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

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## Abbreviations

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

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## Abstract

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

## Keywords

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

## 1. Introduction

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

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

## 2. Materials and Methods

### 2-1 Animals

Experiments were performed with 20–22-day-old male Sprague-Dawley rats having undergone no treatment. Testes from six to ten rats were pooled. The experimental protocol was designed in compliance with recommendations of the European Economic Community (EEC) (86/609/EEC) for the care and use of laboratory 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% CO<sub>2</sub>. 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<sup>-4</sup>M vitamin C,  
91       10µg / ml vitamin E, 10<sup>-7</sup> M testosterone, 3.3 10<sup>-7</sup> M retinoic acid, 3.3 10<sup>-7</sup> M retinol, 10<sup>-3</sup> M pyruvate (Sigma)  
92       and 1ng / ml FSH (NIH, Bethesda, MD).

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## 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 1,3dinitrobenzene (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).

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## 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 cm<sup>2</sup>), 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.

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

## 2-5 RT-qPCR

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

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

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

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

**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	CGACCAGCCGCAAACTAAAG	ATCATCGCCCCGTTTCCTAC	NM_017056.2
TP2	GGCCTCAAAGTCACACCAAT	CTTGCTCACTTTCCCTTCCA	NM_017057.2
PRM3	GTGGCCTGTGTGAGTCAAGA	CCTCAGCACCATCTTGCTTT	NM_001002855.2
AR	GTGTCGTCTCCGAAATGTT	GGAATCAGGCTGGTTGTTGT	NM_012502.1
ER $\alpha$	CATCGATAAGAACCGGAGGA	AAGGTTGGCAGCTCTCATGT	NM_012689.1
Aromatase	TCTCCAGATTCGGCAGCAAG	ATAAGAACAGGGCCCGTCAG	NM_017085.2
TJP1	TGCTCCAGCAGGTCTAAGT	TGGTAGCTGAGGGCAGAACT	NM_001106266.1
Claudin-11	CTGGTGGACATCCTCATCCT	GCGCATACAGGAAACCAGAT	NM_053457.2
Occludin	GAGGGTACACAGACCCAGAG	CAGGATTGCGCTGACTATGA	NM_031329.2
Connexin 43	TCCTTGGTGTCTCTCGCTTT	GAGCAGCCATTGAAGTAGGC	NM_012567.2
Clusterin	TTCATTCCCTCCAGTCCAAG	CACGAGAGGGGACTTCTGAG	NM_053021.2
Vimentin	AGATCGATGTGGACGTTTCC	CACCTGTCTCCGGTATTCTGT	NM_031140.1
16S	AGTCTTCGGACGCAAGAAAA	AGCCACCAGAGCTTTTGAGA	NM_001169146.1

### 3. Results

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

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

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

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

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

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

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

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

As shown in Fig. 3a, the TEER of tap water 1 was higher than the control on days 8 and 10 (123 and 127% 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  $p \leq 0.001$ ). By contrast, the TEER of tap water 2 ranged between 90% and 98% of the value of the control water until day 13, but was slightly lower from day 15 until day 20 (between 75 and 78% of control  $p \leq 0.05$  to  $p \leq 0.01$ ). For plastic bottled mineral waters (Fig. 3b), the TEER of water 3 was never less than 85% of the 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  $p \leq 0.001$ ).



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

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

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

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

## 4. Discussion

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

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

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

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

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

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

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

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

## **7. Declarations**

### **-Ethical Approval**

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

### **-Consent to Participate**

All authors consented to participate to this work.

### **-Consent to Publish**

All authors consented to publish this work.

### **-Availability of data and materials**

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

### **-Competing Interests**

The authors declare no competing interest.

### **-Funding**

The company KALLISTEM funded all the experiments described in this Manuscript.

### **-Authors Contributions**

Conceived and designed the experiments: LP, MHP and PD. Performed the experiments: AB, GM and PD. Analyzed the data: AB, GM, MHP and PD. Contributed reagents/materials/analysis tools: LP. Contributed to the writing of the manuscript: MHP and PD.

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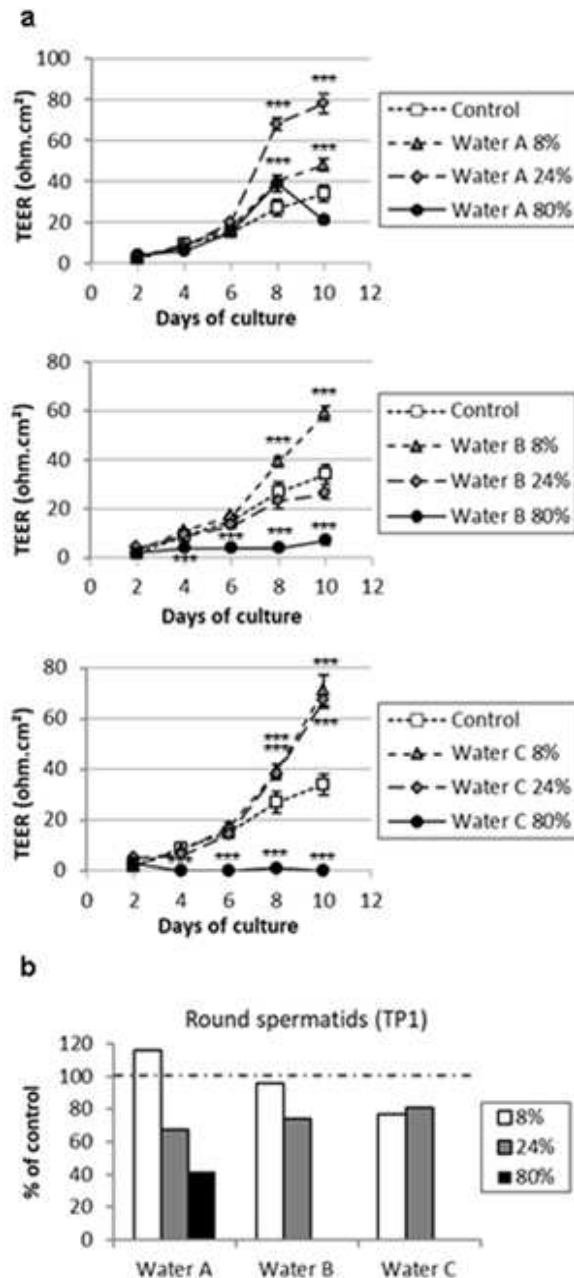
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**Figure 1:** Effects of three hospital effluent waters on (panel a) the TEER of rat seminiferous tubules in culture and on (panel b) the mRNA-related qPCR product levels (% of control) of TP1 (specific of round spermatids) after 10 days of culture.

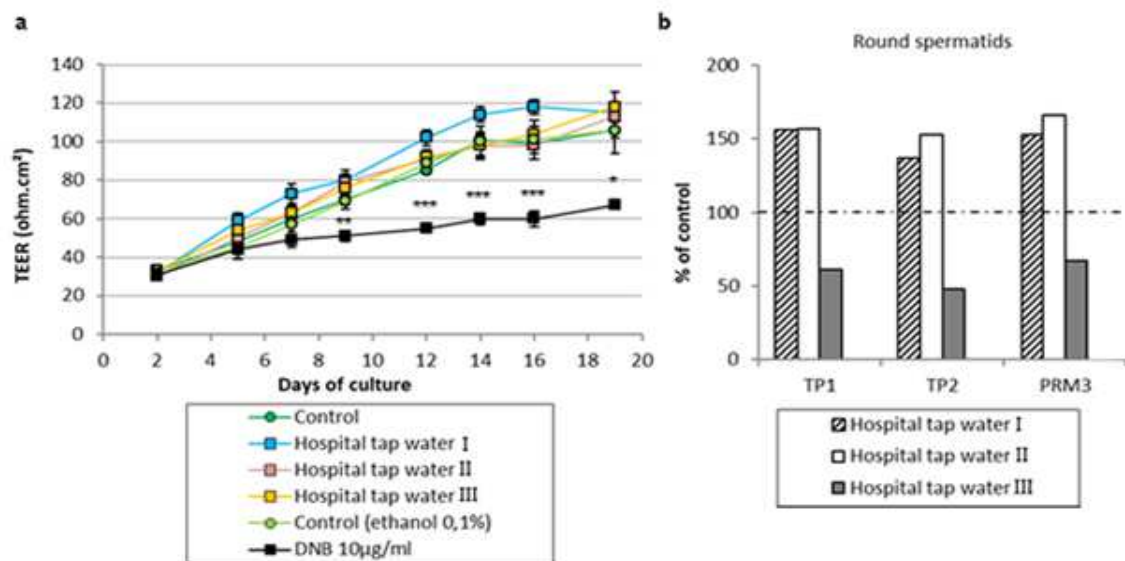
Seminiferous tubule segments were cultured in control medium or in the presence of media made with A, B or C waters at 8%, 24% or 80%.

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

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

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





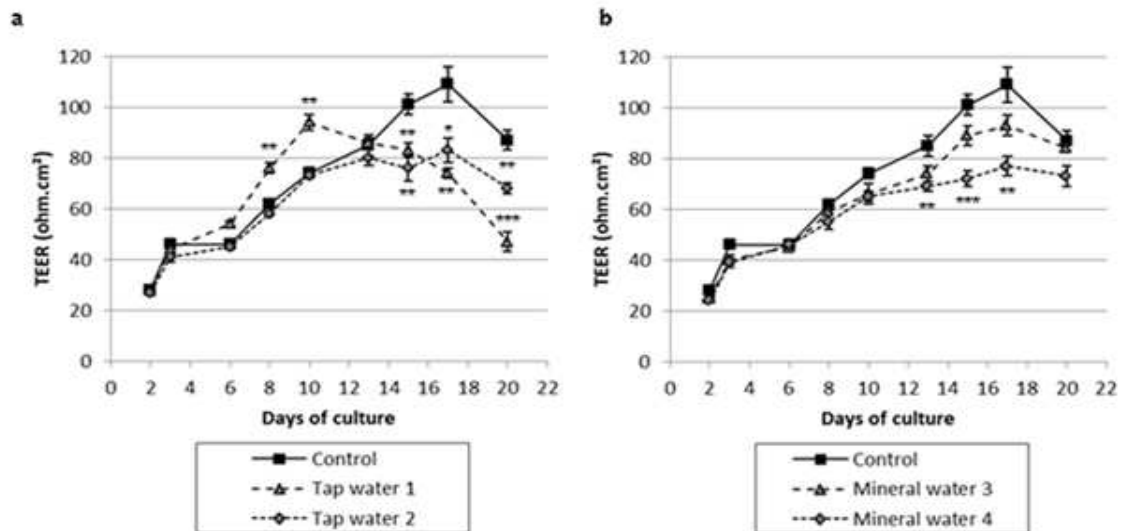
**Figure 2:** Effects of three hospital tap waters on (panel a) the TEER of rat seminiferous tubules in culture and on (panel b) the mRNA-related qPCR product levels (% of control) of TP1, TP2 and PRM3 (specific of round spermatids) after 19 days of culture.

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

(panel a): results are means  $\pm$  SEM of 7 wells per day for each condition.

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

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



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

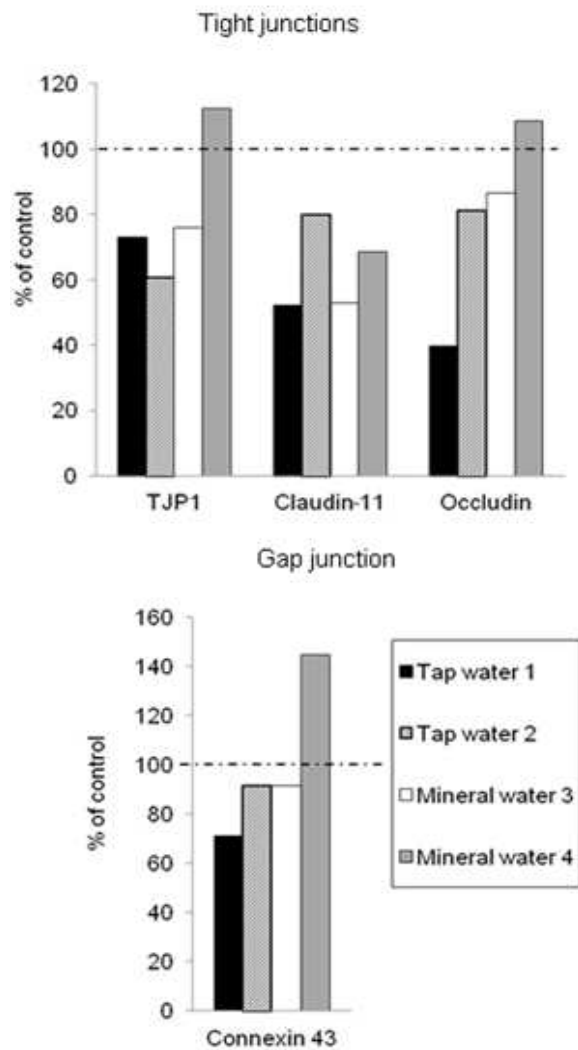
Seminiferous tubule segments were cultured in control medium or in the presence of media made with the tested waters.

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

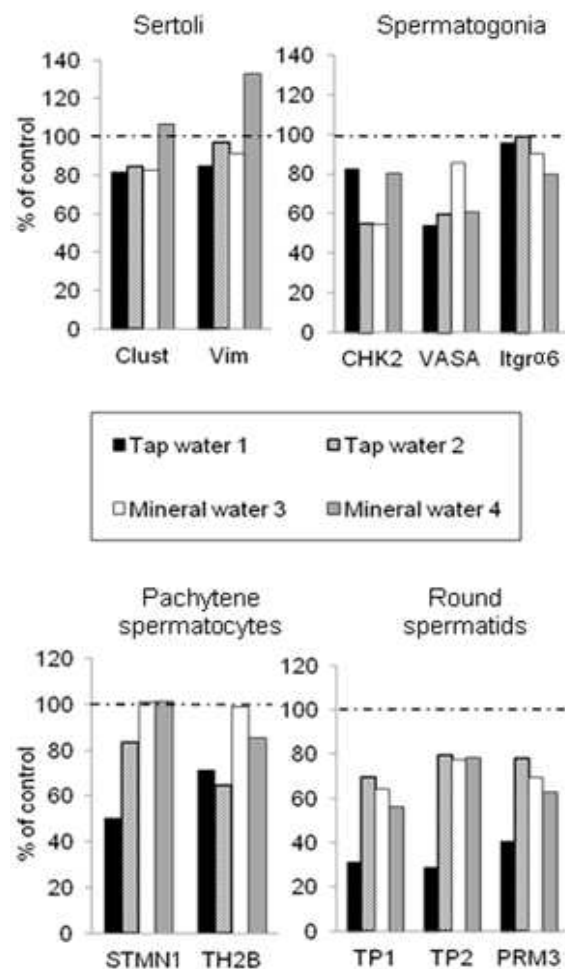
(panel b): mineral water 3, mineral water 4

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

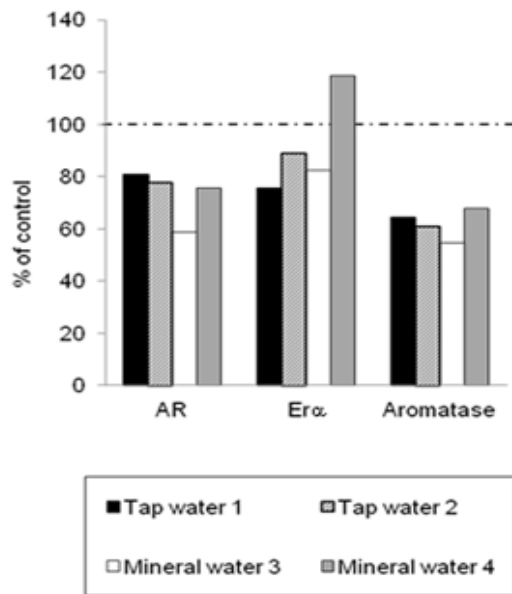
Error bars reflect the standard error of the mean ( $n = 4$ ). \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  by one-way ANOVA.



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



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



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