TÜBİTAK

Turkish Journal of Medical Sciences

http://journals.tubitak.gov.tr/medical/

Research Article

Turk J Med Sci
(2016) 46: 1240-1248

doi:10.3906/sag-1501-101

The consequences of aluminium intake on reproductive function in male rats: a three-generation study

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Received: 21.01.2015 • **Accepted/Published Online:** 13.09.2015 • **Final Version:** 23.06.2016

Background/aim: The effects of aluminium exposure on reproductive biomarkers in male rats were followed in a three-generation study.

Materials and methods: Forty Wistar male rats (F_0) were divided into the following groups: control (C), receiving only tap water, and three experimental (E) groups, receiving aluminium sulphate (AS) (E_1: 200 ppb, E_2: 400 ppb, and E_3: 1000 ppb) in drinking water for a 6-month exposure period. To obtain F_1, three males from each group were mated with previously unexposed females (1:2 sex ratios) that during gestation and lactation were exposed to the same AS levels as males. The F_1 generation male offspring were divided as described and exposed to the same AS levels. The protocol to obtain F_2 was similar to that described for F_1.

Results: Significantly lower testosterone levels in rats exposed to AS and in generations F_1 and F_2 compared to the parental one, luteinising hormone (LH) fluctuations in F_0 and a significant LH decrease in F_2 and F_3 generations, testis weight decrease, increased immobile and abnormal sperm, and histoarchitecture alterations in the testes were observed. Moreover, interval between deliveries increased.

Conclusion: Chronic exposure to AS was significantly deleterious, producing a pronounced decrease in the sperm count and testosterone levels in all experimental groups.

Key words: Aluminium exposure, reproductive disruptor, rat model

1. Introduction

Aluminium (Al) is an abundant element in the earth's crust, existing primarily as polymorphous alumina silicates in combination with oxygen, silicon, fluorine, and other elements in soil, rocks, clays, and gems. Aluminium utensils are widely used throughout the world and for a long time it has been considered to exist predominantly in forms not biologically available to humans and animals. When aluminium cutlery is used with salty, acidic, or alkaline foods, the individual's aluminium exposure can increase significantly (1–3).

In the medicine field, aluminium compounds are now widely used being in the composition of numerous pharmaceutical conditionings (e.g., antacids, phosphate binders, buffered aspirins, vaccines, or antiperspirants), making them a potential threat (4–6). To date, the main known toxicological effects of aluminium included anaemia (7–9), neurodegenerative disorders such as Alzheimer disease and dementia (10–17), amyotrophic lateral sclerosis (18–20), hepatotoxicity (21–23), or diverse reproductive disorders (24–30). The toxic effects associated with aluminium are due, in most situations, to generation of reactive oxygen species (ROS) (31,32), conducing to cellular lipidic proteinic and/or DNA oxidative deterioration (33–35).

As aluminium exposure is still present, our research aim was to provide answers to some controversial opinions on aluminium's disrupting effects on male reproductive parameters after long-term exposure. We considered that a three-generation study can generate a complete and

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certain image of aluminium's deleterious activity in male rat reproductive biology. In this respect our rat model can be considered a helpful risk assessment tool, because it could be usefully extrapolated to humans.

2. Material and methods

2.1. Animals

Healthy Wistar albino rats (280–330 g) were purchased from the authorised Biobase of University of Medicine and Pharmacy "*Victor Babeș*" Timișoara, Romania. The rats were housed in standard polycarbonate cages (l × w × h = 750 × 720 × 360 mm) and fed ad libitum with standard diet. As bedding, wood shavings were used. The environmental temperature was maintained at 20 ± 2 °C and relative humidity was 55 ± 10%. During the experimentation period, the light cycle was 12 h light and 12 h dark. Before the start of the experiment the animals were kept in cages for 1 week to acclimatise and were handled in accordance with Directive 2010/63/EU on the handling of animals used for scientific purposes (36) and guidelines of the National Research Council, Institute of Laboratory Animal Research) (NRC) (37). The experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine from Banat's University of Agricultural Science and Veterinary Medicine from Timișoara (no. 3558/05.06.2012).

2.2. Animal grouping and experimental protocol

The age of rats was 28 days (immediately after weaning) at commencement of the study and the exposure period was 6 months. This rodent model was carried out over three generations (Figure 1).

Step I: Forty Wistar male rats for F_0 (parental) generation were divided in four groups (n = 10/each group): control (C), receiving only tap water, and three experimental groups receiving aluminium sulphate (AS) (aluminium sulphate octadecahydrate (purity ≥98%), from Sigma-Aldrich Chemie GmbH, Steinheim, Germany) in drinking water per experimental group as follows: E_1: 200 ppb; E_2: 400 ppb, and E_3: 1000 ppb.

Step II: In order to obtain the F_1 generation, three rat males randomly selected from each experimental group were mated with young female rats unexposed to aluminium in the ratio of 1 male to 2 females. During gestation and lactation, the female rats were exposed to the same levels as the male rats. The male rats from the F_1 generation were divided as described in step I and exposed to the same aluminium levels.

Step III: The protocol and conditions to obtain the F_2 generation were identical to those described in step II. The exposure for F_1 and F_2 rats was in utero, during lactation until weaning for a 6-month period.

At the end of each experimental step the unmated male rats (n = 7/each group) were euthanised by exsanguination,

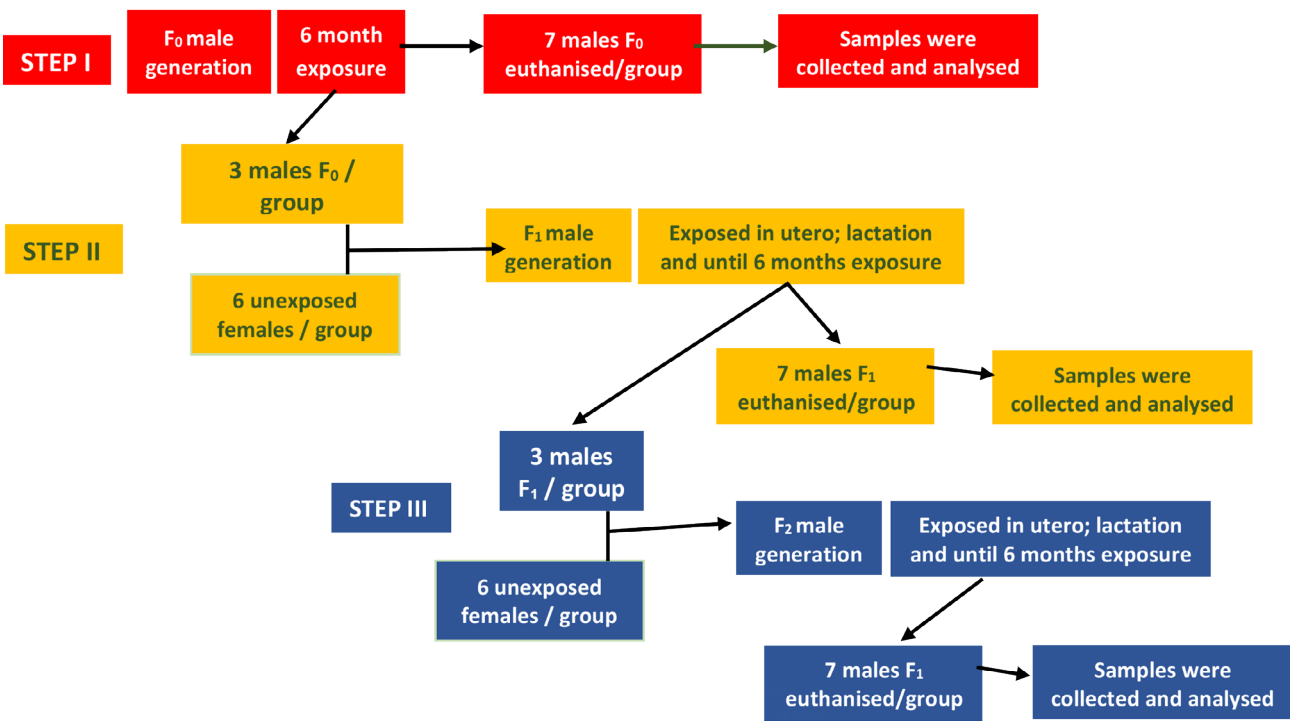

Figure 1. The experimental protocol steps.

after anaesthesia with ketamine (Ketaminol 10%, Intervet International BV, Boxmeer, Holland) at dose of 50 mg/kg.bw (38) and samples for the histologic assay were collected.

2.3. The hormonal assay

To obtain serum samples, blood was collected in a Serum Plain BD Vacutainer (BD ref no. 367837), without anticoagulant, using the cardiac puncture technique at the same time, from 0700 to 0800 in the morning, for each group. The serum testosterone and luteinising hormone (LH) level were determined by chemiluminescence using the Randox Evidence Evolution Biochip Array (Randox Laboratories, UK) in Tody Laboratories, Bucharest, Romania (ISO 15189 Certified laboratory).

2.4. Sperm assay

Immediately after euthanasia, fresh right and left vas deferens were excised; the luminal fluid content was collected and used for the sperm evaluation assay. An aliquot from this fluid was diluted in Tyrode's saline solution (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), prewarmed at 37 ℃ (at 1:20 dilution rate), gently mixed, and incubated for 3 min at 37 °C. For sperm motility evaluation, 3 µL of diluted sample was placed on a Leja slide chamber (Leja Products B.V., Netherlands) and computer-assisted analysed (IVOS HTB, version 12.3, Hamilton Thorne Biosciences, USA), using the HTB IVOS parameter settings, recommended for rat semen.

The ratio of motile sperm to the total sperm examined was determined. For sperm morphology analysis, an aliquot of 5 µL of the diluted sample was placed on a microscope slide, mixed with an equal volume of eosin 1% stain solution, smeared, air-dried for 5-10 min, and finally washed. An average of 200 sperm cells were examined on each slide sample using conventional light microscopy and classified as morphologically normal or abnormal. The sperm concentration of a vas deferens fluid sample was determined based on the haemocytometer cell counting method using a Neubauer counting chamber (Celeromics, France). An aliquot from the collected vas deferens fluid was diluted in 3% NaCl solution (at a 1:200 dilution) and sperm cells were counted according to the known WHO guidelines (39).

2.5. Histological assay

Being rapid and more suited for this tissue, Bouin's fixation by immersion and paraffin embedding was used: the testes were removed, washed in saline buffer, immersed and fixed in Bouin's solution, and embedded in paraffin medium. The blocks were sliced at 5 µm and then stained by Mallory's trichrome method. The histoarchitecture was studied using an Olympus CX 41 microscope (Olympus, Europe) with image capture software and data interpretation at 200× and 400× magnifications, to follow the structural changes and the morphometry and stereometry of the seminiferous tubules/experimental groups compared with the control group.

In addition, the testis weight dynamics was examined by histocytometry and the interval between deliveries was determined, and they were analysed comparatively for all three generations.

2.6. Statistical analysis

The statistical software used was GraphPad Prism 5.0 for Windows (GraphPad Software, San Diego, CA, USA). The results were expressed as mean ± SEM. For the evaluation of differences between groups, two-way ANOVA with Bonferroni correction was used, with statistical difference set at $P < 0.05$ or lower.

3. Results

3.1. General observations

The general health status of the control and experimental groups was good; the water and fodder intakes were normal for the entire experimental time. Over the 6-month study we did not observe any visible medical or behavioural modification, illness signs, or mortality in any rat group.

3.2. Testosterone and luteinising hormone dynamics

A significantly ($P < 0.01$) decreased serum testosterone (T) level in all three experimental groups compared to the control group was observed. An increase in AS intake level was followed by a T level decrease, but not strictly proportional.

Comparing the generations, T levels were significantly lower ($P < 0.01$) in the F_1 and F_2 generations compared to the F_0 generation. Making a comparison between the F_1 and F_2 generations, no significant ($P > 0.05$) differences were noted for the value of 200 ppb AS, but in the groups exposed to 400 and 1000 ppb AS, the decrease in testosterone levels was significant ($P < 0.01$) (Figure 2).

In the F_0 generation, exposure to AS was followed by a statistically significant ($P < 0.05$) increase in LH level in all experimental groups compared with the control one.

In the F_1 generation, an increase in LH levels not significant ($P > 0.05$) at 200 and 400 ppb AS but significant ($P < 0.05$) at 1000 ppb AS was noted. For male rats from the F_2 generation the LH levels were significantly lower ($P < 0.01$) in exposed groups compared to the C group.

In male rats from the F_1 generation a significant decrease ($P < 0.05$) in LH levels compared to the F_0 generation was observed at 200 and 400 ppb AS, but in the 1000 ppb AS exposed group a nonsignificant increase ($P > 0.05$) was observed. In comparison with the F_0 and F_1 groups, the LH levels from F_2 male rats were significantly lower ($P < 0.01$) (Figure 3).

3.3. Sperm quality assessment

In the F_0 generation, exposure to AS decreased the sperm counts nonsignificantly ($P > 0.05$) at 200 and 400 ppb but

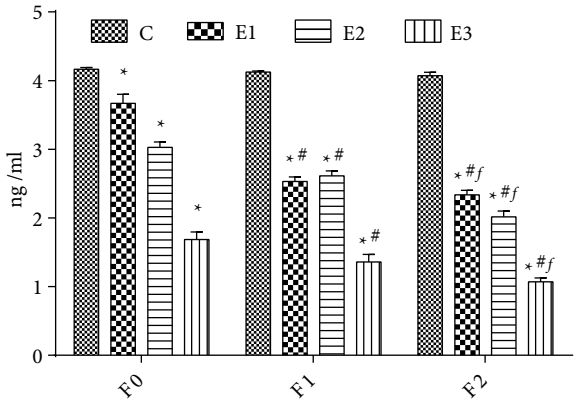

Figure 2. Serum testosterone dynamics in rats exposed to aluminium sulphate.
*significantly different compared to control group, $P < 0.01$
significantly different compared to F_0 generation, $P < 0.01$
f significantly different compared to F_1 generation, $P < 0.01$

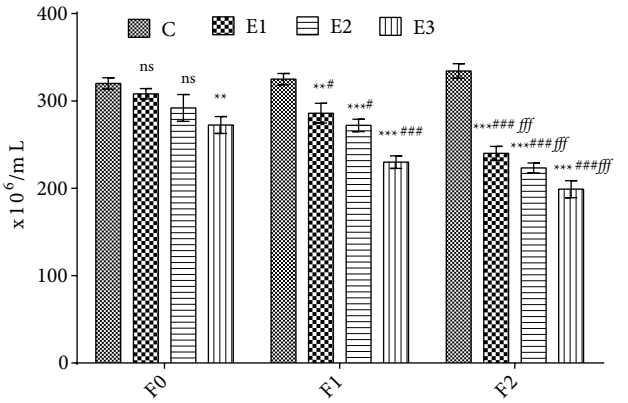

Figure 4. Sperm count dynamics in male rats exposed to aluminium sulphate.
ns - not significantly different compared to control, $P > 0.05$
, * significantly different compared to control group, $P < 0.01$, $P < 0.001$
#, ### significantly different compared to F_0 generation, $P < 0.05$, $P < 0.001$
fff significantly different compared to F_1 generation, $P < 0.001$

significantly ($P < 0.01$) at 1000 ppb. In F_1 male rats, the sperm count decreased significantly in all experimental groups at 200 ($P < 0.01$), 400, and 1000 ppb ($P < 0.001$), in the third generation, the same dynamics of the sperm count as in the F_1 generation being ascertained (Figure 4).

In all three generations the percentage of immobile sperm was significantly higher in AS exposed groups than in the C group ($P < 0.01$) (Figure 5).

Comparing the generations it was observed that the percentage of immobile sperm in male rats from the F_1 generation was increased more than of those from the F_0 generation, significantly only at 400 ppb AS. In individuals from the F_2 generation the comparative percentage of immobile sperm was significantly ($P < 0.01$) higher than of those from the F_0 and F_1 generations at all exposure levels: 200 ppb: F_2/F_0: +159.28%; F_2/F_1: +107.77%; 400 ppb: F_2/F_0: +167.46%; F_2/F_1: +55.17%; and 1000 ppb: F_2/F_0: +93.72%; F_2/F_1: +76.62%.

The percentage of abnormal sperm was significantly increased ($P < 0.01$) in all experimental groups from all generations compared to the control group, there being an increase in abnormal sperm percentage, but not strictly proportional to the dose or generation. A nonsignificant

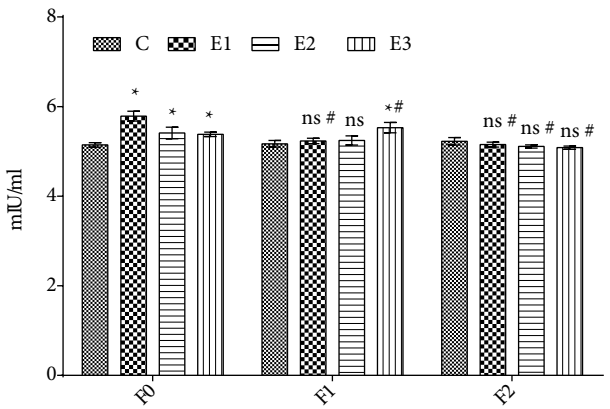

Figure 3. Serum LH dynamics in rats exposed to aluminium sulphate.
ns - not significantly different compared to control or F_0 and F_1 generation, $P > 0.05$
* significantly different compared to control group, $P < 0.01$
# significantly different compared to F_0 generation, $P < 0.01$

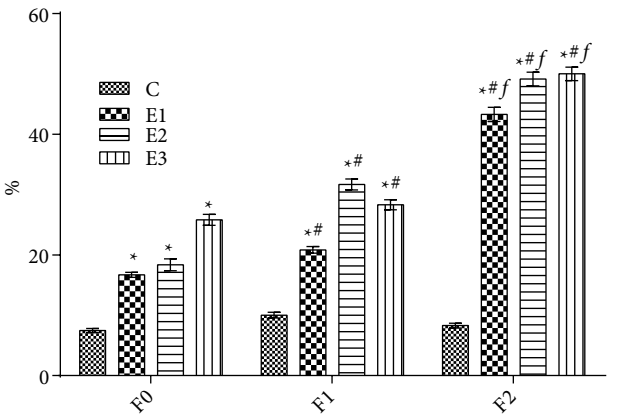

Figure 5. Dynamics of immobile sperm in male rats exposed to aluminium sulphate.
* significantly different compared to control group, $P < 0.01$
# significantly different compared to F_0 generation, $P < 0.01$
f significantly different compared to F_1 generation, $P < 0.01$

($P > 0.05$) increase in the abnormal sperm percentage was observed between the F_0 and F_1 generations. The increase in abnormal sperm percentage was significantly higher in male rats from the F_2 generation ($P < 0.01$) compared to those from the F_0 generation (Figure 6).

The main spermatozoid abnormalities registered were head without tail, broken tail, and flexed head (Figures 7A–7C), while the histological lesions observed were interstitial oedema, seminiferous epithelial necrosis and exfoliation, basal membrane disintegration, and Leydig cell necrosis and disintegration (Figures 7D–7F).

3.4. Testis weight dynamics

After exposure of rats to AS in the F_0 generation we observed that this was followed by a decrease in testis weight, not statistically significant ($P > 0.05$) at 200 ppb but significant ($P < 0.01$) at 400 and 1000 ppb.

In F_1 male rats, the testis weight decreased significantly in all experimental groups ($P < 0.001$); in the third generation, the same dynamics of the testis weight as in the F_1 generation was recorded. A significant decrease in testis weight was recorded in the F_1 and F_2 generations compared to the parental generation F_0 ($P < 0.001$) (Figure 8).

3.5. Interval between deliveries

The interval between deliveries increased in all three generations of the experimental groups compared to the control group, not significantly ($P > 0.05$) in the F_0 generation, but significantly in the F_1 and F_2 generations ($P > 0.05$ at 200 ppb in the F_1 generation and $P > 0.001$ in the other experimental groups). In the F_1 and F_2 generations, the intervals between deliveries were increased significantly compared to the F_0 generation ($P > 0.001$) (Figure 9).

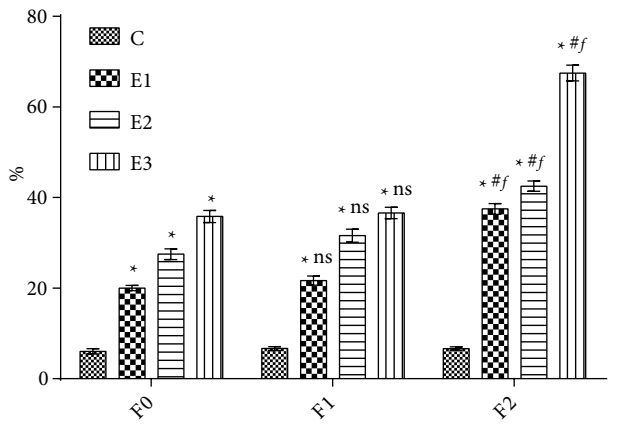

Figure 6. Abnormal sperm mean percentage dynamics in rats exposed to aluminium sulphate.
ns - not significantly different compared F_0 generation, $P > 0.05$
* significantly different compared to control group, $P < 0.01$
# significantly different compared to F_0 generation, $P < 0.01$
f significantly different compared to F_1 generation, $P < 0.01$

4. Discussion

Testicles are specialised gonad organs having two basic functions, to produce germinal cells and to produce steroid hormones. The LH stimulates the Leydig cells in males to synthesise and secrete testosterone, playing a decisive role in spermatogenesis (40).

Physiologically, in males, the LH levels are regulated and controlled by testosterone negative feedback and, according to this, the decrease in testosterone will determine the increase in LH level by hypophysis stimulation. In this respect, testosterone is a key hormone that regulates spermatogenesis, and so the decrease in testosterone has as a consequence the impairment of sperm count and quality. In the conditions of the present study we observed in all exposed individuals a testosterone level decrease (and appropriate to this, the LH level should increase).

In our research this phenomenon was present in the F_0 and F_1 generations, but in the F_2 generation was absent, even at 200 ppb AS, denoting, in our opinion, a lack of hypophysis response, and confirming others authors' results who proved that aluminium after absorption passes easily through the rat placenta and via milk in the suckling period, and so can be found in the next generation's tissues and organs (41,42).

Sun et al. observed also a significant decrease in testosterone and LH levels and a decrease in androgen receptor protein and nRNA expression, which weakened the binding of androgen with the specific receptors in rats exposed to 128.36 mg/kg.bw and 256.72 mg/kg.bw aluminium chloride for 120 days (40).

Guo et al. observed that exposure to aluminium significantly decreased testosterone levels in mice treated with different intraperitoneal doses of aluminium chloride for 12 or 16 days (26). Significantly reduced testosterone levels were recorded also by Khattab et al., who ascertained a highly significant decrease in sperm count, motility, and sperm viability and also a highly significant increase in sperm abnormalities in rat males exposed to 20 mg/kg.bw aluminium chloride for 70 days (43). In contrast to these authors, Mayyas et al. observed an increase in testosterone and LH levels in male mice exposed to 1000, 1200, and 1400 ppm/day aluminium chloride in drinking water for a 12-week exposure period (44).

Our study revealed with certainty a significant decrease in sperm count, especially in the F_1 and F_2 generations in the case of the highest aluminium exposure levels. We also found an increase in immobile and abnormal sperm percentages in all exposed groups, compared with the control group, and in all generations, our finding being sustained by other published research (24,29,45,46).

Another possible mechanism for low sperm count (aluminium can block the voltage-gated calcium channels,

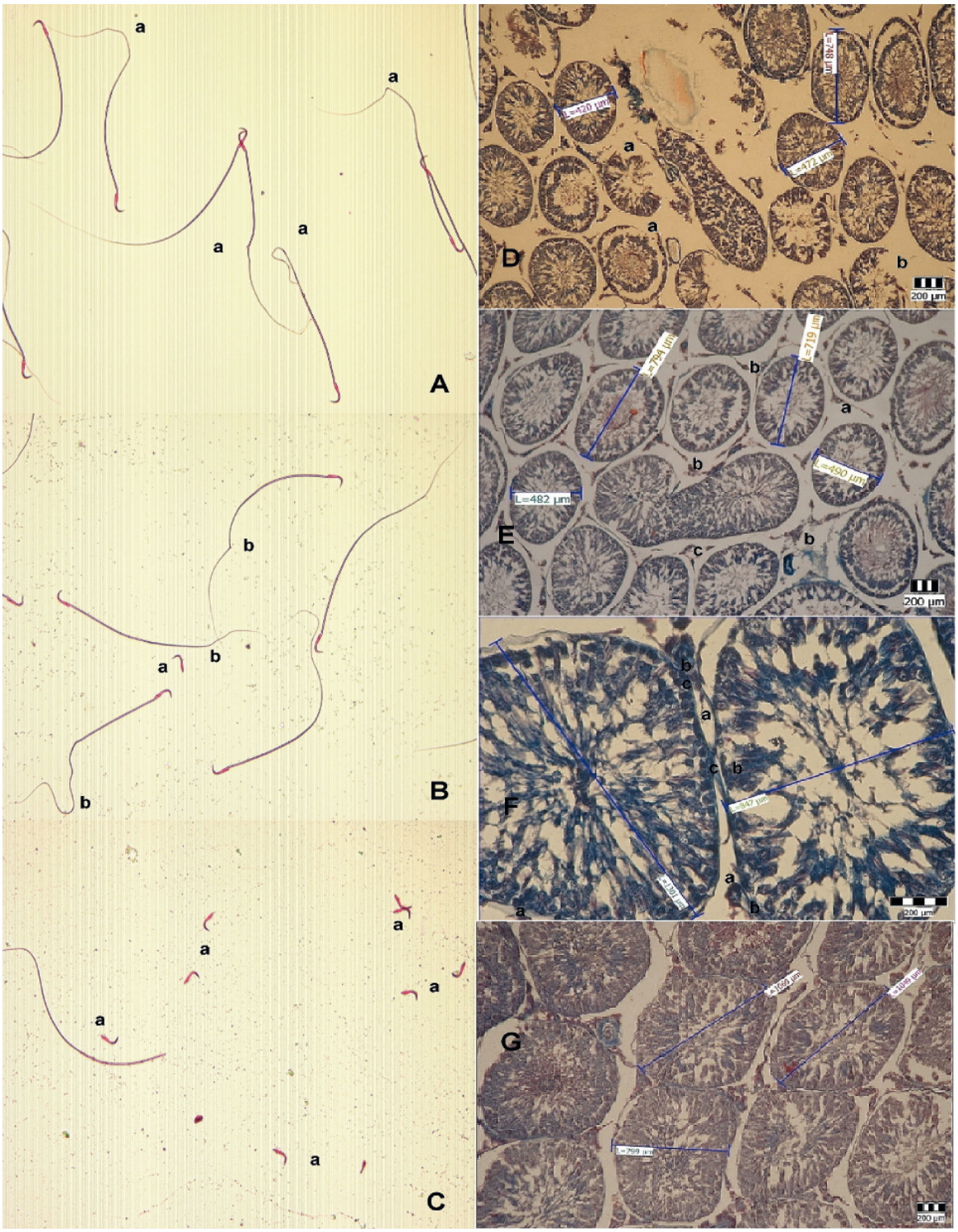

Figure 7. Sperm abnormalities (A, B, C) and histoarhitectonics lesions (D, E, F). in rats exposed to aluminium sulphate.
A. Sperm abnormalities in F_0 rats exposed to aluminium sulphate: a – bent tail (Mag. 200×); B. Sperm abnormalities in F_1 rats exposed to aluminium sulphate: a – head without tail, b – bent tail; (Mag. 200×) C. Sperm abnormalities in F_2 rats exposed to aluminium sulphate: a – head without tail; D. Testis section in F_0 generation (400 ppb, T.M. 100×) a – slight interstitial oedema, b – seminiferous tubules epithelial necrosis and exfoliation; E. Testis section in F_1 generation (1000 ppb, T.M. 100×) a – interstitial oedema, b – extended seminiferous epithelial necrosis and exfoliation, c – slight Leydig cell disintegration; F. Testis section in F_2 generation (1000 ppb, T.M. 400×) a – large areas of interstitial oedema, b – large zones with seminiferous tubules necrosis and basal membrane disintegration, c – Leydig cell necrosis and disintegration; G. Testis section in control group, normal image and dimensions of the seminiferous tissue (T.M. 200×).

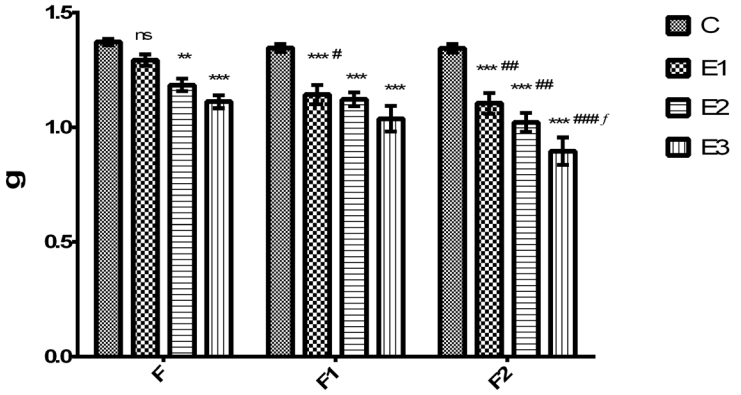

Figure 8. Testis weight dynamics in rats exposed to aluminium.
, * significantly different compared to control group,
ns – not significant, P < 0.01, P < 0.001
#, ##, ### significantly different compared to F_0 generation, P < 0.05, P < 0.01, P < 0.001
f significantly different compared to F_1 generation, P < 0.05

causing impairment of gonadotropin secretion in the hypophysis, and resulting in a decrease of sperm count) was also suggested (46).

Buraimoh et al. observed a significant decrease in sperm count in rats that received 475 mg/kg.bw, 950 mg/kg.bw, 1425 mg/kg.bw, and 1900 mg/kg.bw aluminium chloride via intubation for 8 weeks. The reduced sperm counts in this case may be caused by interference by this element with the sperm's maturation and storage in the epididymis or by interference with sperm production in the testis (47).

A similar dynamics of sperm count and semen morphology was observed in rats exposed even to smaller

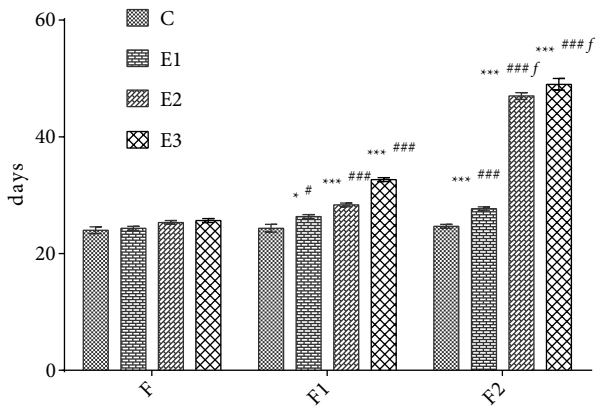

Figure 9. Interval between deliveries in rats exposed to aluminium.
*, *** significantly different compared to control group,
ns – not significant, P < 0.05, P < 0.001
#, ### significantly different compared to F_0 generation, P < 0.05, P < 0.001
f significantly different compared to F_1 generation, P < 0.001

aluminium chloride doses (64.18 mg/kg.bw, 128.36 mg/kg.bw, and 256.72 mg/kg.bw) (48). These aluminium levels overlapped to a great extent the values previously found by our collective from areas surrounding the aluminium industry in Romania (49).

Hirata-Koizumi et al., in a two-generation study, affirmed that they did not identify any changes in caudal epididymis sperm numbers or in the percentage of abnormal sperm in rats exposed to aluminium sulphate in doses of 50, 500, and 5000 ppm. On the other side, in our study, we have observed with certitude, an increase in immobile sperm in rats exposed to aluminium sulphate, compared to the control group, the most significant increase being ascertained especially in the second and third generations (50).

Authors even found a possible cause of reduced motility and viability of sperm. It may be due to a protein, the aconitase that binds citrate and so catalyses its isomerisation to isocitrate, via the intermediate cis-aconitase in the Krebs cycle, which, in the presence of aluminium, showed decreased activity. This could be influenced by the mitochondrial enzymes and consequently the changes in mitochondrial function may influence sperm quality parameters (29,30).

The sperm count, morphology, and motility alteration and testosterone decrease could be also a consequence of the testis histoarchitecture alteration that we recorded. We found severe and alarming changes in the testes' histoarchitecture, modifications that are supported also by other researchers' findings (46,51).

Those modifications are the consequences of the oxidative stress in the testes, being reported also by other authors (27,43).

From a histologic point of view, we agree with authors who reported that aluminium can produce a marked degeneration and necrosis of the germ cells lining,

interstitial oedema, and testicular degeneration with complete absence of germ cells in male rats treated with aluminium chloride at dose of 75 mg/kg.bw (26,27), or marked distorted seminiferous tubules with loss of normal distribution of epithelial lining and vacuolar cytoplasm (47), all these being solid indicators of testis degeneration.

The present study confirmed the indisputable role of aluminium as reproductive disruptor and toxic after chronic exposure to aluminium sulphate. The main observed modifications were a pronounced decrease in sperm count and testosterone levels in all experimental groups, an increase in immobile and abnormal sperm percentage and testis histoarchitecture alteration in all groups and all three generations (being more evident in the subsequent generation than in the parental one), and testis weight decrease in all experimental groups.

Acknowledgements

This work was carried out during the project POSDRU/89/1.5/S/62371, co-financed by the European Social Fund through the Sectorial Operational Programme for the Human Resources Development 2007–2013.

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