Aluminum Exposure for 60 days at Human Dietary Levels Impairs Spermatogenesis and Sperm Quality in Rats

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Abstract

Concerns about environmental aluminum (Al) and reproductive health have been raised. We investigated the effects of Al exposure at a human relevant dietary level. Experiment 1 (Lower level) rats were treated orally for 60 days: a) control – ultrapure water; b) aluminum at 1.5 mg/kg bw/day and c) aluminum at 8.3 mg/kg bw/day Experiment 2 (High level) rats were treated for 42 days: a) control – ultrapure water; b) aluminum at 100 mg/kg bw/day Al decreased sperm count, daily sperm production, sperm motility, normal morphological sperm and impaired testis histology. Al increased oxidative stress in testis, epididymis and prostate and inflammation in testis. Our study shows for the first time the specific presence of Al in the germinative cells and, that low concentrations of Al in testes (3.35 μg/g) are sufficient to impair spermatogenesis and sperm quality. Our findings provide a better understanding of the reproductive health risk of Al in the environment. However, further studies are necessary to fully address these discoveries.

Keywords: metal; reproductive adverse effects; sperm quality.

Introduction

Human exposure to aluminum (Al) is inevitable, and its real consequence is largely unknown. After oxygen and silicon, Al is the third most abundant element in the Earth´s crust and the increased biological availability of this metal is due to natural and anthropogenic actions over the years (Exley 2012, 2013).

People are exposed to Al through dietary and non-dietary sources. Al salts are added to various commercially-available foods, are used as a flocculants in the treatment of drinking water and in the storage of food products (Fekete et al. 2013). Humans are also exposed to considerable amounts of Al by non-dietary sources such as Al adjuvant in vaccines, medicines, cosmetics, sunscreens, deodorants and make up products (Bondy 2015).

In 2007, the tolerable weekly intake of Al for humans was adjusted to 1 mg Al/kg body weight (b.w.) (Food and Agriculture Organization of the United Nations / World Health Organization, FAO/WHO, 2007). However, it is known that humans may exceed health-based guidance values (Fekete et al. 2013; Gonzalez-Weller et al. 2010; Yang et al. 2014).

Even with a low rate of Al absorption through the gastrointestinal tract (Powell and Thompson 1993), taking account the overall sources of Al exposure, humans are continuously exposed to considerable and partly estimated amounts of Al every single day. Benefits are lacking between the interaction of this non-essential metal with normal biomolecules, making this body burden of Al potentially toxic (Exley 2013).

Over the last years, concerns have increased about Al exposure and its relation with reproductive health (Dawson et al. 1998; Hovatta 1998; Jamalan et al. 2016). The decline of sperm quality and increases in infertility have been observed over recent decades (Carlsen et al. 1992; Nelson and Bunge 1974; Sheiner et al. 2003), which suggests the involvement of environmental contributors to this phenomenon. Sperm health after Al exposure has been investigated; however, the findings, to date, are inconsistent (Dawson et al. 1998; Mur et al. 1998). Recently, Al content in human sperm was related to reduction in sperm quality. Specifically, patients with oligozoospermia had higher Al concentration than others (Klein et al. 2014). Experimental studies in animal models of Al intoxication support the human studies and show that Al exposure seems to be related to hormonal imbalance, decreases in sperm quality, histological abnormalities in reproductive organs and infertility (Ige and Akhigbe 2012; Mohammad et al. 2015)

91 However, studies addressing reproductive effects of Al have been conducted with doses of Al
92 higher than might commonly be found among human populations (Oda 2016; Sun et al. 2011; Zhu et al.
93 2014). Moreover, due to the suggested biphasic effect of Al (Exley and Birchall 1992), it is urgent to
94 investigate the effects of Al exposure at human dietary levels and then to compare with Al effects at high
95 levels. Herein we investigated the effects of Al exposure at three different doses: two low doses representing
96 human Al exposure through the diet and, one model of exposure at a high Al level known to produce
97 toxicity.

98 **Methods**

99 *Animals*

100 Three-month-old male *Wistar* rats (362.5 ± 11.7 g) were obtained from the Central Animal
101 Laboratory of the Federal University of Santa Maria, Rio Grande do Sul, Brazil. During treatment, rats
102 were housed at a constant room temperature, humidity, and light cycle (12:12h light-dark), giving free
103 access to water and fed with a standard chow *ad libitum*. All experiments were conducted in compliance
104 with the guidelines for biomedical research stated by the Brazilian Societies of Experimental Biology and
105 approved by the Ethics Committee on Animal Use Experimentation of the Federal University of Pampa,
106 Uruguaiana, Rio Grande do Sul, Brazil (Process Number: 028/2014).

107 Rats were divided into two major groups, according to Martinez et al. (2017): Experiment 1 - low
108 aluminum levels, and Experiment 2 - high aluminum level. For group 1, 18 rats were subdivided (in groups
109 of six animals) and treated for 60 days as follows: a) the control groups received ultrapure drinking water
110 (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) the second group
111 received aluminum at 1.5 mg/kg bw/day based on human dietary levels according to a published protocol
112 described by Walton (2007), at the reduced Al exposure for 60 days, and c) the third group drank aluminum
113 at 8.3 mg/kg bw/day which corresponds to the same aluminum human dietary levels (1.5 mg/kg) when
114 translated to an animal dose based on body surface area normalization method (Reagan-Shaw et al. 2008).
115 For experiment 2, (the high aluminum level), 12 rats were subdivided (N=6/each) and treated for 42 days
116 as follows: a) the control group received ultrapure water through oral gavages; b) aluminum at 100 mg/kg
117 bw/day (Prakash and Kumar 2009).

118 Rat body weights, feed, water and Al intakes were measured weekly. At the end of the treatments,
119 animals were euthanized by decapitation and the weights of testis, epididymis, prostate, vas deferens and
120 seminal vesicle (empty, without coagulation gland), were determined. The right testis, epididymis and left

121 vas deferens were used for sperm parameter analysis. Left testis and epididymis were divided in two
122 segments, one of each was processed for histological and or immunohistochemical studies and the other
123 part together with the prostate were quickly homogenized in 50 mM Tris HCl, pH 7.4, (5/10, w/v) for
124 biochemical determinations. Afterwards, samples were centrifuged at 2400g for 10 min at 4°C and the
125 resulting supernatant fraction was frozen at −80°C for further assay.

126 $AlCl_3$. 6 H_2O was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure
127 water (Milli-Q © 2012 EMD Millipore, Billerica, MA). The concentration of each stock solution was 0.008
128 mol/L, 0.034 mol/L and 0.331 mol/L, respectively from Al 1.5, 8.3 and 100 mg/kg bw. Salts and reagents
129 were of analytical grade obtained from Sigma and Merck (Darmstadt, Germany).

130 Sperm Parameters Analysis

131 *Daily sperm production per testis, sperm number and transit time in epididymis*

132 Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the
133 caput/corpus epididymis and cauda epididymis were counted as described by Robb et al. (1978). To
134 calculate daily sperm production, the number of spermatids at stage 19 was divided by 6.1, which is the
135 number of days these spermatids are present in the seminiferous epithelium. The sperm transit time through
136 the epididymis was determined by dividing the number of sperm in each portion by the daily sperm
137 production (Robb et al. 1978).

138 *Sperm morphology*

139 Sperm were obtained from the vas deferens and stored with 1 mL of 10% formal-saline until
140 analysis. For the analysis, smears were prepared on histological slides and 200 spermatozoa per animal
141 were evaluated under 400X magnification (Binocular, Olympus CX31). Morphological abnormalities were
142 classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail),
143 according to Filler (1993).

144 *Sperm motility*

145 Sperm were removed from the vas deferens by internal rising with 1 mL of Human Tubular Fluid
146 (DMPBS-Nutricell-SP-Brazil) pre-warmed to 34ºC. Then, a 10 µL aliquot was transferred to a histological
147 slide. Under a light microscope (20X magnification, Binocular, Olympus CX31, Tokyo, Japan), 100
148 spermatozoa were analyzed and classified as type A: motile with progressive movement, type B: motile
149 without progressive movement and type C: immotile. Sperm motility was expressed as % of total sperm
150 (Martinez et al. 2014).

151 Biochemical Assay

152 *Reactive oxygen species levels*

153 The levels of reactive species (RS) in testis, epididymis and prostate were determined by a
154 spectrofluorometric method, as described by Loetchutinat et al. (2005). This method is unspecific for
155 reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The supernatant fraction
156 of the sample was diluted (1:10) in 50 mM Tris-Hcl (pH 7.4) and 2′, 7′-dichlorofluorescein diacetate
157 (DCHF-DA; 1mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular
158 esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2′,7′-
159 dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the
160 amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480
161 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS
162 levels were expressed as fluorescence units.

163 *Lipid peroxidation*

164 The levels of lipid peroxidation in testis, epididymis and prostate were measured as malondialdehyde
165 (MDA) using a colorimetric method, as previously described by Ohkawa et al. (1979), with modifications.
166 An aliquot of each tissue was incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1%
167 (H_3PO_4), and sodium dodecil sulphate 0.8% (SDS) at 100ºC for 60 min. The color reaction was measured
168 at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as
169 nanomoles of MDA per mg of protein.

170 *Ferric Reducing/Antioxidant Power (FRAP) Assay*

171 The total antioxidant capacity was measured in testis, epididymis and prostate by FRAP assay
172 (Benzie and Strain 1996). This method is based on the ability of the sample to reduce ferric ion (Fe^{3+}) to
173 ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s- triazina (TPTZ) the chelate complex Fe^{+2}-TPTZ.
174 Briefly, 10 μL of the supernatant fraction of each tissue was added to 1 mL freshly prepared and pre-
175 warmed (37◦C) FRAP reagent (300mM acetate buffer (pH = 3.6), 10mM TPTZ in 40mM HCl, and 20mM
176 $FeCl_3$ in the ratio of 10:1:1) in a test tube and incubated at 37◦C for 10min. The absorbance of the blue-
177 colored complex was read against a blank reagent (1 mL FRAP reagent + 10 μL distilled water) at 593 nm
178 (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50-1000 µM
179 – water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented
180 with particular reference to Trolox equivalents.

Testis and epididymis histology

To carry out the histological studies. Epididymis tissues were dehydrated, ~~in~~ xxxx NO HACE FALTA DECIR NADA fixed in 10 % formaldehyde and testis in Bouin's solution for 1–2 days. After several intensive washings, tissues were embedded in paraffin, sectioned at 5 µm and stained with hematoxylin/eosin. Tissues were studied under a Zeiss Axioskop 2 microscope (Zeiss, Jena, Germany) equipped with the image analysis software package AxioVision 4.6 to evaluate the morphometric parameters in testis: thickness of the seminiferous epithelium (µm) and the average number of empty seminiferous tubules/field as well as in the epididymis the average number of efferent ~~ductos~~ ducts /field. The analysis was made in 10 random fields of 8 samples for each group, analysing approximately 7 seminiferous tubules per field and 5 efferent ~~ductos~~ ducts per field of epididymis, in 20X magnification per section.

Testis immunohistochemistry

Testis immunohistochemistry was performed on paraffin-embedded sections of 5 µm thickness. De-~~paraffinzed~~ 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, used as a negative control. AQUÍ BASTARÍA DECIR: as a negative control, preparations were incubated without the primary antibody.

Aluminum content in testis and epididymis

The Al content of testis and epididymis were determined using an established method (House et al. 2012). Briefly, approximately 0.5g and 0.3g of testis and epididymis, were dried to a constant weight at 37 ºC. Dried and weighed tissues were digested in a 1:1 mixture of 15.8M HNO_3 and 30% *w/v* H_2O_2 in a microwave oven (MARS Xpress CEM Microwave Technology Ltd). Upon cooling each digest was diluted

211 to a total volume of 5 mL with ultrapure water (cond<0.067 <µS/cm) and the Al content of digests measured
212 by TH GFAAS (Transversley Heated Graphite Furnace Atomic Absorption Spectrometry) using matrix-
213 matched standards and an established analytical programme (House et al. 2012). Briefly, the TH GFAAS
214 was calibrated by automated serial dilution of 40, 60 and 100 mg µg L^{-1} solution of Al with 1% HNO_3.
215 Non-linear zero intercept WinLab 32-generated fits were applied (Perkin Elmer, UK). Instrument detection
216 limits (IDL) were estimated from three times the standard deviation on the 1% HNO_3 calibration blank
217 absorbance (n = 3 injections) divided by the Winlab32 generated calibration slope. Mean IDL for Al was
218 0.13 µg L^{-1} (SD 0.13 µg L^{-1}, n=62). Concentrations of Al in NIST SRM1566B oyster tissue and IAEA-407
219 fish homogenate were used as spike samples and standard reference material. Results were expressed as µg
220 Al/g tissue dry weight. Each determination was the arithmetic mean of a triplicate analysis.

221 *Lumogallion staining*

222 Lumogallion staining was performed in bouin and formalin-fixed testis and epididymis using a
223 recent validated method to identify the presence of Al in tissues (Mirza et al. 2016; Mold et al. 2014).
224 Briefly, re-hydrated tissues sections were immediately placed into either 1 mM lumogallion (TCI Europe
225 N.V. Belgium) buffered in 50 mM PIPES, pH 7.4 or the PIPES-buffer alone for auto-fluorescence analyses
226 for 45 minutes. Slides were carefully washed 6 times with PIPES-buffer, after rinsed in ultra-pure water for
227 30 seconds, finally mounted using an aqueous mounting media and stored horizontally at 4ºC overnight
228 prior to imaging. Sections of tissues were imaged using a Zeiss Axioskop 2 microscope (Zeiss, Jena,
229 Germany) equipped with the image analysis software package AxioVision 4.6.

230 *Statistical analysis*

231 Data are expressed as mean ± SEM. Data of group 1 were analysed by ANOVA followed Bonferroni
232 post hoc tests when appropriate and for sperm motility analysis Kruskal-Wallis test followed by Dunn's
233 multiple comparisons test. Data of group 2 were analysed by Student´s t-test and Mann-Whitney test for
234 motility data. Values of $p < 0.05$ were considered significant.

235 **Results**

236 *Body and organs weights, fluid and feed intake*

237 Body weight of rats was similar between groups at the start and end of treatments (362.2 ± 11.7;
238 434.7 ± 11.1g means at the start and end, respectively). Al exposure at low levels (group 1) did not change
239 the absolute and relative reproductive organ weights. However, Al at 100 mg/kg bw/day decreased the
240 weight of the ventral prostate (control: 415.8 ± 21.4 *vs* Al 100 mg/kg bw/day: 351.1 ± 21.7 mg, *P < 0.05

241 - Table 1). The quantity of water, Al intakes and feed intake were not different between groups (P > 0.05;
242 one-way ANOVA / t-test, data not shown). STATE MEAND AND SD

243 *Daily sperm production per testis, sperm number and transit time in epididymis*

244 To investigate the effect of Al on sperm count, group 1 rats were treated for 60 days with Al at 1.5
245 or 8.3 mg/kg bw/day and group 2 rats were exposed to Al at 100 mg/kg bw/day for 42 days, and the control
246 rats were treated with ultrapure water. Chronic exposure to Al at different doses altered sperm parameters
247 in testis, there was a reduction in daily sperm production per testis and in sperm count (Table 2). In the
248 epididymis of group 1 rats, Al increased the sperm transit time in the caput/corpus and there was an apparent
249 decrease in sperm number, which was not statistically significant (mean of total sperm in epididymis for
250 group 1 control: 318.8, Al 1.5 mg/kg bw/day: 272.3, Al 8.3 mg/kg bw/day: 279.7 $x10^6$; group 2 control:
251 308.3, Al 100 mg/kg bw/day: 273.2 $x10^6$, P > 0.05, see more details in - Table 2).

252 *Sperm morphology and motility*

253 Sperm analysis revealed a significant decrease in sperm with normal morphology in rats exposed to
254 Al when compared with the control group (group 1: control: 92.5 (92 – 94.3), Al 1.5 mg/kg bw/day: 89.2
255 (85.6 – 92.2)* Al 8.3 mg/kg bw/day: 83 (74.8 – 88)*; group 2: control: 94 (89.63 – 96.13), Al 100 mg/kg
256 bw/day: 84 (81.38 – 87.75)*, - Table 3). Group 1 rats treated for 60 days with Al 8.3 mg/kg bw/day and
257 group 2 rats exposed to Al at 100 mg/kg bw/day, for 42 days, showed specific abnormalities. Within head
258 phenotypes, amorphous, banana and detached head were observed; concerning tail morphology, the bent
259 tail was the most frequency abnormality in rats exposed to Al at major doses (mean of total sperm
260 abnormalities for group 1 control: 6.18, Al 1.5 mg/kg bw/day: 10.58, Al 8.3 mg/kg bw/day: 15.33; group 2
261 control: 6.58, Al 100 mg/kg bw/day: 14.41% *P < 0.05, see more details in - Table 3).

262 Regarding sperm motility, for group 1, Al exposure at the lowest dose of 1.5 mg/kg bw/day did not
263 affect the motility (Figure 1A). On contrast, Al exposure at 8.3 mg/kg bw/day, for 60 days, and rats exposed
264 to Al at 100 mg/kg bw/day, for 42 days, decreased type A sperm (motile with progressive movement)
265 accompanied by an increase in type B (motile without progressive movement) and type C sperm (immotile)
266 (mean of total motile sperm for group 1 control: 85.66, Al 1.5 mg/kg bw/day: 75, Al 8.3 mg/kg bw/day:
267 59.67; group 2 control: 85.16, Al 100 mg/kg bw/day: 64% *P < 0.05, see more details in - Figure 1A and
268 B).

269 *Reactive species and lipid peroxidation levels*

270 Al treatment at different doses increased the levels of reactive species (RS) in epididymis (Figure
271 2C and 2D) and in prostate (Figure 2E and 2F), while in testis only Al at 8.3 mg/kg bw/day and 100 mg/kg
272 bw/day altered this oxidative stress parameter (Figure 2A and 2B).

273 There was a significant increase in lipid peroxidation in testis of Al treated rats at all doses evaluated
274 (Figure 3A and 3B). In epididymis and prostate, the major doses of Al increased MDA levels (Figure 3C,
275 3D, 3E and 3F) and no differences were observed in epididymis and prostate lipid peroxidation after Al
276 exposure at 1.5 mg/kg bw/day (Figures 3C and 3E).

277 *Total antioxidant capacity - Ferric Reducing/Antioxidant Power (FRAP)*

278 Al at 1.5 mg/kg bw/day decreased the total antioxidant capacity in testis, while at the highest dose
279 of 100 mg/kg bw/day there was the opposite effect (Figure 4A and 4B). In the epididymis, only Al at the
280 middle dose of 8.3 mg/kg bw/day decreased the antioxidant capacity (Figure 4C) and, the prostate total
281 antioxidant capacity was reduced after Al exposure at minor and major doses (Figure 4E and 4F).

282 *Testis and epididymis histology*

283 Histopathological studies of testes showed that aluminum exposure for 60 days at the lower levels
284 (Gp.1) or for 42 days at higher levels (Gp.2) impaired testis architecture. In Al-treated rats the thickness of
285 the seminiferous tubules were reduced from 70.56 µm in the control group to 53.96 µm after Al exposure
286 at 8.3 mg/kg and 52.04 µm after Al exposure at the highest dose. There was a decrease in the number of
287 spermatogenic cells in the lumen of the seminiferous tubules in Al-treated rats, which was observed by the
288 increased seminiferous tubules with less or absence of mature spermatogenic cells, classified as empty
289 seminiferous tubules. For Al exposure at 8.3 mg/kg bw/day the average number of empty seminiferous
290 tubules was almost three times the number found in the control group (Figure 5B, 5D, 5E and 5F). However,
291 Al exposure at the higher dose of 100 mg/kg bw/day did not decrease the number of spermatogenic cells
292 (Figure 5G and 5H). In the control groups, the structure of seminiferous tubules was normal (Figure 5A
293 and 5C). The epididymis histology revealed no differences between the structure of epididymis from control
294 and Al-groups. Both showed similar number of empty efferent ducts with the means varying from 7.4 to
295 9.5 per field (Figure 6).

296 *Testis immunohistochemistry*

297 Immunohistochemical analysis showed an increase in the number of activated macrophages in testes
298 of rats treated with Al at the low dose of 8.3 mg/kg bw/day when compared with the control group (ranging

299 from 5 to 15 in the control group and from 21 to 40 in the Al-treated rats - Figure 7A, 7B and 7E). Al
300 exposure at the higher dose did not stimulate inflammation in testes (Figure 7C, 7D and 7F).
301 *Aluminum content and lumogallion staining in testis and epididymis*
302 We investigated the Al content in testis and epididymis of rats exposed to Al at the low dose of 8.3
303 mg/kg bw/day. The mean Al concentration in testis of Al-exposed rats was found to be almost twice the
304 amount found in the control group (control 1.79 ± 0.41 vs Al 3.35 ± 0.47 μ/g * $p < 0.05$ Student's t-test).
305 While, the Al content in the epididymis was not statistically different between groups (control 6.38 ± 0.75
306 vs Al 6.10 ± 1.13 μ/g - n = 5)
307 The presence of Al was confirmed using lumogallion and fluorescence microscopy. Testis and
308 epididymis showed green autofluorescence in the absence of lumogallion (Figures 8A, 8C, 8E and 8G).
309 Lumogallion fluorescence identified Al in the germinative cells in the seminiferous tubules as evidenced
310 by bright orange fluorescence (Figure 8D). In the epididymis Al seemed associated with blood cells. In this
311 organ we are not able to identify differences between control and Al-treated rats, which is in accordance
312 with the quantification of Al by TH GFAAS (Figures 8F and 8H).
313 **Discussion**
314 The decline in semen quality, including in countries that previously boasted good sperm
315 characteristics, highlights the male reproductive system as one of the major targets of environmental
316 toxicants (Nordkap et al. 2012). It seems likely that the cumulative effects of various low-dose exposures
317 to environmental contaminants are responsible for male reproductive effects. Synergistically, the
318 continuous increase in human exposure to Al challenged us to investigate the male reproductive effects
319 regarding Al exposure at human dietary levels. Our results suggest that Al should be considered as a hazard
320 to the male reproductive system even at low Al doses. Here we show that Al exposure for 60 days at human
321 dietary levels impairs sperm quality, as observed by suppression of sperm production and count reduction
322 followed by motility and morphological abnormalities in rats. This functional impairment appears together
323 with a redox imbalance, with increased ROS production, lipid peroxidation and altered antioxidant capacity
324 in reproductive organs. Surprisingly, these effects are similar to those found in rats exposed to Al at a dose
325 more than 60 times higher. Based on these first findings, we decided to go further to better understand the
326 effects of Al on the male reproductive system. For this, we have chosen a dose of Al exposure at a lower
327 level, one that better characterized the reproductive dysfunction, and then we have compared with Al at a
328 higher dose. Unexpectedly, but in accordance with recent discoveries about Al neurotoxicity (Crépeaux et

329 al. 2017), Al at the lower dose of 8.3 mg/kg bw/day had worse effects on the reproductive system.
330 Specifically, the testis histoarchitecture of rats exposed to Al at 100 mg/kg bw/day was better organized
331 with a larger number of sperm cells and without concomitant inflammation. However, further studies are
332 necessary to go further and better understand such discoveries.

333 Recently, using the same model of Al exposure at low levels, we showed that once Al achieved a
334 threshold its toxicity is almost the same. We developed the same behavioral evaluations in rats exposed to
335 low Al doses and the neurotoxicity effects were practically the same as those induced by the highest dose
336 (Martinez et al. 2017).

337 Crépeaux et al. (2017), by investigating the effects of the adjuvant aluminium oxyhydroxide
338 (Alhydrogel®) in female mice, only found neurocognitive impairments at the lowest dose of 0.2 mg Al/kg
339 and not at 0.4 or 0.8 mg Al/kg. In the current study, we have found adverse effects after Al exposure at the
340 higher dose. However, Al at 8.3 mg/kg, the amount equivalent to human Al exposure, showed worse effects.
341 Which seems that the dose is not the most important issue regarding Al toxicity, but the exposure conditions
342 and, consequent distribution and bioavailability through the body. Our results could raise the concern about
343 current safety values (e.g. WHO) relating to human exposure to Al.

344 The male reproductive system, especially the testes and spermatozoa, are very susceptible to
345 oxidative damage, mainly because of their high content of polyunsaturated fatty acids in membranes, their
346 limited antioxidant capacity and the ability of spermatozoa to generate reactive oxygen species (Aitken
347 1995). Overproduction of reactive oxygen species, however, can be detrimental to sperm and, appears to
348 be a common feature underlying male infertility (Turner and Lysiak 2008). Al^{3+} toxicity has been related
349 with its pro-oxidant activity in several organs and tissues (Exley 2004; Prakash and Kumar 2009; Ruipérez
350 et al. 2012; Yu et al. 2016), and more recently in male reproductive toxicity (Jamalan et al. 2016;
351 Mohammad et al. 2015; Oda 2016). In the present study, Al exposure increased oxidative stress in testis,
352 epididymis and prostate, as evident from an increase in RS generation and MDA levels. The oxidative stress
353 came together with an inflammatory process with large number of macrophage activated in testis of rats
354 exposed to Al at 8.3 mg/kg bw/day. The suppression of spermatogenesis and sperm impairments as well as
355 the histopathological changes observed, could be partially attributed to peroxidation of polyunsaturated
356 fatty acids in the sperm membrane, needed for sperm viability (Kistanova et al. 2009), and, to inflammation
357 within the testis.

358 Regarding the cell's defense and protection against increased oxidative stress, the total antioxidant
359 capacity was contrastingly changed among Al exposure models and according to the organ evaluated. For
360 example, Al exposure at the low doses of 1.5 and 8.3 mg/kg bw/day decreased the antioxidant capacity in
361 testis while at the highest dose an increase in the antioxidant profile was observed. This suggests that Al
362 does not have a classical toxicological pattern, but that the adverse effects of this metal are dependent on
363 the duration of exposure and contamination threshold and bioavailability that is achieved, but that a low
364 dose is able to promote male reproductive dysfunction.

365 Data regarding Al and human semen quality are scarce. Studies of Hovatta (1998) and Dawson
366 (1998) showed relationships between Al in seminal plasma and sperm motility. More recently, this
367 association was also found in human sperm samples exposed to $AlCl_3$, cadmium or lead, in which Al
368 showed the worst effects (Jamalan et al. 2016). In a recent study by Klein et al. (2014), semen of 62 patients
369 were investigated and revealed high concentration of Al in individuals with low sperm count.

370 Experimental animal studies addressing Al exposure and the male reproductive system are more
371 numerous. A single intraperitoneal injection of $AlCl_3$ at 25 mg/kg in mice was associated with germ cell
372 degeneration, tubular atrophy, apoptotic cell death of spermatogonia and primary spermatocytes and,
373 mitochondrial damage in Leydig cells (Abdel-Moneim 2013). $AlCl_3$ intragastrically for 4 weeks at 100
374 mg/kg bw/day induced histopathological alterations in testes and epididymis, increased MDA levels and
375 promoted a reduction in glutathione levels in rats (Oda 2016). $AlCl_3$ administrations at doses ranging from
376 34 mg/kg bw/day to 256.72 mg/kg bw/day have been related with a reduction in reproductive organs
377 weights, sperm count and motility, decreased libido and ejaculate volume, increased sperm abnormalities
378 and hormonal imbalance such as decrease in plasma testosterone, luteinizing hormone and follicular
379 stimulating hormone in rats and rabbits (Ige and Akhigbe 2012; Sun et al. 2011; Zhu et al. 2014).

380 However, these studies have been addressing the effects of Al on male reproductive system at
381 considerable high levels of Al exposure. Also these studies failed to consider the amount of Al from the
382 animal´s feed. In our experimental model, we have measured the amount of Al from the feed (Martinez et
383 al. 2017) and, all rats including controls received 1.88 mg/Al/day from their standard feed. Therefore, taking
384 into account the animals mean body weights of 300g, the total amount of Al exposure for experiment 1,
385 low aluminum levels, was: a) 1.5 mg/Al/kg bw/day - 2.33 mg/Al/day (0.45 mg/Al from water plus 1.88
386 mg/Al from feed); b) 8.3 mg/Al/kg bw/day - 4.37 mg/Al/day (2.49 mg/Al from water plus 1.88 mg/Al from

387 feed), and for group 2, High Aluminum Level: c) 100 mg/Al/kg bw/day -31.88 mg/Al/day (30 mg/Al from
388 gavage plus 1.88 mg/Al from feed).

389 In the current study, Al exposure for 60 days at relevant human dietary levels was able to impair
390 sperm quality and spermatogenesis and the Al induced oxidative stress and inflammation in the testis.
391 Relating to our findings about Al concentrations, it is shown for the first time that concentrations of Al
392 around 3 µg/g in testis are sufficient to induce male reproductive dysfunction. According to our knowledge,
393 other studies showing male reproductive toxicity induced by Al were performed with high levels of Al,
394 finding Al concentrations in testes between 35 µg/g and 140 µg/g (Guo et al. 2005; Guo et al. 2001;
395 Mohammad et al. 2015).

396 The identification of Al in tissues or cells using lumogallion and fluorescence microscopy was
397 shown to be specific for Al with no interference from any other metals and no issues relating to
398 autofluorescence (Mirza et al. 2016; Mold et al. 2014). We have used lumogallion staining to show the
399 presence of Al in testes of rats and, we are the first to show Al associated with unidentified structures and
400 among germinative cells, which could reinforce its interference on the spermatogenesis process.

401 **Conclusions**

402 Our study shows that 60-day exposure to low doses of Al, which aimed to mimic human exposure
403 to Al by the dietary route, are able to impair male reproductive health. Strikingly, the reproductive
404 impairment was, sometimes, less-marked at the higher dose of Al, suggesting a non-linear effect of Al in
405 this system. The current study shows, for the first time, the specific presence of Al in the germinative cells
406 and, that low concentrations of Al in testes are sufficient to impair spermatogenesis and sperm quality. The
407 elevation of oxidative stress and inflammation highlight pathways of toxic actions for this metal on the
408 male reproductive system. Our findings provide a better understanding of the reproductive health risk after
409 Al exposure. However, further studies are necessary to fully address the effects of Al in the reproductive
410 system.

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545 **Table 1** Effect of chronic aluminum exposure to low (group 1) and high (group 2) doses on body weight,
546 absolute and relative weights of reproductive organs.

Parameters	Group 1			Group 2	
	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Initial body weight (g)	360.10 ± 10.29	391.9 ± 14.87	396.4 ± 9.56	301.7 ± 9.86	315. 6 ± 14.01
Final body weight (g)	424.6 ± 9.54	450.7 ± 15.91	462.7 ± 10.58	410.1 ± 7.58	415.4 ± 11.78
Testis (g)	1.7 ± 0.13	2.01 ± 0.05	2.07 ± 0.14	1.9 ± 0.05	1.9 ± 0.06
Testis (g/100g)	0.4 ± 0.03	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01	0.4 ± 0.01
Epididymis (mg)	653.8 ± 23.15	703.2 ± 34.08	690.7 ± 25.86	662.2 ± 34.99	616.2 ± 35.13
Epididymis (mg/100g)	151.6 ± 5.14	148.7 ± 5.36	142.1 ± 6.59	144.0 ± 4.71	141.7 ± 5.63
Ventral prostate (mg)	482.7 ± 42.88	429.8 ± 33.60	458.8 ± 58.61	415.8 ± 21.44	351.1 ± 21.79*
Ventral prostate (mg/100g)	111.4 ± 9.09	91.4 ± 8.31	92.1 ± 8.16	104.3 ± 8.95	77 ± 5.31*
Full seminal vesicle (g)	1.6 ± 0.11	1.6 ± 0.21	1.6 ± 0.20	1.2 ± 0.15	1.3 ± 0.12
Full seminal vesicle (g/100g)	0.3 ± 0.04	0.3 ± 0.04	0.3 ± 0.03	0.2 ± 0.04	0.3 ± 0.02
Empty seminal vesicle (g)	0.5 ± 0.10	0.6 ± 0.11	0.6 ± 0.19	0.4 ± 0.05	0.4 ± 0.05
Empty seminal vesicle (g/100g)	0.1 ± 0.02	0.1 ± 0.02	0.1 ± 0.03	0.1 ± 0.02	0.1 ± 0.01
Vesicular secretion (g)	0.9 ± 0.14	0.9 ± 0.13	1.1 ± 0.13	0.7 ± 0.17	0.9 ± 0.14
Vas deferens (mg)	112 ± 14.7	97.2 ± 13.74	113.8 ± 10.44	99.6 ± 12.65	89.1 ± 9.4
Vas deferens (mg/100g)	26.1 ± 3.56	20.1 ± 2.33	23.6 ± 2.69	21 ± 2.93	20.4 ± 1.84

547 Data are expressed as mean ± SEM. The relative organ weight was calculated by use of the formula: organ weight/body weight x 100.
548 Units: g: gram, mg: milligram. * $p < 0.05$ compared with controls from the corresponding group 2 (Student's t-test)
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569 **Table 2** Effect of chronic aluminum exposure to low (group 1) and high (group 2) doses on sperm counts
570 in testis and epididymis of rats.

Parameters	Group 1			Group 2	
Sperm count	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Testis					
Sperm number (x10^6)	142.7 ± 8.42	104.8 ± 2.60**	93.43 ± 6.89**	148.1 ± 8.72	115.8 ± 11.84*
Sperm number (x10^6/g)	86.13 ± 5.43	60.58 ± 0.88**	54.48 ± 5.44**	97.81 ± 6.76	65.79 ± 5.95**
DSP (x10^6/testis/day)	23.40 ± 1.38	17.19 ± 0.42**	15.32 ± 1.13**	24.30 ± 1.21	18.98 ± 1.64*
DSPr (x10^6/testis/day/g)	14.12 ± 0.89	9.92 ± 0.14**	8.93 ± 0.89**	16.04 ± 1.10	10.79 ± 0.97**
Epididymis					
Caput/ Corpus					
Sperm number (x10^6)	140.2 ± 12.16	132.7 ± 4.61	129.7 ± 7.58	142 ± 5.97	133.7 ± 7.53
Sperm number (x10^6/g)	402.5 ± 28.82	351.9 ± 12.69	354.7 ± 20.10	416.0 ± 18.41	369.2 ± 10.97
Sperm transit time (days)	6.03 ± 0.45	7.74 ± 0.34*	9.77 ± 0.77*	6.21 ± 0.46	7.33 ± 0.67
Cauda					
Sperm number (x10^6)	178.6 ± 17.81	139.6 ± 9.29	150.0 ± 11.89	166.3 ± 10.48	139.5 ± 14.88
Sperm number (x10^6/g)	823.7 ± 62.56	642.1 ± 49.22	701.3 ± 31.66	737.7 ± 26.43	645.4 ± 35.91
Sperm transit time (days)	7.61 ± 0.62	8.11 ± 0.46	10.03 ± 1.09	7.03 ± 0.81	7.51 ± 0.81

571 DSP: daily sperm production; DSPr: daily sperm production relative to testis weight. Data are expressed as mean ± SEM. Units: g:
572 gram. * $p < 0.05$ ** $p < 0.01$ compared with their corresponding controls (ANOVA or Student's t-test)
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594 **Table 3** Effect of chronic aluminum exposure to low (group 1) and high (group 2) doses on sperm
595 morphology of rats.

Parameters	Group 1			Group 2	
Sperm morphology	Control	Al 1.5 mg/kg	Al 8.3 mg/kg	Control	Al 100 mg/kg
Normal	92.5 (92 – 94.3)	89.2 (85.6 – 92.2)*	83 (74.8 – 88)**	94 (89.63 – 96.13)	84 (81.38 – 87.75)**
Head Abnormalities					
Amorphous	2 (1.6 – 2.5)	3.5 (1.3 – 8.1)	6 (3.8 – 10) **	1.5 (0.8 – 2.5)	7.2 (6.8 – 11.1) **
Banana Head	0.5 (0 – 0.6)	1 (0 – 2.2)	3 (1.6 – 4.8) *	1.5 (1 – 2)	0 (0 – 0.6)
Detached Head	1 (0.5 – 3)	1.2 (0.5 – 2.5)	1.5 (0.8 – 2.3)	1.7 (0.5 – 4.2)	3.2 (1.2 – 6)*
Total of Head Abnormalities	3.7 (2.8– 5.3)	6.7 (3 – 12.8)	10.7 (9 – 16.1)**	5.5 (3.5 – 9.6)	11.7 (9.3 – 15.1)*
Tail Abnormalities					
Bent Tail	1 (0.5 – 1.8)	1 (0.5 – 2.3)	2.5 (2 – 3)**	0.0 (0.0 – 0.0)	1 (0.5 – 1.5)**
Broken Tail	0 (0.0 – 0.5)	0.2 (0 – 0.75)	0.5 (0.3 – 1)	0.2 (0.0 – 0.6)	1.2 (0.3 – 4.8)
Total of Tail Abnormalities	1.5 (1.2 – 3.2)	2.5 (1.6 – 4.2)	3 (2.2 – 4.2)	0.2 (0.0 – 0.6)	2 (1.5 – 2.7)**

596 Data are expressed as median (Q1 – Q3). *$p < 0.05$ **$p < 0.01$ compared with their corresponding controls (Kruskal-Wallis test
597 followed by Dunn's or Mann – Whitney).
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Figure legends

Figure 1. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm motility: motile with progressive movement, motile without progressive movement and immotile. Data are expressed as median (Q1 – Q3), n=6, * $p < 0.05$ compared with their corresponding controls (Kruskal-Wallis test followed by Dunn's or Mann – Whitney).

Figure 2. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on reactive oxygen species levels (ROS). Values of ROS on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's t-test). UF: Units of fluorescence.

Figure 3. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on lipid peroxidation measurements. Values of MDA (malondialdehyde) on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's t-test)

Figure 4. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on total antioxidant capacity. Values of FRAP (Ferric Reducing/Antioxidant Power) on testis (A and B), epididymis (C and D) and prostate (E and F). Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (ANOVA followed by Bonferroni or Student's t-test)

Figure 5. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis histopathology. Control group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D). Average number of empty seminiferous tubules per field (X20) for group 1 (E) and for group 2 (F) in absolute numerical values. Testes sections of Al-treated rats showing reduction of spermatozoa in the lumen of the seminiferous tubules (arrows). Thickness of the seminiferous epithelium (µm) for group 1 (G) and for group 2 (H), showing a reduced thickness in testes of Al-treated rats (double arrows). Scale bars: 50 µm. Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (Student's t-test)

Figure 6. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on epididymis histopathology. Control group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D). Average number of empty efferent ducts per field (X20) for group 1 (E) and for group 2 (F). Scale bars: 50 µm. Data are expressed as mean ± SEM (n = 6).

659 **Figure 7.** Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis
660 immunohistochemistry. Activate macrophages (arrows) in testis of controls group (A and C), Al at 8.3
661 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D) detected by immunohistochemistry. Scale bars: 50 µm.
662 Average numbers of activated macrophages per field (objective X20) for group 1 (E) and for group 2 (F).
663 Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls
664 (Student's t-test)

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666 **Figure 8.** Aluminum presence in reproductive tissues. Representative images of aluminum in testis and
667 epididymis: autofluorescence in control groups (A and E) and in Al-treated rats (C and G); lumogallion
668 fluorescence for aluminum in control group (B and F) and in Al-treated rats (D and H). The specific
669 presence of Al is indicated by arrows. Scale bars: 50 µm.

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