The Effect of a Mixture of Amino Acids and Endogenous Lipids on *in vitro* Oocyte Maturation of Cows

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ABSTRACT

Understanding the effects of amino acid (AA) and endogenous lipid (EL) metabolism during *in vitro* oocyte maturation is important; however, most studies use undefined routine maturation media supplemented with glucose or an oxidative agent. The present study was conducted in two phases, with each phase consisting of two stages, and aimed to use supplemented AA (a mix of the 20 amino acids and glutamine) or EL (triacylglycerols) as unique oxidative substrates for oocytes during *in vitro* maturation (IVM) to understand the oocyte requirements during maturation. Cumulus-oocyte complexes (COCs) obtained from slaughtered Aberdeen Angus cows were matured in five groups to evaluate AA utilization and in the other five groups to evaluate EL utilization. The COCs were incubated in a defined medium either without oxidative substrates (negative control) or supplemented with AA, AA and salicylate as deamination inhibitor, AA and glucose (Glc), and Glc alone (positive control) to evaluate AA catabolism. To assess EL utilization, COCs were cultured with L-carnitine (β-oxidation activator), etomoxir (β-oxidation inhibitor), L-carnitine with Glc, Glc alone (positive control), and without oxidative substrates (negative control). Nuclear maturation, ammonia production, and oocyte lipid content were evaluated after maturation. Cleavage and blastocyst rates were assessed at 48 hours and seven days after fertilization. The results indicated that oocytes matured in media with AA and Glc (51% and 52%, respectively) or AA with salicylate (38%) had higher nuclear maturation rates than the control group (11.76 %), and a synergistic effect was observed in the group supplemented with AA and Glc (70 %). When AA was added to the maturation media as a unique oxidative substrate, ammonia production increased, while embryo development was observed only in the AA and Glc-supplemented group. Media with Glc (68 %) or L-carnitine with Glc (61%) increased the nuclear maturation. A higher lipid content was observed with Glc. Glc (63%) and L-carnitine with Glc (57.9%) increased cleavage rates compared with the other groups, but no group reached the blastocyst stage. Catabolism of AA, EL, or Glc alone served as an oxidative substrate and sustained bovine oocyte nuclear maturation, but they were insufficient to induce oocyte developmental competence.

Keywords: Amino acid, Endogenous lipid, Glucose, *In vitro* oocyte maturation, Nuclear maturation

INTRODUCTION

The roles of amino acid and endogenous lipid metabolism during *in vitro* maturation are essential. Bovine embryo development rates are still between 30-40 % of blastocyst production (de Avila Ferronato et al., 2023; Gebreyesus et al., 2024; Guan et al., 2024). Metabolic studies carried out on bovine gametes primarily focused on carbohydrates, which are the main substrates in the media currently used for oocyte *in vitro* maturation. In cattle, an adequate concentration of glucose in the maturation medium supports the maturation process (Rose-Hellekant et al., 1998; Lim et al., 1999; Khurana and Niemann, 2000). It has been demonstrated that glucose is used as an oxidative substrate to provide energy for regulating meiosis in bovine Cumulus-oocyte complexes (COCs; Sutton et al., 2005). In previous studies in the bovine species, glucose metabolism during maturation was investigated, establishing glycolysis as the main pathway for glucose utilization (Sutton et al., 2003; Gutnisky et al., 2013). Alternatively, glucose may be oxidized via the pentose phosphate pathway, a metabolic pathway implicated in nuclear maturation (Gutnisky et al., 2014).

Studies investigating amino acid metabolism during *in vitro* oocyte maturation are limited. Amino acid catabolism begins with the removal of α-amino nitrogen by activating aminotransferases, followed by oxidizing the resulting carbon skeletons to produce energy. In the next step, the receptor metabolite (commonly glutamate) is deaminated, yielding ammonia. This process is catalyzed by glutamate dehydrogenase, which mediates the reversible oxidative deamination of glutamate to α-ketoglutarate, thereby linking carbon and nitrogen metabolism (Rife and Cleland, 1980). Inhibiting glutamate dehydrogenase by salicylate has been reported in rodents, bovine, and porcine species, suggesting that amino

acids are key determinants of oocyte maturation (Cetica et al., 2003; Hong et al., 2004). Supplementing the maturation media with different amino acids had increased the *in vitro* fertilization efficiency, pronucleus, and blastocyst rates, improving the cytoplasmic maturation of the oocytes (Rose-Hellekant et al., 1998; Lim et al., 1999; Cetica et al., 2003; Hong et al., 2004). It was reported that bovine and porcine oocytes can use amino acids as an energy source (Rieger and Loskutoff, 1994; Alvarez et al., 2012).

There are limited studies about lipid metabolism during oocyte *in vitro* maturation. Unlike human oocytes, equine, rodent, and bovine oocytes contain abundant lipid droplets in their cytoplasm (Kikuchi et al., 2002). It has been noted that these lipid droplets are associated with the endoplasmic reticulum and mitochondria, and their redistribution throughout maturation may be linked to metabolic shifts.

After maturation, lipid contents decrease, with triglycerides being the most predominant lipid (Nagano et al., 2006), suggesting that this compound could be used as an energy source during this process (Kim et al., 2001; Sturmey and Leese, 2003).

 Although the hydrolysis of triglycerides releases fatty acids, β-oxidation is the pathway through which fatty acids are oxidized to obtain energy (Nelson and Cox, 2005). It has been described that the inhibition of fatty acid β-oxidation of murine, bovine, and porcine COCs compromises nuclear maturation in these species (Paczkowski et al., 2013). The L-carnitine acyl transferase 1 (CAT 1) is the primary regulatory component of fatty acid β-oxidation. The CAT 1 transfers the activated fatty acids into the mitochondrial matrix, where oxidation occurs. The CAT 1 can be stimulated by L-carnitine and inhibited by etomoxir, therefore stimulating and inhibiting fatty acid β-oxidation (Paczkowski et al., 2013).

Studies conducted to understand amino acid and endogenous lipid metabolism in bovine oocytes were performed in routine media containing glucose and other oxidative substrates. This medium, Tissue Culture Medium 199 (TCM-199), is generally supplemented with fetal bovine serum, resulting in an undefined medium. Therefore, the results may be partly affected by culture conditions, making conclusions relative and difficult to interpret. The specific metabolic requirements of COCs during *in vitro* maturation, and their subsequent impact on embryo development, remain poorly understood. The present study aimed to examine the utilization of amino acids or endogenous lipids as distinctive oxidative substrates for bovine oocytes during *in vitro* maturation.

MATERIALS AND METHODS

Ethical approval

The present study was conducted in accordance with the general guidelines of the Institutional Committee for Care and Use of Experimental Animals, Faculty of Veterinary Sciences, Buenos Aires University (CICUAL; 2003). No animal experiments were performed during the present study. Ovaries were donated to the laboratory by a local slaughterhouse, and *Centro de Reproducción Bovina* donated semen.

Materials and reagents

Reagents used in the present study were purchased from Sigma (Sigma Chemical Co., St. Louis, MO, USA). The minimum essential medium (MEM) animo acids solution (L-arginine hydrochloride (29.95 mM), L-cystine (5.0 mM), L-histidine hydrochloride monohydrate (10.0 mM), L-isoleucine (20.0 mM), L-leucine (20.0 mM), L-lysine hydrochloride (19.81 mM), L-methionine (5.07 mM), L-phenylalanine (10.0 mM), L-threonine (20.0 mM), L-tryptophan (2.5 mM), L-tyrosine (9.94 mM), and L-valine (20.0 mM) with the indicated concentrations, catalog number 11130036, MEM- Non essential amino acids solution (Glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid, L-proline, and L-serine each added at a concentration of 10.0 mM, catalog number 11140050) were purchased from GIBCO (Thermofisher Scientific, USA).

Experimental design
Nuclear maturation and ammonia production (Experiment 1)

Immature COCs obtained by aspiration of antral follicles were classified under a light microscope. Only COCs surrounded by cumulus cells and homogeneous cytoplasm were used (Cetica et al., 1999). Obtained COCs were divided into five randomized groups of 80-100 COCs based on the maturation media, including without oxidative substrates (negative control), supplemented with amino acids, amino acids with 5 mM salicylate (to inhibit oxidative deamination), amino acids plus glucose, and glucose only. After 22 hours of culture, COCs were removed from the maturation medium, and oocytes were denuded. They were then stained with Hoechst 33342 to evaluate the metaphase II plate (n = 80-100 oocytes per treatment across five replicates). Ammonia production was measured during the remaining maturation, with 10 replicates per treatment (Alvarez et al., 2012).

Nuclear maturation and endogenous lipid content (Experiment 2)

To evaluate endogenous lipid content (Genicot et al., 2005), 80-100 immature COCs were divided into five random groups of the defined maturation media without oxidative substrates (negative control), supplemented with L-carnitine (to stimulate fatty acid β-oxidation), etomoxir (to inhibit fatty acid β-oxidation), L-carnitine with glucose, and glucose only. After 22 hours of culture, a group of oocytes was stained with Hoeschst 33342 for the metaphase II plate evaluation (n = 90-100 oocytes per treatment in five replicates). The other group of denuded oocytes was stained with Nile Red to determine endogenous lipid content (n = 55-65 oocytes per treatment in five replicates).

In vitro fertilization and embryo production (Experiment 3)

The COCs were matured in the groups described in experiment 1. After 21 hours of maturation, COCs were cultured in *in vitro* fertilization (IVF) media and then in *in vitro* culture (IVC) media. Cleavage and blastocyst rates were evaluated at 48 hours and seven days after fertilization under a microscope. Embryos with two or more blastomeres were considered cleaved. Each treatment involves 80-85 oocytes across four replicates.

In vitro fertilization and embryo production (Experiment 4)

The COCs were matured in the groups described in experiment 2. After 21 hours of maturation, the COCs were cultured in IVF media and then in IVC media. Cleavage and blastocyst rates were evaluated at 48 h and 7 days after fertilization by the proportion of cleaved embryos and blastocysts, respectively. Each treatment involves 80-85 oocytes across four replicates.

Recovery of cumulus-oocyte complexes

Bovine ovaries were collected from slaughtered 20-24-month-old Aberdeen Angus cows, at an abattoir within 30 minutes of slaughter, and kept warm (30º C) during the 2-hour transport to the laboratory. Ovaries were washed with physiological saline containing 100,000 IU L-1 penicillin and 100 mg L-1 streptomycin. Cumulus-oocyte complexes were recovered by aspiration of antral follicles (3-5 mm in diameter) and collected directly in the maturation medium. The COCs were washed three times before being placed in the definitive maturation medium. Only oocytes surrounded by a compact and multilayered cumulus oophorus, selected under a microscope, were used (Gutnisky et al., 2014).

In vitro maturation of cumulus-oocyte complexes

To assess amino acid utilization during oocyte *in vitro* maturation, COCs were cultured in a defined medium lacking oxidative substrate, mSOF (an artisanal medium without pyruvate and lactate, Takahashi and First, 1992). The medium was supplemented with 0.2 mg/ml FSH (Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg/ml LH (Lutropin-V; Bioniche), 10 ng/ml EGF, 5 mg/ml insulin, 1 mg/ml polyvinyl alcohol (PVA, replacing bovine serum albumin), and 50 μg/ml gentamicin sulfate. Cultures were kept under mineral oil at 39°C, with 5% CO_2 in a humidified atmosphere (negative control). All the groups that contain amino acids were supplemented with amino acids (3% (v/v) amino acids MEM (GIBCO) + 1% (v/v) non-essential amino acids MEM (GIBCO) + 2 mM glutamine), AA with 5 mM salicylate, AA with 5.5 mM glucose, and 5.5 mM glucose only. Supplementation with MEM amino acids (GIBCO), non-essential amino acids (GIBCO), and glutamine ensures a supply of the 20 amino acids.

When endogenous lipids, triacylglycerols, were examined during oocyte *in vitro* maturation, the maturation conditions remained consistent with the negative control. The medium was supplemented with 0.6 mg/ml L-carnitine, 50 mM etomoxir (Etomoxir), 0.6 mg/ml L-carnitine combined with 5.5 mM glucose, or solely 5.5 mM glucose.

To verify the *in vitro* embryo production, a standard protocol for bovine embryo production was used. This protocol utilized an undefined maturation medium enriched with oxidative substrates. Cumulus-oocyte complexes were cultured in Medium 199, which included Earle's salts, L-glutamine, 2.2 mg/L sodium bicarbonate (GIBCO, Grand Island, NY, USA), supplemented with 5% v/v FBS (GIBCO), 0.2 mg/L porcine FSH (Folltropin-V; Bioniche, Belleville, Ontario, Canada), 2 mg/L porcine LH (Lutropin-V; Bioniche), and 50 mg/L gentamycin sulfate. Cultures were maintained under mineral oil at 39°C for 22 hours in a 5% CO_2 atmosphere, humidified air (Gutnisky et al., 2013).

Evaluation of oocyte nuclear maturation

To evaluate oocyte nuclear maturation, COCs were incubated in 1 mg/mL hyaluronidase in phosphate-buffered saline (PBS) medium for five minutes at 37°C, and the oocytes were mechanically denuded by gentle pipetting. Oocytes were fixed for 15 minutes with 2% glutaraldehyde in PBS, cultured with 1% Hoechst 33342 in PBS for 15 minutes, washed in PBS containing 1 mg/mL polyvinylpyrrolidone, and mounted on glass slides. Oocytes were examined under an epifluorescence microscope using 330 to 380 nm (excitation) and 420 nm (emission) filters at 250 and 400 magnifications. Oocytes were considered mature upon the observation of metaphase II chromosome configuration (Gutnisky et al., 2013).

In vitro fertilization and embryo production

To evaluate oocyte development, its ability to be fertilized and develop to the blastocyst stage was analyzed by *in vitro* fertilization. *In vitro* fertilization was performed using frozen–thawed semen from a Holstein bull of proven fertility. Semen was thawed at 37 ºC in a water bath, then resuspended in mSOF with theophylline 3.6 gL^{-1} (Takahashi and First, 1992), centrifuged twice at 500 × g for five minutes, and then resuspended in fertilization medium to a final concentration of 1×10^6 motile spermatozoa L^{-1} after the swim-up procedure (Hallap et al., 2004). Fertilization was performed in 500 μL IVF-mSOF, consisting of modified synthetic oviductal fluid (mSOF) supplemented with bovine serum (5gL-1albumin, 1500 USP UL-1heparin, and 50 μg/ml gentamicin sulfate), under mineral oil at 39ºC, in 5% CO_2 in air and 100% humidity for 20 hours. Putative zygotes were denuded by repeated pipetting and placed in 500 μL IVC-mSOF, consisting of mSOF supplemented with 30 mLL^{-1} amino acid MEM (GIBCO), 10 mLL^{-1} non-essential amino acid MEM (GIBCO), 2 mM glutamine, 6 gL^{-1} fatty acid-free BSA, 5% (v/v) fetal bovine serum (GIBCO), and 50 μg/ml gentamicin sulfate, under mineral oil at 39º C in a humidified atmosphere with 90% N_2, 5% CO_2, 5% O_2 for 24 hours. The proportion of cleaved embryos after 48 hours was evaluated by the number of embryos that presented two or more blastomeres.

In vitro embryo development was performed in IVC-mSOF, under mineral oil at 39ºC in a humidified atmosphere with a tri-gas mixture of 90% N_2, 5% CO_2, and 5% O_2. The proportion of blastocysts produced was determined at day 7 following insemination.

Ammonia production by cumulus-oocyte complexes

Ammonia production was measured by incubating groups of 50 COCs in 500 μL microdrops of the maturation medium. The ammonia concentration was measured in 10 μl of the remaining media after 22 hours of incubation, using a spectrophotometric assay based on NADPH oxidation by glutamate dehydrogenase during ammonia uptake. Microdrops of maturation media without cells served as controls for the experiment. Data were expressed as moles of ammonia produced per COC over 22 hours.

Oocyte endogenous lipid determination

To determine the oocyte lipid content after *in vitro* maturation, matured oocytes were denuded and fixed in a 3 % glutaraldehyde for 15 minutes. Then, oocytes were transferred to a 0.9 % NaCl solution with 10 μg/ml Nile Red and 0.1 % PVA, pH 7 (Genicot et al., 2005) and incubated for 2 hours in the dark at room temperature. Then the oocytes were washed, mounted on a slide, and observed under an epifluorescence microscope with an excitation filter at 510 nm and an emission filter at 590 nm. A digital photograph was taken to analyze the individual luminescence of each oocyte. The microphotographs were analyzed with the software ImageJ, and the lipid content was expressed as arbitrary units of luminescence/oocyte.

Statistical analysis

The proportions of mature oocytes, cleaved embryos, and blastocysts were compared using a chi-squared test for nonparametric data. Data for ammonia production and endogenous lipid determination were expressed as the mean ± standard deviation. For evaluating ammonia production and endogenous lipid content, comparisons were made using analysis of variance followed by the Bonferroni post hoc test. All statistical tests were performed using the InfoStat software (Di Rienzo et al., 2008).

RESULTS

Oocyte nuclear maturation

The media supplemented with mixed amino acids had a higher oocyte nuclear maturation rate (51.52%) than the negative control (11.76%) and amino acids with salicylate (37.66%; $p < 0.05$). No differences in nuclear maturation were observed between the media containing amino acids or glucose. However, a synergistic effect on the nuclear maturation rate was observed in the maturation media supplemented with amino acids in combination with glucose (70 %; $p < 0.05$; Figure 1). To evaluate the significance of endogenous oocyte lipids as sole oxidative substrates during bovine oocyte *in vitro* maturation, COCs were matured without external oxidative substrates and supplemented with either L-carnitine, an activator of CAT1, or etomoxir, an inhibitor of CAT1. Additionally, glucose was added alone or in combination with L-carnitine to the maturation medium as positive controls. The medium supplemented with L-carnitine demonstrated a significantly higher oocyte nuclear maturation rate (21%) than the media without oxidative substrates (0%) or etomoxir (4%; $p < 0.05$). Media supplemented with glucose (69%) or with L-carnitine plus glucose (62%) increased nuclear maturation compared with the other groups ($p < 0.05$), with no significant difference between these two groups (Figure 2).
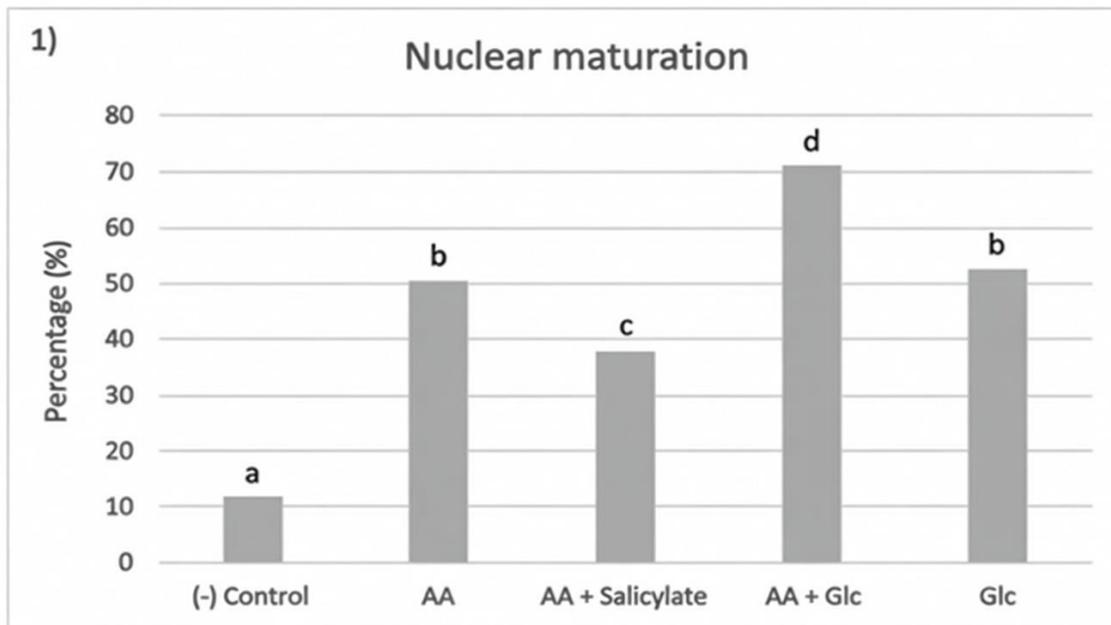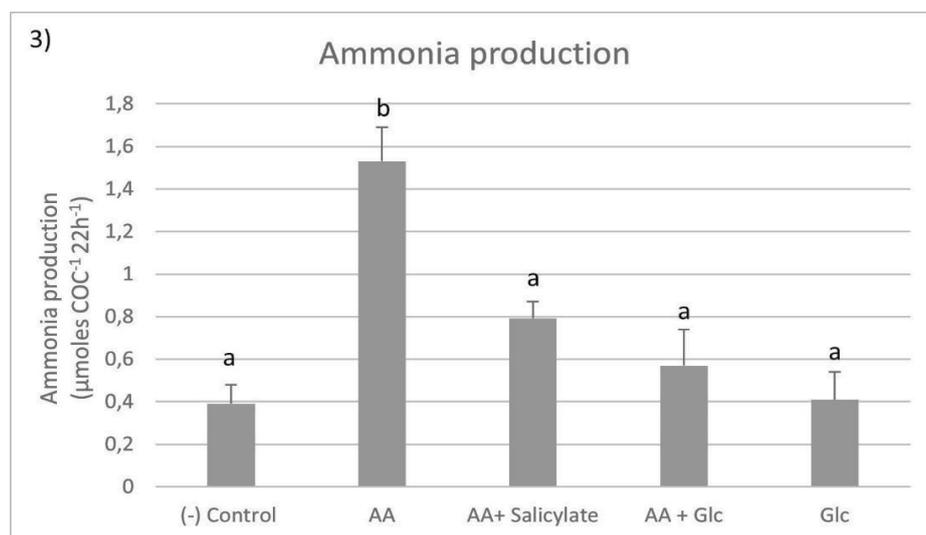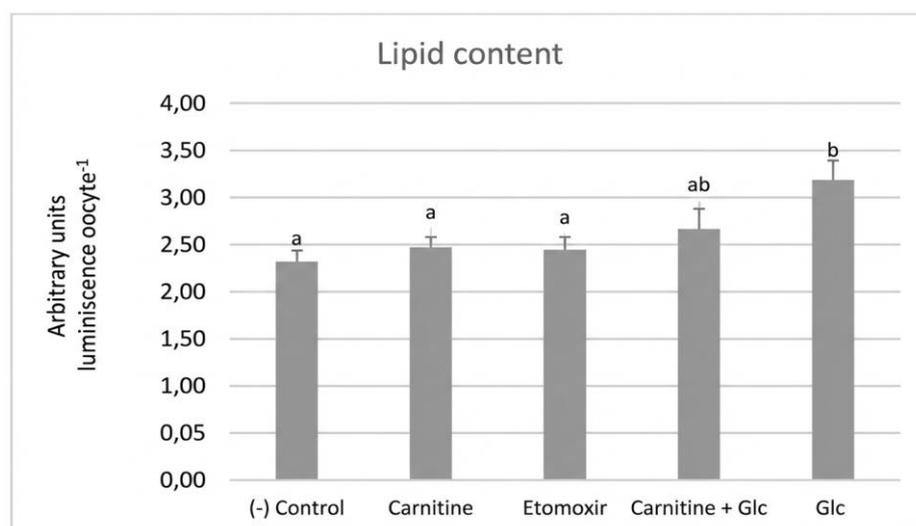
Figure 1. Evaluation of bovine oocyte *in vitro* maturation rates of cumulus-oocyte complexes matured in a defined medium to assess the use of amino acids from culture media. Cumulus-oocyte complexes were matured in a defined medium without oxidative substrates as a negative control (-), supplemented with amino acids (AA), amino acids + salicylate as an oxidative deamination inhibitor (AA + Salicylate), amino acids + glucose (AA + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as percentages (%). [a,b,c,d] Bars with different letters differ significantly (p < 0.05, n=80-100 oocytes for each treatment in five replicates).
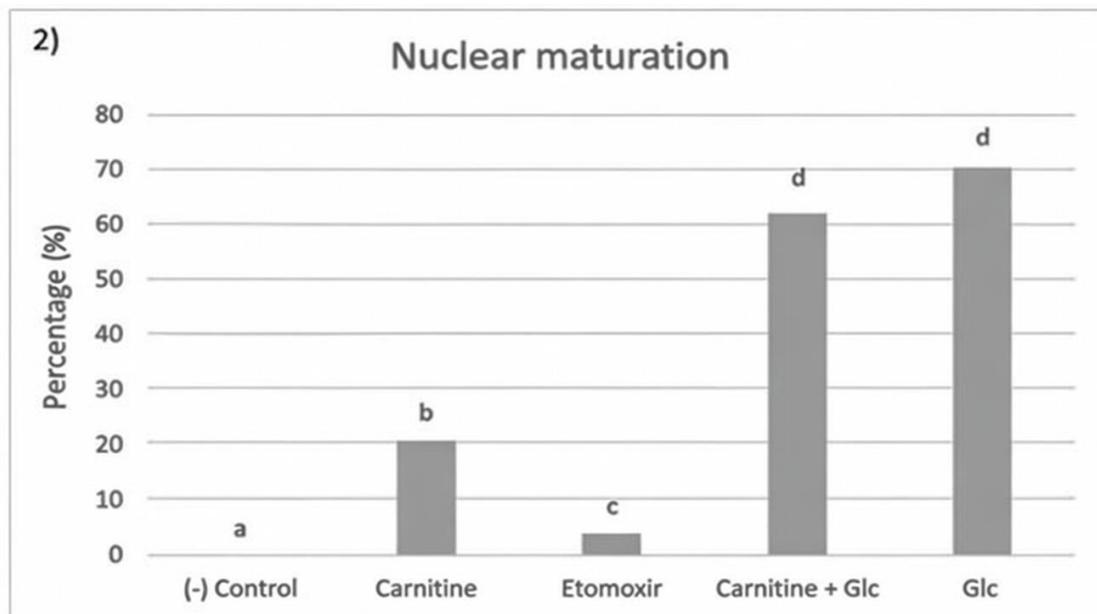
Figure 2. Evaluation of bovine oocyte *in vitro* maturation rates of cumulus-oocyte complexes matured in a defined medium to assess the use of oocyte endogenous lipids. Cumulus oocyte complexes were matured in a defined medium without oxidative substrates as a negative control (-) Control, supplemented with L-carnitine as fatty acid β-oxidation activator (Carnitine), etomoxir as fatty acid β-oxidation inhibitor (Etomoxir), L-carnitine + glucose (Carnitine + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as percentages (%). [a,b,c,d] Bars with different letters differ significantly (p < 0.05, n = 90-100 oocytes for each treatment in five replicates).

Ammonia production

A significant increase in ammonia production was observed in the group supplemented with amino acids (1.53 µmoles/COC^{22h}) compared to those without oxidative substrates (0.39 µmoles/COC^{22h}) or amino acids with salicylate (0.79 µmoles/COC^{22h}; p < 0.05), indicating a greater use of amino acids in the medium as a unique oxidative substrate. The presence of glucose with amino acids in the maturation media significantly reduced ammonia production from amino acids (p < 0.05; Figure 3).

Figure 3. Evaluation of the use of amino acids as oxidative substrates from culture media during bovine oocyte *in vitro* maturation by assessing ammonia production by cumulus-oocyte complexes after 22 h of maturation. Ammonia production by COCs matured in a defined medium without oxidative substrates as a negative control (-) Control, supplemented with amino acids (AA), amino acids + salicylate as an oxidative deamination inhibitor (AA + Salicylate), amino acids + glucose (AA + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as μmoles of ammonia production/COC/22 h (μmoles COC^{-1} 22h^{-1}). [a,b] Bars with different letters differ significantly ($p < 0.05$, n = 10 in three replicates for each treatment).

Figure 4. Evaluation of the use of oocyte endogenous lipids as oxidative substrates during bovine oocyte *in vitro* maturation by assessing neutral lipid content in oocytes after 22 h of maturation. Lipid content in arbitrary units of luminescence/oocyte of oocytes matured in a defined medium without oxidative substrates as negative control (-) Control, supplemented with L-carnitine as fatty acid β-oxidation activator (Carnitine), etomoxir as fatty acid β-oxidation inhibitor (Etomoxir), L-carnitine + glucose (Carnitine + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as arbitrary units of luminescence/oocyte (arbitrary units luminescence^{-1}). [a,b] Bars with different letters differ significantly ($p < 0.05$, n = 55-65 oocytes for each treatment in five replicates).

Endogenous lipid content

The endogenous lipid content was evaluated individually in each oocyte after its maturation. No significant differences were observed across the groups supplemented with L-carnitine, etomoxir, and the negative control. A higher lipid content was observed in the group supplemented only with glucose compared to the other groups ($p < 0.05$). Supplementing the medium with L-carnitine and glucose yielded an intermediate value that did not differ significantly from L-carnitine alone (Figure 4).

### *In vitro* fertilization and embryo production

To assess the importance of amino acids in maturation media as distinct oxidative substrates for supporting embryo development, cleavage rate patterns reflected nuclear maturation rates. The maturation medium supplemented with amino acids (46.3%) indicated a significantly higher cleavage rate than that with amino acids and salicylate (25.6%) or without oxidative substrates (0%; $p < 0.05$), in which no embryos were obtained. No significant differences in cleavage rates were observed between media supplemented with amino acids or glucose. The maturation medium supplemented with amino acids and glucose demonstrated a significantly higher cleavage rate than that supplemented with amino acids or glucose as single oxidative substrates ($p < 0.05$, Figure 5). Regarding embryo production, only the group supplemented with the amino acid and glucose exhibited embryo development to the blastocyst stage (Figure 5). To

verify the IVP system, a group of COCs was matured in the undefined media alongside each experience. The oocyte maturation rate was 90.7% (88 oocytes with metaphase II out of 97 cultured COCs), the cleavage rate was 77.8% (70 cleaved embryos out of 90 inseminated COCs), and the blastocyst rate was 38.8% (35 blastocysts out of 90 inseminated COCs).

When endogenous lipids were used as the sole energy source during oocyte maturation, cleavage rates followed patterns similar to those for nuclear maturation. The maturation medium supplemented with L-carnitine indicated a higher cleavage rate (17%) than the media with etomoxir (4%) or without oxidative substrates (0%), in which no embryos were obtained (p < 0.05). Cleavage rates were highest in media supplemented with glucose (63%) or with L-carnitine and glucose (57.9%), with no significant difference between these two groups (p < 0.05; Figure 6). When embryo production was analyzed, none of the groups reached the blastocyst stage. However, when analyzing the terminal developmental stage, embryos from the L-carnitine and glucose group reached the 8-cell to morula stage exclusively. A group of COCs was matured in an undefined maturation medium as a control for the embryo production system. The oocyte maturation rate was 89.4% (85 oocytes with metaphase II out of 95 cultured COCs), the cleavage rate was 79.3% (73 cleaved embryos out of 92 inseminated COCs), and the blastocyst rate was 38.9% (37 blastocysts out of 95 inseminated COCs).
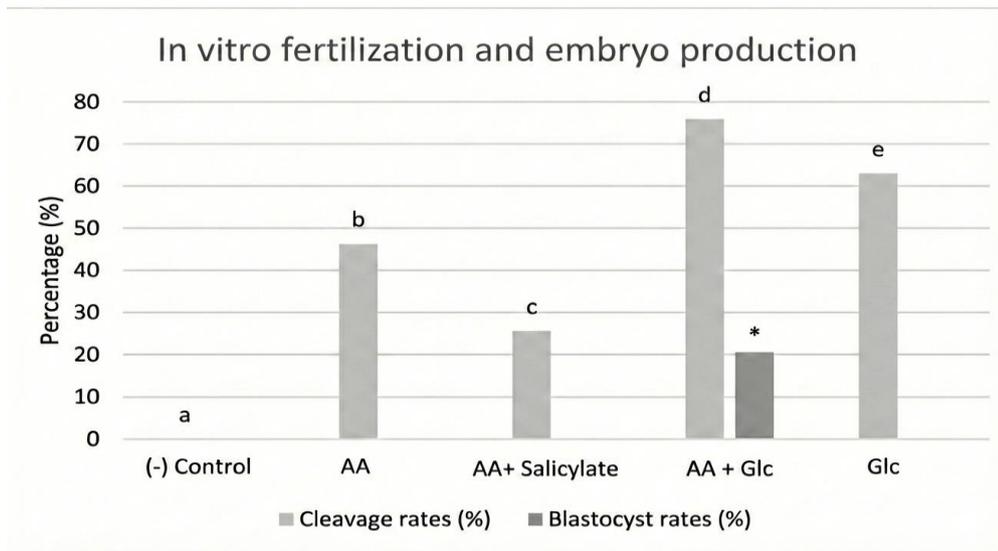
Figure 5. Evaluation of embryo development of bovine cumulus-oocyte complexes matured *in vitro* in a defined medium to assess the use of amino acids from culture media. Embryo cleavage (light gray) and blastocyst (dark grey) rates of oocytes matured in a defined media without oxidative substrates as negative control (-) Control, supplemented with amino acids (AA), amino acids + salicylate as an oxidative deamination inhibitor (AA + Salicylate), amino acids + glucose (AA + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as percentages (%). [a,b,c,d,e] Bars with different letters for cleavage differ significantly (P < 0.05, n=80-85 oocytes for each treatment in four replicates).
* Blastocysts were only obtained in the group added with amino acids + glucose (n = 80-85 oocytes for each treatment in four replicates).
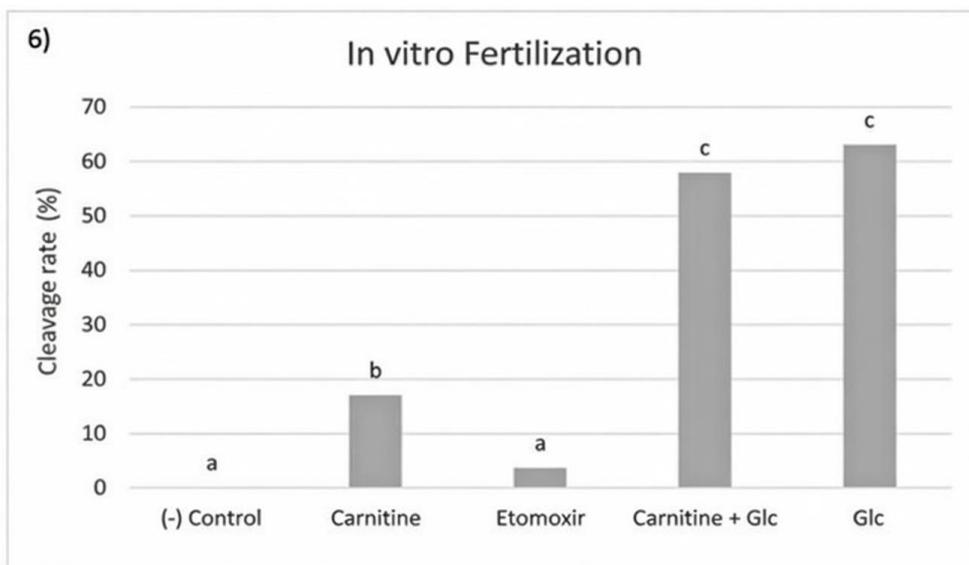
Figure 6. Evaluation of embryo development of bovine cumulus-oocyte complexes matured *in vitro* in a defined medium to assess the use of oocyte endogenous lipids. Embryo cleavage rates of oocytes matured in a defined medium without oxidative substrates as a negative control (-) Control, supplemented with L-carnitine as fatty acid β-oxidation activator (Carnitine), etomoxir as fatty acid β-oxidation inhibitor (Etomoxir), L-carnitine + glucose (Carnitine + Glc), or glucose (Glc). Glucose was added as a positive control. Data are expressed as percentages (%). [a,b,c] Bars with different letters differ significantly (P < 0.05), n = 80-85 oocytes for each treatment in four replicates.

DISCUSSION

The current findings suggest that, in order to produce competent oocytes, the maturation medium should contain amino acids and glucose. Therefore, COCs were matured in a defined medium without oxidative substrates, and amino acid catabolism was assessed by measuring ammonia production and changes in endogenous lipid content in response to specific inducers or inhibitors of metabolic pathways. The combined effects of these oxidative substrates with glucose were assessed due to glucose's established role as the primary substrate in routine maturation media.

The present findings demonstrated that amino acids alone could sustain nuclear maturation to a level equivalent to glucose, reinforcing their critical role as oxidative substrates. This finding is consistent with that of Cetica et al. (2003), indicating that specific amino acids, such as aspartate and alanine, enhance the maturation of denuded bovine oocytes. Furthermore, inhibiting oxidative deamination with salicylate slowed nuclear maturation rate, confirming that COCs can utilize amino acids during this process. In accordance, ammonia production was higher in the group matured with amino acids as the sole oxidative substrates than in the group supplemented with salicylate, where amino acid catabolism was diminished. Moreover, ammonia production decreased when both glucose and amino acids were added to the maturation medium, indicating that in the presence of the hexose, amino acids were partially protected from catabolism. The present results suggest that bovine COCs can utilize amino acids to support nuclear maturation by using their carbon skeletons as oxidative substrates. Consistent with the present findings, high enzymatic activities of alanine aminotransferase and aspartate aminotransferase were detected in bovine COCs (Cetica et al., 2003); in addition, a considerable increase of all detectable amino acids in cumulus cells during maturation has been reported (Uhde et al., 2018).

In vitro fertilization analysis revealed that cleavage rates following maturation in media supplemented with amino acids, glucose, or both, mirrored the patterns observed for nuclear maturation. The present results suggested that amino acids and glucose were implicated in early cytoplasmic maturation, with their combined presence exerting synergistic, positive effects. Despite these findings, only the combination of amino acids and glucose supported embryo development to the blastocyst stage, indicating that the presence of oxidative substrates during *in vitro* maturation was necessary to maintain oocyte developmental competence. The current results align with previous suggestions that amino acids, in the presence of glucose, contribute to the bovine oocytes' cytoplasmic maturation (Lim et al., 1999). Furthermore, total protein content in bovine COCs has been shown to increase during oocyte *in vitro* maturation (Gutnisky et al., 2007) a critical factor given the direct link between oocyte protein stores and developmental competence and blastocyst formation potential (Krisher, 2004). This increase in ammonia production during *in vitro* maturation in medium supplemented with amino acids might be due to higher deamination resulting from increased cellular protein turnover or the increased amino acid catabolism. The present data are aligned with cross-species observations of upregulated amino acid metabolism during oocyte maturation, including increased transporter activity in mice (Pelland et al., 2009) and elevated ammonia output coupled with greater protein accrual in porcine COCs (Alvarez et al., 2012). In the absence of other oxidative substrates, such as glucose, amino acids are primarily used as an energy source, and excessive catabolism could be detrimental to oocyte developmental competence.

The bovine oocyte had a high lipid content (McEvoy et al., 2000). A decrease in total lipid content was observed in oocytes cultured in media without other oxidative substrates after *in vitro* maturation, during the present study. In contrast, oocytes matured in a medium supplemented with glucose exhibited a higher lipid content, suggesting that glucose was utilized as an energy source, while triglycerides were conserved as chemical reserves. Media without oxidative substrates and supplemented with either L-carnitine or etomoxir, did not modify the lipid content of oocytes. However, analysis of oocyte nuclear maturation rates revealed that the proportion of oocytes reaching the metaphase II stage was higher in the L-carnitine-supplemented medium than in the other groups. This finding suggested that triglyceride hydrolysis occurred in all groups, but fatty acid β-oxidation was specifically stimulated only in the presence of L-carnitine. Consistent with the present findings, Cetica et al. (2002) reported lipase activity in bovine oocytes. Furthermore, treatment with etomoxir at concentrations that did not impair nuclear maturation in bovine and murine COCs led to increased glucose consumption. These findings indicated that when fatty acid metabolism is inhibited, glucose metabolism is upregulated to meet the COCs' metabolic demands (Paczkowski et al., 2013). On the other hand, the media supplemented with glucose achieved the highest maturation rates, and no synergistic effect was observed with the combination of L-carnitine, suggesting that oocyte endogenous lipids alone were unable to support nuclear maturation. Thus, carbohydrates are necessary for oocyte completion.

The *in vitro* fertilization results demonstrated that cleavage rates of oocytes matured in medium with L-carnitine, glucose, or both were similar to those with nuclear *in vitro* maturation, and no blastocysts were obtained under these conditions. These results suggested that oocyte endogenous lipids, by themselves or combined with glucose in maturation media, are insufficient for the oocyte to acquire complete developmental competence. However, only the group of oocytes supplemented with L-carnitine and glucose reached the 8-cell morula stage. Including amino acids in

the media for bovine oocytes during in vitro maturation may be a crucial factor for their developmental capacity. Thus, the beneficial effect of L-carnitine supplementation on embryo development may be attributed to its role as a crucial cofactor, enabling the oocyte to metabolize endogenous lipid sources (Nelson and Cox, 2005).

CONCLUSION

Catabolism of amino acids, endogenous lipids, or glucose individually supported oxidative metabolism and bovine oocyte nuclear maturation; however, none was sufficient to promote oocyte developmental competence. Further in vitro experiments using combinations of different substrates and/or stimulators should be conducted to determine the metabolic requirements for bovine COCs during maturation.

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Availability of data and materials

All data supporting the findings of this study are available within the manuscript.

Ethical considerations

All authors confirmed that this study is an original article and has not been published before, nor is it under consideration elsewhere. No AI tools were used for preparing the current study.

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Authors` contributions

Cynthia Gutnisky has contributed to the investigation, methodology, formal analysis, writing- original draft, and reviewing. Paula Gagneten, Santiago Martinez, and Tomás Gadze contributed to investigation and formal analysis. Elizabeth Breininger contributed to the investigation, and Pablo Cetica contributed to supervision, project administration, funding acquisition, writing-review, and editing. All authors have read and approved the final edition of the manuscript.

Competing interests

The authors declared no conflicts of interest.

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