Antioxidant effect of vitamin C on porcine oocytes maturated *in vitro*

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Abstract
Cells are vulnerable to oxidative stress during in vitro culture systems. The objective of the present study was to determine the effect of vitamin C addition in in vitro culture media on porcine oocytes maturation rate based on morphological changes. Porcine COC's were matured according to their morphological class (class I, II and III) in two groups: control (M) and supplemented with vitamin C (0.5 mM, C) in TCM 199 HEPES (M2520) modification media with hormones (0.88UI/ml FSH, F8174) at 38.5^{0}C in 5% CO_2 humidified air atmosphere for 44h. The rates of oocytes with cumulus cells expansion were higher with addition of vitamin C as compared to control group, with 7.83% (C1), 70.59% (C2) and 6.04% (C3). It could be concluded from this preliminary study that addition of vitamin C in in vitro maturation medium has a beneficial effect on porcine oocytes especially in C2 group.
***Key words**: antioxidants, porcine oocyte, in vitro maturation*

Introduction

Assisted reproduction technique (ART) as *in vitro* fertilization (IVF) it is used in porcine reproduction research mainly for study the mammalian embryogenesis, xenotransplantation, transgenesis and genome editing (Abeydeera, 2002).

The large scale implementation of porcine IVF techniques is still poor due to polyspermy and low embryo development, even if there are studies with promissing results (Batista et al., 2016; Romar et al., 2016).

Successful ART is influenced by many factors, among which reactive oxygen species (ROS) has a significant role. Sources of ROS during ART procedures could be either endogenously (immature spermatozoa, leukocytes, oocyte, cumulus mass cells, follicular fluid, embryos) or exogenous environmental factors (visible light, culture media, pH, temperature, oxygen concentration, centrifugation, cryopreservation)(Agarwal et al., 2014).

Studies indicates that supplementing maturation media with different antioxidants such as cysteine, cystamine, β-mercaptoethanol (Mahanta et al., 2016; Mircu et al., 2015, Beheshti et al., 2011, Sadeesh et al., 2014), vitamin C (Sovernigo et al., 2017; Comizzoli et al. 2003), rosmarinic acid (Marc et al., 2017, Malo et al., 2010; Luno et al., 2014; Luno et al., 2015; Olaciregui et al., 2017) or plant antioxidants – flavonoids (Kang et al., 2016, Mbemya et al., 2017) can improve oocytes maturation based on morphological changes, nuclear changes and on gene expression.

Ascorbic acid, a powerful antioxidant, it is used in porcine somatic cell nuclear transfer to enhance the porcine embryos development, results based on increased transcript levels of reprogramming genes, such as Pou5fl, Sox, Klf (Zhao et al., 2017). In feline improves COC maturation rate, even if the COC are retrieved during non-breeding season (Comizzoli et al. 2003), in buffalo also improves the development competence of oocytes (I-Nabi et al., 2017). Tatemoto et al. (2001) suggested that a critical intracellular concentration of ascorbic acid would be necessary for normal cytoplasmic maturation of oocytes.

The purpose of this present research was to evaluate the effect of vitamin C in 0.5mM concentration on porcine oocytes maturation based on morphological changes.

Materials and methods

Sow ovaries (n=46) were collected from local slaughterhouse and transported to the laboratory in containers containing 0.9% NaCl solution supplemented with antibiotics (Pen/Strep, 17-602F, Lonza), at 37^0C within two hours. Handling medium for COC (cumulus -oocytes-complexes) was Dulbecco-PBS (D-8662) supplemented with 100 µl Pen/Strep; 3.6 mg sodium piruvate, 30 mg BSA (A9647, Sigma-Aldrich), 100 mg glucose (G7021, Sigma-Aldrich). COCs were aspirated by puncture procedure from medium to large follicles with 18G needle attached to a 5 ml syringe.

Classification of COCs based on morphological aspects was done under stereomicroscope (Stemi 2000-C, ZEISS) with hot plate (33.4^0C): *Ist class* - CI (COCs with cumulus compact and unexpanded, with full or at least 5 layers of cumulus cells, cytoplasm clearly seen, dense and homogenous, *IInd class* – CII (COCs with cumulus compact, thick, 2-4 layers of cumulus cells, covering all of zona pellucida, cytoplasm dense, with uniform granulation) and *III^{d} class* - CIII (oocytes partially denuded of cumulus cells, or with 1-2 complete layers of cumulus cells and/or with irregular shrunken cytoplasm).

The maturation culture medium, prepared in our: TCM 199 HEPES modification media, (M2520) with 10% ECS and 0.88UI/ml FSH (F8174, Sigma-Aldrich) - *group M* (control), in experimental group we added vitamin C (0.5 mM)- *group C*. Pools of 15-20 COCs were maturated in 400µl media in 4 well dishes (Nunc, Germany) covered with mineral oil at 38.5^0C in 5% CO_2 humidified air atmosphere for 44h. After 44h of culture, all COC were examined for maturation, signs as expansion and mucification of cumulus cells were observed. The COC's were maturated according to there their morphological class (M1, M2, M3, C1, C2, C3).

Results and discussions

The results of supplementation of *in vitro* media with ascorbic acid on porcine oocytes morphological aspects are presented in figure 1-3.

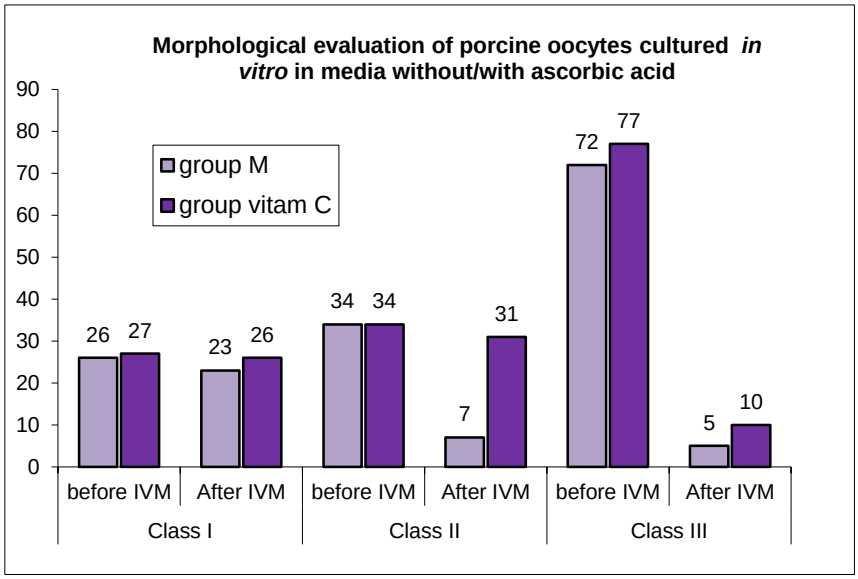

Figure 1. Morphological evaluation of bovine COC's before and after IVM

After *in vitro* maturation of porcine oocytes in the medium without antioxidant (M group) we noticed at the morphological assessment that 88.46% of class I COCs (M1), 20.58% of class COC II (M2) and 6.94% of class III COCs (M3) were matured. In group C, supplemented with vitamin C (C group), 96.29% of class I COCs (C1) were matured after 44 hours, 91.17% of class II (C2) and 12.98% of class III (C3).

Comparing the groups, relative to the number of COC's matured, a increase in their maturation sign is observed, with 7.83% % (C1), 70.59% (C2) and 6.04% (C3), respectively.

From these results we can observe that the oocyte class is associated with their capacity to mature *in vitro.* In order to improve development competence of oocytes it is not sufficient to have a proper culture media, but also to use high quality oocytes. These results are sustained also by BAX/BCL2 gene expression (unpublished data). Similar results we obtained in bovine oocytes cultured in medium supplemented with rozmarinic acid (Marc et al., 2017).

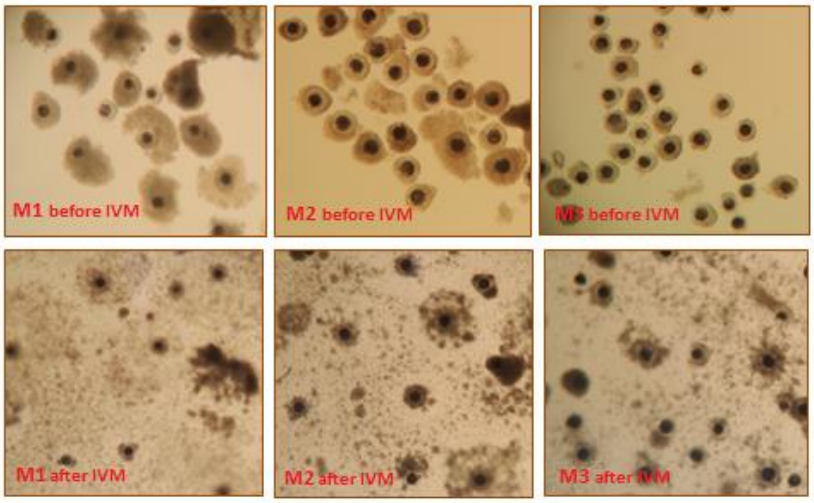

Figure 2. Aspects of porcine oocytes from control group (M)classified according to their morphological aspects before and after IVM (3.2X)

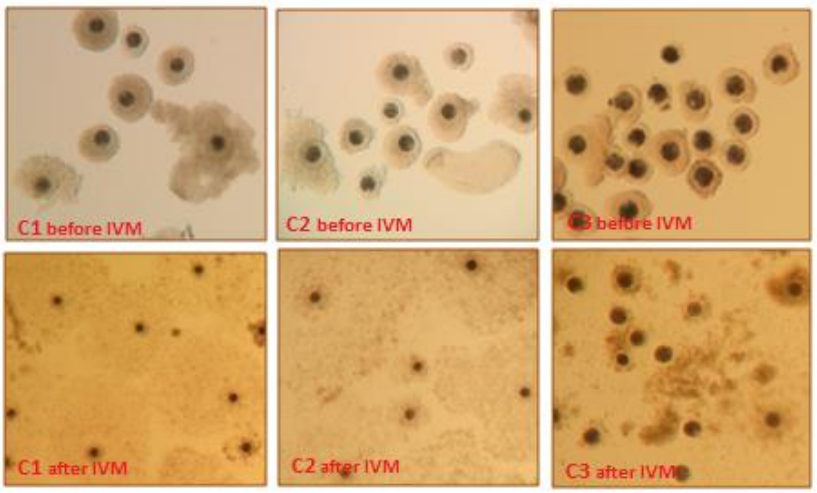

Figure 3. Aspects of porcine oocytes from vitamin C group (C) classified according to their morphological aspects before and after IVM (3.2X)

Vitamin C is no longer only an enzymatic cofactor, an antioxidant, an extracellular promoter of matrix formation by stabilizing collagen structure, but also it is studied for its potential to modulate gene expression (Belin et al., 2009; Belin et al., 2010, Ivanyuk et al., 2015, Duran et al., 2019). Belin et al. (2009) demonstrated that vitamin C stops proliferation *in vivo* and *in vitro* by down-regulation of 30 genes among these 12 belonging to families such as tRNA synthetases and translation initiation factors, involved in cell division. Other researchers demonstrated the activity of vitamin C as a signaling molecule in development and differentiation. For example Duran et al.(2019) demonstrated that ascorbic acid influence the mechanisms of fish pacu (*Piaractus mesopotamicus*) myogenesis by increasing *in vitro* the expression of *myog* and *mtor* - molecular marker of skeletal muscle myogenesis and protein synthesis respectively; probable through its role as an antioxidant agent, that decrease ROS levels and consequently induce upregulation of *igf* – molecular marker of protein synthesis. Vitamin C can enhance cardiac differentiation if applied on day 0-2 in murine CGR8 embryonic stem cell line with promising results in production of sufficient cardiomyocytes for research and potential application in regenerative medicine (Ivanyuk et al., 2015).

The reviewed literature suggest also good results of vitamin C on different animal species oocytes used in different concentration (0-25-50-100 µM)(Kere et al., 2012; I-Nabi et al., 2017), 100-250-500-750 µM (Tatemoto et al. (2001), but not in large concentration that can reduce oocyte development by inducing apoptosis. Kere et al. (2012) obtained good results during *in vitro* maturation of porcine oocytes with relatevely low concentration of vitamic C (50µg/ml), others with 50 µM in buffalo (I-Nabi, 2017) or 0.5mM in cats (Comizzoli, 2003). Our results also indicates that addition of vitamin C in 0.5mM concentration improves porcine oocytes maturation, results sustained also by BAX/BCL2 gene expression were BCL2 gene had higher levels in Ist class COC's from C1 group (unpublished data).

Our preliminary results suggests that antioxidant properties of vitamin C in 0.5mM concentration is effective on porcine oocytes *in vitro* maturation.

Conclusions

1. Supplementation of the porcine oocyte culture media with vitamin C generates a higher proportion of porcine oocytes matured *in vitro* based on morphological evaluation.
2. Quality of the COC used for *in vitro* techniques has an important role in the success of this approach.

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