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Effect of Alpha Lipoic Acid as an Antioxidant Supplement during In Vitro Maturation Medium on Bovine Embryonic Development

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ABSTRACT

This study was conducted to investigate the effects of alpha-lipoic acid (aLA) as an antioxidant that decrease the reactive oxygen species (ROS) in bovine embryonic development. Slaughterhouse derived bovine immature oocytes were collected and 4 different concentrations (0, 5, 10 and 20 mM) of aLA was supplemented in bovine in vitro maturation (IVM) medium. After 20 hrs of IVM, maturation rates, levels of ROS and glutathione (GSH), and further embryonic development after parthenogenetic activation (PA) and in vitro fertilization (IVF) was investigated according to aLA concentrations. Maturation rate was significantly higher in 10 mM group than other groups (80.5% vs. 62.9, 73.9, 64.2%; $P<0.05$). In the levels of ROS and GSH in matured oocytes as an indicator of oocyte quality, significantly better results were shown in 5 and 10 mM groups compared with other 2 groups. After IVM, significantly higher rates of blastocyst formation were shown in 10 mM groups in both of PA (27.9% vs. 18.8, 22.3, 14.2%; $P<0.05$) and IVF (32.6% vs. 23.9, 27.3, 16.2%; $P<0.05$) embryos. In addition, significantly more cell total cell number and higher inner cell mass ratio in 10 mM PA and IVP blastocysts showed developmental competence in 10 uM groups. Therefore, based on the entire data from this study, using 10 μM of aLA confirmed to be the optimal concentration for bovine oocyte maturation and embryonic development.

(Key words: Alpha Lipoic Acid, antioxidant, bovine, oocytes, development)

INTRODUCTION

In vitro production (IVP) of bovine embryos is a profitable technique to improve the genetic trait of cows within short time and considered as one of the important component of livestock industry. The IVP embryos are achieved by 3 major processes including in vitro maturation (IVM), in vitro fertilization (IVF), and in vitro culture (IVC) (Lonergan et al. 2006). Environmental factors including the culture medium, number of embryos cultured, temperature and gas atmosphere can directly affect the embryonic development (Lonergan et al. 2006). For instance, the serum content in the culture medium accelerate the embryonic development rate, but upon not optimal concentration, it decreases the normal morula compaction and the earlier occurrence of blastocysts (Van Langendonckt et al. 1997; Rizos et al. 2003). It has been shown that in vitro derived bovine embryos are prone to impairment in the mitochondrial volume density and intra-cellular communication in comparison to the in vivo process (Crosier et al. 2000; Crosier et al. 2001). Poor environmental conditions have a detrimental effect on the pre-and postnatally development (Fleming et al. 2004). It is now proved by several studies on animal models that embryos are very susceptible to environmental conditions (i.e. (Lonergan et al. 2006; Dennery 2007; Agarwal et al. 2014)).

Reactive Oxygen Species (ROS) are a group of highly reactive chemicals that contain oxygen (for instance peroxide, superoxide and hydroxyl radical). They are natural products produced by the living cells during cell signaling and homeostasis. Their extreme reactivity leads more molecule to become reactive and unstable (Choi et al. 2013). In large amount, they contribute to pathological conditions leading to cell damage and impairment (Lampiao 2012). This takes place when there is an imbalance between the production of the ROS and the detoxifying antioxidant levels (Choi et al. 2013; Agarwal et al. 2014). One of the direct effects of ROS is not only damaging the DNA and the lipid content, but also

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initiating apoptosis. The oocyte and the early embryo stages are more sensitive and vulnerable to the ROS damage. In excess, ROS leads to chromosomal scattering and cytoskeleton damage for the oocyte (Agarwal et al. 2014). It further decreases the number of the blastocyst development rate and leads to embryos death. At the end, it can completely fail the IVF process (Choi et al. 2013). Even though IVF is performed as close as possible to the in vivo procedure, the typical physiological conditions cannot be fully produced. For instance, the control over the ROS level by antioxidant is tightly regulated in vivo, however, it is hard to maintain the ROS level as in the in vitro experiment design (Rakhit et al. 2013). Therefore, one obvious solution for enhancing the IVF is by using antioxidants to quench the ROS.

Antioxidants, natural or synthetic, are substances that inhibit the oxidation processes (Gupta & Sharma 2006). IVF success is adversely affected by many factors, this includes but not limited to ROS as mentioned above. Many researchers claimed that ROS significantly affect the quality of oocyte and the embryo development (i.e. (Agarwal et al. 2014; Dehghani-Mohammadabadi et al. 2014)). This had paved the way toward improving the IVF techniques by using antioxidants to improve the process (Kably et al. 2004). Authors in (Ali et al. 2003) demonstrated that addition of antioxidant during IVM and IVF can improve the embryonic development.

Alpha Lipoic Acid (aLA) is a universal natural antioxidant that quenches ROS in aqueous and lipid environments (Packer et al. 1995; Rosini et al. 2011; Zhang et al. 2013). It has a beneficial effect on genes and proteins related to the cell growth and metabolism. Moreover, it works in synergies with other antioxidants including vitamin C and E by regenerating them (Packer et al. 1995). Recently, scientific research has uncovered the potential of aLA in the medical field, particularly in IVF. Zhang *et al*. demonstrated that optimal concentration of aLA supplemented to the goat oocytes IVM could potentially improve the maturation rate and the blastocyst development (Zhang et al. 2013). Furthermore, several studies indicated the therapeutic effect of aLA in mouse animal models as it increases the levels of other natural in vivo antioxidants (Maitra et al. 1995; Amudha et al. 2006). Additionally, Talebi A *et al*. demonstrated that aLA improves the in vitro development of mouse follicles by reducing the intracellular ROS levels (Talebi et al. 2012).

Even though aLA benefit was not fully explored in bovine embryonic development, one could assume the potential of this antioxidant in improving the embryonic development during IVM. Therefore, we examined the effect of different concentration of aLA on bovine embryonic development through supplementation in IVM medium in this study.

MATERIALS and METHODS

All chemical and reagent were purchased from Sigma Aldrich (USA) unless stated otherwise.

1. Oocyte collection and in vitro maturation (IVM)

Ovaries were collected from the slaughterhouse before keeping them in a container with a normal saline and controlled temperature of 35^{0}C. Ovaries were brought to the lab without delay (i.e. less than 2 hours after collection). Using a 10-mL sterile syringe, the Cumulus-oocyte complexes (COCs) fluid was collected from follicles with 2-8 mm diameter size. Under the microscope, the COCs with at least 3 layers were collected and washed 3 times with HEPES-buffered tissue culture medium-199 supplemented with 0.1%(w/v) polyvinyl alcohol (TH-PVA). Afterward, 30-50 COCs were transferred into four-well dish with 500 μL of IVM medium (TCM 199) that was supplemented with 10% FBS, 75 μg/mL of kanamycin, 10 ng/mL EGF (epithelial growth factor),0.5 sodium pyruvate,10 mg/mL of human chorionic gonadotrophin (hCG; Intervet International BV, Holland), 10 IU/mL pregnant mare serum gonadotrophin (PMSG) and 1μg/ml 17β-estradiol. At the end, the four-well dish was incubated at 39°C in a humidified atmosphere of 5% CO_2 for 24h.

2. Measurement of intracellular ROS and GSH levels in Oocyte

After 24h of IVM, the intracellular ROS and GSH levels in oocytes were measured following the protocol used in references (Kwak et al. 2012; Lee et al. 2014). In brief, the intracellular ROS of the oocytes was detected by di-chlorohydrohydro-fluorescein diacetate (H2DCFDA, Invitrogen) as green fluorescence, while the GSH was detected by 4-choloromethyl-6-8-difluoro-7-hydroxycoumarin (CMF2HC, Invitrogen) as blue fluorescence. Ten oocytes from each treated class were incubated for 30min under a dark condition with TCM 199-HEPES 0.1% (w/v), polyvinyl alcohol (PVA) containing 10 μM H2DCFDA and 10 μM of CMF2HC. The oocytes then washed with Dulbecco's

phosphate buffered saline (DPBS, Invitrogen) supplemented with 0.1% (w/v) PVA. Then, a sample from each washed oocyte was suspended into 10 µl of 1% DPBS before examination under the epifluorescent microscope (Leica DM IRB, Wetzlar, Germany) using UV filter (460 nm and 370 nm) for ROS and GSH respectively. After image acquisition, the image files were transferred in Tagged Image File Format (TIFF) before measuring the fluorescent intensity of the oocyte and analyzing it with Image J software (Version 1.41, NIH, Bethesda, MD, USA). Normalization of the data was performed in comparison with control oocytes.

3. Production of IVF Embryos

Spermatozoa were prepared similarly to authors in (Machado et al. 2009). In brief, Percoll discontinuous gradient solution of (45-90%) was prepared by mixing 1 mL of 90% Percoll with 1 mL of capacitation-TALP (Tyrode's albumin lactate pyruvate). In a 15 ml Tube, 2 mL of 45% ml, percoll was added to 2 ml of 90% Percoll solution before adding the spermatozoa on top. This discontinues gradient mix was centrifuged for 15 min at 1680 rpm. The spermatozoa pellets were washed with capacitation-TALP by centrifuged down at 1680 rpm for 10 min twice. Around 100 µL of the sperm, the pellet was transferred to microtubes. The matured oocytes from IVM were transferred to IVF-TALP medium supplement with 1 µg/ml heparin. This is done by adding 5-7 oocytes to 50µL of the IVF medium mounted with mineral oil to avoid evaporation. Then, directed inseminated with 1-2 ′ 10^6 spermatozoa/ml before incubated at 39°C in a humidified atmosphere of 5% CO_2 for 18-22h. After fertilization, the zygotes were denuded by adding 0.1% hyaluronidase while pipetting gently. Afterward, zygotes were cultured in a modified synthetic oviduct fluid medium (mSOF) containing 8 mg/mL BSA, overlaid with mineral oil and incubated at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The cleavage rate and the blastocyst rate was determined after 2 and 8 days, respectively.

4. Production of Parthenogenetic Activated (PA) Embryos

After 22-24 hours of IVM, the oocytes were denuded by gentle pipetting in HEPES-buffered TCM-199 medium with 0.1% of hyaluronidase. Only the first polar oocytes were activated by 5 mM of Ca^{2+} Ionomycin in the HEPES-buffered TCM-199 medium for 4min. Afterwards, the oocytes were transferred to TCM-199 medium containing 2 mM of 6-dimethylaminopurine and incubated at 38.5º C in a humid atmosphere with 5% CO2 for 4 hr. The activated oocytes cultured in (mSOF) and incubated at 39°C in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. The cleavage and the blastocyst rates were assessed and recorded after 2 and 8 days respectively.

5. Differential staining

After 8 days, the differential staining was performed on the blastocysts similar to the method reported by authors in (Selokar et al. 2012). After day 8, the blastocysts were washed once in PBS before staining with Hoechst 33342 for 15 min. After one washing step with PBS, the blastocysts were incubated in 500 µl of phosphate buffer saline (PBS) sublimated with 0.1% of Triton X-100 and 25 µg/ml propidium iodine (PI) for 30-40s. After another PBS washing step, the blastocysts were mounted with glycerol droplets and examined under an epifluorescent microscope (Leica DM IRB, Wetzlar, Germany). The ICM nuclei were stained blue while the TE nuclei stained red due to the Hoechst and PI respectively.

6. Experiment design

In the 1st experiment, the antioxidant activity of aLA was tested by inoculating different concentrations in the IVM medium (5, 10 and 20 µM) and compared with aLA-free IVM culture. Afterwards, the maturation rate, ROS and GSH levels were measured. On the other hand, in the 2nd experiment, the effect of the aLA on the development of the embryo during IVM was determined by the cleavage rate, blastocyst formation and blastocyst cell number in parthenogenesis (PA) and IVF embryos. Each experiment was run in 8 replicates.

7. Statistical analysis

All data in this study were analyzed by IBM SPSS Statistics (Version 24.0.). The data are designated as the mean values ± standard error of the mean (SEM). $P<0.05$ was considered statistically significant. Image J software (Version 1.41, NIH, Bethesda, MD, USA) was used to for measuring the fluorescent intensity of the oocyte.

RESULTS

1. Effect of aLA on oocyte maturation

The evaluation of bovine oocytes' nuclear maturation was

measured by the rate of the first Polar Body (PB) extrusion. Furthermore, 1964 oocytes were considered in 8 replicates. Result in Fig. 1 shows that maturation rate for oocytes (after IVM) was significantly higher (P<0.05) when supplemented with 5 μM and 10 μM aLA (73.9±1.5 and 80.5±1.3 respectively) in comparison to the control group (62.9±1.6) (Fig 1). However, the 20 μM concentration has no significant effect on the maturation rate (64.2±1.3).

2. Effect of aLA on intracellular levels of GSH and ROS

Results in Fig. 2 and 3 show the effect of aLA on intracellular levels of GSH and ROS. The GSH levels in the aLA oocyte treated groups were significantly higher in 5 μM and 10 μM (1.27 and 1.30 pixels/oocyte respectively) than the control group (1.00). While no significant difference was observed in the 20 μM (1.11). On the other hand, the ROS levels were significantly lower for 5 μM and 10 μM (0.71 and 0.67 pixels/oocyte respectively) in comparison to the control group (1.0 pixels/oocyte) whereas the 20 μM group was insignificantly different from the control group (0.93 pixels/oocyte).

3. Effect of aLA on the PA embryonic development

We can notice from Table 1 that the treated groups with 5 μM and 10 μM of aLA show significantly higher development rate of parthenogenetic embryos in comparison to the non-treated group. This was indicated by the high cleavage rate (75.5±1.7 and 81.1±2.2 respectively) and the blastocyst rate (22.3±1.7 and 27.9±1.2 respectively). On the contrary, the 20 μM treated group showed no significant difference in the cleavage and the blastocyst rate compared to the control group (P>0.05).

4. Effect of aLA on the blastocyst traits

The differential staining of the bovine parthenogenetic blastocysts treated with 5 and 10 μM aLA significantly improved the quality of the embryos compared to the control groups as we can see in Table 2. That was demonstrated by high ICM, TE and ICM/TE ratio. Whereas, the 20 μM treated group has no significant difference from the control group.

5. Effect of aLA on the IVF embryonic development

Results in Table 3 show that the development rate of the IVF embryos was significantly elevated with 5 and 10 μM

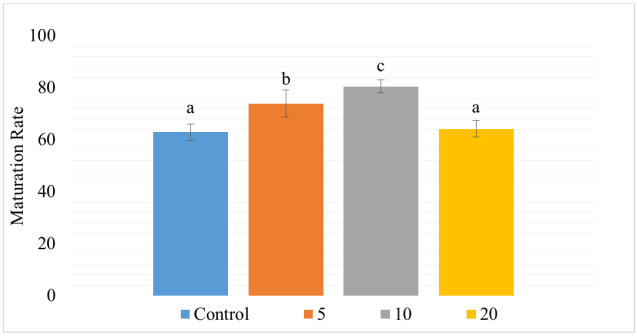

Figure 1. Maturation rates of bovine oocytes after supplementation of aLA into IVM media. The total of 1964 oocytes were considered in 8 replicates. Bars with different letters (a, b and c) are significantly different (P<0.05).

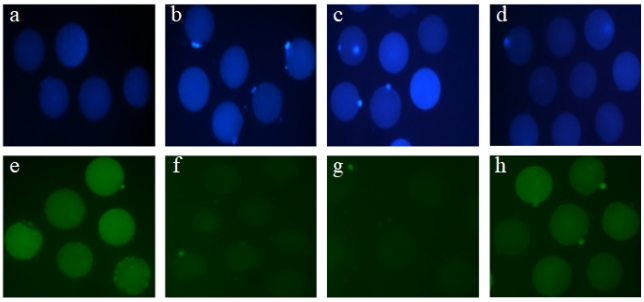

Figure 2. Epifluorescence photomicrographic images of in vitro matured bovine oocytes. Oocytes were stained with Cell Tracker Blue (a-d) and 2', 7'-dichlorodihydrofluorescein diacetate (H2DCFDA) (e-h) to detect intracellular levels of glutathione (GSH) and reactive oxygen species (ROS), respectively, whereas, (a and e) control and (b and f) aLA 5mM and (c and g) aLA 10mM and (d and h) aLA 20mM oocytes. Note that, cumulus cells and other substances are not considered (negligible).

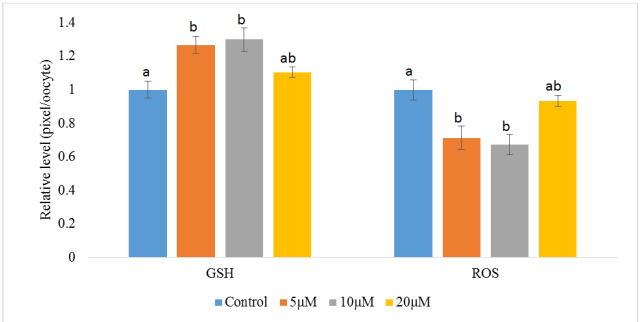

Figure 3. Effects of aLA supplementation during in vitro maturation (IVM) on intracellular GSH and ROS levels in in vitro matured bovine oocytes. Within each group (GSH and ROS) of end point, bars with different letters (a, b and ab) are significantly different (P<0.05). GSH samples, 10 oocytes with five replicates; ROS samples, 10 oocytes with five replicates.

Table 1. Effect of aLA supplementation into IVM media with 4 different concentrations on preimplantation development of bovine parthenogenetic embryos.

Treatment (μM)	No. of embryos cultured	Cleavage (± SEM%)	Blastocyst (± SEM%) in the embryo
Control	218	151 (68.8±1.6)^{a}	41 (18.8±0.7)^{a}
5	223	168 (75.5±1.7)^{b}	50 (22.3±1.7)^{b}
10	242	194 (81.1±2.2)^{b}	66 (27.9±1.2)^{c}
20	226	149 (67.0±2.2)^{a}	32 (14.2±0.7)^{d}

Values with subscript (a, b and c) indicate significant different (P<0.05). Experiment was replicated 8 times.

Table 2. Differential staining of bovine parthenogenetic blastocysts developed by 4 different aLA concentration in IVM media.

aLA conc. (mM)	No. of cells				% of ICM/TE
	No. of blastocyst	Total	Inner cell mass (ICM)	Trophectoderm (TE)	
Control	14	128.5±10.5^{a}	29.7±1.8^{ab}	98.8±8.9^{ab}	31.6±2.0^{ab}
5	12	145.7±7.8^{ab}	34.9±2.0^{ab}	110.8±7.4^{ab}	32.9±2.8^{ab}
10	15	150.9±3.3^{b}	39.6±1.7^{a}	111.3±2.7^{a}	35.8±1.8^{a}
20	13	132.6±6.1^{ab}	29.1±2.5^{b}	103.4±4.7^{b}	28.2±2.3^{b}

Values with subscript (a, b) indicate significant different (P<0.05)). Experiment was replicated 8 times.

Table 3. Effect of aLA supplementation into IVM media with 4 different concentrations on preimplantation development of bovine in vitro fertilized embryos.

Treatment (μM)	No of embryos cultured	Cleavage (± SEM%)	Blastocyst (± SEM%)
Control	279	204 (74.2±2.2)^{a}	64 (23.9±2.1)^{a}
5	244	195 (81.0±2.3)^{b}	63 (27.3±2.8)^{ab}
10	270	228 (84.9±2.6)^{b}	83 (32.6±3.5)^{b}
20	279	194 (66.6±1.9)^{c}	43 (16.2±1.9)^{c}

Means in the same column with different superscripts were significantly different (P<0.05). Values are listed as Mean ± S.E.M. Experiment was replicated 8 times.

Table 4. Differential staining of bovine in vitro fertilized blastocysts developed by 4 different aLA concentration in IVM media.

aLA conc. (mM)	No. of cells				% of ICM/TE
	No. of blastocyst	Total	Inner cell mass (ICM)	Trophectoderm (TE)	
Control	12	146.5±8.2^{a}	37.0±2.8^{a}	109.5±6.2^{a}	32.5±3.5^{a}
5	14	160.5±6.2^{ab}	41.0±1.0^{a}	119.5±1.0^{a}	33.1±1.7^{a}
10	15	173.0±8.0^{a}	48.0±2.1^{a}	125.0±2.1^{a}	34.4±3.7^{a}
20	14	133.0±4.0^{b}	35.0±1.2^{a}	98.0±1.2^{a}	31.3±2.2^{a}

Values with subscript (a, b) indicate significant different (P<0.05). Experiment was replicated 8 times.

aLA treated groups compared to the non-treated group. The 20 μM treated group as contrary has an insignificant difference from the control group. In comparison to the control group, the differential staining of IVF blastocysts supplemented with aLA, showed significantly higher ICM, TE, and ICM/TE ratio, except for 20 μM treated group as we can see in Table 4. Based on all of reported results, the 10 μM of aLA proved to be an optimal concentration to improve the bovine embryonic development.

DISCUSSION

A powerful antioxidant must meet at least one of the following criteria to be a potential compound for medical use: scavenging free radical species, metal chelating activity, synergetic act with other antioxidants, and impact on gene expression (Packer et al. 1995). aLA is a universal and ideal antioxidant since it fulfills all the above criteria (Packer et al. 1995). Research on animal models (e.g. (Maitra et al. 1995; Amudha et al. 2006)) reveals that the injection of aLA leads to significant increase in the GSH level, vitamin E, and vitamin C.

aLA has proved antioxidant activity that improves the embryonic development of goats and mice (Zhang et al. 2013; Zavareh et al. 2016). Our knowledge about aLA is largely based on very limited data as no study reported its effect on bovine embryonic development. The aim of this research is thus to reveal the beneficial effect of different concentrations of aLA in bovine embryonic development during the IVM. By exploiting the ROS and GSH levels we can able to investigate the antioxidant activity of aLA in matured oocytes.

During the IVM, the oocytes are sensitive to the ROS which leads to oocyte disruption and later embryo development. In this study, we demonstrate that during the IVM, the addition of aLA can significantly improve the oocyte maturation rate. Medium containing 10 μM of aLA was statistically higher in comparison to other groups. Thus, in bovine, the addition of 10 μM of aLA to the IVM provides the optimal antioxidant activities to improve the oocyte maturation rates. The experiments of this study are in line with previous results (Bilska & Wlodek 2005; Çakatay 2006; Talebi et al. 2012) where aLA is proved to be a powerful inhibitor for oxidation process inside the cells.

Studies have reported that ROS are produced during the cellular metabolic process as well as in in-vitro manipulation e.g. light and oxygen concentration (Agarwal *et al*., 2014). Upon excess of ROS, the antioxidant defense will be impaired leading to early embryonic development failure (Ruder et al. 2008). Moreover, the ROS levels directly decrease the GSH levels (Gardiner et al. 1998). Along with measuring the ROS levels, we used the GSH level as a biomarker to predict the bovine oocyte maturation, since it is proved to be the most relevant indicator for the mammalian oocyte maturation (Zuelke et al. 2003). Our data showed that a medium with 10 μM of aLA has statistically higher levels of GSH and lower levels of ROS in comparison to other groups demonstrating the powerful antioxidant properties for aLA. These results correlate fairly well with several findings and further support the role of aLA in improving the GSH synthesis while maintaining the intracellular ROS level (Packer et al. 1995; Sen et al. 1997; Sen & Packer 2000).

The blastocyst quality is one of the most important factor for successful IVF. Therefore, supplements to the culture medium that can improve the blastocyst quality are of high demands for IVF technology.

The positive impact of the aLA extended to improve the bovine embryonic development competence as demonstrated by the high blastocyst quality and blastocyst formation rate after PA and IVF. IVM treated group with aLA showed a significant increase in the blastocyst rate. However, at a very high concentration (i.e. 20 μM of aLA) a reverse effect is detected. Several studies proposed that very high concentration of aLA induces the apoptosis leading to programmed cell death (Marsh et al. 2005; Wenzel et al. 2005; Simbula et al. 2007; Yamasaki et al. 2009). This explains the reverse effect of 20 μM of aLA concentration on the bovine embryonic development. On the other hand, it has been suggested in the literature that small amount of ROS is required for oocyte development competence (Blondin *et al.*, 1997). This might justify that very high concentration of aLA might decrease embryonic development as its powerful antioxidant activity scavenges all of the ROS. Therefore, based on the entire data from this study, using 10 μM of aLA confirmed to be the optimal concentration for bovine oocyte maturation and embryonic development.

CONCLUSION

In summary, the treatment of bovine oocyte with 10 μM of aLA during the IVM, improves the oocyte maturation thoroughly till the embryonic development of the blastocysts. This proves the superior potential of aLA as an antioxidant for improving the bovine IVP technology. Further studies are needed to explore genes related to apoptosis and oxidative stress using molecular tools.

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