

# Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation

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**Abstract.** In this study, a dose-response assessment was performed to understand the relation between supplementation of media with L-ascorbic acid or vitamin C and porcine oocyte maturation and the *in vitro* development of parthenotes (PA) and handmade cloned (HMC) embryos. Various concentrations (0, 25, 50 and 100 µg/ml) of vitamin C supplemented in *in vitro* maturation (IVM) and culture (IVC) media were tested. None of these vitamin C additions affected nuclear maturation of oocytes, yet supplementation at 50 µg/ml led to significantly increased intracellular glutathione (GSH) levels and reduced reactive oxygen species (ROS). When cultured in IVM- and/or IVC-supplemented media, the group supplemented with 50 µg/ml of vitamin C showed improved cleavage rates, blastocyst rates and total cell numbers per blastocyst ( $P < 0.05$ ) compared with other groups (control, 25 µg/ml and 100 µg/ml). In contrast, supplementation with 50 µg/ml vitamin C decreased ( $P < 0.05$ ) the apoptosis index as compared with the groups supplemented with 100 µg/ml. In addition, even with a lower blastocyst rate to start with (37.6 vs. 50.3%,  $P < 0.05$ ), supplementation of HMC embryos with vitamin C ameliorated their blastocyst quality to the extent of PA embryos as indicated by their total cell numbers (61.2 vs. 59.1). Taken together, an optimized concentration of vitamin C supplementation in the medium not only improves blastocyst rates and total cell numbers but also reduces apoptotic indices, whereas overdosages compromise various aspects of the development of parthenotes and cloned porcine embryos.

**Key words:** Culture system, Embryo development, Handmade cloning, Parthenote, Vitamin C

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In an attempt to improve the efficiency of *in vitro* production (IVP) of embryos, we have come to a better understanding of the environmental factors that impact embryo development. In reality, *in vitro* culture superimposes onto embryos a number of stress factors, which nonetheless can be avoided *in vivo* [1]. Direct consequences of these *in vitro* stresses are physiological compromises of embryonic gene expression and development [2]. Today, the task of maintaining some mammalian embryos in culture is challenging, particularly in species such as the canine and porcine [3–6]. Recent studies have been reported with great improvement of *in vitro* culture systems by manipulating defined media via various combinatory supplements such as vitamins ([7–12], growth factors [13–17], cytokines [18, 19], hormones [20] and other selective intracellular and extracellular modulators for biochemical processes [9] in several species. Despite efforts to make improvements, the yield and quality of IVP porcine embryos are still low when compared with their *in vivo* counterparts [13, 21, 22]. In a long-standing practice, the hydrosoluble antioxidant vitamin C has often been used as a supplement in *in vitro* culture

systems for oocytes and embryos [7, 9, 10, 12]. As a result of reduced O<sub>2</sub> tension, IVP embryos acquired improved development evident by a decreased reactive oxygen species (ROS) content and DNA fragmentation [9, 23–25]. Glutathione (GSH), a major intracellular free thiol group, is involved in cellular proliferation, amino acid transport, DNA and protein syntheses and free radical scavenging in the oxidative microenvironment. It has been reported that GSH synthesis during oocyte maturation is beneficial to pronuclear formation and subsequent development [23, 26–28]. However, none of the past studies elucidated the functions of supplemented vitamin C in porcine oocyte maturation and further development *in vitro* or in the development of porcine parthenotes and handmade cloned (HMC) embryos. The objective of this study was to investigate the effects of vitamin C (L-ascorbic acid) when supplemented in media for 44 h at various doses on *in vitro* maturation (IVM) and during the overall culture of porcine embryos. Improved oocyte maturation rates and subsequent embryonic development after parthenogenetic activation and nuclear transplantation were demonstrated in this study.

## Materials and Methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

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### *Oocyte recovery and IVM*

Oocyte recovery and IVM were performed as described in previous studies [15, 29, 30]. Briefly, ovaries obtained from a local slaughterhouse were transported to the laboratory within 2 h in physiological saline containing penicillin (600 IU/ml). Oocytes were aspirated from follicles (3 to 7 mm in diameter), and cumulus-oocyte complexes (COCs) with at least two layers of cumulus cells and homogeneous ooplasm were selected for maturation in North Carolina State University 23 (NCSU-23) medium. Twenty to 30 oocytes were randomly allocated to each 100  $\mu$ l droplet of IVM medium containing different concentrations of vitamin C (0, 25, 50 and 100  $\mu$ g/ml), covered by mineral oil and cultured at 39 C in an incubator containing 5% CO<sub>2</sub>. For the first 22 h, the COCs were cultured in NCSU-23 medium supplemented with 10% porcine follicular fluid, cysteine (0.1 mg/ml), equine chorionic gonadotrophin (10 IU/ml) and human chorionic gonadotrophin (10 IU/ml), and then the COCs from all treatment groups were switched to the medium without hormones for another 22 h.

### *Measurement of ROS and intracellular GSH levels*

The matured oocytes were sampled to determine intracellular ROS and GSH levels by methods described previously [24, 28]. Briefly, H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate; Invitrogen, Eugene, OR, USA) and CellTracker Blue CMF2HC (4-chloromethyl-6.8-difluoro-7-hydroxycoumarin; Invitrogen) were used to detect intracellular ROS by green fluorescence and GSH level by blue fluorescence, respectively. Ten oocytes from each treatment group were incubated (in the dark) for 30 min in NCSU-23 supplemented with 10  $\mu$ M H2DCFDA and 10  $\mu$ M CellTracker. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA and placed into 10  $\mu$ l droplets, and the fluorescence was observed under an epifluorescence microscope (TE300; Nikon, Tokyo, Japan) with UV filters (460 nm for ROS and 370 nm for GSH). Fluorescent images were saved as graphic files, and their fluorescence intensities were analyzed with ImageJ software (Version 1.44p; National Institutes of Health, Bethesda, MD, USA). All images data were normalized by the untreated control oocytes for statistical analysis.

### *In vitro production of HMC embryos*

The procedures for cloning HMC embryos were mainly based on previous studies [30, 31] with some modifications [16]. Briefly, *in vitro* matured oocytes with a first polar body were incubated in HEPES-buffered TCM-199 containing pronase (3.3 mg/ml) and FBS (33%) for 10 sec and then washed 2 times in HEPES-buffered TCM-199 supplemented with 10% FBS (T10). After washing, oocytes were lined up in 40  $\mu$ l T10 containing 2.5 mg/ml cytochalasin B (16 oocytes in each droplet). Oocytes were rotated with a fire-polished glass pipette to identify the membrane protrusion or first polar body for oriented bisection with a microblade (ESE020, Bioniche Animal Health, Pullman, WA, USA) under a stereomicroscope. The demi-ooplasts were transferred to a T10 drop after bisection.

Cell fusion was performed with a two-step protocol containing two consecutive fusions. First, the enucleated cytoplasm was transferred to the HEPES-TCM-199 droplet containing 1 mg/ml phytohaemagglutinin (PHA) for 5 sec and then put into the T10 droplet holding

the fibroblasts, in which each cytoplasm was allowed to pair with one fibroblast cell. The cytoplasm-fibroblast pairs were incubated in fusion medium (0.3 M mannitol and 0.01% PVA) for 20 sec and transferred to a fusion chamber (with two electrodes separated by 1 mm). Under 0.6 kV/cm AC, the cell pairs were aligned between the wires with the fibroblasts farthest from the wires. Fusion was performed with one DC pulse at 2.0 kV/cm for 9  $\mu$ sec, and the pairs were then transferred to the T10 drop and incubated for 1 h before the second fusion.

For the second fusion, the remaining cytoplasm and the fused cytoplasm-fibroblast pairs were transferred to activation medium (0.3 M mannitol, 0.1 mM MgSO<sub>4</sub>, 0.1 mM CaCl<sub>2</sub> and 0.01% PVA) for equilibration. Then they were aligned with the fused pairs farthest from the wires and then subjected to DC pulse (0.80 kV/cm, 80  $\mu$ sec) fusion and initial activation simultaneously. After fusion and activation, the cytoplasm-fibroblast triplets were incubated in T10 for 20 min to allow complete fusion prior to activation with 6-dimethylaminopurine (6-DMAP).

### *Oocyte activation and embryo culture*

After 44 h of IVM, cumulus cells were removed from matured oocytes by gentle pipetting in DPBS containing 0.1% hyaluronidase. The oocytes with an extruded polar body were chosen under stereomicroscope and then washed twice in activation medium (0.28 M mannitol; 0.01% polyvinyl alcohol; 0.05mM HEPES; 0.1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O and 0.1 mM MgCl<sub>2</sub>) before parthenogenetic activation. For activation, an electrical pulse (2.04 kV/cm, 30  $\mu$ sec) generated by a BTX Electro-Cell Manipulator 2001 (BTX, San Diego, CA, USA) was applied to oocytes. After being washed twice with 2.5 mM 6-DMAP, activated oocytes were transferred to porcine zygote medium (PZM-3) containing 2.5 mM 6-DMAP for 4 h and then washed six times with PZM-3 medium. The activated oocytes were cultured continuously in PZM-3 medium following the same pattern of treatment as described above for 7 days to evaluate their developmental competence [4, 15, 30].

### *Apoptosis assays and total cell counts*

Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated d-UTP nick end-labeling (TUNEL) assay as described by Hao *et al.* [32] and Nguyen *et al.* [16] with minor modifications. Briefly, 7-day-old embryos were washed three times in DPBS/PVP (DPBS supplemented with 0.1% polyvinylpyrrolidone) and fixed in 4% (v/v) paraformaldehyde solution for 1 h at room temperature. Membranes were made permeable in 0.1% Triton X-100 in 0.1% citrate solution for 1 h at room temperature. Fixed embryos were incubated in TUNEL reaction medium (In Situ Cell Death Detection Kit, Fluorescein; Roche, Mannheim, Germany) for 1 h at 38.5 C in the dark. So, the broken DNA ends of the embryonic cells were labeled with TdT and fluorescein-dUTP. After the reaction stopped, the embryos were washed in DPBS/BSA (DPBS supplemented with 0.1% bovine serum albumin) and mounted on glass slides with DAKO Fluorescent Mounting Medium (S3023, Dako North America, Carpinteria, CA, USA) containing Hoechst 33342 for total cell counts. Whole-mount embryos were examined under an epifluorescence microscope (Nikon) by TUNEL assay and Hoechst staining. The numbers of apoptotic nuclei (by TUNEL assay) and

**Table 1.** Maturation of porcine oocytes cultured in IVM medium supplemented with various concentrations of vitamin C

Vitamin C (µg/ml)	Total No. oocytes	GV (%)	GVBD (%)	MI (%)	MII (%)
0	68	5.3 ± 3.0	9.2 ± 3.3	13.7 ± 4.4	71.8 ± 7.6
25	73	8.3 ± 8.3	4.6 ± 2.3	4.4 ± 2.2	82.6 ± 7.6
50	82	5.2 ± 3.5	4.9 ± 1.3	5.9 ± 3.1	79.8 ± 7.8
100	69	6.5 ± 2.6	6.1 ± 1.7	7.2 ± 1.0	80.3 ± 3.3

No significant differences were detected among treatment groups: values of three replicates are shown as the mean ± SEM. IVM, *in vitro* maturation; GV, germinal vesicle; GVBD, germinal vesicle break down; MI, metaphase I; MII, metaphase II.

**Table 2.** Relative levels of GSH and ROS in porcine oocytes cultured in IVM medium supplemented with vitamin C

Vitamin C (µg/ml)	Total No. oocytes	Relative level (pixels/oocyte) of	
		GSH	ROS
0	36	1.0 ± 0.1 <sup>a</sup>	1.0 ± 0.2 <sup>a</sup>
25	44	2.2 ± 0.6 <sup>ab</sup>	0.8 ± 0.1 <sup>a</sup>
50	49	3.4 ± 0.7 <sup>b</sup>	0.4 ± 0.1 <sup>b</sup>
100	44	2.5 ± 0.6 <sup>ab</sup>	0.7 ± 0.1 <sup>a</sup>

<sup>a,b</sup> Within the same column, numbers without the same superscripts differ ( $P < 0.05$ ). Values of four replicates are shown as the mean ± SEM. GSH, glutathione; ROS, reactive oxygen species; IVM, *in vitro* maturation.

total numbers of nuclei were determined from optical images. The apoptotic index was calculated as follows: apoptotic index = (number of TUNEL-positive nuclei / total number of nuclei) × 100.

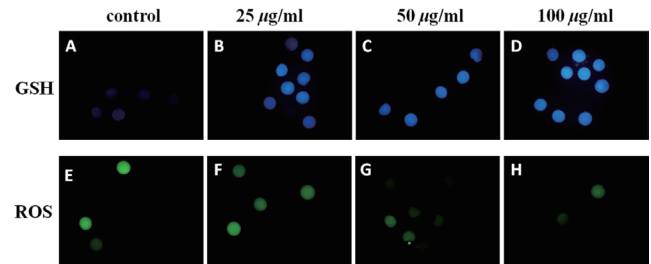
### Statistical analysis

Data for the percentage of apoptotic blastomeres were normalized by arcsine transformation prior to analysis. Statistical differences in embryo development among treatment effects on cleavage rates, total cell numbers, percentages of blastocysts and apoptotic indices were tested by the general linear model procedure (SAS Institute, Cary, NC, USA). Differences between treatment means were determined by using Tukey's test and were considered significant at  $P < 0.05$ . All data are expressed as the mean ± SEM.

## Results

### Effects of vitamin C on oocyte maturation and intracellular levels of GSH and ROS

To optimize the effective concentration of vitamin C in IVM medium, oocytes were cultured in medium supplemented with 0, 25, 50 and 100 µg/ml of vitamin C. The results showed that there was no significant difference between treatment groups in various aspect of nuclear maturation such as proportion of oocytes at the germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI) and metaphase II (MII) stages of meiosis as presented in Table 1. However, 50 µg/ml vitamin C in the maturation medium increased ( $P < 0.05$ ) the intracellular GSH level and decreased ( $P < 0.05$ ) ROS accumulation in MII oocytes (Table 2 and Fig. 1).



**Fig. 1.** Epifluorescent microphotographic images of *in vitro* matured porcine oocytes. Oocytes were stained with CellTracker Blue (A–D) and H2DCFDA (E–H) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II oocytes derived from the culture in medium supplemented with (B, C, D, F, G and H) or without (A and E) vitamin C. Matured oocytes from the group treated with 50 µg/ml of vitamin C treated group showed an increased cytoplasmic level of GSH (C) but a reduced ROS level.

### Effects of vitamin C in the maturation medium on the development of parthenotes

For further developmental assessment (Table 3), our results showed that there was no significant difference in the cleavage rate regardless of the concentration of vitamin C used. The percentage of parthenogenetic embryos reaching the blastocyst stage was higher in the 50 µg/ml group (53.8%) than in the control group (39.8%). As for total cell numbers per blastocyst, embryos cultured at 50 µg/ml (55.2%) exhibited an improved total cell number as compared with those cultured at 100 µg/ml (39.9%). None of the treatments showed a significant difference in apoptotic indices ( $P > 0.05$ ).

### Effects of vitamin C in the culture medium on the development of parthenotes

This study aimed to identify the best working concentration of vitamin C for embryo development in the IVC medium. The results (Table 4) showed that different treatments had similar cleavage rates ( $P > 0.05$ ). The blastocyst rate was higher with 50 µg/ml supplementation (46.1%) than with the other treatments (28.5% for the control, 30.5% for 25 µg/ml and 24.5% for 100 µg/ml). Total cells numbers per blastocyst at 50 µg/ml (50) were also significantly higher than those of the control (41) and the 100 µg/ml group (41). As for the apoptotic index, supplementation at 50 µg/ml resulted in a significantly lower value (3.9%) than supplementation at 100 µg/ml (7.3%).

### Effects of vitamin C addition in both IVM and IVC media on the development of porcine parthenotes

The developmental competence of the porcine parthenotes under the influence of vitamin C is shown as Table 5. Parthenotes supplemented with vitamin C at 50 µg/ml had better cleavage rates than those with 100 µg/ml (95.4 vs. 84.9%,  $P < 0.05$ ). Regarding the blastocyst rates, the rate was significantly higher (59.1%) for parthenotes supplemented with 50 µg/ml of vitamin C than for parthenotes in the control group and in 100 µg/ml group (40.8 and 36.0%,  $P < 0.05$ ). Embryos grown in 50 µg/ml vitamin C had the highest total cell number per blastocyst (55.9) among the groups (46, 45 and 44 for the control and 25 and 100 µg/ml groups respectively). Concomitantly,

**Table 3.** Development of porcine parthenotes derived from oocytes matured in IVM medium supplemented with vitamin C

Vitamin C ( $\mu\text{g/ml}$ )	Total No. oocytes	Cleavage rate (n) (%)	BL rate (n) (%)	TCN/BL (n) (%)	Apoptotic index (%)
0	155	87.79 $\pm$ 3.11 (126)	39.82 $\pm$ 1.70 <sup>b</sup> (62)	43.82 $\pm$ 3.94 <sup>ab</sup> (41)	7.02 $\pm$ 1.12
25	149	90.53 $\pm$ 2.59 (135)	43.16 $\pm$ 4.25 <sup>ab</sup> (64)	47.37 $\pm$ 3.63 <sup>ab</sup> (47)	5.36 $\pm$ 0.57
50	144	92.53 $\pm$ 1.35 (133)	53.87 $\pm$ 4.10 <sup>a</sup> (76)	55.23 $\pm$ 2.58 <sup>a</sup> (54)	4.19 $\pm$ 0.32
100	145	84.91 $\pm$ 4.84 (122)	44.13 $\pm$ 3.09 <sup>ab</sup> (63)	39.91 $\pm$ 2.34 <sup>b</sup> (45)	4.17 $\pm$ 1.18

<sup>a,b</sup> Within columns, means without the same superscripts differ ( $P < 0.05$ ). Values from six replicates are shown as the mean  $\pm$  SEM. IVM, *in vitro* maturation; TCN, total cell number; BL, blastocyst.

**Table 4.** Development of porcine parthenotes cultured in IVC medium supplemented with vitamin C

Vitamin C ( $\mu\text{g/ml}$ )	Total No. oocytes	Cleavage rate (n)	BL rate (n)	TCN/BL (n) (%)	Apoptosis index (%)
0	132	97.03 $\pm$ 0.94 (128)	28.51 $\pm$ 7.59 <sup>a</sup> (38)	41.17 $\pm$ 0.84 <sup>a</sup> (34)	6.91 $\pm$ 0.87 <sup>ab</sup>
25	123	91.31 $\pm$ 4.12 (112)	30.48 $\pm$ 5.09 <sup>a</sup> (37)	44.55 $\pm$ 2.31 <sup>ab</sup> (31)	6.19 $\pm$ 0.95 <sup>ab</sup>
50	123	93.59 $\pm$ 2.05 (115)	46.11 $\pm$ 6.07 <sup>b</sup> (56)	49.83 $\pm$ 2.21 <sup>b</sup> (45)	3.88 $\pm$ 0.69 <sup>a</sup>
100	126	90.40 $\pm$ 3.80 (114)	24.48 $\pm$ 3.41 <sup>a</sup> (31)	40.41 $\pm$ 2.31 <sup>a</sup> (25)	7.29 $\pm$ 0.75 <sup>b</sup>

<sup>a,b</sup> Within the same column, means without the same superscripts differ ( $P < 0.05$ ). Values from six replicates are shown as the mean  $\pm$  SEM. IVC, *in vitro* culture; BL, blastocyst; TCN, total cell number.

**Table 5.** Development of porcine parthenotes cultured consecutively in IVM and IVC media supplemented with vitamin C

Vitamin C ( $\mu\text{g/ml}$ )	Total No. oocytes	Cleavage rate (n) (%)	BL rate (n) (%)	TCN/BL (n) (%)	Apoptosis index (%)
0	151	90.15 $\pm$ 3.01(136) <sup>ab</sup>	40.86 $\pm$ 4.2 <sup>a</sup> (62) <sup>a</sup>	45.78 $\pm$ 1.81 <sup>a</sup> (57)	6.31 $\pm$ 0.64 <sup>ab</sup>
25	154	90.08 $\pm$ 2.36 (139) <sup>a</sup>	44.39 $\pm$ 4.54 <sup>ab</sup> (64)	45.12 $\pm$ 2.43 <sup>a</sup> (56)	5.93 $\pm$ 0.98 <sup>ab</sup>
50	141	95.36 $\pm$ 2.25 (135) <sup>b</sup>	59.14 $\pm$ 8.46 <sup>b</sup> (76)	55.86 $\pm$ 1.61 <sup>b</sup> (61)	4.87 $\pm$ 0.37 <sup>a</sup>
100	149	84.94 $\pm$ 5.53 (126) <sup>a</sup>	36.04 $\pm$ 5.83 <sup>a</sup> (63)	43.99 $\pm$ 0.98 <sup>a</sup> (61)	7.60 $\pm$ 0.75 <sup>b</sup>

<sup>a,b</sup> Within the same column, means without the same superscript differ ( $P < 0.05$ ). Values from six replicates are shown as the mean  $\pm$  SEM. IVM, *in vitro* maturation; TCN, total cell number; BL, blastocyst.

**Table 6.** Developmental competence of porcine parthenotes and handmade cloned (HMC) embryos cultured in media supplemented with (50  $\mu\text{g/ml}$ ) or without vitamin C

	Total No. embryos	Cleavage rate (n) (%)	Blastocyst rate (n) (%)	Total cell number (n)
PA (+)	99	89.6 $\pm$ 1.2 (88)	50.3 $\pm$ 1.9 <sup>a</sup> (49)	61.2 $\pm$ 1.7 <sup>a</sup> (44)
HMC (-)	111	92.6 $\pm$ 1.4 (104)	27.0 $\pm$ 4.8 <sup>b</sup> (34)	43.8 $\pm$ 4.6 <sup>b</sup> (34)
HMC (+)	114	95.1 $\pm$ 1.6 (109)	37.6 $\pm$ 4.1 <sup>c</sup> (45)	59.1 $\pm$ 4.6 <sup>a</sup> (40)

<sup>a,b,c</sup> Numbers with different superscripts in the same column differ ( $P < 0.05$ ). Values from four replicates are shown as the mean  $\pm$  SEM. PA, parthenotes; HMC, handmade cloned embryos; +, with (50  $\mu\text{g/ml}$ ) in *in vitro* culture medium; -, without vitamin C in *in vitro* culture medium.

embryos supplemented with vitamin C at 50  $\mu\text{g/ml}$  had a reduced apoptotic index compared with those supplemented with 100  $\mu\text{g/ml}$  (4.8 vs. 7.6%,  $P < 0.05$ ).

#### Effects of vitamin C on the development of parthenotes and HMC embryos

As shown in Table 6, cleavage rates and total cell numbers did not differ between PA and HMC embryos regardless to the treatment group, despite the PA embryos showing a higher ( $P < 0.05$ ) blastocyst rate (50%) than HMC embryos (38%) in IVC supplemented with vitamin C (50  $\mu\text{g/ml}$ ), which in turn showed a higher blastocyst rate than the HMC embryos grown in medium without vitamin C. In terms of cell number per blastocyst, PA and HMC embryos for

50  $\mu\text{g/ml}$  supplementation of vitamin C had higher cell numbers (61 and 59, respectively) compared with HMC embryos (44) cultured in medium without vitamin C.

#### Discussion

Ameliorating the culture system is of great importance for gamete and embryo manipulation and production *in vitro*. One of the major problems encountered in the culture system has been oxidative stress and its adverse effects on oocytes and embryos [1, 23, 24, 33]. To alleviate this situation, special attention has been accorded to the addition of antioxidants to the culture system [7, 9, 10, 33–35], and vitamin C is one of the antioxidant being tested [7, 9, 10, 33, 34,

36]. Evaluating it based on its chemical structure, vitamin C is an electron donor and therefore a reducing agent. It thus has two different biochemical roles: as an antioxidant and an enzymatic cofactor [12]. In the present study, vitamin C did not affect the nuclear maturation status regardless of the concentrations used. Similarly, Tatemoto *et al.* [7] and Córdova and coworkers [37] found that the addition of vitamin C to the oocyte maturation medium exerted no effect on the maturation rates of bovine oocytes. Our results are also in agreement with those found in murine and bovine [11] oocytes. Nevertheless, Tao *et al.* [9, 10] showed that L-ascorbic acid promoted polar body extrusion in denuded porcine oocytes. Some antioxidants such as vitamin C, vitamin E and Trolox had no effect on oocyte maturation, but others such as propyl gallate and 2,4,5-trihydroxybutrophenone inhibited the spontaneous resumption of meiosis [38]. In the same study, denuded oocytes were shown to be less sensitive to an antioxidant in terms of resumption of meiosis. It is suggested that the effect of vitamin C supplemented in the IVM medium lies mainly in its capability to promote ooplasmic maturation.

High levels of ROS during early embryonic development caused lipid peroxidation of cell membranes [39, 40] and DNA fragmentation, and its presence also perturbed RNA transcription and protein synthesis [23, 41]. It has been reported that the inability of embryos to develop normally *in vitro* is caused by a cytoplasmic deficiency of protective enzyme activity, such as those of superoxide dismutase, catalase or the glutathione peroxidase/reductase couple [39]. Glutathione is one of the naturally synthesized antioxidants that protect cells from ROS toxicity and regulate the intracellular redox balance [28]. The intracellular level of GSH increases during oocyte maturation [23, 27, 42]. Its concentration in a matured oocyte has been used as a parameter for assessing ooplasmic maturation [28], and it has been in turn associated with the capability to form the male pronucleus after fertilization [7, 27] and the developmental competence of developing embryos [43]. Recent reports also showed that addition of anthocyanin,  $\beta$ -mercaptoethanol or cysteine addition to IVM media improved the cytoplasmic maturation of oocytes and embryo development [28, 43–45]. In the present study, 50  $\mu\text{g/ml}$  of vitamin C supplementation during IVM exerted a strong antioxidant effect by decreasing the ROS content and increasing the GSH level in matured oocytes, which presumably allowed better ooplasmic maturation and subsequent embryo development. Concomitantly, we should notice that 100  $\mu\text{g/ml}$  of vitamin C supplementation during IVM did not produce noticeable amelioration of the GSH or ROS status, allowing us to draw the conclusion that there is an existing dose-response window. These results differ in part from those of Tatemoto *et al.* [7], who found no effect on intracellular GSH content when ascorbic acid was added but obtained an increase in the intracellular GSH content. In addition, vitamin C did have some antioxidant effects, but it also impose some side effects by decreasing the maturation rate at 750  $\mu\text{M/ml}$  [7] and increasing the ratio of abnormal oocytes for COCs at 250  $\mu\text{M/ml}$  and denuded oocytes at 750  $\mu\text{M/ml}$  [9].

*In vitro* produced embryos with a large number of cells are more likely to successfully implant and give rise to live births [46]. It has been shown that the quality of PA blastocysts is not better than that of IVF blastocysts in terms of their total cell numbers and apoptotic nuclei per blastocyst [9]. In this study, we demonstrated that embryos treated with vitamin C (50  $\mu\text{g/ml}$ ) had improved cleavage rates,

blastocyst rates and total cell numbers per blastocyst and a reduced apoptotic index. Clearly, a suboptimal concentration of free-radical scavengers in the culture system would also contribute to poor embryo viability and, in turn, to progressive embryo losses during IVP [16, 47]. Nonetheless, supplementation of 100  $\mu\text{g/ml}$  of vitamin C to the culture medium increased the apoptotic index, suggesting the existence of a narrow window for the concentration dependency [10, 48] of vitamin C treatment.

In the present study, parthenotes only showed improvement in their blastocyst rate (Table 6) after vitamin C addition. Supplementation of the medium with 50  $\mu\text{g/ml}$  of vitamin C improved the blastocyst formation rate compared with that of the untreated group and ameliorated the embryo quality compared with that of the PA considering the total cell numbers per blastocyst. Our results are in agreements with those found by Kragh and coworkers [49], but the cell numbers were still low compared with those of embryos (cell numbers = 77) derived from the piglet previously obtained by Du and colleagues [6]. The quality of the developing HMC blastocysts was not impaired compared with those of the parthenogenetic embryos in medium supplemented with 50  $\mu\text{g/ml}$  of vitamin C. The beneficial effect of vitamin C supplementation may be ascribed to an improved culture condition and/or a better cytoplasmic maturation of the oocytes due to a reduced intracellular oxidative status for embryo development [50]. Moreover, the *in vitro* environment can alter the delicate interdependency of the epigenetic state, metabolisms and development of the embryo [12, 51]. Huang and colleagues [36] related the enhanced acH4K5 signal to more efficient genome activation during reprogramming, as supported by the elevation of Oct4 and Sox2 expression levels observed in vitamin C-treated blastocysts.

In conclusion, the addition of a relatively low concentration of vitamin C during *in vitro* maturation of porcine oocytes enhanced their cytoplasmic maturation and subsequent development, whereas high concentrations of vitamin C induced apoptosis. Our study clearly also demonstrated that supplementation of 50  $\mu\text{g/ml}$  of vitamin C in IVC medium improved, at least partially, the suboptimal culture conditions for porcine parthenotes and cloned embryos. Further studies are required to elucidate more in-depth mechanisms underlying oocyte maturation in the presence of vitamin C as well as potential additives in the culture medium.

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