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Combinations of Trolox and ascorbic acid have a beneficial effect on *in vitro* maturation of pig oocytes

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Abstract: The poor *in vitro* development of pig oocytes and embryos has been blamed on oxidative stress. We sought to find out if combinations of Trolox (T), a synthetic and cell-permeable derivative of vitamin E, and ascorbic acid (AA) could improve the maturation rates of *in vitro* cultured pig oocytes. Pig oocytes underwent maturation for 44–45 h in medium M 199 supplemented with 0 μM T + 0 μM AA, 100 μM T + 250 μM AA, 300 μM T + 250 μM AA, 100 μM T + 750 μM AA or 300 μM T + 750 μM AA. These combinations were chosen based on previous research conducted in our laboratory and on the available literature. After maturation several parameters were assessed: cumulus oophorus expansion, oocyte viability (based on the presence of metabolic activity versus membrane damage), extrusion of the first polar body, mitochondrial membrane potential (MMP), pronucleus formation and embryo development after fertilization. All antioxidant combinations significantly improved cumulus expansion and formation of the first polar body. The best were 300 μM T + 250 μM AA for the first characteristic and 300 μM T + 750 μM AA for the second. Antioxidant presence in the maturation media increased the percentages of viable oocytes but not significantly. MMP was not significantly modified by the addition of antioxidant combinations. We also found that a low concentration of T (100 μM) mixed with a high concentration of AA (750 μM) in the oocyte maturation media led to significantly higher rates of both female and male pronuclei formation and also enhanced embryo development to the morula stage. Therefore, we recommend this combination to improve the *in vitro* maturation media of pig oocytes.

Keywords: antioxidant; pig female gametes; developmental potential

Although substantial progress has been made to improve the competence of *in vitro* produced pig embryos, there is a lack of consistency in the success rate of *in vitro* matured oocytes compared to *in vivo* matured ones (Khazaei and Aghaz 2017). Oocyte developmental competence is considered a limiting factor for the quality of *in vitro* produced embryos. Oxidative stress, which is the result of an imbalance in the production and elimination of the intracellular reactive oxygen species (ROS), is considered one of the main factors affecting the outcome of *in vitro* embryo production (Soto-Heras and Paramio 2020). Oocytes themselves generate ROS through regular cell metabolism, mainly due to electron leakage during oxidative phosphorylation. The physiological level of ROS is beneficial during folliculogenesis, oocyte maturation and embryogenesis because they act as second messengers in physiological cell signalling and control pathways (Jamil et al. 2020).

In vitro ROS excess is responsible for oocyte mitochondrial dysfunction, aberrant calcium homeostasis and damage to proteins, lipids and DNA (Lord and Aitken 2013) and it triggers apoptosis in the majority of germ cells within the ovary and even in ovulated oocytes (Prasad et al. 2016). Therefore, maintaining balance between ROS production and scavenging is very important to create

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a suitable environment for correct oocyte maturation and embryo development. *In vitro*, this balance can be attained by adding antioxidants to the culture media.

Culture media for oocytes and embryos have been supplemented with many types of antioxidant substances such as resveratrol, melatonin, coenzyme Q10, thiol compounds and vitamins such as C and E (reviewed by Soto-Heras and Paramio 2020).

Vitamin E is represented by a group of lipophilic compounds that includes tocopherols and tocotrienols. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (T) is a synthetic, water-soluble and cell-permeable derivative of vitamin E that could be incorporated both into the water and the lipid compartments of a cell (Hamad et al. 2010). It accumulates in the cell cytosol and can effectively scavenge intracellular radicals in human cells protecting them from damage induced by oxidants (Guo et al. 2012).

The water-soluble ascorbic acid (AA) (vitamin C) reduces potentially damaging ROS and forms the relatively stable ascorbate free radical that can then be reduced back to ascorbate by cell enzymes (Grosso et al. 2013). AA inhibits lipid peroxidation of cellular membranes including those surrounding and those within intracellular organelles, thus preventing cell damage (Ngo et al. 2019). Based on this AA protects ovarian cells from oxidative damage (Kere et al. 2013).

The aim of this research was to investigate the effects of antioxidant combinations on pig oocyte maturation. We combined a water-soluble substance (AA) with one that can access both the water and the lipid cellular compartments (T). To achieve our goal we supplemented the culture medium of cumulus-oocyte complexes (COCs) with four T and AA combinations and after maturation we analysed cumulus oophorus expansion, oocyte viability, extrusion of the first polar body, mitochondrial membrane potential (MMP), formation of the pronuclei and embryo development after fertilization.

MATERIAL AND METHODS

Chemicals

Unless otherwise stated, all chemicals used in the experiment were purchased from Merck KGaA (Darmstadt, Germany).

Culture media

COCs were collected in medium M 199 containing Earle's salts, 5.958 g/l HEPES, 100 IU/ml penicillin and 100 µg/ml streptomycin.

The maturation medium was M 199 containing Earle's salts, 10 IU/ml Chorulon (MSD Animal Health, Madison, NJ, USA), 10 IU/ml Folligon (MSD Animal Health, Madison, NJ, USA), 10% foetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin. The culture medium was supplemented with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid also known as Trolox (T) and ascorbic acid (AA) using the following combinations: 0 µM T + 0 µM AA (CTRL), 100 µM T + 250 µM AA (100T-250AA), 300 µM T + 250 µM AA (300T-250AA), 100 µM T + 750 µM AA (100T-750AA) or 300 µM T + 750 µM AA (300T-750AA). The used concentrations were decided based on published research (Miclea et al. 2010) and unpublished data from previous experiments in our laboratory.

The medium used for fertilization was Tyrode's albumin lactate pyruvate medium (TALP; Coy et al. 2002) supplemented with the antibiotics 75 µg/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin.

The embryo culture medium was NCSU-23 (Petters and Wells 1971) supplemented with 504.27 mg/l sodium lactate, 36.31 mg/1 mM sodium pyruvate, 2 mg/l β-mercaptoethanol, 0.1 mg/ml cysteine, 4 mg/ml bovine serum albumin (BSA), 75 µg/ml penicillin, 50 µg/ml streptomycin, and 50 µg/ml gentamicin.

Collection of COCs and oocyte *in vitro* maturation

Collection of COCs and maturation of oocytes were performed using the method described by Tao et al. (2010) with a few modifications. Pig ovaries were obtained from pre-pubertal gilts at a nearby slaughterhouse (Primacom SRL, Targu Mures, Romania) and transported to the laboratory in sterile saline solution (9 g/l NaCl) at 37 °C supplemented with 100 IU/ml penicillin and 100 µg/ml streptomycin. Follicles between 2 mm and 6 mm in diameter were aspirated with a 10 ml syringe connected to a 16-gauge needle and placed in harvest medium. COCs with uniform ooplasm and compact cumulus cell mass

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were washed twice and distributed in 45 µl droplets of maturation medium (five oocytes in each droplet) covered with paraffin oil and incubated for 44–45 h at 38.5 °C under 5% CO_2 in air (21% O_2).

Assessment of cumulus oophorus expansion

Cumulus oophorus expansion was analysed after 44–45 h using an IX51 Olympus microscope (Olympus Corporation, Tokyo, Japan) for all experimental groups. The employed subjective assessment method scored no response as 0, a minimal response as 1, expansion of outer cumulus layers as 2, expansion of all cumulus layers except the corona radiata as 3, and expansion of all cumulus layers as 4 (Tao et al. 2004). Images of the COCs were taken with a 10× objective using the Cell^R software (Olympus Corporation, Tokyo, Japan) and a score (0 to 4) was assigned to each COC.

Evaluation of oocyte viability and polar body extrusion

COCs were mechanically denuded in phosphate buffered saline (PBS) supplemented with 5 mg/ml BSA. Cells were stained in PBS containing 1 µg/ml 3',6'fluorescein diacetate (FDA), 50 µg/ml propidium iodide (PI), and 20 µg/ml Hoechst 33258, incubated for 10 min and viewed at their respective wavelengths with an IX51 Olympus microscope equipped with a 100 W mercury lamp (Olympus Corporation, Tokyo, Japan). Live oocytes were metabolically active and had green fluorescence when viewed at 488 nm excitation and 530 nm emission (Gupta et al. 2007). Their chromosomes were labelled with Hoechst 33342 being blue at 346 nm excitation and 460 nm emission. Because the loss of plasma membrane integrity is a common event in all forms of cell death (Darzynkiewicz et al. 1997) and propidium iodide can penetrate dead cells which have compromised membrane integrity, it is used as an indicator of this phenomenon. In our experiment dead oocytes were stained with PI, appearing red at 536 nm excitation and 617 nm emission.

Mitochondrial membrane potential

Mitochondrial membrane potential (MMP) was assessed for both mature and immature cells. To obtain immature oocytes COCs were collected from ovaries and processed immediately afterwards in a manner similar to the cultured COCs. Following maturation cumulus cells were removed mechanically as described above and oocytes were incubated in hormone and antioxidant free maturation medium to which 300 nM MitoTracker Green FM (Thermo Fisher Scientific, Loughborough, UK) and 200 nM MitoTracker Orange CMTMRos (Thermo Fisher Scientific, Loughborough, UK) had been added. Incubation time was 30 min at 37 °C in an atmosphere with 5% CO_2 and it was followed by three washes in PBS, each lasting 3 minutes. Oocytes were placed in warm PBS drops, viewed with an IX51 Olympus microscope equipped with a 100 W mercury lamp at 490 nm excitation and 516 nm emission wavelength for MitoTracker Green FM and 554 nm excitation and 576 nm emission for MitoTracker Orange CMTMRos and photographed using Cell* software (Olympus Corporation, Tokyo, Japan). To measure cytoplasmic fluorescence intensity images were analysed with Fiji software (Schindelin et al. 2012). Briefly, the "mean grey value" was measured for both MitoTracker Green FM (total mitochondria) and MitoTracker Orange CMTMRos (active mitochondria) in each analysed oocyte. After subtracting background fluorescence, the ratio between the fluorescence of active and total mitochondria was calculated (Hammond 2014). This represents a reliable measurement of MMP independently of the mitochondrial mass (Pendergrass et al. 2004).

In vitro fertilization and embryo culture

After maturation oocytes were mechanically denuded and 10–15 oocytes were placed in 30 µl droplets of fertilization medium. Semen was collected from a Landrace boar using the manual method. Ejaculates were used (one for each replicate in the fertilization experiment) if motility was scored above 80% and the percentage of morphological abnormalities was below 20%. Sperm capacitation was performed by centrifugation at 800 × *g* for 10 min for three times. After the first two centrifugations sperm cells were re-suspended in Beltsville thawing solution (Pursel and Johnson 1975) and after the third in TALP. Sperm cell concentration was measured immediately after the third centrifugation using a haemocytometer and adjusted at 1×10^6 spermatozoa/ml for fertilization.

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Spermatozoa were added to the TALP droplets and cells were incubated at 37 °C under 5% CO_2 in air (21% O_2). At the end of 16–18 h sperm cells were removed mechanically and the presumed zygotes were cultured in NCSU-23 at 38.5 °C under 5% CO_2 in air (21% O_2). Embryo development was assessed 120 h after fertilization by counting the number of embryos that had reached the 2-cell, 4–8 cell, 8–16 cell and morula stages.

Pronuclei formation

Approximately 10 hours after fertilization presumed zygotes were fixed in methanol/PBS (60%) for at least 1 h at 4 °C. DNA staining was performed with 50 µg/ml PI and cells were assessed using a BX53 Olympus microscope equipped with a 100 W mercury lamp (Olympus Corporation, Tokyo, Japan). DNA appeared red at 536 nm excitation and 617 nm emission and cells with two pronuclei were counted.

Statistical analysis

Five replicates were performed for each experiment and for all of them the various antioxidant combinations were contrasted against each other and the control. One-way ANOVA with the Tukey post-hoc test was performed using GraphPad InStat v3.05 for Windows 95 (GraphPad Software, San Diego, CA, USA). Values were considered statistically significant when $P < 0.05$.

RESULTS AND DISCUSSION

In the present study we investigated the effects of T and AA combinations on pig oocyte developmental competence following maturation. We assessed cumulus oophorus expansion, oocyte viability, formation of the first polar body and MMP, pronucleus formation and embryo development after co-incubation with spermatozoa.

According to the method we employed the 3rd and the 4th stage of expansion are believed to represent fully matured oocytes (Tao et al. 2004). If oocytes scored as 3 and 4 (Figure 1) were counted as one group, average percentages were above 80% for all antioxidant combinations (Table 1). Their presence significantly improved cumulus expansion beyond what the culture medium without antioxidants could support. This improvement ranged from 11.59% for 100 µM T-750 µM AA to 20.15% for 300 µM T-250 µM AA.

When stained with FDA, the cytoplasm of viable oocytes was bright green while cells with membrane damage had red DNA. The presence of blue stained DNA in the first polar body signalled that nuclear maturation had taken place (Figure 2).

Percentages of live oocytes increased in the presence of all four antioxidant combinations but not significantly (Table 1), the most favourable treat-

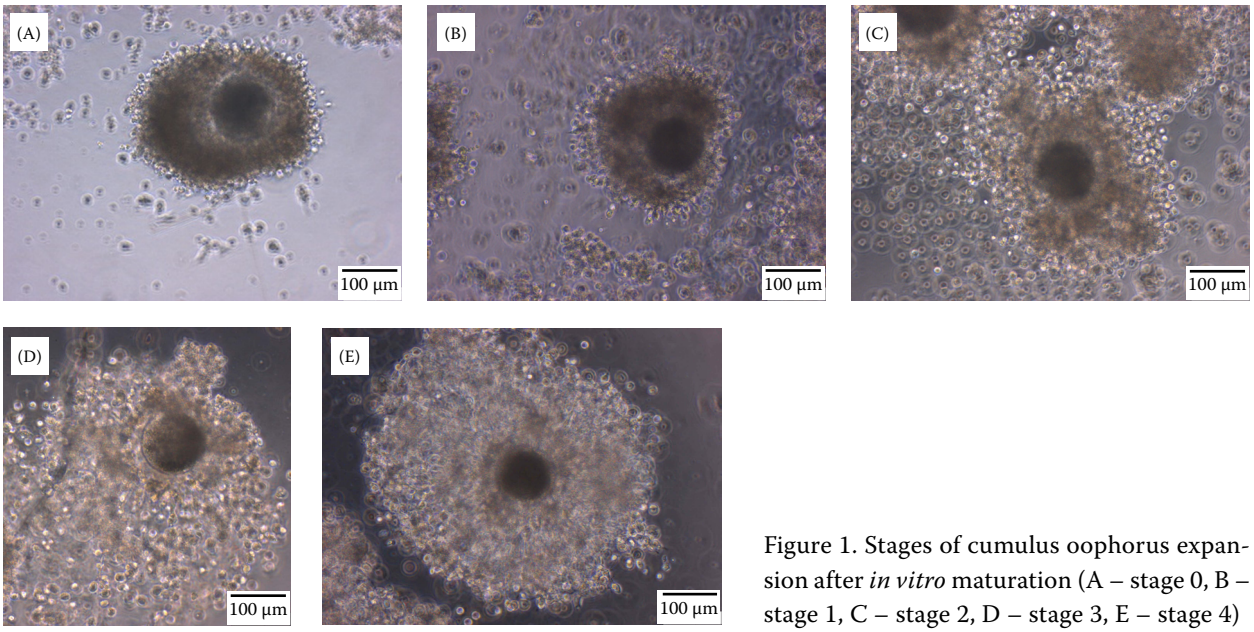

Figure 1. Stages of cumulus oophorus expansion after *in vitro* maturation (A – stage 0, B – stage 1, C – stage 2, D – stage 3, E – stage 4)

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Table 1. Effect of Trolox (T) and ascorbic acid (AA) on cumulus oophorus expansion, oocyte viability and nuclear maturation

Antioxidant combinations	*n*	Expanded cumulus oocyte complexes (%) (3^{rd} + 4^{th} stages)	*n*	Live oocytes (%)	1^{st} polar body (%)
Control	564	68.71 ± 2.24^{a}	265	97.18 ± 0.82	47.65 ± 1.61^{a}
100T-250AA	563	87.28 ± 1.09^{b}	272	98.30 ± 0.57	55.21 ± 1.77^{b}
300T-250AA	578	88.86 ± 1.19^{bc}	314	98.40 ± 0.86	55.60 ± 1.61^{b}
100T-750AA	586	80.30 ± 1.41^{d}	256	98.44 ± 0.32	54.92 ± 1.91^{b}
300T-750AA	606	84.28 ± 2.18^{bcd}	308	99.01 ± 0.99	58.10 ± 1.87^{b}

Values represent the mean ± standard error

$^{a–d}$Within the same column, means not sharing any letter are significantly different ($P < 0.05$)

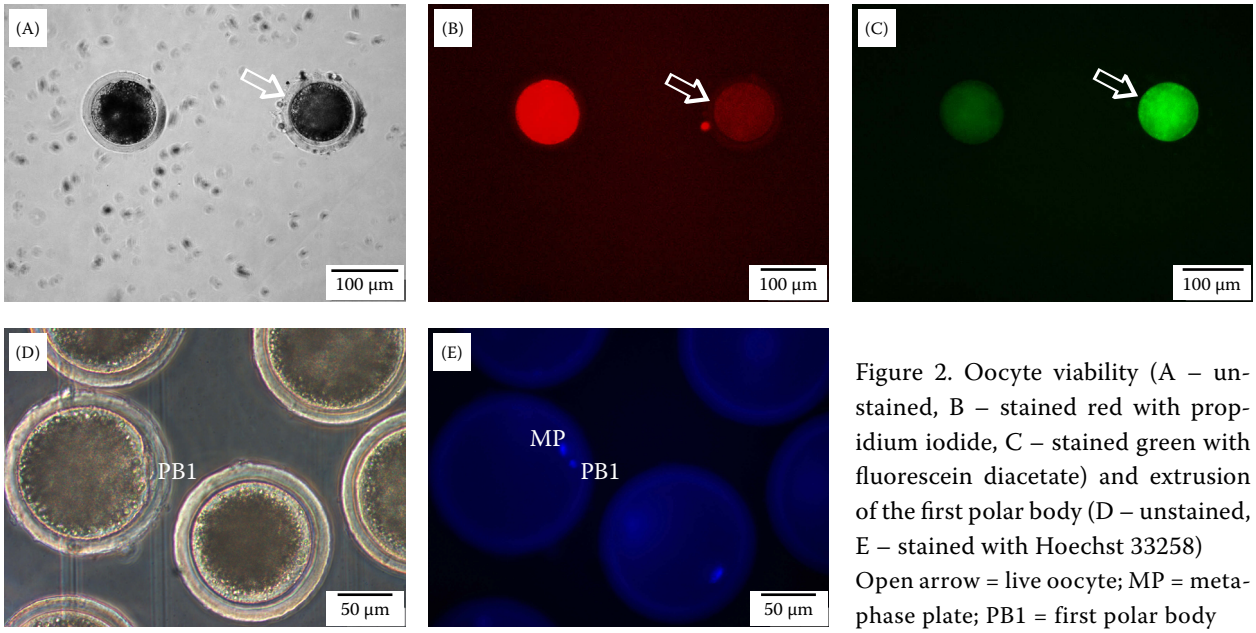

Figure 2. Oocyte viability (A – unstained, B – stained red with propidium iodide, C – stained green with fluorescein diacetate) and extrusion of the first polar body (D – unstained, E – stained with Hoechst 33258)
Open arrow = live oocyte; MP = metaphase plate; PB1 = first polar body

ment being 300 µM T-750 µM AA. The difference between our results and the slight negative alteration in viability encountered by Tao et al. (2004) could be due to the fact that the antioxidant action was modulated by the presence of cumulus cells.

The first polar body was detected for a significantly larger number of oocytes when COCs were cultured together in media supplemented with T and AA. This situation was similar for all investigated combinations and the differences were statistically significant. The highest percentage was scored for 300 µM T-750 µM AA.

Because it is generated by the electron transport, the membrane potential is a direct expression of mitochondrial function and activity but also of cytoplasm quality. In our experiment MMP increased significantly during transition from GVBD stage to MII stage which correlates with increased metabolic activity after the maturation process (Figure 3).

This can be corroborated by the increase in the number of mitochondrial DNA copies during maturation (Mao et al. 2012). A separate statistical analysis was performed to investigate differences between the control and antioxidant combinations without immature oocytes. No statistically significant differences between the control and the antioxidant combinations could be detected but the highest MMP was recorded for 300 µM T-750 µM AA. Antioxidant combinations had a slight influence on this parameter suggesting that their presence did not have a major impact on mitochondrial activity.

Formation of both female and male pronuclei together with embryo development is the best evidence for the quality of oocyte cytoplasm.

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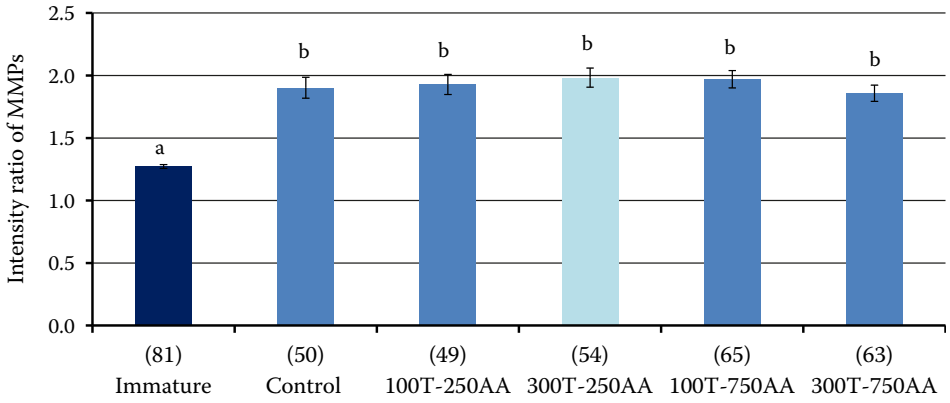

Figure 3. Effect of Trolox (T) and ascorbic acid (AA) on the oocyte mitochondrial membrane potential (MMP)
Data are presented as the mean ± standard error. Values in parentheses represent the number of analysed oocytes
[a,b]Columns not sharing any letter are significantly different ($P < 0.05$)

Formation of two pronuclei was significantly better if oocytes were cultured in antioxidant supplemented media (Figure 4). Among the four treatments the largest percentages were recorded for 100 µM T-750 µM AA.

The rate of embryo formation was better in a medium without antioxidants (Table 2). However, their presence resulted in an increase in the percentage of embryos that reached more advanced stages of development such as 8–16 cells or morula (Figure 5).

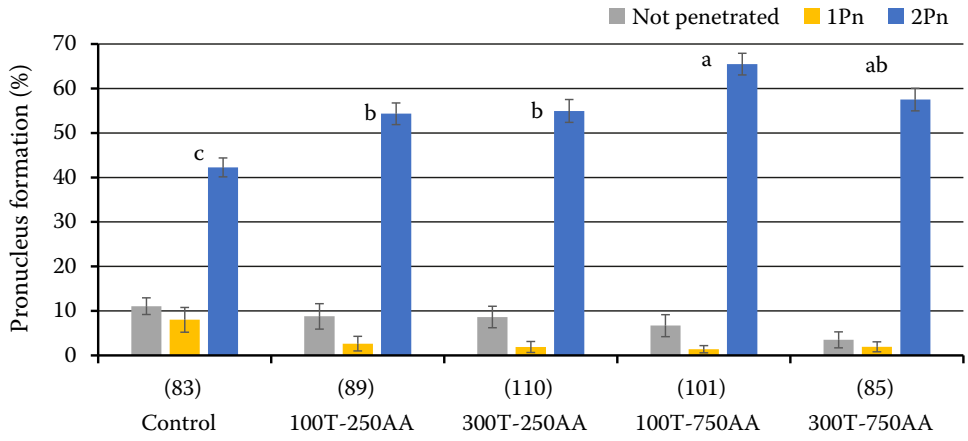

Figure 4. Effect of Trolox (T) and ascorbic acid (AA) on the formation of pronuclei
Data are presented as the mean ± standard error. Values in parentheses represent the number of presumed zygotes
1Pn = one pronucleus; 2Pn = two pronuclei
[a–c]For the same parameter columns not sharing any letter are significantly different ($P < 0.05$)

Table 2. Effect of Trolox (T) and ascorbic acid (AA) on embryo formation and development

Antioxidant combinations	*n*	Embryo formation (%)	2 cells (%)	4–8 cells (%)	8–16 cells (%)	Morula (%)
Control	258	84.86 ± 2.93[a]	6.16 ± 1.99[a]	28.65 ± 5.74[a]	24.20 ± 2.02[a]	41.33 ± 2.52[a]
100T-250AA	200	82.48 ± 3.65[a]	7.00 ± 3.13[a]	21.60 ± 3.45[a]	22.29 ± 0.90[ab]	42.48 ± 2.78[a]
300T-250AA	189	79.91 ± 3.14[a]	3.98 ± 2.17[a]	21.91 ± 4.75[a]	29.36 ± 1.88[a]	46.50 ± 2.83[ab]
100T-750AA	226	79.49 ± 3.89[a]	5.26 ± 1.53[a]	19.11 ± 3.41[a]	16.93 ± 0.89[bc]	58.36 ± 0.98[b]
300T-750AA	270	79.54 ± 3.67[a]	6.91 ± 3.03[a]	27.06 ± 2.87[a]	16.86 ± 1.28[bc]	45.19 ± 4.29[a]

Values represent the mean ± standard error
[a–c]Within the same column, means not sharing any letter are significantly different ($P < 0.05$)

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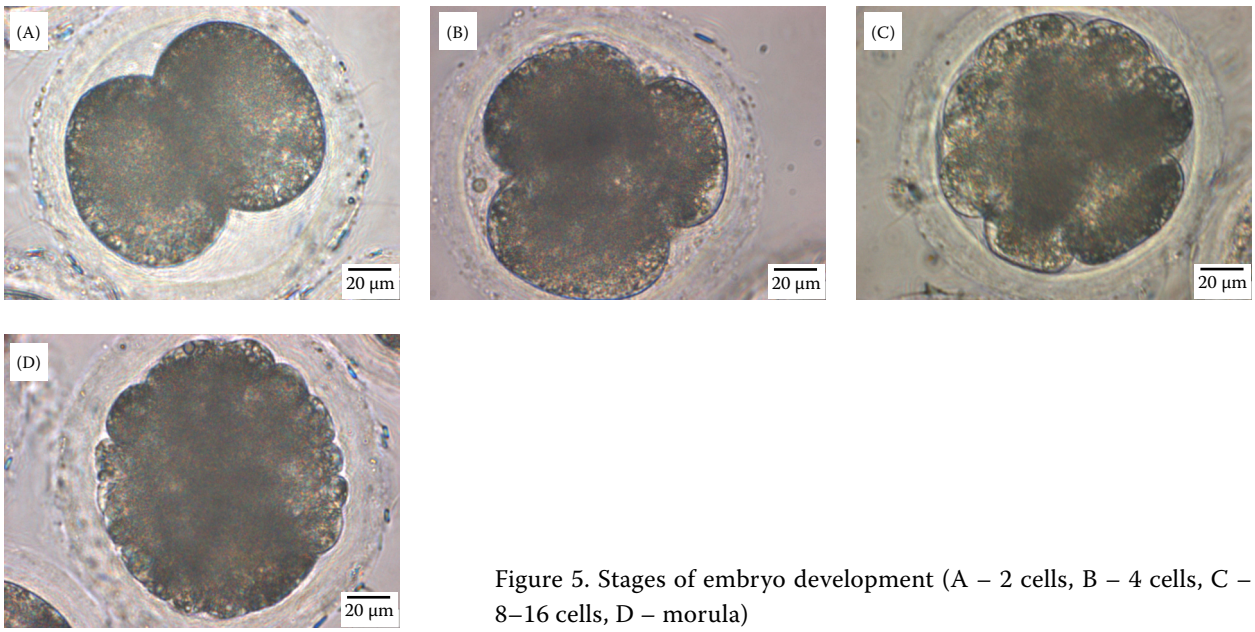

Figure 5. Stages of embryo development (A – 2 cells, B – 4 cells, C – 8–16 cells, D – morula)

Both progression to the MII with extrusion of the first polar body and formation of the male and female pronuclei benefited from antioxidant supplementation. The former is evidence of improved nuclear maturation while the latter could be considered a sign of higher cytoplasmic maturation. These were accompanied by an increased rate of morula formation. The same mixture of antioxidants that supported the formation of both pronuclei (100 µM T-750 µM AA) significantly increased the percentage of embryos that reached the morula stage.

Several studies have investigated the effect of vitamin E or vitamin C on oocytes and embryos in various situations (Tao et al. 2010; Kere et al. 2013) but only a few have concentrated on investigating combinations (Jeong et al. 2006; Hossein et al. 2007).

Given the current interest in vitamins and their involvement in reproduction physiology but also that pig oocytes have been proposed as a research model for human gametes because they are similar in developmental characteristics (Mordhorst and Prather 2017) this research will be useful in understanding and managing both animal and human reproduction.

Our research showed that combinations of T and AA can significantly promote meiotic maturation and developmental competence of porcine oocytes. AA could act as an antioxidant to counteract DNA damage induced by ROS which would lead to the decline of oocyte quality and fertilization ability (Collins et al. 2015) or it could improve pig oocyte meiotic maturation and developmental competence by reprogramming the global methylation status of DNA, histone and RNA (Yu et al. 2018). The antioxidant strength of Trolox is related to its ability to interact with and to penetrate the lipid bilayers (Lucio et al. 2009). It has also been shown to be a powerful hydroxyl and alkoxyl radical scavenger both in aqueous and lipid environments (Alberto et al. 2013). According to Niki (2014), the synergistic interaction of vitamin E and vitamin C is effective for enhancing the antioxidant capacity of vitamin E. The beneficial effect of Trolox and ascorbic acid may be the result of improved culture conditions for cumulus cells and indirectly for oocytes. These translate into better nuclear and cytoplasmic maturation.

CONCLUSION

The addition of Trolox and ascorbic acid during *in vitro* maturation of pig COCs enhanced their nuclear and cytoplasmic maturation. Based on our study, supplementation of culture media with 100 µM Trolox and 750 µM ascorbic acid can improve the culture conditions of pig COCs. Further studies are necessary to fully explain the mechanisms that govern interactions between Trolox and ascorbic acid on the one hand and cell components on the other.

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Conflict of interest

The authors declare no conflict of interest.

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