Neurotoxicity evaluation of meloxicam in the alternative *in vivo* model, *Caenorhabditis elegans*

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Abstract

Inflammatory processes cause changes in the permeability of the blood brain barrier. Non-steroidal anti-inflammatory drugs (NSAID) are most commonly used to treat these inflammatory processes, including meloxicam, and they can reach the central nervous system (CNS) and cause neurotoxicity. Since there are no studies evaluating the neurotoxicity of NSAID in alternative models of toxicity, the aim of this study was to evaluate the acute neurotoxicity (through nematodes changes in behavior) of meloxicam in an alternative in vivo model, Caenorhabditis elegans, as well as, to determine meloxicam toxicity through LD_{50} and development assessments. Meloxicam LD_{50} was high (50.03 mg/mL) and only the highest dose (100 mg/mL) caused a decrease in the nematode body size, indicating low toxicity in this alternative model. Besides, a neurological effect was observed only in the highest dose. Meloxicam showed neurotoxicity only at a very high dose, suggesting low potential to cause toxicity in the CNS. However, further studies are necessary to evaluate meloxicam neurotoxicity.

Keywords: *C. elegans;* neurotoxicity; meloxicam; NSAID.

1. INTRODUCTION

Inflammation is a defense response of the organism to an infection or tissue damage and it might cause changes in the permeability of the blood brain barrier [1]. Non-steroids anti-inflammatory drugs are most commonly used to treat these inflammatory processes, including meloxicam [2,3]. Thus, anti-inflammatory drugs can reach the central nervous system (CNS) and cause neurotoxicity. However, the literature lacks

studies evaluating the neurotoxicity of these drugs in an alternative model of toxicity.

According to the Interagency Committee on Neurotoxicology (ICON), neurotoxicity comprehends a broad concept, which includes adverse effects on the structure or function of the central or peripheral nervous system, caused by biological, chemical or physical agents. Neurotoxic effects can be permanent or reversible, resulting in direct or indirect action in the nervous system. Then, the nervous system represents a challenge to the development of risk assessment strategies of the neurotoxic effects in view of the complexity of the mechanisms involved in their triggering [4].

Caenorhabditis elegans is an alternative model for assessing neurotoxicity, since these nematodes do not have blood brain barrier, becoming a good choice to assess the toxicity of drugs that arrives in the CNS [5,6]. Furthermore, they have 302 neurons representing 118 characterized neuronal subtypes, providing an *in vivo* model for studying mechanisms of neuronal injury with resolution of single neurons [7]. In addition, it presents strong genetic homology with mammals, being possible to evaluate drugs effects and mechanism of action with the use of this model [8].

*C. elega*ns is an advantageous model organism to be used as a biosensor, since it has a sensorial and response system against xenobiotic compounds, which facilitates the detection and evaluation of toxic compounds, discovery of new molecules that can reduce or neutralize toxic compounds, besides of evaluating compounds that improve health or increase longevity [9,10].

Regarding the above, the aim of this study was to evaluate the acute neurotoxicity of the meloxicam in an alternative *in vivo* model, *Caenorhabditis elegans*. Also, to determine the toxicity of the NSAID through of LD_{50} and development assessments.

2. MATERIALS AND METHODS

2.1 Caenorhabditis elegans strain

The N2 wild type *C. elegans* strain was obtained from the Caenorhabditis Genetics Center (CGC) and was maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 at 20 °C.

2.2 Synchronization and treatment

The nematodes were synchronized according to Ávila et al. [8], the pregnant nematodes were treated with a solution of 0.25 M NaOH and 1% NaClO to break the cuticles thereof, and the eggs were separated by flotation with 30% sucrose solution. The eggs were stored in an incubator at 20 °C and after 14 hours the animals were obtained in the L1 stage, in which experiments were performed.

Meloxicam solution (100 mg/mL) was prepared in DMSO. 2,500 nematodes were treated with 5 different concentrations of meloxicam (10 to 100 mg/mL) in liquid medium by constant agitation in a rotator for 1 hour at 20 °C, after which the nematodes were placed in NGM medium seeded with *E. coli* OP50. 24 hours later, the scoring of survival nematodes per treatment was evaluated and it was compared with the control group treated with DMSO. All concentrations were tested in five independent experiments in replicates within each experiment.

2.3 ***Determination of lethal dose 50% (LD_{50})***

After exposure to meloxicam, the worms were placed on a new NGM/OP50 plate. For the survival assays, they were counted in stereomicroscope and compared to the control group (DMSO) in order to plot a survival curve and calculate the LD_{50} [11].

2.4 Development

Development was assessed by sampling. After reaching the adult stage, 20 nematodes per group were evaluated by measuring the body surface area. This procedure was performed through photos acquired in the stereomicroscope coupled with a camera. Subsequently, the measurement of the surface area was performed in ImageJ software [12,13].

2.5 Behavioral test

The behavioral evaluation assessed the motor activity of the worms and the sensorial activity. The nematodes were transferred to NGM plates without OP50 and stood for 1 minute to get used to the environment. Subsequently, the number of times with which the animal moves its head up was evaluated for 1 minute. Data were always compared to the control group and the experiment was repeated at least five times [14].

2.6 Statistical analysis

One-way ANOVA followed by Tukey was performed for the evaluation of *C. elegans* body area and head-thrashes frequency. The lethal dose 50% was determined by the log dose–response curve. $P < 0.05$ was considered as significant. Statistical analysis was performed at Graph Pad Prism 5.0.

3. RESULTS AND DISCUSSION

Meloxicam demonstrated a high LD_{50}, since the dose increase resulted in an increase in mortality rate of the nematodes. Figure 1 shows the percentage of survival versus logarithmic dose of meloxicam. The LD_{50} for meloxicam was 52.51 mg/mL. All the tested doses were compared to the control group, which did not receive the treatment.

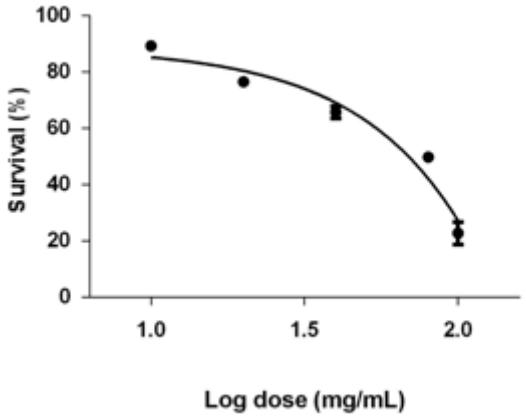

Figure 1. Log dose-response curve for lethal dose 50% determination of meloxicam after acute treatment.

Data are expressed as mean ± S.D (n=5).

It was verified that the LD_{50} was half the highest tested dose, suggesting the low toxicity of meloxicam. According to Ura *et al*. [15], the LD_{50} is only one of the parameters to be considered in toxicological tests, since the mortality rate shows only the acute effect [16]. Then, development may be more sensitive than the mortality rate and it is necessary to consider other aspects such as growth and movement [15].

The size of the nematodes after treatment with meloxicam, assessed by the measurement of surface area of the worms, showed that only the higher dose caused a decrease in body size compared to the control group (Figure 2).

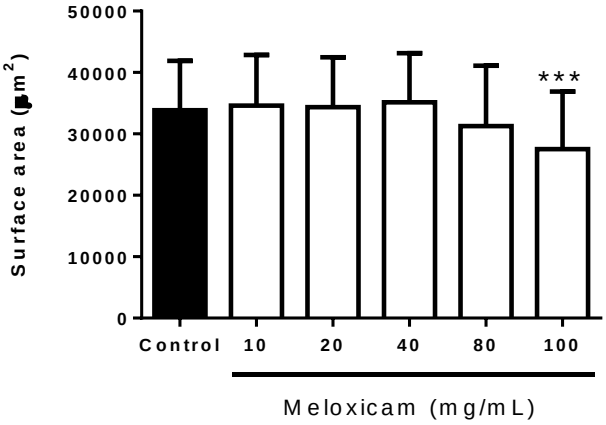

Figure 2. Body area of *Caenorhabditis elegans* after acute treatment with meloxicam in different concentrations. ANOVA post hoc Tukey. Data are expressed as mean + S.D. $F(5, 354) = 6.860$, $P < 0.0001$. Different from Control ***$P < 0.001$ (n=5).

Since *C. elegans* growth is determined by a conservative genetic regulatory pathway, this endpoint test is a good parameter to evaluate toxic effects [17,11]. Jiang et al. [18] conducted an experimental study with *C. elegans* to verify toxicity endpoints of heavy metals, and evaluated the growth, as a physiological endpoint. They demonstrated that this evaluation has high sensitivity and it could be a good parameter in toxicological studies in *C. elegans*. In the present study, we observed that only the highest dose caused a decrease in the nematode development. Regarding that the effect of a toxicant in the development of the nematode can be evaluated by measuring the body length or surface area of synchronous worms [12,19,20], the results suggest that meloxicam presented low toxicity. Jacques and Avila [21] also used this endpoint to assess toxicity of the commercial compound glyphosate. They observed that the worms' exposure to this compound caused significant changes in brood size and worm body length. Moreover, Charão et al. [13] evaluated the development of nematodes as a toxicity endpoint in *C. elegans* through surface area measurement and demonstrated the low toxicity of lipid core nanocapsules.

Moreover, the acute exposure to 100 mg/mL of meloxicam decreased *C. elegans* basic movements (Figure 3), suggesting neuronal damage.

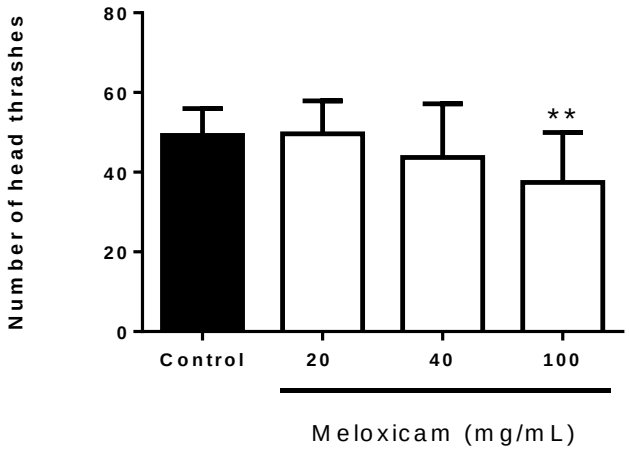

Figure 3. *C. elegans* head-thrashes frequency. ANOVA post hoc Tukey. Data are expressed as mean + S.D. $F(3, 83) = 6.198$, $P < 0.0007$ (n=5). Different from Control $^{**}P < 0.01$.

Jiang et al. [18] evaluated the behavior through body bends and head thrash frequencies. They demonstrated that there is a great concentration response between the parameters evaluated and the four metals tested in *C. elegans* and the determination of behavioral and physiological tests (as growth evaluation) presented similar results in terms of toxicity endpoint. The same was observed in our study, where only in high doses of meloxicam it was observed a decrease in *C. elegans* head thrashes, the same doses that presented reduction in growth. According to Yu et al. [21] effects on the locomotion of nematodes have been linked to a deterioration of the neural network, which can be evaluated based on several criteria, such as head thrash, body bend frequency and basic movements, suggesting neuronal damage caused by meloxicam at 100 mg/mL. Furthermore, a defect in locomotion reflects an impairment of the neuronal network formed by the interneurons AVA, AVB, AVD, and PVC providing input to the A and B-type motor neurons (responsible for forward and backward movement) and the inhibitory D-type motor neurons involved in the coordination of movement [22].

Considering that the inflammatory process can cause alterations in the permeability of the blood brain barrier [1], the present study evaluated for the first time the neurotoxicity of the anti-inflammatory meloxicam in an alternative *in vivo* model of toxicity, using the nematode *C. elegans*. The use of alternative methods is an important aspect in the toxicity study [19] and *C. elegans* presents many advantages, such as oral absorption of drug administration in worms, as demonstrated by Charão et al. [13], who evaluated oral absorption and potential toxicity of biodegradable nanocapsules in the same alternative model. In addition, *C. elegans* is well suited for neurophysiology of neurotoxicity evaluation [5,6]. According to the results obtained it is possible to infer that meloxicam presents low toxicity in the *C. elegans* model. In addition, meloxicam demonstrated low potential to cause toxicity in the Central Nervous System in the nematode.

4. ACKNOWLEDGEMENTS

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5. CONFLICTS OF INTEREST

The authors declare that there is not any conflict of interest.

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