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Exposure to tolerable concentrations of aluminum triggers systemic and local oxidative stress and global proteomic modulation in the spinal cord of rats

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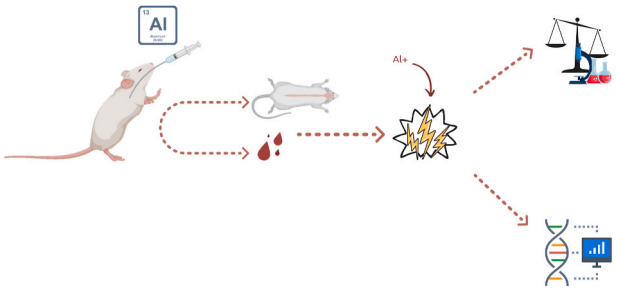
HIGHLIGHTS

- Aluminium exposure promoted lipid peroxidation with repercussions on biochemical homeostasis in blood and spinal cord.
- The proteomic profile of spinal cord was significantly altered after prolonged exposure to aluminum.
- The ORA analysis identified proteins associated with important biological processes in CNS.

GRAPHICAL ABSTRACT



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ABSTRACT

The tolerable aluminum (Al) intake levels for humans are constantly under review by regulatory agencies due to novel pre-clinical evidence on the neurotoxicity of prolonged Al exposure; however, little is known about the effects of Al on the spinal cord. This study aimed to investigate potential adverse effects on both spinal cord and systemic biochemical balance after prolonged exposure to a low dose of Al. Twenty adult rats were distributed in the control (distilled water) and exposed group (8.3 mg of $AlCl_3$/kg/day). After 60 days, both blood and spinal cord samples were collected for oxidative stress and proteomic analyses. In plasma and erythrocytes, glutathione level was not different between groups; however, exposure to $AlCl_3$ significantly decreased glutathione level in the spinal cord. Thiobarbituric acid reactive substances levels in the plasma and spinal cord of animals from the

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control group were significantly lower than those animals exposed to $AlCl_3$. Exposure to $AlCl_3$ significantly modulated the expression of proteins associated with the cell cycle, stimulus-response, cytoskeleton, nervous system regulation, protein activity, and synaptic signaling. Therefore, prolonged exposure to a low dose of Al triggered oxidative stress and proteomic changes that may affect spinal cord homeostasis.

1. Introduction

Aluminum (Al) is the third most abundant metal found in the environment (particularly in soils and waters) and comprises about 8% of the Earth's surface. It forms several compounds due to its high affinity for oxygen and other ionic elements (Kumar and Gill, 2014a; Goullé and Grangeot-Keros, 2020). Al is widely used as an adjuvant to improve the immune response of vaccines, and as raw material to produce vehicles, electronics, construction, cosmetics, personal care products, and packages (Fig. 1) (Exley, 2013; Gherardi et al., 2016; Lin et al., 2018; Principi and Esposito, 2018; Sanajou et al., 2021). Welders and workers in some industries are particularly exposed to Al and/or its compounds (Ogawa and Kayama, 2015). Rivers and lakes can be contaminated by Al due to rock erosion, as well as acid rain can solubilize soil minerals with high Al content (Driscoll and Schecher, 1990; Niu, 2018). Although some medications such as antacids and buffered aspirin contain Al, it is mainly ingested through food and drinking water (Krewski et al., 2007; Kumar and Gill, 2014b). Al chloride ($AlCl_3$) is the most well-known salt used as a low-cost coagulant for water treatment (Krewski et al., 2007; Exley, 2013).

Chronic exposure to Al can cause detrimental effects on both environment and the human central nervous system (CNS) (Rondeau et al., 2009; Exley, 2013). Al has been suggested as a severe neurotoxicant that leads to behavioral, neuropathological, and neurochemical alterations associated with cognitive damage (Bondy, 2016; Wahby et al., 2017; Nie, 2018). Our research group recently demonstrated oxidative stress, morphological, and functional changes in the CNS caused by long-term exposure to Al (Fernandes et al., 2020).

The Al-induced neurotoxicity in several CNS areas has been reported; however, potential adverse effects on the spinal cord still need further investigation. Thirty-one pairs of spinal nerves (anterior and posterior short roots) emerge from this very important anatomical structure. The anterior roots mainly control skeletal muscle movements, while the posterior roots carry sensory information (pain, temperature, vibration, and limb position) from the skin, muscles, joints, and viscera to the brain (Hardy, 2021). The sympathetic center is located in the lateral horns of the spinal cord (Th_1-L_2) and modulates cardiovascular and respiratory activities. It has been recently shown that exposure to Al may negatively affect the development of fine motor skills in infants (Ma et al., 2021). Considering the key role of the anterior horns of the spinal cord on body movement and the continuous human exposure to Al, potential pathophysiological mechanisms need to be detailed. Therefore, this animal study aimed to investigate potential adverse effects on both the spinal cord and systemic biochemical balance after prolonged exposure to a low dose of Al.

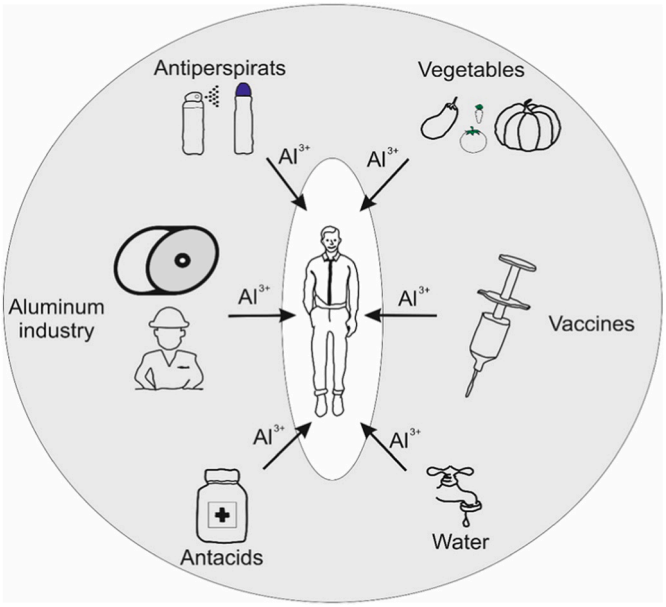

Fig. 1. Environmental and occupational exposure to Al^{3+}.

2. Materials and methods

2.1. Animals and experimental groups

This study was approved by the Ethics Committee for Animal Research of the Federal University of Pará (file number 5923210617) and followed the ARRIVE guidelines 2.0 and the NIH Guide for the Care and Use of Laboratory Animals. Twenty 90-day-old male Wistar rats (*Rattus norvegicus*) weighing about 175–250 g were housed in individual cages at 25 ± 2 °C with lights on from 6:00 to 18:00. Half of the animals received only distilled water (control group), while the other half daily received 8.3 mg of $AlCl_3$ per kg for 60 days (exposed group) through intragastric gavage. The Al exposure dose was determined by the extrapolation method described by elsewhere (Reagan-Shaw et al., 2008) and based on previous studies that used human-relevant dietary levels (Martinez et al., 2017a, 2017b). All animals were weekly weighed to adjust the Al dose.

2.2. Sample collection

The animals were anesthetized with a combination of ketamine hydrochloride (90 mg/kg) and xylazine hydrochloride (10 mg/kg) until total loss of corneal reflexes. Then, blood samples were collected from the left cardiac ventricle by intracardiac puncture and placed in heparinized tubes for oxidative stress analyses. Spinal cord samples were obtained through laminectomy of the dorsal region and immediately stored in liquid nitrogen at −80 °C for further proteomic analysis. All analyses were performed in triplicate (Fig. 2).

2.3. Oxidative stress analyses

2.3.1. Blood analysis

Blood samples were centrifuged at 3500 rpm for 10 min to separate plasma from the red blood cell pellet. Cell debris and remaining plasma were removed through three washes with 0.9% saline solution at room temperature and three centrifugations at 3500 rpm for 10 min. Plasma and erythrocytes were stored at −80 °C in different microtubes and thiobarbituric acid reactive substances (TBARS) and glutathione (GSH) levels were measured. All biochemical tests were done in triplicate.

2.3.2. Spinal cord analysis

Spinal cord samples were thawed, resuspended in 20 mM Tris-HCl buffer solution (pH 7.4) and homogenized through sonication over an ice bath to avoid heat damage in lipids and proteins. The homogenates were centrifuged at 3500 rpm for 10 min at 4 °C to collect the supernatants for further oxidative stress analyses.

2.3.3. Measurement of thiobarbituric acid reactive substances (TBARS)

After filling with 0.5 mL of 10 nM TBA reagent and 250 μL of plasma, erythrocytes, or spinal cord samples, each tube was placed in a water bath at 94 °C for 1 h, cooled to room temperature for 15 min, and then received 4 mL of butyl alcohol. Next, the samples were vortexed to extract polyunsaturated lipid peroxidation products such as malondialdehyde (MDA), which is a subproduct of cell membrane fatty acid degradation. The tubes were centrifuged at 2500 rpm for 10 min and the MDA concentration (nM/g and % of control) was determined by spectrophotometry at 535 nm (Kohn and Liversedge, 1944; Percário, 1994).

2.3.4. Glutathione (GSH) measurement

The modified protocol of Ellman (1959) and Miranda et al. (2018) was used to measure GSH concentration. The supernatant of plasma, erythrocytes, and spinal cord samples was solubilized in a tube with 20 μL of distilled water and 3 mL of PBS-EDTA buffer solution (pH 8.0) and the GSH concentration (μM/μg and % of control) was measured by spectrophotometry at 412 nm. Next, 0.47 mmol 5,5′-dithiobis (2-nitrobenzoic acid) was added to the solution and GSH concentration was measured after 3 min.

2.3.5. Protein concentration

Five μl of each sample (plasma, erythrocytes, and spinal cord) was vortexed with 250 μL of Bradford's reagent (Bradford, 1976). The protein concentration (μg) was measured by spectrophotometry at 595 nm. Bovine serum albumin solutions with known concentrations were used as standards.

2.4. Proteomic analysis

The proteomic analysis followed sample homogenization, protein extraction, reduction, alkylation, digestion, desalination, and purification (Bittencourt et al., 2019; Corrêa et al., 2020). The samples were cryofracture in liquid nitrogen and a lysis buffer at 4 °C [7 M urea and 2 M thiourea diluted in ammonium bicarbonate (AMBIC); BioRad, USA] was added to extract proteins, which were quantified by using the Bradford assay. Then, each sample was incubated at 37 °C for 30 min with 10 μL of 50 mM AMBIC and 25 μL of 0.2% RapiGEST™ (Waters Co., Manchester, UK). Next, each sample was added with 2.5 μL of 100 mM dithiothreitol and incubated at 37 °C for 60 min. Finally, 2.5 μL of 300 mM iodoacetamide (BioRad, USA) was added to each sample and incubated at room temperature for 30 min.

Protein digestion was induced by adding 10 μL of trypsin (Thermo Fischer, USA) and 10 μL of 5% trifluoroacetic acid (Sigma-Aldrich, USA) for 90 min at 37 °C. After 14-h incubation at 37 °C, the samples were centrifuged at 14,000 rpm for 30 min at 6 °C and porous spin columns (Pierce C18, Thermo Fischer, USA) were used to collect and purify the supernatants. Peptides were read by using a UPLC-Xevo QTof MS system and the Protein Lynx Global Server (PLGS) software (Waters, Manchester, UK), and proteins were identified through the Uniprot.org database. Unique, up-, and down-regulated proteins were identified in each group. Biological interpretations based on Gene Ontology (GO) were performed by using the ClueGO plug-in (Cytoscape v. 3.8.2, Java®) (Bindea et al., 2009).

2.4.1. Over-representation analysis (ORA)

Proteins observed in the exposed group with expression 50% higher or lower than the control group were analyzed (log2 ratio values ≤ -0.58 or ≥ 0.58). Proteins with absolute changes were assigned as −1 (only expressed in the control group) and 1 (only expressed in the exposed group). The ORA analysis was performed by using the R studio software with the EGSEA package (Alhamdoosh et al., 2017). The Uniprot.org database available by Bader Lab was consulted to identify proteins and associated biological processes. The enrichment map plug-in (Cytoscape v. 3.8.2, Java®) was used to group proteins and select the main biological processes for graphic analysis. The Network Analyst (https://www.networkanalyst.ca/) was used to observe protein-protein interactions (Xia et al., 2014), which were graphically represented with aid of GOplot plug-in and R studio software.

2.5. Statistical analyses

The Shapiro-Wilk test was used to assess the normality of oxidative stress analysis data and the Student's t-test was used to compare groups at a significance level of $p < 0.05$ (GraphPad Prism 7.0). The results were expressed as mean (± standard error of the mean) and converted to the percentage of control for graphing. All identified proteins were tabulated and used for GO analysis (Excel, Microsoft). The Monte-Carlo algorithm was used in the PLGS software to observe protein expression differences in the groups: negatively ($p < 0.05$) and positively regulated proteins ($1 - p > 0.95$).

3. Results

3.1. Results on oxidative stress in blood and spinal cord

In both plasma and erythrocytes, GSH level was not significantly different between groups ($p = 0.8558$ and $p = 0.9932$, respectively).

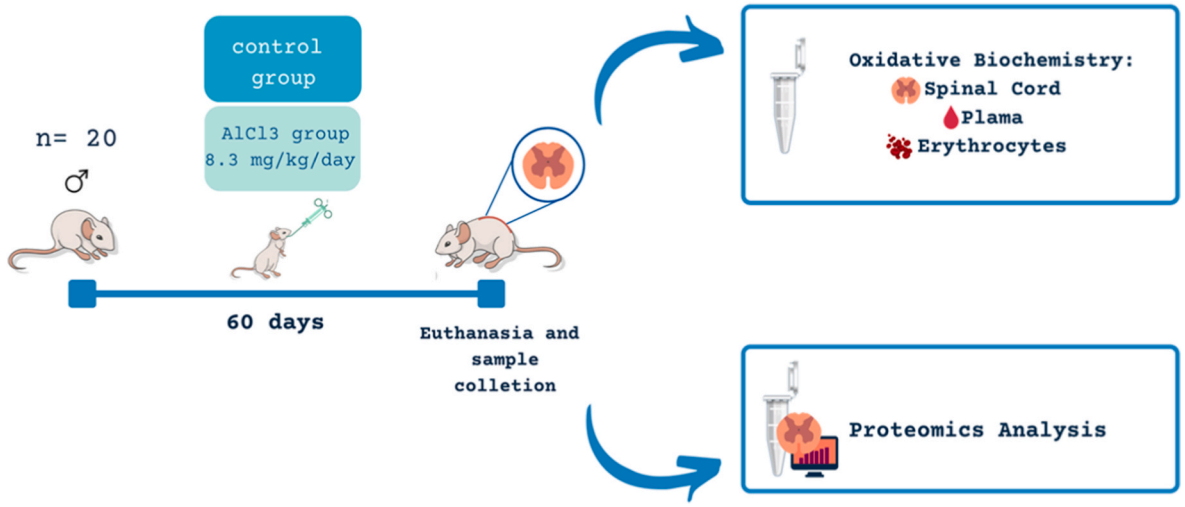

Fig. 2. Schematic explaining the study setup. Adult rats received distilled water (control) or $AlCl_3$ through intragastric gavage. After 60 days, plasma and spinal cord samples were collected for oxidative stress and proteomics analysis.

Conversely, exposure to $AlCl_3$ significantly decreased the GSH level in the spinal cord (48.82%; $p = 0.0004$). TBARS level in the erythrocytes of animals exposed to $AlCl_3$ was similar to the control group ($p = 0.4058$); however, TBARS levels in plasma (82.2%; $p = 0.0014$) and spinal cord (39.1%; $p = 0.0003$) of animals from the control group were significantly lower than those animals exposed to $AlCl_3$ (Fig. 3).

3.2. Results on proteomic profile in spinal cord

A total of 65 and 78 proteins were found up- and down-regulated, respectively (Supplementary Table 1). Moreover, 69 and 50 proteins were exclusively expressed in the control and exposed groups, respectively. Some relevant proteins with altered expression are listed in Table 1. The GO-based analysis revealed the 12 biological processes shown in Fig. 4.

The GO-based analysis of spinal cord biological processes revealed 12 functional categories (Fig. 4): Generation of precursor metabolites and energy (15%), Export from cell (14%), Organelle localization (13%), Regulation of exocytosis (10%), Axonal development (10%), Rab protein signal transduction (8%) Pyruvate metabolic process (7%), Negative regulation of endopeptidase activity (6%), Positive regulation of ion transmembrane transport (5%), MAPK cascade (4%), Regulation of blood vessel diameter (4%), and Postsynapse organization (4%).

3.3. ORA analysis

The ORA analysis revealed 52 proteins with a high level of protein-protein interactions related to the following biological processes: cell cycle, cytoskeleton regulation, stimulus-response, nervous system regulation, protein activity, and synaptic signaling (Fig. 5).

4. Discussion

To the best of our knowledge, this is the first study to bring the global proteomic approach of the spinal cord of rats exposed to urban-like and tolerable Al concentrations to which humans are exposed, besides the

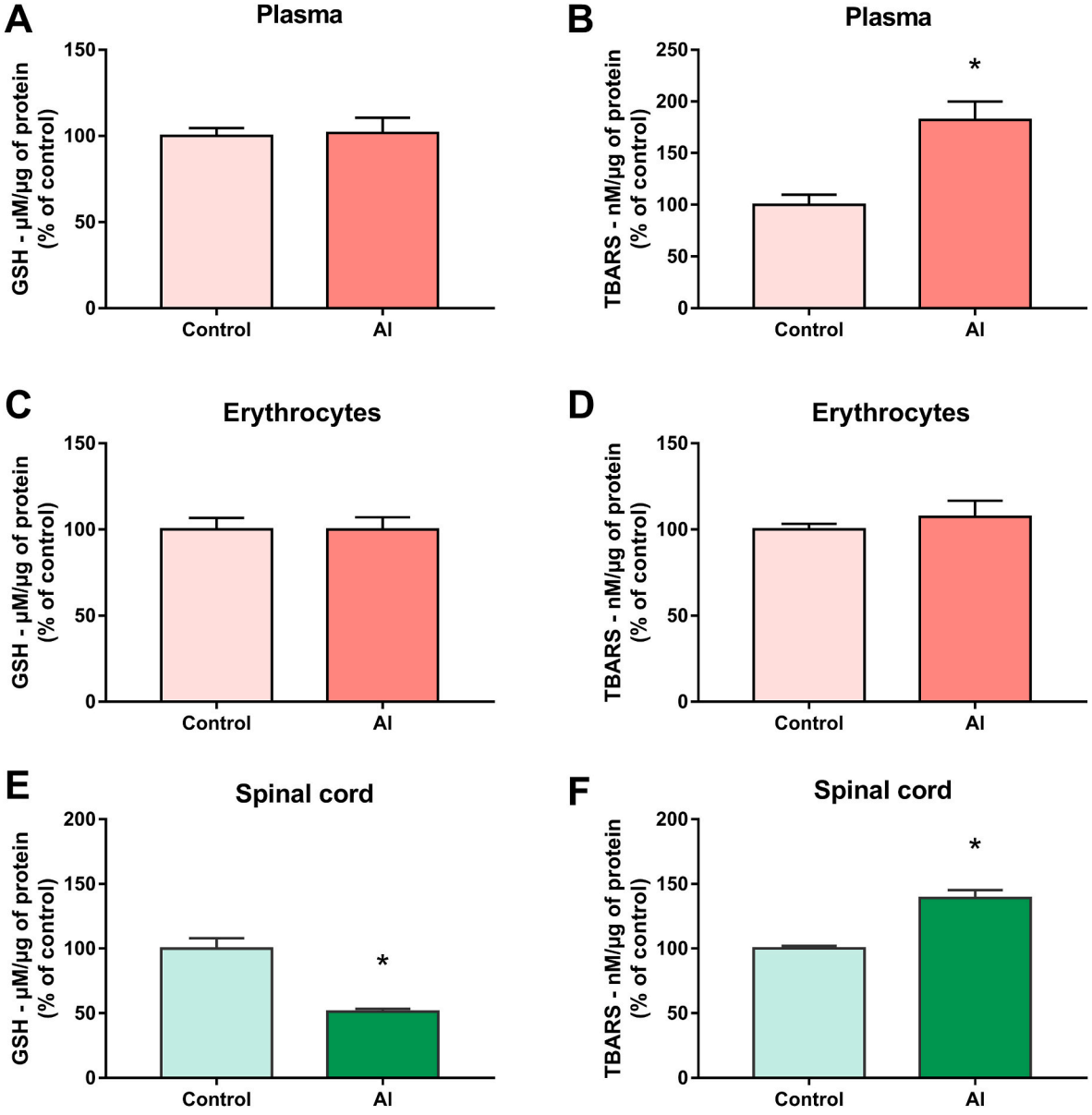

Fig. 3. Oxidative stress analyses of the blood and spinal cord of rats exposed to $AlCl_3$ for 60 days. The graphs represent, as relative percentage of the control group, the means and standard errors of the oxidative stress 'parameters: A- GSH levels and B- TBARS levels (plasma); C- GSH levels and D- TBARS levels (erythrocytes); E– GSH levels and F- TBARS levels (spinal cord). n = 10. * Student's *t*-test, $p < 0.05$.

Table 1
Altered proteins obtained from the spinal cord of rats exposed or not to $AlCl_3$.

Accession ID [a]	Protein Name	PLGS Score	Ratio Al: control [b]
P09812	Glycogen phosphorylase_ muscle form	45	2.32
P60203	Myelin proteolipid protein	12,280	2.25
Q63345	Myelin-oligodendrocyte glycoprotein	1888	1.88
P13233	2'_3′-cyclic-nucleotide 3′-phosphodiesterase	12,721	1.60
P85108	Tubulin beta-2A chain	17,640	1.60
Q3KRE8	Tubulin beta-2B chain	17,536	1.60
P48500	Triosephosphate isomerase	3189	- 0.85
P61765	Syntaxin-binding protein 1	1081	- 0.84
P04906	Glutathione S-transferase P	1230	- 0.84
P06907	Myelin protein P0	1792	- 0.82
P02688	Myelin basic protein	17,541	- 0.76
P11951	Cytochrome *c* oxidase subunit 6C-2	313	+
Q63754	Beta-synuclein	271	+
Q63544	Gamma-synuclein	163	+
P60192	SNARE-associated protein Snapin	122	+
O88600	Heat shock 70 kDa protein 4	105	–
Q63537	Synapsin-2	92	–
P35289	Ras-related protein Rab-15	65	–
+244 proteins with different status of regulation			

[b] Positive and negative ratio values indicate up- and down-regulated proteins, respectively. Positive (+) and negative (−) signs indicate exclusive expression in the exposed and control groups, respectively.

characterization of oxidative stress state in the organ and systemically by analyzing the blood. In fact, little is known about how susceptible the spinal cord is to xenobiotics, including metals such as Al, therefore, we addressed important and novel results regarding molecular targets of Al neurotoxicity. Our results pointed that Al, in this model of exposure, modulated proteins of synaptic signaling, morphology maintenance, cell metabolism and cell death in the spinal cord of rats, suggesting a generalized compromise of the neural microenvironment and the organ homeostasis.

Since oxidative stress seems a common pathway of Al toxicity (Ćirović et al., 2021; Fernandes et al., 2021), this study first evaluated whether the exposure model could modulate the local and systemic oxidative parameter known as reduced GSH, an important non-enzymatic antioxidant that helps to control the oxidative stress, and the lipid oxidation through TBARS levels both at plasma, erythrocytes, and spinal cord. The results suggested that as well as the cerebellum, the spinal cord is more sensitive to Al toxicity than the blood since the GSH level only decreased in the spinal cord. Conversely, TBARS levels were increased in both the plasma and spinal cord, which is corroborated by a previous study (Fernandes et al., 2021). The alterations in the metabolism of lipids may result in detrimental consequences in the structure and functions of cell membranes and represent a potential mechanism of neuronal damage (Ledesma et al., 2012).

The oxidative stress diagnosed in the spinal cord in this work was also found at the same dose, in papers that evaluated the hippocampus (Martinez et al., 2019; Fernandes et al., 2020). Although we did not perform tissue and functional analyzes of the spinal cord, another study showed that Al was able to promote an increase in ROS levels and lipid peroxidation, in addition to decreasing the antioxidant capacity of the hippocampus, which corroborates our results (Martinez et al., 2019). Furthermore, Al was able to significantly increase its levels in hippocampal tissue parenchyma associated with a deficit in long-term memory (Fernandes et al., 2020).

The proteomic approach revealed the modulation of proteins related to metabolism and energetic status such as Glutathione S-transferase P (P04906), cytochrome *c* oxidase subunit 6C-2 (P11951), Glycogen phosphorylase, muscle form (P09812), and Triosephosphate isomerase (P48500). The P04906 detoxifies reactive chemical species after xenobiotics exposure (Bocedi et al., 2019). This metabolic enzyme acts in combination with reduced GSH to protect cells from oxidative stress (Niu et al., 2009). The decreased expression or inhibition of this enzyme activity has been associated with the increased oxidative imbalance and decreased cell antioxidant mechanism (Circu and Aw, 2012). This study revealed that A1 exposure decreased both GSH levels in the spinal cord and expression of P04906; in addition, the modulation of P09812 was increased after Al exposure. The Al-induced oxidative stress suggested cellular energy impairment; thus, the increased expression of this

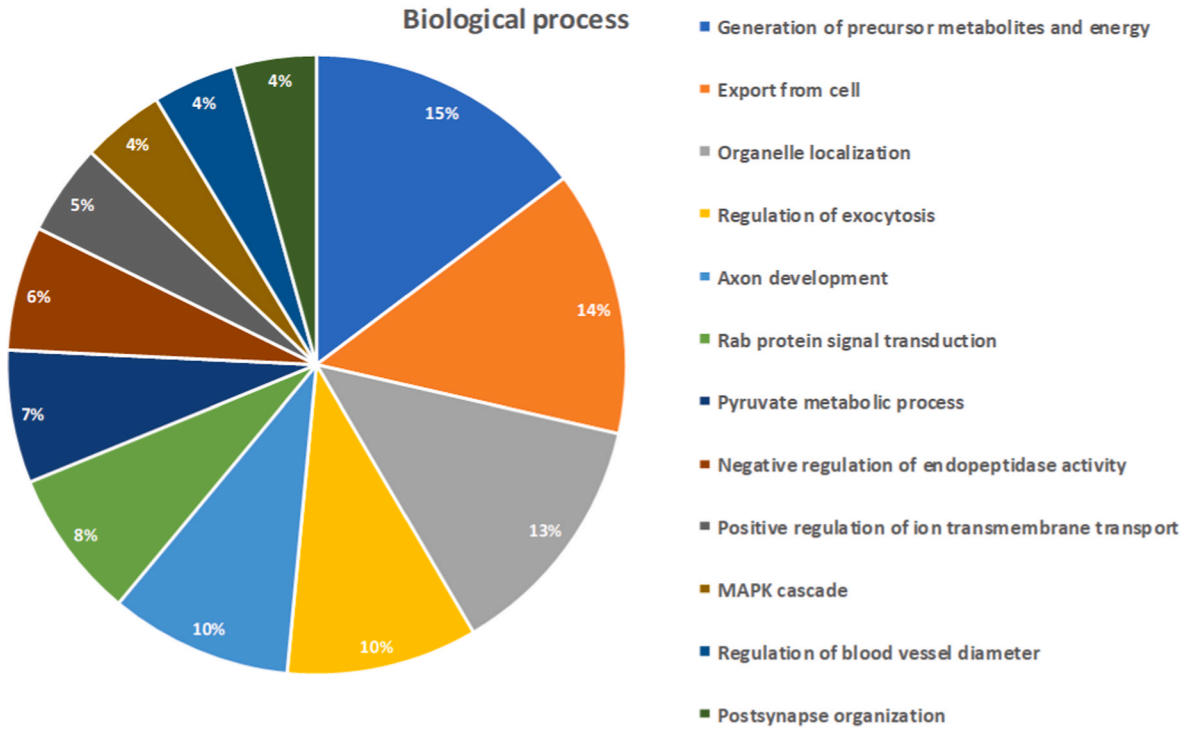

Fig. 4. Biological processes of proteins expressed in the spinal cord of rats exposed to $AlCl_3$ for 60 days.

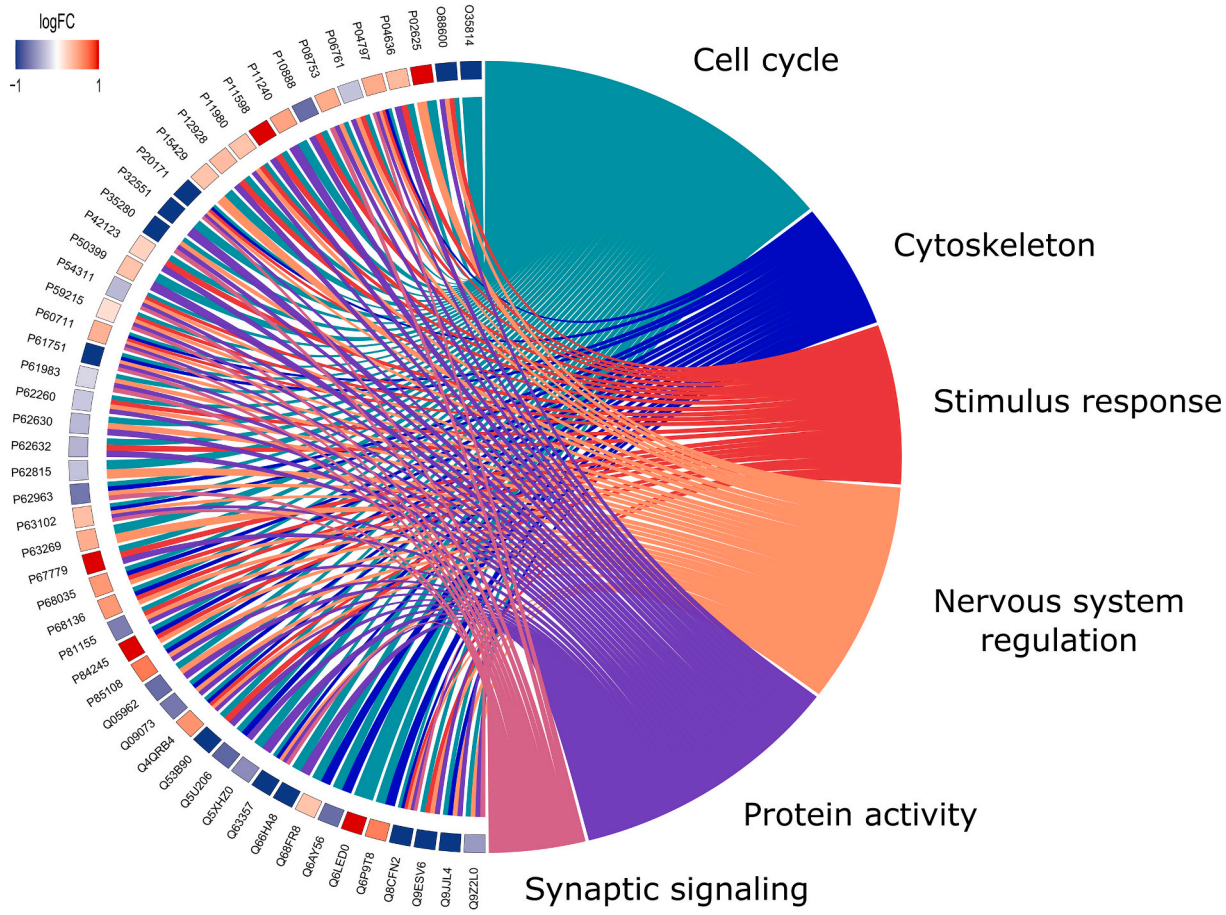

Fig. 5. Circos plot of 52 proteins with a high level of protein-protein interactions related to cell cycle (green), cytoskeleton regulation (blue), stimulus-response (red), nervous system regulation (orange), protein activity (purple), and synaptic signaling (pink).

protein may indicate an attempt to maintain the cell alive through the breakdown of stored glycogen for energy production (Dua et al., 2010). In addition, it was also observed a reduced regulation of P48500, which plays a key role in mediating a protective energy metabolism during oxidative stress (Grüning et al., 2014). Therefore, overall data confirms that even 8.3 mg/kg/day of $AlCl_3$ induced oxidative stress in the spinal cord of rats.

Oxidative stress has been suggested as a marker for spinal cord injury and is one of the most important mechanisms involved in amyotrophic lateral sclerosis that is characterized by a significant neurodegenerative pattern (Jia et al., 2013; Pollari et al., 2014). Interestingly, this proteomic data also revealed significant modulation of nervous system regulation pathways, such as axon development, post-synapse organization, synaptic vesicle maturation, neurotransmitter secretion, exocytic process, neurogenesis, and others. Hence, the maintenance of myelinated axons in the spinal cord is crucial for somatosensorial functions (Bercury and Macklin, 2015). The proteomic analysis revealed the upregulation of both Myelin-oligodendrocyte glycoprotein (Q63345) and Myelin proteolipid protein (P60203), which are constituents of the myelin sheath. The Q63345 is also found in the membrane of oligodendrocytes and plays an important role as a cell surface receptor or cell adhesion molecule during the inflammatory process of demyelinating disorders (Peschl et al., 2017). Moreover, the P60203 is the most abundant protein in the CNS and mainly maintains the lamellar structure of myelin sheath; however, its gene overexpression has been associated with neurodegenerative diseases characterized by demyelination or hypomyelination (Lin and Goodman, 1994). Furthermore, the downregulation of both Myelin basic protein (P02688) and Myelin protein P0 (P06907), which are important to myelin compaction, has been previously associated with neurodegenerative processes (Martini et al., 1995; Bittencourt et al., 2021). In this perspective, the modulation of these proteins may indicate spinal cord damage triggered by a toxicant. It must be emphasized that the metal would initially contact the lipidic myelin sheath and easily cross the blood-brain barrier; thus, the damage could be even aggravated by lipid peroxidation and affect nerve signal conduction and neuronal communication (Bican et al., 2013).

The morphology of neurites is determined by microtubules, actin filaments, and neurofilaments that are abundant in axons and dendrites and cross-linked by the heterogeneous class of microtubule-associated proteins (MAPs) (Kirschner, 1978). Studies have shown a variety of neuropathological disorders that affect the cytoskeleton such as Alzheimer's disease and amyotrophic lateral sclerosis, in which occur neurofilament aggregation (Gordon, 2020). Tubulin beta-2A chain (P85108) and Tubulin beta-2b chain (Q3KRE8) were observed in this study. These proteins are the major components of microtubules that in turn form the cytoskeleton. The cytoskeleton forms a large protein network that is highly dynamic and involved in plasticity, transport, and cell signaling; in addition, it is the main determinant of cell morphology (Roll-Mecak, 2020). The neuronal cytoskeleton is involved in mechanisms for the anterograde axonal transport of molecules synthesized in the perikaryon, particularly the microtubules and their associated motor proteins. Whereas actin filaments seem fundamental for short-distance anterograde axonal transport (Kuznetsov et al., 1992), the role of neurofilaments is not fully understood. Thus, redox imbalance can alter several cell structures such as those associated with the cytoskeleton (Aragão et al., 2017).

Finally, the limitations of this study may encourage further investigations. It has been shown in the present study that the systemic

and local oxidative stress seems to be intrinsically associated with the modulation of molecular components involved in biochemical and morphological aspects in spinal cord. Although the potential effects of these biochemical and proteomic alterations on neuronal and glial cells viability and/or death were not evaluated, the literature shows that biochemical balance is crucial for key functions of the nervous system. Novel questions arise from the alterations observed in this study that may trigger other functional and morphological damage.

5. Conclusion

The spinal cord of rats has undergone modulation on different several biological levels after even by prolonged oral exposure to Al at a low dose. This study provided novel evidence about to the scarce literature on the effect of Al in the spinal cord, and modulation of proteomic profile modulation and through the oxidative stress triggering pathway activation; thus, specific mechanisms of Al-induced neurotoxicity in the spinal cord were better understood, albeit the repercussions of this damage on the spinal cord morphology and function remain unclear, showing that the spinal cord is susceptible to Al-induced neurotoxicity and points out the need for more studies to elucidate the possible neurodegenerative and functional repercussions of the results showed here.

Credit author statement

Conceptualization: L.E.Q., W.F.L., W.A.B.A.; Methodology: R.M.F., L. E.Q., W.A.B.A., A.D., L.O.B., P.F.S.M. C.A.R.; Formal analysis: L.O.B., R. R.L., R.M.F. W.A.B.A. P.F.S.M.; Investigation: L.E.Q., L.O.B., R.M.F. W.A. B.A. P.F.S.M. R.R.L.; Resources: R.R.L., M.A.R.B.; Data curation: L.E.Q., L.O.B., R.M.F. W.A.B.A. P.F.S.M..; Writing – original draft preparation: L.E.Q., L.O.B., W.A.B.A. P.F.S.M., B.P.; Writing, Writing – review & editing: L.E.Q., L.O.B., W.A.B.A. P.F.S.M., B.P., A.C., A.C.; Visualization: R.R.L., B.P., M.C.M.; Supervision: R.R.L.; Project administration: R.R.L.; All authors have read and agreed to the published version of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.137296.

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