

Evaluation of the mutagenic potential in a mouse model of peripheral neuropathic pain**Avaliação do potencial mutagênico em um modelo de dor neuropática periférica em camundongos**

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ABSTRACT

Objective: This study to evaluate the mutagenic potential of a neuropathic pain model induced by partial compressive ligation of the sciatic nerve after 30 hours, 7, 14, 42 and 70 days post-lesion, through micronucleus test. **Methods:** Eighteen male adult mice were equally divided into negative control group (NC); positive control group (PC), who received 50 mg/kg of cyclophosphamide; and partial sciatic nerve ligation group (SNL). *In vivo* micronucleus test in peripheral blood sample was performed by counting 2,000 polychromatic erythrocytes; It was carried out in NC at first; PC was tested after 30h of cyclophosphamide administration; SNL groups were tested 30 hours (SNL30h), 7 (SNL7d), 14 (SNL14d), 42 (SNL42d) and 70 (SNL70d) days post-lesion. **Results:** Thirty hours after surgery, animals presented with an increased micronuclei frequency (5.89 ± 0.92), which slightly falls on the 7th post-lesion day. On the 14th day, however, the greatest mean of 8.11 ± 0.59 was attained, though another continuous decrease can be observed on both 42nd and 70th days post-injury. **Discussion:** Neuropathic pain induced by partial compressive ligation of the sciatic nerve is capable of triggering DNA damage, as suggested by the increase micronuclei incidence rates on the SNL groups.

Keywords: Neuropathy, Peripheral nerve injuries, Micronucleus test, Genomic instability, Pain, Sciatic nerve.

RESUMO

Objetivo: Este estudo para avaliar o potencial mutagênico de um modelo de dor neuropática induzida por ligadura compressiva parcial do nervo ciático após 30 horas, 7, 14, 42 e 70 dias de pós-lesão, através de teste de micronúcleo. **Métodos:** Dezoito ratos adultos masculinos foram divididos igualmente em grupo controle negativo (NC); grupo controle positivo (PC), que recebeu 50 mg/kg de ciclofosfamida; e grupo de ligação parcial do nervo ciático (SNL). O teste in vivo de micronúcleo em amostra de sangue periférico foi realizado contando 2.000 eritrócitos policromáticos; foi realizado em NC no início; PC foi testado após 30h de administração da ciclofosfamida; grupos SNL foram testados 30 horas (SNL30h), 7 (SNL7d), 14 (SNL14d), 42 (SNL42d) e 70 (SNL70d) dias pós-lesão. **Resultados:** Trinta horas após a cirurgia, os animais apresentaram um aumento da frequência de micronúcleos ($5,89 \pm 0,92$), que cai ligeiramente no 7º dia pós-lesão. No 14º dia, entretanto, a maior média de $8,11 \pm 0,59$ foi atingida, embora outra diminuição contínua possa ser observada tanto no 42º como no 70º dia pós-lesão. **Discussão:** A dor neuropática induzida pela ligadura compressiva parcial do nervo ciático é capaz de desencadear danos no DNA, como sugerido pelo aumento das taxas de incidência de micronúcleos nos grupos SNL.

Palavras-chave: Neuropatia, Lesões do nervo periférico, Teste do micronúcleo, Instabilidade genômica, Dor, Nervo ciático.

1 INTRODUCTION

Peripheral neuropathies affect 2% to 8% of world's population, being related to multiple etiological factors, e.g. trauma, diabetes mellitus, vasculitis and Human Immunodeficiency Virus (HIV) infection [1]. These disorders may trigger sensory abnormalities, such as allodynia, hyperalgesia, spontaneous activation of nociceptors and expansion of hypersensitive areas [2,3]. Among those dysfunctions, neuropathic pain remains as an important concern, as long as it negatively impacts a broad spectrum of physiological, mental and work-related aspects, besides increasing healthcare costs [4,5].

Regarding pathophysiological mechanisms of neuropathic pain, functional, chemical and structural changes of neurons have been described, such as high levels of inflammatory mediators, nerve growth factor (NGF) and reactive oxygen species (ROS) [6,7]. Overproduction of this latter factor leads to oxidative stress, capable of activating transcription factors, thus interfering in gene expression, besides increasing the likelihood of genomic damage and changes in transcriptional regulation of cell [8–11]. Those cytogenetic damages may trigger chromosome abnormalities in daughter cells throughout mitosis, being even a risk of neoplasia development [11].

A well-established method for assessing the effect of potential genotoxic agents is observing the frequency of micronuclei in cells [12]. Overall, micronuclei are chromosome fragments that are not

properly integrated into daughter cells' nuclei throughout mitosis, being a cytogenetic marker of chromosome breakage (clastogens) or loss (aneugens) [13,14].

Considering neuropathic pain as a possible stress-generator factor, the present study aims to evaluate the mutagenic potential of a neuropathic pain model induced by partial compressive ligation of the sciatic nerve after 30 hours, 7, 14, 42 and 70 days post-lesion, through micronucleus test.

2 MATERIALS AND METHODS

2.1 ANIMALS AND GROUPS

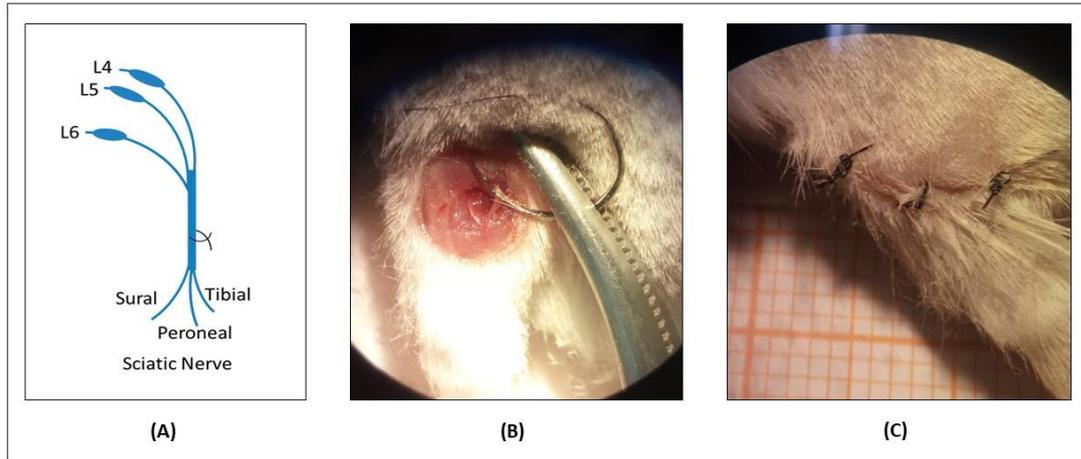
Eighteen male Swiss mice (*Mus musculus*) aged 9-11 weeks and weighing from 20 g to 30 g were housed in standard polyethylene boxes, under 12:12 light/dark cycle, in a temperature-controlled environment (22 °C and 60% humidity), where *ad libidum* access to food and water was provided.

The animals were assigned in three different groups (n=6 for each group): (I) Negative control group (NC), comprising naïve animals; (II) Positive control group (PC), whose animals were tested for verifying their reactivity to a substance with well-known mutagenic properties, by receiving 50 mg/kg of cyclophosphamide, a cytotoxic agent that induces micronucleus formation, administered by intraperitoneal injection [15,16,17]; and (III) Partial sciatic nerve ligation (SNL) group, which was tested 30 hours (SNL30h), 7 days (SNL7d), 14 days (SNL14d), 42 days (SNL42d) and 70 days (SNL70d) post-lesion.

2.2 NEUROPATHIC PAIN MODEL

The neuropathic pain model is based on a partial unilateral nerve ligation [18] (Figure 1A). Once the anaesthesia was performed with a mixture of ketamine (60 mg/kg) and xylazine (8 mg/kg), a tight ligature with a 8-0 silk non-absorbable suture around approximately half of the left sciatic nerve diameter (Figure 1B) was carried out. Skin incisions were closed with the same suture material (Figure 1C).

Figure 1. (A) Experimental model of peripheral neuropathic pain; (B) Sciatic nerve being exposed for partial ligation; (C) Closure of skin incision with silk non-absorbable suture after surgical procedure.



Source: Data from the own researchers, 2017.

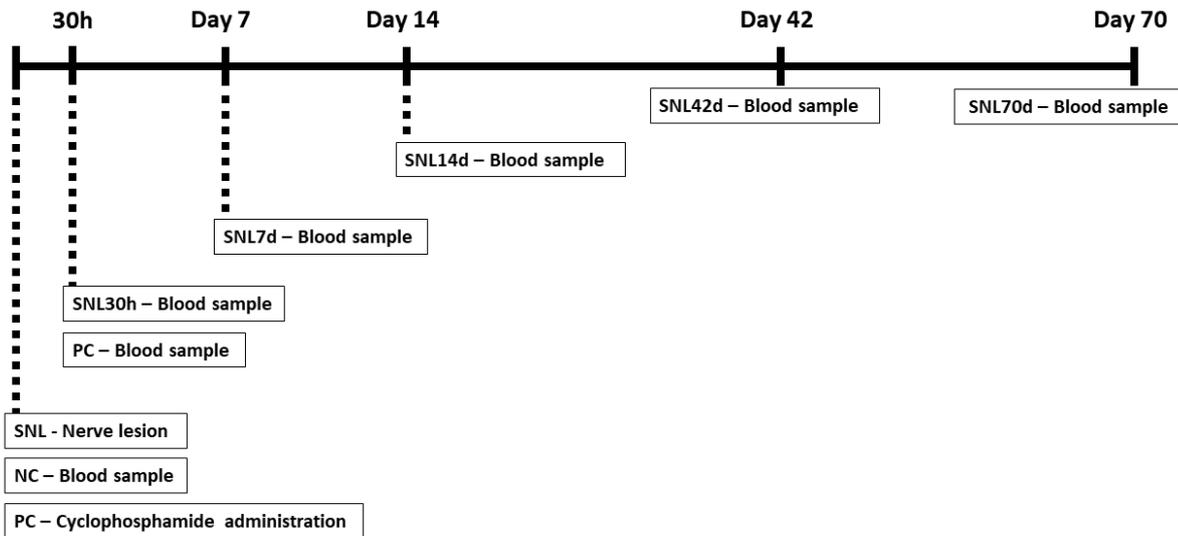
2.3 IN VIVO MICRONUCLEUS TEST

The *in vivo* micronucleus test in peripheral blood was performed in accordance to the protocol described by Hayashi et al ⁽¹⁹⁹⁴⁾ [19].

Blood samples collection schedule was carried out as shown in Figure 2. It occurred through distal minimal section of the mouse's tail. The blood was dripped in small amounts (~5 μ L) on dry slides, in which the sample was smeared. The smears were air-dried for 24 hours, then the fixation was performed by dipping them into 100% methanol for 10 minutes and dried again. After that, the slides were stained with Giemsa diluted in phosphate buffer solution, pH 6.8, in a 1:10 proportion, for 15 minutes. Excess dye was carefully washed out with distilled water.

The analysis was performed under light microscopy by two independent and blinded researchers, who assessed the frequency of micronuclei in blood cells by counting 2,000 polychromatic erythrocytes.

Figure 2. Peripheral blood sample collection schedule.



Source: Data from the own researchers, 2017.

2.4 STATISTICAL ANALYSIS

Data analysis was conducted through IBM Statistical Package for Social Sciences (SPSS) software, version 22. Data were presented as mean \pm standard error of the mean.

All variables were tested for normality through Shapiro-Wilk test, followed by either Student's t-test or Mann-Whitney U test, comparing each post-injury time interval to NC group. A significant level of $p < 0.05$ was established.

2.5 ETHICS

This study was approved by the Ethics Committee for Animal Experimentation of Biological and Health Sciences Center of Paraíba State University, Brazil (protocol number: 5102042015), and all experimental procedures were carried out following the ethical guidelines of Brazilian College of Animal Experimentation.

3 RESULTS

All post-lesion intervals exhibited a statistically significant ($p < 0.05$) higher micronuclei frequency when compared to NC group.

Thirty hours after surgery, animals presented with an increased micronuclei frequency (5.89 ± 0.92), which slightly falls on the 7th post-lesion day. On the 14th day, however, the greatest mean of 8.11 ± 0.59 was attained, though another continuous decrease can be observed on both 42nd and 70th days post-injury. These results are demonstrated in Table 1.

Table 1. Frequency of micronuclei by each post-surgery interval.

Group	Mean	SE	p-value
NC	2.00	0.26	
PC	18.33	0.21	0.002*†
SNL30h	5.89	0.92	0.012*†
SNL7d	4.22	0.57	0.002*†
SNL14d	8.11	0.59	0.000*
SNL42d	5.67	0.23	0.000*†
SNL70d	5.43	0.20	0.001*†

NC = Negative control group; PC = Positive control group; SNL30h = Partial sciatic nerve ligation after 30h of surgery; SNL7d = Partial sciatic nerve ligation after 7 days of surgery; SNL14d = Partial sciatic nerve ligation after 14 days of surgery; SNL42d = Partial sciatic nerve ligation after 42 days of surgery; SNL70d = Partial sciatic nerve ligation after 70 days of surgery; SE = Standard error; p-value: significance level for either Student's t-test or Mann-Whitney U test comparing each group with NC; *Significance level < 0.05; †Mann-Whitney U test.

4 DISCUSSION

The present study provided a novel approach on the study of neuropathic pain through the prism of mutagenesis, with evidence that a neuropathic pain model induced by partial compressive ligation of the sciatic nerve is capable of triggering cytogenetic damage, as suggested by the micronuclei frequency increments on the SNL groups.

A previous investigation regarding the effect of SNL on pain-like behavior demonstrated that this model may result in long-lasting mechanical allodynia and thermal hyperalgesia, besides mechanical hypersensitivity from the 7th to the 70th day after injury [20].

Neuropathic pain is also associated with profound inflammatory responses, which may play an important role on the persistence of nociceptive symptoms [21]. This complex process is mediated by several chemical mediators, such as vasoactive amines, prostaglandins, cytokines (e.g. interleukin 1- β (IL-1), interleukin 6, tumor necrosis factor α (TNF- α), which sensitize and stimulate nociceptor, thus promoting long-term deleterious plastic alterations [21–23].

Apart from the uncomfortable effects generated by the inflammatory response, it is worth recognizing that it plays a significant role on enabling nerve repair [21]. To illustrate this, nerve functional recovery depends on TNF- α and IL-1 expression [24], and the release of chemical mediators triggers the secretion of growth factors, thus contributing on nerve regenerating process [25,26].

Overall stress provoked by neuropathic pain and its concurrent inflammatory response has been linked to a large number of persisting adaptations at both cellular and molecular levels [27]. Among these, we highlight the occurrence of profound gene expression changes, though the underlying mechanisms by which these alterations are induced remain poorly understood [27,28]. Some studies, however, suggest that the inflammatory response is one of the main factors responsible for such DNA

damage [21,29], while other authors have demonstrated that even neuronal activity can be enough to induce highly toxic DNA lesion, which may alter the expression of late response genes [30].

Considering DNA as an extremely vulnerable structure to endogenous and environmental sources of damage [31], our findings reinforce the hypothesis that neuropathic pain may be a powerful source of stress, capable of triggering micronuclei formation. We observed that all post-injury groups presented with increases in frequency of micronuclei, with a statistically significant difference when compared to NC group.

The first post-surgery period examined was 30h, which corresponds to the first mitotic cycle after lesion. The visible increment in micronuclei formation at this period can possibly be associated with the post-surgical inflammatory responses themselves rather than with neuropathic pain, as this symptom may not be completely established at such point.

On the 7th post-lesion day, the first signs of mechanical hypersensitivity to normally innocuous stimuli emerge [20], resulting in alterations in micronuclei frequency as well. The mean frequency was though smaller than SNL30h, thus suggesting that the immediate surgical stress and inflammatory responses may have a more substantial impact when compared to the first signs of neuropathic pain.

The SNL14d group presented the highest micronuclei incidence rate among the analyzed timepoints. Our previous study indicate that, at such timepoint, the first signs of nerve degeneration become microscopically visible,³² and this finding may be associated with peripheral late immune response, such as the upregulation of IL-13, accumulation of macrophages [33] and T-cells infiltration on dorsal root ganglia [21].

A downward trend in micronuclei incidence rate can be observed after 14 days, though SNL42d and SNL70d groups present with a quite stable result. This finding seems to be in consonance with our previous results regarding pain-like behavior in animals submitted to partial SNL, which demonstrated no significant difference in mechanical allodynia and thermal hyperalgesia induced by nerve injury from the 42nd to the 70th post-injury days [20].

Taken together, our data points to the need for careful neuropathic pain and inflammation management therapies following peripheral nerve injuries, as the chromosomal instability culminating in micronuclei formation induced by these endogenous and exogenous factors may play a role in the emergence of malignant cells in general [34]. Nevertheless, we strongly recommend that future studies consider a larger follow-up period after SNL in order to ensure whether micronuclei incidence rates will normalize after some time or not.

In summary, our study suggests that neuropathic pain induced by partial compressive ligation of the sciatic nerve is capable of triggering DNA damage, as suggested by the increase micronuclei incidence rates on the SNL groups.

5 DISCLOSURE OF INTEREST

No potential conflict of interest was reported by the authors.

6 FUNDING

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