Alpha-tocopherol and Ascorbic Acid Combinations Influence the Maturation of Sheep Oocytes

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Abstract
The goal of this research was to establish whether supplementation with combinations of α-tocopherol and ascorbic acid could improve the maturation and expansion of sheep cumulus-oocyte complexes. Sheep oocytes were cultured for 24 hours at 37°C in 5.4% CO_2 atmosphere in M199 containing 20 µM α-tocopherol+750 µM ascorbic acid or 5 µM α-tocopherol+250 µM ascorbic acid. Afterwards, *cumulus oophorus* expansion was assessed and oocytes were denuded. The presence of the first polar body was assessed by fluorescent staining with Hoechst 33258. Differences between treatments were analyzed by the analysis of variance and interpreted using the LSD test. Supplementation with the combination of 20 µM α-tocopherol+750 µM ascorbic acid resulted in significantly greater ($p<0.05$) percentages of COCs that were scored as 3. However, the number of COCs scored at 4 decreased. The same dynamic could be seen when oocytes were checked for the presence of the first polar body. Percentages decreased with the increase in antioxidant concentration. This indicates that although antioxidants, in these particular concentrations have been proven to have a positive influence on swine oocyte maturation the same cannot be said for ovine female gametes.

Keywords: α-tocopherol, ascorbic acid, maturation, oocyte, sheep

1. Introduction

Embryo developmental competence is built on the quality of oocyte maturation. Therefore, diminished oocyte developmental competence has been suggested as a primary cause for the reduced potential of in vitro-produced embryos. This has called for intensified efforts to successfully mature and inseminate oocytes in vitro [1, 2].
Although, the in vitro production of mammalian embryos has been greatly improved, it is still less efficient than its in vivo version. That can be in part ascribed to the composition of the oocyte which in sheep contains high levels of lipids (89 µg fatty acids), second only to the pig [3]. Protection of the oocyte lipid components that render them susceptible to oxidative injury may prevent some of the damage currently associated with in vitro culture.
The negative effects of free radicals are cause by endogenous overproduction and exogenous sources that lead to an imbalance in redox metabolism and therefore to oxidative stress.
Cells possess enzymatic as well as non enzymatic systems that can neutralize free radicals. They are also scavenged by exogenous antioxidants such as Cu, Se, Mg, Zn, ascorbic acid (vitamin C) and α-tocopherol (vitamin E) [4].
Tocopherols (vitamin E) are the most important lipid soluble antioxidants in the cell. They protect polyunsaturated fatty acids in membranes against free radicals [5] and improve the development of bovine embryos [6].

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Ascorbic acid is the most important antioxidant outside the cell [7]. It functions as a reducing agent of oxygen and cytocromes c and a, but can also protect membranes against peroxidation.
It is known that ascorbic acid can regenerate α-tocopherol from tocopheroxyl radicals, thereby acting in synergy with α-tocopherol [8]. Although combinations of antioxidants have been previously used in the culture media of mouse [9], buffalo [10] and pig [11, 12] embryos their effect had not been studied in the oocyte maturation environment.
The goal of ours research was to establish whether supplementation with combinations of α-tocopherol and ascorbic acid could improve viability and maturation of sheep oocytes.

2. Materials and methods

Collection and maturation media:
The medium used for harvest was M 199 supplemented with L-glutamine (3.4 g/l), penicillin (100 μg /ml) and streptomycin (100 IU/ml).
For oocyte maturation M 199 was supplemented with L-glutamine (3.4 g/l), sodium pyruvate (2 mM), Chorulon (10 IU/ml), Folligon (10 IU/ml), 10% foetal bovine serum, penicillin (100 μg /ml) and streptomycin (100 IU/ml). Alpha-tocopherol dissolved in 95% ethanol solution was added to the maturation medium in order to arrive at concentrations of 5 and 20 μM. Ascorbic acid dissolved in ultrapure water was added to the same maturation medium in order to arrive at concentrations of 250 and 750 μM. The employed combinations were 5 μM α-tocopherol+250 μM ascorbic acid and 20 μM α-tocopherol+750 μM ascorbic acid.

Oocyte collection and maturation:
Sheep ovaries were collected and transported to the laboratory in a thermal container containing sterile saline solution (NaCl 0.9%) at 37°C supplemented with penicillin (100 μg /ml) and streptomycin (100 IU/ml). The contents of follicles were aspired with a 10 ml syringe equipped with a 21-gauge needle and collected in Petri dishes containing harvest medium. Oocytes with a uniform ooplasm and compact cumulus cell mass were washed 2 times with harvest medium and then placed in 30 μl droplets of maturation medium containing the various antioxidant concentrations. All the droplets were covered in paraffin oil and incubated for 24 hours at 37°C in an atmosphere with 5,4% CO_2. Cumulus oocyte complexes (COCs) were evaluated using an Olympus inverted phase contrast microscope, in order to assess *cumulus oophorus* expansion. Cumulus expansion was assessed by a subjective scoring method [13]. Briefly, no response was scored as 0, minimum observable response as 1, expansion of outer cumulus-enclosed oocyte layers as 2, expansion of all *cumulus*-enclosed oocyte layers except the *corona radiata* as 3, and expansion of all *cumulus*-enclosed oocyte layers as 4. Each group was compared to the control in order to establish whether any differences existed between the degrees of *cumulus* expansion and if they were significant.
After *cumulus* expansion assessment the COCs were transferred to PBS with 5 mg/ml bovine serum albumin (BSA) and mechanically denuded using a micropipette. Then, they were transferred to PBS containing 20 μg/ml Hoechst 33258, incubated for 15 minutes and viewed under ultraviolet illumination with an Olympus inverted microscope. The chromosomes and first polar body of mature oocytes were labelled with the Hoechst 33258, being blue under UV light.
For both experiments differences between treatments were analyzed by the analysis of variance and interpreted using the LSD test and the program Excel (Microsoft). For all comparisons, the values were considered statistically significant when $p < 0.05$.

3. Results and discussion

Our goal was to establish the influence of two α-tocopherol and ascorbic acid concentrations on sheep oocyte maturation.
Maturation was assessed by indirect means such as *cumulus oophorus* expansion (Figure 1) indicating cytoplasmic maturation and direct means, namely fluorescent staining of the first polar body which is believed to be the sign for nuclear maturation.

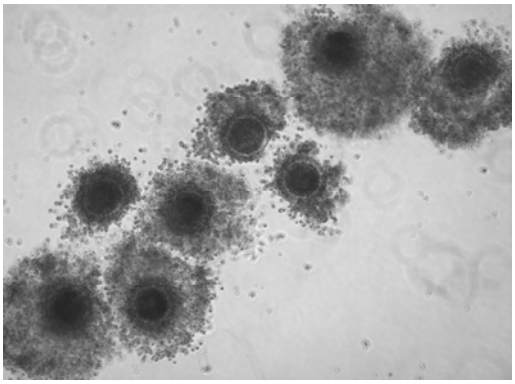

Figure 1. Several stages of cumulus oophorus expansion; magnified 40x.

Cumulus oophorus expansion:
Supplementation with the combination of 20 µM α-tocopherol+750 µM ascorbic acid resulted in significantly greater ($p<0.05$) percentages of COCs that were scored as 3 (Table 1). Their *cumulus oophorus* was expanded, except for the *corona radiata* and they were considered to be mature and ready for fertilization.

Table 1. Percentages of COCs at each stage of cumulus expansion after culture in medium .supplemented with antioxidant combinations

Treatment	Number of COCs	Percentage of COCs at each stage of cumulus expansion				
		0	1	2	3	4
Control	154	0	2.60	16.88	45.45	35.06
E5+C250	177	0.56	4.52	12.99	45.76	36.16
E20+C750	149	0	7.38	18.12	50.34 (*)	24.16

* - significant and positive ($p<0.05$)

This was accompanied by a decrease below control level in the percentages of COCs scored at 4, meaning a completely expanded *cumulus oophorus*.
The other antioxidant combination did not prove as effective as it induced only a small increase in percentages of COCs scored at both 3 and 4.
Antioxidant mixes increased percentages of COCs in categories 1 and 2 above what could be seen for the control, but not significantly. When correlated with the other two expansion stages this suggests a slightly detrimental effect of both mixtures on sheep oocytes.

Staining of the first polar body:
When oocytes were checked for the presence of the blue stained first polar body, it became apparent that percentages decreased with the increase in antioxidant concentration and they remained lower than the control for both antioxidant mixtures (Table 2).

Table 2. Nuclear maturation of oocytes after culture in antioxidant supplemented medium

Treatment	Number of assessed oocytes	Oocytes with a visible first polar body (%)
Control	41	43.90
E5+C250	45	31.11
E20+C750	42	30.95

Various concentrations of α-tocopherol and ascorbic acid have been shown to improve developmental competence of ovine embryos [14, 15, 16].
Our results indicate that the combination 20 µM α-tocopherol+750 µM ascorbic acid has a positive effect on oocyte nuclear maturation as indicated by *cumulus oophorus* expansion. However, polar body formation as a sign of nuclear maturation was not influenced in a beneficial manner.
Although antioxidant combinations have been proven to have a positive effect on swine oocyte maturation [17] the same cannot be said for the ovine female gamete. This could be the result of differences between lipid compositions in the gametes of the two species but also because of

specific interactions between fatty acids and the two antioxidants.
Further research is needed in order to refine this knowledge, establish more concentrations with a beneficial effect and further define the mechanisms by which antioxidants act in the cell.

4. Conclusions

This research shows that the mixture 20 µM α-tocopherol and 750 µM ascorbic ascorbic has a beneficial effect on cytoplasmic maturation and a slightly detrimental one on nuclear maturation.

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