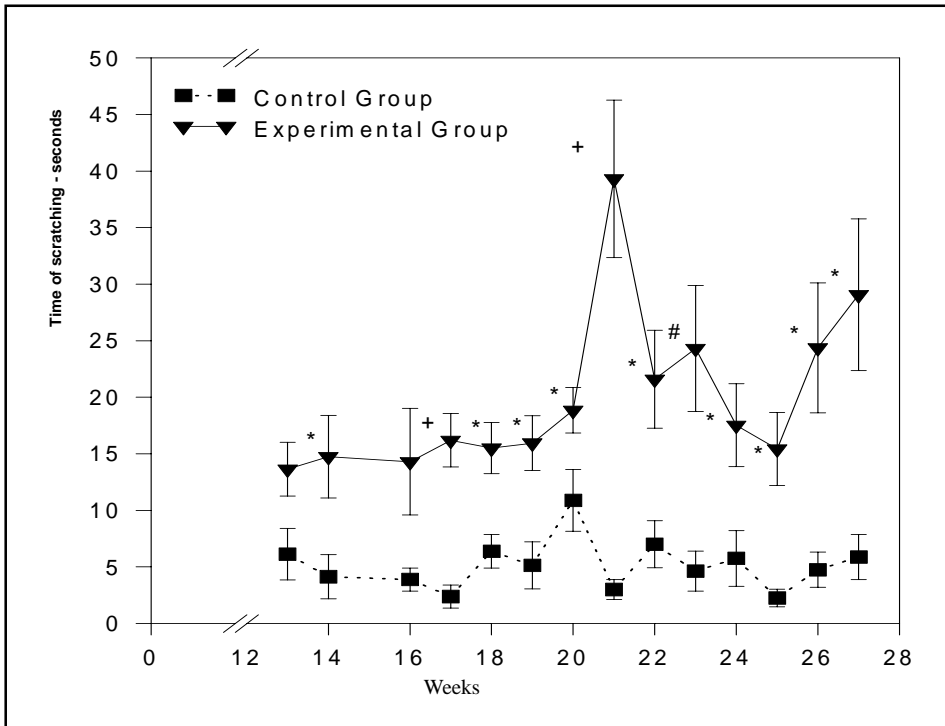


Fig 1. Time-course pattern of scratching in Wistar rats with experimentally-induced diabetic neuropathy between 12th and 27th week of diabetes. Control group: non-diabetic rats ($n = 8$). Subgroup 3: diabetic rats with time of scratching above 12 seconds in more than 4 consecutive occasions ($n = 20$). The values correspond to the averages, and to std. error of the average. + $p < 0.0001$; # $p < 0.01$; * $p < 0.05$ – test “t” and Mann-Whitney Rank Sum test.

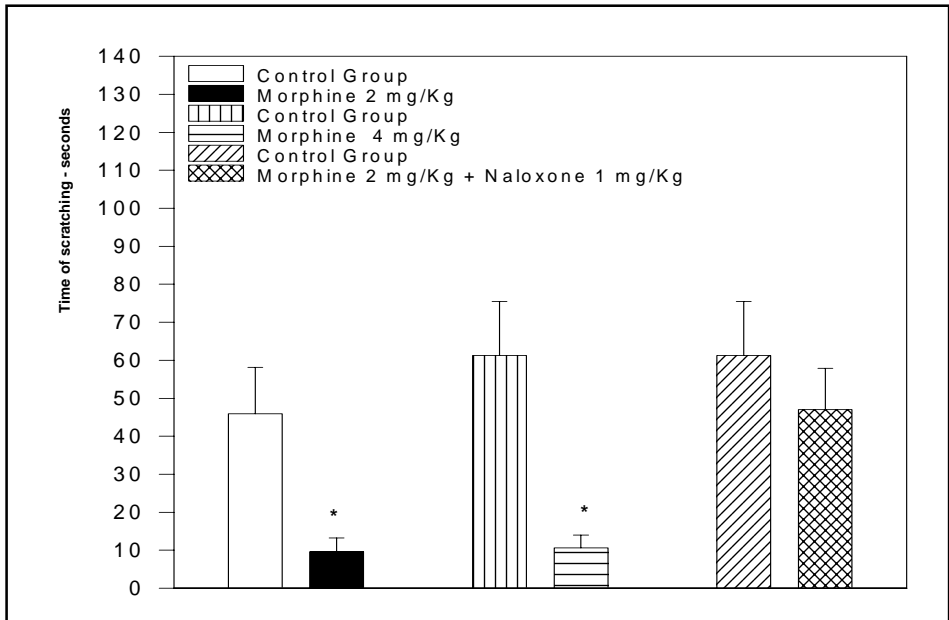


Fig 2. Influence of morphine treatment in the scratching behavior of diabetic rats in the 27th week. Morphine 2 and 4 mg/kg ($n=9$) and morphine 2 mg/kg plus naloxone 1 mg/kg ($n=9$) in diabetic rats in the 27th week. Values correspond to the average and std. error of the average. * $p<0.01$ – Wilcoxon Sign Rank test.

The time spent with the scratching behavior decreased significantly ($p<0.01$) after treatment with morphine in the doses of 2 and 4 mg/kg, when compared with the same animals before treatment. When we used morphine simultaneously in the dosage of 2 mg/kg and naloxone in the dosage of 1 mg/kg, there was not significant decrease of the scratching behavior in relation to the same animals before treatment (Fig 2). In relation to the other behaviors, the pharmacological tests with morphine showed an increase of the motor and alimentary activities, and a decrease of the rest. Naloxone antagonized the morphine effects.

DISCUSSION

The diabetic animals presented clinical alterations similar to the human diabetes, such as polyuria, progressive cataract, weight loss, slight to moderate muscular atrophy (mainly in the hindpaws), confirming it as a model of experimental diabetes.

The secondary motor changes, due to diabetic neuropathy in this model, were, then, slight to moderate. This offered some advantages, such as: (1) observation of the spontaneous behaviors, because the animal was not disabled for the daily life; (2) pain quantification, through motor evoked response; (3) animal self care, decreasing, in some way, the morbidity of the disease, and increasing the survival. The use of the parameters of the Sciatic Functional Index confirms these motor observations.

The diabetic animals did not present abnormal response in the thermal test with temperature of 40°C, which is a non-noxious stimulus. Courteix¹¹ did not also find allodynia in the thermal test of tail withdrawal to a stimulus of 40°C in rats with diabetes of 4 weeks, but they found allodynia to the stimulus of 42°C. Moreover, these studies of experimental neuropathic diabetes were time-limited, and no long-term study of this model has been described.

In our study, we clearly visualized two types of response of the diabetic animals to the noxious stimulus of 46°C. A subgroup of animals presented hindpaw withdrawal latency below 8 sec.,

evidencing this way hyperalgesia in relation to the normal animals. The other subgroup presented the same or higher latency to 8 sec., denoting hypoalgesia in relation to the normal animals. These data may substantiate the condition of painful diabetic neuropathy.

It is possible that the controversy in literature, relative to the hyperalgesia of diabetic rats, be due to the non-separation of the animals in these two subgroups. Courteix et al.¹¹ suggested separate the “responder” rats (animals that present variation larger than 10% in the scores of the pain tests in relation to the scores of those same tests accomplished before diabetes) from the “non-responder.” However, we cannot discard the possibility of these “non-responder” animals to have pain for the only fact they do not present an evoked behavior of pain. Such animals may have painful anesthesia, also seen in humans¹², where tactile or painful sensations are lost even in the presence of spontaneous pain. This may explain why some animals do not react to a noxious stimulus of 46°C, or other, although they feel spontaneous pain. We believed that this fact is one of the factors for the contradictory results found in the literature on the model of the diabetic rat. Moreover, evoked behaviors induced in the thermal tests are more prone to be influenced by stressful condition of test performing. In this sense, the quantification of spontaneous behaviors that suggest pain (as scratching or others, for example) seems more interesting because the observed animals are under less stressful conditions.

In relation to scratching behavior, the subgroup 1 (animals with time of latency below 8 sec in the thermal test) presented increase of the scratching behavior in a significant level ($p < 0.05$) during almost all the duration of the study. This correlated with the values of the thermal test suggestive of hyperalgesia. The subgroup 2 (animals with time of latency equal or above 8 sec in the thermal test) also presented increase of the scratching behavior, even so the results are not as important as in the subgroup 1. There is, therefore, indication of suggestive behavior of pain in the subgroup 2, in spite of the hypoalgesia evidenced to the thermal test.

The subgroup 3 (animals with time of scratching behavior above 12 sec in more than 4 consecutive occasions) showed increase of this behavior, which was significant ($p < 0.05$) in relation to the control group, what may possibly be related to pain.

The significant increase of scratching in the diabetic rats seems not to be related to stress for isolation in the observation cage, since the control rats were also submitted to the same conditions and did not express a significant increase of this behavior, as we can depict from Figure 1. Moreover, coincidental modifications in the curve between the experimental and control animals may perhaps occur due to external experimental noise, but these are random and not persistent and significant behavioral changes.

The decrease of the exploratory activity (rearing and sniffing) could be an indirect indicative of chronic pain. In the same way, the increase of the time spent in “resting/sleeping” as well as the decrease in “eating” could be another indication of chronic pain.

The scratching behavior decreased significantly ($p < 0.01$) with the use of morphine in the doses of 2 and 4 mg/kg. The treatment of the animals with morphine 2 mg/kg, and naloxone 1 mg/kg did not change the scratching behavior significantly, showing, this way, an analgesic effect of morphine on the animals with painful diabetic neuropathy. The decrease in resting/sleeping behavior and the non-interference on the motor behaviors indicate that there was not important sedative effect of morphine, moreover increase of the time spent in the eating behavior corroborates the analgesic effect of morphine. Possibly with the decrease in pain, the animals increased the interest in feeding. These results confirm the nociceptive meaning of the scratching behavior.

According to Kupers⁵, the induction of animal behavioral changes that can be quantified and affected by an analgesic, is a necessary request for an adequate animal model to the pain study. Moreover, it would be important that the changes induced by the analgesic drug should be reverted by a specific antagonist.

The model of the diabetic mouse has presented contradictory results, and it has evaluated only evoked behaviors. Moreover, most of the studies on the model were accomplished in short periods of disease. Yoon¹³ separated the neuropathic pain into two components: spontaneous and evoked pain. The distinction among those two components could help to elucidate the mechanisms of neuropathic pain. Courteix¹¹ did not find any clear change in the behaviors that could suggest spontaneous pain in the diabetic rat, but just evoked pain. Our results, based on changes in evoked behaviors as pain parameter, showed some aspects that agree with the findings of Courteix¹¹. However, in our study, we could use the quantification of the scratching spontaneous behavior as pain parameter, with the advantage of quantifying pain without observer's direct interference.

The scratching behavior, main parameter of our observations, has been used in several models of nociceptive and neuropathic chronic pain. According to Kupers et al.⁵, the behavior of scratching is the first pain parameter described to the moment that happens in many presumably experimental models of pain. Moreover, this behavior is easy to quantify, it is a very objective measure and it allows the use of conventional statistical analysis. This behavior has been evidenced in rats that received an intrathecal injection of substance P agonists¹⁴, as well as in rats that received direct intraventricular substance P¹⁵. Besides these evidences related to the substance P, an already well defined pain mediator, the behavior of scratching is also evoked by intrathecal injection of excitatory substances¹⁶, and by the administration of gamma-aminobutyric acid (GABA) receptor antagonists. On the other hand, the intrathecal administration of glycine and gamma-aminobutyric acid (GABA) receptor agonists attenuates or blocks the scratching behavior induced by substance P in rats¹⁷.

Another aspect to be discussed is the influence of induced diabetes on the hypothalamic-pituitary-adrenal axis, and the consequences on the pain behavioral repertoire of the experimental animals. The findings on this are controversial. Forman et al.¹⁸ found decreased levels of β -endorphins, centrally and peripherally, suggesting dysfunction of neuro-endocrine system in diabetes. They also think that this may contribute, partially, for the diminished capacity of tolerating pain in these diabetic animals; however, not only β -endorphins, and also enkephalins, substance P etc, may also be involved. Nevertheless, some authors have found diminished sensitivity to morphine action in diabetic animals¹⁹. Such decrease would be due to hyperglycemia and not due to changes in opioid receptors, since other opioid drugs do not have the same effect of morphine. Kamei et al.²⁰ suggested that the mechanism of mexiletine would be through an opioid receptor $\delta 1$, and Mao et al.²¹ suggested that the tolerance to morphine in neuropathic pain would be mediated by NMDA receptors.

Our results have then shown (1) a long-term clinical and behavioral profile of experimental diabetic neuropathy, (2) that the scratching behavior is increased in diabetic rats with painful neuropathy and (3) that this behavior may be used as possible spontaneous parameter of chronic pain in this animal model, for its decrease by morphine and antagonism by naloxone. Moreover, hyperalgesia found in the animals support the meaning of his behavior, as a pain symptom, so that it may possibly be useful as a parameter for further studies aiming at defining possible mechanisms of this painful neuropathic condition.

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