ORIGINAL ARTICLE

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

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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 ($2OHE_2$), 4-hydroxyoestradiol ($4OHE_2$), 2-methoxyoestradiol ($2ME_2$) and 4-methoxyoestradiol ($4ME_2$) 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 $2OHE_2$ and $2ME_2$ decreased cell viability inducing DNA fragmentation. In addition, ICI182780 did not block the effect of $2OHE_2$ and $2ME_2$, while N-Acetylcysteine and Z-VAD-FMK only blocked the effect of $2OHE_2$. Moreover, $2OHE_2$ but not $2ME_2$ induced PARP and caspase-3 cleavage. Finally, lower $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ or $2ME_2$ concentrations could be related to male infertility since $2OHE_2$ by apoptosis and $2ME_2$ by undetermined mechanisms induce DNA fragmentation in Sertoli cells.

Introduction

Oestradiol (E_2) 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). E_2 is mainly hydroxylated in position 2 by the enzyme Cytochrome P450 isoform 1A1 (CYP1A1) which generates 2-hydroxyoestradiol ($2OHE_2$), and also in position 4 by the isoform CYP1B1 which generates 4-hydroxyoestradiol ($4OHE_2$) (Dawling *et al.*, 2004). Subsequently, the enzyme Catechol-O-Methyltransferase (COMT) replaces the hydroxyl group by a methyl group to produce 2-methoxyoestradiol ($2ME_2$) from $2OHE_2$ and 4-methoxyoestradiol ($4ME_2$) from $4OHE_2$ (Dawling *et al.*, 2004).

In recent years, it has been demonstrated that $2OHE_2$, $4OHE_2$, $2ME_2$ and $4ME_2$ are not inactive metabolites, but rather participants in various physiological processes (Parada-Bustamante *et al.*, 2013, 2015). For instance, $2ME_2$ modulates angiogenesis in endothelial and granulosa cells regulating the expression of endothelin and VEGF, respectively (Dubey *et al.*, 2001; Kohen *et al.*, 2013), while $2OHE_2$ 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 $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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, E_2 is normally produced from Testosterone by the Aromatase enzymatic complex, which is expressed in Leydig, Sertoli and germ cells (Lardone *et al.*, 2010). E_2 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, E_2 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 E_2 since CYP1A1, CYP1B1 and COMT are expressed in germ and somatic testicular cells (Revel *et al.*, 2001; Choudhary *et al.*, 2003; Leung *et al.*, 2009; Overbye & Seglen, 2009; Deb *et al.*, 2010). Moreover, the mammalian testis is capable of producing $2OHE_2$ from E_2 (Watanabe & Yoshizawa, 1987) and $2ME_2$ from $2OHE_2$ (Axelrod & Goldzieher, 1962) *in vitro*. In addition, the enzyme Aromatase isolated from testes has also the ability to produce $2OHE_2$ from E_2 *in vitro* (Almadhidi *et al.*, 1996). On the other hand, it has been hypothesised that elevated intratesticular concentrations of hydroxyoestradiols are associated with male infertility, given that high $2OHE_2$ and $4OHE_2$ concentrations decrease the viability and motility of human spermatozoa *in vitro* (Bennetts *et al.*, 2008; Aitken *et al.*, 2013). However, the direct effects of hydroxyoestradiols and methoxyoestradiols on testicular somatic cells have not been evaluated. The aim of this study was to explore the effects of different concentrations of $2OHE_2$, $2ME_2$, $4OHE_2$ and $4ME_2$ on Sertoli cell viability *in vitro*.

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 hydroxyoestradiols and methoxyoestradiols on TM4 cell viability

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 treated with different concentrations (0.0001–20 μmol l^{-1}) of $2ME_2$, $4OHE_2$, $4ME_2$ (Steraloids, Newport, RI, USA), $2OHE_2$ (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 E_2 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).

Effects of $2OHE_2$ and $2ME_2$ on DNA integrity in TM4 cells

TM4 cells (3×10^3) 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} $2OHE_2$, μmol l^{-1} $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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} $2OHE_2$ or $2ME_2$ plus 20 μmol l^{-1} ICI182780 (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 hours post-treatment, cell viability was evaluated by MTS assay as previously described.

PARP and Caspase-3 activation by $2OHE_2$ and $2ME_2$ in TM4 cells

TM4 cells (2.5×10^5) previously treated with 20 μmol l^{-1} $2OHE_2$ or $2ME_2$ for 24 h were incubated with a lysis solution containing Hepes (50 mM, pH 7.5), NaCl 150 mM, $MgCl_2$ 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 electroblotted 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 $2OHE_2$ and $2ME_2$ in TM4 cells

Possible H_2O_2 generation induced by $2OHE_2$ or $2ME_2$ 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 (1×10^6 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 $2OHE_2$ 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 H_2O_2 was also prepared.

Evaluation of cell death by necrosis in TM4 cells treated with $2ME_2$

In order to evaluate whether $2ME_2$ decreases TM4 cell viability inducing necrosis, we determined Lactate Dehydrogenase enzymatic activity in culture medium from TM4 cells (2×10^3) treated with 20 μmol l^{-1} $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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 CO_2-incubator with 5% CO_2 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 Versene™ 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). 1×10^3 Sertoli cells were plated in coverslips covered with poly-L-lysine in order to perform TUNEL assay and 0.5×10^4 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 $2OHE_2$ and $2ME_2$ 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

$2OHE_2$ and $2ME_2$ decrease Sertoli cell viability 24 h post-treatment

In order to explore the *in vitro* effects of $2OHE_2$, $2ME_2$, $4OHE_2$ or $4ME_2$ 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 $2OHE_2$ 20 μmol l^{-1} (43.6 ± 5.1%), $2ME_2$ 10 μmol l^{-1} (41.5 ± 2.4%) and $2ME_2$ 20 μmol l^{-1} (39.3 ± 2.8%). Interestingly, $4OHE_2$ and $4ME_2$ did not alter cell viability at any tested concentrations (Fig. 1c,d).

Oestrogen receptor-independent mechanisms mediate the deleterious effects of $2OHE_2$ and $2ME_2$ in Sertoli cells

To determine whether the deleterious effects of $2OHE_2$ and $2ME_2$ require a functional oestrogen receptor, TM4 cells were treated for 24 h with $2OHE_2$ or $2ME_2$ and concomitantly with ICI182780, a specific oestrogen receptor antagonist (Robertson, 2001). The effects of $2OHE_2$ and $2ME_2$ on Sertoli cell viability were not blocked when these metabolites were co-incubated with ICI182780 (Fig. 2). Thus, the deleterious effects of $2OHE_2$ and $2ME_2$

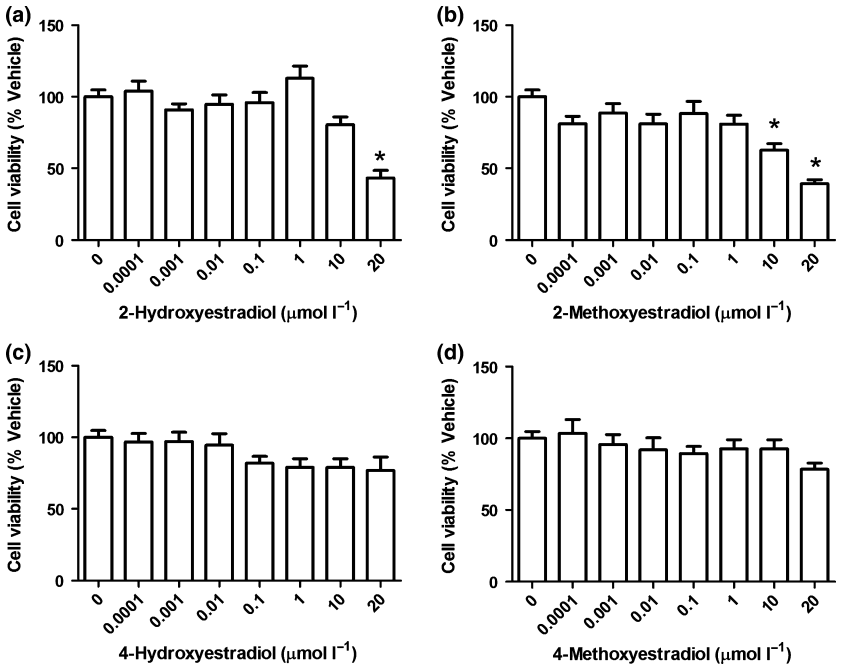

Fig. 1 Effects of hydroxyoestradiols and methoxyoestradiols on TM4 Sertoli cell viability. TM4 cells were treated for 24 h with different doses of $2OHE_2$ (a), $2ME_2$ (b) $4OHE_2$ (c) or $4ME_2$ (d) and then cell viability was evaluated by MTS assay. *$P < 0.05$ compared with the vehicle group; Mann–Whitney test.

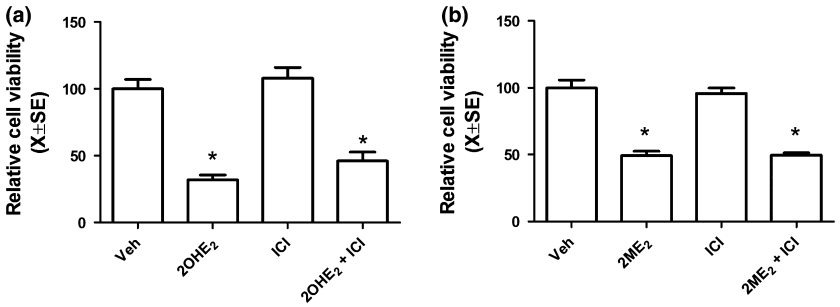

Fig. 2 Effects of $2OHE_2$ and $2ME_2$ are not mediated by the classical oestrogen receptor. TM4 cells were treated for 24 h with $2OHE_2$, $2ME_2$ or vehicle and concomitantly with the oestrogen receptor antagonist ICI182780 (ICI) to then evaluate cell viability by MTS assay. The effect of ICI on $2OHE_2$ (a) and $2ME_2$ (b) is separately shown. $^*P < 0.05$ compared with the vehicle group; Mann–Whitney test.

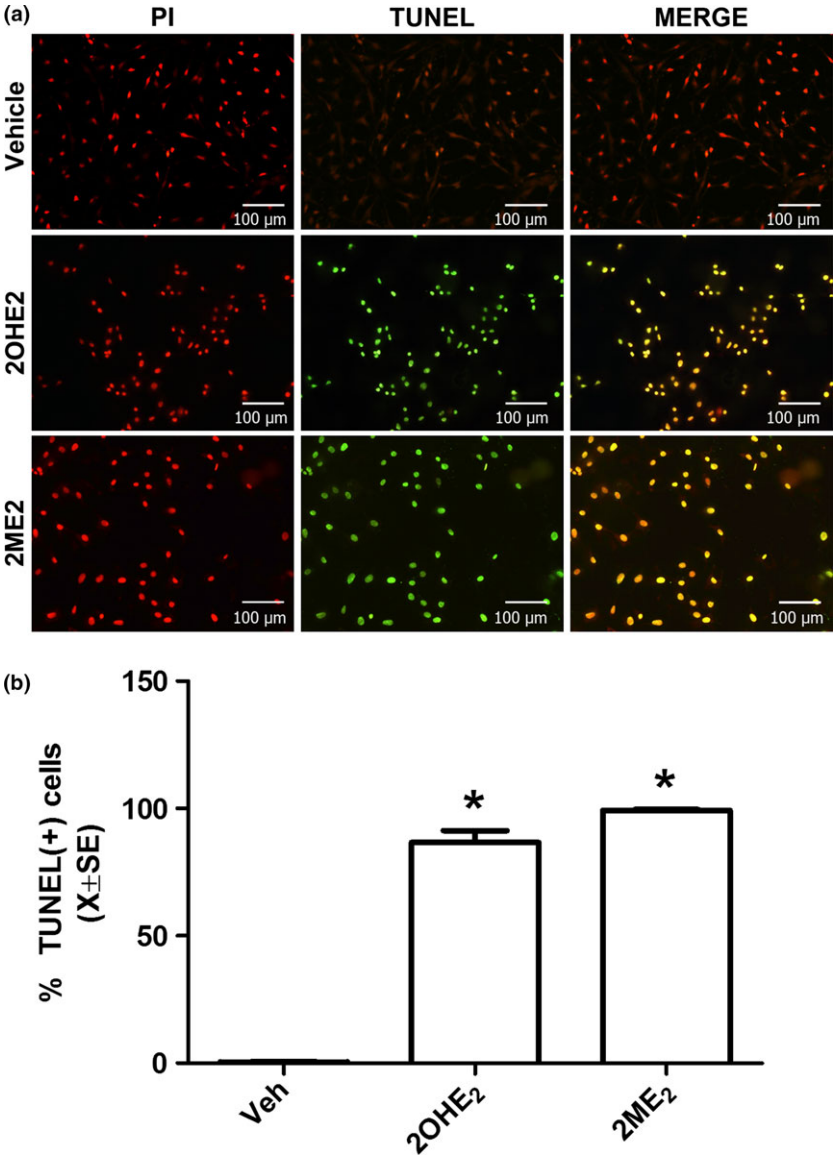

Fig. 3 $2OHE_2$ and $2ME_2$ induce DNA fragmentation in TM4 Sertoli cells. TM4 cells were treated for 24 h with $2OHE_2$, $2ME_2$ or vehicle to evaluate DNA fragmentation by TUNEL Assay (a). Nuclei were counterstained with Propidium Iodide (PI). The percentage of cells with fragmented DNA in relation 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.

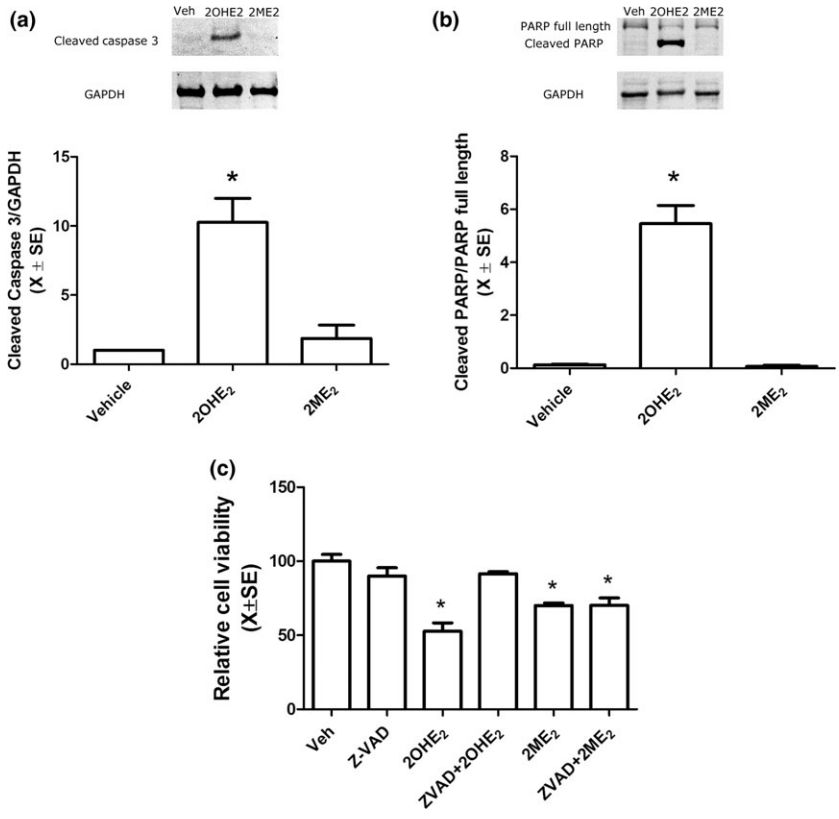

Fig. 4 $2OHE_2$, but not $2ME_2$, induces apoptosis in TM4 Sertoli cells by a caspase-dependent apoptotic mechanism. TM4 cells were treated for 24 h with $2OHE_2$, $2ME_2$ or vehicle and then total protein was isolated to evaluate cleavage of caspase-3 (a) and PARP (b) by western blot. Besides, TM4 cells were treated for 24 h with $2OHE_2$, $2ME_2$ 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.

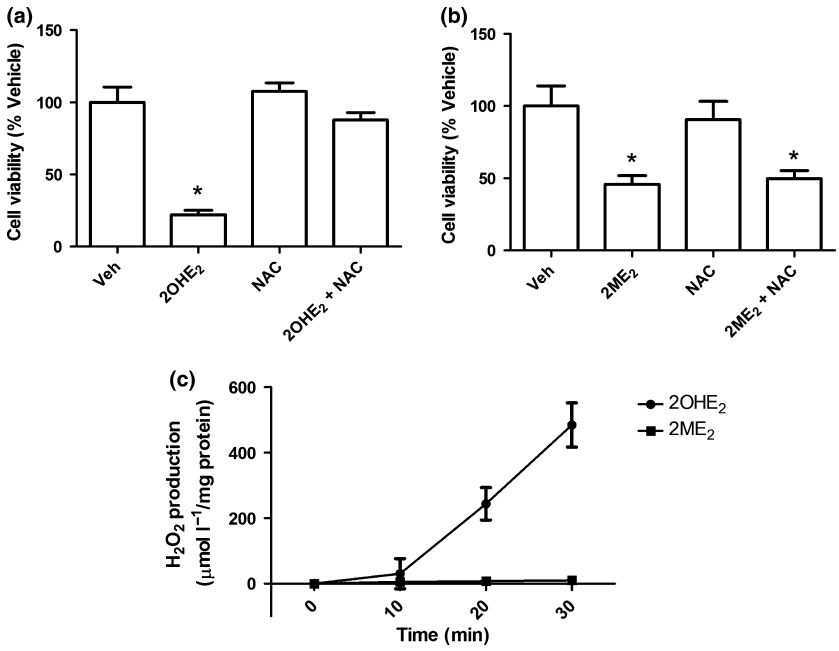

Fig. 5 Oxidative stress mediates $2OHE_2$ deleterious effects on TM4 Sertoli cell viability. TM4 cells were treated for 24 h with $2OHE_2$, $2ME_2$ or vehicle and concomitantly with the antioxidant N-AcetylCysteine (NAC) to then evaluate cell viability by MTS assay. The effect of NAC on $2OHE_2$ (a) and $2ME_2$ (b) is separately shown. Besides, H_2O_2 generation was measured in lysates protein obtained from TM4 cells treated with $2OHE_2$ and $2ME_2$ for different times (c). *$P < 0.05$ compared with the vehicle group; Mann–Whitney test.

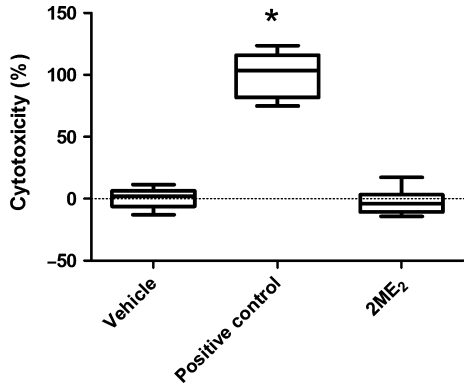

Fig. 6 $2ME_2$ does not activate a necrotic process in Sertoli cells. TM4 cells were incubated with $2ME_2$ or vehicle for 24 h to then measure Lactate Dehydrogenase enzymatic activity in culture medium. Culture medium incubated with LDH (0.1 μg ml^{-1}) was included as a positive control. $^*P < 0.05$ compared with the vehicle group; Mann–Whitney test.

on Sertoli cell viability are not mediated by the classical oestrogen receptor.

$2OHE_2$ and $2ME_2$ induce DNA fragmentation in Sertoli cells, but only the effect of $2OHE_2$ is mediated by a caspase-dependent apoptotic mechanism

To explore the mechanisms by which $2OHE_2$ and $2ME_2$ decrease Sertoli cell viability, TM4 cells were incubated with $2OHE_2$ 20 μmol l^{-1}, $2ME_2$ 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 $2OHE_2$ (86.6 ± 4.6%) and $2ME_2$ (99.3 ± 0.3%). Then, we evaluated whether $2OHE_2$ and $2ME_2$ induced PARP and caspase-3 cleavage. We found that $2OHE_2$, but not $2ME_2$, induced caspase-3 and PARP cleavage in TM4 cells (Fig. 4a,b). Interestingly, the effect of $2OHE_2$ 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 $2ME_2$ (Fig. 4c).

Oxidative stress mediates the effect of $2OHE_2$ on Sertoli cell viability

To determine whether the deleterious effects of $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ (Fig. 5a), but it did not prevent the effect of $2ME_2$ (Fig. 5b). In order to corroborate this result, TM4 cells were treated with $2OHE_2$ or $2ME_2$ and generation of hydrogen peroxide (H_2O_2) was evaluated. Results showed that $2OHE_2$, but not $2ME_2$, increased H_2O_2 generation in TM4 cells (Fig. 5c).

$2ME_2$ does not induce necrosis in Sertoli cells

In order to determine whether $2ME_2$ decreases Sertoli cell viability activating a necrotic process, Lactate Dehydrogenase (LDH) activity was evaluated in culture medium from TM4 cells treated with $2ME_2$ or vehicle. LDH activity was not significantly different between the cells treated with $2ME_2$ or vehicle (Fig. 6).

$2OHE_2$ and $2ME_2$ decrease the viability of human Sertoli cells inducing DNA fragmentation

To determine whether the deleterious effects of $2OHE_2$ and $2ME_2$ observed in TM4 cells are replicated in human Sertoli cells, we used a commercially available human primary Sertoli cell (hSEC). $2OHE_2$ 20 μmol l^{-1} (54.2 ± 5.2%, $P < 0.05$) and $2ME_2$ 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 $2OHE_2$ and $2ME_2$ decreased the viability of these cells inducing DNA fragmentation, hSEC cells were incubated with $2OHE_2$ 20 μmol l^{-1}, $2ME_2$ 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 $2OHE_2$ (99.7 ± 0.2) and $2ME_2$ (76.5 ± 9.6%).

Lower concentrations of $2OHE_2$ and $2ME_2$ decrease Sertoli cell viability 48 h post-treatment

In order to explore whether lower doses of $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ and $2ME_2$ (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 $2OHE_2$ 0.01 μmol l^{-1} (57.4 ± 3.0%), $2OHE_2$ 0.1 μmol l^{-1} (62.1 ± 8.1%), $2OHE_2$ 1.0 μmol l^{-1} (59.5 ± 1.3%), $2ME_2$ 0.01 μmol l^{-1} (64.6 ± 6.1%), $2ME_2$ 0.1 μmol l^{-1} (48.3 ± 1.6%) and $2ME_2$ 1.0 μmol l^{-1} (56.1 ± 5.2%).

Discussion

Although $2OHE_2$ and $2ME_2$ concentrations in the mammalian testis have not been experimentally determined, studies indicate that $2OHE_2$ and $2ME_2$ are normally

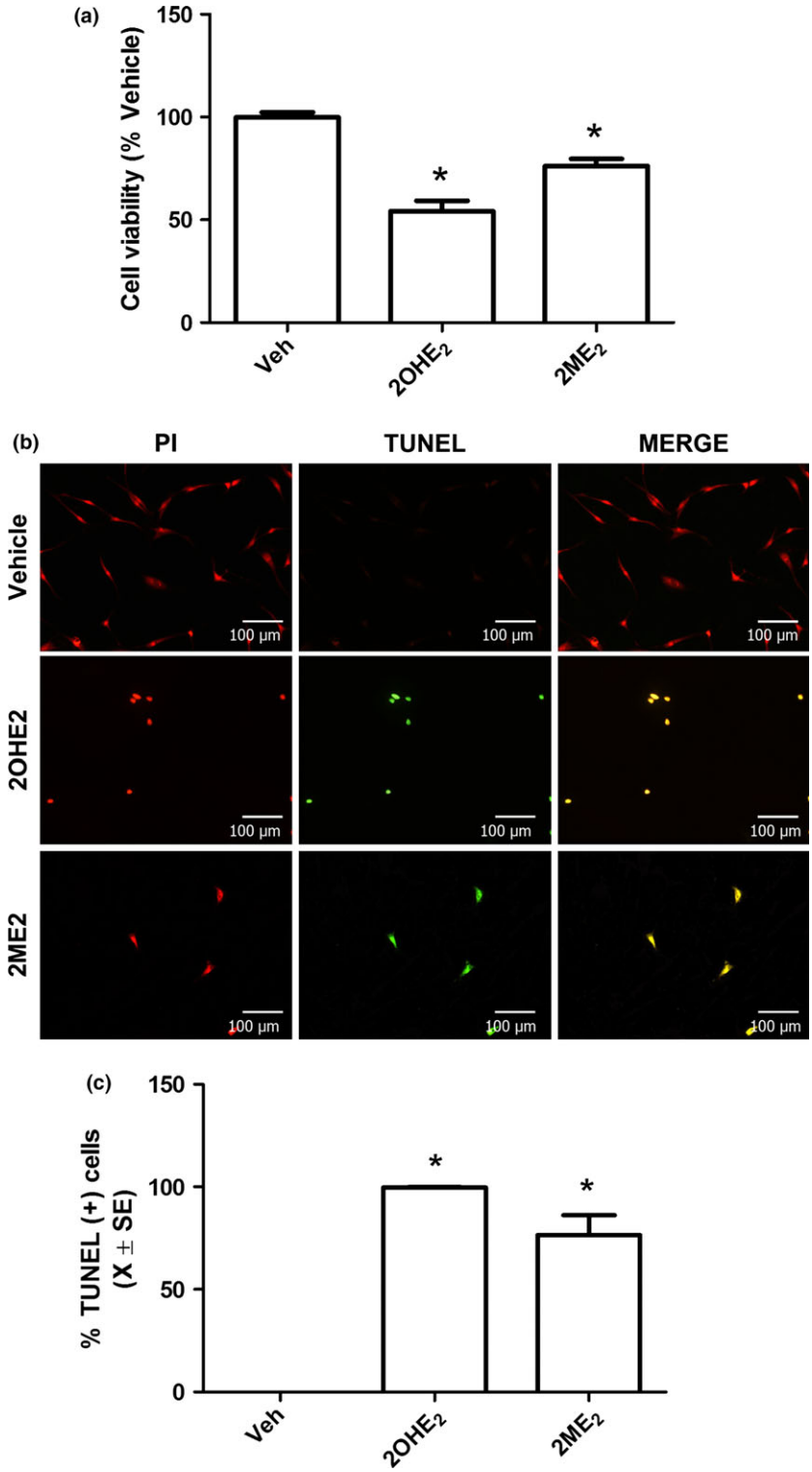

Fig. 7 $2OHE_2$ and $2ME_2$ also decrease the viability of human Sertoli cells inducing DNA fragmentation. hSECs cells were treated for 24 h with $2OHE_2$, $2ME_2$ or vehicle and then cell viability was evaluated by MTS assay (a). Besides, other group of hSEC cells were also treated for 24 h with $2OHE_2$, $2ME_2$ 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 E_2 in this organ, with similar efficiency and kinetic parameters as those reported for the ovary (Axelrod & Goldzieher, 1962; Watanabe & Yoshizawa, 1987), where $2OHE_2$ and $2ME_2$ coexist and can reach a concentration of 5.7 and 10.0 µg kg^{-1} respectively (Bianchi *et al.*, 2007). However, an elevated intratesticular $2OHE_2$ generation may be related to male infertility, since high concentrations of $2OHE_2$ exert deleterious effects on

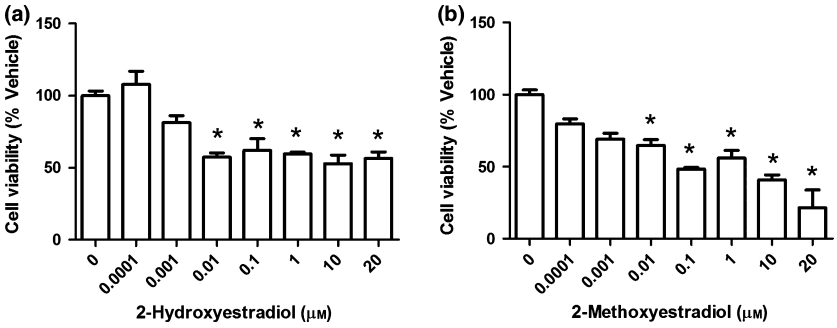

Fig. 8 Lower doses of $2OHE_2$ and $2ME_2$ decrease TM4 Sertoli cell viability 48 h post-treatment. TM4 cells were treated for 48 h with different doses of $2OHE_2$ and $2ME_2$ and then cell viability was evaluated by MTS assay. *$P < 0.05$ compared with the vehicle group; Mann–Whitney test.

human spermatozoa *in vitro* (Bennetts *et al.*, 2008; Aitken *et al.*, 2013). The results of our study support this concept, given that high $2OHE_2$ concentrations induced Sertoli cell death.

Regarding the mechanisms by which $2OHE_2$ 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 $2OHE_2$ would be mediated by an increased oxidative stress in Sertoli cells since the antioxidant NAC blocked the effect of $2OHE_2$. Our results are in accordance with the fact that NAC is also capable of blocking the harmful effects of $2OHE_2$ in human mammary epithelial cells (Hurh *et al.*, 2004). Moreover, we confirmed that hydrogen peroxide concentration is quickly increased in Sertoli cells exposed to $2OHE_2$. On the other hand, we showed that $2OHE_2$ 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 $2OHE_2$, indicating that $2OHE_2$ activates a caspase-dependent signalling pathway. Altogether, our results show that $2OHE_2$ induces DNA fragmentation in Sertoli cells by an increased oxidative stress in these cells which triggers a caspase-dependent apoptotic mechanism.

$2ME_2$ 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 & Gerk, 2010). However, the facts that $2ME_2$ induced Sertoli cell death in our study and that high ovarian $2ME_2$ concentrations are associated with the aetiology of polycystic ovarian syndrome (Salih *et al.*, 2008) suggest that elevated $2ME_2$ concentrations exert negative effects in the male and female gonad. In this study, we discarded that the mechanisms by which $2ME_2$ 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, $2ME_2$ 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 $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ and $2ME_2$ 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 E_2 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 E_2. Furthermore, the impacts of $2OHE_2$ and $2ME_2$ 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 $2OHE_2$ and $2ME_2$; 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 $2OHE_2$ and $2ME_2$ decreased Sertoli cell viability when cells were exposed to these metabolites for 48 h. This latter result also suggests that $2ME_2$ has a relatively long half-life in testis. $2ME_2$ 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 $2OHE_2$, this compound exerts its deleterious effects mainly by its biotransformation to E_2-2-3-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 $2OHE_2$ is continuously transformed to E_2-2-3-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 $2OHE_2$ on Sertoli cells are mediated by its biotransformation to $2ME_2$, 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 E_2 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 E_2 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 $2OHE_2$ and/or $2ME_2$ intratesticular levels may involve increased expression of the enzymes responsible for transforming E_2 into $2OHE_2$ and $2ME_2$.

In summary, our results support the concept that an increased $2OHE_2$ 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 $2ME_2$ 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.

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