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

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Highlights

- Our study shows that 60-day exposure to low doses of aluminum, which aimed to mimic human exposure to dietary aluminum is able to impair male reproductive health. Strikingly, the reproductive impairment was, sometimes, less-marked at the higher dose of Al, raising concerns regard to safe values for human exposure to aluminum.

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 and a high level exposure to Al. Experiment 1 (Lower level) rats were treated orally for 60 days: a) controls - 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) controls - ultrapure water; b) aluminum at 100 mg/kg bw/day. Al decreased sperm count, daily sperm production, sperm motility, normal morphological sperm, impaired testis histology; increased oxidative stress in reproductive organs and inflammation in testis. Our study shows 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.

Abbreviations: ROS, reactive oxygen species; RNS, reactive nitrogen species; DCF, dichlorofluorescein; mesenteric resistance arteries; MDA, malondialdehyde; TBA, thiobarbituric acid.

Keywords: metal; reproductive adverse effects; sperm quality.

1. 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 [1,2].

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 packaging and storage of food products [3]. 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 [4].

In 2007, the tolerable weekly intake of Al for humans was adjusted to 1 mg Al/kg body weight (b.w.) [5]. However, it is known that humans may exceed health-based guidance values [3,6,7].

Even with a low rate of Al absorption through the gastrointestinal tract [8], 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 [2].

Over the last years, concerns have increased about Al exposure and its relationship to reproductive health [9-11]. The decline of sperm quality and increases in infertility have been observed over recent decades [12-14], 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 [9,15]. Recently, Al content in human sperm was related to reduction in sperm quality. Specifically, patients with oligozoospermia had higher Al concentration than others [16]. 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 [17,18].

However, studies addressing reproductive effects of Al have been conducted with doses of Al higher than might commonly be found among human populations [19-21]. Moreover, due to the suggested biphasic effect of Al [22], it is urgent to investigate the effects of Al exposure at human dietary levels and then to compare with Al effects at high levels. Herein we investigated the effects of Al exposure at three different doses: two low doses representing human Al exposure through the diet and, one model of exposure at a high Al level known to produce toxicity.

2. Material and methods

2.1 Animals

Three-month-old male *Wistar* rats (362.5 ± 11.7 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. [23]: Experiment 1 - low aluminum levels, and Experiment 2 - high aluminum level. For group 1, 18 rats were subdivided (in groups of six animals) and treated for 60 days as follows: a) the control groups received ultrapure drinking water (Milli-Q, Merck Millipore Corporation. © 2012 EMD Millipore, Billerica, MA); b) the second group received aluminum at 1.5 mg/kg bw/day based on human dietary levels according to a published protocol described by Walton [24], at the reduced Al exposure for 60 days, and c) the third group drank aluminum at 8.3 mg/kg bw/day which corresponds to the same aluminum human dietary levels (1.5 mg/kg) when translated to an animal dose based on body surface area normalization method [25]. For experiment 2, (the high aluminum level), 12 rats were subdivided (N=6/each) and treated for 42 days as follows: a) the control group received ultrapure water through oral gavages; b) aluminum at 100 mg/kg bw/day [26].

Rat body weights, feed, water and Al intakes were measured weekly. At the end of the treatments, animals were euthanized by decapitation and the weights of testis, epididymis, prostate, vas deferens and seminal vesicle (empty, without coagulation gland), were determined. The right testis, epididymis and left vas deferens were used for sperm parameter analysis. Left testis and epididymis were divided in two segments, one of each was processed for histological and or immunohistochemical studies and the other part together with the prostate were quickly homogenized in 50 mM Tris HCl, pH 7.4, (5/10, w/v) for biochemical determinations. Afterwards, samples were centrifuged at 2400g for 10 min at 4°C and the resulting supernatant fraction was frozen at −80°C for further assay.

$AlCl_3$. 6 H_2O was purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in ultrapure water (Milli-Q © 2012 EMD Millipore, Billerica, MA). The concentration of each stock solution was 0.008 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 were of analytical grade obtained from Sigma and Merck (Darmstadt, Germany).

2.2 Sperm Parameters Analysis

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

Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were counted as described by Robb et al. [27]. To calculate daily sperm production, the number of spermatids at stage 19 was divided by 6.1, which is the number of days these spermatids are present in the seminiferous epithelium. The sperm transit time through the epididymis was determined by dividing the number of sperm in each portion by the daily sperm production [27].

2.2.2 Sperm morphology

Sperm were obtained from the vas deferens and stored with 1 mL of 10% formal-saline until analysis. For the analysis, smears were prepared on histological slides and 200 spermatozoa per animal

were evaluated under 400X magnification (Binocular, Olympus CX31). Morphological abnormalities were classified into head (amorphous, banana and detached head) and tail morphology (bent and broken tail), according to Filler [28].

2.2.3 Sperm motility

Sperm were removed from the vas deferens by internal rising with 1 mL of Human Tubular Fluid (DMPBS-Nutricell-SP-Brazil) pre-warmed to 34ºC. Then, a 10 µL aliquot was transferred to a histological slide. Under a light microscope (20X magnification, Binocular, Olympus CX31, Tokyo, Japan), 100 spermatozoa were analyzed and classified as type A: motile with progressive movement, type B: motile without progressive movement and type C: immotile. Sperm motility was expressed as % of total sperm [29].

2.3 Biochemical Assay

2.3.1 Reactive oxygen species levels

The levels of reactive species (RS) in testis, epididymis and prostate were determined by a spectrofluorometric method, as described by Loetchutinat et al. [30]. This method is unspecific for reactive oxygen species (ROS), also measuring reactive nitrogen species (RNS). The supernatant fraction of the sample was diluted (1:10) in 50 mM Tris-Hcl (pH 7.4) and 2′, 7′-dichlorofluorescein diacetate (DCHF-DA; 1mM) was added to the medium. DCHF-DA is enzymatically hydrolyzed by intracellular esterases to form nonfluorescent DCFH, which is then rapidly oxidized to form highly fluorescent 2´,7´-dichlorofluorescein (DCF) in the presence of ROS. DCF fluorescence intensity is proportional to the amount of ROS that is formed. The DCF fluorescence intensity emission was recorded at 520 nm (with 480 nm excitation) (SpectraMax M5 Molecular Devices, CA, USA) for 60 min at 15 min intervals. The ROS levels were expressed as fluorescence units.

2.3.2 Lipid peroxidation

The levels of lipid peroxidation in testis, epididymis and prostate were measured as malondialdehyde (MDA) using a colorimetric method, as previously described by Ohkawa et al. [31], with modifications. An aliquot of each tissue was incubated with thiobarbituric acid 0.8% (TBA), phosphoric acid buffer 1% (H_3PO_4), and sodium dodecil sulphate 0.8% (SDS) at 100ºC for 60 min. The color reaction was measured at 532 nm against blanks (SpectraMax M5 Molecular Devices, CA, USA). The results were expressed as nanomoles of MDA per mg of protein.

2.3.4 Ferric Reducing/Antioxidant Power (FRAP) Assay

The total antioxidant capacity was measured in testis, epididymis and prostate by FRAP assay [32]. This method is based on the ability of the sample to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which forms with 2,4,6-Tri(2-piridil)-s- triazina (TPTZ) the chelate complex Fe^{+2}-TPTZ. Briefly, 10 microliters of the supernatant fraction of each tissue was added to 1 mL freshly prepared and pre-warmed (37◦C) FRAP reagent (500 microliters of 300mM acetate buffer (pH = 3.6), 250 microliters of 10mM TPTZ in 40mM HCl, and 250 microliters of 20mM $FeCl_3$) in a test tube and incubated at 37◦C for 10min. The absorbance of the blue-colored complex was read against a blank reagent (1 mL FRAP reagent + 10 microliters distilled water) at 593 nm (SpectraMax M5 Molecular Devices, CA, USA). A standard dose-response curve of Trolox (50-1000 µM – water soluble analog of vitamin E) was prepared and the FRAP assay is described. Results are presented with particular reference to Trolox equivalents.

2. 4 Testis and epididymis histology

To carry out the histological studies. Epididymis tissues were dehydrated, fixed in 10% formaldehyde and testis in Bouin's solution for 1–2 days. After several intensive washings, tissues 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 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 ducts per field of epididymis, in 20X magnification per section.

2.5 Testis immunohistochemistry

Testis 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. As a negative control, preparations were incubated without the primary antibody. 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).

2.6 Aluminum content in testis and epididymis

The Al content of testis and epididymis were determined using an established method [33]. 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 to a total volume of 5 mL with ultrapure water (cond<0.067 <µS/cm) and the Al content of digests measured by TH GFAAS (Transversley Heated Graphite Furnace Atomic Absorption Spectrometry) using matrix-matched standards and an established analytical programme (House et al. 2012). Briefly, the TH GFAAS was calibrated by automated serial dilution of a 60 µg L^{-1} solution of Al with 1% HNO_3. Non-linear zero intercept WinLab 32-generated fits were applied (Perkin Elmer, UK). Instrument detection limits (IDL) were estimated from three times the standard deviation on the 1% HNO_3 calibration blank absorbance (n = 3 injections) divided by the Winlab32 generated calibration slope. Mean IDL for Al was 0.13 µg L-^{1} (SD 0.13 µg L^{-1}, n=62). Concentrations of Al in NIST SRM1566B oyster tissue and IAEA-407 fish homogenate were used as spike samples and standard reference material. Results were expressed as µg Al/g tissue dry weight. Each determination was the arithmetic mean of a triplicate analysis.

2.7 Lumogallion staining

Lumogallion staining was performed in bouin and formalin-fixed testis and epididymis using a recent validated method to identify the presence of Al in tissues [34,35]. 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.

2.8 Statistical analysis

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

3. Results

3.1 Body and organs weights, fluid and feed intake

Body weight of rats was similar between groups at the start and end of treatments (362.2 ± 11.7; 434.7 ± 11.1g means at the start and end, respectively). The quantity of water, Al intakes and feed intake were not different between groups (P > 0.05; one-way ANOVA / t-test - Table 1). Al exposure at low levels (group 1) did not change the absolute and relative reproductive organ weights. However, Al at 100 mg/kg bw/day decreased the 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 - Table 2).

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

To investigate the effect of Al on sperm count, group 1 rats were treated for 60 days with Al at 1.5 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 rats were treated with ultrapure water. Chronic exposure to Al at different doses altered sperm parameters in testis, there was a reduction in daily sperm production per testis and in sperm count (Table 3). In the epididymis of group 1 rats, Al increased the sperm transit time in the caput/corpus and there was an apparent decrease in sperm number, which was not statistically significant (mean of total sperm in epididymis for 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: 308.3, Al 100 mg/kg bw/day: 273.2 $x10^6$, P > 0.05, see more details in - Table 3).

3.3 Sperm morphology and motility

Sperm analysis revealed a significant decrease in sperm with normal morphology in rats exposed to Al when compared with the control group (group 1: control: 92.5 (92 – 94.3), Al 1.5 mg/kg bw/day: 89.2 (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 bw/day: 84 (81.38 – 87.75)*, - Table 4). Group 1 rats treated for 60 days with Al 8.3 mg/kg bw/day and group 2 rats exposed to Al at 100 mg/kg bw/day, for 42 days, showed specific abnormalities. Within head phenotypes, amorphous, banana and detached head were observed; concerning tail morphology, the bent tail was the most frequency abnormality in rats exposed to Al at major doses (mean of total sperm 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 control: 6.58, Al 100 mg/kg bw/day: 14.41% *P < 0.05, see more details in - Table 4).

Regarding sperm motility, for group 1, Al exposure at the lowest dose of 1.5 mg/kg bw/day did not affect the motility (Figure 1A). On contrast, Al exposure at 8.3 mg/kg bw/day, for 60 days, and rats exposed

to Al at 100 mg/kg bw/day, for 42 days, decreased type A sperm (motile with progressive movement) accompanied by an increase in type B (motile without progressive movement) and type C sperm (immotile) (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: 59.67; group 2 control: 85.16, Al 100 mg/kg bw/day: 64% *P < 0.05, see more details in - Figure 1A and B).

3.4 Reactive species and lipid peroxidation levels

Al treatment at different doses increased the levels of reactive species (RS) in epididymis (Figure 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 bw/day altered this oxidative stress parameter (Figure 2A and 2B).

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

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

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

3.6 Testis and epididymis histology

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

3.7 Testis immunohistochemistry

Immunohistochemical analysis showed an increase in the number of activated macrophages in testes of rats treated with Al at the low dose of 8.3 mg/kg bw/day when compared with the control group (ranging from 5 to 15 in the control group and from 21 to 40 in the Al-treated rats - Figure 7A, 7B and 7E). Al exposure at the higher dose did not stimulate inflammation in testes (Figure 7C, 7D and 7F).

3.8 Aluminum content and lumogallion staining in testis and epididymis

We investigated the Al content in testis and epididymis of rats exposed to Al at the low dose of 8.3 mg/kg bw/day. The mean Al concentration in testis of Al-exposed rats was found to be almost twice the

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). While, the Al content in the epididymis was not statistically different between groups (control 6.38 ± 0.75 vs Al 6.10 ± 1.13 μ/g - n = 5)

The presence of Al was confirmed using lumogallion and fluorescence microscopy. Testis and epididymis showed green autofluorescence in the absence of lumogallion (Figures 8A, 8C, 8E and 8G). Lumogallion fluorescence identified Al in the germinative cells in the seminiferous tubules as evidenced by bright orange fluorescence (Figure 8D). In the epididymis Al seemed associated with blood cells. In this organ we are not able to identify differences between control and Al-treated rats, which is in accordance with the quantification of Al by TH GFAAS (Figures 8F and 8H).

4. Discussion

The decline in semen quality, including in countries that previously boasted good sperm characteristics, highlights the male reproductive system as one of the major targets of environmental toxicants [36]. It seems likely that the cumulative effects of various low-dose exposures to environmental contaminants are responsible for male reproductive effects. Synergistically, the continuous increase in human exposure to Al challenged us to investigate the male reproductive effects regarding Al exposure at human dietary levels. Our results suggest that Al should be considered as a hazard to the male reproductive system even at low Al doses. Here we show that Al exposure for 60 days at human dietary levels impairs sperm quality, as observed by suppression of sperm production and count reduction followed by motility and morphological abnormalities in rats. This functional impairment appears together with a redox imbalance, with increased ROS production, lipid peroxidation and altered antioxidant capacity in reproductive organs. Surprisingly, these effects are similar to those found in rats exposed to Al at a dose more than 60 times higher. Based on these first findings, we decided to go further to better understand the effects of Al on the male reproductive system. For this, we have chosen a dose of Al exposure at a lower level, one that better characterized the reproductive dysfunction, and then we have compared with Al at a higher dose. Unexpectedly, but in accordance with recent discoveries about Al neurotoxicity [37], Al at the lower dose of 8.3 mg/kg bw/day had worse effects on the reproductive system. Specifically, the testis histoarchitecture of rats exposed to Al at 100 mg/kg bw/day was better organized with a larger number of sperm cells and without concomitant inflammation. However, further studies are necessary to go further and better understand such discoveries.

Recently, using the same model of Al exposure at low levels, we showed that once Al achieved a threshold its toxicity is almost the same. We developed the same behavioral evaluations in rats exposed to low Al doses and the neurotoxicity effects were practically the same as those induced by the highest dose [23].

Crépeaux et al. [37], by investigating the effects of the adjuvant aluminium oxyhydroxide (Alhydrogel®) in female mice, only found neurocognitive impairments at the lowest dose of 0.2 mg Al/kg 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 higher dose. However, Al at 8.3 mg/kg, the amount equivalent to human Al exposure, showed worse effects. This may seem as though the dose is not the most important issue regarding Al toxicity, but the exposure conditions, intrinsic and individual characteristics and, consequent distribution and bioavailability through

the body. Our results suggest that current safety limits (e.g. WHO) relating to human exposure should be reviewed.

The male reproductive system, especially the testes and spermatozoa, are very susceptible to oxidative damage, mainly because of their high content of polyunsaturated fatty acids in membranes, their limited antioxidant capacity and the ability of spermatozoa to generate reactive oxygen species [38]. Overproduction of reactive oxygen species, however, can be detrimental to sperm and, appears to be a common feature underlying male infertility [39]. Al^{3+} toxicity has correlates with pro-oxidant activity in several organs and tissues [40,26,41,42], and more recently in male reproductive toxicity [11,18,19]. In the present study, Al exposure increased oxidative stress in testis, epididymis and prostate, as evident from an increase in RS generation and MDA levels. The oxidative stress came together with an inflammatory process with large number of macrophage activated in testis of rats exposed to Al at 8.3 mg/kg bw/day. The suppression of spermatogenesis and sperm impairments as well as the histopathological changes observed, could be partially attributed to peroxidation of polyunsaturated fatty acids in the sperm membrane, needed for sperm viability [43], and, to inflammation within the testis.

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

Data regarding Al and human semen quality are scarce. Studies of Hovatta [10] and Dawson [9] showed relationships between Al in seminal plasma and sperm motility. More recently, this association was also found in human sperm samples exposed to $AlCl_3$, cadmium or lead, in which Al showed the worst effects [11]. In a recent study by Klein et al. [16], semen of 62 patients were investigated and revealed high concentration of Al in individuals with low sperm count.

Experimental animal studies addressing Al exposure and the male reproductive system are more numerous. A single intraperitoneal injection of $AlCl_3$ at 25 mg/kg in mice was associated with germ cell degeneration, tubular atrophy, apoptotic cell death of spermatogonia and primary spermatocytes and, mitochondrial damage in Leydig cells [44]. $AlCl_3$ intragastrically for 4 weeks at 100 mg/kg bw/day induced histopathological alterations in testes and epididymis, increased MDA levels and promoted a reduction in glutathione levels in rats [19]. $AlCl_3$ administration at doses ranging from 34 mg/kg bw/day to 256.72 mg/kg bw/day have been related with a reduction in reproductive organs weights, sperm count and motility, decreased libido and ejaculate volume, increased sperm abnormalities and hormonal imbalance such as decrease in plasma testosterone, luteinizing hormone and follicular stimulating hormone in rats and rabbits [17,20,21].

However, these studies have been addressing the effects of Al on male reproductive system at considerable high levels of Al exposure. Also these studies failed to consider the amount of Al from the animal´s feed. In our experimental model, we have measured the amount of Al from the feed [23] and, all rats including controls received 1.88 mg/Al/day from their standard feed. Therefore, taking into account

the animals mean body weights of 300g, the total amount of Al exposure for experiment 1, 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 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 feed), and for group 2, High Aluminum Level: c) 100 mg/Al/kg bw/day -31.88 mg/Al/day (30 mg/Al from gavage plus 1.88 mg/Al from feed).

In the current study, Al exposure for 60 days at relevant human dietary levels was able to impair sperm quality and spermatogenesis and the Al induced oxidative stress and inflammation in the testis. Relating to our findings about Al concentrations, it is shown for the first time that concentrations of Al around 3 μg/g in testis are sufficient to induce male reproductive dysfunction. Previous studies showing male reproductive toxicity were performed with unrealistic high doses of Al (from 34 mg/kg to 400 mg/kg/ bw), showing higher Al concentration in testes, between 35 μg/g and 140 μg/g [45,46,18].

The identification of Al in tissues or cells using lumogallion and fluorescence microscopy was shown to be specific for Al with no interference from any other metals and no issues relating to autofluorescence [34,35]. We have used lumogallion staining to show the presence of Al in testes of rats and, we are the first to show Al associated with unidentified structures and among germinative cells, which could reinforce its interference on the spermatogenesis process.

5. Conclusions

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

Conflict of Interest: The authors declare that they have no conflict of interest.

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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)

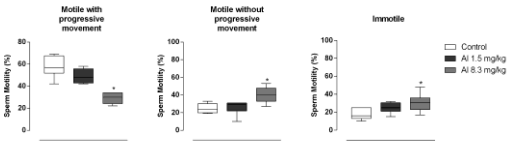
Figure 5. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis histopathology. Control group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D). Average number of empty seminiferous tubules per field (X20) for group 1 (E) and for group 2 (F) in absolute 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).

Figure 7. Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on testis immunohistochemistry. Activate macrophages (arrows) in testis of controls group (A and C), Al at 8.3 mg/kg b.w. (B) and Al at 100 mg/kg b.w. (D) detected by immunohistochemistry. Scale bars: 50 µm. Average numbers of activated macrophages per field (objective X20) for group 1 (E) and for group 2 (F).

Data are expressed as mean ± SEM (n = 6). * $p < 0.05$ compared with their corresponding controls (Student's t-test)

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



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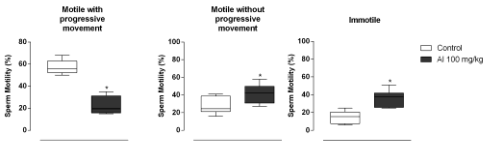
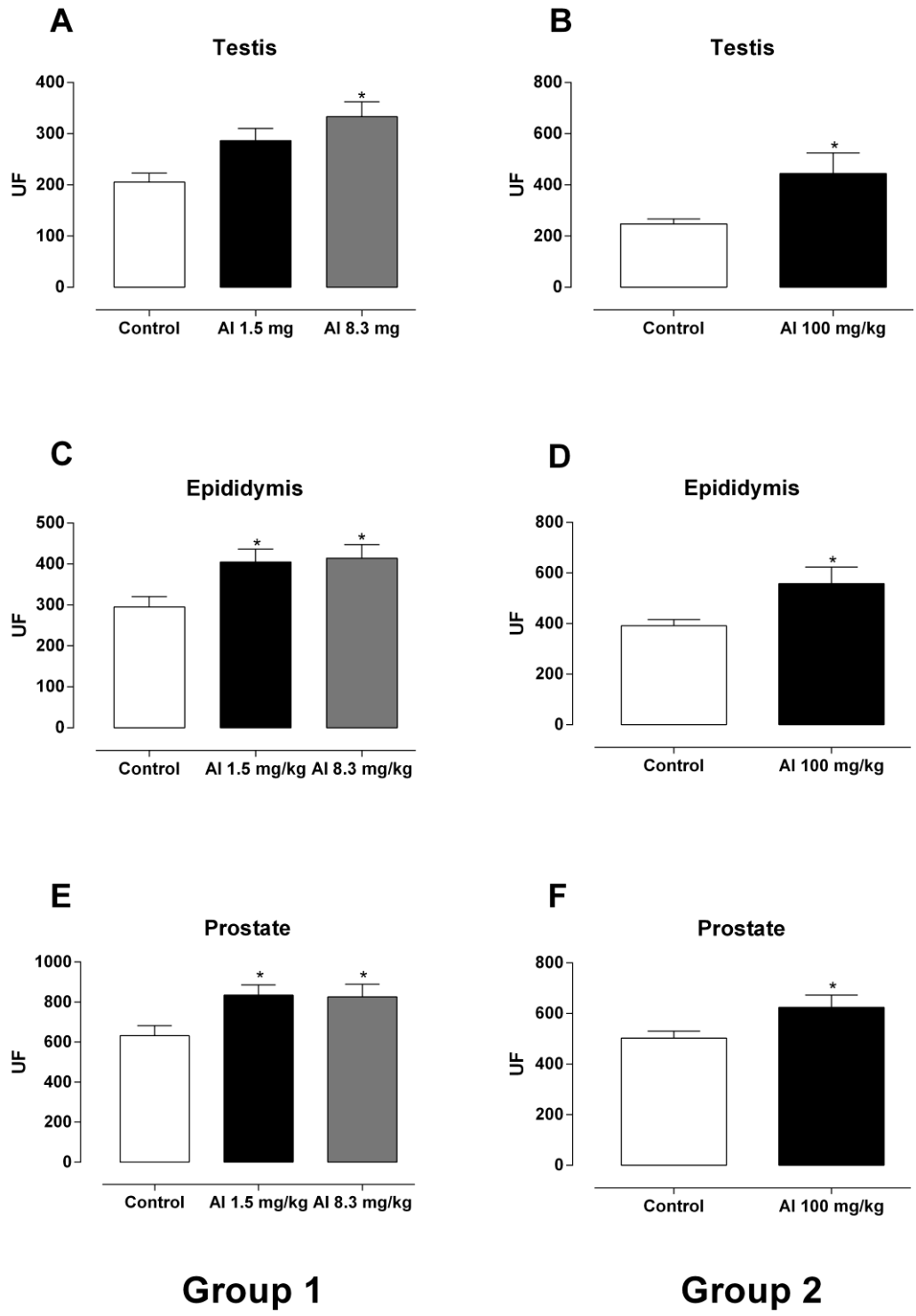
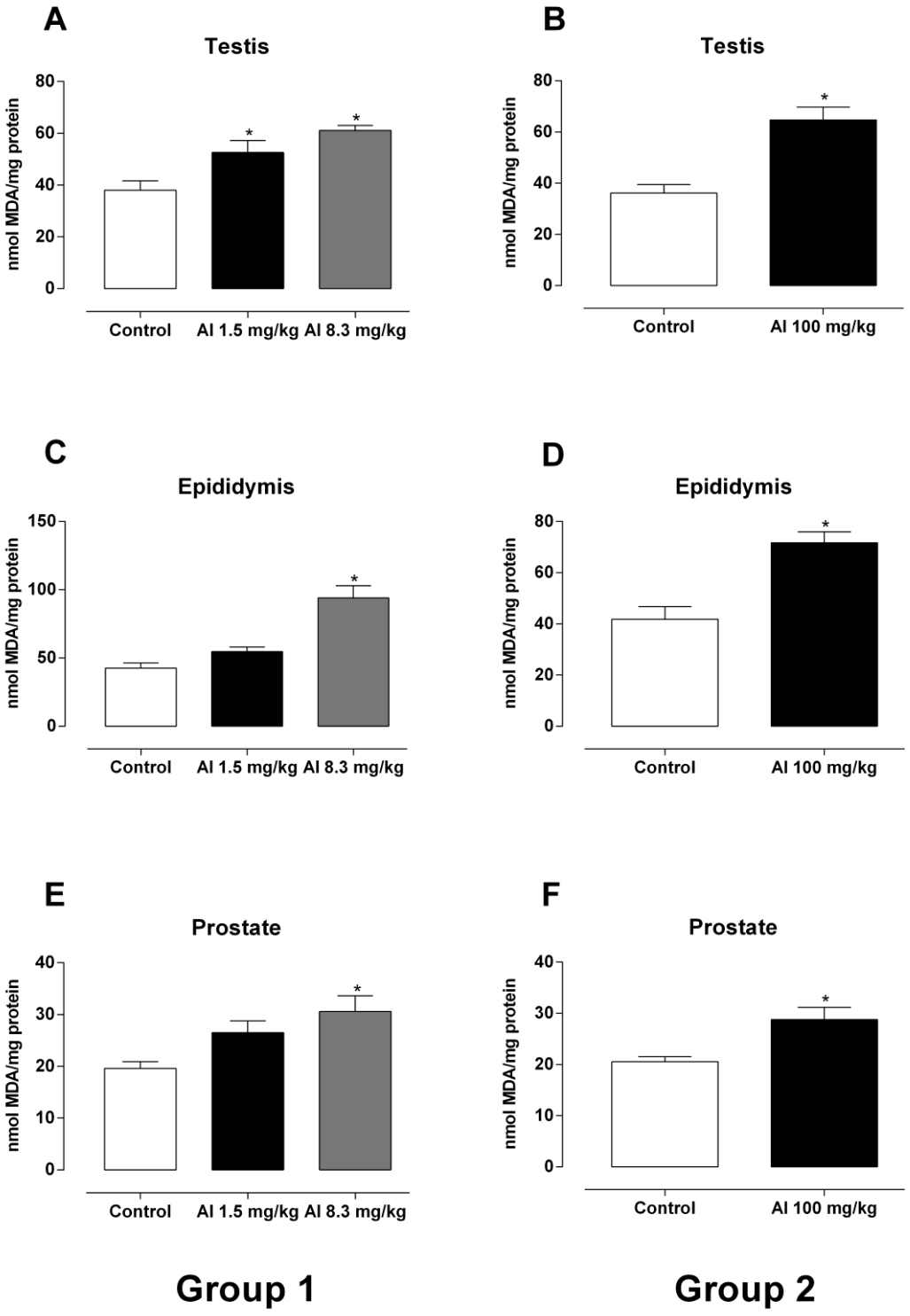
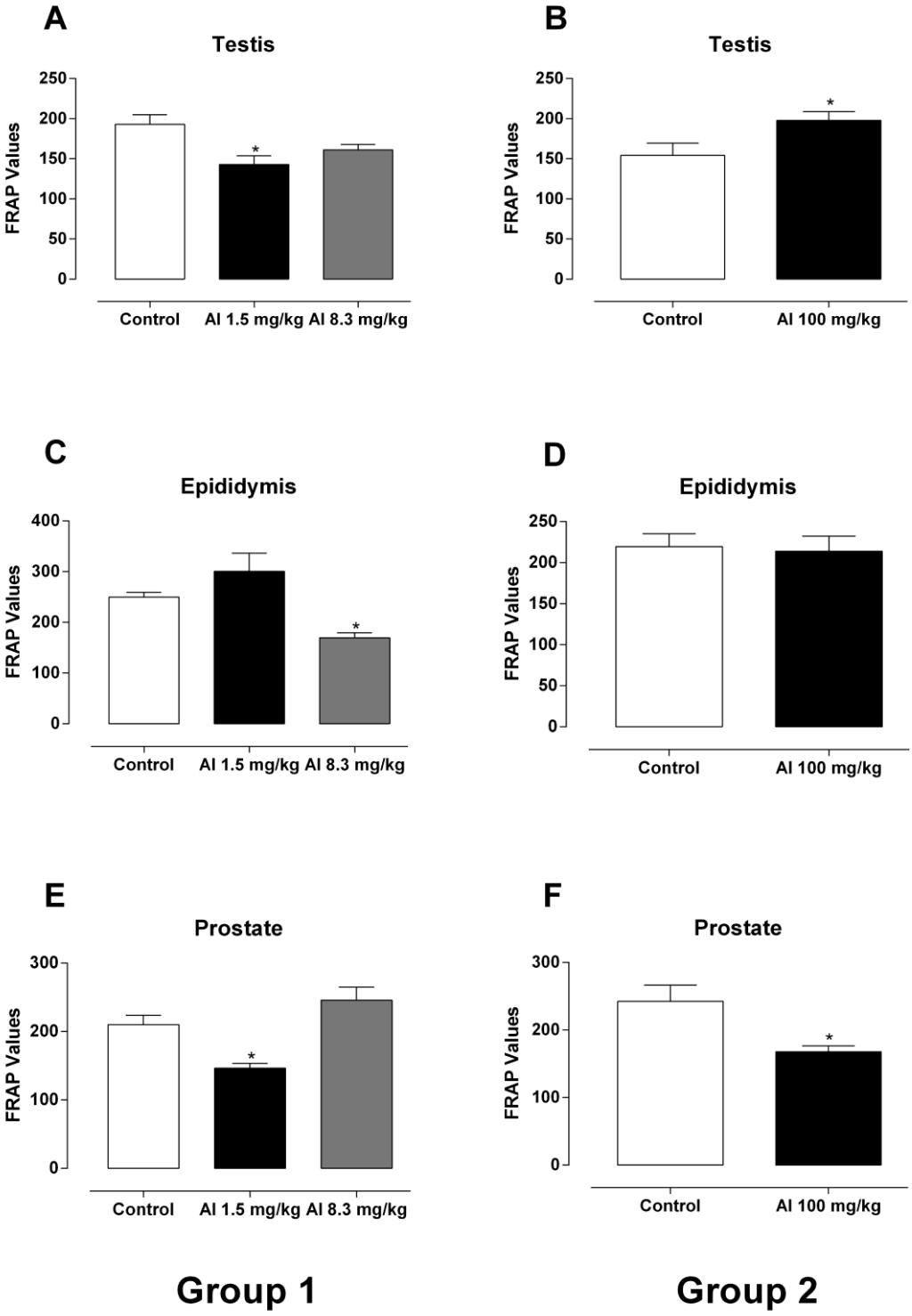
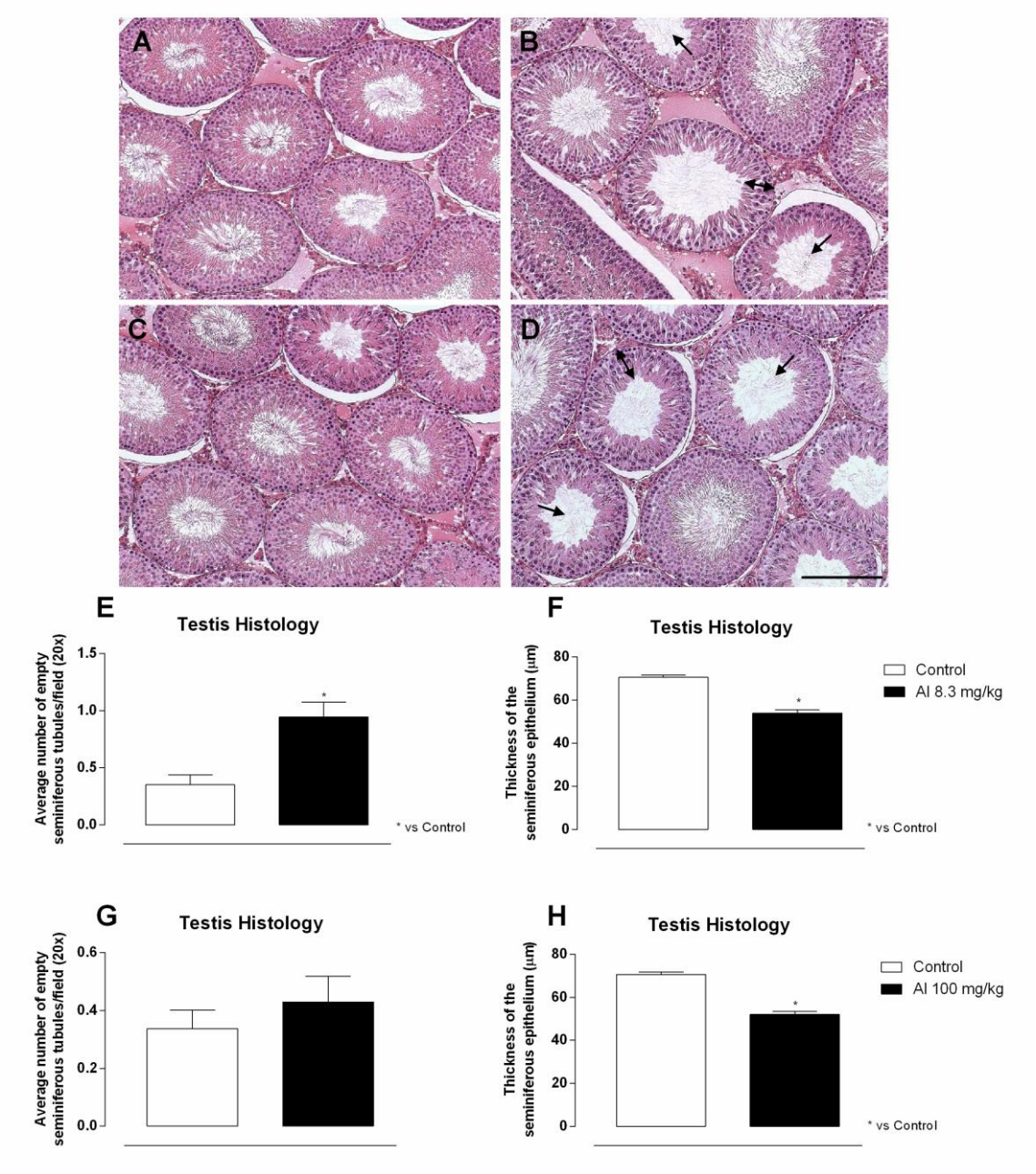


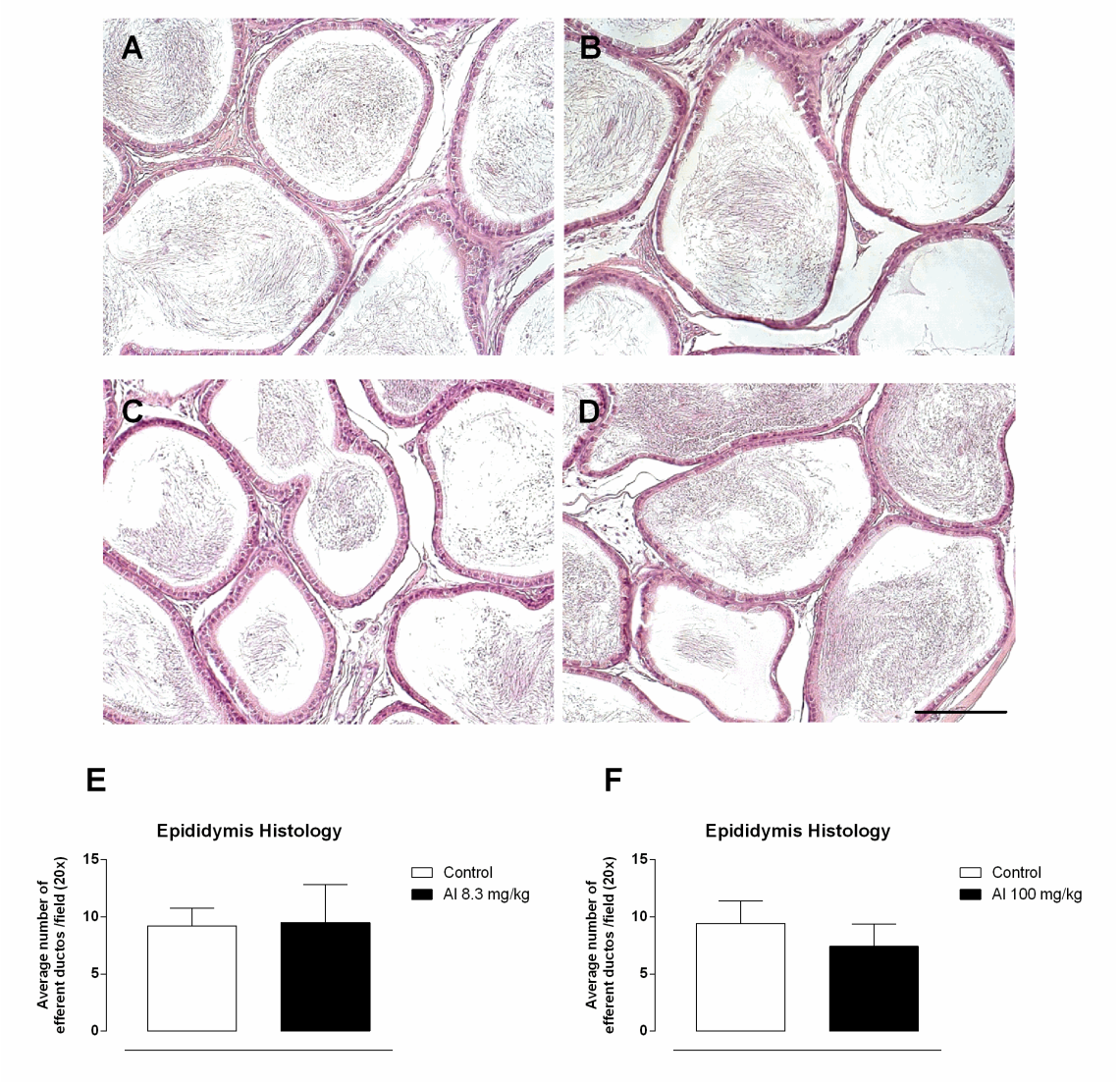
Group 2

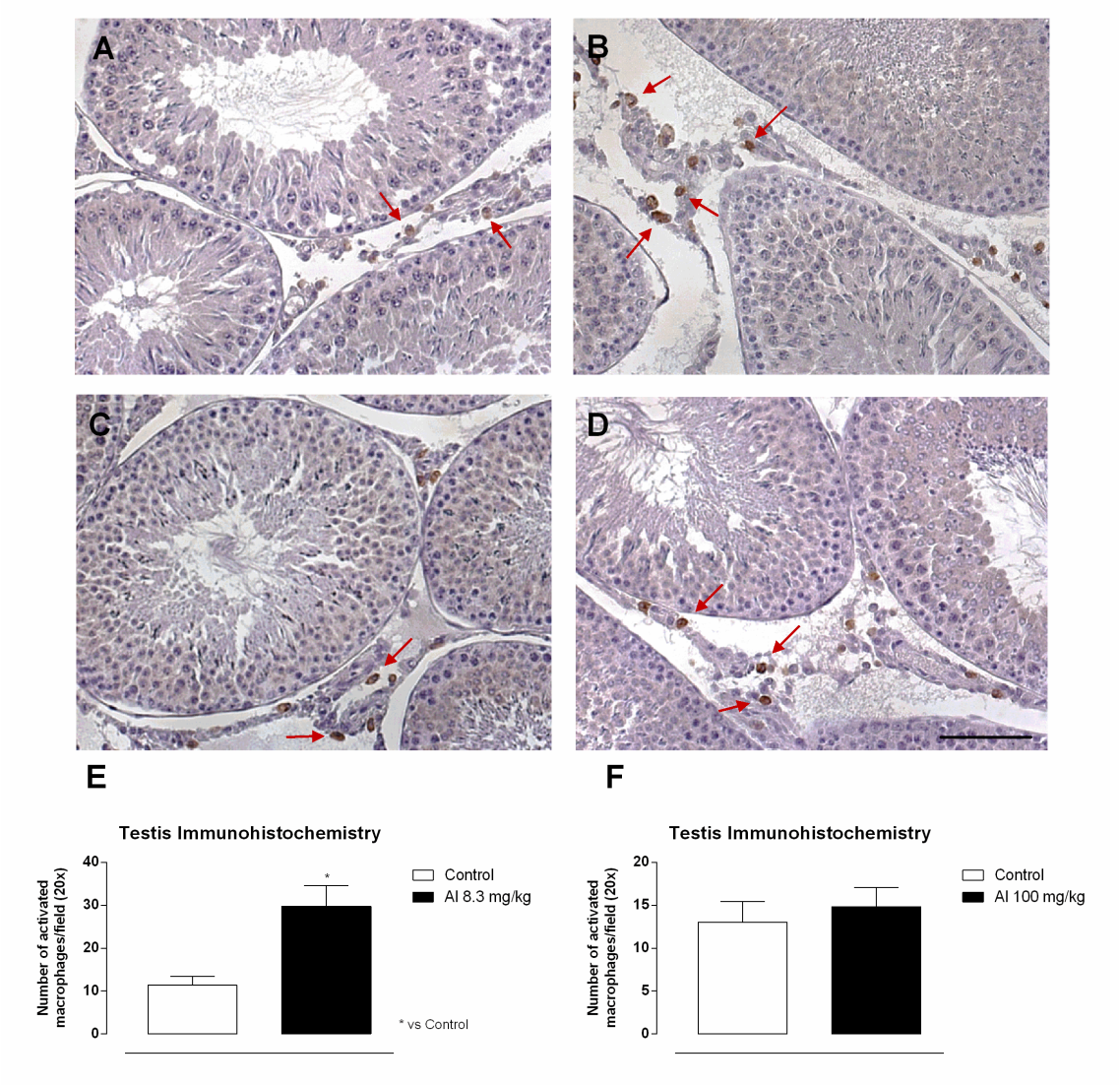












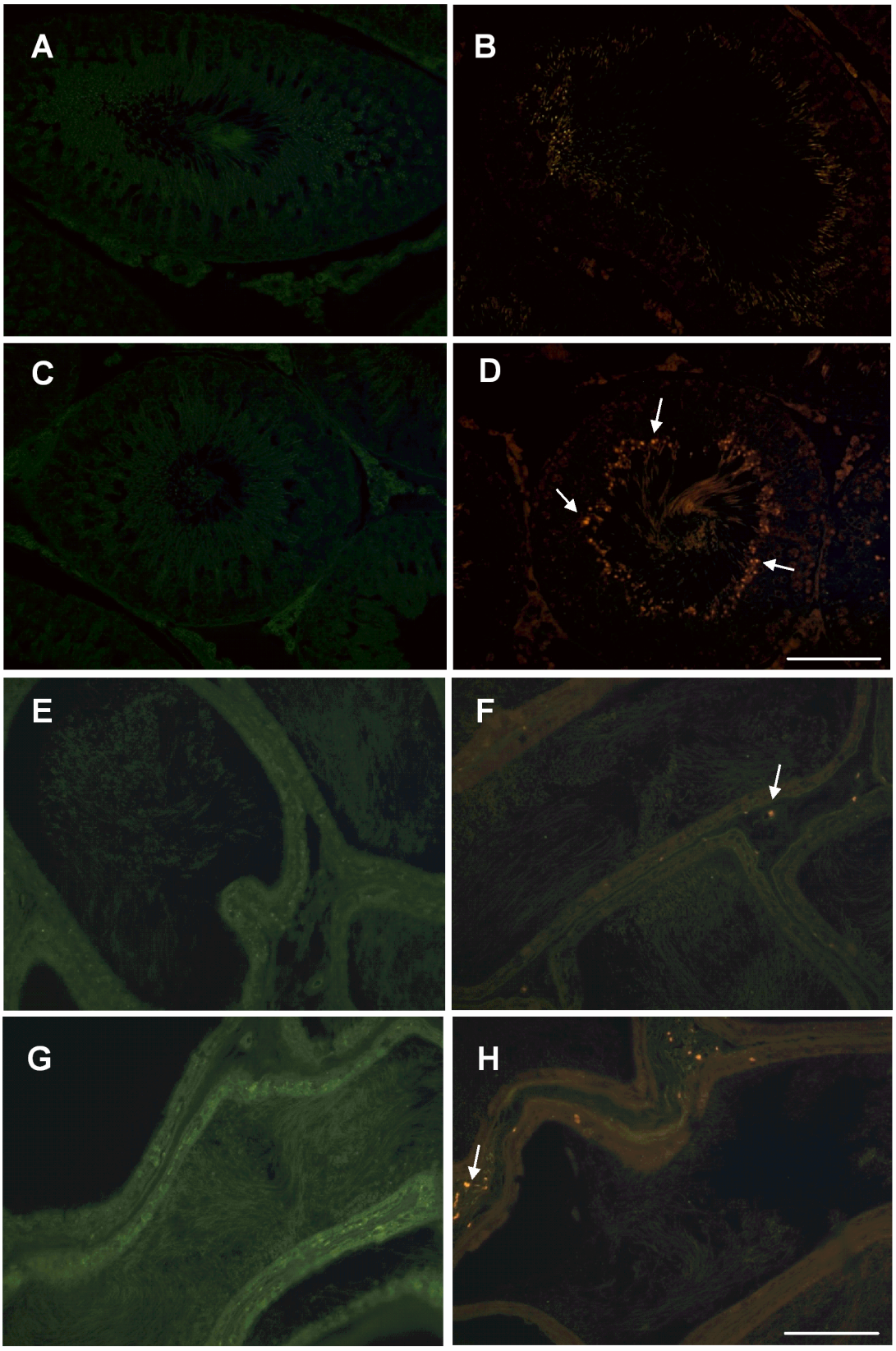


Table 1 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on daily feed and drink intakes ($p > 0.05$).

Feed / fluid intakes	**Group 1**			**Group 2**	
	Control	**1.5 mg Al /kg bw/d**	**8.3 mg Al /kg bw/d**	**Control**	**100 mg Al/kg bw /d**
Feed intakes	21.54 ± 0.27 g	22.16 ± 0.34 g	22.89 ± 0.41 g	22.23 ± 0.43 g	21.98 ± 0.34 g
Fluid intakes	35.24 ± 0.76 ml	34.99 ± 0.59 ml	35.67 ± 0.47 ml	34.32 ± 0.69 ml	35.67± 0.57 ml

Data are expressed as mean ± SEM. $p > 0.05$ (ANOVA or Student's t-test)

Table 2 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on body weight, 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

Data are expressed as mean ± SEM. The relative organ weight was calculated by use of the formula: organ weight/body weight x 100.

Units: g: gram, mg: milligram. * $p < 0.05$ compared with controls from the corresponding group 2 (Student's t-test)

Table 3 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm counts 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

DSP: daily sperm production; DSPr: daily sperm production relative to testis weight. Data are expressed as mean ± SEM. Units: g: gram. * $p < 0.05$ ** $p < 0.01$ compared with their corresponding controls (ANOVA or Student's t-test)

Table 4 Effect of subchronic aluminum exposure to low (group 1) and high (group 2) doses on sperm 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)**

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