α -Tocopherol modifies the expression of genes related to oxidative stress and apoptosis during *in vitro* maturation and enhances the developmental competence of rabbit oocytes

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Abstract. The developmental competence of *in vitro* maturation (IVM) oocytes can be enhanced by antioxidant agents. The present study investigated, for the first time in the rabbit model, the effect of adding α -tocopherol (0, 100, 200 and 400 μ M) during IVM on putative transcripts involved in antioxidant defence (superoxide dismutase 2, mitochondrial (*SOD2*), glutathione peroxidase 1 (*GPX1*), catalase (*CAT*)), cell cycle regulation and apoptosis cascade (apoptosis tumour protein 53 (*TP53*), caspase 3, apoptosis-related cysteine protease (*CASP3*)), cell cycle progression (cellular cycle V-Akt murine thymoma viral oncogene homologue 1 (*AKT1*)), cumulus expansion (gap junction protein, alpha 1, 43 kDa (*GJA1*) and prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase) (*PTGS2*)) and metabolism (glucose-6-phosphate dehydrogenase (*G6PD*)). Meiotic progression, mitochondrial reallocation, cumulus cell apoptosis and the developmental competence of oocytes after IVF were also assessed. Expression of *SOD2*, *CAT*, *TP53*, *CASP3* and *GJA1* was downregulated in cumulus–oocyte complexes (COCs) after IVM with 100 μ M α -tocopherol compared with the group without the antioxidant. The apoptotic rate and the percentage of a non-migrated mitochondrial pattern were lower in COCs cultured with 100 μ M α -tocopherol, consistent with better-quality oocytes. In fact, early embryo development was improved when 100 μ M α -tocopherol was included in the IVM medium, but remained low compared with *in vivo*-matured oocytes. In conclusion, the addition of 100 μ M α -tocopherol to the maturation medium is a suitable approach to manage oxidative stress and apoptosis, as well as for increasing the *in vitro* developmental competence of rabbit oocytes.

Introduction

In vitro maturation (IVM) has become an effective method to produce competent oocytes for a wide range of applications in assisted reproductive technologies (ARTs), such as human ARTs, animal cloning, genetic resources and fertility preservation, as well as for basic research. However, the current potential of IVM oocytes remains lower than for *in vivo*-matured

oocytes (Rizos *et al.* 2002; Arias-Álvarez *et al.* 2017). Oxidative stress has recently appeared as one of the most important factors contributing to low oocyte quality *in vitro*. Standard culture conditions induce oxidative stress in the cell due to an imbalance between oxidants and antioxidant defence mechanisms, subsequently increasing concentrations of reactive oxygen species (ROS). ROS production is a normal internal process of cells,

including in oocytes and embryos. However, excessive ROS concentrations induce a cascade of chain reactions that can ultimately lead to lipid peroxidation in membrane phospholipids, affecting the function and permeability of cell membranes, and generating DNA damage, meiotic arrest and mitochondrial dysfunction, which, in turn, activate caspase cascades and culminate in irreversible cell death (Combelles *et al.* 2009; Tripathi *et al.* 2009). Oxidative oocyte injury is detrimental to fertility potential (Guérin *et al.* 2001) and early embryo development (Kitagawa *et al.* 2004), and may increase the risk of miscarriage (for a review, see Agarwal *et al.* 2012). Therefore, the presence of antioxidant substances in the oocyte microenvironment would effectively minimise oxidative stress and benefit *in vitro* oocyte maturation processes.

Vitamin E is one of the most widely studied non-enzymatic antioxidant agents because it is the primary free radical scavenger in the mammalian cell membrane (Chow 1991). α -Tocopherol is the most active form of vitamin E and a natural antioxidant. It is a lipid-soluble vitamin that protects polyunsaturated fatty acids in membranes and inhibits lipid peroxidation, maintaining cell membrane function. Thus, α -tocopherol protects against cell damage induced by oxidative stress (Tao *et al.* 2010), and acts as a modulator of many intracellular or extracellular biochemical processes (Azzi and Stocker 2000). In the mammalian ovary, α -tocopherol is the most important antioxidant that provides cell defence against ROS. It is present in follicular fluid in both animals and humans (Cassano *et al.* 1999; Palini *et al.* 2014), and it plays an important role as a local precursor of retinoids, which may be relevant for follicular development and oocyte maturation (Schweigert *et al.* 2003). These findings make α -tocopherol a candidate non-invasive agent for improving IVM efficiency. During IVM, α -tocopherol content in the membranes of cumulus–oocyte complexes (COCs) diminishes by 50%, suggesting a partial loss of its antioxidant activity (Dalvit *et al.* 2005). Supplementation of culture media with α -tocopherol improved granulosa cell viability (McCluskey *et al.* 1999), folliculogenesis, oocyte quality, fertilisation rates and *in vitro* early embryo development in cattle (Olson and Seidel 2000), gilts (Kitagawa *et al.* 2004; Tao *et al.* 2010; Tareq *et al.* 2012) and sheep (Natarajan *et al.* 2010). α -Tocopherol also protects oocytes during IVM against the deleterious effects of cryoprotectants in vitrified ovaries (Farzollahi *et al.* 2016; Yashiro *et al.* 2015), and increases the blastocyst rate of somatic cell nuclear transfer (SCNT) embryos (Jeong *et al.* 2006; Wongsrikeao *et al.* 2007). Therefore, although the relationship between α -tocopherol and oocyte maturation and embryo development is well established in different species, no studies to date have been performed in the rabbit.

Rabbits are used as livestock and as a model for basic reproduction research (Fischer *et al.* 2012; López-Tello *et al.* 2017) because they share many biochemical and physiological features with humans (Fan and Watanabe 2003; Ogonuki *et al.* 2005). The development of suitable IVM, IVF and *in vitro* culture (IVC) techniques in this species is crucial because these techniques are useful in biotechnology to produce SCNT embryos for animal breeding, the preservation of genetic resources, developmental biology studies and as model system

for human reproduction. However, the IVM method in rabbits is poorly developed compared with other species due to the difficulty of achieving optimal rates of *in vitro* oocyte maturation, and currently there is limited information about the cellular and molecular mechanisms controlling oocyte maturation. Oxidative stress could be a key factor determining the low developmental competence in this species. Therefore, understanding cellular and molecular mechanisms during rabbit maturation in response to antioxidants could provide important information in order to find optimal media for use in animal and human ARTs.

In the present study, a dose–response experiment was performed to determine whether α -tocopherol has a direct effect on meiotic and cytoplasmic *in vitro* oocyte maturation events. Furthermore, the effects of α -tocopherol on the apoptotic index in cumulus cells, the mRNA expression patterns of some important genes related to crucial mechanisms in oocytes and cumulus cells (CCs) and early embryo development after IVF were examined in the rabbit model.

Materials and methods

Unless stated otherwise, all chemicals were purchased from Sigma Chemicals.

Experimental design

Experiment 1: effects of α -tocopherol on markers of nuclear and cytoplasmic oocyte maturation

A dose–response experiment was performed to assess the direct effect of α -tocopherol on markers of nuclear and cytoplasmic oocyte maturation. Serum-free maturation medium was supplemented with or without α -tocopherol (0, 100, 200 or 400 μ M). Experiment 1a assessed: (1) oocyte meiotic progression by investigating the MII rate and phosphorylation of extracellular signal-regulated kinase (ERK) 1/2; and (2) cytoplasmic maturation, based on determination of mitochondrial distribution patterns. Experiment 1b analysed apoptosis in CCs and the expression of putative transcripts involved in essential events for oocyte maturation, namely cumulus expansion (gap junction protein, alpha 1, 43 kDa (*GJA1*), prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase) (*PTGS2*)), antioxidant defence (superoxide dismutase 2, mitochondrial (*SOD2*), glutathione peroxidase 1 (*GPX1*), catalase (*CAT*)), cell cycle regulation and the apoptosis cascade (apoptosis tumour protein 53 (*TP53*); caspase 3, apoptosis-related cysteine protease (*CASP3*)), cell cycle progression and viability (cellular cycle V-Akt murine thymoma viral oncogene homologue 1 (*AKT1*)) and metabolism (glucose-6-phosphate dehydrogenase (*G6PD*)) in rabbit oocytes and their CCs.

Experiment 2: effects of α -tocopherol on the developmental competence of oocytes

IVM oocytes that had been cultured with 100 μ M α -tocopherol (which showed the best results in Experiments 1a and 1b) or without α -tocopherol (control) were used for IVF and IVC. In addition, *in vivo*-matured oocytes were used for IVF and IVC. Early embryo development was evaluated until the blastocyst stage.

Oocyte collection and IVM

Oocytes for IVM were recovered from adult New Zealand × California white rabbit dams (*Oryctolagus cuniculus*) located in the facilities of the Polytechnic University of Madrid.

Ovaries were obtained by mid-ventral laparotomy of dams after they had been sedated with 35 mg kg⁻¹ ketamine (Imalgene 1000; Merial); dams were killed with an intravenous bolus of barbiturate (30 mg kg⁻¹; Doletal; Vetoquinol). All procedures were performed in accordance with the policies of the University Scientific Ethics Committee and the Spanish Policy for Animal Protection (Royal Decree 53/2013), and approved by the Animal Ethics Committee of the Community of Madrid (Ref. PROEX 302/15) and met the European Union directive about the protection of animals used in experiments.

Ovarian follicles ≥ 1 mm in diameter were aspirated under a stereoscopic microscope (Nikon), and COCs were selected based on their morphological appearance, as described previously (Arias-Álvarez *et al.* 2010). COCs were cultured in four-well dishes in groups of 30–40 COCs per well for 16 h at 38°C under an atmosphere of 5% CO₂ in air with maximum humidity.

The maturation medium consisted of tissue culture media (TCM)-199 with 2 mM L-glutamine, 0.1 mg mL⁻¹ sodium pyruvate and 0.3% w/v bovine serum albumin (BSA). For Experiments 1a and 1b, a serum-free medium was used, as previously reported (Arias-Álvarez *et al.* 2017). The IVM medium included 10 ng mL⁻¹ epidermal growth factor (EGF) and 0, 100, 200 or 400 µM α-tocopherol (T3251; Groups 0E, 100E, 200E and 400E respectively). α-Tocopherol was dissolved in ethanol and stored at 4°C in the dark. Prior to culture, appropriate dilutions of this stock solution were made in maturation medium by vortexing for 2 h. The final ethanol concentration was <0.05% in all experimental groups. Final concentrations of α-tocopherol in the maturation medium were checked by HPLC.

To assess the beneficial effect of α-tocopherol on blastocyst yield (Experiment 2), oocytes were matured *in vitro* with or without 100 µM α-tocopherol in a serum-free medium (Groups 100E and 0E (control) respectively). In order to improve the rate of *in vitro* embryo development, a suitable IVM medium used previously in rabbit species because of its results (Lorenzo *et al.* 1996; Arias-Álvarez *et al.* 2010) was used in the present study. This medium contained 10 ng mL⁻¹ EGF, 100 ng mL⁻¹ insulin growth factor 1 (IGF1) and 10% v/v fetal calf serum (FCS). The IVM oocytes cultured in this medium with or without 100 µM α-tocopherol are referred to Groups 100E-FCS and 0E-FCS (control) respectively.

To obtain *in vivo*-matured COCs, 12 dams were killed 15 h after the induction of ovulation by intramuscular injection of gonadorelin (20 mg; Inducel-GnRH; Ovejero), a synthetic analogue of gonadotrophin-releasing hormone that stimulates the synthesis and release of endogenous LH from the anterior pituitary gland. Ovulated oocytes were recovered by flushing the reproductive tract with phosphate-buffered saline (PBS) supplemented with 1 mg mL⁻¹ polyvinylpyrrolidone (PVP).

Oocyte quality markers during oocyte maturation in Experiments 1a and 1b were analysed in five replicates (*n* = 731 COCs). Oocyte developmental competence following IVM,

IVF and IVC in Experiment 2 was assessed in three replicates (*n* = 618 COCs).

Nuclear maturation and mitochondrial distribution in oocytes

Nuclear maturation, measured as the MII rate, and cytoplasmic maturation (assessed on the basis of mitochondrial patterns) were evaluated in 100 oocytes after IVM (*n* = 46, 16, 20 and 18 in Groups 0E, 100E, 200E and 400E respectively) by confocal scanning microscopy. Briefly, CCs were mechanically removed, and denuded oocytes were incubated with 200 µM MitoTracker Orange probe (Invitrogen) at 38°C for 30 min for mitochondrial staining, followed by 1 µg mL⁻¹ Hoechst 33342 dye for 30 min at room temperature for nuclear staining. Samples were observed under a laser scanning confocal microscope (TCS SP2; Leica) using 546 nm and 351/364 nm excitation lasers to visualise mitochondria and chromosomes respectively. Five sections taken every 5 µm were made for each oocyte and a maximum projection was made for each one. The mitochondrial migration pattern was observed at a magnification of ×40 and classified according to Arias-Álvarez *et al.* (2017) as follows: migrated homogeneous (MH), in which there is high fluorescence in the cortical area and moderate fluorescence throughout the cytoplasm; migrated clustered (MC), in which there is fluorescence in the cortical area with aggregates; diffused homogeneous (DH), in which the fluorescence is dispersed throughout the cytoplasm; and diffused clustered (DC), in which aggregates of fluorescence are dispersed throughout the cytoplasm.

Western blotting analysis of ERK1/2 phosphorylation in oocytes

Total protein was extracted from 387 COCs after IVM (*n* = 100, 96, 90 and 101 from Groups 0E, 100E, 200E and 400E respectively). COCs were denuded by gentle pipetting and washed four times in PBS with 0.1% PVP. CCs were recovered and centrifuged twice in PBS with 0.1% w/v PVP at 700g for 5 min at room temperature. The supernatant was removed and the cells and oocytes were homogenised in cold lysis buffer containing an EDTA-free protease inhibitor cocktail (Roche Diagnostics SL, Applied Science) in centrifuge tubes, snap frozen in liquid nitrogen and stored at -80°C until assay. The western blot procedure followed the protocol reported by García-García *et al.* (2018). Briefly, samples were dissolved in protein sample buffer (5% 2-β-mercaptoethanol and Laemmli), boiled for 5 min and centrifuged at 3000g for 1 min at room temperature. The supernatant containing soluble proteins was divided into two aliquots and each one was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12% acrylamide plus bisacrylamide) and electrotransferred to nitrocellulose membranes. The membranes were probed with the primary antibody and incubated at 4°C overnight. The antibodies used were anti-ERK1/2 (MK1; Santa Cruz Biotechnology) and anti-phosphorylated (p) ERK1/2 (Santa Cruz Biotechnology) diluted 1 : 200 in blocking solution (LiCor Biotechnology) containing 0.2% v/v Tween-20. After incubation, membranes were washed four times for 5 min each time at room temperature in PBS

+ 0.2% v/v Tween-20 with gentle shaking. Then, membranes were incubated for 45 min at room temperature in the dark with the secondary antibodies (goat anti-mouse for ERK and donkey anti-mouse for pERK1/2; 1:15 000 dilution; LiCor Biotechnology). β -Actin (Sigma Aldrich A2228) was immunodetected with mouse monoclonal antibody as a loading control. The integrated density of bands was quantified using ImageJ (<http://rsbweb.nih.gov/ij/>, verified 13 June 2018). The level of ERK activation in each treatment group was measured as the ratio of activated pERK1/2 to total ERK1/2 after normalisation against β -actin.

Cumulus cell apoptosis

The presence of apoptotic cells was detected by terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end-labelling (TUNEL) in 41 COCs in total ($n = 10, 11, 10$ and 10 in Groups 0E, 100E, 200E and 400E respectively) after IVM. Strand DNA breaks that had occurred during the cell apoptosis process were detected using an *In Situ* Cell Death Detection Kit, POD (Roche Diagnostics SL, Applied Science), as described previously (Arias-Álvarez *et al.* 2010, 2017). COCs were counterstained with $0.25 \mu\text{g mL}^{-1}$ propidium iodide (PI) for 15 min at 37°C , and mounted between a coverslip and glass slide in incubation in mounting solution (ProLong Gold Anti-fade Reagent; Invitrogen). Samples were examined with laser scanning confocal microscopy (TCS SP2; Leica) using a 488-nm excitation laser to visualise TUNEL-positive cells and a 546-nm excitation laser to assess red fluorescence. Images were taken every $5 \mu\text{m}$ and then analysed individually using ImageJ software (<http://rsbweb.nih.gov/ij/>, verified 13 June 2018). The apoptosis index was calculated as the ratio of green (TUNEL-positive) to red (PI-positive) areas multiplied by 100. Maximal projection was obtained with all sections taken in each COC to visualise the whole apoptotic area.

RNA extraction and reverse transcription in COCs

The abundance of mRNAs was analysed in 203 COCs ($n = 51, 50, 50$ and 52 in Groups 0E, 100E, 200E and 400E respectively) as previously described (Bermejo-Alvarez *et al.* 2010). Briefly, CCs were mechanically removed by gentle repeated pipetting, and groups of 10–12 oocytes and their corresponding CCs were analysed separately. Both oocytes and CCs were snap frozen in liquid nitrogen and stored at -80°C . Poly(A) RNA was prepared using the Dynabeads mRNA Direct Extraction Kit (DynaL Biotech, Invitrogen) according to the manufacturer's instructions with minor modifications (see Arias-Álvarez *et al.* 2013, 2017). The reverse transcription (RT) reaction (Bioline, Eco-gen) was performed using poly(T) primer, random primers and Moloney murine leukaemia virus reverse transcriptase enzyme (MMLV high performance reverse transcriptase; Epicentre Biotechnologies) in a total volume of $40 \mu\text{L}$ to produce cDNA. Tubes were heated to 70°C for 5 min to denature the secondary RNA structure. RT was completed with the addition of 50 units of reverse transcriptase. Then, the tubes were incubated at 25°C for 10 min to induce annealing of random primers, at 37°C for 60 min to allow for RNA retrotranscription and at 85°C for 5 min to denature the enzyme.

Quantitative real-time polymerase chain reaction in COCs

The mRNA transcripts were quantified using real-time quantitative RT-polymerase chain reaction (qRT-PCR), as described previously (Arias-Álvarez *et al.* 2013, 2017). The level of expression of each transcript is given relative to that of the housekeeping gene H2A histone family member Z (*H2AFZ*). Briefly, all qPCR were performed in duplicate on a Rotorgene 6000 Real Time Cyclor (Corbett Research) by adding $2 \mu\text{L}$ of each sample to the PCR mix (GoTaq qPCR Master Mix; Promega) containing specific primers to amplify the housekeeping gene and genes related to important events in oocyte maturation, namely *SOD2*, *GPX1*, *CAT*, *TP53*, *CASP3*, *AKT1* and *G6PD*. *GJA1* and *PTGS2* were quantified only in CCs because their expression is related to cell-to-cell communication and cumulus expansion (Feuerstein *et al.* 2007; Shao *et al.* 2016). Primer sequences and the approximate sizes of the amplified fragments of all transcripts are given in Table 1. Each pair of primers was previously validated (Arias-Álvarez *et al.* 2017) to achieve efficiencies close to 1, and the comparative cycle threshold (CT) method was used to quantify expression levels as described by Schmittgen and Livak (2008). In each cycle, fluorescence was acquired at a temperature above the melting temperature of primer dimers to avoid primer-dimer artefacts. The cycle during the log-linear phase of the reaction at which fluorescence increased above background (threshold cycle) was determined for each sample. Within this region of the amplification curve, a difference of one cycle is equivalent to a doubling of the amplified PCR product. According to the comparative CT method, the ΔCT value was calculated by subtracting the CT value for the housekeeping gene (endogenous control) from the CT value for each gene. The $\Delta\Delta\text{CT}$ value was determined by subtracting the highest treatment ΔCT value from all other ΔCT values. Fold changes in the relative gene expression of the target were calculated using the formula $2^{-\Delta\Delta\text{CT}}$.

Assessment of the developmental competence of oocytes

The developmental competence of oocytes matured *in vitro* with $100 \mu\text{M}$ α -tocopherol was evaluated by IVF and IVC procedures as described previously (Arias-Álvarez *et al.* 2017).

IVF was performed using a pool of heterospermic fresh semen collected using an artificial vagina from five adult studs. The sperm samples were diluted 1:50 in fertilisation medium (Tyrode's medium with 2 mM sodium bicarbonate, 36 mM sodium lactate, 1 mM sodium pyruvate, 0.01% BSA and 10 mg mL^{-1} heparin), washed with fertilisation medium and centrifuged twice at $1000g$ for 5 min at room temperature each time.

Afterwards, spermatozoa underwent a swim-up procedure in 2 mL Tyrode's medium for 20 min under an atmosphere of 5% CO_2 in air with maximum humidity at 38°C . The spermatozoa recovered from the supernatant were incubated for a further 6 h under the same conditions. Then, IVM and *in vivo*-matured oocytes were coincubated with spermatozoa at a final concentration of 1×10^6 spermatozoa mL^{-1} for 4 h at 38°C in four-well dishes containing 50 COCs in 500 mL Tyrode's

Table 1. Oligonucleotide sequences used for amplification of genes of interest in rabbit (*Oryctolagus cuniculus*) oocytes and cumulus cells by quantitative reverse transcription–polymerase chain reaction

Abbreviations: bp, base pairs; *H2AFZ*, H2A histone family member Z; *GJA1*, gap junction protein, alpha 1, 43 kDa; *PTGS2*, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase); *AKT1*, cellular cycle V-Akt murine thymoma viral oncogene homologue 1; *G6PD*, glucose-6-phosphate dehydrogenase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *SOD2*, superoxide dismutase 2, mitochondrial; *GPX1*, glutathione peroxidase 1; *CAT*, catalase; *TP53*, apoptosis tumour protein 53; *CASP3*, caspase 3, apoptosis-related cysteine protease

Gene	Primer sequence (5'–3')	Fragment size (bp)	GenBank Accession no.
<i>H2AFZ</i>	Forward: AGGACGACTAGCCATGGACGTGTG Reverse: CCACCACCAGCAATTGTAGCCTTG	212	NM_016750
<i>GJA1</i>	Forward: TGCCTTTCGTTGTAACACTCA Reverse: AGAACACATGAGCCAAGTACA	142	NM_001198948.1
<i>PTGS2</i>	TCCAAGCTGGCCTCACTGATGG AGCATGTGTGTGGCCCGACTTG	169	NM_001082388.1
<i>AKT1</i>	Forward: CACCACTGGATTCTCTGCCT Reverse: GGTACCGTTTGTGACTGTGC	230	XM_008257300.1
<i>G6PD</i>	Forward: CTGATCCTGGGTCGCTTCAT Reverse: ACGTACATGGGCACAAAACCA	68	NM_174602.2
<i>GADPH</i>	Forward: CGCTGGGACGGGGTGCCCTTCATC Reverse: CGCCAGGCCTCCCGCAGTTCATCA	347	XM_583628.4
<i>SOD2</i>	Forward: GCTTACAGATTGCTGCTTGT Reverse: AAGGTAATAAGCATGCTCCC	101	S67818.1
<i>GPX1</i>	Forward: CTGCCC GCCTGGGAAATGCTGT Reverse: CAGTCTCTCCTCCCGGGCTGG	328	NM_001034349.2
<i>CAT</i>	Forward: TGA CTGTTGCTGGAGACTGG Reverse: TGTGCTTCTTCCTGTCGATG	250	NM_001076085.1
<i>TP53</i>	Forward: GTGCTGACCAAGGACACGGC Reverse: CTGCACCAGGGCAGACCAGC	223	NM_001082404.1
<i>CASP3</i>	Forward: ACCCAGAAAGACTGTGGATGG Reverse: AYGCTGCTTCACCACTTC	247	BC102589

medium per well. After coincubation, presumptive zygotes were denuded by gentle pipetting and cultured in TCM-199 supplemented with 20% FCS. Then, groups of 25 zygotes were placed in 25- μ L droplets of culture medium overlaid with mineral oil and incubated for 96 h under an atmosphere of 5% CO₂, 5% O₂ in air with a maximum humidity at 38.5°C. Differences in oocyte developmental competence between *in vivo*-matured and IVM oocytes were scored based on embryo development at 24, 48, 72 and 96 h post-insemination (h.p.i.). Embryos were classified on the basis of conventional morphological criteria according to their developmental stage following the guidelines of the International Embryo Transfer Society (www.iets.org, verified 13 June 2018). Morulae and blastocyst rates were expressed as a percentage of the total number of cleaved zygotes.

Statistical analysis

Data were analysed using SPSS ver. 19s (IBM, SPSS Inc.). The Shapiro–Wilk test was used to assess normality. MII rate, mitochondrial patterns and *in vitro* early embryo development were compared using Pearson's Chi-squared test. Comparisons of the apoptosis rate, ERK1/2 phosphorylation and gene expression between groups were made using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test. Non-parametric samples were analysed by the Kruskal–Wallis test. All data are expressed as the mean $\pm$ s.e.m. Differences were considered significant at two-tailed $P < 0.05$.

Results

Nuclear maturation and ERK1/2 phosphorylation in oocytes

The MII rate increased in the 100 μ M α -tocopherol-supplemented compared with control group, but there were no significant differences in MII rate between the 0E, 100E, 200E and 400E groups (63.4%, 81.2%, 65.0% and 77.8% respectively). ERK1/2 activation was evaluated in oocytes and their corresponding CCs. The ratio of pERK1/2 to total ERK1/2 in CCs and oocytes did not show significant concentration-dependent changes following antioxidant supplementation of the culture medium during IVM (Fig. 1).

Mitochondrial relocation in the oocyte

Mitochondrial migration patterns are compatible with cytoplasmic oocyte maturation, with diffuse patterns compatible with cytoplasmically immature oocytes in the rabbit. As indicated in Table 2, the percentage of oocytes with a non-migrated pattern was significantly lower in the 100E group compared with all other α -tocopherol concentrations tested ($P < 0.05$). The 200E group showed a lower percentage of migrated patterns compared with the other groups ($P < 0.05$) and the highest rate of non-migrated oocytes ($P < 0.05$), due primarily to an increase in the DH pattern ($P < 0.05$). Groups 0E and 400E showed similar results.

Fig. 1. Effect of different concentrations of α -tocopherol in the IVM medium on extracellular signal-regulated kinase (ERK) 1/2 activation of rabbit cumulus–oocyte complexes. Kinase activation after each treatment is shown as the ratio of phosphorylated (p) to total ERK1/2 in (a) cumulus cells and (b) oocytes matured in the presence of 0, 100, 200 or 400 μ M α -tocopherol (0E, 100E, 200E and 400E respectively). Data are the mean $\pm$ s.e.m.

Table 2. Mitochondrial migration patterns observed in rabbit (*Oryctolagus cuniculus*) oocytes after IVM with 0, 100, 200 and 400 μ M α -tocopherol (Groups 0E, 100E, 200E and 400E respectively)

Within columns, different letters indicate significant differences ($P < 0.05$). MC, migrated clustered; MH, migrated homogeneous; DC, diffused clustered; DH, diffused homogeneous

	0E	100E	200E	400E
No. of oocytes	46	16	20	18
Migrated (%)	54.3 ^a	75.0 ^a	30.0 ^b	44.4 ^a
MC (%)	15.2	50.0	20.0	27.8
MH (%)	39.1	25.0	10.0	16.7
Non-migrated (%)	45.7 ^a	25.0 ^b	70.0 ^c	55.6 ^a
DC (%)	19.6	12.5	10.0	22.2
DH (%)	26.1 ^a	12.5 ^a	60.0 ^b	33.3 ^a

Apoptotic rate in cumulus cells

All concentrations of α -tocopherol added to the IVM medium significantly reduced the mean apoptotic rate in CCs compared with the control group without antioxidant ($P < 0.05$). TUNEL- and PI-positive areas in COCs are shown in Fig. 2.

Abundance of quality-related gene transcripts in COCs

In oocytes, genes related with the antioxidant response, such as *SOD2* and *CAT*, were significantly reduced when the IVM medium was supplemented with 100 μ M α -tocopherol compared with the control group without antioxidant (Fig. 3; $P < 0.05$). In addition, the abundance of *TP53* mRNA (related to DNA damage and the apoptosis cascade) was downregulated in oocytes in Group 100E compared with those in Group 0E ($P < 0.05$).

The relative abundance of the genes studied in CCs is shown in Fig. 4. *SOD2* mRNA expression was significantly lower in Group 100E than in the control group ($P < 0.05$), whereas *SOD2* mRNA expression in the rest of the groups was intermediate. Expression of *CASP3*, associated with activation of the apoptotic cascade, in CCs was significantly downregulated in groups

with intermediate concentrations of the antioxidant (100E and 200E) compared with Groups 0E and 400E ($P < 0.05$). *TP53* gene expression was slightly lower in Group 100E than Group 0E ($P = 0.07$). The relative abundance of genes related to cumulus expansion, namely *GJA1* and *PTGS2*, tended to down-regulated ($P = 0.1$) after the addition of α -tocopherol compared with the control group without antioxidant. The relative abundance of the other transcripts in oocytes and their CCs was similar among experimental groups.

Developmental competence of IVM oocytes with α -tocopherol

As indicated in Table 3, the kinetics of early embryo development were affected by embryo origin (*in vitro* or *in vivo*) and the IVM medium used. Both cleavage rate after IVF and blastocyst rate increased significantly ($P < 0.01$) in embryos derived from ovulated oocytes compared with those derived from IVM oocytes. No differences in cleavage rate were found between groups of *in vitro*-derived oocytes. However, when α -tocopherol was included in the serum-free IVM medium (Group 100E), a higher number of morulae was found at 48 and 72 h.p.i. ($P = 0.06$ and $P < 0.01$ respectively), compared with the IVM group without α -tocopherol (0E). Blastocyst percentage was similar between both groups (see Fig. S1, available as Supplementary Material to this paper).

The use of an IVM medium with FCS and growth factors improved the kinetics of early embryo development *in vitro*. The percentage of early morulae at 48 h.p.i. ($P = 0.06$), morulae at 72 h.p.i. ($P < 0.01$) and compacted morulae at 96 h.p.i. ($P = 0.06$) was enhanced in the 0E-FCS compared with 0E group. However, when α -tocopherol was included in the IVM medium enriched with FCS and growth factors (100E-FCS), blastocyst yield was significantly improved ($P < 0.01$) compared with the IVM group without α -tocopherol (0E-FCS) and with both IVM groups without FCS (100E and 0E).

Discussion

The present study provides a novel description of some important nuclear and cytoplasmic events associated with the maturation process in oocytes and their surrounding cumulus cells when the IVM medium is supplemented with different concentrations of α -tocopherol. The results indicate that α -tocopherol can regulate cell function during oocyte maturation and protect against the harmful effects of ROS produced by IVM. Under the conditions in the present study, α -tocopherol supported oocyte nuclear and cytoplasmic maturation. It also reduced the apoptotic rate in CCs and induced changes in mRNA transcripts related to antioxidant response, cell cycle regulation and the apoptotic cascade and cumulus expansion in COCs, thereby improving the blastocyst rate after IVF and IVC.

In accordance with the unchanged pattern of nuclear maturation rate, ERK1/2 phosphorylation was not substantially modified in oocytes matured with in the presence of different concentrations of α -tocopherol. Meiotic maturation of mammalian oocytes (transition to MII) goes together with changes in the protein phosphorylation pattern (Kastrop *et al.* 1990; Gall *et al.* 1993). One of the most important kinases or kinase

Fig. 2. (a) Apoptotic rate in rabbit cumulus cells after IVM with 0, 100, 200 or 400 μ M α -tocopherol (0E, 100E, 200E and 400E respectively). Data are the mean $\pm$ s.e.m. Different letters above columns indicate significant differences ($P < 0.05$). (b–e) Cumulus cell apoptosis visualised by the terminal deoxynucleotidyl transferase-mediated dUTP–digoxigenin nick end-labelling (TUNEL) assay in rabbit cumulus–oocyte complexes (COCs) after IVM. (b) All cell nuclei are stained red with propidium iodide in the mid section; (c) TUNEL-positive (green) staining of cells considered apoptotic in the mid section; (d) maximal projection obtained with all sections acquired in each COC showing the nuclei of all cumulus cells stained with propidium iodide; (e) maximal projection of the same COCs showing TUNEL-positive labelled cells considered apoptotic. Photographs were taken under a $\times 40$ objective.

Fig. 3. Relative poly(A) mRNA abundance of seven candidate genes related to the cell cycle (cellular cycle V-Akt murine thymoma viral oncogene homologue 1 (*AKT1*)), glucose metabolism (glucose-6-phosphate dehydrogenase (*G6PD*)), oxidative response (superoxide dismutase 2, mitochondrial (*SOD2*), glutathione peroxidase 1 (*GPX1*), catalase (*CAT*)) and apoptosis (apoptosis tumour protein 53 (*TP53*), caspase 3, apoptosis-related cysteine protease (*CASP3*)) in rabbit oocytes after IVM with different concentrations of α -tocopherol (0, 100, 200 or 400 μ M; Groups 0E, 100E, 200E and 400E respectively). Data are the mean $\pm$ s.e.m. Different letters above columns indicate significant differences ($P < 0.05$).

cascades supporting this process is ERK, as well as the kinase Akt. ERK1/2 act as intrafollicular mediators to stimulate CC expansion and play a key role in the acquisition of nuclear oocyte competence (Motlik *et al.* 1998; Duggavathi and Murphy 2009; Fan *et al.* 2009). In contrast, Akt is involved in the transition from MI to MII, polar body extrusion and organisation of microtubules (Hoshino and Sato 2008). The similar percentage of MII achieved and ERK1/2 phosphorylation observed in all experimental groups could be due to the effect of the similar concentrations of EGF included in the IVM medium, because EGF can activate ERK1/2 phosphorylation to support oocyte maturation and cumulus expansion (Fan *et al.* 2009). These results were confirmed with similar

AKT1 mRNA expression in CCs and oocytes. Therefore, the findings of the present study suggest that this natural antioxidant does not improve meiosis throughout IVM, as reported previously in COCs from rat (Takami *et al.* 1999), cattle (Dalvit *et al.* 2005) and sheep (Adelrud *et al.* 2015).

In the present study, two genes related to the cumulus expansion process were evaluated (*PTGS2* and *GJA1*). COCs matured *in vitro* with α -tocopherol showed slightly lower *GJA1* and *PTGS2* gene expression than the control group. *GJA1*, also known as connexin 43, has been proposed as a major mediator of cell-to-cell communication via gap junctions (Gittens and Kidder 2005). As demonstrated previously, downregulation of *GJA1* in CCs after oocyte maturation is associated with cumulus

Fig. 4. Relative poly(A) mRNA abundance of eight candidate genes related to cumulus expansion (gap junction protein, alpha 1, 43 kDa (*GJA1*) and prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclo-oxygenase) (*PTGS2*)), cell cycle (cellular cycle V-Akt murine thymoma viral oncogene homologue 1 (*AKT1*)), glucose metabolism (glucose-6-phosphate dehydrogenase (*G6PD*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*)), oxidative response (superoxide dismutase 2, mitochondrial (*SOD2*), glutathione peroxidase 1 (*GPX1*)) and apoptosis (apoptosis tumour protein 53 (*TP53*), caspase 3, apoptosis-related cysteine protease (*CASP3*)) in rabbit cumulus cells after IVM with different concentrations of α -tocopherol (0, 100, 200 or 400 μ M; Groups 0E, 100E, 200E and 400E respectively). Data are the mean $\pm$ s.e.m. Different letters above columns indicate significant differences ($P < 0.05$). Data with * differs at $P = 0.1$, and with $\dagger$ differs at $P = 0.07$.

Table 3. Early development of rabbit (*Oryctolagus cuniculus*) embryos from oocytes after IVM in medium containing fetal calf serum (FCS) or not and supplemented with or without 100 μ M α -tocopherol (100E and 0E (control) respectively)

Unless indicated otherwise, data are given as n (%). Within columns, different letters indicate significant differences ($P < 0.05$); * indicates significant differences at $P = 0.06$

	Presumptive zygotes (n)	Total cleaved at 24 h	Early morulae at 48 h	Morulae at 72 h	Compacted morulae at 96 h	Blastocysts at 96 h
<i>IVM COCs</i>						
0E	128	25 (19.5)a	0 (0.0)a*	0 (0.0)a	3 (12.0)a*	0 (0.0)a
100E	132	26 (19.7)a	4 (15.38)a*	6 (23.0)b	6 (23.1)a	2 (7.6)a
0E-FCS	128	24 (18.8)a	4 (16.6)a	8 (33.3)b	9 (37.5)a*	3 (12.5)a
100E-FCS	127	28 (22.0)a	1 (3.5)a	6 (21.4)b	7 (25.0)a	5 (17.8)b
<i>In vivo</i> -matured COCs	103	48 (46.6)b	28 (58.3)b	36 (75.0)c	32 (66.6)b	13 (27.1)c

expansion and reflects higher oocyte developmental competence in different species (Edry *et al.* 2006; Feuerstein *et al.* 2007; Shao *et al.* 2016; Arias-Álvarez *et al.* 2017). In contrast, *PTGS2* is the key enzyme in prostaglandin E biosynthesis and is involved in the regulation of COC expansion during the ovulatory process in mice (Davis *et al.* 1999; Jang *et al.* 2015). *PTGS2* mRNA abundance in CCs is positively correlated with *in vitro* oocyte quality in bovine (Assidi *et al.* 2008) and human (McKenzie *et al.* 2004), but not rabbit (Arias-Álvarez *et al.* 2017) COCs.

Mitochondrial activity and distribution are also associated with oocyte developmental competence, because mitochondria are the major intracellular source of energy through ATP production by oxidative phosphorylation and produce enzymes involved in the antioxidant defence of the cell. Analysis of mitochondrial migration revealed that 100 μ M α -tocopherol reduced the percentage of mitochondrial patterns, consistent

with cytoplasmically immature oocytes in rabbit. Abnormal dynamics of mitochondrial reorganisation in the maturing oocyte are related to anomalous ATP content (Zhuang *et al.* 2012), mitochondrial dysfunction and increased ROS concentrations, and subsequently to cell damage (Torner *et al.* 2004; Lord and Aitken 2013). The primary ROS generated by the mitochondria, superoxide anion ($\cdot\text{O}_2^-$), is converted by superoxide dismutase (*SOD2*) enzymes into H_2O_2 (Kowaltowski *et al.* 2009), followed by CAT transformation of H_2O_2 into water and oxygen (Khan *et al.* 2015). The results of the present study show that the inclusion of 100 μ M α -tocopherol reduces *SOD2* and *CAT* poly(A) mRNA abundance in oocytes compared with the control group. *SOD* and *CAT* activities are lower in oocytes with lower levels of oxidative stress (Waiz *et al.* 2016). In fact, in mice and human MII oocytes, no *CAT* transcripts have been detected (El Moutassim *et al.* 1999), and downregulation of *SOD2* mRNA transcripts is associated with higher oocyte

developmental competence (Arias-Álvarez *et al.* 2017). In contrast, it has been reported that oocytes (Khalil *et al.* 2013) and embryos (Marques *et al.* 2018) with a better antioxidant defence show higher levels of endogenous antioxidant enzymes. These apparent discrepancies could be due to the addition of different types of antioxidants to the IVM medium and differences between species. In the present study, *SOD2* gene expression was downregulated in CCs from the group supplemented with 100 µM α -tocopherol compared with the group without antioxidant. This is in contrast with previous results in rabbit oocytes (Arias-Álvarez *et al.* 2017), in which increased *SOD2* gene expression was related to a higher developmental competence. We hypothesise that the presence of α -tocopherol in the IVM medium may enhance extrinsic antioxidant protection, which could possibly reduce the synthesis of endogenous antioxidant enzymes, such as *SOD2*, in CCs to basal levels to maintain the redox equilibrium. Future work should determine additional pathways to elucidate the mechanism of action of antioxidant protection on CCs.

The lower abundance of *TP53* poly(A) transcripts in oocytes from Group 100E compared with Group 0E also supports the notion that this concentration of α -tocopherol could protect the oocyte during maturation in rabbits. *TP53* is involved in cell cycle regulation and DNA repair, leading to cell cycle arrest and apoptosis by regulating genes such as *CASP3*. The downregulation of both *TP53* and *CASP3* in CCs following the addition of 100 µM α -tocopherol to the IVM medium, together with the reduced apoptotic rate observed in this group, may suggest a higher quality of IVM CCs with an intermediate concentration of α -tocopherol. Earlier studies found that α -tocopherol could prevent follicular cells from undergoing apoptosis (Martín-Romero *et al.* 2008; Tao *et al.* 2010; Asadi *et al.* 2012), but this effect depended on the concentration. In ruminants, higher concentrations of α -tocopherol in the maturation medium decreased cleavage rates (Adeldust *et al.* 2015). In the present study, a significant increase in *CASP3* gene expression was observed in Group 400E, but there was no increase in the apoptotic rate in these CCs. Previous studies have shown higher *CASP3* gene expression is associated with a greater apoptotic rate (Yuan *et al.* 2005). However, activation of the caspase cascade is a previous step to morphological alterations visualised by TUNEL (for a review, see Savitskaya and Onishchenko 2015).

Changes in oocyte quality markers seen following 100 µM α -tocopherol supplementation resulted in a better embryo production *in vitro*. The percentage of morulae increased when α -tocopherol was included in the IVM medium. However, these results only appeared to be consistent when α -tocopherol was included in our previously tested IVM medium with FCS and growth factors (Lorenzo *et al.* 1996; Arias-Álvarez *et al.* 2010), with which a better blastocyst yield was found. Although FCS may contain a variety of compounds with antioxidant properties, present study demonstrates that embryo production was significantly enhanced only when α -tocopherol was added to the FCS-enriched IVM medium. These results are consistent with several reports of improvements in embryo development after the inclusion of antioxidants during IVM (Thiyagarajan and Valivattan 2009; Tao *et al.* 2010; Tareq *et al.* 2012; Farzollahi *et al.*

2016) or IVC (Olson and Seidel 2000; Natarajan *et al.* 2010). In addition, SCNT outcome is higher in the presence of α -tocopherol, because it enhances cytoplasmic maturation of recipient oocytes, increases blastocyst formation and blocks DNA damage in transgenic embryos (Wongsrikeao *et al.* 2007). Recently, Yashiro *et al.* (2015) demonstrated the beneficial effects of α -tocopherol on the revivability of vitrified-warmed oocytes, in which both blastocyst rate and quality were improved. However, some findings regarding the inclusion of antioxidants are controversial, because other studies have reported that antioxidant supplementation during IVM does not affect cleavage rates and embryo development towards the blastocyst stage (Dalvit *et al.* 2005; Adeldust *et al.* 2015), even though a decreased apoptotic rate was reported. This discrepancy may be related to the animal species used, the percentage oxygen tension during IVM, the source of oocytes or the composition of the maturation media used.

Conclusions

This study indicates that supplementation of the IVM medium with 100 µM α -tocopherol improves oocyte quality in different ways: (1) it modifies mRNA transcripts, which reflect reduced oxidative stress (*SOD2* and *CAT*), cell damage and apoptotic cascade activation (*TP53* and *CASP3*) in oocytes and CCs; (2) it decreases the apoptotic rate in CCs; (3) it reduces the rate of cytoplasmically immature oocytes; and (4) it increases oocyte developmental competence, because early embryo development *in vitro* was improved. We propose that the presence of α -tocopherol in rabbit IVM medium may reduce *in vitro*-derived oxidative stress in non-damaged rabbit oocytes. However, the blastocyst rate for embryos derived from IVM oocytes remains lower than that for embryos from *in vivo*-matured oocytes. Further work is warranted in order to explore the modulation of intracellular ROS production with a view to improving the IVM medium in this species.

Conflicts of interest

The authors declare no conflicts of interest.

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