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ABSTRACT

In vitro embryo production has grown in recent decades due to its great potential for cattle production. However, the quality of *in vitro*-produced embryos is lower compared with those produced *in vivo*. The postfertilization culture environment has a major influence on bovine embryo quality. We hypothesize that the inclusion of the inclusion of alpha-lipoic acid (ALA) in the *in vitro* culture (IVC) medium during the first 24 h would have positive effects on embryo development *in vitro* and cryotolerance. The aims of this study were to evaluate the antioxidant effect of ALA in IVC medium for 24h on bovine zygotes (21 h post *in vitro* fertilization, IVF), day 2 cleaved embryos (46 h post-IVF), and to assess embryo quality, developmental competence, and cryotolerance after vitrification. In all experiments, IVC medium was the Control, and 2.5 μM ALA was the treatment implemented. Viability and reactive oxygen species (ROS) levels in zygotes and day 2 embryos did not differ from the Control ($P > 0.05$). Supplementation with ALA increased total blastocyst and hatching rates ($P < 0.05$). It also improved embryo quality, evidenced by the increased blastocyst total cell number and the percentage of excellent-quality embryos observed ($P < 0.05$). In embryos cultured with ALA and then vitrified, ALA reduced intracellular ROS levels in warmed blastocysts ($P < 0.05$). In conclusion, ALA supplementation to IVC medium during 24 h is a new advantage in improving embryo quality for assisted bovine reproduction.

Keywords: antioxidant; vitrification; cattle; embryo development; *in vitro* culture; embryo quality

1. Introduction

In spite of recent progress in embryo culture protocols, the quality of *in vitro*-produced embryos is lower compared with those produced *in vivo* [1]. The postfertilization culture environment has a great impact on bovine embryo quality [2]. In *in vitro* embryo culture (IVC), the media composition and the embryo's needs are asynchronous. The changing demands of the developing embryo are not fulfilled, which is manifested by various embryonic features, particularly ultrastructural alterations [3], limited compaction at the morula stage [4], and lower cryotolerance [5].

There has been a substantial increase in the use of *in vitro* production (IVP) of bovine embryos worldwide. Since it is an effective and efficient technology, methods for embryo cryopreservation are gaining increasing practical relevance [6]. In parallel with improvements in vitrification protocols, higher quality *in vitro*-derived bovine embryos must be obtained by improving laboratory culture techniques and medium [7]. It has been demonstrated that vitrification has negative effects on oocytes and embryos by disturbing the reduction-oxidation status, reducing glutathione content (GSH), and increasing reactive oxygen species (ROS) levels [8, 9, 10]. Moreover, repairing the cryo-induced damage to the cell structure and function involves the generation of energy, leading to increased ROS production [11, 12]. Also, it has been shown that vitrification induces alterations in mitochondrial function and distribution and decreases the membrane potential of oocytes and embryos [13, 14, 15]. Therefore, the higher the quality of the embryo, the better it will withstand the adverse effects of vitrification [16]. In mammalian embryos, individual antioxidants are effective to ameliorate oxidative stress [11]. Alpha-lipoic acid (ALA), which is synthesized from octanoic acid and sulfur sources through the action of lipoic synthetase acid [17], is a coenzyme in mitochondrial multienzyme complex reactions in charge of recycling other cellular antioxidants – such as GSH [18] – and regulating mitochondrial function. It also assists in ATP production for energy provision [19]. The

antioxidant properties of ALA have been previously described, showing that it protects mouse embryos against oxidative stress by stimulating the expression of antioxidant genes [20, 21]. Additionally, the beneficial effect of ALA in assisted reproductive technologies in different species has been reported. The maturation rate of cloned goat embryos was improved by supplementing ALA to *in vitro* maturation (IVM) medium [22]. Furthermore, the resulting enhanced developmental competence in this species was mediated through the reduction of cellular apoptosis by inhibiting apoptotic activator genes [22]. In mouse embryo production, *in vitro* fertilization (IVF) and IVC media supplemented with ALA improved subsequent embryo development (by decreasing oxidative stress) and increased embryo viability [23, 24]. Besides, ALA concentration is a crucial factor for obtaining beneficial or detrimental effects on bovine blastocysts [25]. It was demonstrated that 2.5 µM ALA supplemented at the beginning of IVC and maintained during the embryo development increased blastocyst total cell number, while 7.5 µM ALA decreased the hatching rate in bovine [25]. Therefore, the multifactorial effects of ALA make it one potential candidate substance to improve embryo quality, contributing to reducing oxidative stress in bovine blastocysts [11].

The largest drop in IVP efficiency takes place between the 2-cell and blastocyst stages, indicating that the culture stage after fertilization is fundamental in the process [26, 27]. Several main developmental events occur between the zygote and blastocyst formation: i) the first cleavage division [28]; ii) the activation of the embryonic genome at the 8 to 16 cell stage [29]; iii) compaction of the morula on day 5 [30]; iv) the formation of blastocyst on day 6-7 [31]. Thus, any modifications of the culture condition during the 6-day window of post-fertilization, which could affect any or all of these processes could have a major influence on the embryo quality. *In vitro* embryos show a peak of ROS production at 2- to 4-cell stages [32]. In addition, a lower amount of antioxidants is generated in this window [33]. For this reason, the aim of this study was to evaluate the antioxidant effect of ALA during the first 24 h of IVC on bovine embryo quality, developmental competence, and cryotolerance after vitrification. Considering

the economic relevance of the livestock industry, the improvement of assisted reproductive technologies becomes increasingly important.

2. Materials and methods

2.1. Reagents and media

All reagents for media preparation were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Follicle-stimulating hormone (FSH) was purchased from Bioniche (Belleville, Ontario, Canada). (±)-α-lipoic acid (ALA) (CAS 1077-28-7), 2′,7′-dichlorofluorescein diacetate (H2DCFDA) (CAS 4091-99-0) and fluorescein diacetate (FDA) (CAS 596-09-8) were purchased from Sigma Chemical Co. (St. Louis). Hoechst 33342 (CAS 875756-97-1) was obtained from Life Technologies (Carlsbad, CA, USA). Ethanol (CAS 64-17-5) was purchased from Merck KGaA (Darmstadt, Germany).

2.2. Experimental design

The study included three experiments (Fig. 1). The treatments implemented were 0 µM ALA (Control) and 2.5 µM ALA in IVC medium. The chosen concentration was based on our previous studies [25]. ALA was diluted in ethanol 0.1 % whose toxicity had been previously verified [34, 35].

Experiment 1: Effect of ALA on viability and ROS production in putative zygotes (21 h post-IVF) and day 2 cleaved embryos (46 h post-IVF)

The effect of ALA as an antioxidant compound on early embryo development was investigated by supplementing the IVC medium with ALA to measure viability and acute production of ROS in putative zygotes at 3 h of treatment [36] and in day 2 cleaved embryos at 24 h of treatment [37]. After IVF, putative zygotes were denuded by gentle pipetting and culture in IVC medium supplemented with ALA for 3 h, immediately half of the random samples were used to evaluate viability and ROS production. The remaining zygotes were kept in culture with ALA (24 h) to evaluate day 2 cleaved embryos in the same form. The levels of ROS were

evaluated using H2DCFDA stain and viability was determined with the FDA technique. The combination of two assays was performed following the criteria proposed by Lane et al. [38]. A total of 30-40 COCs were matured per treatment per replicate per assay. Three replicates were performed for each assay.

Experiment 2: Effect of ALA on embryo developmental competence and quality

The effect of ALA supplemented during the first 24 h in IVC medium on embryo kinetics and quality was evaluated with the IVP technique. The rates of cleavage on day 2 (48 h post-IVF), blastocysts on days 7 (B7) and 8 (B8), total blastocysts (BT), and hatching on days 8 and 9 were recorded. Also, the total cell number per blastocyst on day 8 was measured with Hoechst 33342 following the criteria chosen by Fabra et al. [25]. A total of 95-100 COCs were matured per treatment per replicate. Treatments were repeated in six replicates.

Experiment 3: Effect of ALA on embryo cryotolerance

To measure this effect, ALA was supplemented during the first 24 h in IVC medium. The quality grades of B7 and B8 were verified and then only grade 1 blastocysts were vitrified. After warming, re-expansion at 3 h and hatching rate at 24, 48, and 72 h were evaluated. The production of ROS in warmed embryos was also assessed. A total of 323-341 COCs were matured per treatment per replicate. Treatments were repeated in two to five replicates.

2.3. Procedures

2.3.1. Embryo IVP

Embryo IVP was carried out as described in our previous study [25, 39]. Briefly, ovaries came from an abattoir. Within 3 h of slaughter, they were sent to the laboratory in a sterile solution of NaCl (0.9% w/v) with streptomycin (100 mg/L) and penicillin (59 mg/L) at 37 °C. The ovaries were pooled irrespective of the estrous cycle stage of donors. Cumulus-oocyte complexes (COCs) were aspirated from 3-8 mm follicles with an 18-G needle connected to a sterile test tube and vacuum line (50 mm Hg). Only cumulus-intact oocytes with an evenly

granulated cytoplasm were selected using a low-power (20-30 x) stereomicroscope (Diaphot, Nikon, Tokyo, Japan). The IVM medium was bicarbonate-buffered tissue culture medium (TCM-199) with 10% v/v fetal bovine serum (FBS), 0.2 mM sodium pyruvate, 1 mM glutamine, 1 μg/ml FSH, and 1 μg/ml 17β-estradiol. The COCs were washed in TCM-199 buffered with 15 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and twice with IVM medium. Then, groups of 20-25 COCs were transferred into 100 μL of IVM medium under mineral oil and matured for 24 h. Fertilization medium consisted of Tyrode's albumin lactate pyruvate (TALP) medium [40] supplemented with 2% (v/v) minimal essential medium-essential amino acids (MEM-EAA), 1% (v/v) MEM-nonessential amino acids (MEM-NEAA), 6 mg/mL bovine serum albumin fatty acid-free (BSA-FAF), 20 μM penicillamine, 10 μM hypotaurine, and 10 mg/mL heparin sulfate. After IVM, expanded COCs were washed twice in HEPES-TALP supplemented with 3 mg/ml BSA-FAF and twice in 100 μL IVF medium. In all experiments, frozen semen from the same bull and batch with proven fertility was used. Spermatozoa were washed in a discontinuous Percoll gradient prepared by depositing 2 mL of 90% Percoll under 2 mL of 45% Percoll in a 15 mL centrifuge tube. Semen samples were deposited on the top of the Percoll gradient and centrifuged at 500 *g* for 20 min. The pellet was removed and resuspended in 300 mL HEPES-TALP solution and centrifuged at 300 *g* for 10 min. After removal of the supernatant, spermatozoa were resuspended in IVF medium and counted in a hemocytometer chamber. Each drop of 80 μL of IVF medium with 20-25 COCs was inseminated with 20 μL at a final concentration of 2 x 10^6 sperm/mL. Gametes were co-incubated for 18 h. The IVC medium consisted of synthetic oviduct fluid (SOF) [41] supplemented with 1 mM glutamine, 2% (v/v) MEM-EAA, 1% (v/v) MEM-NEAA, and 8 mg/ml BSA-FAF (274–276 mOsm/kg) [42]. All embryos were first cultured with IVC medium supplemented with ALA during 3 h (Experiment 1) or 24 h (Experiment 1, Experiments 2 and 3). A total of 20-25 COCs were cultured in a drop of 80 μL under mineral oil. At the end of the ALA treatment, a new culture dish was used in both treatments with IVC medium in the

presence of 1.5 mM glucose under mineral oil. Then, half of the culture medium, of each treatment, was renewed through a feeding procedure with IVC medium fresh every 48 h of culture. This procedure was performed two times. At the end of incubation (days 7-8), the morphological stage of embryo development was evaluated with low power (20-30 X) stereomicroscope (Diaphot, Nikon, Tokyo, Japan). Both IVM and IVF were carried out at 39 °C with 5% CO_2 in air and humidity at saturation. The IVC was carried out in an atmosphere of 7% O_2, 5% CO_2, and 88% N_2 at 39 °C with saturated humidity. To study embryo kinetics, cleavage rates (48 h post-IVF) were evaluated as embryos with 2-4 cells or embryos with > 4 cells, and blastocyst rates were determined as blastocysts appear on days 7 and 8 of development. The rates of cleavage, B7 and B8 were calculated from the total COC matured. Then, the blastocysts obtained were homogeneously divided into two groups. In group one, hatching from the total blastocyst on days 8 and 9 was determined. In group two, at least 20-30 blastocysts on day 8 per treatment were fixed and evaluated with Hoechst 33342.

2.3.2. Quantification of intracellular ROS

Intracellular ROS levels in presumptive zygotes, day 2 cleaved embryos, and warmed blastocysts were quantified using the fluorescent probe H2DCFDA. Only warmed blastocysts were cultured for 3 h in IVC medium until ROS levels were evaluated. The H2DCFDA probe is oxidized directly by H2O2, its derivatives, and

other peroxides, and is also oxidized indirectly by the superoxide anion, thus providing a reliable tool to assess intracellular ROS production. A stock solution of H2DCFDA dissolved in dimethyl sulfoxide (DMSO) was diluted in PBS to a working concentration of 5 µM. Samples were washed twice in PBS and immediately incubated with 5 µM H2DCFDA in a dark, humidified 5% CO_2 atmosphere at 38.5 °C for 5 min (day 2 cleaved embryos or warmed blastocysts) and 20 min (presumptive zygotes). Then, they were washed twice with fresh PBS and imaged immediately using an epifluorescence microscope equipped with UV filters (460

nm) to quantify embryo area and fluorescence intensity (pixels) in fluorescence arbitrary units (FAU). Fluorescence intensities were analyzed using ImageJ software (version 1.46r; National Institutes of Health, Bethesda, MD, USA). Data were expressed as mean H2DCFDA intensity in FAU ± standard deviation (SD).

2.3.3. Viability

After IVC, presumptive zygotes and day 2 cleaved embryos were incubated for 10 min at 37 °C in PBS medium containing 2.5 µg/L FDA. The samples were observed using an epifluorescence microscope (Olympus BX40) equipped with a 420 nm excitation filter. For evaluation, each sample was photographed and analyzed with Image J software 1.48v (Wayne Rasband, National Institutes of Health, USA). For this purpose, the intensity of each pixel was divided by the number of pixels of each cell. Data were expressed as mean FDA intensity (FAU ± SD) [43].

2.3.4. *Blastocyst total cell number analysis*

The total cell number of blastocysts on day 8 was measured using Hoechst 33342. First, the embryos were fixed in 4% formaldehyde. Then, they were washed three times in PBS with 5% FBS and incubated in 1 mg/mL Hoechst 33342 for 10 min at 37 °C. After that, the embryos were washed and mounted in a glass slide in a 20 µL glycerol drop and examined under an epifluorescence microscope (Olympus BX40) with an appropriate combination of filters (460 nm) at 200x magnification by one blind researcher to determine the total number of cells.

2.3.5. Embryo vitrification and warming

The quality grades of B7 and B8 were verified according to the classification of the International Embryo Transfer Society [44]. Only grade 1 blastocysts were vitrified. Embryo vitrification was performed according to the technique described previously [45, 46]. The medium and reagent used for vitrification were a basic maintenance medium (MM) (TCM 199-

214 HEPES + 20% FBS) and a sucrose medium (SM) (0.5 M sucrose and TCM 199-HEPES).
215 Briefly, embryos were handled in MM. Then, the blastocysts selected for vitrification were
216 exposed to 850 µL MM with 7.5% ethylene glycol (EG) + 7.5% DMSO (Vitrification Solution
217 1; VS1) for 3 min and then moved into a well containing 670 SM µL with 16.5% EG + 16.5%
218 DMSO (Vitrification Solution 2; VS2). The blastocysts were vitrified with the surface device
219 Cryotech® (ex Cryotop®). Then, the straws were plunged into liquid nitrogen. The time spent
220 by blastocysts in VS2 (including loading) was 20–25 seg.

221 The medium and reagent used for warming were a solution consisting of 800 µL MM + 400
222 µL SM (0.25 M) (MS1) and another solution consisting of 800 µL MM + 200 µL SM (0.15 M)
223 (MS2). Warming was conducted by immersing the pulled end of the straws directly in 1.2 mL
224 in MS1 for 5 min. Then, straws were transferred to the MS2 for another 5 min and subsequently
225 transferred to MM for 5 min. Finally, blastocysts were washed in SOF + 5% FBS and placed
226 into the culture microdroplet. All procedures were performed in a warm room (30 °C) on a
227 heated surface (41 °C). Blastocysts were cultured in an incubator with an atmosphere of 7% O_2,
228 5% CO_2, and 88% N_2 at 39 °C with saturated humidity. Warmed blastocysts were used to
229 evaluate re-expansion at 3 h and then they were randomly divided into two groups: one was
230 used to evaluate hatching at 24, 48, and 72 h [47], and the other to evaluate intracellular ROS
231 levels.

232 *2.4. Statistical analysis*

233 Data were analyzed using the RStudio Software. We used a Bayesian approach with normal
234 and non-informative prior distribution to calculate the 95% Bayesian Credible Intervals
235 (95%BCIs). The Bayesian statistical approach is progressively being used in many fields of
236 science as an alternative to analyzing data with small samples [48, 49, 50]. Cleavage, B7, B8,
237 BT, hatching rates, and vitrification data were analyzed with Beta distribution for the
238 comparison of two proportions. Blastocyst total cell number, viability, and ROS levels were
239 analyzed using Bayesian comparison of two media adapted to the normal distribution

frequentist approach. *P* values < 0.05 were regarded as significant, and *P*-values < 0.1 as a tendency.

3. Results

3.1. Effect of ALA on viability and ROS production in putative zygotes and day 2 cleaved embryos

Table 1 shows viability data and intracellular ROS levels obtained after treating putative zygotes or day 2 cleaved embryos with ALA during IVC. No differences in viability and ROS production between Control and ALA in putative zygotes ((*P* = 0.22); (*P* = 0.15), respectively). Similarly, we did not detect differences in viability and ROS production between Control and ALA in day 2 cleaved embryos ((*P* = 0.17); (*P* = 0.30), respectively).

3.2. Effect of ALA on embryo developmental competence and quality

In Experiment II, there were no significant differences in the rates of total cleavage between treatments (*P* = 0.14). While the rates of cleaved 2-4 cells did not differ between the Control and ALA (*P* = 0.37), an increasing trend in the rate of > 4 cleaved cells was observed in the Control group (*P* = 0.10; Table 2). The results demonstrated that the rate of total blastocysts increased with ALA treatment (*P* = 0.01) due to an increase in the rate of B7 (*P*=0.002), while the B8 rate did not differ (*P* = 0.22; Table 2). The hatching rate and blastocyst total cell number were evaluated as quality parameters of embryos. Results of hatching are expressed as a percentage in respect of the total blastocysts observed (Table 2). We observed that the total hatching rate increased with ALA (*P*=0.02) (Table 2). In the same way, when evaluating total blastocyst cell number, this parameter was greater in ALA compared with the Control (*P* = 0.05) (Table 2).

3.3. Effect of ALA on embryo cryotolerance

The results of embryo quality demonstrated that grade 1 embryos were greater in ALA on day 7 (P = 0.001) and trend on day 8 (P = 0.06) (Table 3). No differences were observed between treatments when grade 2 embryos were evaluated on days 7 and 8 ((P = 0.42); (P = 0.26), respectively) (Table 3).

Table 4 shows the total embryos vitrified on days 7 and 8 of culture. We observed an increase in the total embryos vitrified in ALA as compared with the Control (P = 0.01) on day 7. Also shows the percentages of embryo re-expansion, hatching, and intracellular ROS levels after the vitrification–warming process of day 7 and 8 blastocysts. Re-expansion rates 3 h after warming did not differ on days 7 and 8 (P = 0.35 and P = 0.48, respectively). No differences in hatching rate were observed between ALA and Control at 24 h. However, the groups were different in hatching rate at 48 h on days 7 and 8 (P = 0.05; P = 0.03, respectively) and in hatching rate at 72 h on day 8 (P = 0.05) increasing this parameter in the Control group. Regarding intracellular ROS levels, they were reduced in the ALA group on day 7 (P = 0.05).

4. Discussion

In the present study, the effect of 2.5 µM ALA supplementation at the beginning of bovine IVC (first 24 h) was analyzed. We studied intracellular ROS level and viability of zygotes and day 2 cleaved embryos. Additionally, early embryo development, blastocyst quality, and cryotolerance after vitrification were evaluated.

The current results indicate that the inclusion of ALA did not modify viability and ROS levels evaluated after an acute treatment (3 h) in zygotes, and at the end of 24 h of treatment in day 2 cleaved embryos. This would indicate that despite the effect of ALA was not quickly evidenced, it was key to embryonic development. Our results demonstrated that ALA increased the rate of the total blastocyst and that the number of ALA-treated embryos appearing on day 7

was higher than the control. We have previously reported that the percentage of embryos developing to the blastocyst stage was not modified after a long treatment (7 days) with 2.5 µM ALA in IVC [25]. Agreeing with this result, Mokhtari et al. [51] found no difference in cleavage, morula compaction, and blastocyst rates in mice when IVC was supplemented with 1 and 10 µM ALA for 5 days. This coincides with the fact that the mammalian preimplantation embryo is most sensitive to its environment during the cleavage stages [52, 53]. Hence, supplementation of antioxidants during this stage, as we did in our investigation, may be beneficial. During embryo development after fertilization, different critical events regulated by a harmonized expression of genes occur under ideal culture conditions [54]. For instance, the switch from using the maternal genome mRNA to that generated from embryonic genome activation (EGA). Researchers suggest that in bovine embryos, transcriptional activity starts between the zygote- and late 4-cell stages (minor GA) and the developmental block occurs at the 8-cell stage [54]. The timing of the 'developmental block' indicates that embryonic transcription is remarkably responsive to culture conditions. This process can be relieved by reducing glucose in the culture medium. Non-use of glucose would be due to the lack of activity of the enzyme phosphofructokinase [55]. So, the little glucose used in the embryonic first stages would be metabolized mainly through the pentose phosphate pathway. This pathway produces, among other things, reduced glutathione (GSH), which protects against peroxidation, and nucleotide precursors [56]. It has been reported that ALA plays a role in intracellular GSH recycling [57]. Supplementation of ALA during the first 24 h of IVC could participate in this process, increasing GSH synthesis and improving embryo development. In bovine embryos, GSH increased during IVM; such concentrations persisted during fertilization and were still present during the initiation of early embryo development [58]. An increased store of GSH probably protects blastomeres from oxidative stress during early embryo development, especially during the maternal-zygotic genome switch of the embryo [59, 60]. Future work is needed to verify our hypothesis.

Our results demonstrated that ALA improved blastocyst quality. We showed that blastocyst total cell number, the number of grade 1 blastocysts, and the hatching rate increased with ALA treatment. In line with these results, when we studied the effect of ALA during IVC, on day 8, the total cell number was greater in blastocyst cultured with 2.5 µM ALA [25]. In the same way, Truong et al. [24] described an increase in total cell number in mouse blastocyst culture with 5 µM ALA during 5 days and showed a significant increase in blastocyst total cell number of embryos grown in media with antioxidants (acetyl-L-carnitine, N-acetyl-L-cysteine, and ALA), supplemented only in the pre-compaction period. Hassan et al. [61] confirmed that adding 10 µM ALA during bovine IVM resulted in a significantly greater total cell number and higher inner cell mass ratio.

Tolerance to cryopreservation has been used routinely as an embryo quality parameter. Slow freezing and vitrification are normally used to cryopreserve bovine embryos. Once blastocyst-stage embryos overcome the embryonic block, a first selection has been made. Since these embryos have a greater nuclear-cytoplasmic ratio, they are more suitable for cryopreservation [16]. Several antioxidants have been effective in ameliorating oxidative stress in mammalian embryos during cryopreservation [38, 62, 63]. Different authors have used ALA as a cryopreservation strategy. The addition of ALA to the extender resulted in a higher percentage of post-thaw and motile boar spermatozoa and an improvement in the activities of superoxide dismutase, lactate dehydrogenase, glutamic-oxaloacetic transaminase and catalase [64]. Buffalo bull spermatozoa treated with ALA during cryopreservation revealed higher sperm survival function and time of sperm attributes [65]. Mouse pre-antral follicles cryopreserved with ALA also showed higher rates of survival, antrum formation, and metaphase II oocytes [66]. The combination of ALA with acetyl-L-carnitine and N-acetyl-L-cysteine supplemented in vitrification and/or warming solutions in mice led to significantly increased inner cell mass number and total cell number and increased outgrowth area, which correlated with the increased fetal weight, crown-rump length and limb development following the transfer, as compared

with embryos with no antioxidants [63]. The number of grade 1 - blastocysts vitrified increased and the level of ROS was lower in day 7 ALA vitrified-warmed blastocysts than in Control, however, we observed that the hatching rate post-vitrification did not improve in the ALA group**.** Recently, Truong and Gardner [63] suggested that antioxidants should be present during exposure to increase the oxidative stress associated with vitrification and that prior exposure was not enough to protect cells against cryo-induced injury. For this reason, the potential effects of ALA on vitrification solutions and whether ALA may ameliorate the oxidative stress caused by the vitrification–warming process should be assessed in future studies of bovine embryos.

5. Conclusions

In conclusion, these results suggest that ALA added during early stages of culture increases grade 1- blastocysts rate. Moreover, ALA improves embryo quality, in terms of cell number per blastocyst as well as hatching rate. Also, ALA decreases ROS level in vitrified-warmed blastocysts. This supplementation may be useful to increase the efficacy of bovine *in vitro* embryo production.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on reasonable request.

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Table 1. Effect of 2.5 µM Alpha-lipoic acid (ALA) supplementation during 3 and 24 h in *in vitro* culture of bovine embryos on viability and intracellular reactive oxygen species (ROS) levels in putative zygotes and in day 2 cleaved embryos.

	Putative zygotes			Day 2 embryos		
Treatment	**Viability % FAU (*n*)**	**ROS level % FAU (*n*)**	**ROS/FDA**	**Viability % FAU (*n*)**	**ROS level % FAU (*n*)**	**ROS/FDA**
Control	38.60 ± 3.24 (62)[a]	23.88 ± 2.89 (81)[a]	0.61[a]	106.55 ± 12.64 (47)[a]	21.22 ± 1.62 (53)[a]	0.19[a]
ALA	35.27 ± 2.91 (51)[a]	19.83 ± 2.64(83)[a]	0.56[a]	122.79 ± 12.28 (61)[a]	20.08 ± 1.58 (79)[a]	0.16[a]

Results are expressed as % FAU ± SD (*n*); FAU: Fluorescence arbitrary units. *n*: number of putative zygotes or day 2 embryos analyzed. Viability was measured with the fluorescein diacetate (FDA) assay. ROS level was evaluated with the 2′,7′-dichlorofluorescein diacetate (H2DCFDA) assay. A total of 30-40 COCs were matured and fertilized per treatment per assay. All cleaved embryos and putative zygotes were analyzed except degenerate and non-fertilized oocytes. Treatments were repeated in three replicates for each assay. [(a–b)] Values with different superscripts within each column differ ($P \leq 0.05$).

Table 2. Effect of 2.5 µM alpha-lipoic acid (ALA) supplementation during *in vitro* culture of bovine embryos on embryo development rate and total blastocyst cell number

Treatment	Matured Oocyte (*n*)	% (*n*)										% (h/b)		% (*n*)
		Cleaved 2-4 cells		Cleaved > 4 cells		B7		B8		BT		Hatched		Total cell number
Control	567	49.41 [a] (281)	mpd=-0.00 IL= -0.05	34.14 [a] (189)	mpd=-0.03 IL=-0.08	19.38 [a] (108)	mpd=10.06 IL=0.02	5.51 [a] (35)	mpd= -0.01 IL= -0.03	24.90 [a] (143)	mpd= 0.05 IL= 0.01	37.5[a] (12/32)	mpd= 0.20 IL= 0.03	54.54 ± 3.73[a] (30)
ALA	546	49.65 [a] (276)	*P*= 0.37	30.86 [a] (163)	*P*= 0.10	25.72 [b] (142)	*P*=0.00	4.07 [a] (28)	*P*= 0.22	29.79 [b] (170)	*P*= 0.01	59.57[b] (28/47)	*P*= 0.02	64.39 ± 4.77[b] (20)

Results of embryo development are expressed as a percentage with respect to matured oocytes (*n*). Mean posterior difference (mpd); inferior limit (IL). B: blastocyst; B7: blastocyst that appeared on day 7 of culture; B8: blastocyst that appeared on day 8 of culture. BT: total blastocyst. Results of hatching (h) rate are expressed as a percentage with respect to total blastocysts (b) (% (h/b)). Results of total cell number of blastocysts on day 8 were evaluated with Hoechst 33342 and expressed as % mean ± SD (*n*). A total of 95-100 COCs were matured per treatment per replicate. Treatments were repeated in six replicates. [(a–b)] Values with different superscripts within each column differ ($P \leq 0.05$).

Table 3. Effect of 2.5 µM Alpha-lipoic acid (ALA) supplementation during *in vitro* culture of bovine embryos on the blastocyst quality at day 7 (D7) and day 8 (D8) of culture

	Treatment	Blastocyst Quality % (*n*)			
		1		2	
D7	Control	2.16 (32)	mpd=0.04;	4.64 (70)	mpd=0.003;
	ALA	7 (53)	IL= 0.02;	4.98 (37)	IL= -0.02;
			P = 0.001		*P* = 0.42
D8	Control	1.5 (23)	mpd=0.01;	8.66 (130)	mpd=-0.01;
	ALA	3.51 (26)	IL= -0.001;	7.33 (55)	IL= -0.04;
			P = 0.06		*P* = 0.26

Results are expressed as % (n). D7: day 7 of embryo culture. D8: day 7 of embryo culture. The quality grade was classified as: 1) excellent, 2) good. A 323-341 COCs were matured per treatment per replicate. Treatments were repeated in two to five replicates. $^{(a–b)}$ Values with different superscripts within each column differ ($P \leq 0.05$).

Table 4. Effect of 2.5 µM alpha-lipoic acid (ALA) supplementation during *in vitro* culture of bovine embryos on re-expansion rates and intracellular reactive oxygen species (ROS) levels of vitrified/warmed blastocysts.

	Treatment	Vitrified embryos (*n*)		Expanded % (*n*)		Hatched % (*n*)						Intracellular ROS levels %FAU (*n*)
				3h		Embryos (*n*)	24h		48h		72h		
D7	**Control**	**22**	mpd=0.05 LI= 0.01	**68.18 (15)[a]**	mpd=-0.04 IL=-0.247	**11**	**18.18 (2)[a]**	mpd=0.08 IL=-0.15	**54.54 (6)[a]**	mpd=-0.26 IL=-0.53	**9.00 (1)[a]**	mpd=-0.06 IL=-0.24	**137.35 ± 18.44 (9)[a]**
	ALA	**41**	*P*= 0.01	**62.85 (22)[a]**	*P*=0.35	**21**	**30.00 (6)[a]**	*P*=0.27	**25.00 (5)[b]**	*P*=0.05	**5.00 (1)[a]**	*P*=0.28	**100.45±14.18(10)[b]**
D8	**Control**	**33**	mpd=0.00 LI=-0.03	**81.25 (26)[a]**	mpd=-0.00 IL=-0.16	**17**	**29.41 (5)[a]**	mpd=0.11 IL=-0.13	**29.41 (5)[a]**	mpd=-0.22 IL=-0.42	**17.64 (3)[a]**	mpd=-0.16 IL=-0.33	**121.90± 8.90(15)[a]**
	ALA	**37**	*P*=0.39	**80.55 (29)[a]**	*P*=0.48	**19**	**42.10 (8)[a]**	*P*=0.22	**5.20 (1)[b]**	*P*=0.03	**0.00 (0)[b]**	*P*=0.05	**127.30±8.35(10)[a]**

Blastocyst were vitrified/warmed on day 7 (D7) and day 8 (D8) of development. Results of re- expanded blastocysts and hatching are expressed as percentages (*n*). Results of expanded and hatching are expressed with respect to the embryos evaluated. Mean posterior difference (mpd); inferior limit (IL). ROS level data are expressed as % mean ± SD (*n)* and expressed as fluorescence arbitrary units (FAU). [(a–b)] Values with different superscripts within each column differ ($P \leq 0.05$).

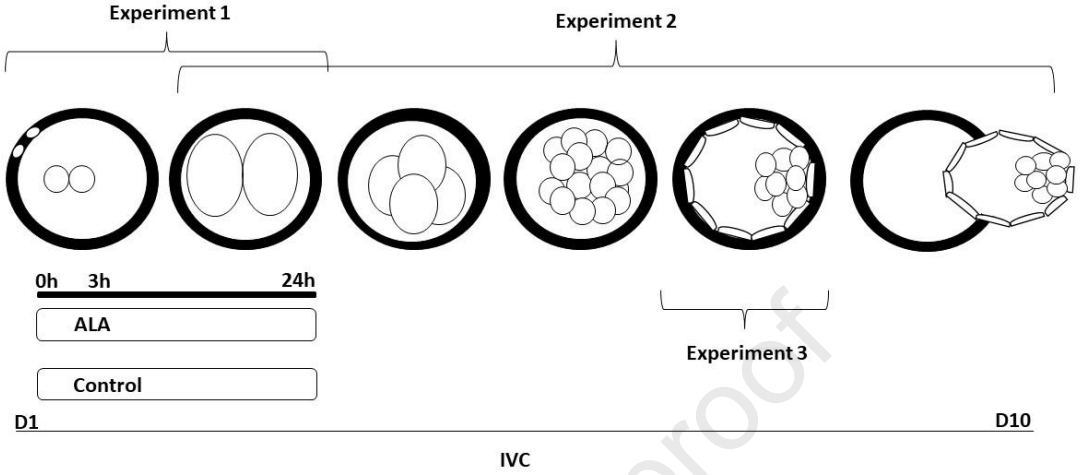

Figure 1. Experimental design. Alpha-lipoic acid (ALA) was diluted in ethanol 0.1% and supplemented during bovine cultured *in vitro* (IVC). The IVC medium consisted of synthetic oviduct fluid (SOF). The treatments were Control: media in the absence of ALA or ALA: presence of 2.5 µM ALA**.** Experiment 1: Reactive oxygen species (ROS) levels and viability were evaluated in presumptive zygotes and day 2 embryos (46 h post-*in vitro* fertilization) after 3 or 24 h ALA exposure, respectively. Experiment 2: Presumptive zygotes were cultured in the absence (Control) or presence of ALA for 24 h. Then, embryos were culture with SOF media until day 10 (D10). Cleavage, day 7 and 8 blastocysts and hatching rate were recorded and total cell number was evaluated in blastocysts. Experiment 3: Presumptive zygotes were cultured in the absence (Control) or presence of ALA for 24 h. Then, embryos were culture with SOF media until day 7-8. On days 7 and 8, blastocysts in each group were vitrified. Then, embryos were warmed and cultured for 3 h in IVC. Finally, re-expansion, hatching and ROS level were evaluated. D1: day 1 of IVC; D10: day 10 of IVC. The brackets indicate the embryonic stages analyzed in each experiment.

Highlights

- Supplementation of IVC medium with ALA during 24 h improved embryo quality and blastocyst rate
- Supplementation of IVC with ALA during 24 h increased total cell number per blastocyst
- Supplementation of IVC with ALA during 24 h decreased ROS level in vitrified-warmed blastocysts
- Supplementation of IVC with ALA increased the hatching rate 24 h post warmed embryos