Ozone treatment prevents the toxicity of an environmental mixture of estrogens on rat fetal testicular development

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Abstract

Effluents from wastewater treatment plants contain a mixture of estrogens (MIX: 17β-estradiol: E2, estrone: E1, estriol: E3 and 17α-ethinylestradiol EE2). High doses of estrogens have been shown to negatively impact fetal testicular development, but the impact of low doses of estrogens in mixture have yet to be elucidated. Using an organ culture system in which embryonic 15.5 day-old rat testes were grown *ex vivo*, we showed that exposure to the MIX at environmentally relevant concentrations reduces testis growth. No effect was observed on testosterone secretion, but we quantified a significant decrease in the number of Sertoli cells and gonocytes because of higher rates of apoptosis. As ozone (O_3) can be used as a disinfectant during wastewater treatment, we confirmed by HPLC–MS analysis that it removes the four parent compounds. Interestingly, the negative effects of the MIX were not observed when testes were exposed to the MIX treated with O_3.

Key words: fetal testis, estrogen, mixtures, low doses, organ culture, ozone, water treatment

Highlights:

- Water treatment with ozone is efficient to remove the mixture of 17β-estradiol, estrone, estriol, 17α-ethinylestradiol
- A mixture of four estrogens at environmental level can negatively impact fetal testis development in rats
- Water treatment with ozone removes the deleterious effects of the mixture of estrogens on fetal testis development

1. Introduction

Concerns about the impact of estrogenic compounds found in the environment on reproductive health have been raised over the last 60 years [1]. The first warning sign came from observations of reproductive disorders in wild animals in regions where accidental exposure to chemical spills have been reported. These effects varied from subtle changes to more permanent effects such as infertility and feminization [2]. In parallel, data from epidemiological studies have shown increases in disorders of human male reproductive function [1]. It has been suggested that such disorders are correlated to the rising environmental concentrations of xenoestrogens [3]. Indeed, hormone-disrupting chemicals may cause non reversible damage during development, a period of rapid cell division and cell differentiation. A small disturbance in early development can have serious consequences in later life. We and others showed that exposure to estrogenic-like molecules can negatively affect testicular fetal development and function both *in vitro* and *in vivo* (reviewed in [4]). Using primary cell cultures and organ cultures, we showed that when exposure occurs early during testis development, high dose of estrogens negatively affect fetal testosterone production and the number of germ cells, Leydig cells, and Sertoli cells [5-7]. Overall, our results demonstrated that there is a specific time during gestation when rat testicular cells are more sensitive to estrogens.

Many of the experimental data demonstrating negative effects of estrogens on testicular development have been generated using single compound at high doses compared to relevant environmental concentrations and mixtures. Indeed, synthetic and natural estrogens are commonly present as complex mixtures and at low doses in the environment. Therefore, it is important to understand the potential interactive effects between such molecules at low dose, to properly assess the environmental risk. Estrogen receptor agonists can exhibit additivity in mixtures [8], such that when estrogenic compounds do not have any impact at low dose on their own, the sum of actions of each estrogenic chemicals may be able to induce deleterious effects. Several studies have demonstrated that effluents from wastewater plants contain estrogenic substances [9, 10]. Male fish exposed both *in situ* as well as under controlled laboratory conditions show induced levels of vitellogenin (VTG), an estrogen-regulated protein, when exposed to wastewater effluent [11]. These estrogenic effects have been shown to be responsible for inhibiting spermatogenesis and are suspected to be responsible for increases in hermaphrodism observed in fish in a variety of aquatic ecosystems [10, 11]. While several substances in treated wastewater have been identified as estrogenic, the presence of naturally occurring female steroid hormones (17β-estradiol (E2); estrone (E1) and estriol (E3)) and synthetic estrogens used in the contraceptive pills (17α-ethinylestradiol: EE2), are thought to be the most prevalent and significant in terms of estrogenic effects [12].

Ozone (O_3) can be used as a disinfectant during wastewater treatment or as a tertiary treatment. It has been demonstrated that it is capable of removing pharmaceutical compounds and steroids [13-15].

Hébert et al. (2008) showed that it is an efficient disinfection strategy to remove the toxicity of municipal effluents on the immune system of the rainbow trout [16]. We previously showed that O_3 can indeed degrade E2 and EE2 but we identified two EE2 transformation products with open phenolic ring structures [6]. The formation of these products is dose and pH dependant. Most importantly, we showed that the removal of estrogenicity after ozonation of EE2 was incomplete and that the transformation products had a negative impact on testosterone secretion by fetal rat testes [6]. These results lead to the conclusion that the disappearance of the parent compound is not a sufficient endpoint, as the transformation products created may be bioactive and exhibit some level of toxicity. Care should be taken about the formation of transformation products when considering such treatment of wastewater. Nevertheless, these previous studies were conducted at concentration of EE2 much higher than those found in the environment and using a single compound.

The aims of the present study were to use the organ culture of rat fetal testis as a bioassay to monitor the toxicity of a mixture of estrogens (MIX: EE2+E2+E3+E1), at concentrations found in wastewater influent and determine if O_3 treatment could be efficient in removing the parent compounds and potential toxicity.

2. Material and methods

2.1- Chemicals and reagents

17β-estradiol (E2) (CAS 50-28-2) and 17α-ethinylestradiol (EE2) (CAS 57-63-6) were purchased from Sigma-Aldrich Canada Ltd (Oakville, Ontario, Canada). Estrone (E1) (CAS 53-16-7) and estriol (E3) (CAS 50-27-1) were purchased from Steraloids (Newport, RI, USA). Stable isotopically labeled surrogates; ^{13}C2-estrone (90%), ^{13}C2-estradiol (99%), and ^{13}C2-ethinylestradiol (99%), were purchased from Cambridge Isotope Laboratories Inc (Tewksbury, MA, USA)) and were used to monitor the recovery of target compounds during sample preparation prior to chemical analysis. Optima liquid chromatography–mass spectrometry (LC–MS) grade acetonitrile and water as well as trace metal grade ammonium hydroxide (88%) were purchased from Fisher Scientific (Ottawa, ON, Canada). Ultrapure water was obtained using a Milli-Q water purification system from Millipore (Bedford, MA, USA). Anhydrous ethyl alcohol was purchased from Commercial Alcohols (Boucherville, QC, Canada).

Concentrated stock solutions of E1, E2, E3 and EE2 were prepared in anhydrous ethyl alcohol (EtOH) and stored in amber glass bottles at −20 °C. Working solutions and calibration standards were prepared by appropriate dilution of stock solutions in 1L of ultrapure water to obtain the selected final concentrations. The 1X MIX concentration listed in Table 1 was based on a review of 115 international publications establishing the average concentrations found in wastewater influent

[12]. Control waters were made by adding the equivalent volume of EtOH (standardized to be 0.01% of the final volume) and O_3 (Supplemental Table 1). Culture media (DMEM/HamF12 without phenol red – Sigma #D2906) was prepared as previously described [6] using water containing the products to be tested.

2.2- Ozonation procedure

Stock O_3 solutions were prepared by sparging O_3 into ultrapure water contained in a gas-washing bottle at room temperature. O_3 was produced using oxygen as feed gas and an O_3 generator (Ozonia TRIOGEN TOGC2) (Leonia, NJ, USA). The dissolved O_3 concentration in the stock ozone solution was determined spectrophotometrically by measuring the absorbance at 258 nm (ε=2950 $M^{-1}cm^{-1}$) [17]. The O_3 stock solution (~ 20 mg/L) was added to the hormone mixture solution of 1 L at an appropriate ratio in order to achieve desired O_3 doses of 50 µg/L and 500 µg/L O_3 for MIX 10X and 100X, respectively.

2.3- Animals and organ culture

Time-mated Sprague-Dawley female rats were obtained from Charles River (St Constant, Quebec, Canada). The animal studies were conducted in accordance with the guidelines set out by the Canadian Council of Animal Care (CCAC) and as reviewed and approved by the Institutional Animal Care and Use Committee of the INRS. Testes were sampled from fetuses obtained on gestation day (GD) 15.5. Organ culture was maintained as previously described [18] on Millicell culture inserts (Millipore PICM01250, Etobicoke, Ontario, Canada) floating on culture media. For each animal, one testis was placed in control media (with or without O_3) and the other was placed in media containing 1X or 10X MIX pre- or post-exposure to O_3 (Supplemental Table 1). The media was changed every 24 hours for 3 days. At the end of the culture, testes were immerged in Bouin fixative for 2h, dehydrated, embedded in paraffin, and cut into 5 µm sections for histological analysis.

2.4- Immunohistochemistry

Every fifth section of the serial sections was mounted on one slide. Immunostaining for the Anti-müllerian Hormone (AMH) (SC-6886, Santa Cruz Biotechnology (Dallas, TX) 1:200 or 1:100 in double staining), PCNA (ab92552, Abcam (Toronto, Ontario, Canada), 1:250), P450 side chain cleavage (ABS235, Millipore, 1:100) or the cleaved-caspase 3 (9664S, Millipore, 1:100) were done. Briefly, sections were incubated with 3% H_2O_2 in distilled water for 10 min to inactivate endogenous peroxidases. The slides were then washed, blocked for 1 hour with 5% normal goat serum or 5% bovine serum albumin (BSA) followed by an overnight incubation with the primary

antibody at 4°C. The primary antibody was detected using biotinylated anti-rabbit secondary antibody and the avidin-biotin-peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) or anti-goat-HRP (SC-2020, Santa Cruz, 1:200). Peroxidase activity was visualized using 3,3-diaminobenzidine as a substrate (DAB Substrate Kit, SK4100, Vector Laboratories) or Vector VIP Substrate (SK4600, Vector Laboratories) for PCNA in double staining with AMH. For all immunohistochemical staining, negative controls were done by omitting the primary antibody.

2.5- Stereology and cell counting

The person doing the following measurements was blinded to the treatments.

Testis and Cord Volume Measurement: The cross sectional areas of the whole testis and seminiferous cords were measured for all sections mounted using the Zen software (Carl Zeiss, blue version). The sums of the areas were multiplied by 5 and the section thickness to obtain an approximation of their volumes.

Sertoli cells and gonocytes numbers: Identification of cell types was based on their nucleus morphology and AMH staining. As previously described in Delbes, Duquenne [5], the number of cells in each section was added up and multiplied by 5 to give the crude count (CC). The Abercrombie formula was applied to correct for double counting of cells that appear in two successive sections. The formula is $TC=CC \times S / (S+D)$, where TC is true count, S is the thickness of the section, and D is the mean diameter for each testis (DM) divided by $\pi/4$ [19]. DM was obtained by measuring 100 random nucleus diameters for each cell type.

Proliferative index: Sertoli cells and gonocytes proliferation indices were obtained after double staining for AMH and PCNA. The proliferation index (percentage of cells showing a clear positive immunoreaction to PCNA) was obtained by counting at least 100 gonocytes or 400 Sertoli cell nuclei from two different sections per sample.

Apoptotic cells: The number of cleaved caspase-3 positive cells per tubule was monitored. The percentage of tubule containing 0, 1 to 2 or more than 2 apoptotic cells was determined on at least 75 tubules from 5 different sections per sample.

2.6- Testosterone assay

Testosterone concentrations were measured in the culture media using a testosterone ELISA kit following the manufacturer's protocol (IBL America #IB79106, Minneapolis, MN). Each sample was run in duplicate.

2.7- Quantification of hormones using HPLC-MS

Concentrations of all hormones were quantified using a Dionex ICS-5000 system coupled to a MSQ Surveyor mass spectrometer (Thermo Scientific). Samples were eluted on a C-18 Hypersil GOLD column (2.1×50 mm, 1.9 μm particle size) maintained at 50 °C. A mixture of 32% acetonitrile in water with 1mM NH_4OH was used for elution (isocratic). An electrospray ionization (ESI) source was used in the negative ionization mode with cone voltage set at 95 V. Probe temperature was set at 500 °C. Flow rate was 0.3 mL/min and injection volume was 25μl. The mass to charge ratios (m/z) of respective molecular ions and retention times of target hormones and their surrogates can be found in Supplemental table 2. The limits of detection (LOD) of the HPLC-MS system for each compound are also included in supplemental Table 2. 10 ml samples were spiked with surrogates and then dried completely using a Thermo Scientific Speed Vac Concentrator (Savant SPD131DDA) coupled with a Refrigerated Vapor Trap (RVT4104) (50 ◦C, 1 Torr at a ramp of 30 Torr/min). 0.25 mL of reverse osmosis water and 0.25 mL of methanol were then added to the dried samples and vortex mixed vigorously in order to reconstitute a final volume of 0.5 ml for HPLC-MS analysis. Considering the concentration factor of 20X following drying and reconstituting, the overall limits of detection of the chemical analysis method were calculated and also tabulated in supplemental Table 2. The monitoring of concentrations of surrogates allowed correcting for any losses of the hormones during sample preparation and matrix effect during chemical analysis. For E3, no surrogate was available to us and the concentration correction calculations for E3 were performed by taking the average of the correction factors of the remaining three compounds.

2.8- Statistical analysis

All values presented are the means ± standard error to the mean (SEM) and each condition was tested on at least 3 organ cultures from different litters. The significance of the difference between the mean values of the treated and untreated testes from the same animal in organ culture was evaluated using the Student's paired *t*-test. To evaluate the impact of O_3 treatment, one-way analysis of variance was used followed by a Tukey post-hoc test.

3. Results

3.1- Removal of estrogenic compounds by O_3

To monitor the impact of estrogens at environmentally relevant concentrations and their removal by O_3 treatment, we modelled a mixture of the most prevalent and investigated steroids found in wastewater (i.e. EE2, E2, E3 and E1). To assess the efficacy of the O_3 treatment in removing the 4 estrogens contained in the MIX, HPLC-MS analysis were conducted. In order to improve ease of analysis, a more concentrated MIX (100X) was treated with an appropriate amount of dissolved O_3 (500 µg/L) and analyzed by HPLC-MS. The results associated to the removal of target estrogenic compounds can be found in Table 2 and Figure 1. As shown in the chromatograms (Figure 1) and the measured concentrations (Table 2), all target compounds were removed below their detection limits. Based on these limits of detection, the removal efficiencies were at least 97.5%.

3.2- Impact on fetal testis development and cell number

To monitor the toxicity of the combination of four estrogens at doses found in environmental waters, the mixture was tested at a dose equivalent to the average concentration measured in wastewater (MIX 1X) and 10 times higher (10X MIX) (Table 1). In order to assess the toxicity of this MIX, we used an organ culture system that replicates *ex vivo* the *in vivo* rat fetal development. Testes were sampled at GD15.5 and the impact of 72h exposure to the 1X or 10X MIX was first evaluated on testicular histology. Immunostaining for the AMH was done to visualize Sertoli cell cytoplasm and seminiferous cord structure (Figure 2A and 2B). Cross sectional areas of the whole testis (Figure 2A) and of seminiferous cords (Figure 2B) were measured for all sections to determine the total testis and seminiferous cords volumes, respectively. Analysis of successive sections showed that all the treatments tested affected neither seminiferous cord organization nor AMH expression in Sertoli cells (supplemental figure 1A-1C). The interstitium volume was quantified as the difference between the volumes of the testis and that of the seminiferous cords. As shown in Figure 2C, the 10X MIX induces a significant decrease in the total testis volume. This decrease is mostly due to a significant decrease in the volume of seminiferous cord whereas, despite a downward trend, no significant change could be measured in the volume of interstitium. Interestingly, this negative effect was not observed when the MIX 10X was treated with O_3.

Figure 2 shows the effect of the mixtures on Sertoli cells and gonocyte numbers. Sertoli cell cytoplasm is positive for AMH staining and their nuclei are small, dark blue stained and located at the base of the seminiferous cord epithelium (red dots in Figure 3A). Gonocyte cytoplasm is negative for AMH and their nuclei are large, lightly stained and spherical, located in the center of the seminiferous cords (green dots on Figure 3A). As shown in Figure 3B, the MIX at 10X significantly decreased the numbers of gonocytes per testis, when compared to controls. It also

tended to decrease the number of Sertoli cells (p=0.053). Interestingly, this negative effect was not observed at 1X or for the MIX 10X treated with O_3. Note that the average diameters of the nuclei of gonocytes and Sertoli cells were not affected by any of the treatments (Supplemental Table 3).

3.3- Impact on cell proliferation and apoptosis

To further understand the negative impact of the MIX at the 10X dose on the number of Sertoli cells and gonocytes, we measured cell proliferation and death (Figure 4). Proliferation was evaluated based on immunostaining for PCNA. Only PCNA-negative gonocytes could be observed at that stage but the percentage of PCNA-positive Sertoli cells was evaluated (Figure 4A). Sertoli cell proliferation was not affected by the 10X MIX (Figure 4C). Furthermore, no change was observed when the testes were exposed to the ozonated 10X MIX (Figure 4C).

To assess apoptosis, we evaluated the number of cleaved-caspase 3-positive cells per tubule (Figure 3B). We showed that the 10X MIX significantly decreased the percentage of tubules that do not contain apoptotic cells. Indeed, it significantly increased the percentage of tubules containing 1 to 3 cleaved-caspased 3-positive cells when compared to controls (Figure 4D). Interestingly that effect was no longer observed when the 10X MIX was treated with O_3 (Figure 4D).

3.4- Impact on testosterone secretion

To test the impact of the MIX before and after ozonation on fetal testosterone secretion, testosterone was measured in the culture media every 24 h for 3 days. We did not observe any significant effect of the MIX at 1X or 10X, either before or after O_3 treatment (Figure 5). Observation of Leydig cells after imunohistochemistry of the P450 side chain cleavage on successive sections did not reveal major changes in the overall distribution and morphology of individual Leydig cells (Supplemental Figure 1D-1E).

4. Discussion

The present data demonstrate that a mixture of estrogens can have significant impact on fetal rat testis development at doses that are relevant to the environment. The mixture of hormones tested was designed to represent an accumulated estrogenicity that can be found in the environment. We chose to use the four most prevalent compounds known to be present in wastewater. Concentrations used were determined based on average concentrations in wastewater influent, as described by Miege et al. [12] in an analysis of 115 international research papers. Importantly, equivalent concentrations have also been reported in effluents of water treatment plants [12] as well as in surface and grounds waters [20]. Moreover, other man-made chemicals known to have estrogenic activities have been detected in wastewaters and in the environment and could contribute to an overall exposure [21]. We are therefore confident that the MIX tested is representative of level of estrogenicity that can be found in the environment.

We tested the impact of such mixture on rat foetal testis development using an organ culture system, which is a powerful model that reproduces the *in vivo* kinetics of development [18]. The present data confirm that the tissue is viable in control conditions as each cell type expressed their specific markers, the proliferation index of Sertoli cells corresponds to what is expected at the corresponding age in vivo and more than 90% of seminiferous cords do not exhibit apoptotic cells. We previously showed that 50µg/L (~170nM) of EE2 negatively affects testosterone secretion in this organ culture system [6]. These results were in agreement with earlier indications that estrogen can affect the expression of steroidogenic enzymes in Leydig cells [22, 23]. In the present study, no effect of the MIX was observed on testosterone secretion, suggesting that the expression of steroidogenic enzymes is not sensitive to such low doses of estrogens. This is in accordance with other studies using a similar culture model and time of sampling, where diethylstibestrol (another potent estrogen agonist) induced a dose dependant decrease in basal testosterone production with no effect detected at 0.4nM [7]. Consistent with this observation, we did not observe a significant impact of any treatment on the volume of the interstitium (the testicular compartment where the Leydig cells are located) or the distribution and morphology of individual Leydig cells. This strongly suggests that the endocrine function of the fetal testis was not affected by such low doses of estrogens.

We report here the significant decrease of the volume of testes after a 72 hours exposure to the MIX at the 10X but not at the 1X dose. Interestingly, this decrease is associated with a significant decline in seminiferous cords' volume that is most probably attributable to the decrease in the total number of both Sertoli cells and gonocytes. Using estrogen receptor β knock-out mice or baboon treated an aromatase inhibitor during late gestation, it has been shown that endogenous E2 can negatively regulate both proliferation and apoptosis in gonocytes ultimately leading to changes in the number of

gonocytes [24, 25]. Similarly, Lasurguere et al. [7] reported that exposure to 4μM of disthylstibestrol can affect the proliferation index of both gonocytes and Sertoli cells as well as induce apoptosis in gonocytes. In the present study, we could not measure the proliferation index of gonocytes as they enter G0 arrest during the culture period [26]. Nonetheless, these results showed that exposure to the 10X MIX did not affect the entrance into the quiescence phase. Sertoli cell proliferation was not affected by the 10X MIX but the treatment induced a significant increase in the average number of apoptotic cells per seminiferous cords. These results strongly suggest that the 10X MIX decreased the overall number of cells per seminiferous cords by inducing apoptosis.

To our knowledge, this study is the first to demonstrate negative effects of such low-doses of estrogens on the development of the mammalian fetal testis *ex vivo*. In fish, it had been shown that 2 ng/L EE2 can cause feminization of males and that 4 ng/L caused abnormal reproductive development [27, 28]. The mixture studied here contains four compounds in different concentrations, known to have various affinities for the estrogen receptors. Indeed, it has been well described that binding affinities of the four tested compounds for the estrogen receptors (ER) are classified in the following order: EE2>E2>E3>E1 [29]. It is important to note that the present experiments were designed to evaluate the impact of the overall estrogenic activity of the MIX and the contribution of each individual compound is impossible to measure. Therefore we cannot conclude if each compound participates to the overall observed effect. Nevertheless, it has been shown by others that estrogens can exhibit additivity [8, 9, 30]. Moreover, Filby et al. [9], suggested that the response to EE2 can be amplified by the presence of a mixture of chemicals. One can therefore hypothesize that the negative effect observed with the 10X MIX is the results of the additive impact of each compounds on the estrogen receptors present in testicular cells. Nevertheless, the understanding of the precise mechanism by which each compound of the 10X MIX induced the overall observed negative effect on the fetal testis development require further experiments. Moreover, there are important limitations of extrapolating the results to *in vivo* situations, as this would require considering actual exposure of a mammal to this sewage outfall and the pharmacokinetics of these estrogens.

O_3 can be used as a disinfectant during wastewater treatment. In the present study, we showed that ozonation treatment is efficient at removing the four target hormones at concentrations below their limit of detection. This is in accordance with what we, and others, have previously shown for the removal of hormones and other drugs [6, 31]. We had previously described that the O_3 treatment of EE2 at higher initial concentration (50 -500 μg/L) leads to the production of 2 transformation products [6]. However, although the same ratio of ozone:target analyte was used in the present study, no transformation products were detected in the treated solutions. This can be explained by the much lower concentration of hormones used, which limits our ability to detect potential transformation products. Nevertheless, the present data demonstrate that all negative effects

observed in organ culture after exposure to the 10X MIX, were eliminated after pre-treatment with O_3. Therefore we can hypothesize that no transformation product were generated or that they were not produced in concentration sufficient to exhibit the effect previously observed [6].

5. Conclusion

Overall, the present data demonstrate that a mixture of four estrogens at concentrations found in the environment can have biological effect as indicated by impact on testis development, demonstrating a potential risk to the receiving environment. We further demonstrate that O_3 treatment is efficient in removing the toxicity of this mixture. Our findings highlight the importance of water treatment strategies but also the need to consider the interactive effects of estrogens and to better estimate the potential health effects of environmental mixtures on biological systems.

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Figure legends:

Figure 1: HPLC-MS Chromatograms showing the disappearance of target compounds before and after ozone treatment. Initial concentrations of hormones were: EE2: 0.42 µg/L; E2: 2.22 µg/L; E1: 6.71 µg/L and E3 : 11 µg/L. Concentration of ozone was 500 µg/L.

Figure 2: Effect of the MIX before or after ozonation on rat fetal testis development. Testis were explanted from 15.5-day-old rat fetuses cultured for 3 days. Cross sectional areas of the whole testis (A) and seminiferous cords (B) were measured for all sections. The interstitium volume was calculated as the difference between the total testis and the seminiferous cords. Quantitative data are expressed as % of the respective control cultures (C) and are represented as the mean ± SEM from 3 to 4 cultures. *$p<0.05$ in the paired statistical comparison with the corresponding control values.

Figure 3: Effect of the MIX before or after ozonation on the total numbers of Sertoli cells and gonocytes in the 15.5-day-old rat fetal testis cultured for 3 days. Sertoli cells (red dots) and Gonocytes (green dots) were counted on histological sections after immunohistochemical detection of AMH (A). Scale bar represents 20µm. Quantitative data are expressed as % of the control cultures (B) and are represented as the mean ± SEM from 3 to 4 cultures. *$p<0.05$ compared with the corresponding control testis using a paired t-test.

Figure 4: Effect of the MIX before or after ozonation on cell proliferation and apoptosis in testes from 15.5 days old rat fetuses in organ culture for 3 days. A: Proliferative Sertoli cells and Gonocytes were identified using PCNA and AMH double staining. Only PCNA-negative gonocytes (asterix) could be observed, but PCNA-positive (arrowhead) and PCNA-negative (arrow) Sertoli cells (positive for AMH) were counted. B: Apoptotic cells were identified as cleaved-caspase 3- positive (arrowheads). C: the percentage of PCNA-positive Sertoli cells was measured in at least 400 cells and is represented as the mean ± SEM from 3 cultures. D: Apoptosis was quantified as the percentage of tubules containing none, 1-3 cells or more cleaved-caspase 3 positive cells in at least 60 tubules per sample (n=6 and 3 for the Mix 10X and the Mix 10X +O3 respectively)

Figure 5: Effect of the MIX before or after ozonation on testosterone secretion from 15.5 days old rat fetal testes in organ culture for 3 days. Media were changed every 24 h and their testosterone content was measured by ELISA. Values (mean ± SEM) are the relative testosterone secretion, expressed as the percentage of the control value from 6 to 9 cultures.

Table 1: Composition of the 1X Mixture (MIX) adapted from [12].

Compounds	Concentration (µg/L)	Concentration (pM)
17α-Ethinylestradiol (EE2)	0.0042	14.2
17β –estradiol (E2)	0.0222	80.8
Estrone (E1)	0.0672	248.6
Estriol (E3)	0.115	398.8

Table 2: Quantification of estrogenic compounds by HPLC-MS analysis

*Calculated based on the method detection limits found in Supplemental Table 2

Compounds	100X theoretical concentration (µg/L) before O_3	100X measured concentration (µg/L)		% removal*
		before O_3	after O_3	
EE2	0.42	0.48 ± 0.02	< LOD	> 97.5%
E2	2.22	2.27 ± 0.10	< LOD	> 99.6%
E1	6.72	6.26 ± 0.13	< LOD	> 99.8%
E3	11.5	10.81 ± 0.36	< LOD	> 99.9%

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