Aluminum exposure for 60 days at an equivalent human dietary level promotes peripheral dysfunction in rats

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Abbreviations: Al, aluminum; PTWI, provisional tolerable weekly intake; ASIA, Autoimmune/inflammatory Syndrome Induced by Adjuvants; CNS, central nervous system, PNS, peripheral nervous system; AD, Alzheimer Disease; ROS, reactive oxygen species; RNS, reactive nitrogen species; MDA, malondialdehyde; TBA, thiobarbituric acid; FRAP, ferric reducing/antioxidant power; AChE, acetylcholinesterase.

Abstract

Aluminum (Al) is a neurotoxic associated with a number of chronic human diseases. We investigated the effects of Al exposure at doses similar to human dietary levels on the peripheral nervous system over a 60 day period. *Wistar* male rats were divided into two major groups and received orally: 1) Low aluminum level - rats were subdivided and treated for 60 days as follows: a) Untreated - ultrapure water; b) $AlCl_3$ at a dose of 8.3 mg/kg bw for 60 days, representing human Al exposure by diet; and 2) High aluminum level - rats were subdivided and treated for 42 days as follows: C) Untreated – ultrapure water; d) $AlCl_3$ at 100 mg/kg bw for 42 days, representing a high level of human exposure to Al. Von Frey hair and plantar tests were used to verify the tactile and thermal sensitivities, respectively. The presence of catalepsy behavior and the spontaneous motor activity were investigated by "ring test" and using individual photocell activity chambers. Reactive oxygen species, lipid peroxidation and total antioxidant capacity in plasma, were measured. Immunohistochemistry to investigate the nerve inflammation and, the specific presence of Al in the sciatic nerve fibers were investigated. Al exposure at a representative human dietary level promotes the development of mechanical allodynia, catalepsy behavior, increased the number of activated macrophages in the sciatic nerve, systemic oxidative stress and, is able to be retained among the sciatic nerve fibers. The effects of Al in the peripheral nervous system were similar to those found in rats exposed to Al at a dose much higher (100 mg/kg). Therefore, our findings suggest that Al may be considered toxic for the peripheral nervous system, thus inducing peripheral dysfunction.

Keywords: metal; peripheral neuropathy; oxidative stress; inflammation.

1. Introduction

Aluminum (Al) has no known biological function and is potentially toxic (Exley 2009). Humans are exposed to Al through dietary and non-dietary sources, and its real consequence perhaps not entire clear (Exley 2012, 2013). The Provisional Tolerable Weekly Intake (PTWI) of Al for humans has been adjusted to 1 mg Al/kg body weight (bw) by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives (JECFA) and European Food Safety Authority (EFSA) (WHO 2007; EFSA 2008). However, due to myriad sources of Al exposure, this health-based guidance value may be exceeded by humans (Gonzalez-Weller et al. 2010; Fekete et al. 2013; Yang et al. 2014).

Therefore, the increased body burden of Al has been related with several health conditions (Exley 2012). The body presence of this non-essential metal seems to trigger the development of neurological disorders (Mirza et al. 2016), reproductive dysfunction (Klein et al. 2014), Autoimmune/inflammatory Syndrome Induced by Adjuvants (ASIA) such as macrophagic myofascitis (Gherardi et al. 2016), microcytic anemia (Barata et al. 1996), atherosclerosis plaques formation (Lind et al. 2012), osteopenia (Li et al. 2011) and breast cancer (Linhart et al. 2017).

Al is a neurotoxin able to be accumulated and retained in the brain, contributing towards both the onset and the aggressive progression of all forms of Alzheimer Disease (AD) (Mirza et al. 2017). Al accumulation in neurons has been related to cognitive and motor impairments, mostly related with neurodegenerative diseases (Kasbe et al. 2015; Lakshmi et al. 2015). In the peripheral nervous system (PNS) there are evidences of Al-induced neuropathy. People exposed to Al through contaminated water (13.17 to 15.70 ppm) in the Kirazli region (Biga Peninsula, NW Turkey) showed a history of peripheral neuropathy (Bakar et al. 2010). More recently, Al accumulation in the dorsal root ganglion in the course of oxaliplatin treatment of cancer exacerbates neuropathic pain (Park et al. 2015). In agreement, the suggested Al chelation by glutathione, known as an antioxidant and a metal chelator, decreases the concentrations of Al in the dorsal root ganglion and alleviates the neuropathic pain induced by oxaliplatin in mice (Lee et al. 2017).

However, even with the well - known toxicity of Al in the Central Nervous System (CNS), the effects of Al exposure on the PNS remain poorly understood. Moreover, most Al studies have entailed unrealistic high doses of Al which cannot be used as a common level found among human populations.

Herein, we addressed the effects of Al in the PNS and motor behavioral in rats exposed to both a high level of Al and also one that better represents human exposure to Al through the diet.

2. Materials and methods

2.1 Animals

Three-month-old male *Wistar* rats (360 ± 11.2 g) were obtained from the Central Animal Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats were housed at a constant room temperature, humidity, and light cycle (12:12h light-dark), giving free access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa, Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

Rats were divided into two major groups, according to Martinez et al. (2017) and treated orally: 1) Low aluminum level - rats were subdivided (N=10/each) and treated for 60 days as follows: a) Untreated – received ultrapure drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) $AlCl_3$ at 8.3 mg/kg bw per day, a dose based on human dietary levels translated to an animal dose according to the body surface area normalization method (Reagan-Shaw et al. 2008); and 2) High aluminum level - rats were subdivided (N=10/each) and treated for 42 days as follows: a) Untreated – received ultrapure water through oral gavages; b) $AlCl_3$ at 100 mg/kg bw per day, representing a high level of human exposure to Al (Prakash and Kumar 2009).

$AlCl_3.6\ H_2O$ was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water. The concentration of each stock solution was 0.034 M and 0.331 M, respectively from 8.3 and 100 mg/kg bw. Salts and reagents were of analytical grade obtained from Sigma-Aldrich and Merck (Darmstadt, Germany).

2.2 Tactile sensitivity: Von Frey hair test

Mechanical sensitivity was assessed by measuring the withdrawal threshold to calibrated von Frey hairs (Bioseb Instruments, USA) (Vera et al. 2007). The test was realized at the start (0 day), middle (21 and 30 days) and end (42 or 60 days) of the treatments. Rats were placed individually on an elevated iron mesh in a clear plastic cage and the filaments were applied to the plantar aspect of each hind paw, from below the mesh floor. Each stimulus was applied for approximately 1 second with an interstimulus

interval of approximately 3 seconds. A significant decrease in von Frey hair threshold evoked by mechanical stimulus was defined as presence of mechanical allodynia.

2.3 Thermal sensitivity: plantar test

Responses to thermal stimuli were evaluated after mechanical sensitivity using a plantar test apparatus (Ugo Basile, Comerio VA, Italy) (Bennett and Xie 1988). During the testing days rats were placed within a plastic compartment on a glass floor and a light source beneath the floor was aimed at the mid plantar surface of the hind paw. So, the withdrawal reflex interrupts the light and automatically turns-off the light and a timer. The withdrawal latency of each paw was measured during three trials at 2 min intervals and the mean of the three readings was used for data analysis.

2.4 Catalepsy

Catalepsy was measured using a modification of the "ring test" (Fox et al. 2001). Rats were hung by their front paws from a rubber-coated metal ring fixed horizontally at a height that allowed their hind paws to just touch the bench. The time taken for the rat to move-off the ring was measured with a cut-off limit of 30 seconds.

2.5 Spontaneous locomotor activity

Spontaneous locomotor activity was evaluated in the middle (21 or 30 days) and end (42 and 60 days) of the treatments using individual photocell activity chambers (Cibertec S.A., Madrid, Spain) (Vera et al. 2007). For this, rats were placed in the recording chambers and the number of interruptions of photocell beams was recorded over a 30-min period. Total number of activity counts throughout the 30 min of test duration was recorded. The mean number of crossings of the photocell beams was used for comparison.

2.6 Blood and tissue collection

At the end of the treatment period, after the behavioral assessment, rats were anesthetized with an association of ketamine and xylazine (87 mg/kg and 13 mg/kg, respectively, intraperitoneal injection), and after loss of the righting reflex they were submitted to an aorta artery puncture and blood was subsequently collected to obtain plasma for the biochemical experiments. Thereafter, rats were euthanized by decapitation, and the sciatic nerve of the right hind paw were carefully removed for immunohistochemistry analysis.

2.7 Reactive oxygen species levels

148 Biochemical studies of oxidative stress biomarkers were performed in plasma. For that, blood
149 was centrifuged at 2400g for 10 min at 4°C and plasma was obtained for the measurements.
150 Levels of reactive species were determined by the spectrofluorometric method described by
151 Loetchutinat et al. (2005). This method is unspecific for reactive oxygen species (ROS), also measuring
152 reactive nitrogen species (RNS). The plasma was diluted in 50 mM Tris HCl (pH 7.4) and 2′, 7′-
153 dichlorofluorescein diacetate (DCHF-DA; 1mM) was added to the medium. DCHF-DA is enzymatically
154 hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to
155 form highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence
156 intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission
157 was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60
158 min at 15 min intervals. The ROS levels were expressed as fluorescence units.
159 *2.8 Lipid peroxidation*
160 Lipid peroxidation was measured as malondialdehyde (MDA) using a colorimetric method, as
161 previously described by Ohkawa et al. (1979), with modifications (Martinez et al. 2017). An aliquot of
162 plasma was incubated with thiobarbituric acid 0.6% (TBA) and phosphoric acid buffer 1% (H_3PO_4) at
163 100ºC for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular
164 Devices, CA, USA). The results were expressed as nanomoles of MDA per mL of plasma.
165 *2.9 Ferric Reducing/Antioxidant Power (FRAP) Assay*
166 The total antioxidant capacity was measured by FRAP assay (Benzie and Strain 1996), with
167 modifications (Martinez et al. 2017). This method is based on the ability of samples to reduce ferric ion
168 (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s- triazine (TPTZ) the chelate complex
169 Fe^{+2}-TPTZ. Briefly, 10 μL of plasma was added to 1 mL freshly prepared and pre-warmed (37°C) FRAP
170 reagent (300mM acetate buffer (pH = 3.6), 10mM TPTZ in 40mM HCl, and 20mM $FeCl_3$ in the ratio of
171 10:1:1) in a test tube and incubated at 37°C for 10min. The absorbance of the blue-colored complex was
172 read against a blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm (SpectraMax M5
173 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50-1000 µM – water soluble
174 analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular
175 reference to Trolox equivalents.
176 *2.10 Immunohistochemistry*

Sciatic nerve immunohistochemistry was performed on paraffin-embedded sections of 5 μm thickness. De-paraffined slides were washed with phosphate buffered saline (PBS) with 0.05 % Tween 20 (Calbiochem, Darmstadt, Germany). Thereafter sections were incubated for 10 min in 3 % (*v/v*) hydrogen peroxide to inhibit endogenous peroxidase activity and blocked with fetal bovine serum for 30 minutes to minimize nonspecific binding of the primary antibody. Sections were then incubated overnight at 4 °C with a monoclonal antibody against macrophage-associated antigen (CD163, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to quantify the number of activated macrophages, which is consistent with the presence of inflammation. After incubation, samples were washed with PBS-Tween. The peroxidase-based kit Masvision (Master Diagnostica, Granada, Spain) was used as chromogen. Samples were counterstained with hematoxylin and coverslips mounted with Eukitt mounting media (O. Kindler GmbH & Co, Freiburg, Germany). To determine the level of non-specific staining the preparations were incubated without the primary antibody.

2.11 Lumogallion staining for presence of Aluminum

Lumogallion staining was performed in formalin-fixed sciatic nerve using a recent validated method to identify the presence of Al in tissues (Mirza et al. 2016; Mold et al. 2014). Briefly, re-hydrated tissues sections were immediately placed into either 1 mM lumogallion (TCI Europe N.V. Belgium) buffered in 50 mM PIPES, pH 7.4 or the PIPES-buffer alone for auto-fluorescence analyses for 45 minutes. Slides were carefully washed 6 times with PIPES-buffer, after rinsed in ultra-pure water for 30 seconds, finally mounted using an aqueous mounting media and stored horizontally at 4ºC overnight prior to imaging. Sections of tissues were imaged using a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6.

2.12 Statistical analysis

Data are expressed as mean ± SEM. Results were analyzed using unpaired Student's t-test for comparison between groups and repeated measure one-way ANOVA for comparison between evaluations pre, in the middle and in the end of the treatments. Values of $P < 0.05$ were considered significant.

3. Results

3.1 Body weight, fluid and feed intakes

Body weight of rats was similar between groups at the beginning and end of treatments. Water or Al intakes as well as the feed intake were not different between groups (Martinez et al. 2017)

3.2 Aluminum promotes the development of peripheral neuropathy

Exposure to Al at low (8.3 mg/kg bw for 60 days) or at high level (100 mg/kg bw for 42 days) decreased the mechanical sensitivity threshold in the middle (30 and 21 days) and end of the treatments (60 and 42 days - Fig 1A,B). The threshold for mechanical sensitivity before treatment was 21.92 ± 1.20 g (N=64) without differences between groups. In control groups, this threshold did not significantly change with the treatment, whereas for Al-treated rats the mean threshold value after treatments decreased 61.2 % and 40.9 % for Al exposure at low and high doses, respectively (Fig 1A,B). The thermal sensitivity tested by the plantar test was unaffected by Al (Fig 1C,D). However, exposure to Al promoted the development of catalepsy. Rats exposed to low or high levels of Al showed an increased latency time for reaction in the catalepsy test, being necessary from 2 to 4 times more, respectively when compared to their respective control group, suggesting the presence of motor behavioral disorders (Fig 2A,B). Spontaneous motor activity was not modified in the different experimental conditions (Fig 2C,D). Taken together, these results suggest the presence of mechanical allodynia and motor behavioral disorders after Al exposure even at low doses.

3.3 Aluminum induces systemic oxidative stress and peripheral neuro-inflammation

Al treatment at low and high doses promoted an imbalance between pro-oxidant and antioxidant systemic biomarkers, as observed by the raised on ROS levels, lipid peroxidation and total antioxidant capacity in plasma (Fig 3).

Immunohistochemical analysis showed an increase in the number of activated macrophages in sciatic nerve of rats treated with Al at the low dose of 8.3 mg/kg and of rats treated with the higher dose of 100 mg/kg, when compared with the respective control groups (ranging from 10 to 34 in the control group and from 25 to 67 in the Al-treated rats – Fig 4).

3.4 Aluminum seems to be retained in the sciatic nerve fibers

The presence of Al in the sciatic nerve was confirmed using lumogallion and fluorescence microscopy. The nerve showed green autofluorescence in the absence of lumogallion (Fig 5A) and no specific fluorescence in the control rats (Fig 5B). Lumogallion fluorescence identified Al in the in the sciatic nerve of Al-treated rats as evidenced by bright orange fluorescence (Fig 5C,D).

4. Discussion

Aluminum is neurotoxic, and evidences now point to Al as a primary etiological factor in AD (Exley 2017). Recently, we have observed that Al reaches a threshold sufficient to promote cognitive dysfunction and memory impairment even at low doses (Martinez et al. 2017). Here, we show that Al at

human dietary levels could affect the PNS inducing peripheral neuropathy. Rats exposed to Al for 60 days at a representative human dietary Al level (8.3 mg/kg) have developed mechanical allodynia, peripheral nerve inflammation with increased number of activated macrophages in the sciatic nerve and, all of this submerged in an increased systemic oxidative stress. Interestingly, and in agreement with our previous findings (Martinez et al. 2017), these effects are similar to those found in rats exposed to Al at a dose much higher (100 mg/kg). Moreover, our study shows for the first time the specific presence of Al in the sciatic nerve fibers.

The present study supports the concern regarding safety values for human exposure to Al. Herein, rats exposed to Al in a model that aims to mimic human exposure to this metal develop peripheral dysfunction similar as those found in Al exposure at a high level. Our model of exposure together with previous reports in the literature suggest that once achieved a toxic Al threshold or burden in any compartment or tissue, Al could begin its cascade of toxicity in the body, which seems occur at a similar human exposure level (Exley 2014; Martinez et al. 2017; Crépeaux et al. 2017).

Data regarding Al and peripheral nerve dysfunction are scarce. Injection of aluminum in the subepineurial space of the sciatic nerve was used as a model for neuro-degeneration in rabbits (Kihira et al. 1995). Recently, the presence of Al in the dorsal root ganglion of oxaliplatin-treated mice, a platinum-based anticancer drug, was associated with severe side effects in the PNS (Park et al. 2015). It was further confirmed by the use of the antioxidant glutathione as a suggested metal chelator which in turn decreases the concentration of Al in the dorsal root ganglion and the neuropathic pain induced by oxaliplatin (Lee et al. 2017). The effect of high Al in water resources (140 times higher than the maximum allowable limits) on human health was evaluated and, a total of 273 people living in the Kirazli region, Turkey were interviewed. The neuropathy history was about 36% higher in the region, creating a statistically significant difference (Bakar et al. 2010). Important to highlight that serum Al may have toxic effects on hemodialysis patients even when it is in the “acceptable” range (below 20 μg/L), being associated with increased mortality and, disorders of the PNS are among the various neurologic conditions that have been reported in such population (Bansal et al. 2014; Hsu et al. 2016).

In the current study Al-treated rats have developed catalepsy, which is an extreme form of immobility manifested in some Parkinson disease patients and resembles the extrapyramidal side effects that occur in humans exposed to various antipsychotic drugs, some of which acting as acetylcholinesterase (AChE) inhibitors (De Ryck et al. 1980). In experimental animals, the inhibition of

AChE has been related with the catalepsy development (Castelló et al. 1992; Sklan et al. 2006). The catalepsy behavior found in Al-treated rats is in agreement with our previous findings. We have seen that Al exposure at an equivalent human dietary level inhibits the AChE activity in hippocampus of rats (Martinez et al. 2017). Indeed, Al exposure has a large history on cholinergic system, acting either stimulating or inhibiting AChE activity (Kumar 1999; Prakash and Kumar 2009; Lakshmi et al. 2015). However, the neurotoxicity mechanisms of Al need to be better understood.

Al^{3+} is a potent pro-oxidant due to formation of superoxide radical ion (Exley 2004) or by promoting the Fenton reaction by reducing Fe (III) to Fe (II) (Ruipérez et al. 2012). In the present study, rats exposed to Al have shown the features of mechanical allodynia followed by increased oxidative stress in plasma and peripheral inflammation in the sciatic nerve, suggesting the development of peripheral neuropathy in rats. While there are several lines to explain the pathophysiological basis of neuropathic dysfunction in other diseases, oxidative stress and inflammation seem to play an important role in neuronal damage, contributing towards to demyelination of peripheral nerves thus altering the normal conduction (Ellis et al. 2013; Sandireddy et al. 2014). Overall, our findings suggest that the increased ROS, lipid peroxidation and antioxidant capacity in plasma together with the peripheral inflammation with large number of macrophage activated in the sciatic nerve could underlie the pathogenesis of peripheral impairment in Al-treated rats. Moreover, by lumogallion staining and fluorescence microscopy, we have seen for the first time the specific presence of Al in the sciatic nerve highlighting its interference on the neuropathic dysfunction observed after Al exposure. However, further detailed study will be necessary to verify the precise locations of deposits of Al in the nerve.

5. Conclusions

Our study provides evidence that 60-day exposures to low doses of Al, which aimed to mimic human exposure to Al by dietary source, are able to impair the PNS and, those effects are almost the same as observed after Al exposure at a high level. Here we show for the first time the specific presence of Al in the sciatic nerve fibers, which suggest that Al may trigger the onset of the peripheral dysfunction. The elevation of oxidative stress and inflammation highlight pathways of toxic actions for Al in the PNS. Moreover, our findings raise the concern regarding safety values of human exposure to Al and suggest an action of Al on the development of the neuropathic dysfunction.

Conflict of Interest: None.

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426 **Figure legends**

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428 **Figure 1.** Effect of chronic aluminum exposure on mechanical and thermal sensitivities. Mechanical
429 sensitivity was measured using the Von Frey test and thermal sensitivity was measured using the plantar
430 test (see text for details) before (day 0), in the middle (30 or 21 days) and end of the treatments (60 or 42
431 days). Respective values of mechanical and thermal sensitivities of control rats and treated with $AlCl_3$ for
432 60 (8.3 mg/kg bw *per day* – A, C) or 42 days (100 mg/kg bw *per day* – B, D). Data are expressed as mean
433 ± SEM, n=8, * $P < 0.05$ compared with their corresponding controls (Student's t-test).
434 **Figure 2.** Effect of chronic aluminum exposure on catalepsy and spontaneous locomotor activity. Both
435 behavioral analysis were performed before (day 0), in the middle (30 or 21 days) and end of the
436 treatments (60 or 42 days). Respective values of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw
437 *per day* – A, C) or 42 days (100 mg/kg bw *per day* – B, D). Data are expressed as mean ± SEM, n=8, * P
438 < 0.05 compared with their corresponding controls (Student's t-test).

439

440 **Figure 3.** Effect of chronic aluminum exposure on oxidative stress assessments. Reactive oxygen species
441 (ROS), lipid peroxidation and total antioxidant capacity (FRAP- Ferric Reducing/Antioxidant Power)
442 levels of control rats and treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day*) or 42 days (100 mg/kg bw *per*
443 *day*). Data are expressed as mean ± SEM, n=8, * $P < 0.05$ compared with their corresponding controls
444 (Student's t-test).

445

446 **Figure 4.** Effect of chronic aluminum exposure on sciatic nerve immunohistochemistry. Activated
447 macrophages (arrows) in sciatic nerve of controls group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at
448 100 mg/kg b.w. (D) detected by immunohistochemistry (objective X40). Scale bars: 20 µm. Average
449 numbers of activate macrophages per field (objective X20) of control rats and treated with $AlCl_3$ for 60
450 (8.3 mg/kg bw *per day* - E) or 42 days (100 mg/kg bw *per day* - F). Data are expressed as mean ± SEM,
451 n=8, * $P < 0.05$ compared with their corresponding controls (Student's t-test).

452

453 **Figure 5.** Aluminum presence in peripheral nerve. Representative images of aluminum in the sciatic
454 nerve: autofluorescence control (A), lumogallion fluorescence for aluminum in control group (B) and in
455 rats treated with $AlCl_3$ for 60 (8.3 mg/kg bw *per day* - C) or 42 days (100 mg/kg bw *per day* - D). The
456 specific presence of Al is indicated by arrows. Scale bars: 50 µm.

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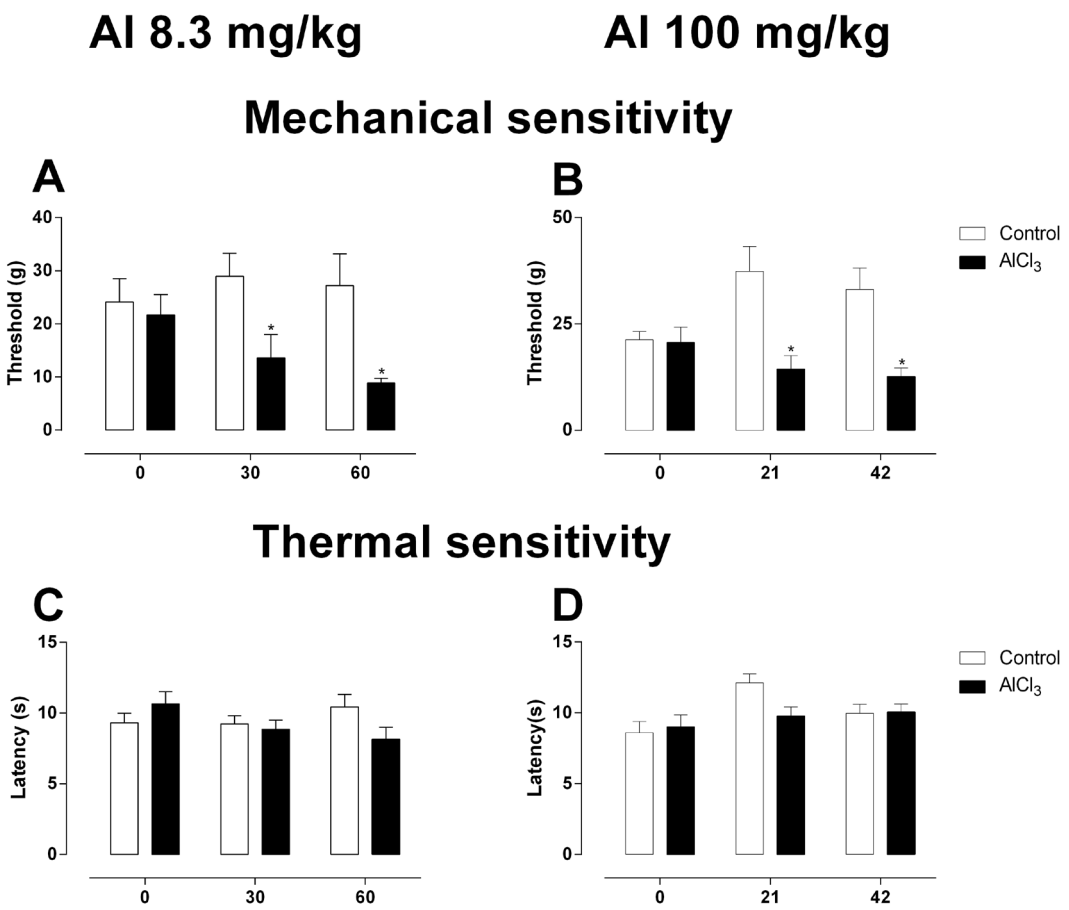
458 Fig 1



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478 Fig 2
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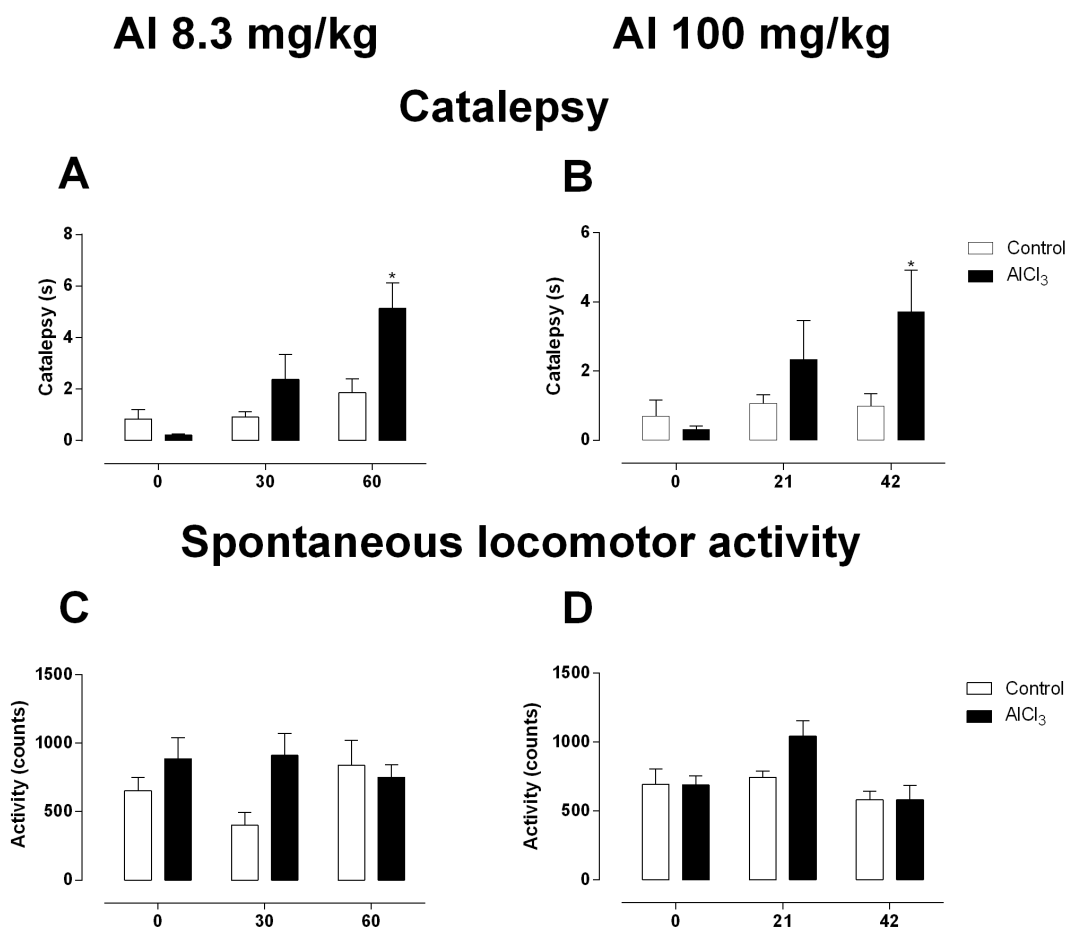

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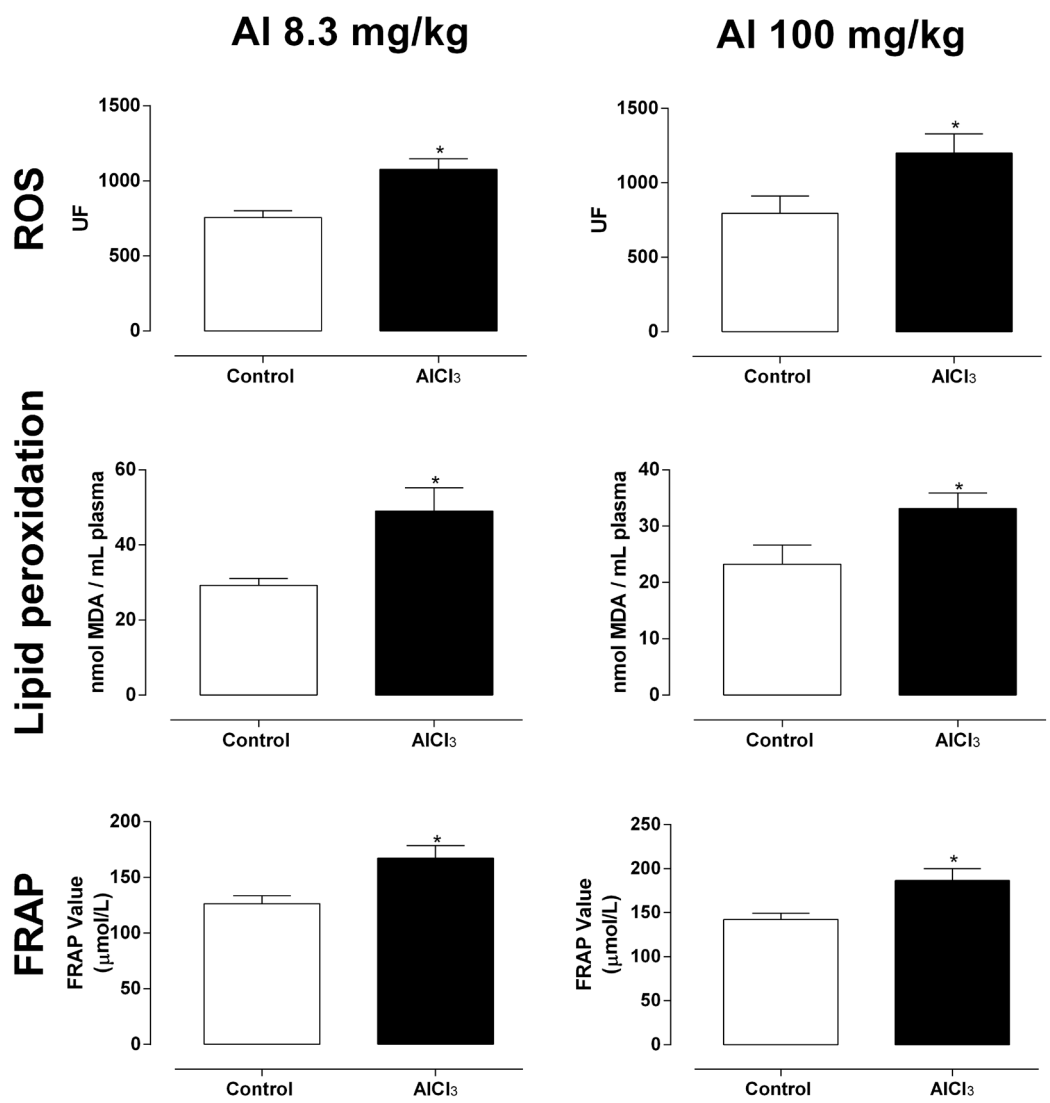
Fig. 3


Al 8.3 mg/kg
Al 100 mg/kg
ROS
UF
1500
1000
500
0
Control
AlCl3
Lipid peroxidation
nmol MDA / mL plasma
60
40
20
0
40
30
20
10
FRAP
FRAP Value (µmol/L)
200
150
100
50
0
250
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515 Fig. 4
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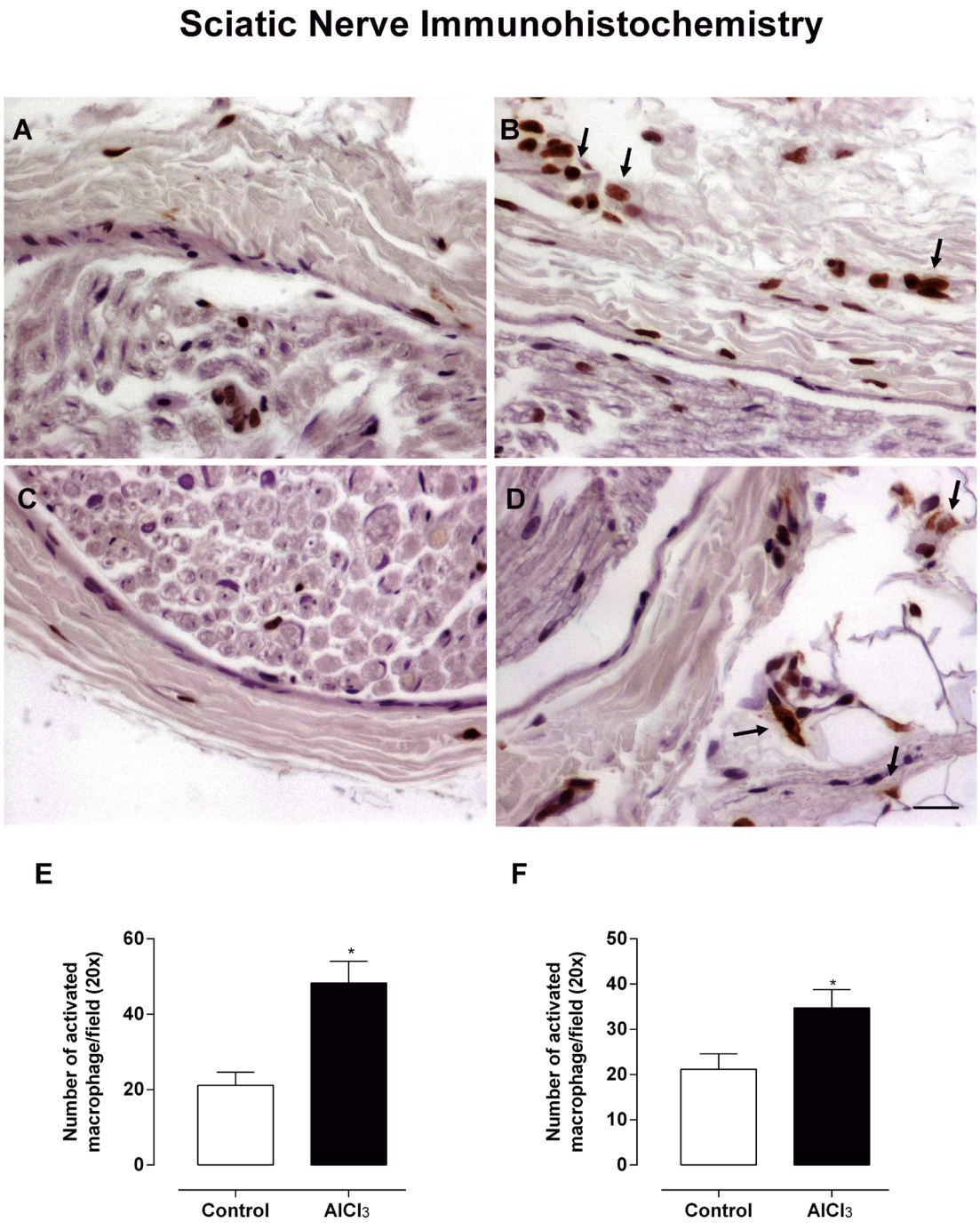

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525 Fig. 5

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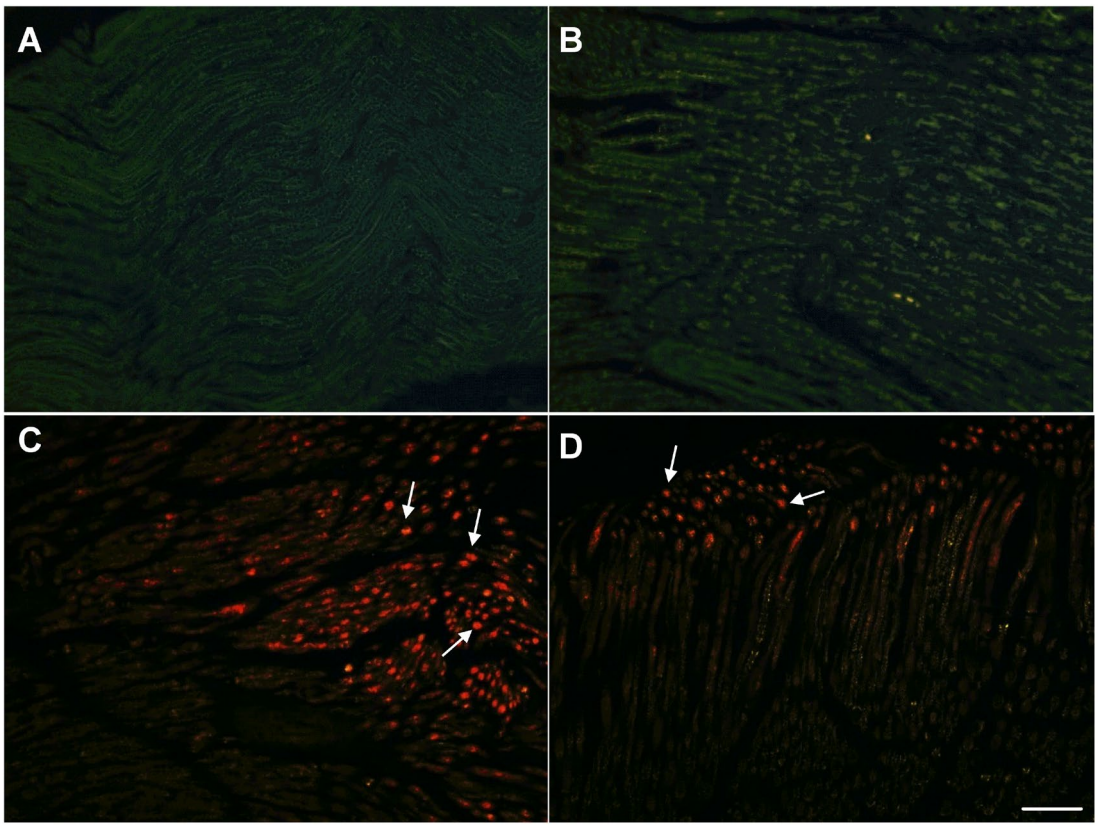

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