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1 **An in-vitro bioassay to assess the potential global toxicity of waters on**
2 **spermatogenesis: a pilot study**

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15 ² Co-senior authors.

16

17

18 **Abbreviations**

19 Trans-epithelial electrical resistance: TEER; 1,3-dinitrobenzene (DNB); checkpoint kinase 2: CHK2;
20 DEAD-box helicase 4: VASA; testis specific histone 2B: TH2B; Stathmin 1: STMN1; Transition Protein 1:
21 TP1; Transition Protein 2: TP2; Protamine-3: PRM3; Androgen receptor: AR; Estrogen receptor alpha: ERα;
22 Tight junction protein-1:TJP1; Ribosomal protein S16: 16S; the glial cell-derived neurotrophic factor
23 (GDNF); Stem Cell Factor (SCF).

24

25

26 **Abstract**

27 Many toxicants are present in water as a mixture. Male infertility is one of the environmental impacts in
28 developed countries. Using our rat seminiferous tubule culture model, we evaluated the effects of waters of
29 different origins, on several parameters of the seminiferous epithelium. Concentrated culture medium was
30 diluted with the waters to be tested (final concentrations of the tested waters were between 8 and 80%). The
31 integrity of the blood-testis barrier was assessed by the trans-epithelial electric resistance (TEER). The levels of
32 mRNAs specific of Sertoli cells, of cellular junctions, of each population of germ cells, of androgen receptor, of
33 estrogen receptor α and of aromatase were also studied. We report, here, the results obtained with ten waters,
34 some of them possessing a negative effect on spermatogenesis. The results showed that according to the tested
35 waters, their effects on the parameters studied might be quite different indicating many different mechanisms of
36 toxicity, including some endocrine disrupting effects. It has been reported that men with impaired semen
37 parameters have an increased mortality rate suggesting semen quality may provide a fundamental biomarker of
38 overall male health. Hence, we have developed a relevant *in vitro* bioassay allowing to evaluate the potential
39 toxicity of different types of waters on male fertility and to assess some aspects of their mechanism of action. In
40 addition to the TEER measure, the number and/or the identity of the studied mRNAs can be largely increased
41 and/or modified, thus enhancing the possibility of using this model as a “warning system”.

42

43 **Keywords**

44 Water quality, *in vitro* bioassay, methods, spermatogenesis, blood-testis barrier integrity, endocrine disrupting
45 effects, global toxicity

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54 **1. Introduction**

55 A large number of environmental factors can pollute air, water and food and may potentiate each other when
56 present as a mixture (Cotton et al. 2016; Durand et al. 2017, 2020). Numerous studies suggest a decline in semen
57 quality in some parts of the world (Carlsen et al. 1992; Auger et al. 1995; Adamopoulos et al. 1996; Van
58 Waeleghem et al. 1996; Lackner et al. 2005; Sripada et al. 2007; Shine et al. 2008; Mukhopadhyay et al. 2010;
59 Geoffroy-Siraudin et al. 2012). This might occur in response to adverse environmental factors (Gabrielsen and
60 Tanrikut 2016).

61 Using our rat seminiferous tubule culture model (Bio-Alter®), which has been carefully validated on both a
62 physiological and a “physio-toxicological” point of view (Geoffroy-Siraudin et al. 2010; Goldstein et al. 2016),
63 we have developed a method to assess the potential impact of waters on spermatogenesis and to explore some
64 aspects of their mechanism of action. For this purpose, the integrity of the blood-testis barrier was determined by
65 measuring the trans-epithelial electric resistance (TEER). Indeed, we, and others, have shown that several
66 toxicants which may be present in the environment can alter the blood-testis barrier (Carette et al. 2015; Gao et
67 al. 2015; Goldstein et al. 2016). Also, the expression of genes specific of Sertoli cells, of cellular junctions, and
68 of each population of germ cells was studied by reverse transcription-quantitative polymerase chain reaction
69 (RT-qPCR). Similarly, the expressions of Androgen receptor (AR), of Estrogen receptor alpha (ER α) and of
70 aromatase were investigated. Hence, in order to validate this *in vitro* bioassay, in the present manuscript we
71 report the results obtained with ten waters from different origins.

72

73

74 **2. Materials and Methods**

75

76 **2-1 Animals**

77 Experiments were performed with 20–22-day-old male Sprague-Dawley rats having undergone no treatment.
78 Testes from six to ten rats were pooled. The experimental protocol was designed in compliance with
79 recommendations of the European Economic Community (EEC) (86/609/EEC) for the care and use of laboratory
80 animals.

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84 **2-2 Preparation and culture of seminiferous tubule segments**

85 Small seminiferous tubule segments were seeded, at a high density, in bicameral chambers and cultured in a
86 serum-free culture medium as previously described (Geoffroy-Siraudin et al. 2010; Carette et al. 2015; Goldstein
87 et al. 2016). Incubations were carried out at 33°C in a water-saturated atmosphere of 95% air and 5% CO₂. Each
88 culture was carried out in four to seven replicates for 10 to 21 days, either in the absence or presence of the water
89 sample to be tested. The complete culture medium was 15mM HEPES-buffered F12 / DMEM supplemented with
90 10µg / ml gentamycin, 10µU / ml nystatin, 10µg / ml insulin, 10µg / ml human transferrin, 10⁻⁴M vitamin C,
91 10µg / ml vitamin E, 10⁻⁷ M testosterone, 3.3 10⁻⁷ M retinoic acid, 3.3 10⁻⁷ M retinol, 10⁻³ M pyruvate (Sigma)
92 and 1ng / ml FSH (NIH, Bethesda, MD).

93

94 **2-3 Preparation of the culture medium**

95 Complete 5X culture medium was made by dissolution of DMEM / F12, 15mM HEPES-buffered, in powder in
96 Versol water. The pH of this 5X medium was adjusted to a physiological pH: 7.1± 0.2 in order to avoid
97 characterizing the effects of a difference in pH. Then, the medium was filtered through a 0.2 µm filter.
98 Concentrated culture medium was diluted five-fold with either Versol water (control) or the water to be tested
99 giving a final concentration of the tested water of 80% (v/v). For those waters to be tested at lower
100 concentrations, they were first diluted with Versol water before being added to the concentrated culture medium
101 (final pH between 6.9 and 7.3). The waters to be tested were present from day 2 of culture and only in the basal
102 compartment of the bicameral chamber. Only the basal media [prepared without (control) or with the water to be
103 tested] were renewed every 2-3 days. To test the effect of 1,3-dinitrobenzene (DNB), a stock solution at 10 mg /
104 ml was prepared in 100% ethanol. The final concentration tested in the culture medium was 10 µg / ml (0.1%
105 ethanol).

106

107 **2-4 Trans-epithelial electrical resistance**

108 TEER measurement was performed as previously (Carette et al. 2015; Durand et al. 2017) with an EVOM2
109 (World Precision Instruments, Florida, USA). The culture was equilibrated at room temperature before the assay.
110 The TEER was calculated according to the following equation: TEER = (R total - R control).A (Ω cm²), where R
111 total is the resistance measured, R control is the resistance of the control insert (insert alone with the culture
112 medium), and A is the surface area of the insert.

113 TEER was measured for 4 to 7 replicate wells. One-way ANOVA was performed
114 (<https://biostatgv.sentiweb.fr/?module=tests/anova>).

115

116 2-5 RT-qPCR

117 Four to seven wells were pooled and total RNA was extracted from cultured seminiferous tubules, at the end
118 of the culture (between day 10 and day 20), using TRI-REAGENT (Euromedex, Souffelweyersheim, France)
119 according to the manufacturer's protocol. All samples were treated by DNase I before the reverse transcription;
120 first-strand cDNA was synthesized from 1 µg RNA with Super Script III in the presence of oligodT and Random
121 Primer (all from Thermo Fisher Scientific, Villebon sur Yvette, France). Real-time PCR was performed in a
122 StepOnePlus instrument (Applied Biosystems) by using SYBR-Green (Qiagen, Courtaboeuf, France).

123 Primer sequences are presented in Table 1. The ribosomal protein S16 (16S) was used as a housekeeping gene
124 control. The results are expressed in % expression for each gene studied: absolute value of the condition treated /
125 absolute value of the control (Carette et al. 2015).

126 The absolute value: $2^{-[\text{moyCT}]}$ was calculated for each gene studied as well as for the housekeeping gene
127 (16S). The value obtained for each gene studied was divided by the value obtained for the 16S mRNA.

128 Pooling of wells did not permit an estimate of measurement error in the RT-qPCR data, thus, statistical analysis
129 was not performed. Based on our experience, we consider that a condition causes (i) an increase in the level of
130 expression when the value of the measure is greater than 130% of the value of the control; (ii) a decrease of the
131 level of expression when the value of the measure is lower than 70% of the value of the control.

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139 **Table 1:** Sequences of the primers used for RTqPCR

	fwd-5'	rev-5'	Reference Sequence
CHK2	TGCTGGAGCCTAGGAGTCAT	TCATGTGCTCATCCTGAAGC	NM_053677.1
VASA	GATGCACCACCGGCAATTTT	TTCCCAGACCCTGTTTGAGC	NM_001077647.1
Integrin $\alpha 6$	TCGGTCTCTGGAGTCGCTAA	GCCGTGCCGAGGTTTTTAAG	NM_053725.1
TH2B	GATGCCGCGAAGAGAGTTAC	GGTCGAGCGCTTGTTGTAAT	NM_022643.1
Stathmin	AAGGATCTTTCCCTGGAGGA	TTCTCCTCTGCCATTTTGCT	NM_017166.1
TP1	CGACCAGCCGCAAATAAAG	ATCATCGCCCCGTTTCCTAC	NM_017056.2
TP2	GGCCTCAAAGTCACACCAAT	CTTGCTCACTTTCCCTTCCA	NM_017057.2
PRM3	GTGGCCTGTGTGAGTCAAGA	CCTCAGCACCATCTTGCTTT	NM_001002855.2
AR	GTGTCGTCTCCGAAATGTT	GGAATCAGGCTGGTTGTTGT	NM_012502.1
ER α	CATCGATAAGAACCGGAGGA	AAGGTTGGCAGCTCTCATGT	NM_012689.1
Aromatase	TCTCCAGATTCGGCAGCAAG	ATAAGAACAGGGCCCGTCAG	NM_017085.2
TJP1	TGCTCCAGCAGGTCCTAAGT	TGGTAGCTGAGGGCAGAACT	NM_001106266.1
Claudin-11	CTGGTGGACATCCTCATCCT	GCGCATAACAGGAAACCAGAT	NM_053457.2
Occludin	GAGGGTACACAGACCCAGCA	CAGGATTGCGCTGACTATGA	NM_031329.2
Connexin 43	TCCTTGGTGTCTCTCGCTTT	GAGCAGCCATTGAAGTAGGC	NM_012567.2
Clusterin	TTCATTCCCTCCAGTCCAAG	CACGAGAGGGGACTTCTGAG	NM_053021.2
Vimentin	AGATCGATGTGGACGTTTCC	CACCTGTCTCCGGTATTCGT	NM_031140.1
16S	AGTCTTCGGACGCAAGAAAA	AGCCACCAGAGCTTTTGAGA	NM_001169146.1

140

141 3. Results

142

143 3-1 Effect of different dilutions of effluent waters on the TEER and the levels of TP1 mRNA

144 In a first set of experiments serial dilutions of three hospital effluent waters (Poitou Charente region, France)
 145 were tested. Water A: a general effluent (which does not contain only hospital effluents); Water B: an effluent
 146 from the main building of the hospital (standard hospitalizations from general medicine: 24% of total flow);
 147 Water C: an effluent from the regional cancer center (14% of total flow).

148 Measurements of the TEER showed clear dose- and time- dependent effects on the integrity of the blood-
 149 testicular barrier (Fig1a). For every water, at the lower concentration tested (8%), a TEER greater than that of the
 150 control was observed on days 8 and 10 (all $p \leq 0.001$). By contrast, for the higher concentration (80%) of waters
 151 B and C, the TEER was lower than the control from day 4 to day 10 ($p \leq 0.001$). These effects were associated to
 152 rather dose-dependent changes in the expression of TP1 mRNA, which is specifically expressed by round
 153 spermatids (Fig. 1b). However, for both Water B and Water C, no mRNA of TP1 could be measured, when they
 154 were tested at 80%, indicating a dramatic effect of these waters on round spermatids.

155

156 **3-2 Effect of 3 different hospital tap waters from the Ile de France region (tested at the concentration of**
157 **80%) on the TEER and the levels of TP1, TP2 and PRM3 mRNAs**

158 In this series of experiments, DNB was included as a positive control in order to validate the responsiveness
159 and the sensitivity of the culture (Fig. 2a). Indeed, DNB is a powerful testicular toxicant. In the Bio-AlteR® rat
160 model, we have shown that DNB exerts a direct toxic effect on Sertoli cells and germ cells and alters the TEER
161 (Goldstein et al. 2016). In the present experiment, DNB at 10 µg / ml reduced the TEER from day 9 to day 19
162 (between 68 and 73% of the control, $p \leq 0.05$ to $p \leq 0.001$), and reduced dramatically the expression of the
163 mRNAs of TP1, TP2 and PRM3 (all less than 1% of the control values, data not shown). For hospital tap waters
164 I, II, and III no change of the TEER was ever observed (Fig. 2a). Nevertheless, hospital tap waters I and II
165 increased the amounts of TP1, TP2 and PRM3 mRNAs (between 137 and 166% of the control). By contrast, the
166 amounts of mRNAs of TP1, TP2 and PRM3 were decreased (61%, 48% and 67% of the control respectively) by
167 hospital tap water III (Fig. 2b).

168

169

170 **3-3 Effect of four drinking waters on the TEER and on the levels of some “specific” mRNAs**

171 In order to refine the above results, in the last set of experiments presented here, we increased the number of
172 mRNAs investigated. For this purpose, we evaluated the effects on spermatogenesis of two tap waters (1 and 2)
173 from different sources of the area of Lyon (France) and of two plastic bottled mineral waters (3 and 4). All these
174 waters were tested at the concentration of 80%.

175

176 **3-3-1 Effect on the TEER**

177 As shown in Fig. 3a, the TEER of tap water 1 was higher than the control on days 8 and 10 (123 and 127%
178 respectively, $p \leq 0.01$), but lower at the end of the culture (54 to 82% from day 15 to day 20, $p \leq 0.01$ to
179 $p \leq 0.001$). By contrast, the TEER of tap water 2 ranged between 90% and 98% of the value of the control water
180 until day 13, but was slightly lower from day 15 until day 20 (between 75 and 78% of control $p \leq 0.05$ to
181 $p \leq 0.01$). For plastic bottled mineral waters (Fig. 3b), the TEER of water 3 was never less than 85% of the
182 control, whereas for water 4 it was lower only from day 13 to day 17 (71 to 81% of control $p \leq 0.01$ to $p \leq 0.001$).

183

184

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186 3-3-2 Effect on the relative levels of some “specific” mRNAs

187 For tap water 1 the clear biphasic modifications of the TEER were associated, on day 20 of culture, to
188 changes in the expression, of some genes specific of tight (claudin-11 and occludin: 52% and 40% of control) or
189 gap junctions (71% for connexin 43) (fig. 4). Smaller changes were observed for tap water 2 and for the two
190 mineral waters 3 and 4 (61% for TJP1 for water 2); (53 and 68%, for claudin-11 for waters 3 and 4 respectively)
191 and 144% for connexin 43 for water 4).

192 The mRNAs of clusterin and of vimentin, specific of the Sertoli cells, were not modified by any water, but a
193 slight increase (133% of the control for vimentin) for mineral water 4 (Fig. 5). Conversely, more changes were
194 observed at the level of germ cells specific mRNAs. In spermatogonia, CHK2 mRNA was decreased by tap
195 water 2 and mineral water 3 (55 and 54% of the control respectively), and VASA mRNA (between 54 and 61%
196 of the control for tap waters 1, 2 and mineral water 4). No change was ever observed for integrin $\alpha 6$ mRNA. In
197 spermatocytes, stathmin mRNA was decreased only by tap water 1 (50% of the control), and TH2B mRNA by
198 tap water 2 (65% of the control). TP1, TP2 and PRM3 mRNAs, specific of round spermatids (Marret et al. 1998;
199 Grzmil et al. 2008) were all greatly decreased by tap water 1 (between 29 and 41% of control). TP1 mRNA was
200 also decreased by tap water 2 and the two mineral waters (between 56 and 69% of control), and PRM3 mRNA
201 by the mineral waters 3 and 4 (70 and 63% of the control respectively).

202 As shown in Fig. 6, only mineral water 3 decreased the mRNA of AR (59% of the control). No change was
203 ever observed at the level of Era mRNA, whereas aromatase mRNA was decreased by all the tested waters
204 (between 55 and 68% of control).

205

206

207 4. Discussion

208 Methods for assessing the total impact, on water quality, of all the chemicals present are clearly needed
209 (Murk et al. 2002; Bain et al. 2014; Escher et al. 2014; Di Paolo et al. 2016; Väitalo et al. 2016, 2017; van der
210 Oost et al. 2017a, b). The present bioassay, which is based on primary mammalian cultured cells, associates a
211 functional approach (TEER), with a quantification of some mRNAs which are specific of the different types of
212 cells.

213 Indeed, in this model, the assessment of the TEER is performed in cultures associating both the somatic cells
214 and the germ cells of the seminiferous epithelium. It has been shown that disruption of Connexin 43 induces an
215 increase of the transcription of some tight junction proteins which results in an increase of the TEER (Carette et

216 al. 2010, 2015) whereas an alteration of tight junctions leads to a decrease of the TEER (Janecki et al. 1992; Hu
217 et al. 2014). This would suggest that at the lower concentrations tested, waters A, B and C disrupted mainly
218 Connexin- based gap junctions, whereas at the higher concentrations they disrupted both gap junctions and tight
219 junctions. Of importance, in this study, we show that some waters may have an impact on some parameters
220 involved in the process and/ or regulation of spermatogenesis, directly at the level of the seminiferous
221 epithelium, including some endpoints related to possible endocrine disrupting effects.

222 It is clear that according to the tested waters, their effects on the parameters studied may be quite different
223 indicating many different mechanisms of toxicity leading to decrease or increase in the mRNA levels of TP1,
224 TP2 or PRM3. Moreover, whereas for the hospital effluent waters A, B and C there was a rather good correlation
225 between the TEER and the amounts of TP1 mRNA, this was clearly not the case for the hospital tap waters I, II
226 and III. The absence of an effect on the TEER suggests either no effect on the blood–testis barrier or the
227 occurrence of an equilibrium resulting from a disruption of Connexin 43 associated with some alteration of the
228 tight junctions. The important impact on the TEER of tap water 1 was associated to rather important
229 modifications of the expression of the mRNAs specific of some cellular junction proteins including a decrease in
230 Connexin 43 mRNA. At the level of the blood testis-barrier, Connexin 43, a constitutive protein of gap junctions,
231 is highly intermingled with proteins of tight (Occludin, Claudin, Zonula occludens-1...) and adherens junctions
232 (N-cadherin) (Pointis and Segretain 2005). Occludin- and Claudin-11-based tight junctions are the major
233 components of the blood–testis barrier (Morrow et al. 2010). Recent studies have revealed that the gap junction
234 protein Connexin 43 is crucial for spermatogenesis (Pointis et al. 2005; Brehm et al. 2007; Sridharan et al. 2007;
235 Vinken et al. 2010). In addition, it has been reported that chemicals could alter Sertoli/germ cell interaction,
236 through disruption of Connexin 43-based gap junctions, and affect the other junction types leading to some
237 destabilization of the blood-testis barrier (Li et al. 2009, 2010; Carette et al. 2010).

238 Therefore, the measurement of TEER in the present model of culture appears as a first, simple and sensitive,
239 but not sufficient, mean to evaluate the incidence of a water sample on the spermatogenic process. The great
240 modifications of the values of the TEER induced by tap water 1 were clearly associated to changes in the
241 expression of genes specifically expressed by meiotic (spermatocytes) and post meiotic (round spermatids) cells
242 which are able to differentiate only in the presence of a functional blood-testis barrier (Mruk and Cheng 2015).
243 Despite lower modifications of the TEER values, tap water 2 and mineral waters 3 and 4 also induced changes in
244 the amounts of mRNAs of several junctional proteins. These changes were associated to decreased levels of
245 some mRNAs specific of post-meiotic cells indicating alteration of the meiotic step. The amounts of the mRNAs

246 of CHK2 and of VASA, specific of the mitotic phase of spermatogenesis, were also decreased by some of the
247 drinking waters 1 to 4. However, it is important to underline that no change was ever observed for those mRNAs
248 of integrin $\alpha 6$ suggesting no alteration of the population of the spermatogonial stem cells (Shinohara et al. 1999).
249 Only very few, if any, changes at the level of the mRNAs of vimentin or clusterin, specific of the Sertoli cells,
250 were induced by either drinking water 1 to 4.

251 Rather surprising was the decrease in mRNAs of aromatase induced by drinking waters 1 to 4. The Sertoli
252 cells are the main source of estrogens in immature rats, whereas in adults, estrogens are synthesized by the
253 Leydig and the germ cells (Carreau et al. 2009). This decrease of the aromatase expression in the adult Sertoli
254 cells has been suggested to result from a negative control of Sertoli cell aromatase expression by germ cells via
255 paracrine regulatory factors (Boitani et al. 1981; Silandre et al. 2007; Bouraïma-Lelong et al. 2010). Hence it is
256 tempting to suggest some endocrine disrupting activity of these 4 drinking waters. Similarly, the decrease in AR
257 mRNAs induced by mineral water 3 might indicate an anti-androgenic effect of this plastic bottled mineral
258 water, and/or of the plastic of the bottle itself.

259 In this work we have developed a global relevant and sensitive biological approach to evaluate the potential
260 global toxicity of waters on male fertility and to assess some aspects of their mechanism of action. Further, it
261 should be underlined that most of the growth factors, cytokines, neurotrophins and steroid hormones produced
262 within the testis, and necessary for spermatogenesis, are widely expressed in the organism and/or necessary for
263 the regulation of other vital functions. For instance, (i) the glial cell-derived neurotrophic factor (GDNF) has
264 been observed in the central nervous system (Du and Dreyfus 2002) and peripheral organs including the kidneys,
265 lungs, blood, and testes (Suter-Crazzolara and Unsicker 1994; Trupp et al. 1995; Suvanto et al. 1996); (ii) the
266 receptor tyrosine kinase c-Kit and its ligand Stem Cell Factor (SCF) are involved in haemopoiesis,
267 melanogenesis and spermatogenesis (Ashman 1999; Rossi et al. 2000). In this line, it has been reported that men
268 with impaired semen parameters have an increased mortality rate suggesting semen quality may provide a
269 fundamental biomarker of overall male health (Jensen et al. 2009; Eisenberg et al. 2014). Taken together, these
270 features suggest the possibility of using this *in vitro* bioassay as a “warning system”.

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276 **7. Declarations**

277 **-Ethical Approval**

278 The experimental protocol was designed in compliance with recommendations of the European Economic
279 Community (EEC) (86/609/EEC) for the care and use of laboratory animals.

280

281 **-Consent to Participate**

282 All authors consented to participate to this work.

283

284 **-Consent to Publish**

285 All authors consented to publish this work.

286

287 **-Availability of data and materials**

288 The datasets used and/or analyzed during the current study are available from the corresponding author on
289 reasonable request.

290

291 **-Competing Interests**

292 The authors declare no competing interest.

293

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296

297 **-Authors Contributions**

298 Conceived and designed the experiments: LP, MHP and PD. Performed the experiments: AB, GM and PD.

299 Analyzed the data: AB, GM, MHP and PD. Contributed reagents/materials/analysis tools: LP. Contributed to the
300 writing of the manuscript: MHP and PD.

301

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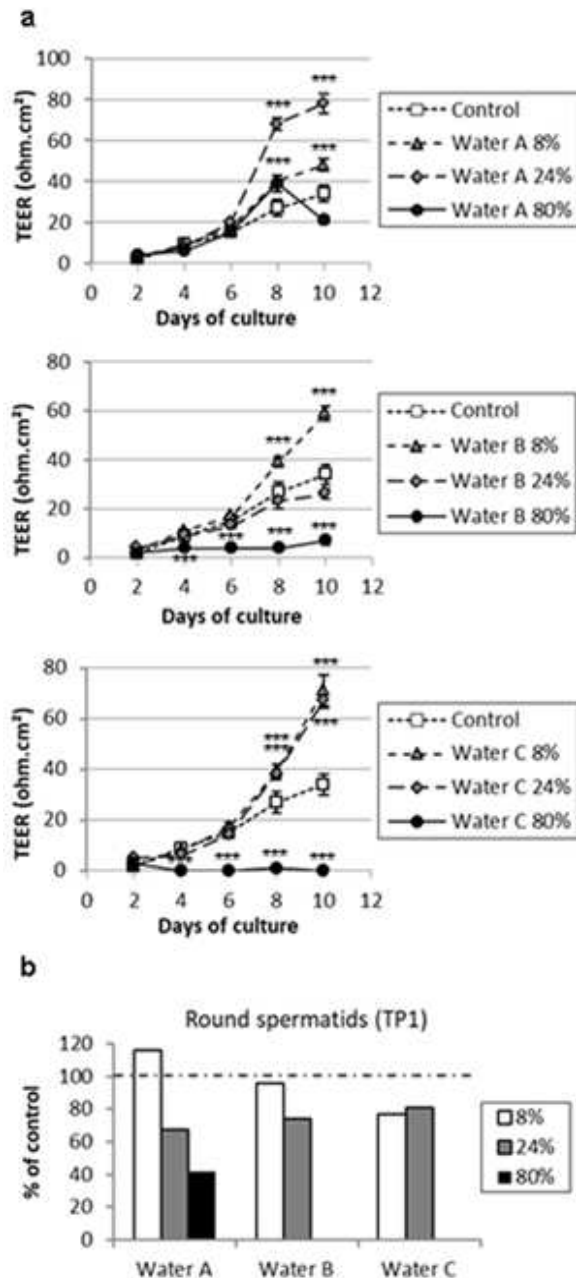
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465 **Figure 1:** Effects of three hospital effluent waters on (panel a) the TEER of rat seminiferous tubules in culture
466 and on (panel b) the mRNA-related qPCR product levels (% of control) of TP1 (specific of round spermatids)
467 after 10 days of culture.

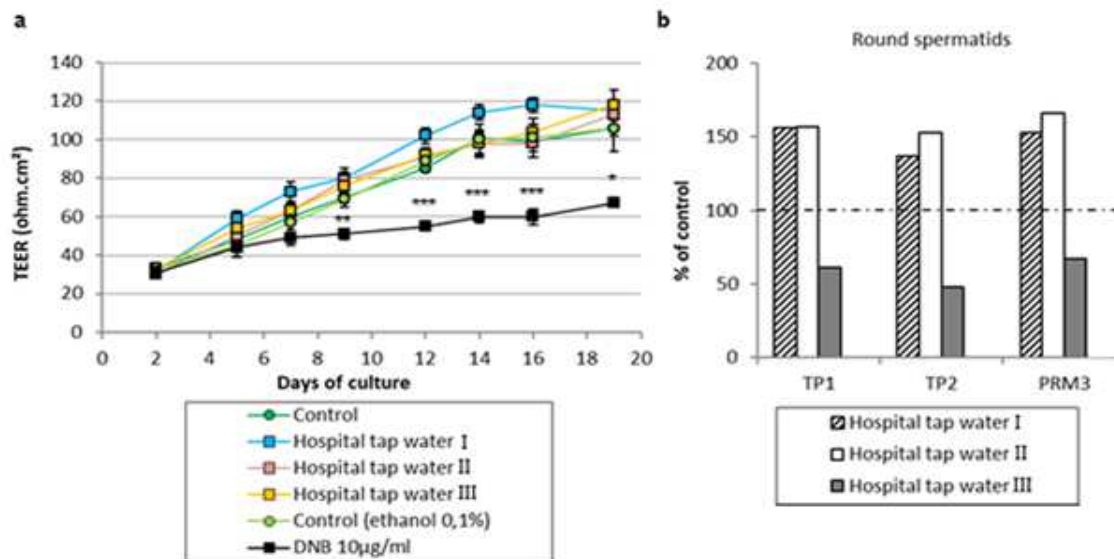
468 Seminiferous tubule segments were cultured in control medium or in the presence of media made with A, B or C
469 waters at 8%, 24% or 80%.

470 (panel a): results are means \pm SEM of 4 wells per day for each condition.

471 Error bars reflect the standard error of the mean (n = 4). **p \leq 0.01, ***p \leq 0.001 by one-way ANOVA.

472 (panel b): results are means of 4 pooled wells.

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478 **Figure 2:** Effects of three hospital tap waters on (panel a) the TEER of rat seminiferous tubules in culture and on
479 (panel b) the mRNA-related qPCR product levels (% of control) of TP1, TP2 and PRM3 (specific of round
480 spermatids) after 19 days of culture.

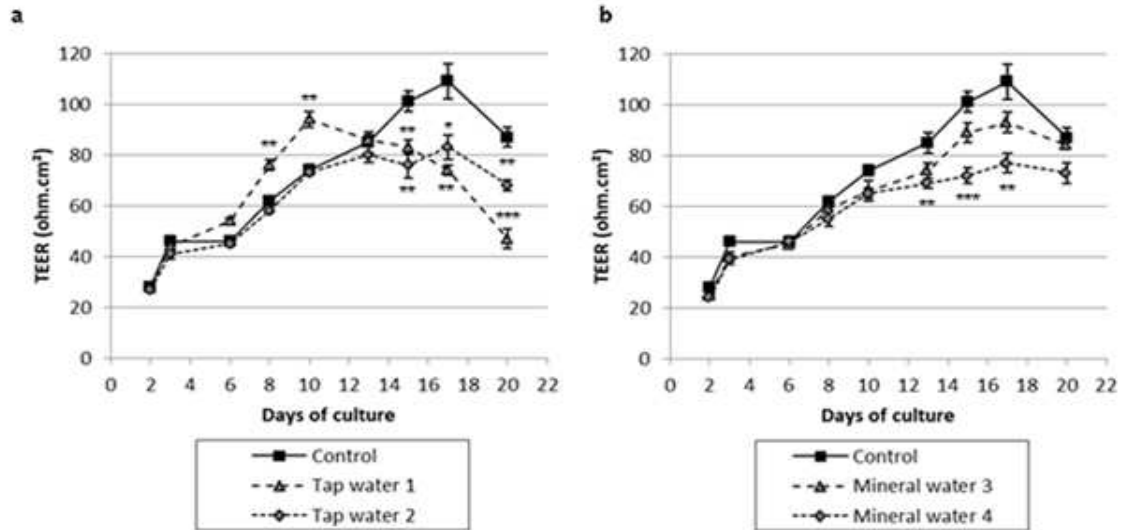
481 Seminiferous tubule segments were cultured in control medium or in the presence of media made with hospital
482 tap waters I, II or III. The effect of DNB at 10µg/ml was studied in parallel (in this case the control contained
483 ethanol at 0.1%).

484 (panel a): results are means ± SEM of 7 wells per day for each condition.

485 Error bars reflect the standard error of the mean (n = 7). **p≤ 0.01, ***p≤ 0.001 by one-way ANOVA.

486 (panel b): results are means of 7 pooled wells.

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495 **Figure 3:** Effect of the four drinking waters on the TEER of rat seminiferous tubules in culture.

496 Seminiferous tubule segments were cultured in control medium or in the presence of media made with the tested
497 waters.

498 (panel a): Tap water 1, Tap water 2

499 (panel b): mineral water 3, mineral water 4

500 Results are means \pm SEM of 4 wells per day for each condition.

501 Error bars reflect the standard error of the mean (n = 4). *p \leq 0.05, **p \leq 0.01, ***p \leq 0.001 by one-way ANOVA.

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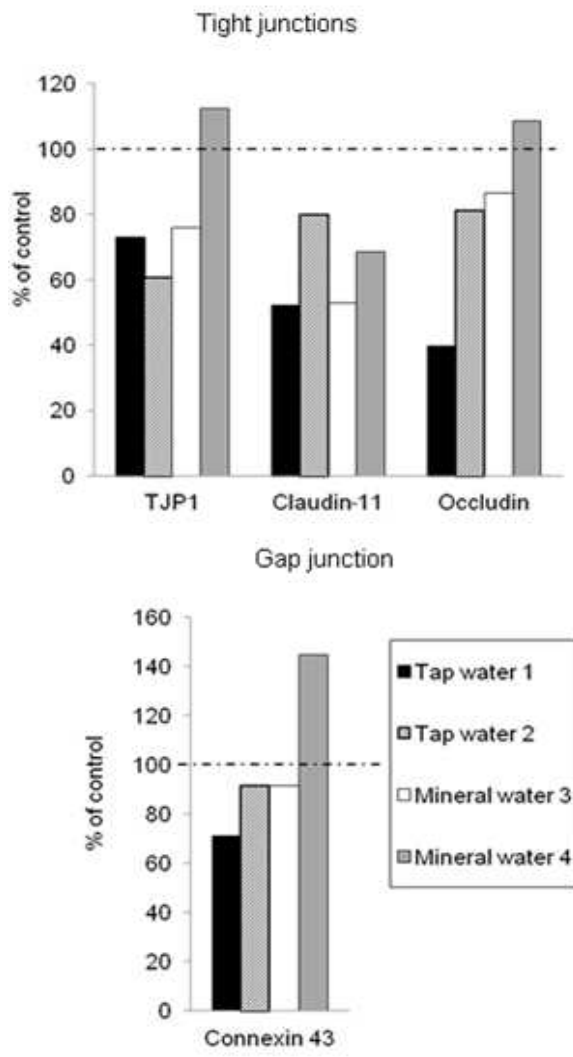
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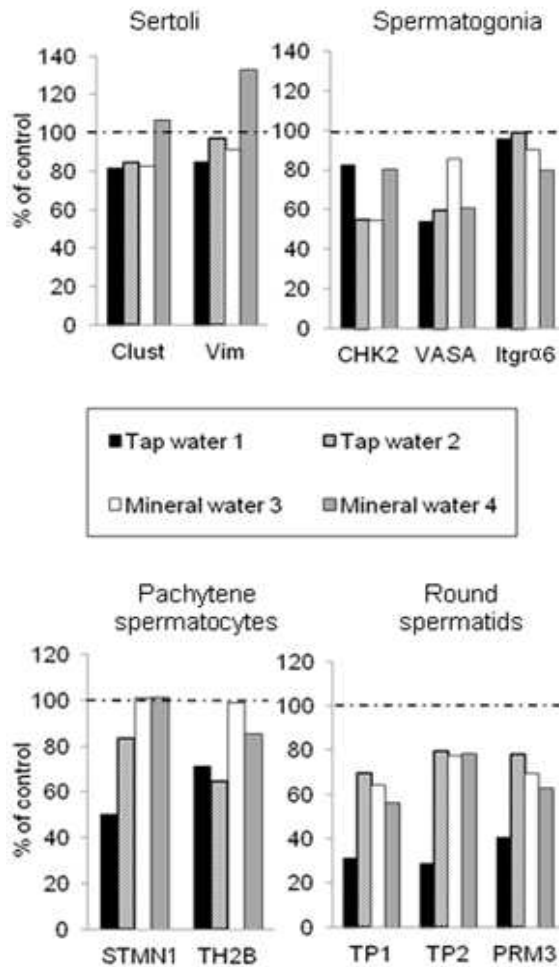


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518 **Figure 4:** mRNA-related qPCR product levels (% of control) of TJP1, claudin-11, occludin (specific of tight
519 junctions) and connexin 43 (specific of gap junctions) in seminiferous tubule segments cultured for 20 days in
520 control medium or in the presence of media made with Tap water 1, Tap water 2, mineral water 3 or mineral
521 water 4. Results are means of 4 pooled wells for each condition.

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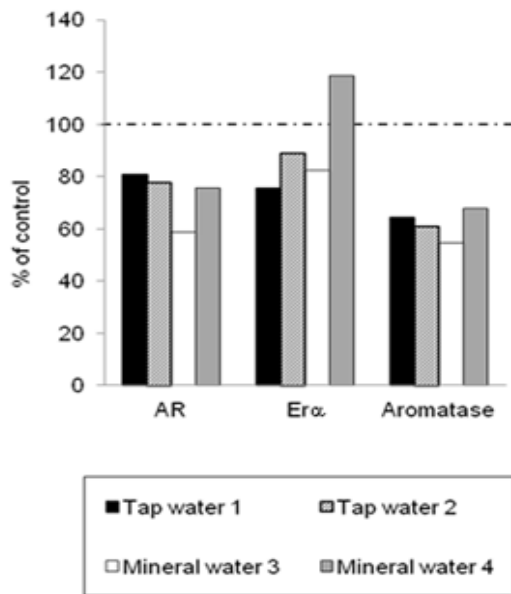
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 537 **Figure 5:** mRNA-related qPCR product levels (% of control) of clusterin (Clust), vimentin (Vim) (specific of
 538 Sertoli cells), CHK2, VASA, integrin $\alpha 6$ (Itgr $\alpha 6$) (specific of spermatogonia), STMN1, TH2B (specific of
 539 pachytene spermatocytes), TP1, TP2 and PRM3 (specific of round spermatids) in seminiferous tubule segments
 540 cultured for 20 days in control medium or in the presence of media made with Tap water 1, Tap water 2, mineral
 541 water 3 or mineral water 4. Results are means of 4 pooled wells for each condition.

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554 **Figure 6:** mRNA-related qPCR product levels (% of control) of AR, Erα and aromatase in seminiferous tubule
555 segments cultured for 20 days in control medium or in the presence of media made with Tap water 1, Tap water
556 2, mineral water 3 or mineral water 4. Results are means of 4 pooled wells for each condition.

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