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1 **Perinatal exposure to Bisphenol A disturbs the early differentiation of male germ**  
2 **cells**

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23 **Declarations of interest:** none.

24 **Abstract:**

25 Understanding the effects of Bisphenol A (BPA) on early germ cell differentiation and  
26 their consequences in adult life is an area of growing interest in the field of endocrine  
27 disruption. Herein, we investigate whether perinatal exposure to BPA affects the  
28 differentiation of male germ cells in early life using a transgenic mouse expressing the  
29 GFP reporter protein under the Oct4 promoter. In this model, the expression of GFP  
30 reflects the expression of the Oct4 gene. This pluripotency gene is required to maintain  
31 the spermatogonial stem cells in an undifferentiated stage. Thus, GFP expression was  
32 used as a parameter to evaluate the effect of BPA on early germ cell development.  
33 Female pregnant transgenic mice were exposed to BPA by oral gavage, from  
34 embryonic day 5.5 to postnatal day 7 (PND7). The effects of BPA on male germ cell  
35 differentiation were evaluated at PND7, while sperm quality, testicular morphology, and  
36 protein expression of androgen receptor and PCNA were studied at PND130.

37 We found that perinatal/lactational exposure to BPA up-regulates the expression of  
38 Oct4-driven GFP in testicular cells at PND7. This finding suggests a higher proportion  
39 of undifferentiated spermatogonia in BPA-treated animals compared with non-exposed  
40 mice. Moreover, in adulthood, the number of spermatozoa per epididymis was reduced  
41 in those animals perinatally exposed to BPA.

42 This work shows that developmental exposure to BPA disturbed the normal  
43 differentiation of male germ cells early in life, mainly by altering the expression of Oct4  
44 and exerted long-lasting sequelae at the adult stage, affecting sperm count and testis.

45

46

47 **Keywords:** Oct4; BPA; germ cell; differentiation

48

49

## 50 **1. Background**

51 Environmental awareness is rising about the consequences of exposure to endocrine-  
52 disrupting chemicals (EDCs) on the reproduction of humans and wildlife [1, 2]. In  
53 this sense, several studies link male and female reproductive disorders to the  
54 disruption of the early gonad development by EDCs [3]. Bisphenol A (BPA), one of the  
55 most highly produced chemicals worldwide, has been demonstrated to act as an EDC  
56 with estrogen-like (xenoestrogen) actions in both *in vivo* and *in vitro* studies [2]. There  
57 is growing scientific evidence that shows that testes are targets of BPA action in  
58 different species [4-7]. Particularly in mice and rats, it has been demonstrated that  
59 perinatal and adult exposure to BPA affects sperm production and quality in a  
60 deleterious way [8, 9]. Moreover, the effect of BPA on early stages of *in vitro* germ  
61 cell differentiation has also been reported [10]. Nevertheless, the effect of *in vivo*  
62 perinatal BPA exposure on early germ cell differentiation has been poorly investigated  
63 so far.

64 Spermatogonial stem cells (SSCs) are essential for the reproductive performance of  
65 males. This cell population is established during prenatal and early postnatal age,  
66 comprising different processes. During the first week after birth, gonocytes relocate  
67 from their original central position in the seminiferous tubules toward the basement  
68 membrane and differentiate into spermatogonia establishing the subpopulation of  
69 SSCs [11, 12]. These SSCs are the reservoir of undifferentiated spermatogonia  
70 residing in the basal layer of the seminiferous epithelium that supports  
71 spermatogenesis by self-renewal and differentiation [13]. Concurrently, epigenetic  
72 changes and differential expression of pluripotency genes occur during the  
73 development of male germ cells. Among the core genes associated with pluripotency  
74 is Oct4, a member of the class V Pic-1 Oct1,2 Unc-86 (POU) transcription factors  
75 [14]. Oct4 is expressed in proliferating gonocytes during the embryonic stage and  
76 in undifferentiated spermatogonia after birth [15, 16]. Oct4 is required to  
77 maintain SSCs in an undifferentiated state and, therefore, to maintain their self-  
78 renewal [17, 18]. It is thus used as a marker of both the population of SSCs [18]  
79 and the differentiation level [19]. Another transcription factor involved in the regulation  
80 of pluripotency is Transcription factor SOX-2 (Sox2), which acts in cooperation with  
81 Oct4 [14]. Sox2 is required to stabilize embryonic stem cells in a pluripotent state by  
82 maintaining the required level of Oct4 expression [20]. Both genes regulate not only

83 downstream genes but also their own expression, revealing an intricate network behind  
84 the spermatogonia development.

85 Previously we proposed the use of the Oct4/GFP transgenic mouse [21] that  
86 expresses GFP under the Oct4 promoter and the flow cytometry analysis as a new  
87 tool to study early-life effects of environmental estrogens on male germ cell  
88 development [22]. Herein, we used the transgenic Oct4/GFP mouse to investigate  
89 whether the perinatal exposure of pregnant mothers to BPA affects the differentiation of  
90 germ cells in their offspring and their impact in adult life. Male germ cell differentiation  
91 was evaluated at postnatal day 7 (PND7) by analyzing Oct4 expression in testicular  
92 cell suspensions. Expression of Oct4 and Sox2 genes were also analyzed. Sperm  
93 quality, testicular morphology, protein expression of androgen receptor and PCNA  
94 were studied to evaluate BPA effects later in life.

95

## 96 **2. Methods**

97

### 98 *2.1. Animals*

99 C57BL/6 Oct4/GFP transgenic mice were a kind gift from Dr. Konstantinos  
100 Anastassiadis, Biotech Technische Universität Dresden, Germany [23]. Animals were  
101 bred under specific pathogen-free conditions at the animal facility of the Transgenic  
102 and Experimental Animal Unit of Institut Pasteur de Montevideo, Uruguay (IPMon).  
103 Mice were housed in individually ventilated cages with positive pressure, at  $20 \pm 1^\circ\text{C}$ ,  
104 relative humidity of 40 – 60 %, and a 14/10 h light-dark cycle. Animals were fed with  
105 standard mouse diet *ad libitum* and had free access to water during the experimental  
106 protocol. The genotype Oct4/GFP of all mice was determined by PCR analysis,  
107 using specific primers and by fluorescence microscopy (Olympus IX81) of the whole  
108 testis. All animal procedures were approved by the Animal Care Committee of the  
109 IPMon, in accordance with national law N° 18.611 and international guidelines of the  
110 Federation of Laboratory Animal Science Associations (FELASA) regarding  
111 laboratory animal protocols.

112

### 113 *2.2. Oral administration of BPA*

114 In order to assess the effect of BPA in the development of male germ cells during  
115 the gestational and early postnatal periods, pregnant female mice were exposed to  
116 BPA (Sigma, St Louis, MO, USA). Six to eight-week-old Oct4/GFP virgin female mice

117 were mated with Oct4/GFP males. Females with a copulation plug were randomly  
118 separated in three groups (three to five females per group): BPA50 [50µg/kg body  
119 weight (bw)/day(d)], BPA400 (400 µg/kg bw/d) and Ethanol 0.1 % (vehicle control  
120 group). The dose of 50 µg/kg bw/d has been referred to as the appropriate safe dose  
121 of BPA by oral route [2, 24]. The dose of BPA 400 µg/kg bw/d has been referred in  
122 previous studies as the amount of BPA to which non-pregnant female rhesus  
123 monkeys and CD-1 mice must be exposed to reach circulating levels of BPA similar  
124 to those measured in humans [25, 26].

125 The compounds were administered daily by oral gavage from E 5.5 to PND7, in a  
126 single dose using a maximum volume of 0.2 mL. Doses were adjusted based on  
127 each 'dams' body weight at E 11.5, 15.5, 19.5, and after delivery. Of the entire  
128 litter born to the treated mothers (six offspring per litter, average), one group was  
129 sacrificed on PND7 by decapitation, and testes were dissected for further analysis.  
130 The other group was sacrificed on PND130 to evaluate late effects in adulthood.

131

### 132 *2.3. Male germ cell preparation for flow cytometry analysis and sorting*

133 Male germ cell maturation was evaluated by the Oct4 flow cytometric expression profile  
134 of testicular cells, using the parameters previously described by our group [22]. Briefly,  
135 isolated testes were decapsulated and centrifuged at 300 x g for 5 min. The pellet was  
136 resuspended in 1 mL collagenase Type II (1 mg/mL, Sigma, St Louis, MO, USA) and  
137 incubated at 32°-35°C for 15 min. After incubation, the cell suspension was centrifuged,  
138 washed with 1 mL PBS-EDTA-2% BSA, and cells were resuspended in 0.8 mL of  
139 0.05% trypsin-EDTA for 10 min at 32-35°C, agitating every 5 min. Enzyme activity was  
140 blocked by the addition of 0.2 mL of fetal bovine serum (FBS, GIBCO, Grand Island,  
141 NY, USA). After centrifugation, cells were resuspended in 1 mL of Dulbecco's Modified  
142 Eagle Medium (DMEM) (Invitrogen, CA, USA) supplemented with 10 % FBS and  
143 further dissociated by pipetting up and down several times. The testicular cell  
144 suspension was fixed by incubation with 4% paraformaldehyde for 0.5 h, centrifuged,  
145 resuspended in PBS-EDTA-2% BSA, and filtered through a 50 µm pore size Nylon  
146 mesh. The resultant single-cell suspension was used for flow cytometry analysis.

147

### 148 *2.4. Flow cytometry analysis*

149 Flow cytometry analysis was performed in a CyAn™ ADP analyzer (Beckman Coulter  
150 Inc., IN, USA) equipped with 488 nm and 635 nm lasers. Data was acquired with

151 Summit v4.3 software (Beckman Coulter Inc., IN, USA), and post-acquisition analysis  
152 was performed using FlowJo vX.0.7 (Tree Star Inc, Ashland, OR, USA) software.  
153 Forward Scatter (FS) *versus* Side Scatter (SS) was used to define the acquisition gates  
154 for intact cells. FS *versus* Pulse Width dot plot, for doublet discrimination, and SS  
155 *versus* Oct4/GFP (530/40 band-pass filter) dot plot for GFP detection. A minimum of  
156 10,000 single intact cells was collected for analysis in each sample.

157 Oct4 expression from BPA50 (n= 10), BPA400 (n= 12) and vehicle (n= 11) was  
158 analyzed by GFP fluorescence, as previously described [22]. Briefly, three parameters  
159 were defined: 1) the percentage of Oct4/GFP positive cells, as an indicator of the  
160 number of immature cells in the gonads; 2) the increase in the mean fluorescence  
161 intensity (MFI) as well as 3) the percentage of Oct4/GFP positive cells with bright  
162 fluorescence intensity (Oct4+ bright), both used as indicators of the maturation of the  
163 cells (Supplementary material, Figure 1).

164

#### 165 *2.5. Fluorescence-activated cell sorting (FACS)*

166 Cellular suspensions from at least three testes from different animals were pooled per  
167 treatment (Vehicle, n= 7; BPA50, n= 6; BPA400, n= 7; being "n" the number of pools).  
168 Male germ cells expressing GFP were sorted using a BD FACSAria Fusion cell sorter  
169 (BD Biosciences) in *purity* mode with a 0.5 drop sort envelope criteria. GFP  
170 excitation was achieved using a 488 nm laser, and fluorescence emission was  
171 detected employing a 530/40 band-pass filter. Sort decision was based on FS *versus*  
172 SS dot plots, excluding doublets and including GFP positive cells on SS *versus*  
173 Oct4/GFP dot plots. The purity of the GFP positive sorted cell populations was  
174 higher than 90% in all cases.

175

#### 176 *2.6. Immunohistochemistry*

177 Testes from PND7 (Vehicle, n= 5; BPA50, n= 5; BPA400, n= 4) and PND130 mice  
178 were isolated and fixed in 4 % PFA for 2 h at RT and embedded in paraffin. At least  
179 three sections (5  $\mu$ m thickness) at different depths from each testis were  
180 immunoassayed for Oct4 protein at PND7, whereas proliferating cell nuclear antigen  
181 (PCNA) (Vehicle, n=4; BPA50, n=3; BPA400, n=3) and androgen receptor (AR)  
182 (Vehicle, n=4 ; BPA50, n=3 ; BPA400, n=3 ) were assayed at PND130. After paraffin  
183 removal, antigen retrieval was performed with citrate buffer. Primary antibodies were  
184 incubated overnight at 4°C at the dilutions shown in Table S1 (see supplementary

185 data). Biotinylated anti-mouse IgG (Sigma-Aldrich Argentina S.A., Argentina) or anti-  
186 rabbit IgG (Zymed Laboratories, Invitrogen Argentina, Argentina) were used as  
187 secondary antibody, and diaminobenzidine, as chromogen substrate (DAB) (Sigma-  
188 Aldrich Argentina). Except for the determination of the integrated optical density (IOD)  
189 of AR-immunostained slides, samples were counterstained with Mayer hematoxylin  
190 (Biopur, Argentina) and mounted with permanent mounting medium (Eukitt, Sigma-  
191 Aldrich, St Louis, MO, USA). For negative controls, the primary antibody was replaced  
192 with non-immune mouse serum (Sigma-Aldrich, St Louis, MO, USA).

193 AR expression levels were quantified by measuring the IOD following protocols  
194 previously described [27, 28]. The images were recorded with a Spot Insight version 3.5  
195 color video camera attached to an Olympus BH2 microscope (all fields for the entire  
196 section were recorded and two sections per animal were evaluated). The microscope  
197 was set up properly for Koehler illumination. Correction of unequal illumination (shading  
198 correction) and calibration of the measurement system were done with a reference  
199 slide. Images were analyzed using the Image Pro-Plus 4.1.0.1 system (Media  
200 Cybernetics, Silver Spring, MD, USA). The images of immunostained slides were  
201 converted to grayscale. The IOD was calculated as a linear combination between the  
202 average gray intensity and the relative area occupied by positive cells. Because the IOD  
203 is a dimensionless parameter, the results were expressed as arbitrary units. Parameters  
204 were determined, averaged per mouse, and then averaged for each treatment group.

205 The total number of Oct4-positive cells per testis and the percentage of positive  
206 seminiferous tubules were quantified. For the last parameter, tubules with at least one  
207 Oct4-positive cell inside the seminiferous tubules were considered as positive. The  
208 total number of Oct4-positive cells per testis was determined as the sum of all the  
209 positive cells within seminiferous tubules in three sections per sample. The sections  
210 were obtained at different depths of the organ.

211 The PCNA-positive cells in each sections were counted in at least 10 tubular cross-  
212 sections per testis from each animal per group. Proliferation index was calculated using  
213 the formula:  $(\text{PCNA-positive cells} / \text{total number of cells in a tubule}) \times 100$  [29].

214

### 215 *2.7 Histological evaluation*

216 At least three sections (5  $\mu\text{m}$  thickness) at different depths from each adult testis  
217 (Vehicle, n=4 ; BPA50, n=3 ; BPA400, n=3 ) were stained with hematoxylin-eosin for  
218 morphological observation (50- $\mu\text{m}$  interval between each evaluated sections).



219 Histological analysis from the seminiferous tubules was performed by determining the  
220 tubular diameter and area, the seminiferous epithelium diameter and area and the  
221 luminal area, following morphometric parameters as previously described [30, 31].  
222 These measurements were performed using the Image J software (National Institutes  
223 of Health, Bethesda, MD, USA)..

224

#### 225 *2.8. RNA isolation and quantitative real time PCR*

226 Total RNA from pooled GFP positive sorted male germ cells at PND7 was extracted for  
227 each treatment (vehicle, n= 5; BPA50, n= 5; BPA400, n= 4) using an RNeasy Mini Kit  
228 (Qiagen, USA). Samples were immediately preserved in buffer RLT and stored at  
229  $-80^{\circ}\text{C}$  before RNA extraction, according to manufacturer instructions. RNA  
230 concentration and quality were assessed using a NanoDrop spectrophotometer  
231 (Thermo Scientific, USA). Residual DNA was digested with DNase I, Amplification  
232 Grade (Sigma-Aldrich). RNA (0.2  $\mu\text{g}$ ) was reverse transcribed to cDNA using M-MLV  
233 reverse transcriptase (Invitrogen, USA) and oligo(dT) primers (Invitrogen, USA).  
234 Quantitative real-time PCR (RT-qPCR) assays were performed using HOT FIREPol  
235 EvaGreen qPCR Mix Plus (Solis BioDyne; Biocientífica, Rosario, Argentina) using a  
236 Rotor-Gene 6000 thermocycler (Qiagen). The comparative cycle threshold ( $\Delta\Delta\text{Ct}$ )  
237 method was implemented for relative quantification using the ribosomal protein L19  
238 gene as an internal control [27]. The sequences of all primers used for RT-qPCR are  
239 provided in Supplementary Material (Supplementary Material, Table S2).

240

#### 241 *2.9. Sperm count, viability and motility*

242 Testes from male mice perinatally exposed to vehicle (n=6), BPA50 (n=4), and BPA400  
243 (n=7) were obtained at PND130. The cauda epididymis was removed, cut three times  
244 with small scissors, and placed in a 1.5 mL eppendorf with 1 mL of PBS. The tissues  
245 were incubated for 10 min at  $37^{\circ}\text{C}$  to release the sperm.

246 Sperm count and viability were carried out using flow cytometry. Viability was evaluated  
247 with propidium iodide (PI) staining using a gate on an SS vs PI (613/20 band-pass  
248 filter) dot plot. Sperm count was assayed at the same time, using fluorescence beads  
249 (CountBright™ Absolute Counting Beads; Life), acquired in the detector with 530/40  
250 band-pass filter (FITC). Sperm suspension was diluted 1:10, stained with PI (final  
251 concentration 2  $\mu\text{g}/\text{mL}$ ) and mixed with 3,000 fluorescence beads in a final volume of  
252 300  $\mu\text{L}$  of PBS. For each sample, 1,000 beads were counted. By comparing the ratio of

253 bead events to cell events, absolute numbers of spermatozoa in the sample was  
254 calculated. Sperm count per epididymis was expressed as the total number of  
255 spermatozoa present in 1 mL of the PBS cell suspension (cells per epididymis = 100 x  
256 average count sperm).

257 Sperm motility was assessed within thirty minutes after epididymis removal to limit the  
258 deleterious effects of dehydration, pH, or changes in temperature on motility. Two  
259 hundred whole spermatozoa (head and tail) were analyzed in blind duplicates per  
260 animal using an optic microscope at 400x magnification. Spermatozoa were classified  
261 according to three types of motility: Progressive motility (spermatozoa moving actively,  
262 either linearly or in a large circle, regardless of speed); non-progressive motility (all  
263 other patterns of motility except progression) and immotile (no movement).

264

#### 265 *2.10. Statistical analysis*

266 The data were analyzed using the GraphPad Prism© software version 5.0 (San Diego,  
267 USA), and results were expressed as the mean and standard error (SEM). One-way  
268 ANOVA was conducted to assess the overall significance, and Bonferroni's multiple  
269 comparison test was performed to compare each experimental group with the control  
270 group. Levels of biological significance were set at  $p < 0.05$ .

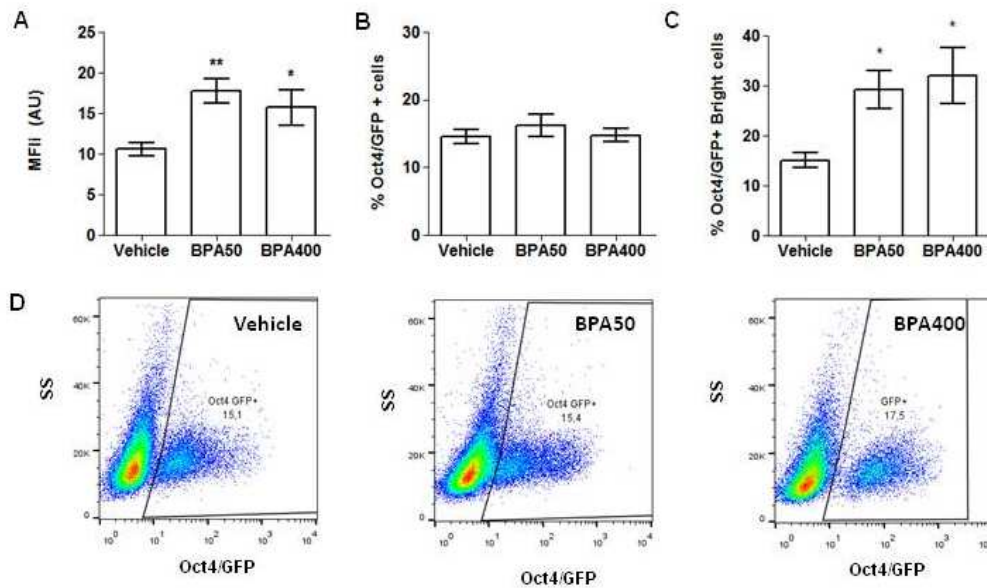
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### 272 **3. Results**

273 In the present work, we studied the effects of prenatal and early postnatal  
274 exposure to BPA on male germ cell development.

275 The alteration of germ cell maturation by BPA treatment was evaluated by the  
276 GFP flow cytometry profiles of testicular suspensions from transgenic Oct4/GFP mice,  
277 using the transcription factor Oct4 as a marker of SSCs [16].

278 Testicular cell suspensions of males born to mothers treated with both doses of BPA  
279 showed an increase in the MFli for Oct4/GFP positive cells (BPA50 = $17.8 \pm 1.5$ ;  
280 BPA400=  $15.7 \pm 2.2$  and vehicle=  $10.6 \pm 0.8$ ), even when the percentage of Oct4/GFP  
281 expressing cells did not differ from the control (Figure 1, A and B). Moreover, in the  
282 BPA-treated Oct4/GFP positive population, it was possible to clearly discriminate two  
283 subpopulations based on its bright or dim MFli (Figure D). Considering the strength of  
284 the signal, the percentage of bright MFli subpopulation was higher in all the BPA  
285 treated groups when compared with the vehicle (Figure 1, C).



286

287

288

**Figure 1: Effect of BPA on early postnatal development of male germ cells.**

289

Maturation profile of germ cells from PND7 mice perinatally exposed to vehicle, BPA50

290

and BPA400 were evaluated by the Oct4/GFP expression pattern of testicular cell

291

suspensions using flow cytometry analysis. A) MFI of Oct4/GFP positive cells; B)

292

Percentage of Oct4/GFP positive cells; C) Percentage of bright Oct4/GFP positive

293

cells; D) Representative dot plots of testicular cell suspensions for each treatment. BPA

294

treated animals showed an increased in the MFI and in the percentage of Bright

295

Oct4/GFP positive cells subpopulation. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$ ;

296

\*\*  $p < 0.01$ .

297

298

To assess whether the changes observed in Oct4/GFP cytometry profiles occur in

299

parallel with changes in the endogenous protein level of Oct4,

300

immunohistochemical analysis was performed in testes of PND7. As shown in figure 2

301

A, Oct4 signal was restricted to cells localized in the basement membrane of the

302

seminiferous tubules, where immature spermatogonia reside. The percentage of Oct4-

303

positive seminiferous tubules, as well as the number of total Oct4-positive cells per

304

testis, were increased in BPA400- compared to vehicle-treated animals (Figure 2, B

305

and C respectively). These findings correlate with the flow cytometry data, which

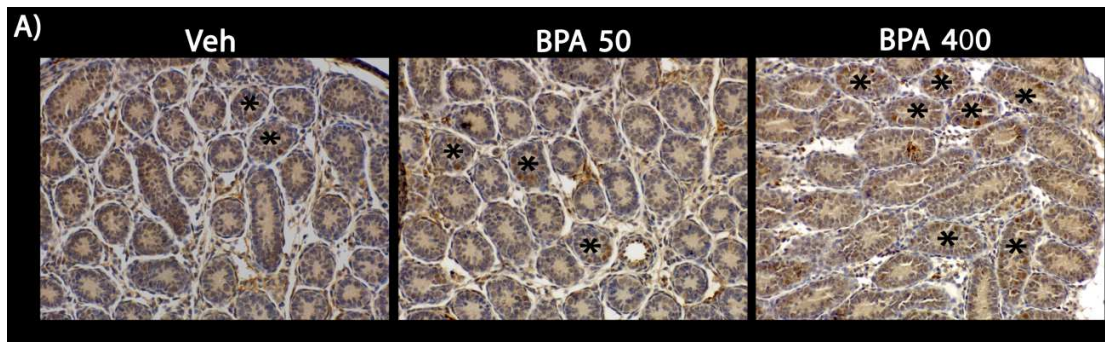
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showed differences in the percentage of brighter Oct4/GFP cells for BPA400- treated

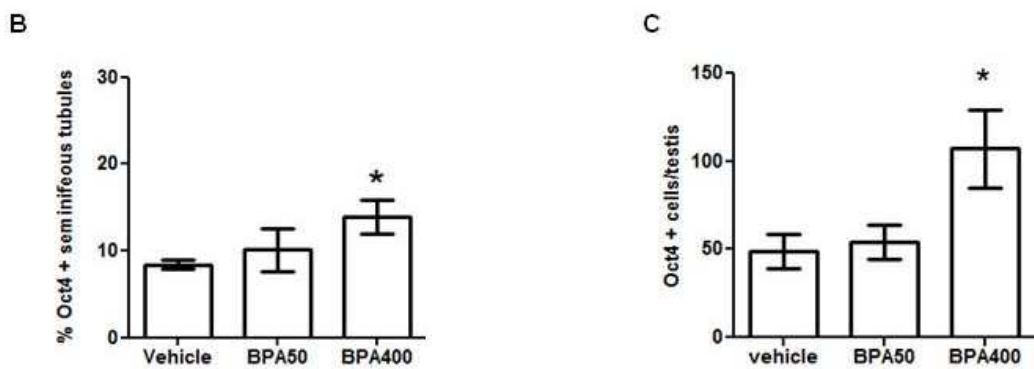
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animals. Otherwise, no significant change was observed in the percentage of

308 positive seminiferous tubules of BPA50-treated mice, even when this group also  
309 showed a brighter MFli subpopulation. Differences in the sensitivity of the readout  
310 technique (fluorescence vs DAB substrate generation) or the affinity of the  
311 antibodies for each antigen could account for this discrepancy.  
312



313

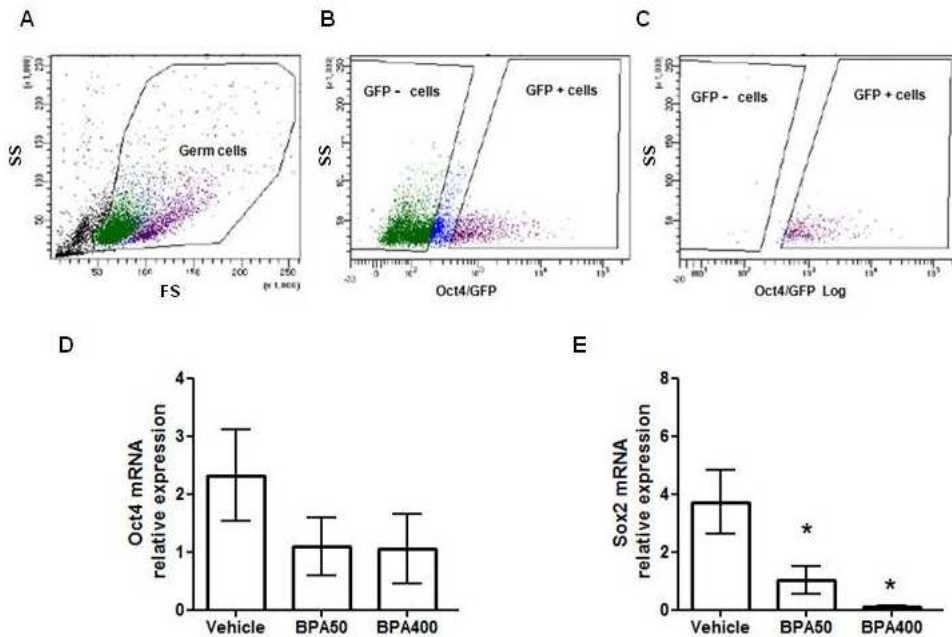


314

315 **Figure 2: Effect of BPA on Oct4 protein expression in testis from PND7 male**  
316 **mice.** Testicular sections from mice born to mothers perinatally exposed to vehicle,  
317 BPA50, BPA400 were analyzed for the expression of Oct4 protein. A) Representative  
318 photomicrographs of testicular sections immunostained for Oct4 protein. Tubules with  
319 at least one Oct4-positive cell inside the seminiferous tubules were defined as positive  
320 (asterisks). Immunohistochemistry was developed by using DAB as chromogen  
321 substrate and counterstaining with Mayer haematoxylin. Magnification 400X. B)  
322 Quantification of the percentage of Oct4-positive seminiferous tubules C) Quantification  
323 of the total number of Oct4-positive cells per testis. Data are expressed as mean  $\pm$   
324 SEM. \*  $p < 0.05$

325 Besides Oct4, which is considered a master pluripotency gene, the transcription factor  
326 Sox2 is also involved in the regulation of pluripotency and self-renewal in stem cells,

327 including SSC. We studied the mRNA expression levels of Oct4 and Sox2 in a pure  
328 population of Oct4/GFP positive germ cells isolated by FACS (Figure 3, A-C). As  
329 observed in Figure 3 D, Oct4 mRNA levels were similar in all groups, whereas Sox2  
330 mRNA relative expression level in the BPA treated groups decreased significantly  
331 (BPA50= 1fold,  $p < 0.05$ ) to nearly undetectable levels (BPA400= 0.1 fold,  $p < 0.05$ ),  
332 compared with vehicle-treated animals (Figure 3, E).



333

334

335

336 **Figure 3: Effect of BPA on Sox2 and Oct4 mRNA expression from Oct4/GFP**  
337 **positive germ cell population isolated by cell sorting.** A-C) Representative flow  
338 cytometry panels of Oct4/GFP positive germ cells sorting: the germ cell population was  
339 selected using SS vs FS dot plot (A); after single cell discrimination (not shown) the  
340 Oct4/GFP positive and negative population were gated using an Oct4/GFP vs SS dot plot  
341 (B). After sorting, the purity of the Oct4/GFP positive sorted population was evaluated by  
342 analysing a fraction of the recovered sample (C). D-E) Pure sorted Oct4/GFP positive  
343 population was analysed for Oct4 (D) and Sox2 (E) genes expression by RT-PCR. The  
344 mRNA levels were normalized to L19 expression. Data are expressed as mean  $\pm$  SEM. \*  
345  $p < 0.05$

346 Finally, to evaluate whether the early-life exposure to BPA had a consequence later in  
347 life, sperm quality, testicular morphology, and protein expression of androgen

348 receptor and PCNA were studied at PND130. For sperm quality, sperm count,  
 349 motility, and viability were analyzed in adult male mice. We found that the number of  
 350 spermatozoa was significantly reduced in BPA50-treated animals, with no difference in  
 351 sperm motility or viability (Table 1).

<b>Group</b>	<b>Spermatozoa count (1x10<sup>6</sup>)</b>	<b>Spermatozoa motility (%)</b>	<b>Spermatozoa Viability (%)</b>
Vehicle (n=6)	7.98 +/- 0.55	34.23 +/-4.62	35.65 +/-3.69
BPA50 (n=4)	5.19 +/-0.69 (*)	53.22 +/-11.55	22.01 +/-3.42
BPA400 (n=7)	6.05 +/-0.79	39.94 +/-10.13	28.81 +/-3.69

352

353 **Table1: Effect of perinatal exposure to BPA in adult male mice.** Sperm quality  
 354 parameters as total spermatozoa count, sperm motility (progressive and nonprogressive  
 355 motility) and viability were analysed in adult males born to mothers exposed perinatally  
 356 to vehicle or BPA. Data are expressed as mean ± SEM. \* p< 0.05, respect to the  
 357 vehicle.

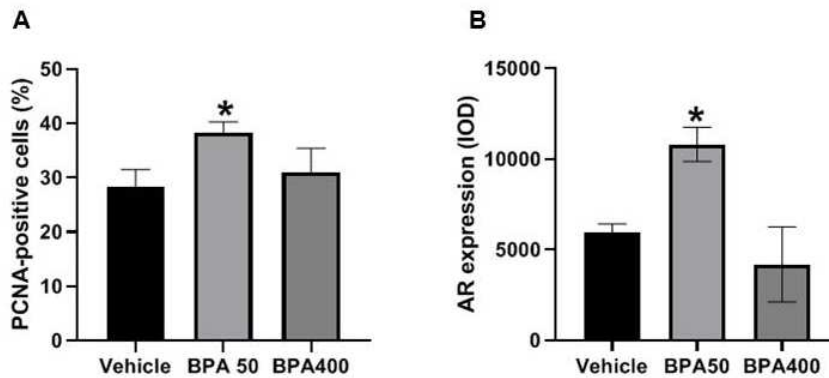
358

359 From histological analysis of testis of PND130, we found that the diameter or the area  
 360 of seminiferous tubules or germinal epithelium and luminal area were unmodified with  
 361 BPA treatment (Table 2). However, the percentage of PCNA-positive cells and the  
 362 expression of AR were increased only in BPA50-treated mice (Figure 4).

<b>Morphometry (µm/ µm<sup>2</sup>)</b>	<b>Vehicle</b>	<b>BPA50</b>	<b>BPA400</b>
<b>Tubular diameter</b>	177 +/- 11	176 +/- 8	186 +/-9
<b>Epithelial height</b>	41 +/- 4	34 +/- 1	51 +/- 9
<b>Tubular area</b>	20940 +/- 2449	19990 +/- 1088	22590 +/- 776
<b>Epithelial area</b>	14453 +/- 1774	13900 +/- 511	16198 +/- 44
<b>Luminal area</b>	6487 +/- 775	6090 +/- 587	6368 +/- 745

363

364 **Table 2: Morphometrical analysis of testicular histology in adult male mice.** The  
 365 diameter or the area of seminiferous tubules or germinal epithelium and luminal area  
 366 were analyzed in adult males born to mothers exposed perinatally to vehicle or BPA.  
 367 Data are expressed as mean ± SEM.



368

369

**Figure 4: Effect of BPA on proliferation and androgen receptor expression in**

370

**testis from PND130 male mice.** Expression of PCNA and AR protein from testicular

371

sections of PND130 mice born to mothers perinatally exposed to vehicle, BPA50,

372

BPA400 were analyzed by Immunohistochemistry as described in *Methods*. A)

373

Quantification of the percentage of PCNA-positive cells B) Quantification of AR

374

expression as IOD. Data are expressed as mean  $\pm$  SEM. \*  $p < 0.05$

375

376

#### 4. Discussion/Conclusion

377

Understanding the effects of environmental estrogens on germ cell differentiation is an area of growing interest in the field of endocrine disruption [32].

379

Oct4 belongs to the Octamer class and, together with Pit and Unc proteins, define the

380

POU family of transcription factors [14]. After gastrulation in the embryo, Oct4

381

expression is confined to the primordial germ cells [33]. In wild-type mice, Oct4 is

382

expressed in mitotically arrested prespermatogonia until birth. Then is detected in

383

newborn male mice at PND1 and PND7 in undifferentiated spermatogonia [16].

384

Previously, using the Oct4/GFP transgenic mouse and the flow cytometry analysis, we

385

were able to define germ cell variables to estimate both the abundance of the

386

undifferentiated cell population (percentage of Oct4/GFP-expressing cells) and the

387

expression levels of Oct4/GFP (MFI of Oct4/GFP and percentage of Oct4<sup>+</sup> bright

388

cells).

389

In the present study, we found an altered differentiation pattern of male germ cells at

390

an earlier stage in development, as evidenced by an increase in the levels of Oct4-

391

driven GFP expression in both doses of BPA. We demonstrated that developmental

392

exposure to BPA did not change the percentage of Oct4/GFP-positive cells but

393

increased the fraction of high-intensity Oct4/GFP-positive germ cells (bright

394 subpopulation) in PND7 pups. These results suggest that males born to mothers  
395 perinatally exposed to BPA have a higher proportion of undifferentiated spermatogonia  
396 than non-exposed males.

397 Moreover, the high proportion of bright Oct4/GFP cells from BPA treated animals  
398 suggests that this compound would promote an immature state in germinal cells.  
399 Interestingly, Yang and colleagues recently demonstrated that exposure to low doses  
400 of BPA up-regulates the expression of the transcription factors required for  
401 maintaining stem cell pluripotency and self-renewal, including Oct4, in a dose-  
402 dependent manner [34]. Based on these and other results, it has been suggested that  
403 BPA disrupts differentiation by promoting a stem cell phenotype [32]. Our results agree  
404 with this idea, adding evidence to the hypothesis that BPA might be able to modify the  
405 stemness state in germ cells.

406 When analyzing the mRNA level of endogenous Oct4 gene in a subpopulation of up-  
407 regulated Oct4/GFP expressing cells, it did not vary between groups, even when BPA  
408 treated animals showed an increase in the MFI of the Oct4 driven GFP. A possible  
409 explanation of this discrepancy could be attributed to the fact that Oct4 expression  
410 during germ cell development is regulated at the post-transcriptional level [35]. Herein,  
411 Oct4 mRNA levels did not change, whereas the protein level did, as indicated by the  
412 flow cytometry and immunohistochemistry analysis.

413 Regarding the Sox2 gene, its crucial contribution appears to be the maintenance of  
414 Oct4 expression by promoting the expression of its positive regulators, while repressing  
415 negative regulators and differentiation inducers [14, 20]. At the same time, Oct4 can  
416 up-regulate itself, Sox2, and other transcription factors involved in self-renewal and  
417 pluripotency [36]. Paradoxically, our results showed a marked reduction in Sox2  
418 mRNA levels in Oct4/GFP positive cells from BPA treated animals. In this respect, it  
419 has been reported that overexpression of Sox2 in embryonic stem cells reduced the  
420 endogenous expression of several Sox:Oct4 target genes and the endogenous Sox2  
421 gene itself [37]. Hence, the reduced mRNA level of Sox2 observed in BPA-exposed  
422 groups could be the result of a negative feedback loop due to the BPA-induced  
423 increase in Oct4 and Sox2 proteins.

424 On the other hand, recent studies reported that expression of Sox2 is reduced by  
425 estrogen [38, 39] or induction of ERalpha [40] and propose that the decreased  
426 expression of Sox2 mRNA is a mechanism for loss of stemness. Thus, the reduced  
427 expression of Sox2 that we observed in BPA-exposed groups could represent an



428 adaptive change, to attenuate the effects on stemness state caused by the high levels  
429 of Oct4 protein induced by BPA. Furthermore, it has been noted that the current  
430 understanding of reprogramming-related regulation of SSCs based on the roles of  
431 individual genes is limited, and that is necessary to reach an expanded view supported  
432 by collective transcriptional regulation [41]. Further studies on the Sox2 protein level as  
433 well as other relevant Oct4-target genes as Nanog would be necessary in order to  
434 confirm this hypothesis.

435 Despite the dose-relationship effect observed in early life in response to BPA, either by  
436 flow cytometry or at histological level, results from PND130 mice did not reproduce that  
437 behavior. In the adult stage, only BPA50-treated mice showed a significant reduction in  
438 sperm count, an increase in the percentage of PCNA-positive germ cells in the  
439 seminiferous tubules and the expression of androgen receptor in the testis, whereas  
440 BPA400 did not.

441 These results suggest that the effect of BPA later in life would respond to a non-  
442 monotonic dose-response, in contrast to the dose-response effect observed at an early  
443 stage. It has been proposed that binding to different receptors may be one of the  
444 mechanisms by which EDCs can exert different patterns of response [42, 43]. We  
445 suggest that the different BPA dose-response relationships observed at PND7 and  
446 PND130 could be explained based on this property of the EDC. For example, at low  
447 doses, BPA almost exclusively binds to the estrogen receptor (ER), but at high doses,  
448 it can also bind weakly to other hormone receptors, like androgen receptor (AR) and  
449 thyroid hormone receptor (THR) [44-47]. All the aforementioned nuclear receptors are  
450 expressed in the testis and exert an important role in its development [48, 49]. Also it  
451 has been shown that BPA exerted non-monotonic dose-response on Ca<sup>2+</sup> entry of  
452 mouse pancreatic B cells by involving ER $\beta$  at low doses whereas at high doses  
453 involved ER $\beta$ -mediated mechanism as well as via ER $\alpha$  simultaneously [50]. So,  
454 considering our results, it is likely that different endpoints could be affected in different  
455 ways.

456 The World Health Organization (WHO) and the United Nations Environment  
457 Programme (UNEP) have reported in their last update on EDCs that human semen  
458 quality has declined, and sperm counts are in the subfertile range in several  
459 countries [51]. However, the question of declining sperm counts continues to cause  
460 controversy, even today [7, 52].

461 Notably, the reduction in mouse and rat sperm quality after BPA treatment has been  
462 reported in several works, using different doses and routes of administration [53, 54].  
463 Herein we reported that adult male mice exposed early in life to BPA 50 µg/kg bw/d (a  
464 dose considered safe [2, 24]), showed a significant reduction in sperm count. Diverse  
465 BPA-mediated mechanisms of action that could account for male reproductive  
466 impairment have been postulated, among them epigenetic modifications, apoptosis  
467 induction, DNA damage, and meiotic errors.

468 In BPA50 group, we found that PCNA-positive cells were significantly increased in the  
469 testis of treated mice. Recently, it was demonstrated that an increase of PCNA and  
470 Ki67 expression occurred as a response to apoptosis of spermatogenic cells in the  
471 testis of rats exposed to Fluoride [55].

472 We also found an increased AR expression in testis from BPA50-treated mice. It is well  
473 known the antiandrogenic activity of BPA and that this compound can interact at several  
474 steps with AR function [46]. Thus, we suggest that this increase in AR signal could be a  
475 compensatory mechanism exerted by testicular cells in response to the antiandrogenic  
476 action of BPA, similar to some mechanisms described in cancer prostate therapy-  
477 resistant cells [56].

478 Whether exist a link between the changes observed at PND7 and PND130 or not, it is  
479 still an unanswered question. Our results suggest that BPA could act on different  
480 pathways and in different ways (dose response vs non-monotonic response). Further  
481 studies on these events are necessary to better understand the molecular mechanisms  
482 by which BPA affects male gonads in mice models. Beyond that, our results highlight an  
483 early disrupting function of BPA that could affect male reproduction, reinforcing the idea  
484 that gonads are sensitive targets of EDC compounds and adding evidence to the  
485 hypothesis of EDCs as a cause of reduced sperm quality worldwide.

486

487

488 In conclusion, the present work shows that perinatal exposure to BPA impairs the  
489 early differentiation of male germ cells at PND7. It has been described that BPA  
490 disrupts differentiation by promoting a stem cell phenotype [32]. Accordingly, results  
491 from the present study indicate that exposure to BPA during the prenatal and postnatal  
492 stages support the persistence of a high undifferentiated spermatogonial population.

493

494

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499

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508

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