THE EFFECT OF SEVERAL ASCORBIC ACID CONCENTRATIONS ON SWINE OOCYTE MATURATION AND EMBRYO CULTURE

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Abstract

Considering that free radicals are easily formed both in vivo and in vitro and that swine oocytes and embryos have a high content of unsaturated and therefore highly oxidizable fatty acids antioxidants must be added to the culture media. Ascorbic acid has been shown to improve swine oocyte maturation and the development of bovine and swine embryos. The goal of this study was to establish the influence of several ascorbic acid and α-tocopherol concentrations on swine oocyte maturation in order to improve oocyte maturation media. Pig oocytes were cultured for 44 hours at 37°C in 5% CO_2 atmosphere, in M199 containing several ascorbic acid (50, 150, 250, 500 and 750 μM) concentrations. Oocytes were fertilized and the embryos were cultured for another 94 hours in TALP medium containing the same antioxidant concentrations. The addition of 50, 150, 500 and 750 mM ascorbic acid to the maturation medium lead to an increase in the number of oocytes assessed as 3 and 4 and a decrease in the number assessed with 0. The number of embryos that developed in medium supplemented with 50 μM ascorbic acid was significantly higher than the contro, thus proving the beneficial influence of ascorbic acid on embryo development.

Key words: antioxidant, swine oocytes, embryos, ascorbic acid

INTRODUCTION

In the cell, free radicals function as signal molecules by activating transcription factors and enzymatic reactions [2]. They are also involved in regulating embryo development and implantation [4]. Their endogenous overproduction and the exogenous sources lead to an imbalance in redox metabolism and therefore to oxidative stress.

In the oocyte and embryo free radical levels are controlled by metabolic pathways mediated by enzymes such as glutathione. However the oocyte contains a large quantity of lipid droplets. This is particularly evident in the oocytes of the domestic pig, where very high levels of lipid have been reported, 161 μg (McEvoy et al., 2000). Triacylglycerol was the major lipid component followed by cholesterol and phosphatidylcholine. Analysis of fatty acids esterified to the individual phospholipids and neutral lipids has shown that in all the classes examined, particularly in the neutral lipid fractions, there are high levels of palmitic acid and oleic acid Triacylglycerol, free fatty acids and most of the phospholipids, particularly phosphatidylethanolamine, are considerably enriched in n-6 polyunsaturated fatty acids, specifically linoleic, arachidonic and adrenic acids [5]. All these unsaturated and therefore easy oxidizable lipids increase their sensitivity to oxidative stress induced by culture conditions. That is why we believe that oocyte maturation and embryo culture can be improved by adding antioxidants to the culture medium in order to reduce the stress [11].

Oocyte maturation encompasses nuclear maturation and maturation of the cytoplasm. While the first refers to the resumption of meiosis and progression to the metaphase II (MII) stage and can be assessed by the presence or absence of the first polar body, the second comprises poorly understood processes [7]. These processes are believed to progress in parallel to one another, and synchronization of nuclear and cytoplasmic maturation is essential for establishing optimal oocyte

developmental potential [8]. The efficiency of this process is indicated by the ability of the oocyte to block polispermic fertilization and allow the formation of the male pronucleus. Whereas nuclear maturation can be evaluated by nuclear staining methods that reveal the first polar body, cytoplasmic maturation can only be determined by indirect means such as cumulus oophorus expansion, the measurement of glutathione content and fluorescent viability coloration. Out of these only the first leaves the oocyte intact and has the least effect on its viability and ability to sustain fertilization. Therefore it becomes the most important mean of assessing maturation.

Ascorbic acid is the most important antioxidant outside the cell [12]. It functions as a reducing agent of oxygen and cytocromes c and a, but can also protect membranes against peroxidation. It can prevent apoptosis in cultured mouse follicles and improve swine oocyte maturation [10] and embryo development of swine embryos [6].

The goal of this research was to establish whether supplementation with ascorbic acid could improve viability and meiotic maturation of porcine oocytes, cumulus cell function and embryo ability to develop *in vitro*.

MATERIAL AND METHOD

Oocyte collection and maturation

Oocytes were harvested in M 199 supplemented with L-glutamine (3.4 g/l), NaHCO3 (2.2 g/l), Hepes (25 mM), penicillin (100 µg /ml) and streptomycin (100 IU/ml) and with the pH adjusted to 7. For oocyte maturation M 199 was supplemented with L-glutamine (3.4 g/l), Chorulon (10 IU/ml), Folligon (10 IU/ml), fetal calf serum 10%, penicillin (100 µg /ml) and streptomycin (100 IU/ml). Ascorbic acid dissolved in ultrapure water was added to the maturation medium in order to arrive at concentrations of 50, 150, 250, 500 and 750 µM active substance. For viability assessment bovine serum albumin (5 mg/ml) and 3'6' flourescein diacetate (1 µg/ml) were added to PBS.

Porcine ovaries were collected from pre-pubertal gilts 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 of 2–6 mm in diameter on the ovarian surface were aspired with a 10 ml syringe equipped with an 21-gauge needle and collected a 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 vitamin concentrations. All the droplets were covered in paraffin oil and incubated for 48 hours at 38°C in an atmosphere with 5% CO_2. Then they were evaluated using an Olympus inverted phase contrast microscope, in order to assess cumulus oophorus expansion and/or the presence of the first polar body. Cumulus expansion was assessed 44 h after incubation by a subjective scoring method [1]. 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. The differences between treatments were analyzed by ANOVA and interpreted using the Student test. For all comparisons, the values were considered statistically significant when $p < 0.05$.

Oocyte viability was certified by using 3'6' fluorescein diacetate (FDA) fluorescent coloration. After morphological evaluation the cumulus-oocyte complexes were transferred to PBS and mechanically denuded. The oocytes were then transferred to PBS supplemented with bovine serum albumin and FDA and incubated at 38°C for 10 minutes. At the end of this time they were examined under ultra-violet light at 495 nm wavelength.

In vitro fertilization and embryo culture

The fertilization medium was TALP-Fert, which is based on Tyrode's saline solution supplemented with bovine serum albumin

(0.004 g/l), glucose (1.00 g/l), sodium lactate (10 µl/ml), sodium pyruvate (0.288 µg/ml) and antibiotics (100 µg /ml penicillin and 100 IU/ml streptomycin). The embryo culture medium had the same composition and ascorbic acid was added in order to make up concentrations of 50, 150, 250, 500 and 750 µM.

Spermatozoa were capacitated in Tyrode using the swim-up technique and a haemocytometer was employed to establish concentration. After cumulus expansion assessment the oocytes were transferred to TALP-Fert and mechanically denuded using a micropipette. Afterwards they were placed in 30 µl droplets and the sperm cells were added. The concentration for fertilization was 1 x 10^6 spermatozoa/ml and it was computed according to the following formula: volume(µl) = 30 x 1 x 10^6 /the concentration of capacitated spermatozoa. After the sperm cells were added the droplets were covered with paraffin oil to prevent media evaporation and incubated at 38°C and 5 % CO_2 in air. After 16 hours the spermatozoa were removed mechanically using a micropipette and the presumed zygotes were cultured in TALP droplets supplemented with ascorbic acid (50, 150, 250, 500 and 750 µM) and covered with mineral oil for 92 hours in the same conditions as described before. During this time the number of embryos that had developed was counted and compared to the control, and the differences analyzed using ANOVA and interpreted using the Student test.

RESULTS AND DISCUSSION

The goal of this study was to establish the influence of several ascorbic acid concentrations (50, 150, 250, 500 and 750 mM) on swine oocyte maturation. At the end of 44 hours, the oocytes were assessed for cumulus expansion (figure 1) and sometimes the presence of the first polar body (figure 1).

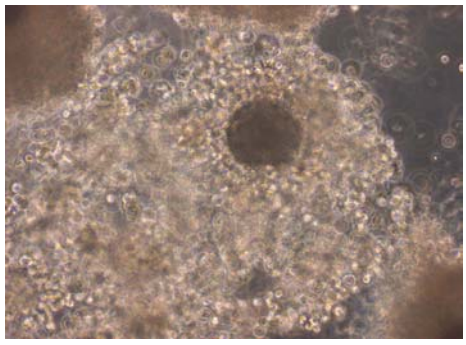

Fig. 1. Oocyte assessed as 4 due to complete cumulus expansion (100x magnification)

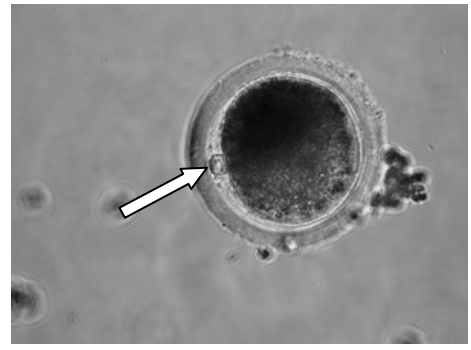

Fig. 2 Oocyte showing the first polar body as a sign of nuclear maturation (100x magnification)

None of the 5 ascorbic acid concentrations led to significant differences in oocyte cumulus expansion when compared to the control. However, the percentage of oocytes to be scored as 3 was higher than the control in several cases amounting to 22.62% for C50, 30.51% for C150, 23.28% for C500 and 30.84% for C750 as compared to 21.91% (table 1). More oocytes reached the 4th grade of cumulus expansion for C50, C150 and C 750 than had done so in the case of control cells.

Because cumulus cells act as a 'go-between' between the oocyte and the follicular or culture environment, their expansion is considered to be reliable sign of this elusive process having taken place. It is also worth noting that fewer oocytes were scored as 0 for all the ascorbic acid concentrations than the control. Therefore, ascorbic acid addition did not interfere with cumulus expansion and oocyte maturation and in view of the results presented above seems to indicate that adding ascorbic acid to the maturation medium diminishes the number of oocytes that show no signs of nmaturation.

Table 1
The differences for ascorbic acid and their significance

Treatment	Total number of oocytes	Stage of cumulus expansion	The percentage of oocytes to have reached a certain stage of cumulus expansion	Significance of differences
M	210			
		0	12,38%	-
		1	10,95%	-
		2	20,48%	-
		3	21,91%	-
		4	34,28%	-
C 50	252			
		0	3,57%	ns
		1	13,49%	ns
		2	25,40%	ns
		3	22,62%	ns
		4	34,92%	ns
C 150	236			
		0	5,93%	ns
		1	10,59%	ns
		2	22,88%	ns
		3	30,51%	ns
		4	30,10%	ns
C 250	215			ns
		0	8,37%	ns
		1	20,47%	ns
		2	15,81%	ns
		3	19,17%	ns
		4	36,28%	ns
C 500	232			
		0	3,02%	ns
		1	25,26%	ns
		2	28,02%	ns
		3	23,28%	ns
		4	25,43%	ns
C 750	227			
		0	3,52%	ns
		1	14,57%	ns
		2	15,86%	ns
		3	30,84%	ns
		4	38,33%	ns

ns - not significant (p>0,05); * - significant

The fact that oocytes that had undergone culture were alive was confirmed by fluorescent staining with FDA. Living cells were highly fluorescent when compared with dead ones, as can be inferred from the two images in figure 3: one taken in visible light (figure 3a) and the other one in ultraviolet light at 495 nm (figure 3b).

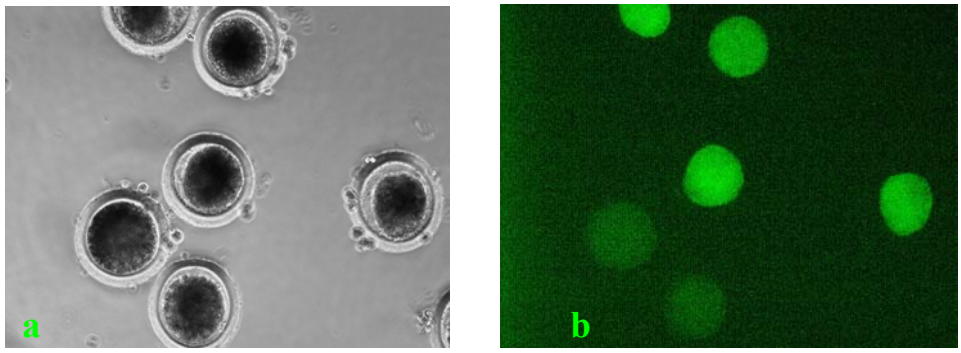

Fig. 3. Flourescent-living and non-fluorescent-dead oocytes: a – visible ligh; b – ultraviolet light at 495 nm (100x magnification)

Oocytes were then incubated with spermatozoa in order for fertilization to occur and the embryos were cultured in medium supplemented with the same ascorbic acid concentrations for 92 hours. The numbers of embryos that had resulted for each treatment was counted, all the stages such as 2 cells (figure 4), 4 cells (figure 4)and morula (figure 5) being taken into account. 233 embryos resulted, the rate being 18.1%.

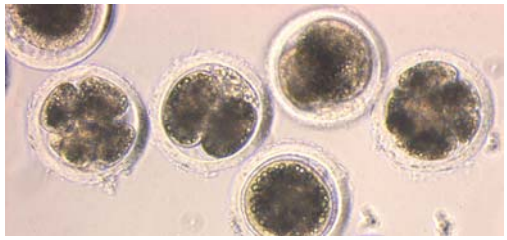

Fig. 4. Embryos at 2 and 4-8 cells stage (100x magnification)

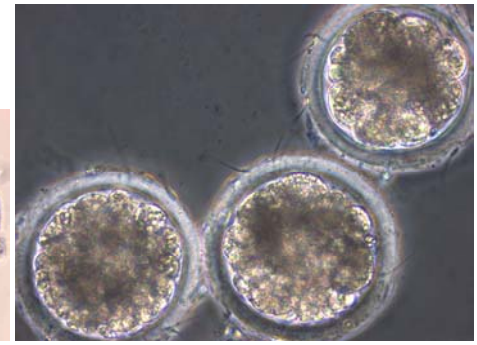

Fig. 5. Embryos at the morula stage (200x magnification)

The number was compared with the control and the differences analyzed using ANOVA and interpreted using the Student test. The results can be seen in table 2.

Table 2
The percentage of embryos that developed during culture with ascorbic acid and the significance of differences

Treatment	Number of fertilized oocytes	Percentage of developed embryos	Significance of differences
M	183	9,83%	-
C 50	243	20,57%	*
C 150	232	19,4%	ns
C 250	195	17,44%	ns
C 500	194	23,08%	ns
C 750	242	16,94%	ns

ns - not significant ($p>0,05$); * - significant ($p<0,05$)

The percentage of embryos that developed in medium supplemented with ascorbic acid was higher than the control (9.83%) for all the treatments. The highest values were registered for 500 μM (23.08%) and 50 μM (20.57%) and the lowest for C750 (16.94%) and C250 (17.44%). The number of embryos for 50 μM was statistically significant. The results are similar to the research conducted by Tatemoto et al. (2001), where the addition of ascorbic acid to the maturation and embryo culture media resulted in a significant increase in the number of embryos.

CONCLUSIONS

The results, as they have been presented above lead to the following conclusions.

Fluorescein diacetate can be used to easily differentiate between living that are fluorescent and dead oocytes, which are dark.

Adding 50, 150, 500 and 750 mM ascorbic acid to oocyte maturation medium had a beneficial effect on cumulus oophorus expansion and resulted in an increase in the number of oocytes that were scored as 3 and 4, therefore being considered mature and in a decrease in the number scored at 0. However this number was not statistically significant.

After fertilization the number of embryos that developed in medium supplemented with 50 μM ascorbic acid was statistically significant and higher than the control, indicating a beneficial effect.

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