Degradation of 17 α -ethinylestradiol by ozonation – Identification of the by-products and assessment of their estrogenicity and toxicity

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ABSTRACT

The presence of the synthetic estrogen 17 α -ethinylestradiol (EE2) in waters at low levels is a concern due to its ability to act as an endocrine disruptor. Ozone (O₃) is commonly used in water treatment and reacts with EE2 to form by-products having characteristics that are mostly unknown. The aim of this study was to identify the by-products of E2 and EE2 ozonation and determine their estrogenicity and toxicity relative to the parent compound. Ozonation by-products were identified via LC–MS analysis. The estrogenicity was measured using the YES assay, and toxicity was determined by monitoring effects on histology of fetal rat testes and testosterone secretion by these tissues. Two EE2 by-products were identified with open phenolic ring structures (masses 302 and 344 u). The Yeast Estrogen Screening (YES) assay showed a decreased but incomplete removal of estrogenicity after ozonation of EE2. Histological analysis of fetal testes revealed that neither E2 nor EE2, with or without ozonation, had any effect on seminiferous cord formation; however, a remarkable negative effect on testosterone secretion was observed, with EE2 by-products after ozonation showing the most rapid and extensive inhibition. These results show that the removal of EE2 via reaction with O₃ resulted in the formation of by-products that are less estrogenic (as demonstrated by the YES assay), but have a greater negative impact on testosterone secretion. Thus, the disappearance of the parent compound is not a sufficient endpoint, as the by-products created may be more toxic. Care should be taken when implementing oxidation applications such as ozone during waste water treatment.

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1. Introduction

Due to the common use of oral contraceptives (Cavallucci, 2007), their excretion and inefficient removal during conventional wastewater treatment, 17 α -ethinylestradiol (EE2) has been detected in surface waters and wastewater effluents at low levels (up to 62 ng/L) (Cicek et al., 2007; Gomes et al., 2003; Huber et al., 2004; Joss et al., 2005; Kolpin et al., 2002; Lai et al., 2002; Miège et al., 2009; Petrovic et al., 2002; Ternes, 1998; Ternes et al., 1999a, 1999b). EE2 can act as an endocrine disruptor on aquatic organisms and mammals through environmental exposures or the food chain and induce reproductive disorders (Aravindakshan et al., 2004; Lange et al., 2009; Vos et al., 2000). For example, it has been shown that adult African clawed male toads exposed to EE2 (10^{−8} M, ~3 μ g/L) exhibited decreased seminiferous tubule mean diameter and occurrence of small oocytes in the testis, suggesting feminization (Cevasco et al., 2008). The hypothesis that estrogen-like molecules could impair adult male fertility

by acting during gonadal development (Sharpe and Skakkebaek, 1993) is well supported by animal studies. Exposure during development (from gestational day 7 to postnatal day 8) to oral micromolar doses of EE2 permanently disrupted the reproductive tract of male rats (Howdeshell et al., 2008). We have also previously shown that ex vivo exposure of the fetal rat testis to exogenous 17 β -estradiol (E2) or diethylstilbestrol (DES) induced a time and dose dependent negative impact on gonocyte number and testosterone secretion (Delbes et al., 2007). Thus, the presence of estrogenic compounds in surface waters has become a major concern.

Ozone (O₃) is gaining popularity as a disinfectant during wastewater treatment, and it has been demonstrated that it is capable of removing compounds of emerging concern such as EE2 (Esplugas et al., 2007; Huber et al., 2003, 2005; Nakada et al., 2007; Reungoat et al., 2010; Schwarzenbach et al., 2006; Ternes et al., 2003). Since the structure of EE2 contains an aromatic ring with a phenolic group, and O₃ is a selective oxidant toward these moieties (Huber et al., 2004), the reaction between EE2 and O₃ occurs very quickly with rate constants estimated experimentally to range from 1.83 $\times$ 10⁵ to 3.65 $\times$ 10⁹ M^{−1} s^{−1}; this is similar for all estrogens including the naturally occurring E2 (Deborde et al., 2005). Past studies investigating the removal of EE2 by O₃ (54% to >99% removal) and the formation

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of by-products show conflicting results. The by-products' identification and the presence of an intact phenolic ring, thought to provide the estrogenic activity (Huber et al., 2004), are unclear (Guedes Maniero et al., 2008; Lee et al., 2008; Vieira et al., 2010; Zhang et al., 2006). Some studies have also shown that the estrogenic activity of EE2-containing waters is reduced after O₃ application from 13% to less than 1/200th of the original amount (Guedes Maniero et al., 2008; Huber et al., 2004; Lee et al., 2008).

The objectives of the present study are to characterize the reaction of O₃ with E2 and EE2 at different pH, to identify the chemical structure of ozonated by-products and to investigate their estrogenicity and toxicity on rat fetal testes.

2. Materials and methods

2.1. Chemicals and reagents

The estrogens 17 β -estradiol (E2) (CAS 50-28-2, purity 98%) and 17 α -ethinylestradiol (EE2) (CAS 57-63-6, purity 98%) as well as formic acid, sodium hydroxide and ammonium acetate were purchased from Sigma-Aldrich (Oakville, Ontario, Canada). The reagent alcohol ($\geq 90\%$ ethanol), acetone, and acetonitrile (ACN) were purchased from Fisher Scientific (Oakville, Ontario, Canada). Deuterated-EE2 (17 α -ethinylestradiol-2,4,16,16-d₄, product number D-4319) was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada).

The estrogen solutions used were made by diluting concentrated stock solutions (1 g/L E2 or EE2 made in ethanol) in reverse osmosis (RO) water. When necessary, the pH of the solutions was adjusted using formic acid or sodium hydroxide. All solutions used for HPLC analysis were vacuum filtered using 0.2 μ m nylon filters and degassed for 30 min.

Stock O₃ solutions were made by sparging O₃ gas produced by an Ozomax generator and a pure oxygen gas feed through RO water contained in a gas-washing bottle chilled to 2 °C on ice. The dissolved O₃ concentration was determined spectrophotometrically by measuring the absorbance at 258 nm ($\epsilon = 2950 \text{ M}^{-1} \text{ cm}^{-1}$) (Bader and Hoigné, 1981). The O₃ stock solution was added to the E2 and EE2 solutions in order to achieve the desired O₃ dose.

Culture media (DMEM/Hamf12 without phenol red) was prepared using 1 L of water containing the products to be tested (Table 1). Briefly, powder media was dissolved in 1 L of water supplemented with 1.2 g of sodium bicarbonate and the pH was adjusted to 7.2. 200 mL was further sampled and supplemented with 15 mM of Hepes and 0.04 mg/mL of gentamycin, filtered through a 0.22 μ m membrane under vacuum and stored at 4 °C.

2.2. Solid phase extraction (SPE)

The estrogen solutions were concentrated using Bond Elut C18 cartridges (Varian, 500 mg/6 mL) and an automated SPE system (Gilson GX-271 ASPEC). The cartridges were conditioned sequentially with 6 mL each acetone, reagent alcohol, and RO water (pH 7.5), followed immediately by the E2 or EE2 solution being concentrated (10 mL/min). The estrogens were then eluted from the cartridge using 6 mL of reagent grade ethanol. The eluted samples were dried completely in

approximately 2.5 h using a Thermo Scientific Speed Vac Concentrator (Savant SPD131DDA) coupled with a refrigerated vapor trap (RVT4104) (50 °C, 1 Torr, ramp = 30 Torr/min). The dried samples were reconstituted in 20% (v/v) alcohol solution prior to HPLC analysis.

2.3. HPLC analysis

E2, EE2 and the O₃ by-products were determined using an Agilent 1200 HPLC equipped with a Zorbax Eclipse Plus C18 column (150 $\times$ 4.6 mm, 3.5 μ m) and a diode array detector using a detection wavelength of 279 nm. A fraction collector was used to collect samples of by-product peaks separately. The HPLC operating conditions were: mobile phase A = 20 mM ammonium acetate (pH = 2.8); mobile phase B = ACN; gradient = 30% ACN (0–5 min), 50% ACN (5–10 min), 70% ACN (10–15 min); flow rate = 1 mL/min; injection volume = 25 μ L; temperature = 28 °C. The limit of detection (LOD) of the HPLC method was calculated to be 60 μ g/L; however, taking into account the SPE concentration (200 $\times$) of the samples, the overall LOD was 0.3 μ g/L.

2.4. Identification of ozone by-products

The by-products present in the ozonated EE2-containing waters and in the by-product peaks collected as fractions were identified using a LC–MS system composed of an Agilent 1200 LC system equipped with a Zorbax Extend C18 column (50 $\times$ 2.1 mm, 1.8 μ m) and an Agilent 6530 quadrupole-time of flight mass spectrometer (QqTOFMS). The LC operating conditions were: mobile phase A = water; mobile phase B = ACN; ACN gradient = 30% (0–0.5 min), 50% (0.6–1.3 min), 70% (1.4–2.1 min), 97% (2.2–3.2 min), 30% (3.3–10 min); flow rate = 0.5 mL/min; injection volume = 10 μ L. Ionization was performed in the negative mode using an atmospheric pressure chemical ionization (APCI) source. APCI conditions were the following: nitrogen gas (N₂), vaporizer temperature = 350 °C, drying gas (N₂) flow = 8 L/min, nebulizer = 45 psig, fragmentor = 150 V, skimmers = 65 V, capillary voltage = 3500 V and corona needle voltage = 4 μ A. The QqTOFMS was operated in the 4 GHz High Resolution with low mass range mode (1700 m/z). Acquisition was performed in the MS (TOF only) mode at a rate of 8 spectra/s. Data analysis was done using an Agilent MassHunter Qual B.03.01, and the products were verified using a deuterated ethinylestradiol (EE2-d₄).

2.5. Yeast Estrogen Screening (YES) assay

The YES assay was conducted as previously described (Routledge and Sumpter, 1996) with minor modifications. The yeast strain was provided by C. Metcalfe (Trent University, Peterborough, Ontario, Canada) and the 96-well plates were incubated for 4 days before measuring the absorbance at 540 nm with a correction for turbidity at 630 nm (Metcalfe et al., 2001) using a BIO RAD Benchmark Plus equipped with Microplate Manager 5.2.1. The degree of color change induced by successive dilutions of the test chemicals provided a measure of their estrogenic potency. The validity of the assay was confirmed by the response of the reference compound used (E2) that showed a median effective concentration (EC₅₀) of 1.5×10^{-10} M ($n = 13$). This EC₅₀ is within the range of previously published values (Balsiger et al., 2010; Guedes Maniero et al., 2008). Replicates of the test compounds (EE2-containing waters before and after O₃ treatment and the by-product peaks collected as fractions) were evaporated and re-dissolved in ethanol and tested ($n = 6$ to 7) in a range of 12 dilutions (1:2) with a row of ethanol blanks and E2 standards in each plate (Supplemental Fig. 1).

2.6. Organ culture

Sprague Dawley rats were housed on a 12L:12D cycle with food and water provided ad libitum. All animal studies were conducted

Table 1
Summary of experimental conditions for the organ culture.

Control media	Treatment media
EtOH (0.005%)	EE2 50 μ g/L
EtOH (0.005%) + 50 μ g/L O ₃	EE2 50 μ g/L + O ₃ 50 μ g/L
EtOH (0.05%)	EE2 500 μ g/L
EtOH (0.05%) + 500 μ g/L O ₃	EE2 500 μ g/L + O ₃ 500 μ g/L
EtOH (0.005%)	E2 50 μ g/L

in accordance with the guidelines outlined in *A Guide to the Care and Use of Experimental Animals* prepared by the Canadian Council on Animal Care. Virgin Sprague Dawley females were mated with males overnight and considered as pregnant (0.5 dpc) when vaginal sperm was detected the next morning. At 15.5 days of gestation, females were asphyxiated with CO₂ and the uteri horns were removed. Fetuses were removed from the uterus and testes were dissected out. Organ cultures were done as previously described (Livera et al., 2006) in 24 well plates (Corning, NY). One testis of each fetus was placed on a filter floating on 400 µL of control medium while the other one was placed on a filter floating on 400 µL of medium prepared with water containing the factors being tested (Table 1). The media were changed every 24 h and the culture was maintained for 72 h. Media (see description of preparation in Section 2.1) were stored at −20 °C until its use in the testosterone assay.

2.7. Testosterone assay

Testosterone concentrations in the culture media were measured using a testosterone ELISA kit following the manufacturer's protocol (IBL Immuno-Biological Laboratories, Hamburg, Germany). Each sample was run in duplicate.

2.8. Histology-immunohistochemistry

At the end of the culture period, testes were fixed for 2 h in Saint Mary's solution (95% ethanol, 1% acetic acid), embedded in paraffin, and cut into 5 µm sections for histological analysis. Serial sections were mounted on slides, deparaffinized, rehydrated, and stained with Hematoxylin–Eosin. The gonocytes were identified by immunocytochemical detection of HSP90 (BD Biosciences – dilution 1:200) as previously described (Culty, 2009).

2.9. Statistical analysis

All values are shown as means ± SEM. To measure the impact of treatment over time in the organ culture system, we performed a 2-way ANOVA analysis using GraphPad Prism version 5.0 for windows (GraphPad software, San Diego, CA). Post-hoc Bonferroni tests were performed to assess the treatment significance compared to controls at each time point. The YES assay results were analyzed using a 4 parameter logistic model to fit the curves and calculate EC₅₀ values (JMP® 9 statistical software).

3. Results and discussion

3.1. Effect of ozone on 17β-estradiol (E2)

A 50 µg/L E2 solution was ozonated according to the batch ozonation procedure described in Section 2.1 to achieve an applied dose of 50 µg/L O₃. After SPE concentration, the HPLC chromatograms show that no E2 or ozonation by-products were detected at the wavelength of analysis (Fig. 1) suggesting degradation of E2 to a level below the limit of detection (LOD) after O₃ treatment.

3.2. Effect of ozone on 17α-ethinylestradiol (EE2)

Solutions containing 50 and 500 µg/L EE2 were ozonated using 50 and 500 µg/L O₃, respectively, at pH 5.3 (unadjusted RO water). After SPE concentration, the HPLC chromatograms show that the EE2 was undetected (Fig. 2A and B). Two ozonation by-product peaks were detected with HPLC retention times of 4.9 and 5.3 min. The same 2 peaks were consistently detected regardless of EE2 concentration (50 µg/L – Fig. 2A and 500 µg/L – Fig. 2B) suggesting that their formation is not dependent on the initial EE2 concentration that is ozonated. However, the ratio of peak 1:peak 2 was almost 10 times smaller at the lower initial concentration of EE2 (50 µg/L vs. 500 µg/L) which suggests that peak 1 may be secondary to the formation of peak 2. Similar experiments were conducted in EE2 solutions with pH levels adjusted to both low (Fig. 3A – pH 3) and high (Fig. 3B – pH 8) values. The HPLC profiles reveal incomplete removal of EE2 at both pH conditions. Two by-product peaks with similar retention times as described at pH 5.3 were detected, with additional small peaks visible in the chromatogram after O₃ addition at pH 8. These results suggest that the pH plays an important role in the

Fig. 1. Effect of ozone (O₃) on 17β-estradiol (E2) in reverse osmosis water at pH 5.3 as determined by HPLC–DAD analysis (50 µg/L E2 + 50 µg/L O₃). The samples were concentrated by a factor of 200 using solid phase extraction (SPE) prior to HPLC analysis. The E2 HPLC retention time was 8.1 min.

removal of EE2 by O₃. However, it does not affect the formation of the two primary by-product peaks resulting from the reaction of EE2 with ozone.

Interestingly, peak 1 was much smaller at both the low and high pH conditions when compared to the same EE2 and O₃ concentration at pH 5.3 (Fig. 2B). The peak

Fig. 2. Effect of ozone (O₃) on 17α-ethinylestradiol (EE2) in reverse osmosis (RO) water at pH 5.3 as determined by HPLC–DAD analysis. The samples were concentrated by a factor of 200 using solid phase extraction prior to HPLC analysis. The retention times were 4.9 and 5.3 min for the two by-product peaks observed after ozonation; and 9.4 min for EE2. A low concentration of EE2 and O₃ was tested (A: 50 µg/L EE2 + 50 µg/L O₃) as well as a high concentration (B: 500 µg/L EE2 + 500 µg/L O₃).

Fig. 3. Effect of ozone (O₃) on 17 α -ethinylestradiol (EE2) in reverse osmosis water at pH = 3 (A) and pH = 8 (B) as determined by HPLC–DAD analysis (500 µg/L EE2 + 500 µg/L O₃). The samples were concentrated by a factor of 200 using solid phase extraction prior to HPLC analysis. The reaction of EE2 with O₃ resulted in the formation of 2 by-product peaks; the EE2 was not completely removed. The retention times were 4.9 and 5.3 min for the two main by-product peaks observed after ozonation; and 9.4 min for EE2. At the higher pH the recovery of analytes obtained via SPE was not as successful as at pH $\leq$ 6 and there were additional tiny by-product peaks observed on the HPLC chromatogram.

1:peak 2 ratio at both pH 3 and pH 8 was similar to the low EE2 concentration ozonated at pH 5.3 (Fig. 2A) reinforcing the idea that the formation of peak 1 is secondary to peak 2. This may be due to the slower reaction rate of EE2 with O₃ at low pH and the increased formation of hydroxyl radicals (OH $\cdot$) at high pH due to O₃ decomposition. Deborde et al. (2005) demonstrated that EE2 reacts slower with O₃ at pH < 5. Therefore, it is expected that at pH 3 the rate of reaction of EE2 with O₃ will be less than at pH 5.3, potentially explaining why there was EE2 remaining after ozonation at the lower pH. As pH increases it has been shown that autonomous ozone decomposition increases (Buffle et al., 2006; Yao and Haag, 1991; Zhang et al., 2006) resulting in the increased formation of hydroxyl radicals (OH $\cdot$). Since OH $\cdot$ is a stronger oxidant than O₃ but less selective to EE2 (Lee and von Gunten, 2009), this may have caused less EE2 removal at the higher pH condition. At pH 5.3 (where EE2 was removed below the LOD after ozonation), the two by-product peaks were approximately equal in size, whereas at both low and high pH (where there was EE2 remaining after ozonation), peak 1 was much smaller than peak 2. As discussed previously, it can be assumed that the formation of peak 1 is secondary to that of peak 2 and that both are a function of the reaction of EE2 with O₃. Thus, the incomplete removal of EE2 after ozonation at the low and high pH conditions would result in peak 1 to be proportionately smaller than peak 2.

3.3. Identification of ozone by-products of EE2

LC–MS analysis showed that the by-product peaks appear to consist of more than one compound each. Although this mixture of ozonated by-products of EE2 was mostly unresolved, [M–H][–] ions corresponding to two compounds with masses 302.1517 and 344.1628 u, respectively (mass accuracy $\leq$ 2.03 ppm) were identified. Both of the by-products are muconic acid derivatives with open phenolic ring structures (Fig. 4B

Fig. 4. EE2 (A) and two of its ozonated by-products with a mass of 302 u (B) and a mass of 344 u (C) as identified with LC–MS analysis (APCI) and verified with deuterated EE2. It should be noted that the phenolic ring was open after ozonation, suggesting a potential decrease in estrogenic activity.

and C). The deuterated congeners of each were also detected (mass accuracy $\leq$ 4.07 ppm), confirming the identified by-products. The by-product with a mass of 344 u was the only identified compound of the several forming peak 2. The formation of these by-products has been observed recently (in addition to four other compounds) during investigations of the reaction pathways of EE2 with O₃ (Vieira et al., 2010). However, we believe this is the first time that the identities of these two by-products (302 and 344 u) have been confirmed with the simultaneous ozonation of a deuterated EE2 compound. The fate of the phenolic ring in the by-products' structures is vital as a closed ring is thought to be directly linked to the binding of compounds to the estrogen receptor (ER) (Anstead et al., 1997). Thus, a disruption of the phenolic moiety is expected to lower its estrogenicity. Although previous studies have predicted the chemical structures of several by-products of EE2 ozonation, only a few of them conducted the YES assay to investigate the simultaneous change in estrogenicity. These previous studies identified EE2 ozonation by-products with the phenolic moiety intact (suggesting unchanged estrogenic activity); however, their YES assay results showed reduced estrogenicity (Guedes Maniero et al., 2008; Lee et al., 2008).

3.4. Estrogenicity of parent compound and by-products

Using the YES assay, we determined the estrogenic activity of EE2 before and after its reaction with O₃ as well as the estrogenicity of each detected ozone by-product

Fig. 5. Estrogenic dose-response using the YES assay (after 4 days incubation) of EE2 before and after reaction with O₃, as well as individual ozonated by-product peaks collected as fractions (peak 1 and peak 2). Waters after ozonation or containing each peak were concentrated 200 times by solid phase extraction (SPE). Data represent the average corrected absorbance $\pm$ SEM from plate replicates (EE2, n = 13; EE2 and EE2 + O₃, n = 6; peaks, n = 7; EtOH, n = 6).

Fig. 6. Effect of the treatment on testicular development. At the end of the 3 days of culture, testes are immersed in Saint Mary's fixative and embedded for further Hematoxylin–Eosin staining (A–E) or HSP90 immunohistochemistry (F–J). Control tissue (A, F) shows the seminiferous cord organization (A) and the gonocyte specific staining (F). After treatment with 50 µg/L E2 (B, G) or its by-products of ozonation (C, H) and with 500 µg/L EE2 (D, I) or its by-products of ozonation (E, J) the seminiferous cord organization was not affected and the gonocyte staining was maintained. Bars represent 50 µm.

peak. Due to low estrogenicity, it was necessary to concentrate the post-ozonation samples (including the by-product peaks collected as fractions) 200 times via SPE. After 4 days of incubation, all samples were plotted alongside the dose–response curve of the E2 standards and the EtOH blanks against the dilution factor of each initial sample; undiluted samples were placed in the first well of each row and successive dilutions (1:2) were done across the remaining 11 wells in the row (Fig. 5). The EE2 sample (500 µg/L initial concentration) tested shows an estrogenic response; however,

after reaction with 500 µg/L O_3 there was a significant decrease in estrogenicity with a response only detected for the undiluted sample in well 1. Only after the ozonated EE2 sample was concentrated 200 times by SPE was the characteristic sigmoidal dose–response curve observed demonstrating an approximate 1-log reduction in estrogenic response compared to the untreated EE2. This suggests that the estrogenicity of the EE2 solution was not completely removed after ozonation but was greatly reduced (by a factor of approximately 2000), which supports the hypothesis that by-products

Fig. 7. Explants from fetuses on 15.5 days post-conception were cultured on floating Millipore filters for 72 h with medium changed every 24 h. For each fetus, one testis was cultured in control medium (black bars) and the other one in medium supplemented with EE2 (gray bars) at 50 µg/L (A) or 500 µg/L (B) or with the ozonated EE2 at 50 µg/L (C) or 500 µg/L (D). Values are mean ± SEM from 9 cultures. * $p < 0.05$, ** $p < 0.01$ in the Bonferroni post-hoc tests comparing with the corresponding control value.

with open phenolic ring structures have lower affinity for the ER. When the two main ozonated EE2 by-product peaks were concentrated 200 times there was no estrogenic response by peak 1 but a weak response induced by peak 2 that was lower than that of the overall ozonated EE2 sample. This suggests that the compounds forming peak 2 play a larger role than those in peak 1 in the estrogenic activity remaining after the ozonation of water containing EE2. Since the by-product with a MW of $344 \text{ g}\cdot\text{mol}^{-1}$ was the most prominent and only identified compound in peak 2, it can be assumed that this by-product exerted the majority of the remaining estrogenic activity of all the EE2 ozonation by-products formed. The same dilutions of the ozonated samples were tested for antagonism using a $1 \times 10^{-8} \text{ M}$ EE2 standard in each well. No significant antagonistic effect was observed (Supplemental Fig. 2).

3.5. Toxicity of parent compound and by-products

In order to assess the toxicity of the EE2 ozonation by-products, we used an organ culture system that replicates *ex vivo* the in vivo rat fetal development (Livera et al., 2006). This system has previously been used in rats (Chauvigne et al., 2009; Delbes et al., 2007) and humans (Lambrot et al., 2009) to assess the impact of endocrine disruptors on testicular development. Because windows of sensitivity to estrogen exposure during rat fetal testis development have been described previously (Delbes et al., 2007), we chose to investigate the impact of the ozonation by-products in a very sensitive period of development, i.e., at 15.5 dpc.

3.5.1. Impact on testicular development

To test the effect of the various compounds and treatments on testicular development, we cultured 15.5 dpc rat testes for 3 days *ex vivo* in media prepared with waters containing E2 or EE2 before and after ozone treatment at pH 5.3. Testes were fixed at the end of the 3 days of culture and sections were observed under a light microscope after Hematoxylin–Eosin staining (Fig. 6A–E). As previously described (Livera et al., 2006), testes grow *ex vivo* similarly to in vivo when cultured in control media, exhibiting the proper seminiferous cord organization (Fig. 6A). After treatment with $50 \mu\text{g/L}$ of E2 (Fig. 6B) or $500 \mu\text{g/L}$ of EE2 (Fig. 6D), the seminiferous cord organization was not affected, suggesting that the treatments did not perturb global testis development. The same was observed after treatment with ozonated waters containing $50 \mu\text{g/L}$ E2 (Fig. 6C) or $500 \mu\text{g/L}$ EE2 (Fig. 6E). The gonocyte specific staining for HSP90 was observed for the same samples (Fig. 6G–J) and was comparable to the control (Fig. 6F), suggesting that the germ cells were not affected by any treatment. To our knowledge, only in vivo gestational exposures to EE2 have been previously reported to show smaller seminiferous cords and Leydig cell hyperplasia but only after 7 days of exposure to a minimum of 0.02 mg/kg/day in mice (Yasuda et al., 1985).

3.5.2. Impact on steroidogenesis

To test the impact of the various compounds on fetal testosterone secretion, testosterone was measured in the culture media every 24 h for 3 days. Throughout the culture period, testosterone production increased significantly, indicating that the tissues were developing properly (Fig. 7). By comparing testosterone production over time from testes cultured in media prepared with RO water containing EtOH and treated with 0, 50 or $500 \mu\text{g/L}$ of ozone (controls as outlined in Table 1), no significant impact was observed (black control bars in Fig. 7), suggesting that ozonated water alone does not affect fetal steroidogenesis in the organ culture system. It was determined that the presence of $50 \mu\text{g/L}$ of E2 (with and without ozone treatment) did not affect steroidogenesis (data not shown) which is in accordance with previous studies showing a negative impact of E2 only at higher concentrations (10^{-6} M , $\sim 250 \mu\text{g/L}$) (Delbes et al., 2007; Lassuguère et al., 2003).

The impact of the presence of EE2 on testosterone production over time was measured using a 2 way ANOVA analysis. In every condition (Fig. 7), both time and treatment had a significant interaction and effect. Testes from 15.5 dpc fetuses cultured for 3 days in the presence of $50 \mu\text{g/L}$ of EE2 exhibited a small but significant decrease in testosterone production after 48 h (Fig. 7A) that was still observed, but was not significant after 72 h. When the testes were cultured in the presence of $500 \mu\text{g/L}$ of EE2, a time-dependent decrease in testosterone production was observed with a significant reduction after 48 and 72 h (Fig. 7B). Testes exposed to $50 \mu\text{g/L}$ (Fig. 7C) or $500 \mu\text{g/L}$ (Fig. 7D) of ozonated EE2 showed a significant decrease in testosterone production from 24 h to 72 h. It is interesting to note that although testosterone production was not affected by the EE2 treatment at 24 h (Fig. 7A and B), exposure to the ozonated EE2 induced a significant reduction at this and subsequent time points (Fig. 7C and D) suggesting that these products are more toxic than the parent compound. Interestingly, these results show for the first time that exposure to EE2 inhibited fetal testosterone production at a concentration where E2 did not ($50 \mu\text{g/L}$), suggesting a higher inhibitory potency of EE2 compared to E2. More importantly, it was demonstrated that ozonation cannot eliminate the EE2 inhibitory effect on steroidogenesis and that the by-products formed may even negatively impact testosterone production faster. Considering our YES assay results (Section 3.4) suggesting that the ozonated EE2 by-products contained in peak 2 have estrogenic activity, it is hypothesized that these by-products are responsible for binding to the ER present in fetal Leydig cells in 15.5 dpc rats (Fisher et al., 1997). The estrogenicity of both peaks being significantly reduced compared to EE2, the toxicity of the by-products may be in part due to action through ERs but other mechanisms need to be investigated. It should be noted that a significant dose dependent effect after ozonation of EE2 was not observed. At the low dose tested ($50 \mu\text{g/L}$ EE2 + O_3), the production of peak 2

was already at a maximum compared to the higher dose ($500 \mu\text{g/L}$ EE2 + O_3) suggesting that peak 1 played a comparatively minimal role in the toxicity of the by-products.

4. Conclusions

Overall, this study illustrates that the removal of EE2 below the LOD via reaction with ozone results in the formation of by-products possessing less estrogenic activity (as assessed by the YES assay) than the parent compound. However, this cannot be used as an indicator of toxicity, as the ozonated EE2 demonstrated a greater negative impact on testosterone secretion by fetal rat testes. Thus, when implementing ozone as a water treatment strategy for removal of recalcitrant compounds such as EE2, the disappearance of the parent compound should not be the sole concern. The by-products created and their characteristics relative to the parent compound must also be considered.

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