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Effect of vitamin E, resveratrol and vitamin C on the *in vitro* development and quality of porcine IVF embryos*

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The aim of this study was to assess the effect of various concentrations of antioxidants such as vitamin E, resveratrol and vitamin C on the development of pig embryos obtained after *in vitro* fertilization of *in vitro* matured porcine oocytes. Furthermore we examined the time of development, the quality of blastocysts and ROS levels in pig embryos cultured with vitamin C. Cumulus-oocyte complexes (COCs) were obtained from gilt ovaries. COCs were matured in the TCM-199 medium (Tissue Culture Medium 199 Hepes) for 42 h at 39°C and 5% CO_2 to metaphase 2. Matured oocytes were fertilized *in vitro*. In the experimental groups the presumptive zygotes were cultured up to the blastocyst stage in the NCSU-23 (IVC) medium supplemented with vitamin E (50 and 100 µM/mL) or resveratrol (0.5 and 1 µM/mL) or vitamin C (10 and 20 µg/mL). In the control group embryos were cultured without any supplements. The addition of vitamin E (50 and 100 µM/mL) or resveratrol (0.5 or 1 µM/mL) to the *in vitro* culture medium of pig embryos did not have a positive effect on embryonic development and did not statistically significantly increase the percentage of cleavage and blastocysts. In contrast, the addition of vitamin C in the amount of 20 µg/mL to the IVC medium significantly accelerated the time of embryo development *in vitro* and statistically significantly decreased the level of ROS in pig blastocysts produced *in vitro*.

KEY WORDS: pig embryos / reactive oxygen species / resveratrol / vitamin C / vitamin E

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To improve the quality of *in vitro* matured oocytes and porcine embryos various antioxidants are used [Qasim *et al.* 2019]. Antioxidants are stable molecules that upon reaction with oxygen species (ROS) do not emit new ones. By breaking the cycle of oxygen species creation antioxidants protect cells from oxidative stress. The damage caused by the activity of ROS accumulates in structures such as cell membranes, DNA and lipids, thereby inhibiting their functions or leading to their degradation. ROS negatively influences oocyte maturation and oocyte fertilization [Budani and Tiboni, 2020]. The oxygen content of *in vitro* production (IVP) environments is higher than that in vivo, which results in an increased production of ROS [Nohalez *et al.* 2018]. Therefore, these specific supplements are needed to protect oocytes or embryos and inhibit the effect of ROS. Antioxidants which are supplemented to the media include phycocyanin, chlorogenic acid, zinc, melatonin, manganese, niacin, resveratrol, vitamin E or vitamin C [Huminiecki and Horbańczuk 2018, Liang *et al.* 2018, Nguyen *et al.* 2019, Yeung et al. 2019, 2021, Ridlo *et al.* 2021, Niu *et al.* 2020, Qasim *et al.* 2019, Almubarak *et al.* 2021, Wang *et al.* 2019, Miclea and Zăhan 2021].

Porcine embryos contain a relatively high concentration of fatty acids [McEvoy *et al.* 2000] and thus can be highly susceptible to their peroxidation. The reaction of lipid peroxidation can inhibit cell functions and initiate the process of apoptosis [Spiteler, 2001]. Vitamin E is an antioxidant that can be dissolved in lipids and that is found in mammal cells. It can inhibit the peroxidation of lipids. It is believed that vitamin E is the main antioxidant in mammal cell membranes [Chow 1991]. During studies of culture medium supplements vitamin E has been identified as having a positive effect on rat [Steele *et al.* 1974] and bovine embryo development [Olson *et al.* 2000]. Other studies have shown that the supplementation of vitamin E has resulted in an increase in the number of bovine embryos that reach the blastocyst stage, as well as a reduction in DNA fragmentation in bovine blastocysts obtained after cloning [Wongsrikeao *et al.* 2007]. While studying the effects of vitamin E on the *in vitro* development of porcine zygotes obtained *in vivo* we have shown its positive influence both on the percentage of produced blastocysts and their quality, evaluated based on the number of nuclei demonstrating DNA fragmentation [Gajda *et al.* 2008]. Additionally, Romek *et al.* [2011] observed a reduced amount of triglycerides in porcine embryos cultured in a medium supplemented with vitamin E. Therefore the continuation of studies on the effects of vitamin E on porcine embryo development seemed justified.

Resveratrol is a very effective antioxidant and a non-toxic anti-fungal compound. It has life prolonging properties, reduces symptoms of aging, as well as reduces inflammations and apoptosis of the vascular endothelium. It has been demonstrated that the addition of resveratrol to the porcine oocytes culture medium has positive effects on their development by increasing the percentage of cleaved embryos and blastocysts [Lee *et al.* 2018]. Other studies [Wang *et al.* 2019] have shown that supplementing the development medium with resveratrol resulted in a decrease of ROS levels in oocytes. However, in the same studies no influence on the development of embryos obtained after parthenogenesis (PA) was observed [Wang *et al.* 2019].

Taking into consideration the antioxidant properties of resveratrol it was decided to use it as a medium supplement for the culture of porcine zygotes produced *in vitro*.

Another antioxidant is vitamin C (ascorbic acid), which by taking part in metabolic processes of proteins and lipids (among others) inhibits lipid peroxidation and neutralizes oxygen species. A positive effect of vitamin C supplementation has been shown on the nucleus maturation of porcine oocytes [Tao *et al.* 2010]. The addition of this antioxidant to the culture medium of porcine embryos has enhanced their development competences and quality [Hossein *et al.* 2007, Hu *et al.* 2012]. Supplementation of embryo culture media with 20 µg/mL vitamin C decreased the number of apoptotic cells and increased the cell number in porcine blastocysts [Hu *et al.* 2012]. In other studies supplementation with 50 µg/mL vitamin C did not improve the embryo production *in vitro* [Nohalez *et al.* 2018].

The above-mentioned modifications to culture media of porcine embryos produced *in vitro* result in an increase in the number of developing embryos; however, the percentage of obtained blastocysts remains unsatisfactory and does not exceed 50% [Nguyen *et al.* 2017, Wang *et al.* 2019, González *et al.* 2021]. Moreover, as has been previously mentioned, the quality of embryos obtained after *in vitro* culture is still much lower than of those developed *in vivo*. Therefore a reliable evaluation of embryo quality is crucial for the development of culture methods. It is assumed that the planned modifications in culture conditions with the addition of antioxidants will result in obtaining *in vitro* embryos of a higher quality.

The purpose of this study was to assess the effect of various concentrations of antioxidants such as vitamin E, resveratrol and vitamin C on the development of pig embryos obtained after *in vitro* fertilization of *in vitro* matured porcine oocytes. The optimal antioxidant variant was selected - vitamin C at a concentration of 20 µg/ml- based on the results of experiments carried out on the effect of antioxidants on embryo development. Embryos cultured in a concentration of 20 mg vitamin C were evaluated in terms of the time of development to the blastocyst stage as well as the ROS level.

Material and methods

Chemicals and culture media

All reagents and chemicals were purchased from Sigma-Aldrich (Sigma Chemical, Saint Louis, Missouri, USA) unless stated otherwise. Some media were prepared in the laboratory, immediately before experiments.

Oocyte collection and *in vitro* maturation

The procedure of *in vitro* maturation was previously described in detail in a study by Samiec and Skrzyszowska [2012] and Kurowska *et al.* [2020]. Ovaries were collected form prepubertal gilts at a local slaughterhouse and transported to the laboratory at 30°C within 2 hours of collection. The cumulus oocyte complex (COC) was aspirated and washed several times in TCM-199 Hepes (Tissue Culture Medium

199 Hepes). Only oocytes with a homogeneous ooplasm and with a multilayered compact cumulus mass were selected for maturation. After 22 hours of maturation oocytes were transferred to the same maturation medium, but without EGF, FGF, FSH, LH and dbcAMP for the next 22 hours under the same conditions.

In vitro fertilization

According to our previous report [Poniedziałek-Kempny *et al.* 2020ab] COCs after maturation were denuded and assessed morphologically. Groups of 10-12 matured oocytes (MII) were placed in 50 µL drops in the IVF medium under mineral oil and were maintained in an incubator (39°C, 5% CO_2 in air) until the addition of spermatozoa. The procedure of capacitation **was** based on our previous study [Poniedziałek-Kempny *et al.* 2021]. The IVF medium was a modified TCM-199 Hepes medium supplemented with 10 µM of caffeine. After incubation a portion of 50 µl sperm (concentration of 1x 10^5/mL) was added to the medium with oocytes. Gametes were co-incubated with spermatozoa for 4 hours in a humidified atmosphere containing 5% CO_2 in the air at 39°C, in a Sanyo incubator.

In vitro culture

After co-incubation presumptive zygotes were washed three times in the North Carolina State University-23 medium (NCSU-23, IVC medium) [Petters and Wells, 1993] supplemented with 4 mg/mL of BSA. Then the presumptive zygotes were placed into four-well plates (Nunc) with an IVC medium and cultured up to the blastocyst stage at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The numbers of morulae and expanded blastocysts were evaluated. The time development of the embryos was assessed on the basis of the number of blastocysts obtained on days 6, 7 and 8 of *in vitro* culture.

TUNEL assay

Cell apoptosis was analyzed using the TUNEL Detection Kit (Tunel reagent, In Situ Cell Detection Kit Roche Diagnostics, Germany). The TUNEL method applied in this study was described previously [Bryła *et al.* 2009].

In the blastocysts the number of cells and the level of nuclear DNA fragmentation were assessed. The apoptotic index was calculated based on the ratio of the number of apoptotic nuclei per blastocyst to the total cell number per blastocyst.

Intracellular ROS

To assess the level of ROS a method described by Romek *et al.* [2005, 2019] was used.

To measure ROS levels blastocysts were stained with 5 mM CM-H_2DCFDA (ThermoFisher Scientific Inc., Waltham, MA, USA) fluorescent dye in the culture medium at 39°C for 30 min under 5% CO_2 in air. Next embryos were washed two times in 20% fetal calf serum in PBS (FCS-PBS), set on the microscope slides in 5 mL drops

of FCS-PBS and analyzed using a Nikon Eclipse E600 epifluorescence microscope equipped with a Plan-Fluor 20 (0.75 NA) lens and a CCD camera. Acquired embryo images were analyzed using the ImageJ software and the amount of fluorescence intensity (DROS) – which is proportional to the ROS level in an individual embryo – was calculated. Then the ROS level per unit volume of the blastocyst cytoplasm (ROS(c)) was calculated using a special formula applied and described by Romek *et al.* [2005, 2019].

Experimental design

Matured oocytes (MII stage) were fertilized *in vitro*. In the experimental groups the presumptive zygotes were cultured up to the blastocyst stage in the NCSU-23 medium supplemented with:

– vitamin E (water soluble α-trolox analogue; 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; Sigma Aldrich) (50 and 100 μM/mL);
– resveratrol (Sigma Aldrich) (0.5 and 1 μM/mL);
– vitamin C (L-ascorbic acid; Sigma Aldrich) (10 and 20 μg/mL).

In the control group presumptive zygotes were cultured in the IVC medium without supplements. In the experimental and control groups the proportion of presumptive zygotes, morulae and expanded blastocysts were calculated. The time of development, total cell number in blastocysts, the number of apoptotic nuclei and ROS levels were assessed.

Statistical analyses

Results are presented as means and standard deviations (SD). For statistical analysis of the percentage data, i.e. development of embryos in all the groups (percentage of cleaved embryos, morulae and expanded blastocysts), cell number and apoptosis levels the Chi-Square test was used, whereas Student's t-test was performed on the ROS level data. Prior to the parametric t-test the normality of data and homogeneity of the group variances were determined using the Shapiro-Wilk and Levene's tests, respectively. Differences at $P<0.05$ were considered to be statistically significant, whereas differences at $P<0.01$ were considered to be highly significant. Statistical analysis was performed using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA) and StatSoft, Inc. (2014) STATISTICA (data analysis software system), version 12 (www.statsoft.com).

Results and discussion

To optimize the results, vitamin E was one of the oxidants which we used as a supplement for the culture medium. We evaluated the *in vitro* development competence of embryo cultured with vitamin E. In our experiments we used Trolox, which is a synthetic analogue of vitamin E. The percentages of cleavage, morulae and blastocysts in the group cultured in the presence of 50 and 100 μM/mL vitamin E were lower when

compared to the control group. We observed no beneficial effect for the addition of both concentrations (50 and 100 μM/mL) of vitamin E to the IVC medium on the embryonic development in pigs (Tab. 1). However, some previous studies reported a positive role of vitamin E. In cattle the addition of 100 μM/mL vitamin E to the embryo culture medium improved *in vitro* development to the early and expanding blastocyst stages [Olson and Seidel 2000]. In pigs supplementation with an analogue of vitamin E (Trolox) during IVC improves preimplantation development of PA embryos by increasing glutathione levels, decreasing ROS levels and regulating apoptosis [Lee *et al.* 2015]. Observations similar to those recorded in our study were made by Wongsrikeao *et al.* [2007], who reported that the addition of 100 μM/mL vitamin E to bovine culture medium decreased the percentage of blastocysts compared to the control group. Moreover, in pigs the addition of 100 μM/mL in the IVC medium had no effect on embryonic development [Hossein *et al.* 2007]. We suspect that the high concentration of vitamin E (100 μM/mL) in the IVC medium of pig and cattle embryos may adversely affect embryonic development, which may be related to its toxicity.

Table 1. Effect of vitamin E on *in vitro* development of porcine embryos obtained after *in vitro* fertilization

Vitamin E (μM/mL)	No. of oocytes/replication	No. of presumptive zygotes (%)	No. of morulae (%)*	No. of expanded blastocysts (%)*
50	98/6	19 (19.3)^{A}	3 (15.7)^{D}	2 (10.6)
100	185/6	61 (32.9)^{c}	37 (60.6)^{F}	26 (42.7)
0 (control)	74/6	33 (44.5)^{B}	21 (63.7)^{E}	18 (54.5)

*In comparison to presumptive zygotes.
$^{aA...}$Within column means bearing different superscripts differ significantly at: small leltters – $P<0.05$; capitals – $P<0.01$.

Resveratrol, known for its antioxidant properties, has also found applications in the *in vitro* culture of mammalian oocytes and embryos. A study by Kwak *et al.* [2012] showed that the addition of 2.0 μM/mL resveratrol to the porcine IVM medium had a positive effect on the oocyte maturation process and the development of embryos obtained as a result of IVF and after PA. Exposure to 5 μM/mL resveratrol during IVM improved nuclear maturation rates and quality of porcine oocytes. Oocytes after maturation with resveratrol showed lower levels of oxidative stress, higher glucose uptake ability and lower incidence of apoptosis. PA-embryos derived from oocytes which matured with resveratrol displayed capabilities to undergo cleavage divisions and complete development up to the blastocyst stage [Wang *et al.* 2019]. Research by Lee *et al.* [2010] showed that with an increasing resveratrol concentration above 1 μM, the percentage of produced porcine blastocysts decreased. The percentages of cleavage and blastocysts were statistically higher in the experimental group with the lower resveratrol concentration (0.5 μM/mL) compared to the control group. The mean number of cells in porcine blastocysts cultured in the presence of 0.5 μM/mL resveratrol was higher compared to embryos in the control group. Similar results were obtained by Zabihi *et al.* [2019] when examining the effect of resveratrol on the *in*

Table 2. Effect of resveratrol on *in vitro* development of porcine embryos obtained after *in vitro* fertilization

Resveratrol (μM/mL)	No. of oocytes/replication	No. of presumptive zygotes (%)	No. of morulae (%)*	No. of expanded blastocysts (%)*
0.5	145/5	17 (11.7)	1 (5.8)^{aA}	1 (5.8)^{c}
1	126/5	6 (4.7)	3 (50.0)^{b}	0 (0.0)
0 (control)	117/5	27 (23.1)	12 (44.4)^{B}	10 (83.8)^{d}

*In comparison to presumptive zygotes.
$^{aA...}$Within column means bearing different superscripts differ significantly at: small leltters – P<0.05; capitals – P<0.01.

vitro development of sheep embryos and the quality of the obtained blastocysts. On the other hand, our results showed that low concentrations of resveratrol (0.5 and 1.0 μM/mL) in the IVC medium do not improve the development of porcine embryos and do not increase the percentage of porcine blastocysts (Tab. 2). Similarly, studies by Salzano *et al.* [2014] showed that the addition of 1.0 μM/mL resveratrol to the IVC medium for bovine embryos reduced the cleavage and blastocyst percentages.

Many previous studies showed the positive role of vitamin C in the development process. From among three tested antioxidants (vitamin E, resveratrol and vitamin C) it was decided to select vitamin C for further tests based on the analysis of porcine embryo development. However, our results demonstrated that the addition of 10 μg/mL or 20 μg/mL vitamin C to IVC medium had no positive effect on the percentage of cleavage, while the percentage of obtained blastocysts was lower in the experimental group when compared to the control group (Tab. 3). Simultaneously it was observed that the percentage of cleavage obtained after parthenogenetic fertilization of oocytes exposed to different concentrations of vitamin C was lower when compared to the control group, but the percentage of blastocysts was higher only in the group of 500 μg/mL vitamin C [Tao *et al.* 2010]. Similar results to ours also showed that IVC medium supplemented with 200 μg/mL ascorbic acid does not improve the frequency of blastocyst formation [Hossein *et al.* 2007]. In contrast, in their study Hu *et al.* [2012] reported a higher percentage of parthenogenetic pig blastocysts after using a lower concentration of vitamin C (20 μg/mL) in the IVC medium when compared to the control group.

Table 3. Effect of Vitamin C on *in vitro* development of porcine embryos obtained after *in vitro* fertilization

Vitamin C (mg/mL)	No. of oocytes/replication	No. of presumptive zygotes (%)	No. of morulae (%)*	No. of expanded blastocysts (%)*
10	237/6	70 (29.6)	28 (40.0)	17 (24.2)
20	223/10	39 (17.5)	23 (59.0)	20 (51.3)^{A}
0 (control)	232/11	54 (23.3)	22 (40.7)	14 (63.6)^{B}

*In comparison to presumptive zygotes.
^{AB}Within column means bearing different superscripts differ significantly P<0.01.

Vitamin C, known for its antioxidant properties, has been used as an additive to culture media also to improve the quality of blastocysts by reducing the level of apoptosis and increasing the number of cells in the blastocyst. However, we observed no statistically significant differences between the number of cells in pig blastocysts cultured in the presence of 20 µg/mL vitamin C and that in the control group (Tab. 4). Our observations support the reports indicating no differences in the mean number of cells/blastocyst in embryos cultured in the presence of 50 µg/mL vitamin C compared to the control group [Huang *et al.* 2011]. Moreover, experiments described by Hossein *et al.* [2007] showed that the addition of 200 µM/mL vitamin C to IVC medium did not improve the quality of obtained porcine embryos (Fig. 1).

Table 4. Cell number and apoptosis levels in porcine blastocysts obtained after culturing with addition of 20 µg vitamin C

Vitamin C (mg/mL)	No. of evaluated blastocysts	Cells/blastocyst		Apoptotic cells/blastocyst		Apoptotic Index (TUNEL, %)
		mean	SD	mean	SD	
20	11	41.4	7.7	1.3	1.2	3.1
0 (control)	7	37.0	6.7	1.9	1.8	5.0

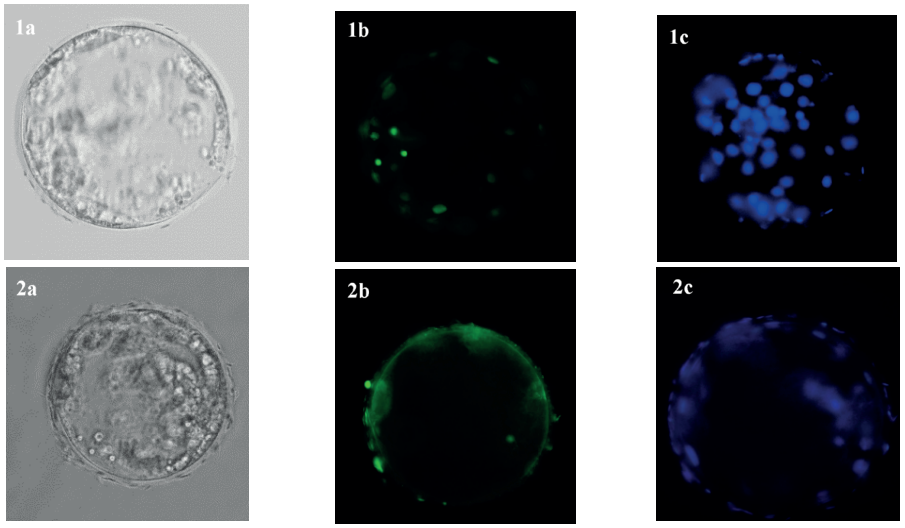

Fig. 1. TUNEL assay of in vitro obtained porcine expanded blastocysts cultured with 20 µg/mL vitamin C (1) and from the control group (2) a) visible light, b) apoptotic nuclei in fluorescein-stained blastocyst, c) total number of cell nuclei in DAPI-stained blastocyst.

Timing of embryo development is a rapid, simple, accurate and non-invasive way to evaluate embryos [Mateusen *et al.* 2005]. To our knowledge, the time of development of pig embryos produced *in vitro* in the presence of vitamin C in the IVC medium has not been evaluated so far. We showed that embryos cultured *in vitro* in

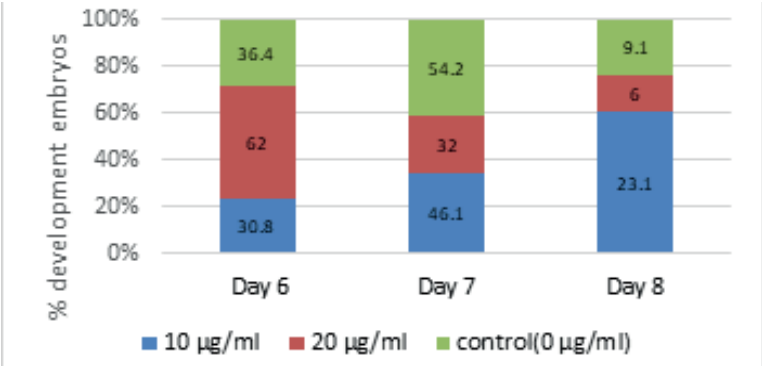

Fig. 2. Effect of vitamin C on the time of development of porcine embryos.

the presence of 20 µg/mL vitamin C showed the fastest development when compared to embryos from the control group or those cultured in the presence of 10 µg/mL vitamin C (Fig. 2). In conclusion, most embryos (62%) cultured with 20 µg vitamin C reached the blastocyst stage on day 6 of the culture. These observations are in agreement with earlier studies that the timing of blastocyst formation is significantly correlated with their quality [Van Soom *et al*. 1997]. Our studies suggest that 6-day blastocysts obtained from embryos cultured with 20 µg vit C were of better quality than those cultured without vitamin C.

Suboptimal *in vitro* culture conditions are one of the main factors leading to the formation of ROS, which adversely affects developing embryos. A study by Hu *et al.* [2012] showed that in pig embryos at the stage of 4-8 blastomeres, grown in IVC medium enriched with vitamin C at various concentrations (2.5, 5, 10, 20 and 40 µg / mL) have lower ROS levels when compared to the control embryos. Similarly, Nohalez *et al.* [2018] observed that the addition of 50 µg/mL vitamin C to vitrification and warming media resulted in an increase of blastocyst survival by decreasing the ROS levels. On the other hand, the authors noted no significant effect of ascorbic acid on the analyzed maturation, fertilization and embryo development parameters. Our team made similar observations, obtaining *in vitro* pig blastocysts with a lower ROS level in the group of embryos cultured in the presence of 20 µg/mL vitamin C compared to the embryos of the control group (Fig. 3 and 4).

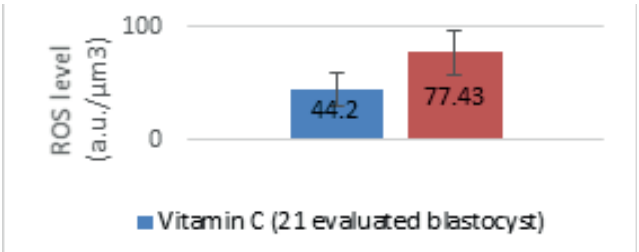

Fig. 3. Effect of 20 µg vitamin C on ROS levels in porcine blastocysts obtained after in vitro fertilization.

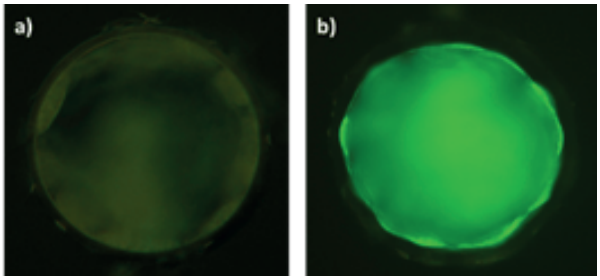

Fig. 4. Reactive oxygen species labeled in porcine expanded blastocysts. Fluorescence photomicrographs of porcine expanded blastocysts stained for ROS with CM-H2DCFDA from a) 20 µg vitamin C and b) control, untreated.

In conclusion, the addition of resveratrol (0.5 and 1.0 µM/mL) and vitamin E (100 µM/mL) to the *in vitro* culture medium of pig embryos had no positive effect on embryonic development and did not increase the percentages of cleavage and blastocysts. On the other hand, the addition of vitamin C in the amount of 20 µg/mL to the IVC medium accelerated the time of *in vitro* embryo development to the blastocyst stage and decreased the ROS level in pig blastocysts produced *in vitro*.

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