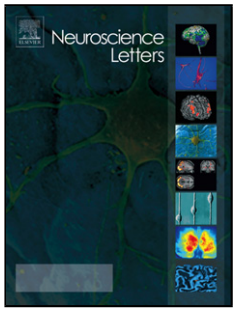

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Lidocaine protects neurons of the spinal cord in an excitotoxicity model

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Highlights

- Lidocaine prevents spinal cord neuronal damage of Kainic acid injected rats
- Lidocaine improves motor and sensitive performance of Kainic acid injected rats
- Lidocaine could be considered as a neuroprotective drug

Abstract

Motor Neuron Disease disorders, described in domestic animals, are characterized by neuronal degeneration at the spinal cord. Excitotoxicity is a crucial factor for the selective loss of these neurons, being the fundamental processes involved in lesion progression after spinal cord injury, where glutamate is one of the main neurotransmitters involved. Kainic acid (KA) resembles the effects induced by the pathological release of glutamate. Lidocaine administered by different routes exerts some neuroprotective effects in the CNS. The aim of the present work was to determine whether lidocaine simultaneously injected with KA into the spinal cord could prevent the excitotoxic effects of the latter. Sprague-Dawley rats were injected by intraparenchymal route with KA or with KA plus 0.5% lidocaine into the C5 segment. *Sham* rats were injected with saline. Animals were motor and sensory tested at 0, 1, 2, 3, 7 and 14 post-injection days and then euthanized. Sections of the C5 segment were used for histological and immunohistochemical analysis. No KA-induced motor and sensitive impairments were observed when lidocaine was simultaneously injected with KA. Moreover, neuronal counting was statistically higher when compared with KA-injected animals. Thus, lidocaine could be considered as a neuroprotective drug in diseases and models involving excitotoxicity.

Keywords

Excitotoxicity; Kainic acid; Neuroprotection; Lidocaine; Spinal cord injury.

1. Introduction

Motor neuron disease (MND) is an entity that encompasses neurodegenerative disorders of α or lower motor neurons in the spinal cord of domestic animals, being excitotoxicity a crucial factor for the selective loss of these neurons [1]. Furthermore, excitotoxicity is one of the fundamental processes involved in lesion progression after spinal cord injury (SCI) being glutamate one of the main neurotransmitters involved [2; 3].

Neurons are more vulnerable to excitotoxicity than glial cells [4]. Within neuronal population dorsal and ventral horn neurons show kainate receptors [5] and can react to KA action with different susceptibility, which may depend on the differential expression of calcium binding proteins and enzymatic system to counteract the deleterious effect of KA or glutamate [6].

Several neurodegenerative disorders were predominantly described in dogs, cats, cows, horses and pigs [7]. They were characterized by neuronal body and axonal degeneration at the spinal cord [1;7]. Pathogenesis of MND might be explained by the great amount of calcium permeable AMPA/kainate receptors in these neurons, which would lead to mitochondrial damage and free radical production. Accumulation of these reactive oxygen species would damage the surrounding astrocytes and reduce the uptake of glutamate by these cells [2;3]. As a result, the extracellular concentration of glutamate would markedly increase and exacerbate the process [1].

Kainic acid (KA), a cyclic analogue of glutamate, induces damage that resembles the effects induced by the pathological release of glutamate. An excitotoxic SCI model that shares similarities with the previously mentioned MND was described [8]. It is based on

a transient excitotoxic stress as the trigger mechanism, followed by secondary phase of damage, mainly induced by the release of glutamate from the affected cells [9].

Local anesthetics are used for spinal, epidural and local anesthesia as well as peripheral nerve block [10]. Also, i*n vitro* and *in vivo* studies have shown that lidocaine is protective against hypoxia and neuronal injury induced by ischemia [11;12].

Although the intrathecal route is usually used to treat insults occurring at the spinal cord, the local drug delivery via intraparenchymal infusions has gained increased consideration for the treatment of some neurodegenerative disorders [13] since it will more precisely determine the effect of the drugs in direct contact with the structures in which their activities are to be determined [14].

The aim of the present work was to determine the effects of a simultaneous injection of lidocaine and KA to analyze whether lidocaine can block the KA induced harmful effects. Here, our hypothesis states that co-administration of lidocaine with KA would block or at least restrict the damage produced by the excitotoxic drug.

All data collected using this approach could serve as a starting point for supporting subsequent therapeutic protocols for the treatment of MND.

2. Materials and methods

2.1. Animals

Young (3-5-month-old, 250-450 g) male Sprague-Dawley rats (n = 90) (**Table 1**), raised in our rat colony, were used. Animals were housed in a temperature-controlled room (22 ± 2°C) on a 12:12 h light/dark cycle. Food and water were available *ad libitum*. All experiments with animals were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH Publications No. 80-23, revised in 1996). The protocol was approved by the

School of Veterinary Sciences, National University of La Plata Institutional Committee for Care and Use of Laboratory Animals (CICUAL), Code 45-1-14 T. All efforts were made to minimize the number of animals used and their suffering.

2.2. Handling of animals and drugs injection

On experimental day 0, rats (**Table 1**) were anesthetized with ketamine hydrochloride (40 mg/kg; i.p.) plus xylazine (8 mg/kg; i.m.) and placed in prone position. Then intraparenchymal injections were performed [15]. Thus, 5 microliters either of the 1 mM KA (KA1 group) [8], 0.5% lidocaine (L05 group) [16], lidocaine + KA (KA1L05) or 0.9% saline (*sham*) were discharged at a rate of 1 µl/min, at the C5 spinal cord segment right side (ipsilateral). Animals were checked periodically until they woke up. No abnormal respiratory frequency nor respiratory noises were detected. In no case, animals required manual emptying of the bladder.

Immediately before surgery both lidocaine (Xylocaine® 2%, AstraZeneca, Haedo, Buenos Aires, Argentina) and KA (Sigma-Aldrich, Inc., St. Louis, MO, USA) were diluted in 0.9% saline solution and kept at 4°C until use.

2.3. High-performance liquid chromatography (HPLC) study

To determine whether there exists chemical interaction between lidocaine and KA that might interfere with the effects of each drug, a HPLC analysis was performed at the established concentrations. The mix solution of KA1 and L05 was compared with 0.5% lidocaine and 1 mM KA. All of them were diluted in saline solution (0.9%), used as vehicle.

Each sample was analyzed using a HPLC device (HPLC-MS Agilent 1100 LC-MSD) with a C-18 column (5 µm particle size, 4.6 x 150 mm), equipped with a mass spectrometer single quadrupole. To ionize samples, ESI/APCI source was used in

positive and negative mode. An isocratic mobile phase mixture of acetonitrile and 0.5% aqueous formic acid, 70:30 were used.

Each analyte was eluted individually and unaltered, which confirm that lidocaine and KA do not interact at the selected concentrations (data no shown).

2.4. Behavioral tests

Behavioral tests were performed the same day, with an interval of 20 minutes between them. All rats were submitted to a set of motor and sensitive tests at the beginning of the experiment (day 0), and at each analyzed time point (days 1, 2, 3, 7, and 14 post-injection - pi) until euthanasia (**Table 1**).

2.4.1. *Heat sensitivity test*

The hot-plate test [17-19] was carried out using a 15 cm diameter hot-plate, set at 55 ± 1°C. The time (measured in sec) between placement (time zero) and licking of their forepaws or jumping (whichever occurred first) was considered as the response latency. A 20 sec cut-off was used to prevent tissue damage of non-sensitive animals. Three measures at 2-min-intervals were taken per session.

2.4.2. Suspension from a horizontal wire mesh pole

The time during which rats could sustain their own weight was determined by placing the animals on a horizontal wire mesh pole [20]. The latency taken by the animals to fall was recorded as the average of three consecutive attempts per sessions.

2.4.3. Ladder rung walking test

This test was used to examine forelimb coordination during skilled walking [8, 21]. Scoring system ranging from 0 (abnormal) to 6 (perfect) was used [21]. Only scores between 0 and 2 were considered as error [22]. Ipsilateral and contralateral limbs were evaluated separately. Errors/no-errors percent of the total number of steps in each trial was calculated. Trials were recorded, and videos analyzed frame-by-frame to classify all the steps per group.

2.5. Euthanasia, specimen collection and processing

Animals of all groups were euthanized either at 1, 2, 3, 7 or 14 pi days (**Table 1**). Immediately before euthanasia rats were anesthesized with ketamine hydrochloride (40 mg/kg, i.p.) plus xylazine (8 mg/kg; i.m.) and then intracardially perfused with PBS followed by PBS-4% paraformaldehyde solution. Tissue processing was done as previously described [8].

2.6. Immunohistochemistry

Immunohistochemistry of C5 sections for neuronal cell counting and astrocytic glial determination was performed as described elsewhere [23]. Anti-NeuN monoclonal antibody (1:200, Millipore, CA, USA) for identification of neurons, or anti-GFAP polyclonal anti-rabbit antibody (Dako Denmark, ready to use) to identify astrocytes were used. The EnVision detection system + HRP system labelled anti-mouse or anti-rabbit (DakoCytomation) was applied during 45 min. Sections were then rinsed threefold in PBS-T. Liquid 3,3-diaminobenzidine tetra-hydrochloride (DAB) (Vector Laboratories Inc., CA, USA) was used as chromogen and Hill's hematoxylin was used for counterstaining. Control negative sections were prepared by omitting primary antibody.

2.7. Image analysis

Images of immunohistochemically stained C5 sections were captured using a digital camera (Olympus DP73, Japan) attached to a microscope (Olympus BX53, Japan). To create a complete map of the entire C5 segment taken with a 40x objective, images were captured using a digital image analyzer (cellSens Dimension, V1.7, Olympus Corporation, Japan) and stitched them using an automatic Multiple Image Alignment process. No further processing was necessary after obtaining the original images.

2.7.1. Morphometry

For determining variations in the area of both ipsilateral and contralateral sides in coronal-sectioned segments, possibly induced by the injected solutions, morphometric analysis was performed using an image analysis software (ImagePro Plus -v6.3, Media Cybernetics, MA, USA). For that purpose, each side of the spinal cord segment was delimited using a ROI (region of interest) and the actual area determined. In each group, the ipsilateral areas were statistically tested against their contralateral areas and against the same side areas in all the experimental groups.

For astrocytes estimation as an indicator of inflammatory process, GFAP immunostained area was considered. For that purpose, color segmentation to detect positively stained cells inside a ROI (as previously defined) was applied and its area was calculated [16, 24]. In all cases, estimation of immunostaining was carried out in sections located up to 500 μm in front of and behind the injection site.

2.7.2. Neuronal counting

To determine the total number of neurons per section, color segmentation was performed. Double-blind observations were done by two independent morphologists.

Immunohistochemically stained images were used to estimate the number of cells present per segment using the following formula [25]:

$$N = \frac{d}{ns} \sum_{i=1}^{n} x_i$$

where, N = total estimated number of cellular bodies; d = length (µm) of the rostro-caudal axis of the cervical segment being assessed (2 mm); n = number of non-contiguous (120 µm apart) slices counted per cervical segment (n = 5); s = thickness of the section (20 µm); x = number of perikarya counted per non-contiguous slice assessed. Therefore, N represents approximately the total number of neurons present in the cervical segment.

8. Statistical analysis

The variables: (A) heat sensitivity test and (B) suspension from a horizontal wire mesh pole were analyzed as repeated measures on the same animal at different times [26]. All models were fitted with SAS software for mixed models (Proc Mixed and Proc Glimmix, SAS v.9.3, USA). Differences between treatments effects at any given day pi were estimated as linear contrasts [27], whenever the test for treatments with degrees of freedom corrected by the procedure of Kenward and Roger [28], deemed the corresponding test to be significant ($P < 0.05$). Therefore, **Figure 1A-B** depict the estimated evolution of the treatment mean response across times for the corresponding response variable.

The analysis of the ladder rung walking test was analyzed using procedures reviewed by Fletcher et al. [29], because one third of the data were measured as zeros. In the first stage, the test was viewed as a Bernoulli variable by coding the non-zero values as 1,

and the zeros were kept as such. A generalized mixed model was fitted to the Binomial response variable with a logit link function. The only fixed effect in the model was treatment and the error covariance structure displayed heteroskedasticity due to day nested within treatment. The second stage considered only the non-zero values of the test. The zeros were taken as missing data [29]. The Gaussian model had treatments as fixed effects and the error covariance structure reflected heteroskedasticity due to treatment effects. The heterogeneity of variance motivated a change in the level of significance of the corrected *F*-test for this variable from 0.05 to 0.10, because differences found between treatments were biologically meaningful. Gaussian models were adjusted with Proc Mixed and the Binomial ladder rung walking test with Proc Glimmix.

The remaining data were analyzed by one-way analysis of variance (ANOVA) and Holm Sidak's test for multiple comparisons was used as a post-hoc test. Significance was assumed at values of $P < 0.05$. All these tests were analyzed using the statistical program GraphPad Prism version 6.00 for Windows (Graph Pad Software, USA).

3. Results

3.1. Behavioral tests

3.1.1. *Heat sensitivity test*

Significant differences between groups for all days pi were found (**Fig. 1A**). KA1 group showed significant differences against *sham*, L05 and KA1L05 groups at all days pi. *Sham* group also showed significant differences against L05 group at day 7 pi, and against L05 and KA1L05 groups at day 14 pi.

3.1.2. *Suspension from a horizontal wire mesh pole*

Rats in KA1L05 group had a significantly longer suspension time than those of the KA1 and L05 groups at days 1, 2, 3 and 7 pi (**Fig. 1B**). *Sham* group was significantly different from KA1L05 group at days 1 and 3 pi. In turn, the KA1 group showed a significantly shorter suspension time in comparison with L05 animals at days 1 and 2 pi, and in comparison with *sham* group at day 2 pi.

3.1.3. *Ladder rung walking test*

For the ipsilateral side, heterogeneity of variances related to the treatments was verified in the Gaussian analysis, being significant the effect of the KA1 treatment in comparison with the other treatments ($P < 0.10$). (**Fig. 1C-D**). For the contralateral side, there were significant differences between the L05 treatment as compared to the other treatments.

3.2. Histopathology

Spinal cord sections of all groups showed some polymorphonuclear cells infiltration and minor hemorrhagic areas at the injection site. This pattern corresponds to the penetration point and the path of the injection needle as was described elsewhere [8]. Slides of *sham* group showed a few swollen neurons in sections relatively closed to the entry point of the needle (from Lamina IV up to Lamina VI). Neurons of L05 group showed a similar aspect to that observed in the *sham* group at the ipsilateral side. Neurons were almost absent at the ipsilateral side of the KA1 group, mostly at the ventral horn, whereas KA1L05 group displayed a similar pattern to that presented by L05 and *sham* groups (**Fig. 2A**). Neurons of the contralateral side remained intact and

showed a similar aspect to that of the *sham* group, independently of the treatment received.

3.3. Morphometry

Comparison of the coronal-sectioned areas of both sides of the C5 sections showed a significant increase of the ipsilateral side area of KA1 animals in comparison to L05 group at day 1 pi (P = 0.0077) (**Fig. 2B-C**). No significant differences were observed for the contralateral section area among groups along the experiment.

GFAP immunostained area showed a significant increase in *sham* animals in comparison to L05 group at day 1 pi (P = 0.0083) at the ipsilateral side (**Fig. 2D**). At day 3 pi, *sham* animals showed a significant increase in the immunostained area in comparison to L05 and KA1L05 groups (P = 0.0235). The immunohistochemical identification of astrocytes showed that KA1 animals increased their immunostained area in comparison to L05 and KA1L05 rats at days 1 pi (P = 0.0435) and 3 pi (P = 0.0235). At the contralateral side the immunostained area of the *sham* group was significantly higher than those of L05 and KA1L05 groups at days 1 and 3 pi (P = 0.0066 and P = 0.0345, respectively) and the immunostained area of KA1 group was significantly higher than those of L05 and KA1L05 groups at day 3 pi (P = 0.0345) (**Fig. 2E**). **Figure 2F$_{1\text{-}2}$** shows the immunostained area of the ipsilateral ventral horn of KA1 and KA1L05 groups at day 2 pi.

3.4. Neuronal counting

Neuronal counting based on NeuN positive stained neurons was performed for both ipsilateral and contralateral sides. No significant differences were found in the number of neurons between *sham*, KA1L05 and L05 groups, whereas KA1 group showed a

significant decrease in the number of neurons at the ipsilateral side in comparison to the other groups at days 1 pi ($P = 0.0005$), 2 pi ($P = 0.0079$), 3 pi ($P = 0.0090$) and 7 pi ($P = 0.0023$) (**Fig. 3A-B**). No differences were found at the contralateral side at any day pi among groups. **Figure 3C** shows the NeuN immunohistochemical staining of neurons of KA1 and KA1L05 groups at day 2 pi.

Estimation of the total number of neurons for the entire segment was performed. Values per each group and day pi are shown in **Table 2**. Significant results were observed for the KA1 group in comparison to the remaining groups at days 1 pi ($P = 0.0024$) and 3 pi ($P = 0.0076$) and against KA1L05 group at day 7 pi ($P < 0.0001$).

4. Discussion

In the present work we evaluated whether lidocaine could prevent the harmful effects of the excitotoxicity induced by KA at the spinal cord when they are simultaneously injected. As previously stated [8], KA-induced sensitive and motor impairments were significant for the first two days after injection of the drug, and progressively reverted towards day 7 pi. Nevertheless, using the simultaneous injection of 0.5% lidocaine and KA, none of those impairments were observed during the entire experiment, suggesting a potential neuroprotective effect of lidocaine.

In our observations, KA mainly affected the middle region and the ventral horn of the C5 spinal cord segment. In the rat spinal cord, the distribution of kainate receptor subunits is primarily found in the utmost Laminae of the dorsal horn and at the level of motoneurons [5, 18]. Since AMPA and NMDA receptor subunits are also abundantly expressed in the dorsal and ventral horns it was suggested that even at lower concentrations than that used in our experiments the effects of KA may contribute to the endogenous glutamate release and action on multiple receptor subclasses and on non-

glutamatergic neurons [5]. However, using the simultaneous injection of lidocaine and KA the previously changes described were not found.

We found that KA-injected rats also showed lower heat sensitivity up to day 7 pi, but this effect disappeared by day 14 pi. These results suggest a possibly reversible excitotoxic effect of KA, as reported by Sun et al. [30]. On the other hand, animals receiving simultaneous injection of 0.5 % lidocaine and KA were more reactive to heat, more resistant in the horizontal wire mesh pole and showed minimal errors in the ladder rung walking test, as compared to KA1 group at almost all pi days. This would also indicate that muscle strength was not compromised, as in some motor neuropathies [1].

In addition, these animals showed no other tissue damage than that inflicted by the introduction of the needle, as it was also observed in *sham* animals. Similarly, when the same anesthetic was intravenously administered 30 min before, during and after a cerebral ischemia, the extent, severity and intensity of the hippocampal lesions were reduced [12]. On the other hand, in our model, KA induced an inflammatory reaction in which an increase in astrocytes was observed, with peaks of reactivity at days 1 and 3 pi [31] on the ipsilateral side, and a reduction on day 2 pi, as was previously described [24]. It is known that astrocytes can detect the presence of injury or stress factors in their microenvironment and therefore be activated [32]. This astrocytic reaction was not observed in animals that simultaneously received KA and lidocaine.

As for the contralateral side response, it may be due to both the release of different cerebral or blood factors as a result of the injury and the migration of activated astrocytes as was discusses elsewhere [16]. This reaction may correspond to a secondary generalized response to limit or protect the still undamaged tissue [24].

In addition, L05 group showed lower astrocytic reaction as compared to *sham* group, as it has been recently reported [16]. These results support the well-known anti-inflammatory effect of lidocaine.

Neuronal counting was statistically higher when lidocaine was simultaneously injected with KA in comparison with those animals receiving only KA, both in individual sections as well as in the whole C5 segment. There is evidence about the reversibility of the effects caused by KA [8, 30] which is noticeable in the functional recovery of the injected animals by day 14 pi. Besides, there is evidence of tissue regeneration and cell repopulation processes in animals injected by the same route and with the same chemical agent [23].

It is known that exposure of neuronal populations to KA triggers a sustained Ca^{2+} intake and an overproduction of reactive oxygen species, thus leading to death of cells by excitotoxicity, which emulates the excessive release of glutamate [33]. Lin et al. [34] showed that lidocaine affects Ca^{2+}-dependent exocytosis *in vitro* and consequently inhibits the release of glutamate from the presynaptic nerve terminals. In addition, Chiu et al. [33] showed the neuroprotective effect of lidocaine *in vivo*.

It is known that lidocaine blocks Na^{+} channels [35] and this effect reduces neuronal depolarization with different beneficial effect, such as the reduction of Ca^{2+} influx and water and chloride uptake [36]. Other studies support the use of Na^{+} channel blockers to provide neuroprotection and functional recovery in neurodegenerative diseases, supporting other beneficial effects of the Na^{+} blockers on other channels or intracellular cascades [37; 38].

4.1 Conclusions

According to our results, 0.5% lidocaine prevents the excitotoxicity induced by KA when both drugs are simultaneously injected. This would allow us to conclude that lidocaine could be considered as a neuroprotective drug in diseases and models that include excitotoxicity as the primary mechanism of injury and used as a promising treatment for localized spinal cord injuries.

Disclosure

Authors declare no Conflict of interests for this article.

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Table 1

Day pi	Sham	L05	KA1	KA1L05	Total n
0	15	25	25	25	90
1	15	25	25	25	90
2	12	20	20	20	72
3	9	15	15	15	54
7	6	10	10	10	36
14	3	5	5	5	18

Table 1. Number of animals (n) participating in each behavioural test.

Numbers express the total number of animals of each group that participated in the behavioural test. After performing the test, some animals of each group were euthanized.

Table 2

Day pi	*Sham*	L05	KA1	KA1L05
0				
1	214300 ± 14278	248100 ± 19919	114300 ± 15500*	288400 ± 12154
2	269000 ± 27628	267700 ± 9200	127700 ± 12400	255525 ± 17456
3	261350 ± 8950	233950 ± 12150	115800 ± 5000*	266600 ± 20061
7	221733 ± 50432	227800 ± 3500	103900 ± 11400*	279167 ± 23354
14	263900 ± 21099	238550 ± 23050	107750 ± 7550	228167 ± 5664

Table 2. Estimation of the total number of neurons for the entire C5 segment. Data are expressed as Mean ± SEM. The total number of neurons for the C5 segment was significantly lower for the KA1 group in comparison with the other groups for days 1 and 3 pi, and significantly lower in comparison with KA1L05 group for day 7 pi (* $P < 0.05$).

Legends to the figures

Fig. 1 Behavioural tests. Estimated mean responses for: A. Heat sensitivity test. B. Suspension from a horizontal wire mesh pole. C. Ladder rung walking test performance on ipsilateral side. Step errors (scores 0-2) average as percent of the total number of steps made in each trial. D. Contralateral side of the same test. In all cases, data are expressed as estimated mean ± SEM. * Days where significant results were observed. *P*-values and significance levels from the repeated measures test are observed at each side of the graph. ** Significant differences among treatments.

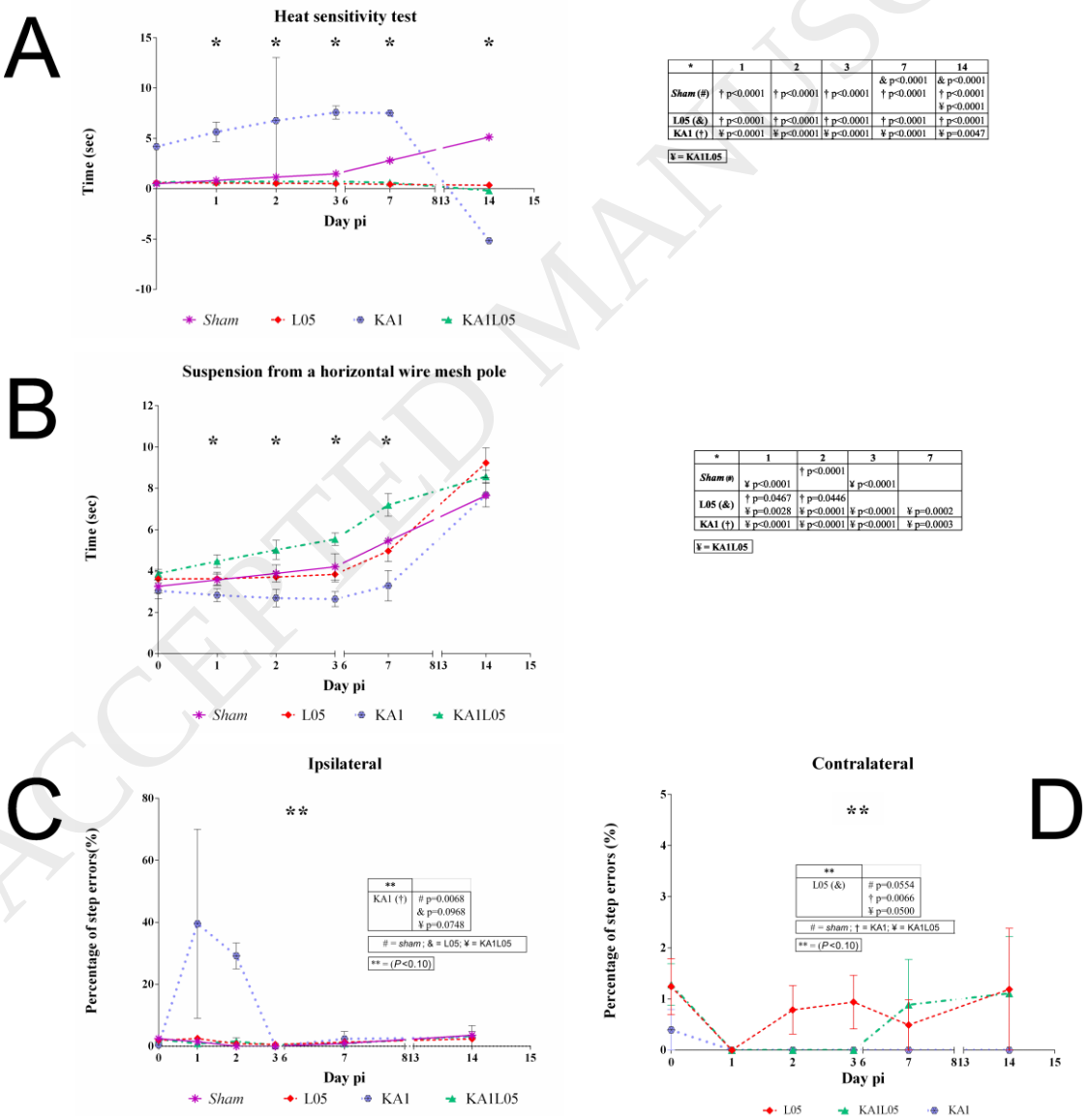
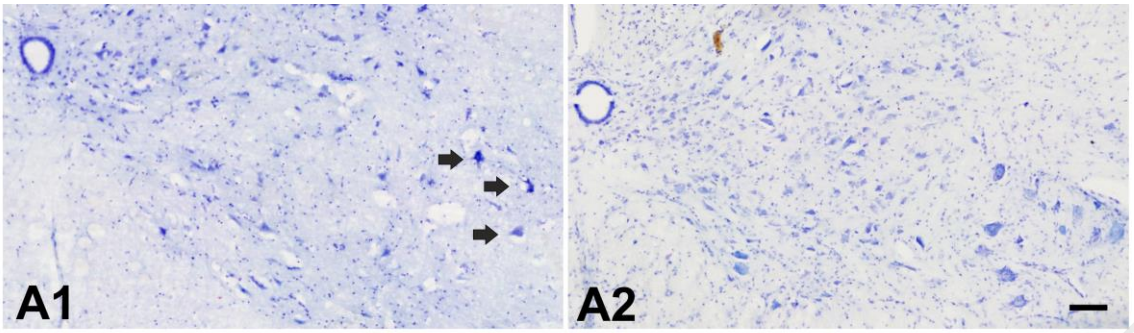
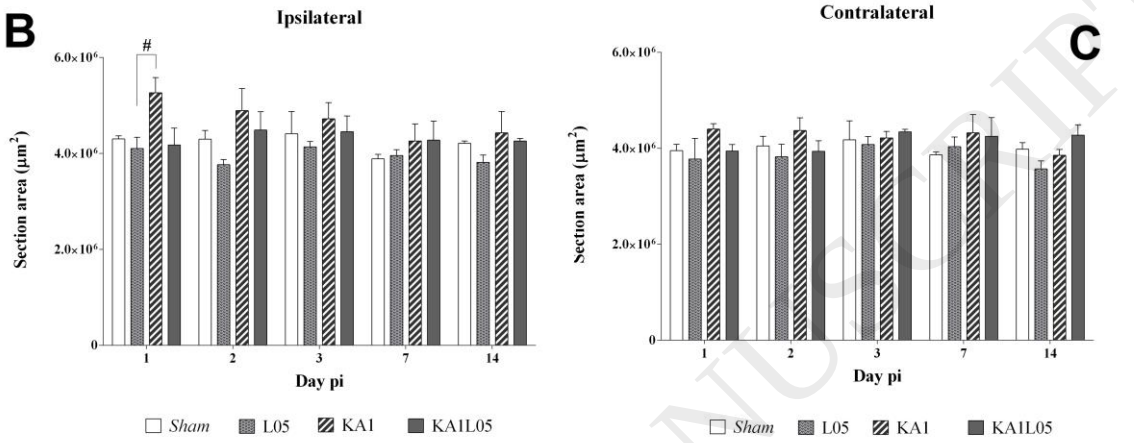
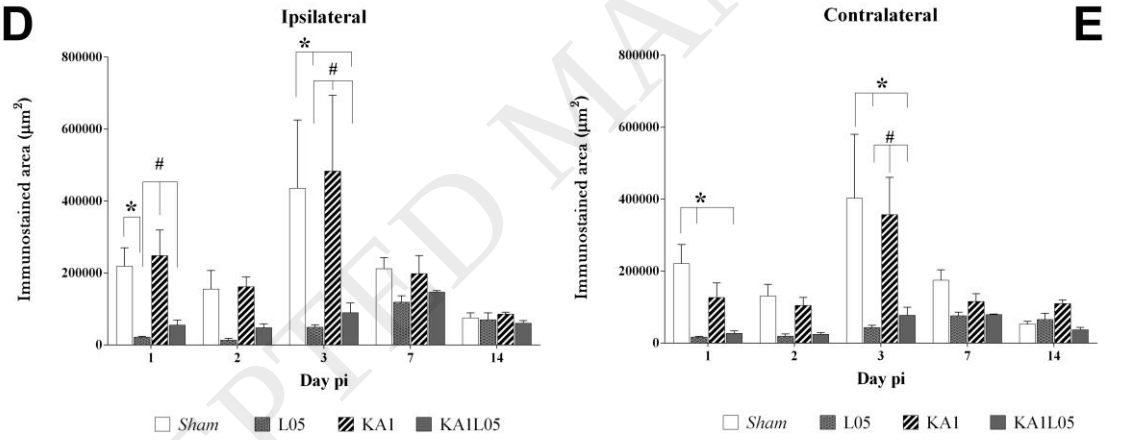


Fig. 2 Inflammatory reactivity of the C5 spinal cord segment. A. Histological aspect of a C5 section of KA1 (A1) and KA1L05 (A2) showing distribution and aspect of neurons at their ipsilateral sides at day 2 pi. At A1, ventral horn neurons are almost missing. The remaining neurons are retracted or with vacuolated cytoplasm (arrows). Bar = 100 µm. B. Ipsilateral section area measurement. # KA1 vs. L05 ($P < 0.05$). C. Contralateral section area measurement. D. Ipsilateral GFAP-immunostained area. * *sham* significance against other groups; # KA1 significance against other groups ($P < 0.05$). E. Contralateral GFAP-immunostained area. * *sham* significance against other groups; # KA1 significance against other groups ($P < 0.05$). In all cases, data are expressed as mean ± SEM. F1 and F2 are an immunohistochemical staining against GFAP positive cells (arrows) at the ipsilateral side of KA1 and KA1L05, respectively, at day 2 pi. Bar = 100 µm.







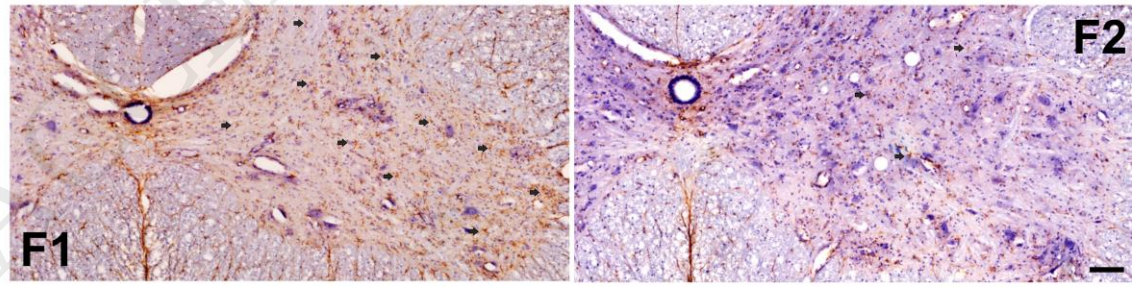


Fig. 3 Neuronal counting. Data are expressed as mean ± SEM. A. Ipsilateral counting #KA1 vs. the remaining groups (P<0.05). B. Contralateral counting. C. Immunohistochemical staining for detection of NeuN positive cells at day 2 pi: C1, ipsilateral side of KA1 group. Few intact neurons can be seen (arrows); C2, ipsilateral side of KA1L05 group. Bar = 50 µm.