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Multigenerational exposure to uranium changes morphometric parameters and global DNA methylation in rat sperm

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1 **Multigenerational exposure to uranium changes morphometric parameters and global DNA**
2 **methylation in rat sperm.**

3
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17 **Running title:**

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19 Effects of uranium on sperm after multigenerational exposure.

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32 **Abstract**

33 There is increasing evidence that environmental exposures early in foetal development
34 influence phenotype and give rise to disease risk in the next generations. We previously found
35 that lifelong exposure to uranium, an environmental contaminant, induced subtle testicular and
36 hormonal defects, however its impact on reproductive system in multiple subsequent generations
37 was unexplored. Herein, rats were exposed to a supra-environmental and non-nephrotoxic
38 concentration of natural uranium (U, 40 mg.L⁻¹ drinking water) from postnatal life to adulthood
39 (F0), during-foetal life (F1) and only as the germ cells from the F1 generation (F2). General
40 parameters (reproductive indices, epididymal weight) and sperm morphology were assessed in
41 the three generations. In order to identify the epigenetic effects of U, we analyzed also the global
42 DNA methylation profile and described for the first time the mRNA expression levels of markers
43 involved in the (de)methylation system in rat epididymal spermatozoa. Our results showed that
44 the F1 generation had a reduced pregnancy rate. Despite the sperm number being unmodified,
45 sperm morphology was affected in F0, F1 and F2 generations. Morphometric analysis for ten
46 parameters was detailed for each generation. No common parameter was detected between the
47 three generations, but the head and the middle-piece were always modified in the abnormal
48 sperms. In the F1 U-exposed generation, the total number of abnormal sperm was significantly
49 higher than in the F0 and F2 generations, suggesting that foetal exposure to uranium was more
50 deleterious. This effect could be associated with the pregnancy rate to produce the F2 generation.
51 Interestingly, global DNA methylation analysis showed also hypomethylation in the sperm DNA
52 of the last F2 generation. In conclusion, our study demonstrates that uranium can induce
53 morphological sperm defects and change in the DNA methylation level after multigenerational
54 exposure. The epigenetic transgenerational inheritance of U-induced reproductive defects should
55 be assessed in further experiments.

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61 **Keywords:** morphology, morphometry, methylation, sperm, fertility, multigenerational,
62 uranium.

63 **Introduction**

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65 Environmental pollution is largely blamed for having a negative influence on human
66 health, particularly male infertility and for the increasing incidence of marital infertility in some
67 parts of the world (Agarwal et al., 2015; Bonde et al., 2016). It is therefore important to identify
68 the environmental factors that could alter the male reproductive function to understand why male
69 human reproductive disorders like cryptorchidism, hypospadias, low sperm counts and testicular
70 cancer may be increasing in incidence in many countries in the last few decades (Virtanen et al.,
71 2017).

72 Male infertility can be induced by multiple mechanisms and in addition, many different
73 factors play a role, for example, in the alteration of sperm parameters (Le Moal et al., 2014;
74 Virtanen et al., 2017). Sperm morphology has long been considered an important factor for
75 evaluating semen and sperm quality. Unfortunately, manual assessments remain problematic
76 because there can be large variations among technicians and laboratories owing to subjective
77 evaluation of sperm morphology. Thanks to computer-aided sperm morphometry analysis
78 (CASMA), automated analysis of rat sperm morphology and morphometry may be useful tools
79 for quantification of the effects of reprotoxicants on sperm morphology (Van der Horst et al.,
80 2018).

81 Epidemiological, clinical and experimental arguments suggest that idiopathic male
82 infertility results from harmful effects caused by pollutants and they **may** act by disrupting the
83 epigenetic mechanisms (Gunes et al., 2016; Strazzullo and Matarazzo, 2017). Indeed, the role of
84 epigenetic modifications, like DNA methylation, is involved in the regulation of spermatogenesis
85 in testis and in sperm function (Gunes and Kulac, 2013; Laurentino et al., 2016). Other
86 environmental factors such as ionizing radiation could alter the DNA methylation in sperm
87 modifying the gene expression and inducing male infertility (Merrifield and Kovalchuk, 2013).
88 In addition, an important question may be raised in regard to whether such modifications in
89 methylome and gene expression **are** transmitted to the next generation. An increasing numbers of
90 environmental pollutants (like endocrine disruptors compounds) are involved in epigenetic
91 transgenerational reproductive disorders confirming the need to perform multi and
92 transgenerational experimental studies (Skinner, 2016).

93 In previous experimental studies, chronic low-dose exposures to uranium, a water
94 environmental pollutant (Souidi et al., 2009), were associated with male reproductive defects
95 (Grignard et al., 2008; Legendre et al., 2016) and modifications of DNA methylation in gonads
96 (Elmhiri et al., 2017). Elmhiri et al. demonstrated that chronic low-dose exposure of uranium
97 affects the global methylation profile in the gonads and this profile has been conserved across
98 generations. The aim of the current investigation was to establish for the first time whether
99 chronic exposure to Natural Uranium (NU) could (i) modify the morphometric sperm parameters,
100 (ii) induce epigenetic effects (DNA methylation) in spermatozoa of rats after multiexposure of
101 F0, F1 and F2 generations.

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121 **Material & Methods**

123 **Experimental design and uranium contamination**

124 All experimental procedures were approved by the Animal Care Committee of the
125 Institute of Radioprotection and Nuclear Safety (IRSN) and complied with French regulations for
126 animal experimentation (Ministry of Agriculture Act No.87-848, October 19, 1987, modified
127 May 20, 2001).

128 NU (Mc Arthur) was obtained from CERCA (Pierrelatte, France). Uranyl nitrate
129 hexahydrate ($\text{UO}_2(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$). The drinking solution was prepared to obtain a final uranium
130 concentration of $40 \text{ mg} \cdot \text{L}^{-1}$ in mineral water (daily uranium intake dose: $1 \text{ mg}/\text{rat}/\text{day}$) (Paquet et
131 al., 2006). The specific activity of the NU was $2.42 \times 10^4 \text{ Bq} \cdot \text{g}^{-1}$, and its isotopic composition
132 was $^{238}\text{U} \sim 99.307\%$, $^{235}\text{U} \sim 0.688\%$, and $^{234}\text{U} \sim 0.005\%$. This concentration of NU is not
133 nephrotoxic (Souidi et al., 2009) and three times higher than the highest uranium concentration of
134 $12.4 \text{ mg} \cdot \text{L}^{-1}$ naturally, found in well water in Finland (Salonen, 1994), to half of the WHO 2011
135 drinking-water guideline for uranium, defined as equal to $0.030 \text{ mg} \cdot \text{L}^{-1}$ (Frisbie et al., 2013). All
136 control groups of rats received *ad libitum* uncontaminated mineral drinking water Evian®
137 (Evian-les-Bains, France).

138 Each experimental group was composed by a number of 20 rats. This number has been
139 calculated to highlight low biological effects associated with low-dose exposure with a sufficient
140 statistical power.

141 Outbred Sprague-Dawley female rats, 12-weeks old and 16-days pregnant, were obtained
142 from Charles River Laboratories (L'Arbresle, France). They were housed individually and
143 maintained in a 12 h light/12 h dark cycle (regular cycle) at 21°C and 50% humidity, with access
144 *ad libitum* to a standard rodent pellet diet and water until birth (Figure 1).

145 The first generation (F0) of the treated group was chronically exposed to NU through
146 drinking water from their birth to 9 months old. During weaning (three first weeks of life), rats
147 (F0) were mostly exposed to NU through lactation (human offspring receives approximately 5%
148 of the mother's daily uranium dose (Wappelhorst et al., 2002)) and through the contaminated
149 drinking water. At weaning, male and female (F0) of different bearing were sorted and randomly
150 housed together. Each experimental group (control *vs* contaminated) was itself divided to obtain
151 breeding and non-breeding subgroups. Finally, four F0 experimental groups were thus created. F0

152 male and female rats were contaminated *via* drinking water supplemented with NU. Rats of the
153 breeding group were mated after 6 months of exposure (a sufficiently long time to chronically
154 expose rats to NU). To compose the next generation F1, one male was housed and mated with
155 two females during 5 days as recommended (Bohacek and Mansuy, 2017). Rats of the non-
156 breeding group were exposed to NU until 9 months old (Figure 1).

157 The next generation (F1), was exposed to NU *in utero* (sensitive foetal-developmental
158 windows (St-Cyr and McGowan, 2018)) and after birth through the mother F0 milk. At weaning
159 the protocol of contamination was stopped and all rats received *ad libitum* uncontaminated
160 mineral drinking water. Males and females F1 of different bearing were sorted and randomly
161 housed (control *vs* contaminated and themselves divided to obtain breeding and non-breeding
162 subgroups.). Breeding groups F1 were mated following the same conditions as previously done
163 for F0 and non-breeding groups housed until 9 months old. Indeed, the last generation F2 was
164 contaminated only from the parental germ cells F1. All animals were euthanized at 9 months of
165 age (Figure 1).

166
167 At 9-months, rats were anaesthetized by inhalation of 5 % isoflurane (Abbot France,
168 Rungis, France) and euthanized by an intracardiac puncture. The paired epididymis were
169 removed and weighed after releasing adipose tissues. Relative organ weight was estimated by
170 calculating the ratio between organ weight and body weight. The organs were deep-frozen in
171 liquid nitrogen, and stored at -80 °C.

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174 **Sperm isolation and staining**

175 Directly after sacrifice, the epididymis was dissected free from blood vessels, fatty tissue
176 and other connective tissue and they were placed in a petri dish containing a 5 mL solution of
177 M199 medium (Sigma-Aldrich, St Louise, MO, USA) at 37°C. The proximal caudal portion of
178 each epididymis was isolated and placed in 5ml of fresh medium in 6 well plates at 37°C. A short
179 length of “cleaned” epididymal tubule was removed by using either scalpel.

180 Sperm flowed freely from the ends of the transected tubule into the medium and a cloud
181 of sperm quickly formed. Media containing sperm were collected from the edges of the sperm
182 ‘cloud’ using a positive displacement pipette (15 µL). For staining sperm, the sperm suspension

183 was placed on a slide and a sperm smear was made by dragging the fluid behind another slide
184 while spreading it at a 45° angle across the slide. After air drying at room temperature, slides
185 were fixed by immersing them horizontally into SpermBlue® fixative (Microptic SL, Barcelona,
186 Spain) for 10 minutes, and stained thereafter with the SpermBlue® stain for 20 minutes,
187 following a similar immersion technique (Van der Horst, 2009). Stained slides were then gently
188 submerged in distilled water for 3 seconds to remove excess stain, and allowed to air dry at a 60-
189 80° angle. Slides were finally mounted using Eukitt (O. Kindler GmbH, VWR, France).

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191 **Sperm morphology and morphometric analysis**

192 Sperm morphology and morphometry were performed using the CASMA Sperm Class
193 Analyser system (SCA® ; Microptic SL) version 5.4 software and using methods previously
194 described (Van der Horst et al., 2018). Analysis used bright field optics employing a 60 x
195 objective, *i.e.*, 600 x magnification, and blue filter on a Nikon E200 microscope. One hundred
196 sperm/animal were analyzed (N= 10 / per group). All spermatozoa were measured randomly, to
197 exclude any bias in favour of a specific morphological form. Images were captured digitally using
198 a Basler 780–75 gigaethernet camera (Basler Microscopy Camera, Germany). The SCA system's
199 morphology module RatTox (Microptic S.L., Barcelona, Spain) automatically analyzes
200 morphometric dimensions of the sperm head and middle-piece (MP). For head morphometry,
201 head length (ARC), width, surface area, perimeter, angle, and roughness (which varies with a
202 range between 0 and 1, and expresses the degree of resemblance of the sperm head to a circle)
203 were measured. Measurements of the MP included width and angle of insertion of the flagellum
204 to the head. The chord length, *i.e.*, distance from the anterior tip of acrosome to the posterior part
205 of head, was measured. Linearity is a derivative expressed as a percentage and refers to
206 $ARC/chord \times 100$ (**Table 1**).

207 To determine morphologically normal *vs* morphologically abnormal sperm in animal
208 species, we used the new approach to determine cut-off points for normal *vs* abnormal rat sperm
209 previously described (Van der Horst et al., 2018). Our method was based on grouping the
210 different morphometric measurements for each percentile, *e.g.*, for each morphometric parameter.
211 The minimum and maximum values for each specific morphometric parameter were derived from
212 the percentile intervals (5–95%) (**Table 2**). Total morphologically abnormal sperm indicating the
213 sum of abnormal head and MP was also determined. The percentage of abnormal sperms was

214 also calculated if sperm has at least one, only one or more than two abnormalities in the head, in
215 MP or in both compartment (head and MP).

216 To determine sperm concentration, epididymis was entirely minced and homogenized.
217 For each animal, sperm concentration was determined in triplicate using Malassez. Counting
218 results are expressed as the total number of sperm cells per mL of epididymis. Sperms were
219 stored at -80°C for subsequent experiments.

220

221 **Epigenetics analysis**

222 **DNA extraction**

223 Isolation of genomic DNA from sperm was optimized according to QIAamp DNA Mini
224 Kit (QIAGEN, France): 100 µL of semen ($5 \cdot 10^6$ cells) was transferred into a new 1.5 mL tube
225 and then 100 µL of buffer X2 (20 mM Tris-Cl pH 8.0; 200 mM NaCl; 20 mM EDTA; 4% SDS),
226 80 mM DTT, 250 µg/mL proteinase K were added and gently mixed. The samples were
227 incubated at 55 °C for 1h. Thereafter 200 µL of Buffer AL and 200 µL ethanol (100%) were
228 added and mixed. The elution of DNA in 50 µL was finally obtained following the tissue
229 protocol (steps 5 to 8) in the QIAamp DNA Mini Kit (QIAGEN, France). The samples were
230 stored at -20 °C until further analysis.

231

232 **Global DNA methylation**

233 Global DNA methylation of epididymal sperm was detected using an ELISA reaction
234 with a monoclonal antibody sensitive and specific for 5-methylcytosine (5-mC) and a horseradish
235 peroxidase conjugate as secondary antibody (5-mC DNA ELISA Kit, Zymo Research, Irvine,
236 CA, USA). The level of 5-mC in DNA is reported as the amount of methylated cytosine relative
237 to the cytosine genomic content (%). An aliquot of the sample containing 100 ng DNA was added
238 to the 5-mC coating buffer and brought to a final volume of 100 µL. All samples and controls
239 were denatured at 98 °C for 5 min in a thermocycler and immediately cooled on ice for 10 min.
240 Controls and samples were added to the ELISA plate and incubated at 37 °C for 1 h. After
241 discarding the coating buffer, the wells were washed three times with the 5-mC ELISA buffer
242 and incubated again with 200 µL of 5-mC ELISA Buffer at 37 °C for 30 min. The buffer was
243 discarded from the wells and the samples were incubated with the antibody mix at 37 °C for 1 h.
244 The antibody mix consisted of the 5-mC ELISA Buffer, anti-5-methylcytosine and the secondary

245 antibody in a ratio 1:2000:1000. After incubation the antibody mix was discarded and 100 μ L
246 HRP was added to each well. The absorbance was measured in duplicate at 405 nm using
247 TECAN reader (Infinite® 200 PRO NanoQuant Plate Reader). The results are expressed as % 5-
248 mC per Total C.

249

250 **RNA extraction and qPCR**

251 Total RNA was extracted from sperm, using mirVana™ miRNA Isolation Kit (Ambion,
252 cat.no.1560). The NanoDrop apparatus (ThermoFisher Scientific, Cergy Pontoise, France) was
253 used for determining the concentration of RNA ng/ μ L. 1 μ g of total RNA was reversely
254 transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems,
255 Courtaboeuf, France) according to the manufacturer's instructions. Real-time qPCR was
256 performed with QuantStudio 12K Flex Real-Time PCR System (ThermoFisher Scientific, Cergy
257 Pontoise, France) using TAQMAN (Applied biosystems), to analyse the mRNA levels of
258 demethyls genes *TET2* (Rn01522037_m1) , *TET3* (Rn01425643_m1), *TDG* (Rn00821513_m1),
259 with three internal controls (*GAPDH* (Rn01775763_g1), *Ywaz* (Rn00755072_m1) and *β 2M*
260 (Rn00560865_m1)) and using SYBR Green green® (Applied biosystems) to analyse the mRNA
261 levels of methyltransferases: *DNMT1*, *DNMT3a* , *DNMT3b* , *DNMT3l* with three internal controls
262 *HPRT*, *β 2M*, and *ACTB* (**Table 3**), relative changes in genes mRNA expression in sperm were
263 calculated using e- Δ CT method and three internal controls (Pfaffl, 2001; Vandesompele et al.,
264 2002). All RTqPCR results are expressed as mean standard deviation, and compared with
265 expression levels of the control group.

266

267 **Statistical Analysis**

268 Results are expressed as mean \pm standard deviation (SD), Student's t-test was routinely
269 performed for statistical analysis of data, and was replaced by Mann-Whitney Rank Sum Test
270 when the equal variance test failed (determined by Sigmaplot Stat software). The Fisher exact test
271 was used to compare fertility index and a quasi-poisson regression was conducted for the
272 anomaly rates modeling.

273 Since the epididymis data consist of a paired observation within the same rat, a reasonable
274 assumption is that these observations are correlated and therefore, statistical methods that
275 recognize and account for the correlation of observations within a subject are appropriate. Thus, a

276 generalized estimating equations (GEE) regression (Zeger and Liang, 1986) was conducted using
277 using the Zelig Package of the R software (R Software, 2016).

278 Differences were considered statistically significant when $p \leq 0.05$.

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307 **Results:**

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309 **Fertility index & Epididymis relative weights**

310 The multigenerational exposure to uranium had no effect on the number of offspring born,
311 male/female sex ratio, or pup mortality in any of the F0, F1 and F2 generations. The average litter
312 size was constant between the control and U-exposed generations (data not shown). However, the
313 pregnancy rate was significantly decreased (55%) in F1 U-exposed generation (35%) in
314 comparison to the pregnancy rate of F0 U-exposed generation (70%) (**Table 4**). The neonatal
315 mortality rate was less than 5% for both control and U generations (data not shown). The
316 epididymis / body weight ratio between the control and U generations was statistically different
317 only in the F1 generation, decreased to -15.4 ± 0.6 % in comparison to the control group (**Table**
318 **4**).

319

320 **Morphometric parameters and sperm morphology**

321 Chronic uranium exposure didn't alter sperm concentration in F0, F1 and F2 generations
322 as compared to control (data not shown). However, morphometric analysis of sperms showed that
323 uranium affected sperm morphology from F0 generation. The angle of the MP was reduced from
324 14.85 ± 2.84 ° to 12.02 ± 3.33 ° in F0 U-generation. The area of the head was statically increased
325 in F1 U-generation (**Table 5**). Using the cut-off values, we determined the percentage of
326 abnormal sperm for each morphometric parameters (**Table 2, Figure 2 & 3**). Furthermore, it was
327 found that uranium significantly induced sperm abnormalities for four parameters in F0 U-
328 exposed generations as ARC, area and perimeter of the sperm head, and angle (for the MP)
329 (**Figure 2A**). Percentage of abnormal sperms head (angle, linearity), and the MP width from the
330 F0 U-generations were significantly different between control and U-exposed groups (**Figure**
331 **2B**). In F2-U generation, there is only an increase in abnormal sperm for the area of the sperm
332 head and the angle of the MP (**Figure 2C**). The percentage of sperm with at least one
333 abnormality in the head, and at least one or only one abnormality in the MP was also increased in
334 the F1 and F2 generations, but no difference was observed in F0 (**Figure 3D to F**). The
335 percentage of sperm with one abnormality in the head and in the MP was modified in the last
336 generations (F1 and F2) (**Figure 3D & 3E**). In addition, we have detected that the percentage of
337 total abnormal sperm was significantly higher on the F1 U-exposed generations, and there was a

338 significant higher percentage of sperms with one abnormality both in the head and in the MP
339 (**Figure 3E**).

340
341 **Global DNA methylation & gene expression of DNA methyltransferases and**
342 **demethylation enzymes**

343 The level of global semen DNA methylation was not significantly modified in F0 and F1
344 generations (**Figure 4A & 4B**). Nevertheless, DNA hypomethylation was observed (-25%) in
345 sperm from F2 generations ($p < 0.005$) (**Figure 4C**).

346 mRNA analysis showed that there were no significant difference in transcripts levels of
347 methylation enzymes, *DNMT1*, *DNMT3a*, and *DNMT3b*, between control and U-exposed groups
348 in F0, F1 and F2 generations (**Figure 4D to F**). However, *DNMT3L* was down-regulated
349 ($p < 0.05$) in comparison with control in F0 generation (**Figure 4D**).

350 The analysis of demethyls genes (*TET2*, *TET3* and *TDG*) in F0 and F1 generations
351 showed that there was no significant difference between control and U-exposed groups (**Figure**
352 **4D to F**). But, the expression rate of *TET2* and *TET3* in F2 generation decreased significantly
353 ($p < 0.05$) (**Figure 4F**).

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357 **Discussion**

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359 Currently, it is well accepted that change in some epigenetic mechanisms marks such as
360 DNA methylation after exposure to certain pollutants could be transmitted through generations
361 with the appearance of deleterious effects such as infertility, reproductive dysfunctions (Gunes et
362 al., 2016; Skinner, 2016; Strazzullo and Matarazzo, 2017). In the field of chronic exposure to
363 radionuclides, very little scientific data are available. Our experimental model allows to study the
364 effects of uranium exposure on morphometric parameters and epigenetics in rat sperms after
365 multigenerational exposure.

366 First, we detected that the pregnancy rate was affected in F1 U-generation. This result
367 could be explained by the exposure period of animals, *i.e* from the foetal life to their weaning,
368 and which includes the time of male sex determination and the development of the male
369 reproductive organs. To study the effects of U on rat sperm, we explored the rat sperm
370 morphometry and morphology in rats in F0, F1 and F2 generations. Using the automated sperm
371 morphology analysis, we compared ten sperm morphometric parameters between control and U-
372 exposed groups in each generation. Interestingly, the analysis shows firstly that the determination
373 of the cut-off values was reproducible with those obtained in Sprague-Dawley rats in the previous
374 study using the CASMA (Van der Horst et al., 2018). We detected that U exposure could affect
375 two compartments of the sperm morphology, *i.e* the head and the middle-piece in F0, F1 and F2
376 generations, but without common parameters between each generation. The percentage of total
377 abnormal sperms was significantly higher only in the F1 U-exposed sperms, when the animals
378 were exposed during their foetal life from ~~the~~ weaning, contrasting with F0 (from birth until 9
379 months of age) and F2 (as germ cell lines of F1) generations. These results confirm the higher
380 susceptibility of rats to U during the foetal and postnatal exposure and the effects of
381 multigenerational exposure to U, which was previously suggested (Angenard et al., 2010; Grison
382 et al., 2018; Legendre et al., 2016). Regarding the Developmental Origins of Health and Disease
383 (DOHaD) hypothesis (Barker et al., 2002), the effects of the exposure to U may be involved in
384 reproductive disorders. Further experiments should be performed in our multigenerational
385 exposure to identify the adverse effects on sperm motility and testicular functions, where the
386 spermatogenesis takes place.

387 No epidemiological studies showed sperm defects in a U-exposed population (McDiarmid
388 et al., 2007; McDiarmid et al., 2006). Only modification in the sex ratio of offspring or in
389 testosterone level was reported (Muller et al., 1967; Zaire et al., 1997). Experimental studies with
390 chronic exposure showed abnormalities of sperm head in adult male rats exposed to 160 mg.L⁻¹
391 and in two-generation study in mice exposed to 64 mg.L⁻¹ (Hao et al., 2009; Linares et al., 2005).
392 Until recent years, little attention had been paid to the potential adverse effects of uranium
393 (including natural and depleted uranium) exposure on human reproduction.

394 In recent years, numerous studies have described the impact of environmental exposures
395 on the reproductive system associated with epigenetic modifications (Gunes et al., 2016; Skinner,
396 2016; Strazzullo and Matarazzo, 2017). In our study, we have also observed epigenetic
397 modifications induced by chronic exposure to uranium. Indeed, contrary to a recent study which
398 showed that DNA was hypermethylated in testis (Elmhiri et al., 2017), in a very surprising and
399 interesting way we observed an overall hypomethylation in sperm of rats exposed to NU and
400 particularly observed from the last generation (F2). As already described in mouse and human
401 (Marques et al., 2011; Ni et al., 2016; Uysal et al., 2016), we analyzed for the first time the
402 mRNA expression of DNA methyltransferase and demethylase enzymes (DNMT, TET and TDG)
403 in rat sperm. We observed only a decrease in DNMT3L expression for the F0 generation.
404 DNMT3L has not catalytic activity but it interacts with DNMT3A and DNMT3B to stimulate
405 their activity (Uysal et al., 2016). Interestingly, DNMT3L knockout phenotype is related to
406 abnormal maternal imprinting and male infertility (Bourc'his and Bestor, 2004). In the F2
407 generation, only the gene expression of the enzymes of the TET family (TET2 and TET3) was
408 modified. In ejaculated spermatozoa from humans with oligozoospermia and/or
409 asthenozoospermia, the expression of these TETs is reduced suggesting their pivotal role in male
410 fertility (Ni et al., 2016). Moreover, together, the mRNA profiles are insufficient to explain the
411 hypomethylation of the global sperm DNA observed from F2 generation. The identification of
412 differential methylated regions needs to be realized using targeted methylation tools as already
413 used (Skinner, 2016; Strazzullo and Matarazzo, 2017).

414 Our study focused on male reproductive disorders, but females could also be involved in
415 the low pregnancy rate observed in F1. Previous study has already demonstrated the modification
416 of the DNA global methylation rate in the ovary of the female exposed in the same
417 multigenerational model (Elmhiri et al., 2017). Moreover in order to explore the pregnancy rate

418 in the F1 U-exposed generation, females which were not pregnant were analyzed regarding their
419 estrus cycle. We detected that the estrus phase was reduced and the diestrus phase was
420 significantly longer in the U-exposed females (data not shown). These results suggest the
421 involvement of both sexes in the multigenerational effects of U exposure. Female reproductive
422 function should be more explored in subsequent studies.

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429 **Conclusion**

430 Thanks to our multigenerational exposure, we showed that uranium induced abnormalities
431 in sperm morphometry and morphology occur, both in the head and the middle piece. These
432 abnormalities were observed in each generation and were more deleterious in the F1 U-exposed
433 generation, suggesting that U exposure during foetal life could have ~~so~~ more adverse effects on
434 reproductive parameters. Consequently, the decrease in the pregnancy rate to generate F2
435 offspring could be the consequences of these sperm morphological defects. Epigenetic change
436 may be one possible consequence of U effects on sperm. Indeed, our results show ~~n~~ epigenetic
437 effects as DNA global methylation (overall sperm hypomethylation) observed in F2. These
438 results raise the question of the biological significance of the epigenetic germinal fingerprints to
439 subsequent generations. There is an increasing proof about a strong link between errors in sperm
440 DNA methylation and male hypofertility (Gunes et al., 2016; Kobayashi et al., 2007), and this
441 study provides also new insights into the sperm morphometry and the epigenetic change induced
442 after uranium exposure and their persistence over generations.

443

444

445 **Conflict of interest**

446 None declared.

447

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450 Radioprotection and Nuclear Safety (IRSN).

451

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Titre long :

Une exposition multigénérationnelle à l'uranium induit des modifications des paramètres morphométriques et de la méthylation globale de l'ADN dans les spermatozoïdes de rats.

Titre court :

Effet de l'uranium sur les spermatozoïdes après exposition multigénérationnelle

Il y a de plus de plus de preuves que les expositions environnementales qui se produisent pendant le développement influencent le phénotype et le risque de développer des maladies chez les générations futures. Nous avons précédemment constaté que l'uranium, un contaminant environnemental, induisait après une exposition durant la vie entière de subtiles anomalies testiculaires et hormonales, mais son impact sur le système reproducteur des générations suivantes restait inexploré. Dans cette étude, des rats ont été exposés à une concentration supra-environnementale et non néphrotoxique d'uranium (U, 40 mg.L⁻¹ dans l'eau de boisson) de la vie postnatale à l'âge adulte (F0), pendant la vie fœtale (F1) et seulement en tant que cellules germinales de la génération F1 (F2). Les paramètres généraux (indices reproductifs, poids) et la morphologie des spermatozoïdes ont été évalués sur les trois générations. Afin d'identifier les effets épigénétiques de l'U, nous avons également analysé le profil global de méthylation de l'ADN et décrit pour la première fois les niveaux d'expression d'ARNm des marqueurs impliqués dans le système de (dé)méthylation des spermatozoïdes épидидymaires de rats. Nos résultats ont montré que la génération F1 avait un taux de naissance réduit. Bien que le nombre de spermatozoïdes n'ait pas été modifié, la morphologie des spermatozoïdes a été affectée chez les générations F0, F1 et F2. L'analyse morphométrique de dix paramètres a été détaillée pour chaque génération. Aucun paramètre commun n'a été détecté entre les trois générations, mais la tête et la pièce intermédiaire ont toujours été modifiées dans ces spermatozoïdes anormaux. Chez les générations F1 exposées à l'U, le nombre total de spermatozoïdes anormaux était significativement plus élevé que chez les générations F0 et F2, ce qui suggère que l'exposition fœtale à l'uranium était plus nocive et pouvait affecter le taux de naissance pour produire la génération F2. Il est intéressant de noter aussi que l'analyse globale de méthylation de l'ADN a montré une hypométhylation dans l'ADN des spermatozoïdes de la dernière génération F2. En conclusion, notre étude démontre que l'uranium peut induire des défauts morphologiques des spermatozoïdes et un changement de méthylation de l'ADN après une exposition multigénérationnelle. La transmission transgénérationnelle épigénétique des anomalies du système reproducteur induites par l'U devra être évaluée dans de futures expériences.

Mots-clés : morphologie, morphométrie, méthylation, spermatozoïdes, fertilité, multigénérationnel, uranium.

Figure 1:

A multi-generational protocol of exposure *i.e.* three generations of rats (F0, F1 and F2) males and females (n=20). F0 were exposed with NU from birth through mother's milk and then through drinking water for 9 months (control animals drank uncontaminated mineral water). F1 were contaminated in utero and through the mother's milk (F0) until weaning. After weaning they drank uncontaminated mineral water. The last F2 generation received only mineral water. The last generation was exposed to uranium only from parental (F1) germ cells.

Figure 2:

The percentage of abnormal sperm for each morphometric parameters in F0, F1 and F2 generations (A, B and C, respectively) (mean \pm SD). There was a significant difference between the control group and the NU40 group (*p < 0.05; **p < 0.005; *** p < 0.001). N=8-10 per group

Figure 3:

The percentage of abnormal sperm for each compartment (head and MP) in F0, F1 and F2 generations (A, B and C, respectively) (mean \pm SD). Total morphologically abnormal sperm indicating the sum of abnormal head and MP was determined. The percentage of abnormal sperms was also calculated if sperm has at least one, only one or more than two abnormalities in the head, in MP or in both compartments (head and MP) for F0, F1 and F2 generations. There was a significant difference between the control group and the NU40 group (*p < 0.05; **p < 0.005; *** p < 0.001). N=8-10 per group

Figure 4:

Examples of abnormal stained rat sperm, automatically analyzed for morphology and morphometric parameters. A: Control Sperm (F1 generation), B : F0 U-exposed sperm with abnormal head parameters in the angle, chord and linearity - (low values), C : F1 U-exposed sperm with abnormal head parameters in the angle, chord and linearity - (upper values) and D : F2 U-exposed sperm with abnormal head parameters in the angle, chord and linearity - (low values)

Figure 5:

The percentage of global DNA methylation assessed with ELISA (mean \pm SD) in the sperm of F0, F1 and F2 generations (A, B and C respectively). mRNA expressions of DNA methyltransferase specific genes (*DNMT1*, *DNMT3a* and *DNMT3b*) and DNA demethyltransferase specific genes (*TET2*, *TET3* and *TDG*) in the sperm of F0, F1 and F2 generations (D, E and F respectively). All results are expressed as mean \pm SD. There was a significant difference between the control group and the NU40 group (*p < 0.05; **p < 0.005). N=8-10 per group.

Figure
Fig 2

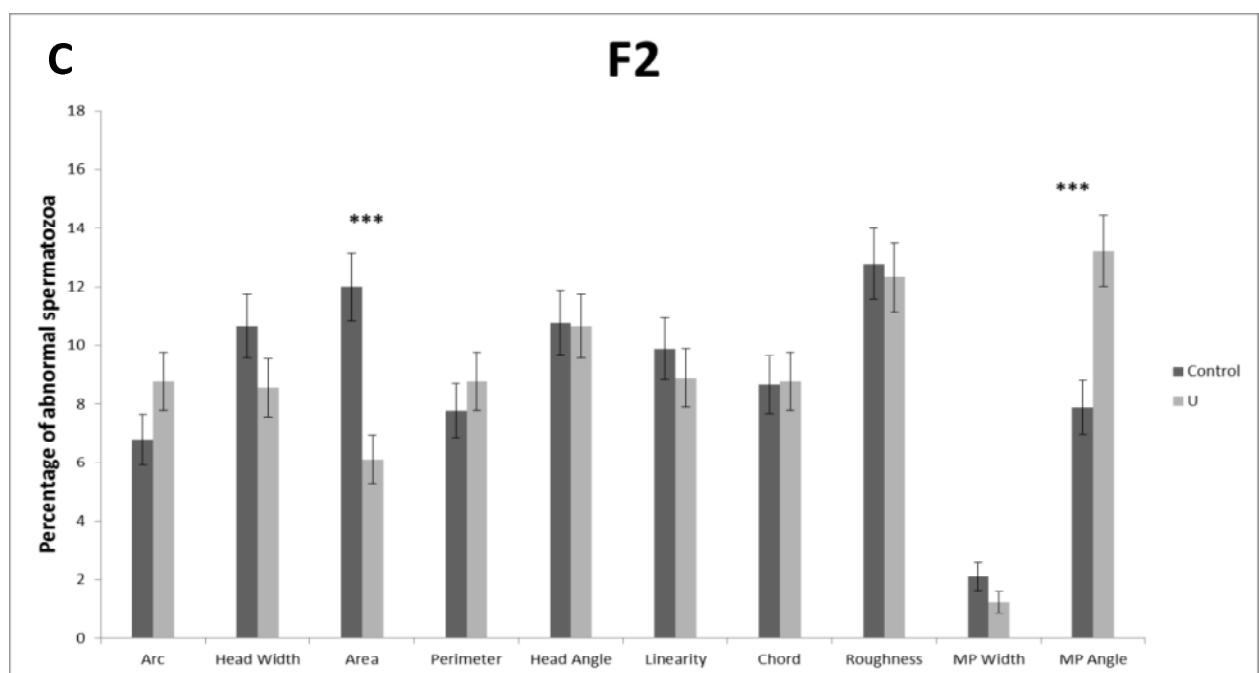
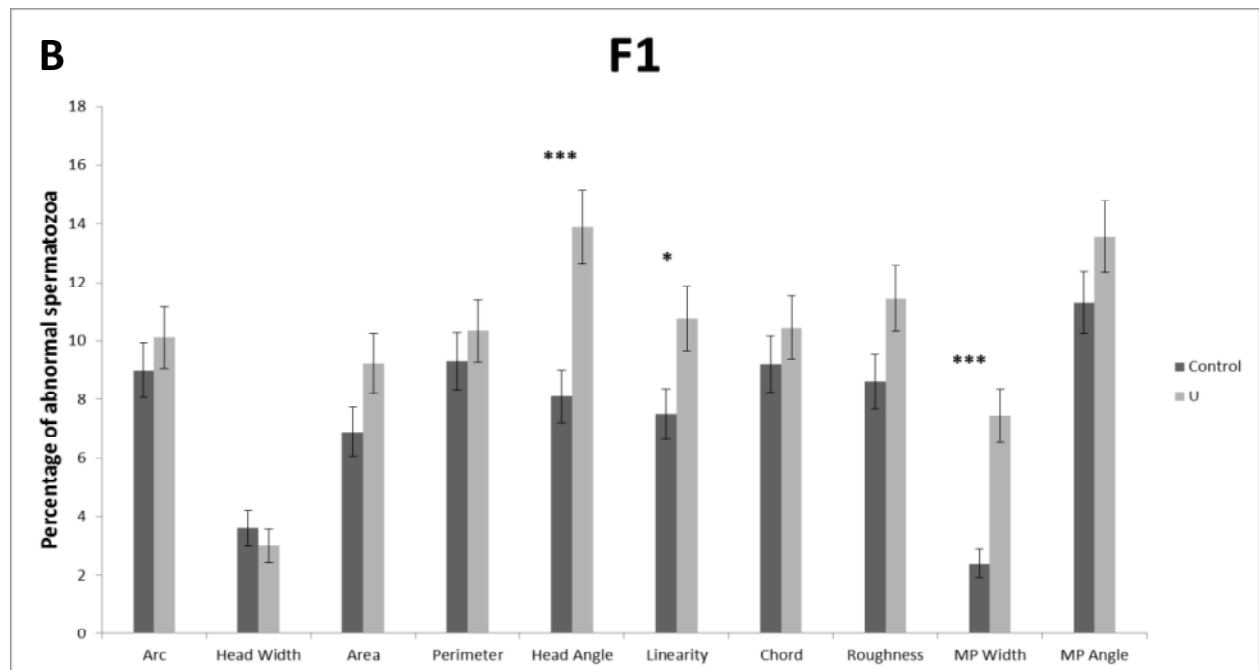
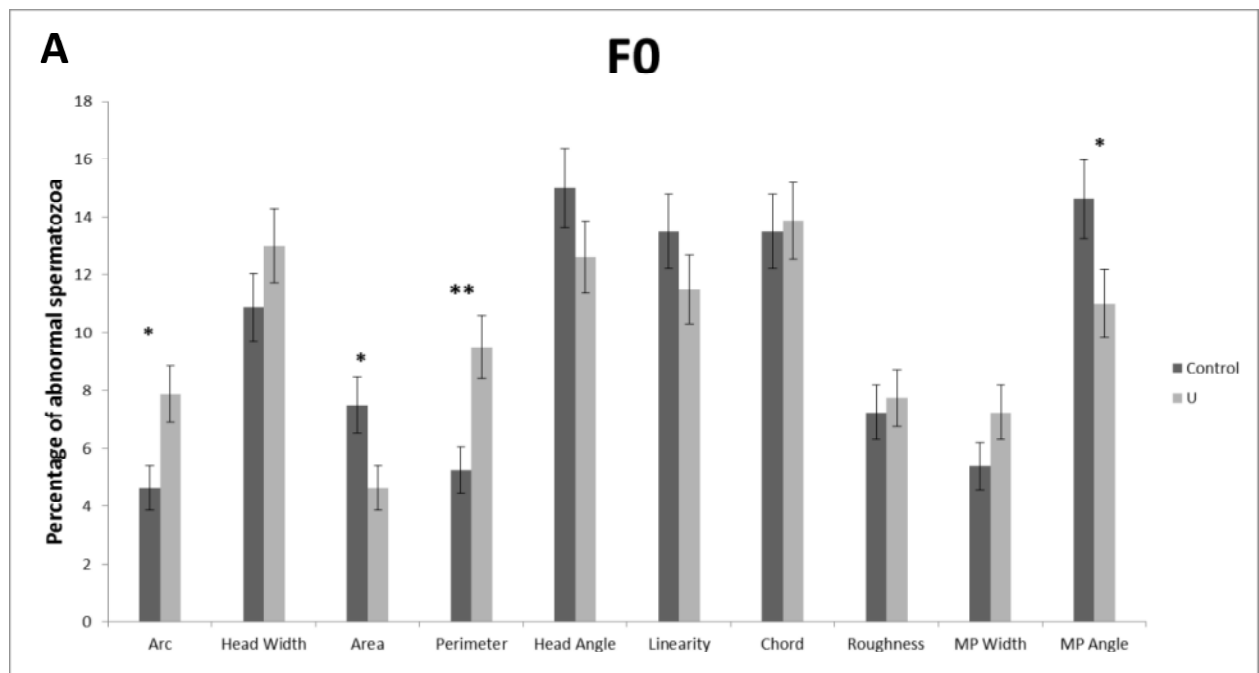


Fig 3

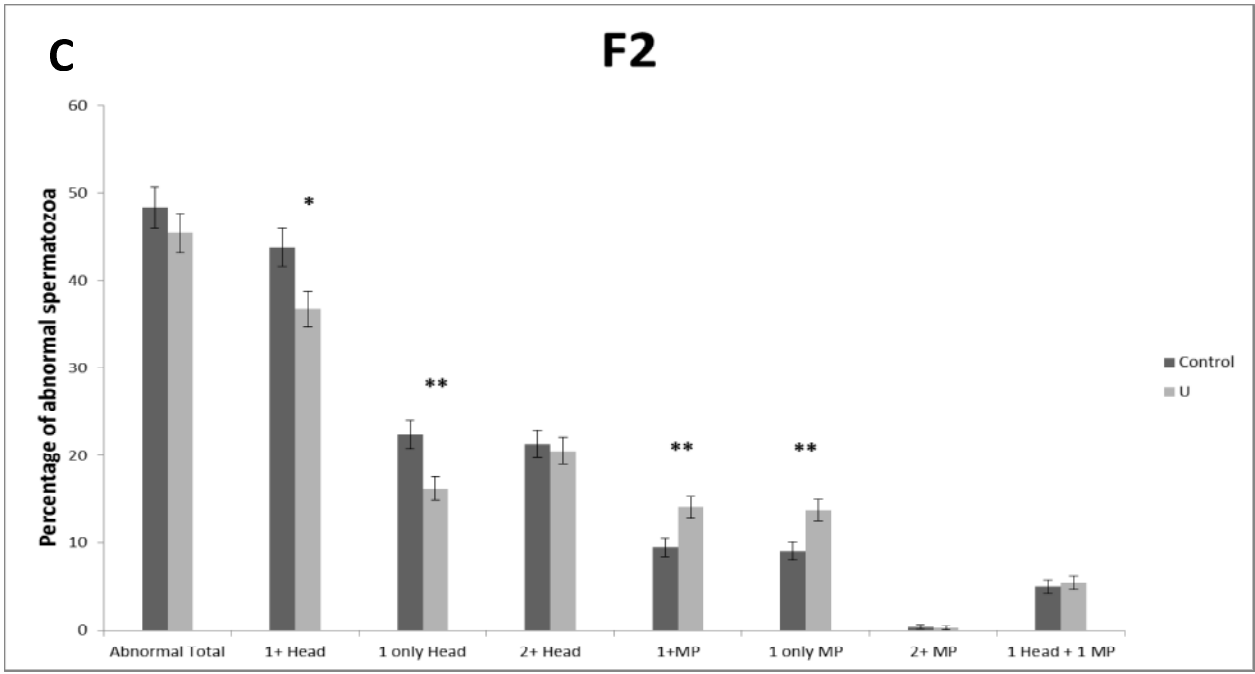
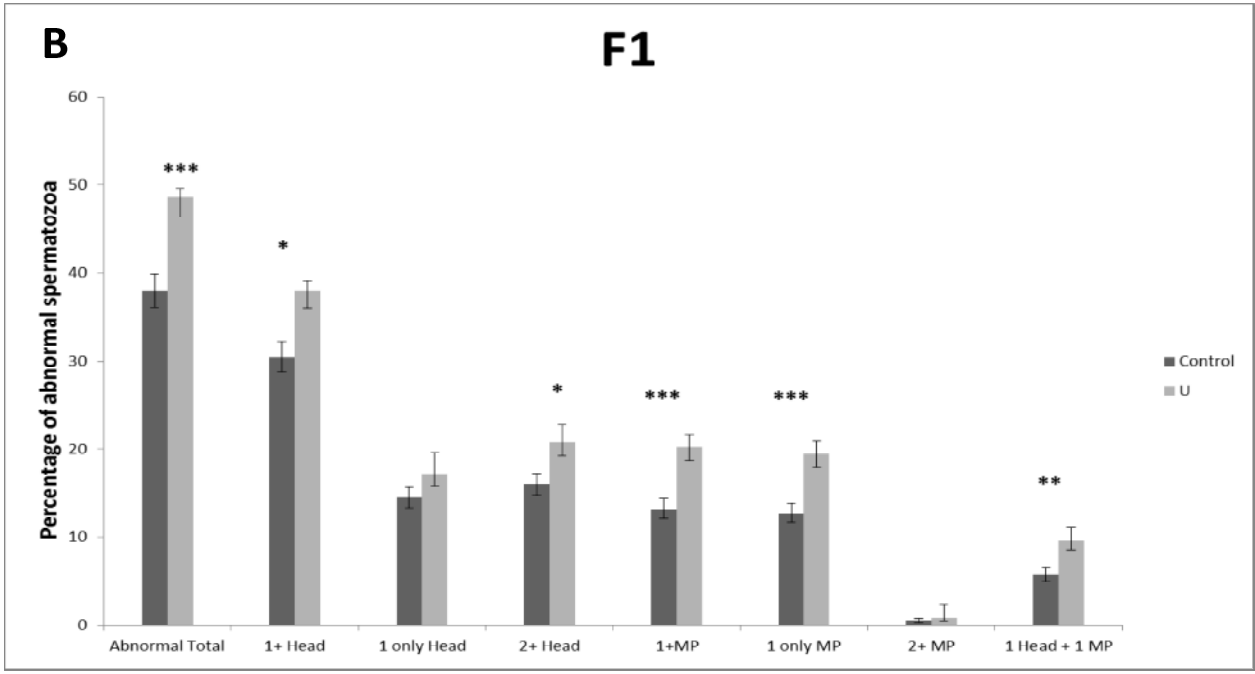
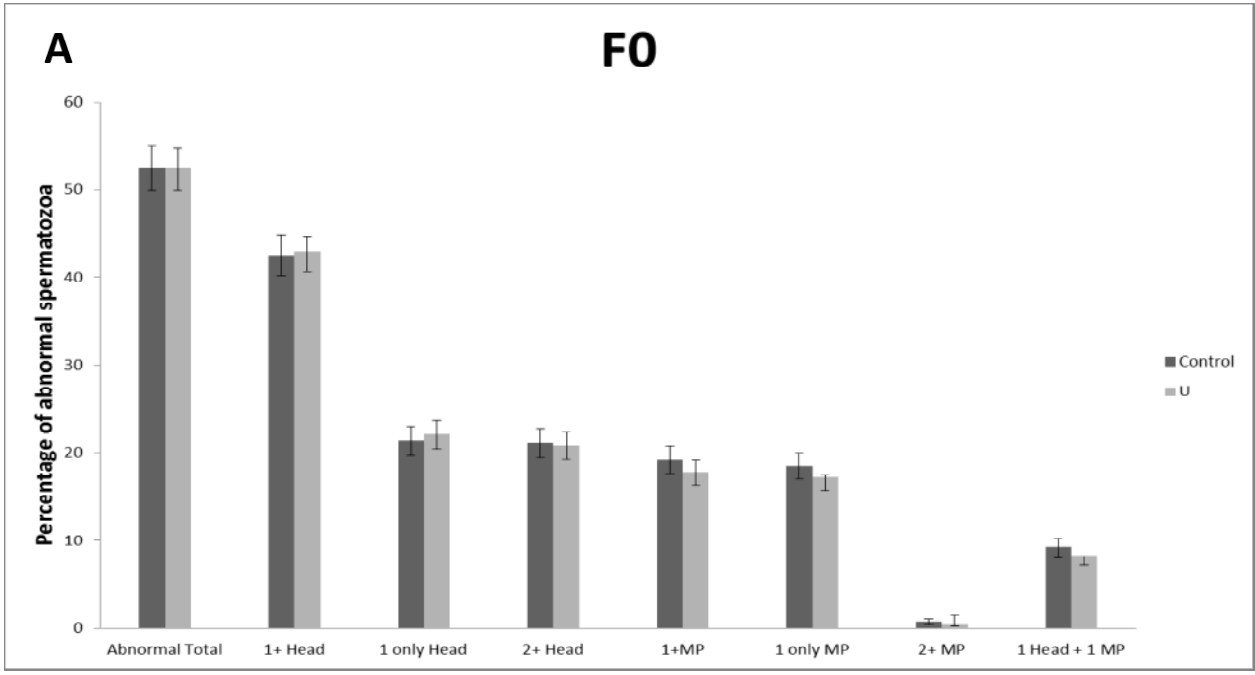


Figure
FIG 1

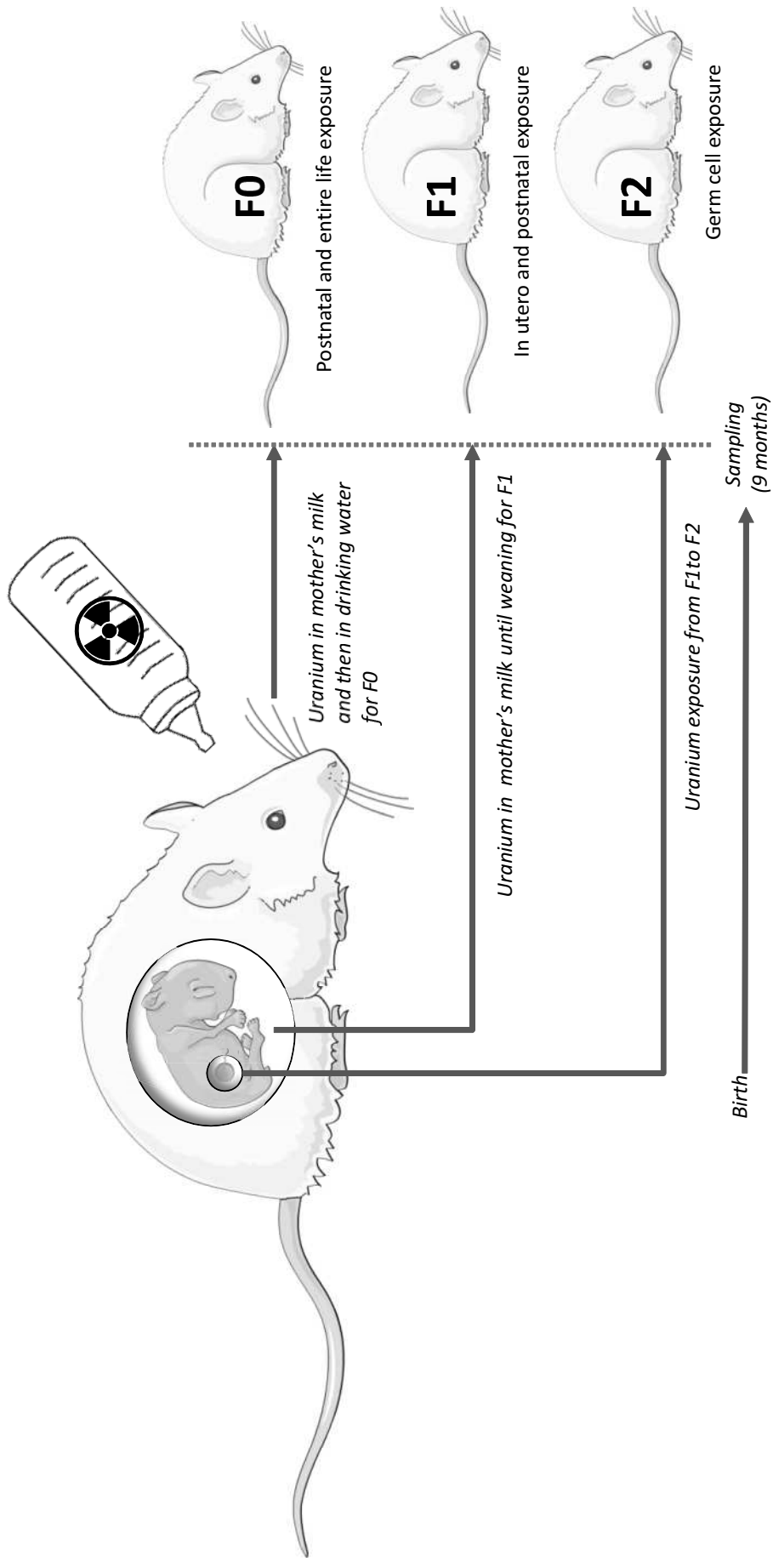


Fig 4

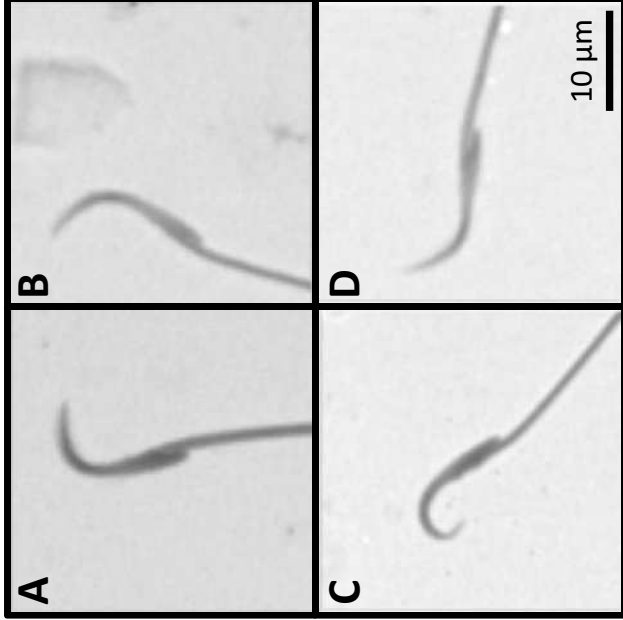
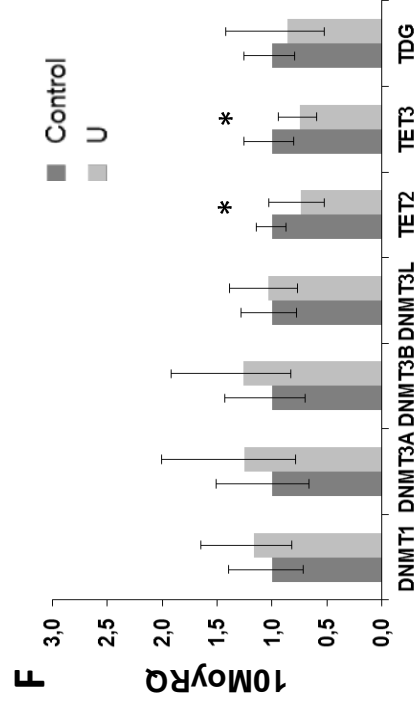
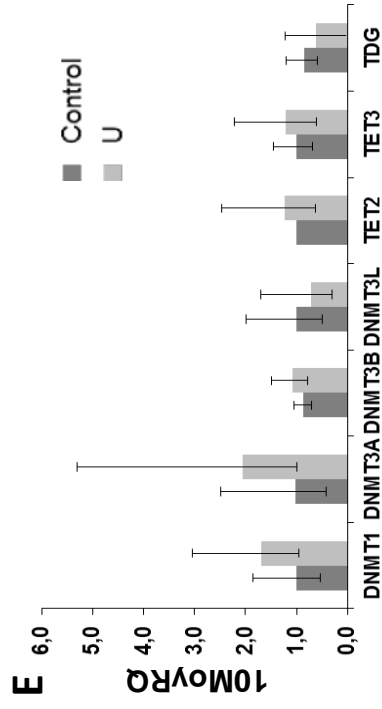
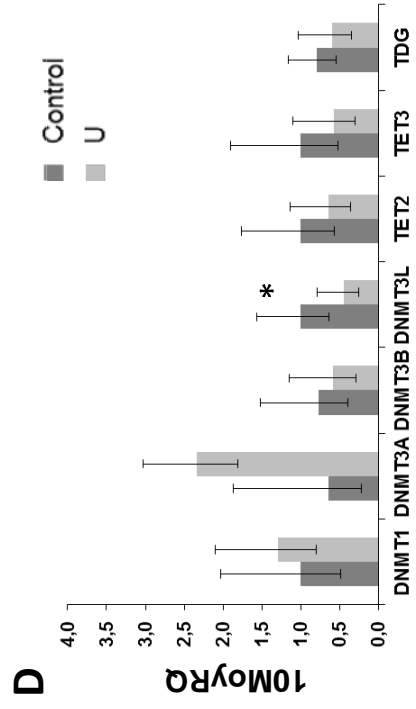
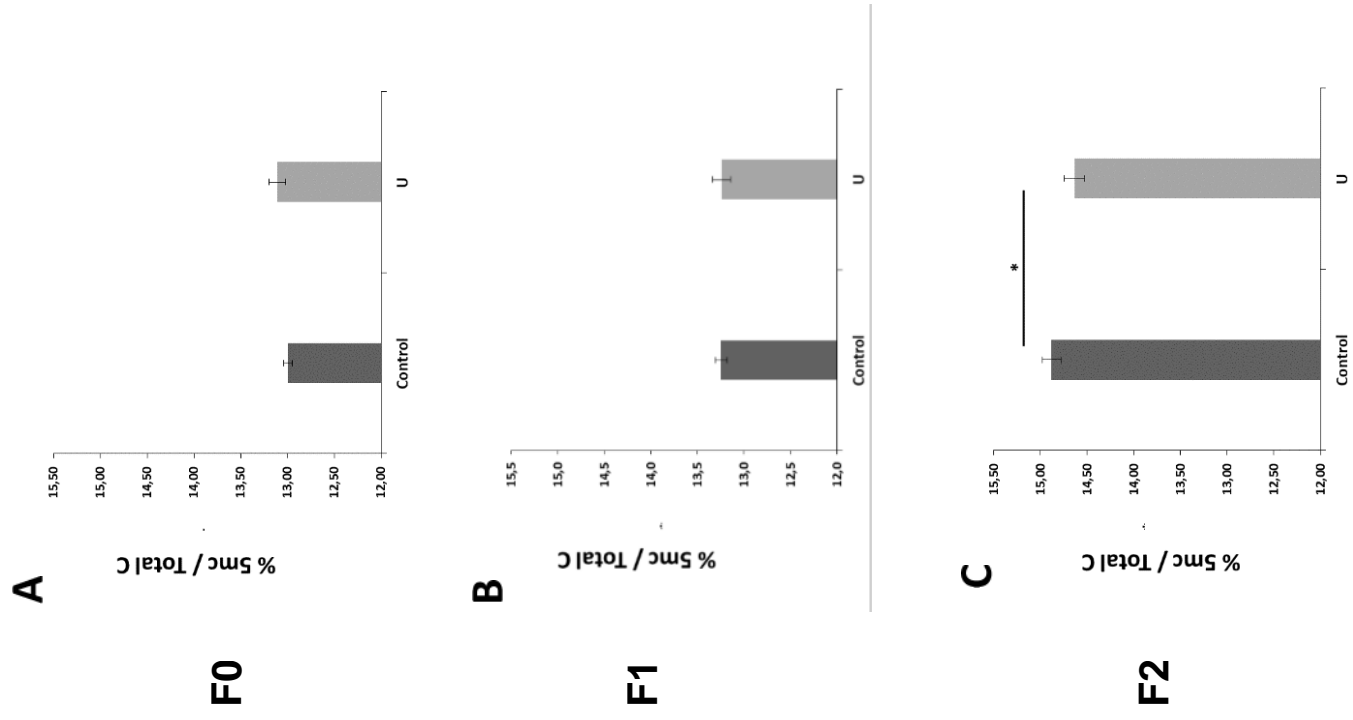


Fig 5



Parameters	Formula
Arc (μm)	L
Area (μm^2)	A
Perimeter (μm)	P
Chord (μm)	C
Linearity (%)	L / C
Roughness	$4 \pi (A/P^2)$

Table 1 : Morphometric variables of the sperm head measured automatically by the SCA®.

Sperm head and middle-piece parameters	Minimum value	Maximum value
H Length = Arc (μm)	18.48	23.62
H Width (μm)	0.84	1.51
H Area (μm^2)	14.12	21.64
H Perimeter (μm)	38.95	49.13
H Angle (degrees)	40.63	84.16
H Linearity (%)	49.06	66.67
H Chord (μm)	10.44	13.53
H Roughness	0.09	0.14
MP Width (μm)	0.28	0.79
MP Angle (degrees)	1.44	29.54

H, head; MP, mid-piece.

Table 2 : Minimal and maximal value for normal head and MP properties selected to determine normal vs. abnormal sperm under the configuration set-up of the SCA 5.4 system using 5-95% confidence interval.

Genes	Sequences
DNMT1	5'-CGGCGGAGGTGTCCTAACTTGGC-3' 5'-GGGTGACGGCAACTCTGGTA-3'
DNMT3a	5'-CGGTAGCGCCTCTTCTTTGAGTTCTAC-3' 5'-GCGATCATCTCCCTCCTTGG-3'
DNMT3b	5'-GGGCCGCTACCACGTTTCAGG-3' 5'-AGGGCCGTCCTGGCTCAAGT-3'
DNMT3l	5'-GCTTTGACGGTGGCGAGAA-3' 5'-TCTGCAAGAACTCGACCACAATC-3'
HPRT	5'-GCTCGAGATGTCATGAAGGAGA-3' 5'-TCAGCGCTTTAATGTAATCCAGC-3'
β 2M	5'-ACATCCTGGCTCACACTGAA-3' 5'-ATGTCTCGGTCCCAGGTG-3'
ACTB	5'-TTCAACACCCCAGCCATGT-3' 5'-TGGTACGACCAGAGGCATACAG-3'

Table 3: Sequence of primers set for gene expression studies in sperm.

Parameters	F0		F1		F2	
	Control	U	Control	U	Control	U
Pregnancy rate	70%	70%	67%	30% *	no reproduction	
Epididymis Relative weight g / 100 g body weight	0.115 ± 0.012	0.113 ± 0.023	0.118 ± 0.011	0.108 ± 0.027 *	0.115 ± 0.020	0.122 ± 0.029

Table 4: Pregnancy rate and epididymis relative weight were compared in F0, F1 and F2 generations. * : p < 0.05. N=17-20 per group.

Sperm head and middle-piece parameters	F0			F1			F2		
	Control	U	P value	Control	U	P value	Control	U	P value
Lenght = Arc (µm)	21.232 ± 0.273	21.457 ± 0.538	0.310	20.972 ± 0.641	21.381 ± 0.416	0.122	21.392 ± 0.349	21.315 ± 0.572	0.736
Width (µm)	1.062 ± 0.093	1.021 ± 0.075	0.345	1.143 ± 0.045	1.174 ± 0.035	0.115	1.223 ± 0.131	1.165 ± 0.139	0.381
Area (µm ²)	16.834 ± 1.248	16.961 ± 0.858	0.817	16.640 ± 0.979	17.946 ± 1.209	0.019 *	18.214 ± 1.516	17.286 ± 1.253	0.176
Perimeter (µm)	44.353 ± 0.597	44.796 ± 1.114	0.339	43.841 ± 1.243	44.707 ± 0.823	0.095	44.912 ± 0.896	44.595 ± 1.086	0.509
Angle (degrees)	64.710 ± 2.025	64.731 ± 2.912	0.987	64.006 ± 1.987	64.007 ± 2.707	0.948	64.663 ± 2.490	65.763 ± 2.124	0.328
Linearity (%)	57.449 ± 1.337	57.447 ± 2.151	0.998	57.263 ± 0.15	56.722 ± 1.586	0.355	56.486 ± 1.756	56.764 ± 1.482	0.722
Chord (µm)	12.210 ± 0.211	12.339 ± 0.247	0.281	12.051 ± 0.391	12.128 ± 0.265	0.626	12.096 ± 0.353	12.101 ± 0.333	0.974
Roughness	0.108 ± 0.006	0.107 ± 0.003	0.587	0.110 ± 0.002	0.114 ± 0.005	0.111	0.114 ± 0.006	0.110 ± 0.008	0.247
Width (µm)	0.568 ± 0.061	0.590 ± 0.078	0.555	0.540 ± 0.050	0.579 ± 0.088	0.153	0.554 ± 0.046	0.533 ± 0.036	0.305
Middle-Piece Angle (degrees)	14.855 ± 2.837	12.025 ± 2.337	0.047 *	12.880 ± 1.926	13.852 ± 2.486	0.352	12.695 ± 2.153	11.088 ± 2.043	0.127

Table 5: Mean value for head and MP properties selected to determine abnormal parameters in each generation . * : p < 0.05. N=8-10 per group.