2-hydroxyoestradiol and 2-methoxyoestradiol, two endogenous oestradiol metabolites, induce DNA fragmentation in Sertoli cells

C. Valencia¹, C. Molina¹, M. Florez¹, J. Buñay², R. D. Moreno², P. A. Orihuela³, A. Castro¹ & A. Parada-Bustamante¹

¹ Instituto de Investigaciones Materno Infantil, Facultad de Medicina, Universidad de Chile, Santiago, Chile; ² Departamento de Fisiología, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago, Chile; ³ Laboratorio de Inmunología de la Reproducción y CEDENNA, Universidad de Santiago de Chile, Santiago, Chile

Keywords
Apoptosis—estradiol metabolites—tests

Correspondence
Alexis Parada-Bustamante, Instituto de Investigaciones Materno Infantil, Facultad de Medicina, Universidad de Chile, Avenida Santa Rosa 1234, segundo piso, Código postal 8360160, Santiago, Chile. Tel.: (56-2)29770869; Fax: (56-2)24247240; E-mail: aparadab@med.uchile.cl

The first two authors contributed equally to this work.

Accepted: February 3, 2016
doi: 10.1111/and.12576

Summary
Elevated intratesticular levels of hydroxyoestradiols and methoxyoestradiols, two classes of endogenous oestradiol metabolites, have been associated with male infertility. The aim of this study was to explore the effects of 2-hydroxyoestradiol (2OHE₂), 4-hydroxyoestradiol (4OHE₂), 2-methoxyoestradiol (2ME₂) and 4-methoxyoestradiol (4ME₂) on Sertoli cell viability. For this, TM4 cells were incubated with different concentrations of these metabolites for 24 h to then evaluate the viability and DNA integrity by MTS and TUNEL assay respectively. The participation of classical oestrogen receptors and the involvement of oxidative stress and apoptotic mechanisms were also evaluated co-incubating TM4 cells with these estradiol metabolites and with the drugs ICI182780, N-acetylcysteine and Z-VAD-FMK respectively. Only high concentrations of 2OHE₂ and 2ME₂ decreased cell viability inducing DNA fragmentation. In addition, ICI182780 did not block the effect of 2OHE₂ and 2ME₂, while N-Acetylcysteine and Z-VAD-FMK only blocked the effect of 2OHE₂. Moreover, 2OHE₂ but not 2ME₂ induced PARP and caspase-3 cleavage. Finally, lower 2OHE₂ and 2ME₂ concentrations (0.01–0.1–1.0 μmol l⁻¹) decreased Sertoli cell viability 48 h post-treatment. Our results support the hypothesis that elevated intratesticular 2OHE₂ or 2ME₂ concentrations could be related to male infertility since 2OHE₂ by apoptosis and 2ME₂ by undetermined mechanisms induce DNA fragmentation in Sertoli cells.

Introduction
Oestradiol (E₂) is a steroidal hormone that is metabolised to hydroxyoestradiols and methoxyoestradiols in several organs, including liver, placenta and prostate (Berg & Kuss, 1987; Tsuchiya et al., 2005; Joubert et al., 2009; Mosli et al., 2012). E₂ is mainly hydroxylated in position 2 by the enzyme Cytochrome P450 isomerase 1A1 (CYP1A1) which generates 2-hydroxyoestradiol (2OHE₂), and also in position 4 by the isomerase CYP1B1 which generates 4-hydroxyoestradiol (4OHE₂) (Dawling et al., 2004). Subsequently, the enzyme Catechol-O-Methyltransferase (COMT) replaces the hydroxyl group by a methyl group to produce 2-methoxyoestradiol (2ME₂) from 2OHE₂ and 4-methoxyoestradiol (4ME₂) from 4OHE₂ (Dawling et al., 2004).

In recent years, it has been demonstrated that 2OHE₂, 4OHE₂, 2ME₂ and 4ME₂ are not inactive metabolites, but rather participants in various physiological processes (Parada-Bustamante et al., 2013, 2015). For instance, 2ME₂ modulates angiogenesis in endothelial and granulosa cells regulating the expression of endothelin and VEGF, respectively (Dubey et al., 2001; Kohen et al., 2013), while 2OHE₂ promotes the resumption of oocyte meiosis modulating the activity of the G protein-coupled oestrogen receptor 1 (Chourasia et al., 2015). On the other hand, deregulated hydroxyoestradiol and methoxyoestradiol generation is related to the aetiology of various diseases, such as pre-eclampsia where a decreased 2ME₂ production is associated with an altered expression of hypoxia inducible factor-1α and a poor trophoblastic invasion (Perez-Sepulveda et al., 2013) and also with...
prostate cancer, since high concentrations of hydroxyoestradiols induce the expression of cyclin D1 and c-myc in prostate epithelial cells which promote proliferation and malignant transformation of these cells (Mosli et al., 2012, 2013). Moreover, high 2OHE₂ and 2ME₂ concentrations induce cell death of osteoclasts (Maran et al., 2006), chondrocytes (Sibonga et al., 2002) and normal mammary cells (Hurh et al., 2004).

In the mammalian testis, E₂ is normally produced from Testosterone by the Aromatase enzymatic complex, which is expressed in Leydig, Sertoli and germ cells (Lardone et al., 2010). E₂ plays a pivotal role in spermatogenesis exerting several of their effects directly on Sertoli cells (Lucas et al., 2011), which provide the physical and nutritional support for developing germ cells. For instance, E₂ regulates in these cells the expression of proteins that regulate energy metabolism (Alves et al., 2012; Martins et al., 2013), ionic homoeostasis (Bernardino et al., 2015) and cell survival (Simoes et al., 2013). However; an increased local or systemic concentration of this hormone or its derivatives induces deleterious effects in testis, affecting the function and viability of Sertoli and germ cells by mechanisms not completely elucidated (Walczak-Jedrzejowska et al., 2008, 2013; Vigueras-Villasenor et al., 2009).

According to previous studies, hydroxyoestradiols and methoxyoestradiols are normally produced in the mammalian testis from E₂ since CYP1A1, CYP1B1 and COMT are expressed in germ and somatic testicular cells (Revel et al., 1996). On the other hand, it has been hypothesised that local or systemic concentration of this hormone or its derivatives induces deleterious effects in testis, affecting the function and viability of Sertoli and germ cells by mechanisms not completely elucidated (Walczak-Jedrzejowska et al., 2008, 2013; Vigueras-Villasenor et al., 2009).

Effects of hydroxyoestradiols and methoxyoestradiols on TM4 cell viability

TM4 cells (0.5 × 10⁵) were grown on 96-well assay plates. When they reached a confluence of 70%, they were serum-deprived for 24 h and then treated with different concentrations (0.0001–20 μmol l⁻¹) of 2ME₂, 4OHE₂, 4ME₂ (Steraloids, Newport, RI, USA), 2OHE₂ (Sigma-Aldrich, Saint Louis, MO, USA) or vehicle (EtOH 0.001%). We selected this broad range of concentrations because the intratesticular levels of these metabolites are unknown and because concentrations included in this range have been often used in studies that explored the effects of these metabolites on the viability of different cell types (Chen et al., 2004; Hurh et al., 2004; Kato et al., 2008). Twenty-four or 48 h post-treatment with E₂ metabolites, 20 μl of MTS reagent provided by the kit CellTitre 96® AQueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) was added to each well. After 30 min of incubation, the absorbance value at 490 nm was obtained using an ELISA plate reader (BioRad, Hercules, CA, USA).

Materials and methods

TM4 cell culture

The TM4 cell line was purchased from the American Tissue Culture Collection (VA, USA). Cells were cultured in growth medium DMEM/F12 (Life Technologies, NY, USA) supplemented with 10% foetal bovine serum (Life Technologies, NY, USA) and a ready-to-use mix of amphotericin B, penicillin and streptomycin (Life Technologies). Next, cells were serum-deprived for 24 h and then incubated with E₂ metabolites and/or the different drugs for 24 or 48 h in DMEM/F12 serum-deprived medium as is indicated below.

Effects of 2OHE₂ and 2ME₂ on DNA integrity in TM4 cells

TM4 cells (3 × 10⁴) were attached to coverslips covered with poly-L-lysine. Then, these cells were serum-deprived for 24 h and subsequently treated with 20 μmol l⁻¹ 2OHE₂, 20 μmol l⁻¹ 2ME₂ or vehicle for 24 h. After treatment, cells were fixed with 4% paraformaldehyde (PFA) for 10 min at room temperature. A commercial TUNEL assay was used to determine the extent of DNA fragmentation following the manufacturer’s instructions (In Situ Cell Death Detection Kit; Fluorescein, Roche Diagnostic Inc, Mannheim, Germany). Finally, the samples were counterstained with propidium iodide, mounted with Vectashield (Vector Laboratories. Inc. Burlingame, CA, USA) and observed under an epifluorescence microscope (Olympus BX-51TF; Olympus Optical Co. Ltd, Tokyo, Japan). The images were obtained using a CoolSNAP-Pro digital camera (Media Cybernetics). The TUNEL-positive cells in random fields were quantified as a percentage with respect to the total
number of Sertoli cells in the field. At least 50 cells were counted each time.

Participation of a classical oestrogen receptor, oxidative stress and caspases activation in the deleterious effects exerted by 2OHE2 and 2ME2 in TM4 cells

TM4 cells (0.5 × 10^4) were grown on 96-well assay plates. When they reached a confluence of 70%, they were serum-deprived for 24 h and then were co-treated with 20 μmol l^-1 2OHE2 or 2ME2 plus 20 μmol l^-1 ICI182780 (Tocris, Ellisville, MO, USA), an oestrogen receptor antagonist; with 3 mmol l^-1 (Tocris, Ellisville, MO, USA), an oestrogen receptor antagonist; with 3 mmol l^-1 N-acetylcysteine (Sigma-Aldrich), a potent antioxidant; or with 20 μmol l^-1 Z-VAD-FMK, a pan-caspase inhibitor (Kamiya Biomedical, Seattle, WA, USA). The doses of each compound were chosen according to previous reports, where these doses of ICI182780, NAC and Z-VAD-FMK are sufficient to block the estradiol effects mediated by a classical oestrogen receptor (Levenson et al., 1998), the production of reactive oxygen species (Park et al., 2009) and the activation of caspases (Yamashita et al., 1999) respectively. Twenty-four hour post-treatment, cell viability was evaluated by MTS assay as previously described.

PARP and Caspase-3 activation by 2OHE2 and 2ME2 in TM4 cells

TM4 cells (2.5 × 10^5) previously treated with 20 μmol l^-1 2OHE2 or 2ME2 for 24 h were incubated with a lysis solution containing Hepes (50 mm, pH 7.5), NaCl 150 mm, MgCl₂ 1.5 mm, EGTA 1 mm, Glycerol 10% (v/v) and Triton X-100 1% (v/v), supplemented with a commercial protease Inhibitor Cocktail (Roche). Cell extracts were sonicated twice for 5 s and then centrifuged at 10 000 g for 10 min at 4 °C. Supernatant was recovered and stored at −20 °C until its use.

Proteins (30 μg) were separated on 10–12% SDS-PAGE slab gels in a Mini PROTEAN electrophoretic chamber (Bio-Rad). Proteins resolved in the gels were electrophoretically transferred onto nitrocellulose membranes (Bio-Rad). The membranes were incubated with blocking solution (TWEEN-20 0.1% in TBS that contained 5% nonfat dry milk) for 1 h and then incubated with antibodies anti-PARP (diluted at 1 : 1000 (v/v); 9542, Cell signalling, Danvers, MA, USA), anti-Cleaved caspase-3 (diluted at 1 : 1000 (v/v); 9661, Cell signalling) or anti-GAPDH (sc-25778; Santa Cruz, Santa Cruz, CA, USA, at 0.14 μg ml^-1) in blocking solution in a humidified chamber overnight at 4 °C. The blots were rinsed five times for 5 min each in PBS and incubated for 1 h in Tween-20 0.1% in PBS that contained horseradish peroxidase (HRP)-conjugated goat anti-rabbit (diluted at 1:5000 (v/v); 31460; Thermo Scientific, IL, USA). HRP activity was detected using enhanced chemiluminescent substrate Western Blotting Substrate (Thermo Scientific, Waltham, MA, USA).

Hydrogen peroxide production induced by 2OHE2 and 2ME2 in TM4 cells

Possible H₂O₂ generation induced by 2OHE2 or 2ME2 in TM4 cells was determined using the Amplex® Red Hydrogen Peroxide/Peroxidase Assay Kit (Life Technologies) according to the protocol previously described by (Fussell et al., 2011). Briefly, TM4 cells were trypsinised, resuspended in PBS (1 × 10⁶ cells) and disrupted on ice. Then, the cell extract was centrifuged at 10 000 g for 10 min at 4 °C and the supernatant was recovered. An amount of 50 μg ml^-1 cell lysates protein was incubated with NaCl 70 mmol l^-1 in potassium phosphate buffer (30 mmol l^-1, pH 7.8) supplemented with 100 μmol l^-1 NADPH, 100 μmol l^-1 Amplex red, 1 U ml^-1 horseradish peroxidase and 2OHE2 20 μmol l^-1, 2ME2 20 μmol l^-1 or vehicle (EtOH 0.001%) in a final volume of 100 μl in 96-well black microtitre plates at 37 °C. The reaction product, resorufin, was recorded at 540 nm in different times (0,10, 20 and 30 min) using an ELISA plate reader (BioRad). A standard curve with cell extracts incubated with known concentrations of H₂O₂ was also prepared.

Evaluation of cell death by necrosis in TM4 cells treated with 2ME2

In order to evaluate whether 2ME2 decreases TM4 cell viability inducing necrosis, we determined Lactate Dehydrogenase enzymatic activity in culture medium from TM4 cells (2 × 10⁵) treated with 20 μmol l^-1 2ME2 or vehicle (EtOH 0.001%) for 24 h, using the LDH-Cytotoxicity assay kit II (Biovision, Milpitas, CA, USA) according to manufacturer’s instructions. This experiment was performed four times in triplicate.

Human Sertoli cell culture

In order to explore whether the deleterious effects induced by 2OHE2 and 2ME2 in TM4 cells were not only a cell line or species-specific effect, we explored if these estradiol metabolites alter the viability and DNA integrity of human Sertoli cells. For this, one vial of human primary Sertoli cells (hSEC) was purchased from Lonza Group Ltd. (Walkersville, MD, USA). These cells were obtained from 12 years African–American male donor. Cells were propagated and subcultured according to the instructions supplied by the manufacturer. Briefly, cryopreserved cells (716 000 cells ml^-1) were thawed in a 37 °C water bath and transferred immediately to five T-
225 flasks. Cells were propagated in Sertoli Cell Basal Medium (Lonza Group Ltd) supplemented with 10% foetal bovine serum (Life Technologies) and a ready-to-use mix of penicillin and streptomycin (Lonza Group Ltd) and then incubated at 37 °C in a CO₂-incubator with 5% CO₂ in a humidified atmosphere. The growth medium was changed the next day and then every 3 days. To subcultures, cells about to reach 70–80% confluence were rinsed with PBS and then incubated with 18 ml of Verse-ne™ at 37 °C for 5 min and then with a trypsin (0.05%)-EDTA (0.02%) solution for 2 min. Then, Sertoli cells were resuspended in medium and were collected (220 g for 5 min at room temperature). 1 × 10⁵ Sertoli cells were plated in coverslips covered with poly-L-lysine in order to perform TUNEL assay and 0.5 × 10⁴ Sertoli cells were plated in 96-well dishes to perform MTS assay. These assays were performed in parallel. When cells reached 60% of confluence (an average of 7 days were necessary to reach this confluence), they were serum-deprived for 24 h and then incubated with 20 μM 2OHE₂ and 2ME₂ for 24 h to then perform MTS and TUNEL assays as previously described. The cells used for all the experiments reported herein (three replicates in each experiment) were from the fourth, fifth or sixth passage to ensure reproducibility.

Statistical analyses
The results are presented as mean ± SE. Differences among groups were evaluated by Kruskal–Wallis test, followed by a Mann–Whitney test using GRAPHPAD PRISM® software version 5.03 (GraphPad Software, Inc. La Jolla, CA, USA). P-values <0.05 were considered statistically significant.

Results

2OHE₂ and 2ME₂ decrease Sertoli cell viability 24 h post-treatment
In order to explore the in vitro effects of 2OHE₂, 2ME₂, 4OHE₂ or 4ME₂ on Sertoli cell viability, mouse TM4 cells were treated with different concentrations of these metabolites (0.0001–20 μmol l⁻¹) and viability was assayed 24 h post-treatment (Fig. 1). Results showed that cell viability was significantly decreased by 2OHE₂ 20 μmol l⁻¹ (43.6 ± 5.1%), 2ME₂ 10 μmol l⁻¹ (41.5 ± 2.4%) and 2ME₂ 20 μmol l⁻¹ (39.3 ± 2.8%). Interestingly, 4OHE₂ and 4ME₂ did not alter cell viability at any tested concentrations (Fig. 1c,d).

Oestregeron receptor-independent mechanisms mediate the deleterious effects of 2OHE₂ and 2ME₂ in Sertoli cells
To determine whether the deleterious effects of 2OHE₂ and 2ME₂ require a functional oestrogen receptor, TM4 cells were treated for 24 h with 2OHE₂ or 2ME₂ and concomitantly with ICI182780, a specific oestrogen receptor antagonist (Robertson, 2001). The effects of 2OHE₂ and 2ME₂ on Sertoli cell viability were not blocked when these metabolites were co-incubated with ICI182780 (Fig. 2). Thus, the deleterious effects of 2OHE₂ and 2ME₂...
Fig. 2. Effects of 2OHE2 and 2ME2 are not mediated by the classical oestrogen receptor. TM4 cells were treated for 24 h with 2OHE2, 2ME2 or vehicle and concomitantly with the oestrogen receptor antagonist ICI182780 (ICI) to then evaluate cell viability by MTS assay. The effect of ICI on 2OHE2 (a) and 2ME2 (b) is separately shown. *P < 0.05 compared with the vehicle group; Mann–Whitney test.

Fig. 3. 2OHE2 and 2ME2 induce DNA fragmentation in TM4 Sertoli cells. TM4 cells were treated for 24 h with 2OHE2, 2ME2 or vehicle to evaluate DNA fragmentation by TUNEL Assay (a). Nuclei were counterstained with Propidium Iodide (PI). The percentage of cells with fragmented DNA in relation to total cells is presented as the mean ± standard error of a total of eight experiments (b). *P < 0.05 compared to vehicle group; Mann–Whitney test.
Fig. 4 2OHE2, but not 2ME2, induces apoptosis in TM4 Sertoli cells by a caspase-dependent apoptotic mechanism. TM4 cells were treated for 24 h with 2OHE2, 2ME2 or vehicle and then total protein was isolated to evaluate cleavage of caspase-3 (a) and PARP (b) by western blot. Besides, TM4 cells were treated for 24 h with 2OHE2, 2ME2 or vehicle (Veh) and concomitantly with the pan-caspase inhibitor Z-VAD-FMK (Z-VAD) to then evaluate cell viability by MTS assay (c). *P < 0.05 compared with the vehicle group; Mann–Whitney test.

Fig. 5 Oxidative stress mediates 2OHE2 deleterious effects on TM4 Sertoli cell viability. TM4 cells were treated for 24 h with 2OHE2, 2ME2 or vehicle and concomitantly with the antioxidant N-AcetylCysteine (NAC) to then evaluate cell viability by MTS assay (a). The effect of NAC on 2OHE2 (a) and 2ME2 (b) is separately shown. Besides, H2O2 generation was measured in lysates protein obtained from TM4 cells treated with 2OHE2 and 2ME2 for different times (c). *P < 0.05 compared with the vehicle group; Mann–Whitney test.
2OHE2 and 2ME2 induce DNA fragmentation in Sertoli cells, but only the effect of 2OHE2 is mediated by a caspase-dependent apoptotic mechanism

To explore the mechanisms by which 2OHE2 and 2ME2 decrease Sertoli cell viability, TM4 cells were incubated with 2OHE2 20 μmol l⁻¹, 2ME2 20 μmol l⁻¹ or vehicle for 24 h and then DNA fragmentation was evaluated by TUNEL assay (Fig. 3). Compared to cells treated with vehicle, the percentage of positive TUNEL cells was significantly increased in TM4 cells treated with 2OHE2 (86.6 ± 4.6%) and 2ME2 (99.3 ± 0.3%). Then, we evaluated whether 2OHE2 and 2ME2 induced PARP and caspase-3 cleavage. We found that 2OHE2, but not 2ME2, induced caspase-3 and PARP cleavage in TM4 cells (Fig. 4a,b). Interestingly, the effect of 2OHE2 on Sertoli cell viability was completely prevented when cells were co-incubated with the cell-permeant pan-caspase inhibitor Z-VAD-FMK (Fig. 4c). However, this inhibitor was unable to prevent the effect of 2ME2 (Fig. 4c).

Oxidative stress mediates the effect of 2OHE2 on Sertoli cell viability

To determine whether the deleterious effects of 2OHE2 and 2ME2 are mediated by an oxidative damage, we evaluated the effect of N-AcetylCysteine (NAC), a well-known free radical scavenger (Samuni et al., 2013). Results showed that NAC 3 mmol l⁻¹ prevented the effect of 2OHE2 (Fig. 5a), but it did not prevent the effect of 2ME2 (Fig. 5b). In order to corroborate this result, TM4 cells were treated with 2OHE2 or 2ME2 and generation of hydrogen peroxide (H₂O₂) was evaluated. Results showed that 2OHE2, but not 2ME2, increased H₂O₂ generation in TM4 cells (Fig. 5c).

2ME2 does not induce necrosis in Sertoli cells

In order to determine whether 2ME2 decreases Sertoli cell viability activating a necrotic process, Lactate Dehydrogenase (LDH) activity was evaluated in culture medium from TM4 cells treated with 2ME2 or vehicle. LDH activity was not significantly different between the cells treated with 2ME2 or vehicle (Fig. 6).

2OHE2 and 2ME2 decrease the viability of human Sertoli cells inducing DNA fragmentation

To determine whether the deleterious effects of 2OHE2 and 2ME2 observed in TM4 cells are replicated in human Sertoli cells, we used a commercially available human primary Sertoli cell (hSEC). 2OHE2 20 μmol l⁻¹ (54.2 ± 5.2%, P < 0.05) and 2ME2 20 μmol l⁻¹ (76.2 ± 3.3%, P < 0.05) significantly decreased the viability of these cells 24 h post-treatment evaluated by MTS assay (Fig. 7a). To explore whether 2OHE2 and 2ME2 decreased the viability of these cells inducing DNA fragmentation, hSEC cells were incubated with 2OHE2 20 μmol l⁻¹, 2ME2 20 μmol l⁻¹ or vehicle for 24 h to then evaluate DNA integrity by TUNEL assay (Fig. 7b,c). Compared to cells treated with vehicle, the percentage of positive TUNEL cells was significantly increased in hSECs cells treated with 2OHE2 (99.7 ± 0.2) and 2ME2 (76.5 ± 9.6%).

Lower concentrations of 2OHE2 and 2ME2 decrease Sertoli cell viability 48 h post-treatment

In order to explore whether lower doses of 2OHE2 and 2ME2 decrease Sertoli cell viability when the cells are exposed to these estradiol metabolites for a longer time, TM4 cells were incubated with different doses of 2OHE2 and 2ME2 (0.0001–20 μmol l⁻¹) and cell viability assayed 48 h post-treatment by MTS assay (Fig. 7). Results showed that cell viability was significantly decreased even by 2OHE2 0.01 μmol l⁻¹ (57.4 ± 3.0%), 2OHE2 0.1 μmol l⁻¹ (62.1 ± 8.1%), 2OHE2 1.0 μmol l⁻¹ (59.5 ± 1.3%), 2ME2 0.01 μmol l⁻¹ (64.6 ± 6.1%), 2ME2 0.1 μmol l⁻¹ (48.3 ± 1.6%) and 2ME2 1.0 μmol l⁻¹ (56.1 ± 5.2%).

Discussion

Although 2OHE2 and 2ME2 concentrations in the mammalian testis have not been experimentally determined, studies indicate that 2OHE2 and 2ME2 are normally
Estradiol metabolites on Sertoli cells

Fig. 7 2OHE₂ and 2ME₂ also decrease the viability of human Sertoli cells inducing DNA fragmentation. hSECs cells were treated for 24 h with 2OHE₂, 2ME₂ or vehicle and then cell viability was evaluated by MTS assay (a). Besides, other group of hSEC cells were also treated for 24 h with 2OHE₂, 2ME₂ or vehicle to evaluate DNA fragmentation by TUNEL Assay (b). Nuclei were counterstained with Propidium Iodide (PI). The percentage of cells with fragmented DNA in relation to total cells is presented as the mean ± standard error of a total of three experiments (c). *P < 0.05 compared to vehicle group; Mann-Whitney test.

generated from E₂ in this organ, with similar efficiency and kinetic parameters as those reported for the ovary (Axelrod & Goldzieher, 1962; Watanabe & Yoshizawa, 1987), where 2OHE₂ and 2ME₂ coexist and can reach a concentration of 5.7 and 10.0 μg kg⁻¹ respectively (Bianchi et al., 2007). However, an elevated intratesticular 2OHE₂ generation may be related to male infertility, since high concentrations of 2OHE₂ exert deleterious effects on
human spermatozoa in vitro (Bennetts et al., 2008; Aitken et al., 2013). The results of our study support this concept, given that high 2OHE2 concentrations induced Sertoli cell death.

Regarding the mechanisms by which 2OHE2 decrease Sertoli cell viability, this estradiol metabolite generates cell death inducing oxidative stress in human spermatozoa (Bennetts et al., 2008) and mammary epithelial cells (Hurh et al., 2004). In this latter cell type, this phenomenon is followed by activation of caspases (Hurh et al., 2004). In our study, we showed that the deleterious effects of 2OHE2 would be mediated by an increased oxidative stress in Sertoli cells since the antioxidant NAC blocked the effect of 2OHE2. Our results are in accordance with the fact that NAC is also capable of blocking the harmful effects of 2OHE2 in human mammary epithelial cells (Hurh et al., 2004). Moreover, we confirmed that hydrogen peroxide concentration is quickly increased in Sertoli cells exposed to 2OHE2. On the other hand, we showed that 2OHE2 induced DNA fragmentation and PARP and caspase-3 cleavage in Sertoli cells. Furthermore, the broad-spectrum caspase inhibitor Z-VAD-FMK (Lin et al., 2013; Sawai, 2013; Thayyullathil et al., 2013) blocked the deleterious effect of 2OHE2, indicating that 2OHE2 activates a caspase-dependent signalling pathway. Altogether, our results show that 2OHE2 induces DNA fragmentation in Sertoli cells by an increased oxidative stress in these cells which triggers a caspase-dependent apoptotic mechanism.

2ME2 has been typically associated with beneficial physiological effects, due to its capacity to induce apoptosis of tumourigenic cells (Mooberry, 2003; Sutherland et al., 2007; Verenich & Gerl, 2010). However, the facts that 2ME2 induced Sertoli cell death in our study and that high ovarian 2ME2 concentrations are associated with the aetiology of polycystic ovarian syndrome (Salih et al., 2008) suggest that elevated 2ME2 concentrations exert negative effects in the male and female gonad. In this study, we discarded that the mechanisms by which 2ME2 induces DNA fragmentation in Sertoli cells involved necrosis and they were mediated by oxidative stress and by activation of a caspase-dependent signalling pathway. However, we did not elucidate the mechanisms by which this estradiol metabolite exerts its deleterious effects on Sertoli cells and this is a limitation of this study. According to the literature, 2ME2 is also able to induce cell death by activating autophagic processes in cervix adenocarcinoma and glioblastoma cell lines (Chen et al., 2008) and by binding to the colchicine site of tubulin in neuroblastoma cells (Manca & Chisu, 2011), suggesting that 2ME2 may induce Sertoli cell death by some of these mechanisms. However, this was not elucidated in this study.

Another limitation is the use of TM4 cells as the experimental model. Although TM4 cells are one of the most extensively studied Sertoli cell lines, some biological properties observed in primary Sertoli cells are not replicated in TM4 cells (Kaur & Dufour, 2012; Reis et al., 2015). In this sense, we explored if the effects of high 2OHE2 and 2ME2 concentrations observed in TM4 cells were mimicked in primary human Sertoli cells in order to discard a species or cell line-specific phenomenon. Our results indicate that 2OHE2 and 2ME2 also induce DNA fragmentation in human Sertoli cells. Moreover, these estradiol metabolites can induce a more severe damage in human Sertoli cells, given that the number of Sertoli cells seems to decrease 24 h after the treatment with these estradiol metabolites compared to control group. However, our results must be interpreted with caution since they were obtained using human Sertoli cells from only one patient.

Regarding the deleterious effects exerted by 2OHE2 and 2ME2 on Sertoli cell viability 24 h post-treatment, it should be noted that the doses required to induce these effects are high, making it improbable that these concentrations are reached at the serum level. However, the intracellular testis concentrations of these estradiol metabolites may be sufficiently high under pathological conditions. Intratesticular concentrations of E2 are almost 100 times higher than serum plasma concentration (Roth et al., 2010) and are even higher in infertile men (Lardone et al., 2010). These estradiol metabolites may be produced via an intracrine pathway in Sertoli and Leydig cells from E2. Furthermore, the impacts of 2OHE2 and 2ME2 on
Sertoli cell viability 24 h post-treatment represent short-term effects of these metabolites. We postulate that under pathological conditions, Sertoli cells may be exposed to high concentrations of 2OHE₂ and 2ME₂; while these levels may not necessarily reach micromolar concentrations, exposure would extend over a longer period of time, potentiating the deleterious effects of these metabolites. This idea is supported by the finding that low concentrations of 2OHE₂ and 2ME₂ decreased Sertoli cell viability when cells were exposed to these metabolites for 48 h. This latter result also suggests that 2ME₂ has a relatively long half-life in testis. 2ME₂ is normally metabolised to 2-methoxyestrone, a reaction mediated by the enzyme 17beta-hydroxysteroid dehydrogenase (Liu et al., 2005; Sweeney et al., 2005; Newman et al., 2006; James et al., 2007), suggesting that the activity of this enzyme is low at least in isolated Sertoli cells. Regarding 2OHE₂, this compound exerts its deleterious effects mainly by its biotransformation to E₂-2-3-Quinone (Parl et al., 2009), a reaction that also generates reactive oxygen species as products (Chen et al., 2005; Fussell et al., 2011). This evidence, together with our results, suggests that 2OHE₂ is continuously transformed to E₂-2-3-Quinone by peroxidases enzymes in Sertoli cells, which induces a constant oxidative stress in these cells. However, we do not discard that the negative long-term effects of low doses of 2OHE₂ on Sertoli cells are mediated by its biotransformation to 2ME₂, a phenomenon that normally occurs in vivo (Zacharia et al., 2004) or by a different direct mechanism.

Male infertility has been related with an increased E₂ intratesticular concentration and it can be triggered by exposure to different toxicants, which induce apoptosis in testicular somatic cells and germ cells by mechanisms not completely elucidated. Interestingly, high intratesticular E₂ concentrations have been reported in men with a primary spermatogenic failure (Lardone et al., 2010) and some toxicants, such as various polychlorinated biphenyls (Fukuzawa et al., 2003; Shimada et al., 2003; Yamamoto et al., 2004), that are capable of severely disrupting the spermatogenic process also increase CYP1A1 levels in germ and somatic testicular cells. These observations suggest that the mechanisms underlying elevated 2OHE₂ and/or 2ME₂ intratesticular levels may involve increased expression of the enzymes responsible for transforming E₂ into 2OHE₂ and 2ME₂.

In summary, our results support the concept that an increased 2OHE₂ intratesticular production may be related with male infertility, since it triggers deleterious effects on Sertoli cells as in germ cells. Besides, we showed that high 2ME₂ concentrations also induce DNA fragmentation in Sertoli cells. Future studies focused on knowing if the intratesticular concentrations of these estradiol metabolites are altered in pathological conditions and if they mediate the deleterious effects of some toxicants that induce male infertility will help to understand the actual role of these molecules on testicular physiology.

**Funding**

This study was funded by CONICYT/FONDECYT from Chilean Government, grant numbers: 11110457 and 1120176, Program U-apoya University of Chile and Proyectos basales and vicerrectoria de investigacion, desarrrollo e innovacion, Universidad de Santiago de Chile.

**References**


Chen ZH, Na HK, Hurh YJ, Surh YJ (2005) 4-


Fukuzawa NH, Ohsako S, Nagano R, Sakaue M, Baba T, Aoki Y, Tohyama C (2003) Effects of 3,3′,4,4′,5-
pentachlorobiphenyl, a coplanar polychlorinated biphenyl congener, on cultured neonatal mouse testis. *Toxicol In Vitro* 17:259–269.


Hurh YJ, Chen ZH, Na HK, Han SY, Surh YJ (2004) 2-


Mooberry SL (2003) Mechanism of action of 2-

Mosli HA, Al-Abd AM, El-Shaer MA, Khedr A, Gazaz FS, Abdel-Naim AB (2012) Local inflammation influences...


