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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, dose-response assessment was performed to understand the relation between supplementation of L-ascorbic acid or vitamin C in the media 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 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 number per blastocyst ($P < 0.05$) compared to other groups (control, 25 μg/mL and 100 μg/mL). In contrast, supplementation of 50 μg/mL vitamin C decreased ($P < 0.05$) apoptosis index as opposed to the groups supplemented with 100 μg/mL. Even with a lower blastocyst rate to start with (32.7 vs. 48.3%; $P < 0.05$), vitamin C-supplemented HMC embryos ameliorated their blastocyst quality to the extent of PA embryos as indicated by their total cell numbers (58 vs. 57). Taken together, optimized concentration of vitamin C supplementation in the medium not only improves blastocyst rates and total cell numbers but also reduces apoptotic indices, whereas over-dosages compromise the development of parthenotes and cloned porcine embryos in various aspects.

Key words: Oocytes · Parthenogenesis · Clone · Embryo · Blastocyst · Antioxident

INTRODUCTION

In the attempt of improving the *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 a number of stress factors on an embryo, which nonetheless can be avoided *in vivo* [1]. Direct consequences of undertaking these *in vitro* stresses are physiological compromises of embryonic gene expression and development [2]. Today, the task to maintain an early mammalian embryo in culture has been significantly challenged, particularly in some species such as canine and porcine [3-5]. Recent studies have reported with great effort improvement of *in vitro* culture system by manipulating defined media via various combinatory supplements such as vitamins [6-11], growth factors [12-16], cytokines [17, 18], hormones [19] and selective intracellular and extracellular modulators for biochemical processes [8] in several species. Despite efforts to make improvement, the yield and quality of IVP embryos are still low or not optimized when compared with their *in vivo*-produced counterparts [20, 21, 12]. In a long-standing practice, the hydrosoluble antioxidant vitamin C has often been used as one supplement to *in vitro* culture system for oocytes and embryos [6, 8, 9, 11]. As a result of reduced O_2 tension, IVP porcine embryos acquired improved development evident by a decreased reactive oxygen species (ROS) content and DNA fragmentation [6, 8, 22, 23]. Glutathione (GSH), a major intracellular free thiol group, is involved in cellular proliferation, amino acid transport, DNA and protein syntheses and free radical

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scavenging against oxidative microenvironment. It has been reported that GSH synthesis during oocyte maturation is beneficial to pronuclear formation and subsequent development [6, 24-26]. However, neither have the past studies elucidated the functions of supplemented vitamin C on porcine oocyte maturation and their further development *in vitro*, nor on the development of porcine parthenotes and handmade cloned (HMC) embryos. A propos, we investigated the effects of vitamin C (L-ascorbic acid) when supplemented in media for 44 h at various doses on *in vitro* maturation (IVM) and for the overall culture of porcine embryos. Improved oocyte maturation rates and subsequent embryonic development after parthenenogenetic activation and nuclear transplantation were demonstrated in this study.

MATERIALS AND METHODS

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

Oocyte Recovery and IVM: The porcine oocytes recovery and IVM were preceded as described in previous studies [14, 27, 28]. 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 (with 3-7 mm in diameter) and cumulus-oocyte complexes (COCs) with at least two layers of cumulus cells and homogeneous ooplasm were selected for maturation in NCSU-23 medium. Twenty to 30 oocytes were randomly allocated to each 100 μL droplet of IVM medium with different concentrations of vitamin C (0; 25; 50 and 100 μg/mL), covered by mineral oil and cultured at 39°C in an incubator containing 5% CO_2. For the first 22 h, the COCs were cultured in the 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 were switched to medium without hormones for another 22 h respecting the different treatments.

Measurement of ROS and Intracellular GSH Levels: The IVM oocytes were sampled to determine intracellular ROS and GSH levels by methods previously described [22, 26]. Briefly, H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate; Invitrogen) 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 μM H2DCFDA and 10 μM CellTracker. After incubation, oocytes were washed with D-PBS (Invitrogen) containing 0.1% (w/v) PVA, placed into 10 μL 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 in tiff format. The fluorescence intensities of oocytes were analyzed by Image J software (Version 1.44p; National Institutes of Health, Bethesda, MD, USA) and normalized to the untreated control oocytes.

***In Vitro* Production of HMC Embryos:** The procedures for cloning HMC embryos were mainly based on previous studies [28, 29] with some modifications [17]. Briefly, *in vitro* matured oocytes with first polar body were incubated in HEPES-buffered TCM-199 containing pronase (3.3 mg/mL) and FBS (33%) for 10 sec and then with 2 times washes in HEPES-buffered TCM-199 supplemented with 10% FBS (T10). After washing, oocytes were lined up in 40 μL 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, USA, INC.) under a stereomicroscope. The demi-ooplasts were transferred into T10 drop after bisection. Cell fusion was performed with a two-step protocol containing two consecutive fusions. First, the enucleated cytoplast 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 where each cytoplast was allowed to pair with one fibroblast cell. The cytoplast-fibroblast pairs were incubated in the fusion medium (0.3 M mannitol and 0.01% PVA) for 20 sec and transferred to the fusion chamber (with two electrodes separate by 1 mm). Under a 0.6 kV/cm AC, the cell pairs were aligned to the wire with the fibroblasts farthest from the wire. The fusion was performed with one DC pulse at 2.0 kV/cm for 9 μsec. The pairs were then transferred from the fusion chamber to the T10 drop and incubated for 1 h before second fusion. For the second fusion, the remaining cytoplasts and the fused cytoplast-fibroblast pairs were transferred to the activation medium droplet (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$ and 0.01% PVA) for equilibration.

Then they were aligned with the fused pairs farthest from the wire followed by a DC pulse (0.80 kV/cm) for 80 µsec for fusion and initial activation. After fusion and activation simultaneously, the cytoplast-fibroblast triplets were incubated in T10 for 20 min to allow complete fusion prior to chemical activation with 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 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 $CaCl_2$•$2H_2O$ and 0.1 mM $MgCl_2$) before parthenogenetic activation. For activation, an electrical pulse (2.04 kV/cm, 30 µs) generated by a BTX Electro-Cell Manipulator 2001 (BTX, San Diego, CA, USA) was applied to oocytes. After being washed twice in 2.5 mM 6-dimethylaminopurine (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 [14, 28].

Apoptosis Assays and Total Cell Numbers per Blastocyst: Apoptosis was detected by terminal deoxynucleotidyl transferase mediated d-UTP nick end-labeling (TUNEL) assay as described by Hao *et al*. [30] and Nguyen *et al*. [15] 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 were mounted on glass slides with DAKO fluorescent mounting medium (S3023, Dako North America, Inc., California, USA) containing Hoechst 33342 for total cell counts. Whole-mount embryos were examined under an epi?uorescence microscope (Nikon, Tokyo, Japan) by TUNEL assays and Hoechst staining. The numbers of apoptotic nuclei (by TUNEL assay) and 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 to overcome heterogeneity of variance. The statistical significance between the embryo development and treatment effects on cleavage rates, total cell numbers, percentages of blastocysts and percentages of apoptotic cells was tested by general linear model procedure (SAS Institute, Cary, NC). Differences among treatment means were determined by using Tukey's test and were considered as significant at $P < 0.05$. All data are expressed as mean ± SEM.

RESULTS

Effects of Vitamin C on Oocyte Maturation and Intracellular Level of GSH and ROS: To optimize the functional 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 germinal vesicle (GV), germinal vesicle break down (GVBD), metaphase I (MI) and metaphase II (MII) 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).

Vitamin C in the Maturation Medium on the Development of Porcine Parthenotes: For further developmental assessment (Table 3), the 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 blastocyst stage was significantly 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 to those cultured at 100 µg/mL (39.9%). None of the treatments showed a significant difference in apoptotic indices.

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

Vitamin C, µg/ML	No. Total oocytes, N	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: three replicates with mean ± SEM

IVM: *in vitro* maturation; GV: germinal vesicle; GVBD: germinal vesicle break down; MI: metaphase; MII: metaphase II

Table 2: Relative levels of GSH and ROS in pig oocytes cultured in IVM medium supplemented with vitamin C

		Relative level (pixels/oocyte) of	
Vitamin C, µg/mL	No. Total oocytes, N	GSH	ROS
0	36	1.0±0.1^{a}	1.0±0.2^{a}
25	44	2.2±0.6^{ab}	0.8±0.1^{a}
50	49	3.4±0.7^{b}	0.4±0.1^{b}
100	44	2.5±0.6^{ab}	0.7±0.1^{a}

^{a,b} Within the same column, numbers without the same superscripts differ ($P < 0.05$); Four replicates; GSH: glutathione; ROS: reactive oxygen species; IVM: in vitro maturation

Table 3: Development of pig parthenotes derived from oocytes matured in the IVM medium supplemented with vitamin C

Vitamin C, µg/mL	No. Total oocytes, N	Cleavage rate, % (n)	BL rate, %(n)	TCN/BL, % (n)	Apoptosis index, %
0	155	87.79 ± 3.11(126)	39.82 ± 1.70 (62)^{b}	43.82 ± 3.94^{ab}	7.02 ± 1.12
25	149	90.53 ± 2.59 (135)	43.16 ± 4.25(64)^{ab}	47.37 ± 3.63^{ab}	5.36 ± 0.57
50	144	92.53 ± 1.35(133)	53.87 ± 4.10 (76)^{a}	55.23 ± 2.58^{a}	4.19 ± 0.32
100	145	84.91 ± 4.84(122)	44.13 ± 3.09 (63)^{ab}	39.91 ± 2.34^{b}	4.17 ± 1.18

IVM: *in vitro* maturation; ^{a,b}: within columns, means without the same superscripts differ ($P < 0.05$); Six replicates with mean ± SEM; TCN: total cell number; BL: blastocyst

Table 4: Development of pig parthenotes cultured in the IVC medium supplemented with vitamin C

Vitamin C, µg/mL	No. Total oocytes, N	Cleavage rate, (n)	BL rate, (n)	TCN/BL, % (n)	Apoptosis index, %
0	132	97.03 ± 0.94(128)	28.51 ±7.59 (38)^{a}	41.17 ± 0.84^{a}	6.91 ± 0.87^{ab}
25	123	91.31 ± 4.12(112)	30.48 ± 5.09(37)^{a}	44.55 ± 2.31^{ab}	6.19 ± 0.95^{ab}
50	123	93.59 ± 2.05(115)	46.11 ± 6.07 (56)^{b}	49.83 ± 2.21^{b}	3.88 ± 0.69^{a}
100	126	90.40 ± 3.80(114)	24.48 ± 3.41 (31)^{a}	40.41 ± 2.31^{a}	7.29 ± 0.75^{b}

IVC: *in vitro* culture; ^{a,b}: within the same column, means without the same superscripts differ ($P < 0.05$); Six replicates with mean ± SEM; BL: blastocyst; TCN: total cell number

Table 5: Development of pig parthenotes cultured consecutively in IVM and IVC media supplemented with vitamin C

Vitamin C, µg/mL	No. Total oocytes, N	Cleavage rate, % (n)	BL rate, %(n)	TCN/BL, % (n)	Apoptosis index, %
0	151	90.15 ± 3.01(136)^{ab}	40.86 ±4.2 (62)^{a}	45.78 ±1.81^{a}	6.31 ±0.64^{ab}
25	154	90.08 ± 2.36 (139)^{a}	44.39 ± 4.54(64)^{ab}	45.12 ±2.43^{a}	5.93 ±0.98^{ab}
50	141	95.36 ± 2.25(135)^{b}	59.14 ± 8.46 (76)^{b}	55.86 ±1.61^{b}	4.87 ±0.37^{a}
100	149	84.94 ± 5.53(126)^{a}	36.04 ± 5.83 (63)^{a}	43.99 ±0.98^{a}	7.60 ±0.75^{b}

IVM: *in vitro* maturation; ^{a,b}: within the same column, means without the same superscript differ ($P < 0.05$); Six replicates with mean ± SEM; TCN: total cell number; BL: blastocyst

Table 6: Developmental competence between the parthenotes and the handmade cloned (HMC) embryos cultured in the media supplemented with 50 µg/mL of vitamin C

	Total No. embryos	Cleavage rate, % (n)	Blastocyst rate, % (n)	Total cell number
PA	120	91.7 ± 3.1 (110)	48.3 ± 1.5^{a} (58)	57.1 ± 2.8
HMC	98	90.8 ± 2.0 (88)	32.7 ± 2.9^{b} (32)	58.4 ± 3.1

^{a,b} Numbers with different superscripts in the same column differ ($P < 0.05$); Six replicates mean ± SEM; PA: pathenotes; HMC: handmade cloned embryos

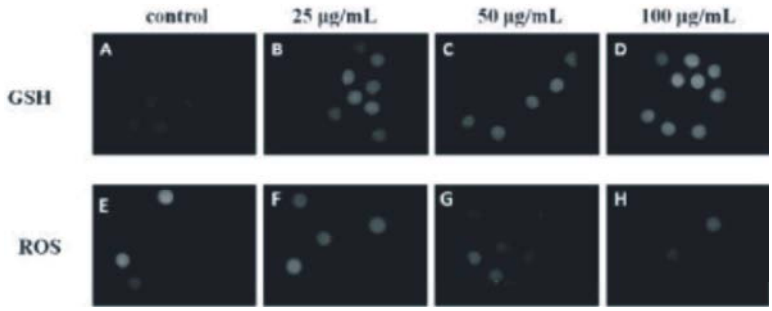

Fig. 1: Epifluorescent microphotographic images of *in vitro* matured porcine oocytes. Oocytes were stained with CellTracker Blue (A-D) and H2DCFDA (E–H) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively. Metaphase II oocytes derived from the culture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

Vitamin C in the Culture Medium on the Development of Porcine 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 no significant difference between each other ($P > 0.05$) in cleavage rates. The blastocyst formation rate was higher with 50 µg/mL supplementation (46.1%) than with other treatments (28.5% for control; 30.5% for 25 µg/mL; 24.5% for 100 µg/mL). Total cells numbers per blastocyst at 50 µg/mL (49.8) were also significantly higher than that of the control (41.2) and the 100 µg/mL group (40.5). As for the apoptotic index, supplementation at 50 µg/mL incurred significantly lower value (3.9%) than that at 100 µg/mL (7.3%).

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 higher cleavage rates than those with 100 µg/mL (95.4 vs 84.9 %; $P < 0.05$). In blastocyt rates, parthenotes supplemented with 50 µg/mL of vitamin C showed significantly higher value (59.1 %) than those in the control group and in the 100 µg/mL group (40.8 and 36.0 %; $P < 0.05$). Embryos grown in 50 µg/mL vitamin C had the greatest total cell number per blastocyst (55.9) among other groups (45.7, 45.1 and 43.9 respectively for control, 25 and 100 µg/mL groups). Concomitantly, embryos supplemented with vitamin C at 50 µg/mL had a reduced apoptosis index compared to those with 100 µg/mL (4.8 vs 7.6 %; $P < 0.05$). Effect of Vitamin C on the Development of the Parthenotes and HMC Embryos: As shown in Table 6, cleavage rates and total cell numbers did not differ between PA and HMC embryos after addition of 50 µg/mL vitamin C in the culture medium, despite the observation that PA embryos showed a higher blastocyst rate (48%) than HMC embryos (32%).

DISCUSSION

Ameliorating the culture system is of great importance for gamete and embryo manipulation and production *in vitro*. One of the major problems in the culture system encountered has been the oxidative stress and its adverse effects on oocytes and embryos [1, 6, 22, 31]. To alleviate this situation, special attention has been accorded to the addition of antioxidants to the culture system [6, 8, 9, 22, 32, 33] and vitamin C is one of those under testing [6, 8, 9, 31, 32, 34]. Evaluating from 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 [11]. In the present study, vitamin C did not affect the nuclear maturation status regardless of the concentrations used. Similarly, Tatemoto *et al*. [6] and Córdova and co-workers [35] found that the addition of vitamin C to the oocyte maturation medium exerted no effect on the maturation rates. Our results are also in agreement with those found in murine and bovine [10] oocytes. Nevertheless, Tao *et al*. [8, 9] 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 [36]. In the same study, denuded

oocytes were shown to be less sensitive to the antioxidant in terms of the resumption of meiosis. It is suggested that the effect of vitamin C supplemented in the IVM medium lies in its capability of promoting ooplasmic maturation. High levels of ROS during early embryonic development caused lipid peroxidation of the cell membranes [37, 38] and DNA fragmentation and its presence also perturbed RNA transcription and protein synthesis [6, 39]. It has been reported that the inability of embryos to develop normally *in vitro* is caused by a deficiency of cytoplasmic protective enzyme activity, such as superoxide dismutase, catalase or the glutathione peroxidase/reductase couple [38]. Glutathione being one of the naturally synthesized antioxidants protects cells from ROS toxicity and regulates the intracellular redox balance [26]. Intracellular level of GSH increases during oocyte maturation [6, 25, 40]. Its concentration in matured oocytes has been used as a parameter for evaluating ooplasmic maturation [26], which is in turn associated with the capability of forming the male pronucleus after fertilization [6, 25] and the developmental competence of developing embryos [41]. Recent reports also proved that anthocyanin, β-mercaptoethanol, or cysteine addition in IVM media improved the ooplasmic maturation of oocytes and embryo development [26, 41, 42]. In this study, 50 μg/mL of vitamin C supplementation during IVM exerted a strong antioxidant effect by a decreased ROS content and an increased GSH level in matured oocytes which allowed a better ooplasmic maturation and subsequent embryo development. These results are in discrepancy with those from Tatemoto *et al*. [6] who found no effect on Intracellular GSH content when ascorbic acid was added but obtained an increase in the intracellular GSH content. However vitamin C showed some antioxidant effects, it also showed some side effect by decreasing the maturation rate at 750 μM/mL [6] and the ratio of atypical oocytes for COC at 250 μM/mL and denuded oocytes at 750 μM/mL [8].

In vitro produced embryos with a large number of cells are more likely to successfully implant and give rise to live offspring [43]. It has been demonstrated that quality of PA blastocysts is not better than IVF blastocysts in terms of their total cell numbers and apoptotic nuclei per blastocyst [30]. In this study, we demonstrated that embryos treated with vitamin C (50 μg/mL) had improved cleavage rates, blastocyte rates, total cell number 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 [44, 15]. Nonetheless, supplementation of 100 g/mL of vitamin C to the embryo culture medium increased the apoptotic index suggesting the existence of a narrow window and the concentration-dependency [9, 45] of Vitamin C treatment.

In the present study, parthenotes only showed improvement in their blastocyst rate (Table 4) after vitamin C addition. Our results are in agreements with those found by Kragh and co-workers [46], but the cell numbers were still low compared to those embryos (cell numbers = 77) derived from the piglet previously obtained by Du and colleagues [5]. The quality of the developing HMC blastocysts was not impaired as opposed to the parthenote embryos. The beneficial effect from vitamin C supplementation may be ascribed to an improved culture conditions and/or a better cytoplasmic maturation of the oocytes due to a reduced intracellular oxidative status for embryo development [47]. Moreover, the *in vitro* environment can alter the delicate interdependence of the epigenetic state, metabolisms and development of the embryo [11, 48]. Huang and colleagues [34] 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.

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 μg/mL of vitamin C in IVC medium relieved the suboptimal culture condition 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.

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