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# Combined epidermal growth factor and hyaluronic acid supplementation of *in vitro* maturation medium and its impact on bovine oocyte proteome and competence

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ABSTRACT

The conditions for *in vitro* oocyte maturation impact on cytoplasmic and nuclear processes in the oocyte. These events are differentially influenced by the nature of the maturation inducer and the presence of intact cumulus in cumulus–oocyte complexes. Epidermal growth factor is the main growth factor promoting oocyte maturation. Also, hyaluronic acid (HA) produced by cumulus cells is known to be responsible for the correct structural and functional organization of the cumulus during oocyte maturation. Therefore, we evaluated the developmental competence of bovine oocytes matured *in vitro* in a maturation medium supplemented with both EGF and HA, compared to FSH and fetal bovine serum (FBS). In addition, the impact of IVM conditions on the proteomic profile of metaphase II bovine oocytes was analyzed by two-dimensional electrophoresis. Cumulus–oocyte complexes were matured in two media: (1) 10 ng/mL EGF, 15 μg/mL HA, and 100-μM cysteamine and (2) 0.01 UI/mL rh-FSH and 10% FBS. The percentages of first polar body and embryo production and the kinetics of embryo development and oocyte proteomic profiles were analyzed. Oocytes matured in the presence of EGF-HA showed an increase (6%, $P < 0.05$) in the percentage of polar body extrusion. The blastocyst rate was 3% ($P < 0.05$) higher in the FSH-FBS group, but no differences were found in the rate of expanded blastocyst neither in total embryo production between IVM conditions. Cleavage rate of oocytes matured with FSH-FBS was 5% higher ($P < 0.05$) with respect to EGF-HA–matured oocytes when evaluated 30 hours after fertilization. However, at Day 7, those inseminated oocytes that underwent division at a correct timing showed that although there are still early blastocysts in the FSH-FBS condition, EGF-HA embryos have developed completely into blastocysts. Still, the production rate of those embryos that achieved expansion was similar between both maturation conditions. On the other hand, non-cleaved presumptive zygotes at Day 7 developed into the different stages with similar rates (~4%) independently of the medium condition. Modifications of IVM medium composition markedly affected protein profile of bovine oocytes in a differential manner. The proteomic approach revealed the presence of 68 spots in both treatments, 41 exclusively found in the FSH-FBS group and 64 exclusive for the EGF-HA group. Taken together, these

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results indicate that combined EGF-HA supplementation of *in vitro* maturation medium could be used to improve oocyte meiotic competence and ensure a better timing to develop into the blastocyst stage.



## 1. Introduction

In domestic animals, the production of embryos using *in vitro*–matured oocytes is a common practice, essential for assisted reproductive technologies including cloning and production of transgenic animals [1,2]. The use of suboptimal oocyte maturation conditions leads to a disruption of the finely balanced cytoplasmic and nuclear processes in the oocyte embittered by its attempt to develop in an artificial environment. In this regard, several studies were aimed at improving the efficiency of the whole IVM system through the use of additives and/or chemically defined media [2–5]. Different media composition and IVM conditions affect the relative abundance of gene transcripts, generating differences in mRNA content and the ability to withstand cryopreservation [6,7]. As transcription activity of the oocyte rapidly decreases during maturation, the information necessary to achieve a successful meiotic and developmental competence has to be managed at the protein level. Even when it has been reported that maturation inducers modify expression of different proteins [8], to our knowledge, no studies have been conducted on the impact of IVM media over bovine oocyte proteome. The only work that deals with the study of the effect of IVM media on the proteome of oocytes has been performed in pig oocytes to discover putative quality biomarkers in terms of their developmental potential with or without added gonadotropins [9]. Cumulus–oocyte complexes (COCs) undergoing IVM are almost universally cultured in the presence of follicle-stimulating hormone (FSH) and less commonly in a media containing EGF [10,11]. Follicle-stimulating hormone and EGF are inducers of cumulus cell EGF-like peptide expression and, consequently, prompt oocyte meiotic resumption and cumulus matrix expansion *in vitro* [12,13]. Epidermal growth factor is considered as one of the main growth factors promoting oocyte maturation, being major factor of gonadotropin signal amplification in COCs [14–16]. *In vivo*, EGF signaling is rapidly amplified and maintained over time to promote and propagate LH pathway throughout the follicle, being essential for ovulation [17]. On the other hand, the production of hyaluronic acid (HA) by cumulus cells during maturation is essential not only for cumulus cell expansion but also for maturation itself and embryo development [5]. Hyaluronic acid is a predominant component of the extracellular matrix and the major glycosaminoglycan present in the follicular fluid, oviduct, and uterine fluids. It is secreted by granulosa cells from the cumulus and synthesized by the HA-synthase enzyme located at the inner cytoplasmic face of the plasma membrane [18]. The functional roles of HA include cell adhesion and migration, regulation of protein secretion, gene expression, and cell proliferation and differentiation [19–21]. Several physiological functions have been assigned to HA as a result of its physicochemical properties, including regulation of protein and water distribution, water-binding capacity, filtering effects, and lubrication [22,23]. Also, because of its chemical and physical properties, it has been successfully used for replacing serum in bovine embryo culture [24]. Serum is widely used in bovine oocyte IVM, although it has been shown to have several disadvantages [25] such as batch-to-batch variability and being a source of animal-transmitted pathogens, e.g., bovine viral diarrhea [26]. Added to culture media, it modifies the lipid composition of bovine oocytes matured *in vitro* [27] and produces embryos with excessive lipid accumulation and reduced cryotolerance [28]. Previously, we have demonstrated that serum added to embryo culture medium affects cryopreservation survival with respect to *in vitro*–produced embryos in the absence of serum [29]. Taking into account the positive effects of HA on *cumulus* remodeling and function and the important role that the EGF network plays in mediating LH function during oocyte meiotic resumption, it seems conceivable that combined EGF and HA supplementation of the maturation medium might increase the developmental competence of oocytes. The objectives of the present study were to (1) evaluate the developmental competence of bovine oocytes matured *in vitro* in a serum- and gonadotropin-free maturation medium supplemented with EGF and HA compared to a standard maturation medium containing FSH and fetal bovine serum (FBS) and (2) assess the impact of the IVM medium composition on the proteomic profile of metaphase II bovine oocytes analyzed by two-dimensional (2D) gel electrophoresis.

## 2. Materials and methods

Unless otherwise stated, cell culture dishes were purchased from Thermo Scientific (Nunc, MA, USA) and reagents and solutions from Sigma–Aldrich (St. Louis, MO, USA).

### 2.1. Oocyte collection and in vitro maturation

Bovine ovaries were collected from a local slaughterhouse and transported within 2 hours in a thermic container to the laboratory at 25 °C to 30 °C. Cumulus–oocyte complexes were aspirated from follicles ranging from 2 to 10 mm in diameter. Cumulus–oocyte complexes with homogeneous ooplasm and more than four complete layers of *cumulus* cells, corresponding to grades 1 and 2 according to de Loos et al. [30], were selected under a stereomicroscope and washed three times in modified M199 supplemented with 0.5% HEPES (w:v). Selected COCs were randomly divided in two different groups according to the composition of the maturation media. The base maturation medium consisted of M199 plus 0.1 mg/mL L-glutamine and 2.2 mg/mL $NaHCO_3$. Group 1 (FSH-FBS) was supplemented with 0.01 IU/mL rh-FSH (Gonal F-75,

Serone, UK) and 10% FBS and group 2 (EGF-HA) with 10 ng/mL EGF, 15 μg/mL HA, and 100-μM cysteamine. For all experiments, 50 COCs per group were placed in four-well dishes (Nunc) in 400 μL of maturation medium for 22 hours at 38.5 °C under 5% $CO_2$ in humidified air.

### 2.2. First polar body determination

To assess nuclear maturation, oocytes were completely denuded of cumulus cells by pipetting in M199-HEPES containing 300 IU/mL hyaluronidase (H3506) for 2 minutes. The percentages of oocytes presenting polar body were analyzed under a stereomicroscope.

### 2.3. Fertilization, embryo production, and embryo development kinetics

*In vitro* fertilization was performed with frozen-thawed semen from a laboratory control bull. Matured COCs were transferred to a new four-well dish containing 400 μL of IVF-SOF solution supplemented with 50 μg/mL heparin. After thawing, semen was subjected to discontinuous Percoll gradient (30%, 60%, and 90%) and added to each well at a final concentration of $2 \times 10^6$ spermatozoa/mL. Cumulus–oocyte complexes and spermatozoa were coincubated at 38.5 °C under 5% $CO_2$ in humidified air for 22 hours. After IVF, presumptive zygotes were transferred to SOF–citrate medium and cultured for 7 days at 38.5 °C in a humidified atmosphere of 5% $O_2$ and 5% $CO_2$. Cleavage rates were assessed on Day 2 and blastocyst yields at Day 7 after fertilization. The kinetics of embryo development was evaluated. For this experiment, a total of 693 COCs (in four independent repeats) were matured and distributed 30 hours after IVF into the following categories: 2-cell embryo (cleaved) and presumptive zygotes (noncleaved). Each group was cultured until Day 7 after fertilization.

### 2.4. Two-dimensional gel electrophoresis

Oocytes matured either in FSH-FBS or EGF-HA were denuded by enzymatic treatment. Zona pellucida was removed by treating oocytes with 1 mg/mL protease type XIV from *Streptomyces griseus* (Protease E, P5147) followed by mechanical shearing. During this process, oocyte morphology was monitored rigorously, and those oocytes with abnormal shape or cytoplasmic abnormalities were discarded. Subsequently, zona pellucida–free oocytes were washed three times in SOF-HEPES and 1% polyvinyl alcohol. Each group, composed by 400 metaphase II (MII) oocytes, was suspended in 10 μL of lysis buffer (9-M urea; 4% (w:v) CHAPS; 65-mM dithiothreitol; 1-mM EDTA, 1% (v:v) inhibitor protease cocktail) and stored at −80 °C until used. Protein extracts were suspended in 2D rehydratation solution, final volume 125 μL (8-M urea, 2% CHAPS, 20-mM dithiothreitol, 1% immobilized pH gradient (IPG) buffer, and traces of bromophenol blue) before loading the IPG strips (pH 3–10 nonlinear, 7 cm, GE Healthcare, USA]. The strips were rehydrated in a swelling tray overnight at 22 °C to 25 °C. Isoelectric focusing was performed in a EttanIPGphor 3 equipment (GE Healthcare, USA) using the following program: voltage set at 300 V for 200 Vh, raised in a linear gradient first to 1000 V for 300 Vh and then to 5000 V for 4500 Vh, and maintained at this voltage until a total run of 7000 Vh was achieved. Focused strips were equilibrated with 2% DDT and then 2.5% iodoacetamide in equilibrium buffer (6-M urea, 0.375-M Tris-HCl, pH 8.8; 2% sodium dodecyl sulfate; 20% glycerol) at 22 °C to 25 °C for 15 minutes each step. The second dimension was performed in 12% polyacrylamide gels [31]. Proteins were visualized by silver staining [32]. The assay was performed by triplicated. The 2D gels were scanned at 300 dpi and analyzed using PD-Quest software (Bio-Rad, USA). The maps obtained for each treatment were evaluated as a single match set, and a master gel was generated, on the basis of a representative gel. Proteins in key regions of the master gel were used as landmarks, and the matching of spots was achieved after several rounds of extensive comparison. Also, final spot matches were performed by checking each spot in each gel with the respective pattern in the master gel. Protein quantities were reported as parts per million of the total integrated optical density of spots in the gels, according to PD-Quest. Spots were analyzed *in silico* (TagIdent tool from ExPASy Bioinformatics Resource; pI range: ±0.5; Mw range, 10%; TaxID: 9913) to predict putative identities on the basis of Mw and pI.

### 2.5. Statistical analysis

Percentage of polar body extrusion, cleavage rate 48 hours after fertilization, and the proportion of blastocyst and expanded blastocysts at Day 7 after fertilization were analyzed by least-squares analysis of variance, using the general linear models with binomial distribution using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). Cleavage rate evaluated at 30 hours after fertilization and cleaved and noncleaved presumptive zygotes groups were analyzed by least-squares analysis of variance, using the general linear models with binomial distribution using SAS 9.1 software (SAS Institute Inc.). Embryo production rates of different developmental stages (early blastocysts, blastocysts, and expanded blastocysts) in both groups were analyzed by least-squares analysis of variance using the general linear models with Poisson distribution using SAS 9.1 software (SAS Institute Inc.). All values reported are mean ± standard error of the mean of untransformed data overall analyses; all treatment effects that were associated with a probability of 0.05 or less are reported.

## 3. Results

### 3.1. Effect of combined EGF and HA supplementation of the maturation medium on nuclear maturation and in vitro embryo development

The effect of an EGF-HA–based maturation medium on oocyte nuclear maturation and embryo developmental competence was evaluated using a standard maturation medium containing FSH and FBS as the control group. Oocytes matured in the presence of EGF-HA showed an increase ($P < 0.05$) in the percentage of the first polar body extrusion (75.8 ± 4.2%, n = 550), compared to oocytes matured in a standard maturation medium (FSH-FBS:

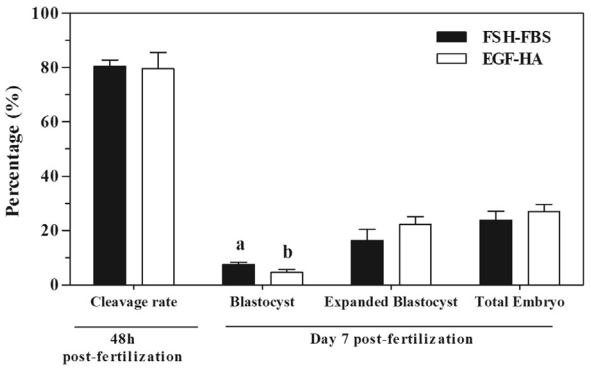



**Fig. 1.** Embryo production of bovine oocytes matured *in vitro* for 22 hours in the presence of EGF combined with hyaluronic acid (HA) or FSH and fetal bovine serum (FBS). Results are shown as percentages ± standard error of the mean from three independent experiments using three wells of approximately 45 to 55 oocytes under each condition. The total number of oocytes used was FSH-FBS: N = 393 and EGF-HA: N = 506. [a,b]Within embryo stage, percentages without a common letter differed ($P < 0.05$).

69.7 ± 6.8%, n = 523). No differences were found in the cleavage rate between both maturation media (Fig. 1). At Day 7, blastocyst rate was 3% higher ($P < 0.05$) in the FSH condition, but no differences were found in the rate of expanded blastocyst neither in total embryo production between maturation conditions. Expanded blastocysts represented 71% of the total embryo production.

### 3.2. Effect of IVM with EGF-HA on in vitro kinetics of bovine embryo development

To assess whether IVM medium composition affects *in vitro* kinetics of embryo development, cleavage rate was evaluated 30 hours after fertilization, and cleaved and noncleaved groups were followed in time until Day 7 at which embryo production rates of different developmental stages were recorded. Cleavage rate of oocytes matured with FSH-FBS was five points higher ($P < 0.05$) in percentage with respect to EGF-HA–matured oocytes (Fig. 2A). At Day 7, those inseminated oocytes that underwent division at a correct timing (cleaved group) showed that although there are still early blastocysts in the FSH-FBS condition, EGF-HA embryos have developed completely into blastocysts and expanded blastocysts ($P > 0.05$; Fig. 2A). On the other hand, noncleaved presumptive zygotes (noncleaved group) at Day 7 developed into early blastocysts, blastocysts, and expanded blastocysts with similar rates (~4%) independently of the medium condition (Fig. 2B).

### 3.3. Effect of combined EGF and HA supplementation of the maturation medium on oocyte proteome

To evaluate the effect of EGF and HA supplementation on oocyte proteins, *in vitro*–matured oocytes were analyzed by 2D gel electrophoresis using a wide-range first dimension. We detected a differential protein profile between treatments, with 109 spots in the FSH-FBS group and 132 spots in the EGF-HA group (Fig. 3A). The comparison of both profiles showed 68 spots present in both treatments, 41 exclusively found in the FSH-FBS group and 64 exclusive for the EGF-HA group (Fig. 3B). Among the spots shared by both treatments, 17 showed differential intensities (≥1.6 fold), most of them reduced in the EGF-HA condition. Differential spots (i.e., present in only one condition or increased/decreased between conditions) were analyzed *in silico* using TagIdent of the Expasy tools to predict putative identities on the basis of Mw and pI. Spots that matched proteins related to oocyte maturation and early development were selected, and the results are summarized in Figure 3C.

## 4. Discussion

Culture conditions, media composition, growth factors, and hormones influence mammalian oocyte IVM and subsequent developmental capacity [33,34]. Successful and reliable oocyte maturation (both cytoplasmic and nuclear maturation) would dramatically improve the efficiency of preimplantation embryonic development and fetal development. Nuclear maturation of mammalian oocytes implies resumption and progression of meiosis to metaphase II with the extrusion of the first polar body. *In vitro*, these events are differentially influenced by the nature of the maturation inducer and the presence of intact cumulus in COCs which is critical for polar body extrusion [35] and meiotic spindle configuration [36]. The physiological function of the expanded cumulus matrix as a molecular filter controlling the supply of soluble metabolites and

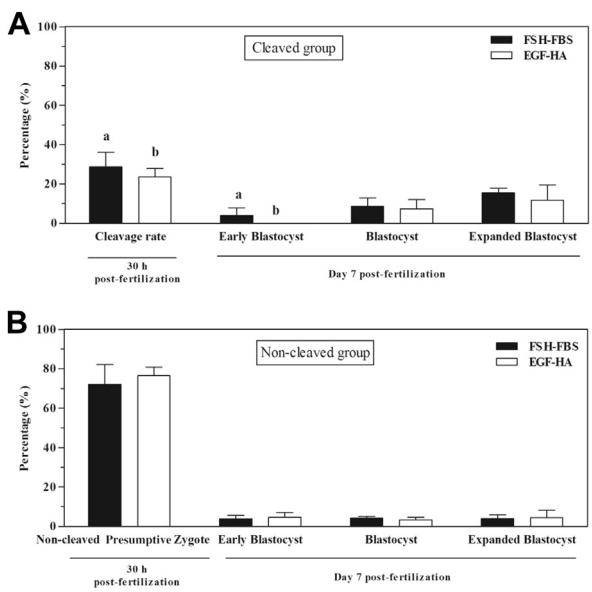
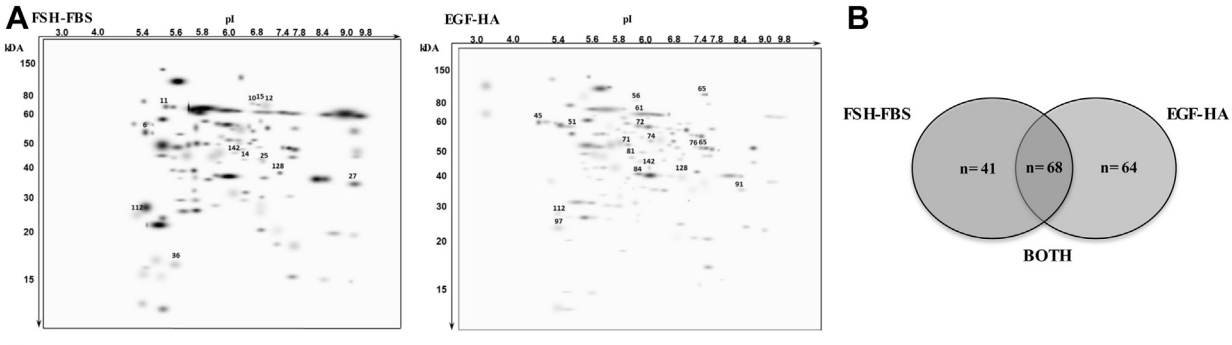



**Fig. 2.** Cleavage rates and embryo production of bovine cleaved zygotes (A) or noncleaved presumptive zygotes (B) at 30 hours after fertilization. Embryos derived from oocytes matured in EGF combined with hyaluronic acid (HA) or FSH and fetal bovine serum (FBS). Results are shown as percentages ± standard error of the mean from four independent experiments using two wells of approximately 40 to 45 oocytes under each condition. The total number of oocytes used was FSH-FBS: N = 376 and EGF-HA: N = 317. [a,b]Within cleavage and embryo stage, percentages without a common letter differed ($P < 0.05$).




C

| Spot number | FSH-FBS | EGF-HA | Fold | Protein name | Mw (kDA) theoret. | pI theoret. | Accession number | Reference |
|---|---|---|---|---|---|---|---|---|
| 6 | x | | | Zinc finger CCCH-type with G patch domain-containing protein | 55 | 5.4 | NP_001019685.2. | [51] |
| 10 | x | | | Solute carrier organic anion transporter family member 3A1 | 75 | 6.7 | NP_001001134.1. | [59] |
| 11 | x | | | Prolactin receptor | 62 | 5.5 | NP_001034815.1. NP_776580.1. | [52] |
| 12 | x | | | Versican core protein | 75 | 6.9 | NP_851378.1. | [21] [48] |
| 14 | x | | | Radical S-adenosyl met.hionine domain- containing protein 2. | 42 | 6.5 | NP_001039406.1 | [49] [50] |
| 15 | x | | | Prostaglandin G/H synthase 1. | 68 | 6.9 | NP_001098793.1. | [46] |
| 25 | x | | | Prostaglandin G/H synthase 2. | 68 | 7.1 | NP_776870.1. | [46] |
| 27 | x | | | Heparanase 50 kDa subunit | 44 | 9.5 | NP_776507.1. | [53] |
| 36 | x | | | Tumor necrosis factor, soluble form. | 18 | 5.55 | NP_776391.2. | [54] |
| 45 | | x | | Disintegrin and metalloproteinase domain 2 | 63 | 5.2 | NP_776653.1. | [55] |
| 51 | | x | | WAP, kazal, immunoglobulin | 60 | 5.45 | NP_001070353.1. | [44] |
| 56 | | x | | Phosphatidylinositol 3-kinase regulatory subunit bet.a | 81 | 5.9 | NP_777001.1. | [43] |
| 61 | | x | | Alpha-fetoprotein | 67 | 5.9 | NP_001029434.1. | [56] |
| 65 | | x | | Plasminogen | 90 | 7.4 | NP_776376.1. | [57] |
| 71 | | x | | Atrial natriuretic peptide receptor 3 Isoforma 1 | 55 | 5.85 | NP_776552.1. | [58] |
| 72 | | x | | Atrial natriuretic peptide receptor 3 Isoforma 2 | 60 | 6 | NP_776552.1. | [58] |
| 74 | | x | | Bile acid receptor | 55 | 6.4 | NP_001029880.1. | [47] |
| 76 | | x | | TGF-beta receptor type-1 | 54 | 7.3 | NP_777046.1. | [42] |
| 81 | | x | | Perilipin-2 | 49 | 5.85 | NP_776405.1. | [60] [61] |
| 84 | | x | | Plasminogen activator inhibitor 1 | 43 | 5.95 | NP_776562.1. | [57] |
| 91 | | x | | Isoform Short of Prolactin receptor | 34 | 8.3 | NP_001034815.1. NP_776580.1. | [62] |
| 97 | | x | | Peroxiredoxin-2 | 22 | 5.4 | NP_777188.1. | [63] [45] |
| 105 | | x | | Activin receptor type-1 | 54 | 7.4 | Q28041 NM_176663.3 | [64] [65] |
| 112 | x | x | 4.1 | EP300-interacting inhibitor of differentiation 2 | 24 | 5.4 | NP_001071517.1. | [66] |
| 128 | x | x | 1.6 | Zona pellucida sperm-binding protein 3. | 36 | 6.9 | NP_776399.1. | [67] [68] |
| 142 | x | x | 5.0 | Sorting nexin-5 | 47 | 6.3 | NP_001029636.1. | [69] |

**Fig. 3.** Differential two-dimensional (2D) map of proteins from oocytes cultured in EGF–hyaluronic acid (HA) and FSH–fetal bovine serum (FBS) media. (A) Master images of 2D gel electrophoresis of oocytes matured in EGF-HA and FSH-FBS media. The position of the molecular mass markers (range, 15–150 kDa) is indicated at the left of each gel, whereas the pI distribution is indicated on the top. Each master gel was performed from three replicates with the same amount of total protein (400 oocytes per group) using Melanie software. Spots present under only one condition and those showing ≥1.6-fold difference between treatments were considered differential proteins. (B) Venn diagram showing the distribution of proteins from MII oocytes cultured in EGF-HA and FSH-FBS media and overlapping proteins present under both conditions. (C) Differential proteins related to oocyte maturation and early development. Scale-fold values refer to FSH-FBS/EGF-HA relation. Spots were analyzed *in silico* (TagIdent tool from ExPASy Bioinformatics Resource; pI range, ±0.5; Mw range, 10%; TaxID: 9913) to predict putative identities on the basis of Mw and pI. Those spots (designated with numbers) that matched proteins related to oocyte maturation and early development were selected. Results are summarized in the embedded table.

concentrating signaling molecules has also been shown to be altered in oocytes matured *in vitro* [23]. In the present study, we observed a positive effect on polar body extrusion of bovine oocytes matured in EGF combined with HA. These results are consistent with Sakaguchi et al. [37], who found that growth factors such as EGF and IGF-I increased the frequency of the first polar body extrusion of oocytes derived from small follicles and mitogen-activated protein kinase activity. However, these growth factors did not affect the timing of polar body extrusion of denuded oocytes, indicating that the mechanism involved is likely mediated by the enclosing *cumulus* cells [38]. On the other hand, HA is considered the main component of the extracellular matrix required not only for cumulus cell expansion but also for oocyte nuclear maturation and further embryo development [5]. In fact, IVM media supplementation with increasing concentrations of HA in combination with hormones as maturation inducers did not affect bovine cumulus cell expansion [5]. In addition, oocyte nuclear maturation was not affected by lower concentrations of HA (0.1 and 0.5 mg/mL) but significantly decreased percentage of oocytes at MII with high HA concentrations (1 mg/mL). It has been shown that EGF signal significantly increases the expression of HA synthase 2 in cumulus cells over FSH [22], thus decreasing the requirements of exogenous HA *in vitro*. The binding of HA to its receptor CD44 promotes multiple intracellular signals *via* cytoskeletal changes that produce transactivation of the EGF receptor and activation of extracellular signal-regulated kinase (ERK) [39]. In this study, because of their synergism as factors involved in signaling mechanisms of oocyte maturation, both EGF and HA could have contributed on COC matrix production and oocyte nuclear maturation. Likewise, cumulus cells constitute an essential requirement for IVM of oocytes that cannot be bypassed even in the presence of EGF and/or FSH [8]. Although EGF and FSH act through a different signal transduction pathway, dependent on tyrosine kinase or cAMP, both activate mitogen-activated protein kinase,

specifically ERK1/2, in cumulus cells [40]. Activation of EGF receptor signaling in cumulus cells triggers oocyte nuclear maturation and acquisition of developmental competence [41]. Our results show that oocytes matured in EGF-HA may have undergone a slight delay of cleavage timing, with no effect on their competence. In addition, EGF-HA embryos developed completely into blastocysts without presenting early blastocysts, thus showing a better developmental timing with respect to FSH-FBS embryos. Recently, morphokinetic parameters have gained notoriety because of their potential as predictors to select embryos with full capacity to implant [42,43]. Therefore, synchronicity of embryonic development provides a criterion for selection of viable and better-quality embryos. In bovines, expression of IGF-I was found in all two-cell embryos that cleaved at 27 and 30 hours after fertilization, but no expression of this gene related to embryo preimplantation development was found in those which cleaved after 36 hours [44]. The maturation status of oocyte cytoplasm plays a major role in reprogramming of gene expression at the onset of embryonic development. Successful embryonic genome activation during embryogenesis is a prerequisite for further development, and thus, oocyte maturation impacts on both preimplantation and postimplantation development [45]. Here, we found that protein profile expression is modified with the medium composition. Oocytes matured with EGF and HA expressed several proteins related with growth and differentiation factors [46–48] and others related to the extracellular matrix, as the peroxiredoxin 2 which has been proposed as an oocyte quality marker [49]. On the other hand, oocytes matured with FSH-FBS expressed proteins related to prostaglandin metabolism such as two types of prostaglandin E synthase [50], and the organic anion transporter OATP-D, central in the translocation of prostaglandins [51]. In addition, Versican core protein found in FSH-FBS group has been proposed as a biomarker of pregnancy by Gebhardt et al. [52]. Radical S-adenosyl methionine domain containing protein 2 also found in FSH-FBS–matured oocytes has been identified as a novel interferon *tau* and progesterone regulated factor [53,54]. According to our knowledge, this is the first study about the impact of culture media conditions on bovine oocyte proteomic profiles analyzed by 2D electrophoresis. Through this approach, we have shown that modifications of IVM medium composition markedly affected protein profile of oocytes in a differential manner. Therefore, our results provide new insights regarding physiology of oocyte maturation and highlight the functional importance of *in vitro* culture conditions.

### 4.1. Conclusions

The present study provides evidence on the effect of combined EGF-HA supplementation of IVM medium on bovine oocyte meiotic competence, developmental competence, and protein profile. Bovine oocytes matured in the presence of EGF-HA improved nuclear maturation. Oocytes matured under these conditions developed completely into blastocysts and showed a differential protein profile. Taken together, findings from our study indicate that EGF-HA supplementation of IVM medium could be used to improve oocyte meiotic competence and ensure a better timing to develop into the blastocyst stage. Future studies will determine the influence and contribution of IVM in the presence of EGF-HA on the quality of the embryos and on cryosurvival and pregnancy rates.

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Author contributions: G.L. Ríos, J. Buschiazzo, N.C. Mucci, G.G. Kaiser, A. Cesari, and R.H. Alberio were responsible for the experimental design and also actively participated on the experimental procedures, analysis, interpretation of data, and on the preparation and revision of the manuscript.

## Competing interests

The authors declare that there is no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

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