ENHANCING THE DEVELOPMENTAL COMPETENCE OF SOMATIC CELL NUCLEAR TRANSFER EMBRYOS DERIVED FROM SMALL BOVINE OOCYTES THROUGH PRE-MATURATION CULTURE WITH L-ASCORBIC ACID

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SUMMARY

Growing oocytes derived from bovine small antral follicles (2-3 mm) exhibit lower developmental competence compared to fully grown oocytes collected from large antral follicles (4-6 mm). We reported that to support the growth of these oocytes in porcine, a pre-in vitro maturation (Pre-IVM) subculture followed by in vitro maturation (IVM) was implemented (Thuy Van *et al*,. 20220). The present study aimed to investigate the effects of Pre-IVM culture on the developmental competence of cloned bovine embryos derived from growing oocytes. Additionally, we examined whether supplementing the Pre-IVM medium with L-ascorbic acid could enhance cloned bovine blastocyst formation. In the first experiment, the effect of Pre-IVM culture, with or without L-ascorbic acid, on parthenogenetic embryos derived from growing oocytes was assessed. Subsequently, the developmental competence of somatic cell nuclear transfer (SCNT) embryos reconstructed from growing oocytes subjected to Pre-IVM with L-ascorbic acid was evaluated. The results indicated that the 8-hour Pre-IVM treatment supplemented with L-ascorbic acid significantly improved the developmental competence, quality, and histone modifications of bovine parthenogenetic blastocyst in the growing oocyte group. In addition, cloned embryos derived from growing oocytes subcultured with Pre-IVM supplemented with L-ascorbic acid showed developmental competence comparable to the fully grown oocyte group. In conclusion, Pre-IVM supplemented with 50 µg/mL L-ascorbic acid improved the developmental competence of bovine cloned embryos derived from growing oocytes.

Keywords: Bovine embryo, In vitro maturation, L-ascorbic acid, Pre–in vitro maturation, somatic cell nuclear transfer.

INTRODUCTION

Somatic cell nuclear transfer (or cloning) is a technique that involves injecting the somatic cell into the cytoplasm of an enucleated egg (the egg nucleus is removed). SCNT plays an irreplaceable role in the study of transgenic animals and human medicine. In vitro maturation (IVM) is a widely used technique for recovering immature oocytes and maturing them to full meiotic competence. However, the number of fully grown oocytes collected for IVM culture from each ovary was limited, while growing oocytes are abundant and can be considered as an alternative source material for the SCNT technique. On the other hand, growing oocytes derived from small antral follicles (2-3 mm) cultured immediately in IVM medium right after being collected showed poor maturation rates and low developmental competence due to its incomplete cytoplasm and nucleus. By inhibiting germinal vesicle breakdown (GVBD) and keeping growing oocytes nuclear at the germinal stage (GV) before IVM, the Pre-IVM technique allows oocytes to acquire complete developmental competence during the meiotic arrest (9). Hypoxanthine, a cyclic nucleotide phosphodiesterase (PDE) enzyme inhibitor found in murine follicular fluid, inhibits germinal vesicle (GV) breakdown in oocytes by maintaining elevated levels of cyclic adenosine monophosphate (cAMP). High cAMP levels help keep the oocytes arrested in the GV stage, preventing premature maturation and allowing for a more controlled and synchronized development process.

Reactive oxygen species (ROS) are produced during the in vitro culturing of oocytes and embryos. An accumulation of ROS is considered oxidative stress. Increased levels of reactive oxygen species (ROS) are often associated with high levels of apoptosis. Antioxidants are compounds that can suppress the formation and reactions of ROS. L-ascorbic acid has been implicated in the biosynthesis of collagen, thus being important during follicular growth, ovulation, and corpus luteum formation, which is needed for the constant modulation of the basement membrane and extracellular matrix (Li *et al*., 2007). In IVM, it improves cytoplasmic maturation by alleviating oxidative stress during oocyte maturation via increasing GSH storage and further protecting the embryo against oxidative aggressions during its early developmental stages (Khazaei *et al*., 2017). In a previous publication, our research group determined that the optimal concentration of L-ascorbic acid for both IVM and in vitro development (IVD) of porcine oocytes is 50 µg/mL (Van *et al*., 2020).

This study was conducted to demonstrate that a pre-IVM environment containing L-ascorbic acid enhances the developmental competence of cloned embryos reconstructed from small bovine oocytes. The conclusion is based on the developmental competence, quality, and histone H3K9 levels in bovine blastocysts derived from small oocytes.

MATERIALS AND METHODS

Unless stated otherwise, all chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA).

Pre-maturation culture and maturation culture

Oocytes- cumulus-granulosa-complexes (OCGCs) isolated from 2-3 mm follicles were washed in HEPES medium 3–4 times to remove infectious factors. OCGCs were then randomly divided into two groups based on L-ascorbic acid concentration: 0 µg/mL and 50 µg/mL, before culturing for 8 hours in a pre-IVM floating drop culture. This medium contained αMEM, 10% fetal bovine serum (FBS), 4 mM hypoxanthine, 50 µg/mL sodium pyruvate, 0.01 IU/mL follicle-stimulating hormone (FSH), 10 ng/mL androgen, with or without 50 µg/mL L-ascorbic acid, and 1 µg/mL estradiol 17β (ES). Following the 8-hour culturing period in the pre-IVM medium, OCGCs were transferred into the IVM medium for 18 to 22 hours. The IVM medium contained TCM199, 10% FBS, 50 µg/ml sodium pyruvate, 0.0065 IU/mL luteinizing hormone (LH), 0.006 IU/mL FSH, 50 ng/ml epidermal growth factor (EGF), and 1 µg/mL ES.

Parthenogenetic activation

After 22 hours of IVM culturing, the OCGs were transferred into a HEPES drop containing the hyaluronidase enzyme at 38.5°C to detach the oocytes from the cumulus cells. They were then moved to other HEPES drops to start the cumulus cell removal. Denuded oocytes were recovered in a recovery medium consisting of TCM 199, 10% FBS, and 50 µg/mL sodium pyruvate for 30 to 45 minutes. After recovery, the oocytes were transferred to modified synthetic oviduct fluid (mSOF) for at least an hour. Following that, the recovered oocytes were treated with calcium ionomycin for 5 minutes and then transferred to drops of 6-dimethylaminopurine (DMAP) for 4 hours. The remaining DMAP on the oocytes was removed by washing with mSOF, and a final transfer to mSOF for IVD culturing was performed. After 2.5 days, the culture medium was changed and replaced with SOF containing 5% FBS to provide the crucial nutrients for the blastocyst stage.

Somatic cell nuclear transfer

The somatic cell was isolated from the lung tissue of the Korean Wagyu cow in preparation for the SCNT process. The cumulus-removing process was the same as described in the parthenogenetic activation section, except the IVM duration was only 18 hours. The XY laser system on the manipulator was used to cut and remove the zona pellucida of denuded and matured oocytes selected by the extrusion of the polar body. Next, using an enucleating pipette on the micromanipulator system, the oocyte's nuclear material and a portion of its cytoplasm were aspirated in drops containing 1.5% cytochalasin B. TCM-free hormone was used to recover the oocytes before the SCNT. The donor cell's nucleus was then extracted, its cell membrane removed, and it was injected into the enucleated oocyte's cytoplasm. Before the freshly developed zygotes were placed in mSOF for 30 minutes, another recovery was conducted in TCM-free hormone. The following were the stages used for activation: five minutes in IO, four hours in DMAP plus scriptaid, and six hours in scriptaid. Lastly, mSOF drops were used to grow and wash the embryos. The culture medium had to be changed after 2.5 days to substitute SOF with 5% FBS, which provides the essential nutrients required for the blastocyst stage.

Immunostaining

After 7 days of culture, the embryos were fixed in 4% paraformaldehyde for 45 minutes and then soaked in Triton X-100 at 4°C overnight. The embryos were subsequently immersed in the primary H3K9 acetylation rabbit antibody for 2 hours. Following three washes, they were incubated with the secondary antibody, Alexa Fluor chicken anti-rabbit 488 IgG, for 1 hour. After an additional three washes, the embryos were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 30 minutes to visualize DNA for cell counting.

Statistical analysis

Statistical analysis was performed by SPSS 20. The data labeling by single-factor analysis of variance (ANOVA) followed by the Turkey test. Each group of experiments was repeated 3 to 4 times to ensure the statistical power of the test. P values less than 0.05 were considered statistically significant. For quantitative analyses, the fluorescent images were subjected to densitometric analysis using NIS Elements BR. software.

RESULTS

Effect of Pre-IVM culture with L-ascorbic acid on developmental competence of bovine parthenogenetic embryo derived from growing oocytes

Overall, the percentage of embryos that can develop up to late blastocyst in small oocytes culturing in Pre-IVM and treading with or without L-ascorbic acid were dramatically higher compared to the negative control, at 21.93%

± 6.08 % for AsA0 group and 30.24% ± 3.09% for AsA50. Additionally, oocytes treated with L-ascorbic acid brought higher results than those without L-ascorbic acid. However, the table showed that the figure in two groups of samples was still lower than the oocytes that came from large antral follicles.

Table 1. Developmental competence of parthenogenetic embryos

	Number of embryos (%) develop up to					
	2 cell	4 cell	8 cell	Morula	Early blastocyst	Late blastocyst
(+)	22	19 (87.83%)^{b}	16 (73.81%)^{b}	14 (64.55%)^{b}	10 (45.77%)^{b}	9 (40.19%)^{b}
(-)	21	15 (67.86%)^{a}	10 (46.49%)^{a}	6 (28.03%)^{a}	3 (15.13%)^{a}	2 (5.13%)^{a}
AsA 0	33	28 (84.73%)^{b}	18 (72.77%)^{b}	15 (41.40%)^{a,c}	11 (30.57%)^{a,b}	8 (21.93%)^{c}
AsA 50	32	23 (83.57%)^{b}	15 (72.14%)^{b}	15 (50.16%)^{b,c}	12 (40.16%)^{b}	8 (30.24%)^{b}

Values with different superscripts are significantly different (^{a,b} < 0.05). The experiments were replicated three times.

The effect of Pre-IVM culture with L-ascorbic acid on quality and epigenetic modification of bovine parthenogenetic blastocyst

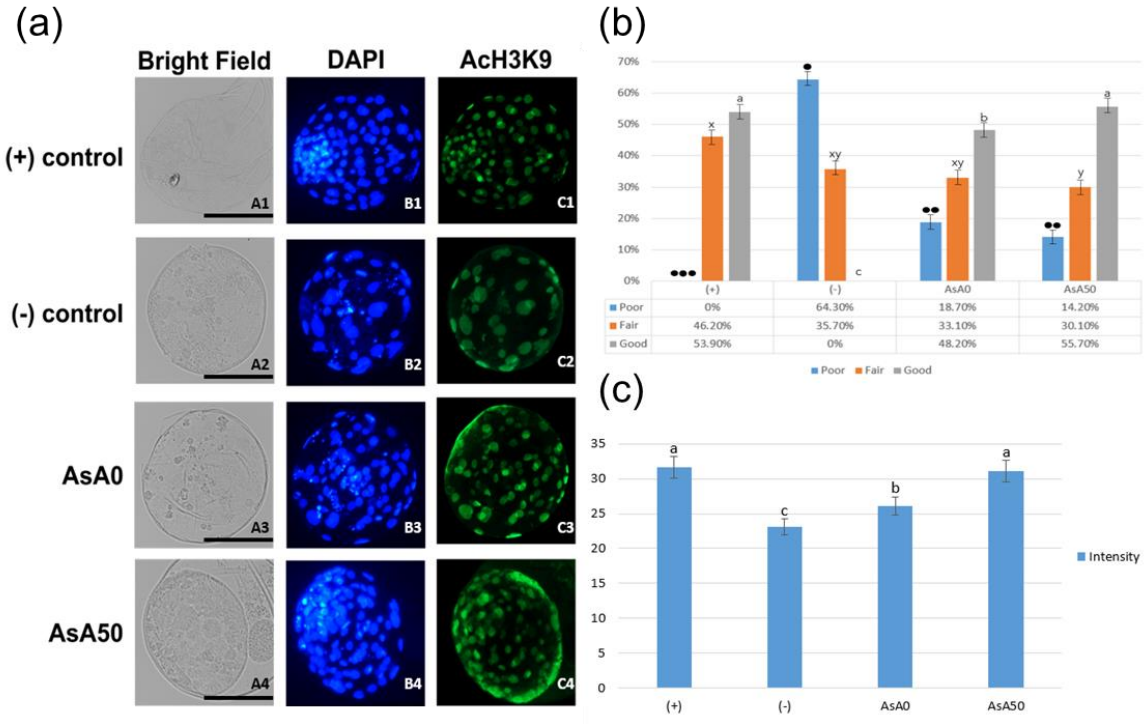

Figure 2. Effect of L-ascorbic acid supplementation during Pre-IVM on quality and epigenetic modification of bovine parthenogenetic blastocyst derived from growing oocytes. (a) DAPI and AcH3K9 staining were performed on Day 7 blastocysts of four groups (scalebar = 100 µm). (b) Effects of L-ascorbic acid on the quality of bovine parthenogenetic blastocyst. (c) Effects of L-ascorbic acid on the histone modification of parthenogenetic blastocyst. Values with different superscripts are significantly different (^{a,b} < 0.05). The experiments were replicated three times.

Late blastocysts of different groups were fixed and stained. With DAPI staining, the number of blastomeres within a blastocyst was able to be counted and the expression of acetylation of histone 3 lysine 9 (H3K9) was measured by antibody staining. Due to the penetration of DAPI into the blastomeres, the cell number of a blastocyst was counted and classified as follows: bad (30 – 50 cells), fair (50 – 80 cells), and good (>80 cells). The results showed that the negative control group had the highest rate of the poorly graded blastocyst and the lowest percentage of well-rated blastocyst, whereas the positive control group had the second highest rate of both at 53.90% and 0%, respectively. When it came to producing high-quality embryos that reached the blastocyst stage, both the pre-IVM groups with and without L-ascorbic acid demonstrated a notable improvement. The proportion of blastocyst that was deemed good without the L-ascorbic acid supplement was 48.20%, while the pre-IVM group

with the additive had a slightly higher percentage of 55.70%, which was also the highest percentage among all the groups. Following antibody staining, histone acetylation was quantified. H3K9 acetylation was lowest in the negative control group and greatest in the positive control group. The pre-IVM medium enriched with L-ascorbic acid produced an exceptionally high acetylation signal of 31.06 ± 12.35, which was similar to the positive control group's (31.59 ± 8.01) results. In contrast to the negative control group, pre-IVM without L-ascorbic acid showed only a slight rise in H3K9 acetylation levels, at 26.07 ± 6.19 and 23.06 ± 5.64, respectively.

Effect of Pre-IVM culture with L-ascorbic acid on developmental competence of bovine cloning embryo derived from growing oocytes

Table 2. Developmental competence of cloning embryos.

	Number of embryos (%) develop up to					
	2 cell	4 cell	8 cell	Morula	Early blastocyst	Late blastocyst
(+)	19	14 (74.29%)^{b}	11 (60%)^{a}	9 (50.48%)^{a}	6 (34.29%)^{a}	4 (22.86%)^{a}
AsA 0	8	7 (91.67%)^{a}	5 (75%)^{a}	1 (8.33%)^{c}	1 (8.33%)^{b}	1 (8.33%)^{b}
AsA 50	13	12 (75%)^{b}	10 (63.10%)^{a}	5 (31.55%)^{b}	5 (31.55%)^{a}	3 (19.64%)^{a}

Values with different superscripts are significantly different (^{a,b} < 0.05). The experiments were replicated three times.

Large antral follicles produced the oocytes in the positive control group, which led to a high rate of late blastocyst formation of cloning embryos (22.86%). With just 8.33% of late blastocysts produced in the pre-IVM group lacking a L-ascorbic acid supplement, the group receiving L-ascorbic acid supplementation produced 19.64% of such embryonic stages. Nevertheless, there was a significant difference between this group's replications, necessitating the conduct of additional tests.

DISCUSSION

The function of the Pre-IVM culture medium is to simply postpone the maturation process or in other words, to maintain the oocyte at GV stage. Once the oocyte undergoes GVBD, it is the indication that the maturation process has begun and this process is strongly influenced by intracellular cAMP level. A high level of cAMP leads to the activation of protein kinase A (PKA) which subsequently keeps the maturation promoting factor (MPF) inactive and ultimately, maintains meiotic arrest (Sen *et al*., 2013). To maintain such cAMP level, any pre-IVM medium requires an essential component termed cAMP modulator which can be used as a single effect (Costa *et al*., 2020) or in combination with two different substances (Buell *et al*., 2015). By hindering the action of PDE and hence preventing cAMP hydrolysis (Ramos *et al*., 2018), hypoxanthine can sustain a high cyclic AMP level, thus, preventing spontaneous maturation and providing more time for growing oocytes to accumulate small regulatory molecules via gap junction. An induced oocyte maturation model is well established for mouse oocytes, whereby meiotic resumption is completely inhibited by purines supplemented with hypoxanthine and then meiotic resumption is induced by FSH or EGF. In this study, the higher blastocyst rate observed in growing oocytes with pre-IVM may be attributed to the progression and synchronization of meiosis to the germinal vesicle (GV) stage in growing oocytes during pre-IVM.

Recently published reports show that endogenous ROS reduces the developmental competence of embryos, thereby lowering the rates of blastocyst formation (Wheeler *et al.,* 2004). ROS also results in decreased pregnancy rates and increased incidence of abnormal offspring upon embryo transfer. High levels of ROS during in vitro embryonic development generate various damages, such as increased lipid peroxides, protein oxidation and DNA strand breakages. Increased concentrations of hydrogen peroxide positively correlate with the number of fragmented embryos or blastomere apoptosis. Hence, ROS may induce apoptosis in embryos. All these observations indicate that ROS generated in embryos negatively affect the development and quality of these embryos. Antioxidants are compounds that can suppress the formation and reactions of ROS. L-ascorbic acid, which is a water-soluble antioxidant, plays a significant role in the ovary. It acts as a cofactor for collagen synthesis and peptide amidation, and it also supports follicular growth. Previous studies have demonstrated that the addition of ascorbic acid to the culture medium can prevent follicular apoptosis in rat and mouse follicles and improve blastocyst production in mice (Eppig *et al.,* 2000). Moreover, ascorbic acid has been found to enhance the developmental competence of porcine oocytes and prevent apoptosis in granulosa cells and ovarian follicular cells. In this study, supplementation of L-ascorbic acid at a concentration of 50 µg/mL slightly improved the developmental competence of cloned embryos derived from growing oocytes. This improvement may be attributed to the ability of L-ascorbic acid to reduce chromosome segregation errors and disrupt mitotic spindles caused by oxidative stress.

CONCLUSION

In conclusion, pre-IVM culture effectively increased the meiotic rate of oocytes generated from small antral follicles. L-ascorbic acid supplementation also improved the quality and developmental competence of parthenogenetic embryos derived from small oocytes. The AsA50 group showed an increase in histone H3K9 acetylation. Furthermore, a late blastocyst was successfully created using this pre-IVM procedure, supporting small oocytes undergoing SCNT.

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CẢI THIỆN KHẢ NĂNG PHÁT TRIỂN CỦA PHÔI BÒ NHÂN BẢN TẠO RA TỪ TẾ BÀO TRỨNG NHỎ ĐANG TĂNG TRƯỞNG ĐƯỢC NUÔI TRƯỞNG THÀNH VÀ CHÍN TRONG ỐNG NGHIỆM BỔ SUNG L-ASCORBIC ACID

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TÓM TẮT

Trứng nhỏ thu thập từ nang 2-3mm có tỷ lệ trưởng thành và khả năng phát triển thấp so với trứng lớn từ nang 4-6mm. Chúng tôi đã báo cáo rằng kỹ thuật nuôi trong ống nghiệm để trứng lợn nhỏ trưởng thành và chín (in vitro maturation, IVM) cần trải qua 2 giai đoạn Pre-IVM và IVM (Thuy Van *et al.,* 2020). Nghiên cứu hiện tại nhằm điều tra ảnh hưởng của quy trình nuôi cấy Pre-IVM lên khả năng phát triển của phôi bò nhân bản được tạo ra từ trứng phân lập từ nang 2-3mm đang tăng trưởng. Ngoài ra, chúng tôi cũng xem xét liệu việc bổ sung L-ascorbic acid vào môi trường Pre-IVM có thể cải thiện sự hình thành phôi nang nhân bản hay không. Trong thí nghiệm đầu tiên, hiệu quả của nuôi cấy Pre-IVM, có hoặc không có L-ascorbic acid, trên phôi trinh sinh được tạo ra từ trứng nhỏ đang tăng trưởng đã được đánh giá. Sau đó, khả năng phát triển của phôi nhân bản tái cấu trúc từ trứng nhỏ đang tăng trưởng đã trải qua Pre-IVM với L-ascorbic acid sẽ được đánh giá. Kết quả cho thấy rằng việc nuôi cấy trong môi trường Pre-IVM trong 8 giờ được bổ sung L-ascorbic acid cải thiện đáng kể khả năng phát triển, chất lượng và sự biến đổi của yếu tố di truyền ngoại gene, histone của phôi nang trinh sản bò trong nhóm trứng đang phát triển. Ngoài ra, phôi nhân bản được tạo ra từ trứng nhỏ đang phát triển được nuôi cấy lại với Pre-IVM bổ sung L-ascorbic acid cho thấy khả năng phát triển tương đương với nhóm trứng đã trưởng thành hoàn toàn. Kết luận, Pre-IVM được bổ sung 50 µg/mL L-ascorbic acid đã cải thiện khả năng phát triển của phôi nhân bản bò được tạo ra từ tế bào trứng nhỏ đang tăng trưởng.

Từ khóa: L-ascorbic acid, nhân bản, nuôi trứng chín trong ống nghiệm, phôi bò, tiền-nuôi trứng chín trong ống nghiệm.

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