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Research Report

Effects of fatty acid binding protein in the maturation media of bovine oocytes on in vitro embryo development

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Abstract

Oocytes matured in vitro have higher lipid content and fatty acid binding protein3 (FABP3) may mediate the inter or intracellular transport of lipids and fatty acids. Our aim was to investigate the effects of varying concentrations of FABP3 during in vitro maturation on blastocyst development of oocytes. In Experiment 1, oocytes from abattoir-derived ovaries were matured in media supplemented with 1, 10 or 20 or 0 μg/ml FABP3 (control). In Experiments 2 and 3, oocytes collected via transvaginal aspiration from mature nonlactating Jersey cows (425-500 kg) were matured in media at concentrations with 10 or 20 and 0 μg/ml FABP3 (control; Experiment 2), 1 or 5 and 0 μg/ml, FABP3 (control; Experiment 3). After fertilization and 8 days of culture, cleavage and blastocyst rates were analysed. In Experiment 1, cleavage rate was higher ($p < 0.05$) in 20 μg/ml group compared to control and 10 μg/ml FABP3 groups. In Experiment 2, cleavage rate was higher ($p < 0.05$) in control and 10 μg/ml groups compared to 20 μg/ml group and blastocyst development was higher ($p < 0.05$) in control compared to 20 μg/ml group. In Experiment 3, cleavage and blastocyst development was higher ($p < 0.05$) in the control group compared to 5 μg/ml group. There was no clear benefit of FABP3 demonstrated among tested concentrations; however, 20 μg/ml during in vitro maturation appeared to be detrimental to cleavage and embryo development in oocytes aspirated from live cows.

Keywords: Fatty acid binding protein, in vitro maturation, bovine embryo development

Introduction

Cattle production has greatly benefitted from assisted reproductive technologies (ART) that enhance the dissemination of superior genetics, reduce generation intervals, and improve productivity, ultimately increasing the efficacy of production systems. These are crucial for meeting the rising global demand for livestock products while also supporting profitability and sustainability of the environment. Among ART, in vitro embryo production (IVP) has been the most impactful in the cattle industry. Worldwide IVP embryos made up 79.7% of all transferrable cattle embryos in 2021.[1] Despite its widespread adoption and potential to address genetic and financial challenges for producers, IVP still faces limitations, particularly in blastocyst development rates. In cattle, only about 20-40% of cultured presumptive zygotes will reach the blastocyst stage,[2] highlighting the need for further optimization of embryo in vitro culture conditions. This broad variation is due to a variety of factors (e.g. follicular profile, health status, endocrine dynamics, possibly stress [heat or handling]). Abnormal development and decreased efficiency is common in ART due to the oocyte source and exposure of gametes and embryos to nonphysiological in vitro conditions.[3] Despite recent advances in IVP biotechniques, the required processes of in vitro maturation, fertilization, and embryo culture remain a source of stress on gametes and embryos that contributed to low and variable embryo development.[4] Embryos derived by IVP differ from their in vivo counterparts in terms of morphological, molecular, and metabolic characteristics.[5] One of the most recognized metabolic abnormalities is increased lipid accumulation during IVP. Although lipids are essential for various cellular functions (e.g. membrane composition, energy storage, cell signalling, and as an energy substrate) excessive lipid accumulation in IVP embryos is associated with reduced embryo quality and poor cryotolerance.[5]

Maturation of oocytes is crucial. the first step of IVP, and requires meiotic and cytoplasmic changes to be completed at the proper timing and in the correct sequence. Ultimately, in vitro maturation bypasses the biological process of selecting follicles destined to ovulate. Lipid accumulation in bovine and mouse embryos occurs during in vitro maturation[6,7] but not during in vivo maturation.[7] Fatty acid binding protein is believed to mediate the transport of lipids and fatty acids

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Citation: Clinical Theriogenology 2026, 18, 13028, http://dx.doi.org/10.58292/CT.v18.13028

between oocytes and cumulous cells via transzonal projections. Fatty acids and their derivatives have several important roles in the cell, including serving as energy sources, contributing to membrane synthesis, and acting as signalling molecules in metabolic pathways.[8] Because fatty acids are hydrophobic, they require transport proteins to move efficiently within the cell. Fatty acid binding proteins are a group of intracellular chaperones that bind fatty acids with high affinity and facilitate their transport to various organelles (e.g. mitochondria, endoplasmic reticulum, nucleus).[8] These proteins are highly expressed in tissues with active fatty acid metabolism and exist in multiple isoforms. Based on results (unpublished data), fatty acid binding protein3 (FABP3) may have a role in effective maturation and development by facilitating lipid movement within the cells and across the plasma membrane; FABP3 was higher in embryos that reached the blastocyst stage compared to those that were arrested at the 8-16 cell stage, from the same culture. Our aim was to investigate the developmental dynamics (cleavage and blastocyst formation) of bovine oocytes of multiple sources matured with varying concentrations of FABP3 supplemented in the maturation media.

Materials and methods

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee (2022-1162), School of Veterinary Medicine, Texas Tech University.

Oocyte collection

Three experiments were conducted in replicate using oocytes obtained either from abattoir-derived ovaries (Experiment 1) or via transvaginal follicle aspiration of Jersey cows (Experiments 2 and 3). For Experiment 1, ovaries from an abattoir (Caviness Beef Packers, Hereford, TX, USA) were transported within 1 hour to the laboratory at room temperature suspended in physiological saline solution. Cumulus oocyte complexes were aspirated from 2-8 mm follicles using an 18 gauge needle and a vacuum pump (flow rate = 18-20 ml/minute) at a pressure of 100 mm Hg. For Experiment 2 and 3, oocytes were collected from reproductively sound, mature, nonlactating Jersey cows (425-500 kg) via transvaginal ultrasound-guided follicular aspiration. Cows were first given a luteolytic dose of prostaglandin $F_{2\alpha}$ and a progesterone device was placed to manage the estrous cycle.

Cows were restrained in a head lock and an epidural (5 ml of 2% lidocaine) anaesthesia was given. The vulva and perineal region was washed and disinfected and the transvaginal ultrasound probe was introduced. Each ovary was located by transrectal palpation and was retracted to the head of the ultrasound transducer. All accessible follicles > 3 mm in diameter were aspirated. A disposable 20 gauge needle was attached to silicone tubing and an aspiration pump was used to create a vacuum pressure of 65 mm Hg. After puncturing several follicles, the system was rinsed with commercial ovum pick-up media (Stroebech Media; Hundested, Denmark) kept at a temperature of 36°C. After recovery, the contents and OPU medium were filtered through an EZ way filter. Cumulous oocyte complexes were located under a stereomicroscope and packaged for transport (20 minutes) to the laboratory for placement into in vitro maturation media. Cows were aspirated every 2-5 days for 5 (Experiment 2) or 8 (Experiment 3) collection sessions.

All in vitro procedures (maturation, fertilization, culture) were conducted under an oil overlay at 38.5°C in a fully humidified (5% CO_2, 5% O_2, 90% nitrogen) environment.

In vitro maturation

All wash, maturation, fertilization, and culture media were from Stroebech Media and FABP3 (Part Number RP-75543) was from Invitrogen (Waltham, Massachusetts, USA). Selected oocytes with compact cumulus cells and homogeneous cytoplasm were washed twice in wash media. Groups of 10-15 cumulus oocyte complexes were placed in 50 µl drops of maturation media and incubated for 18-20 hours. In Experiment 1, oocytes were randomly allocated into 1 of 4 treatment groups: control (without FABP3) and 1, 10 or 20 µg/ml FABP3. In Experiment 2, oocytes were randomly allocated into 1 of 3 treatment groups: control (without FABP3) and either 10 or 20 µg/ml FABP3. In Experiment 3, oocytes were randomly allocated into 1 of 3 treatment groups: control (without FABP3) and either 1 or 5 µg/ml FABP3.

Sperm preparation and in vitro fertilization

Matured oocytes were washed twice in wash media and once in fertilization media. They were then placed in groups of 10-50 oocytes per well in 4 well plates containing 500 µl of fertilization media per well with a 400 µl oil overlay. Sperm from 1 bull were obtained by centrifugation of frozen-thawed semen. Prior to use in these experiments, the selected bull semen was examined for motility, morphology, and concentration for in vitro fertilization. BoviPure solution (BP-100; Nidacon; Mölndal, Sweden) was diluted with BoviDilute (BD-100; Nidacon) to obtain a bottom layer (2 ml) of 80% BoviPure overlaid with a top layer (2 ml) medium at 40%. Frozen semen straws were placed in a water bath at 37°C for 30 seconds and semen was gently layered on top the 40% BoviPure layer. Columns were centrifuged at 1,507 x g for 13 minutes. After centrifugation, fluid above the sperm pellet was carefully removed and a 7 µl sperm pellet (from preliminary data) was added to each fertilization well (~ 10^5 sperm/ml final concentration). Oocytes were coincubated with semen in fertilization wells for 18-20 hours.

In vitro culture

After fertilization, cumulus cells were removed from presumptive zygotes by vortexing in 250 µl of wash media for 2 minutes and 25 seconds or denuding gently with a pipette. The presumptive zygotes were randomly placed by treatment group into 50 µl culture drops on a 60 x 15 mm cell culture dish under a 10 ml oil overlay; 369 presumptive zygotes were cultured for Experiment 1, 234 for Experiment 2, and 304 for Experiment 3. Presumptive zygotes were incubated for 7-8 days of culture, and then cleavage (≥ 2 cells) and blastocyst rates were evaluated.

Data analysis

Contingency tables were constructed for cleavage and blastocyst development outcomes. Cleavage and development to blastocysts were evaluated on days 7 or 8 of culture. Embryo grown alone was the observational unit with a binary outcome recorded (achieved developmental stage or not). Data were analysed via Chi-square. Pairwise comparison was performed using a 2 proportion z-tests. Significance level was set at $p < 0.05$. All analyses were performed on R Statistical Software, R version 4.2.1 (R Core Team 2022).

Results

In Experiment 1, oocytes from abattoir-derived ovaries had a cleavage rate that was higher ($p < 0.05$) for 20 µg/ml (74.1% ± 6.0) group compared to control or 10 µg/ml (55.9% ± 5.1, 51.7% ± 4.6; respectively) group. There was no significant difference between 20 and 1 µg/ml (63.7% ± 4.8), 10 and 1 mg/ml, control and 1 µg/ml, or 10 µg/ml and control. There was no difference in blastocyst development among groups (16.7% ± 3.7, 17.5% ± 3.5, 22.2% ± 5.7, and 21.5% ± 4.3 for 1, 10, 20, and control groups).

In Experiment 2, oocytes from live cows had a cleavage rate that was significantly higher in the control and 10 µg/ml group (75.0% ± 5.0; 64.6% ± 5.4; respectively) compared to the 20 µg/ml group (32.9% ± 5.3). There was no significant difference between 10 µg/ml and control. Blastocyst development was significantly higher in the control (34.2% ± 5.4) compared to 20 (10.1% ± 3.4). No significant difference was detected between 20 and 10 (20.2% ± 4.5) but control tended ($p = 0.07$) to be higher than the 10 µg/ml group.

In Experiment 3, oocytes from live cows had a cleavage rate that was higher ($p < 0.05$) in the control group (78.0% ± 4.0) compared to the 5 µg/ml group (63.4% ± 4.8). The group of 1 µg/ml (75.5% ± 4.4) was not different from either group. Likewise, blastocyst development was significantly higher in control (29.4% ± 4.4) compared to 5 µg/ml (15.8% ± 3.6) but 1 µg/ml (22.3% ± 4.3) was not different from either group. Results are summarized in Table.

Discussion

We utilized in vitro maturation media supplemented with and without FABP3 as an intervention to regulate lipid accumulation. In vitro maturation is the first crucial step in in vitro embryo production that sets up the female gamete for success in embryo development. In cattle, lipid accumulation primarily occurs during in vitro maturation of oocytes but not during in vivo oocyte maturation.[3,7,9] Exact mechanisms by which IVP embryos accumulate more lipids are unclear[5] but lipids in the maturation media may be a source.[7] Compelling evidence for bovine embryos suggested a correlation between lipid-rich culture media and high lipid content, and low cryotolerance of blastocysts.[10,11] Mouse oocytes exposed to lipid-rich follicular fluid from woman of known body mass index during their maturation had increased oocyte lipid content, induction of endoplasmic reticulum stress markers, and impaired oocyte nuclear maturation.[12] Because increased body mass index is associated with elevated triglycerides and free fatty acids in ovarian follicular fluid, oocytes suffer as maturation within lipid-rich environments is detrimental to oocytes.[12] Lipid accumulation was accentuated when bovine oocytes were matured in fetal bovine serum.[7] Many laboratories have moved away from fetal bovine serum due to its undefined composition and high batch to batch variability that contributed to inconsistent outcomes and is suspected to contribute to large offspring syndrome.[13] Serum was not supplemented in any of our 3 experiments (we used serum-free media). Therefore, alternative mechanisms for lipid accumulation must be explored. Oocytes of mammalian species have various lipid contents in absolute terms and in relation to the ooplasm.[14] This may be due to differences in energy metabolism among species; therefore, conclusions about lipid management cannot be applied directly to other species. Oocytes derived from cows contain ~ 5.69×10^{-5} ng/µm^3 fatty acids[15] with almost 50% (~ 2.3×10^{-5} ng/µm^3) stored in the form of lipid droplets.[14] Because of lipid droplets, the oocytes from cows, pigs, and sheep appear darker than those from mice and humans.[14] *Bos taurus* cows naturally have greater lipid accumulation in IVF-derived embryos than *Bos indicus*, and the former still has greater cryotolerance.[16]

Experiments 2 and 3 utilized mature, nonlactating Jersey cows, as their oocytes and embryos are more naturally lipid-rich compared to other *Bos taurus* breeds.[17] Lipid content of in vivo embryos of Jersey cows and beef cows were compared.[18] Embryo weight was correlated with lipid content; beef cattle embryos were 20-27% heavier than Jersey cow embryos, a difference explained by significantly higher lipid content in Jersey cow embryos (~ 36%) compared to beef cattle embryos (~ 8%).[18] As Experiment 1 utilized abattoir-derived oocytes

Table. Effects of FABP3 supplementation during in vitro maturation on cleavage and blastocyst development of oocytes collected from an abattoir or Jersey cows via ultrasound-guided transvaginal follicular aspiration

Experiment	FABP3 supplementation	Number of embryos cultured	Percent cleaved ± SEM	Percent blastocysts ± SEM
1*	Control	93	55.9^{b} ± 5.1	21.5 ± 4.3
	1 µg/ml	102	63.1^{a,b} ± 4.8	16.7 ± 3.7
	10 µg/ml	120	51.7^{b} ± 4.6	17.5 ± 3.7
	20 µg/ml	54	74.1^{a} ± 6.0	22.2 ± 5.7
2#	Control	76	75.0^{a} ± 5.0	34.2^{a} ± 5.4
	10 µg/ml	79	64.6^{a,b} ± 5.4	20.2^{a} ± 4.5
	20 µg/ml	79	32.9^{b} ± 5.3	10.1^{b} ± 3.4
3#	Control	109	78.0^{a} ± 4.0	29.4^{a} ± 4.4
	1 µg/ml	94	75.5^{a,b} ± 4.4	22.3^{a,b} ± 4.3
	5 µg/ml	101	63.4^{b} ± 4.8	15.8^{b} ± 3.6

*abattoir-derived oocytes, #aspirated oocytes (2# = spring, 3# = fall)

^{a,b}within a column and experiment, means without a common superscript differed ($p < 0.05$)

ovaries; without information on the donors of these oocytes, conclusions about breed differences cannot be made. A difference was detected between abattoir-derived oocytes and Jersey cow oocytes potentially because of lipid content or because the ova were recovered from a known source and stage of a follicular wave.

It is of great importance to identify oocytes that are morphologically normal; ooplasm can appear in various patterns and color that can be used as a marker for superior embryo development.[4] Our study utilized a selection criterion of oocytes with brown homogeneous ooplasm, a black periphery, and compact multiple layers of cumulous cells. These morphological patterns are considered developmentally fit and have good developmental potential, along with high density of organelles, high polar body extrusion rates, and intermediate levels of ATP indicating appropriate metabolism.[4]

As oocytes go through maturation and embryos go through differentiation, they undergo major changes in how their internal structures are organized that requires a substantial amount of energy. Oocytes are well-equipped to meet these energy demands because they contain many mitochondria and store energy as lipid droplets. Still, metabolic control is very complex and involves interactions among the follicular fluid, cumulus cells, and the oocyte itself. Energy reserves are of course important, but lipid and energy regulation is also critical and difficult to measure. For example, mitochondria function and movement within the cytoplasm influences how lipid droplets are used are for energy. All these processes rely on a wide range of cellular and molecular mechanisms, each having various energy demands. To support the embryo's potential to develop properly, these processes must be carefully timed and coordinated.[4]

One of the negative impacts of in vitro maturation is asynchronous nuclear and cytoplasmic maturation.[4] Nuclear maturation is easy to evaluate by the appearance of first polar body under a microscope; however, degrees of cytoplasmic and epigenetic maturation are complex and difficult to evaluate. Some research groups have proposed a prematuration step is needed to provide more time for adequate cytoplasmic maturation.

FABP3 was chosen as the intervention in this study because preliminary data (after matrix assisted laser desorption/ionization time of flight analysis) indicated that FABP3 was abundant in embryos that reached the blastocyst stage but not in cohort embryos that arrested at the 8-16 cell stage (unpublished data). Used media, 8-16 cell, and blastocyst embryos were all taken from the same 50 µl culture environments and the fatty acid binding protein (heart subtype 3) was present in the culture media and to a higher degree in the embryos that survived to the blastocyst stage versus those from the same culture that arrested at the 8-16 cell stage of development. It was proposed that excessive lipid accumulation in in vitro-matured oocytes is mediated by the dysregulated transport of FABP3 through transzonal projections between cumulus and oocytes, thus implicating the in vitro maturation process.[3] That study suggested that elevated FABP3 protein concentrations in cumulus cells during in vitro maturation, was associated with elevated lipid accumulation in oocytes.[3] Through comparing immature oocytes with those matured for 9 and 18 hours, they were able to track dynamic changes in FABP3 protein concentrations and lipid concentrations in oocytes during the maturation process. Additionally, disruption of transzonal projections during the first 9 hours of maturation decreased lipid accumulation.[3]

In this study, supplementation with 20 or 5 µg/ml of FABP3 significantly impaired both cleavage and blastocyst development compared to control for oocytes collected from Jersey cows. Although FABP3 supplementation in abattoir-derived oocytes improved cleavage rates, it did not affect blastocyst development. Embryo cleavage has not been a useful estimator of embryo development to blastocysts.[19] Perhaps the arrest at the 8-16 cell stage is indicative of an embryo undergoing apoptosis and though the embryo may cleave a few times, there is limited potential for further development. Further investigation is needed to evaluate the role of lipids in the cleavage or blastocyst development process.

Although not an aim of the experiment, season may have an impact on oocyte quality and developmental potential.[20] In this study, oocytes were collected from a similar group of Jersey cows in spring (Experiment 2) and fall (Experiment 3). Seasonal effects may have impacted the quantity and quality of oocytes, with autumn being favourable for in vitro embryo development.[20] Although Experiments 2 and 3 were not directly compared, blastocyst development for the control group in Experiment 3 (autumn) was numerically higher that the control for Experiment 2 (spring). Heat stress has a direct effect on lipid and energy metabolism systemically in the cow.[21] Elevated temperatures also directly affected the quality of oocytes.[22] However there was not a large difference in average temperature between spring and autumn; according to the National Weather Service, the average temperature in Amarillo, TX where the cows were kept was 68.5 °F in May and 66.7 °F in October.

Our study had several limitations that should be considered when interpreting the results. Some oocytes were abattoir-driven, limiting knowledge of donor-specific variables (e.g. age, health, reproductive status). These factors are known to affect oocyte quality and developmental potential, although oocytes were distributed among treatment groups randomly to mitigate selection bias. Cleavage and blastocyst formation were used as the primary indicators of developmental success and alone may not be sufficient to evaluate the impact of FABP3. These outcomes are influenced by multiple processes that occur between oocyte maturation and blastocyst formation. Since the experimental treatments targeted oocyte maturation, when the transzonal projections are present, the long interval and multiple procedural steps between maturation and final embryo assessment may have promoted confusing results. Thus, in this experiment, the observed differences in blastocyst development appear to be random and uninfluenced by FABP3 treatment. Future studies may involve some sort of assessment immediately following in vitro maturation or alteration of the cumulous cell population to perhaps better understand the effects of FABP3 supplementation. To minimize variation, a single bull was used across all experiments, acknowledging that sire variability can significantly affect blastocyst development.[23] Additionally, external factors (e.g. light exposure, temperature fluctuations, handling time during maturation, fertilization, culture) may adversely affect embryo development.[24] Furthermore, our study did not directly assess lipid content in the oocyte, which is complicated, but may have offered a more direct evaluation of lipid mobilization and the effects of treatment on oocyte competence. Finally, embryos were not transferred to recipients to establish pregnancy rates due to a lack of available recipients that limits the ability to assess subtle treatment effects on developmental potential. Future studies should incorporate more direct measures of oocyte maturation and quality and lipid content of the oocytes and maturation media to strengthen conclusions regarding the effectiveness of maturation media modifications and perhaps a more defined role for FAPB3. Lipidomic profiling will provide deeper insight into lipid regulation mechanisms in vitro and the impact of FABP3 supplementation.

In conclusion, FABP3 supplementation in maturation media produced inconsistent effects on cleavage and blastocyst development, with no clear benefit observed across the tested range. In Experiment 1, using abattoir-derived oocytes, 20 µg/ml FABP3 significantly increased cleavage rates compared to control and 10 µg/ml groups, but blastocyst rates remained unaffected. In contrast, Experiments 2 and 3, using oocytes obtained via transvaginal aspiration in higher doses (5 or 20 µg/ml) were associated with reduced cleavage and blastocyst development. Notably, 1, 5, and 10 µg/ml FABP3 had no negative effect and was comparable to controls in all experiments; however, 20 µg/ml resulted in reduced cleavage and blastocysts development in ova from Jersey cows, suggesting a somewhat toxic dose of FABP3 via an unknown mechanism leading to at least 2 hypotheses. Either, the FABP3 at high doses remains in the maturation media and removes lipid from the oocyte to the extent that metabolic activity is impaired (perhaps due to limited energy sources) or FABP3 passes through the transzonal projections and sequesters excessive lipid within the oocyte, thus limiting developmental potential. Lipids are essential for oocyte maturation and early development, but excessive accumulation can lead to oxidative stress and metabolic dysfunction; therefore, precise regulation is critical. Further research is needed to evaluate lipid regulation by FABP3 (or other substances) and the role(s) that lipids have in IVP to optimize the maturation environment and enhance embryo viability.

Authors' contribution and agreement

JL: methodology, investigation, validation, data curation, writing original draft; **SH:** methodology, investigation, data curation and **JG:** conceptualization funding, methodology, supervision, project administration, formal analysis, reviewing, editing.

Authors have read and approved final submission.

Conflict of interest

None to report.

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