Research Report

Effects of bovine ova density and culture supplements on cleavage and blastocyst development rates of in vitro embryos

Jessica Looman,[a] Zowie Rodriguez,[b] Lyric Waugh,[a] Sydney Hickerson,[a] John Gibbons[a]
[a]Texas Tech School of Veterinary Medicine, Amarillo, TX, USA
[b]Eastern New Mexico University, Portales, NM, USA

Abstract

An optimized in vitro culture system is important to maximize bovine blastocyst development. Four experiments were conducted to explore manipulation of culture environments and investigate the benefits of culturing embryos in groups. Follicles were aspirated from abattoir-derived ovaries and selected oocytes were matured, fertilized, vortexed, and randomly placed in culture groups. Experiment 1 evaluated the effects of ova culture density on blastocyst development among embryos cultured individually or in groups of 5, 10, 20, or 50 embryos. Experiment 2 evaluated the effects of conditioned media from previous replicates on embryo development of the current culture. In Experiment 3, cleaved embryos were grouped together after being in culture for 24 hours. Lastly, in Experiment 4, grouped embryos were placed in 10 µl culture drops to evaluate the effect of high density/low media volume on development. Cleavage and blastocyst rates analyzed via Chi-square indicated that although cleavage rates were similar among culture groups (and experiments), blastocyst development was lower ($p < 0.05$) in 1 embryo culture group compared to other groups. When conditioned culture media from previous replicates were added to original culture media, blastocyst development was similar among original and conditioned culture groups of 20 and 50 embryos. When cleaved embryos were amalgamated and cultured, blastocyst development was higher ($p < 0.05$) in culture groups of 10 than 1 cleaved embryo groups but similar to controls for both culture groups. When embryos were cultured in 10 µl drops, embryos cultured in groups of 5 had lower development to blastocysts compared to groups of 2, 10, 25, and control. In conclusion, these data indicated an apparent 'helper effect' expressed in culture environments of groups, and this effect apparently occurred after cleavage but before blastocyst development. Direct or indirect role(s) that additional cells have on in vitro culture and the mechanism of this helper effect requires further investigation.

Keywords: Single embryo culture, group embryo culture, cleavage, blastocyst

Introduction

In vitro fertilization (IVF) is an important tool in the cattle industry. It enables producers to potentially improve reproductive performance, efficiency, and genetic gains while maintaining their financial goals. According to 2020 data reported by practitioners in the International Embryo Technology Society, USA in vitro embryo production surpassed the number of in vivo produced embryos from multiple ovulation embryo transfer in 2016.[1] With the embrace of this technology by the industry, comes a necessity to improve and advance the techniques to increase blastocyst developmental rates. Achievement of high maturation and cleavage rates but low development to blastocysts could be due to incomplete cytoplasmic oocyte maturation and inadequate culture conditions, leading to impaired embryonic genome activation, or DNA fragmentation.[1]

Embryo culture is an important component in IVF and suboptimal conditions can compromise embryo development. Absence of maternal signaling cues in in vitro media leads to metabolic dysfunction for embryos.[2] With increasing demand for ATP for expansion and proliferation, it is vital for embryos to absorb available nutrients and avoid potential toxins from external environment.[2] Still, there is much work to be done to optimize culture systems for bovine embryos. A positive relationship was observed among bovine embryos cultured together in groups improving development rates.[3–7] Murine embryos cultured in groups also performed better than

CONTACT Jessica Looman **jlooman@ttu.edu**

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embryos cultured alone[8–11] and cooperative effect has also been witnessed in human in vitro produced embryos.[12–15] However, interactions of embryos among each other are not fully understood. Growth factors secreted by embryos were suggested to act in an autocrine fashion to stimulate development of other embryos in the same culture. Embryonic microRNA also were observed in the successful culture media of bovine embryos further suggesting that beneficial factors are shared among embryos in the same environment.[16] Others have concern that embryos cultured in groups may be exposed to negative effects of dying or delayed embryos and favor 1 embryo culture.[17] However, it was speculated that in 1 embryo culture, beneficial factors would be too dilute to be effective and may need to be supplemented into the media.[8]

Embryo density of a culture environment may affect the impacts of autocrine or paracrine interactions between embryos. A universally accepted ideal embryo density has not been established.[18] Embryo density is dependent on the culture drop volume and the number of cultured embryos; alteration of either can change embryo density. Utilization of the culture media and its effectiveness is affected by embryo density; nutrients in the culture media are absorbed and utilized by embryos. Toxic metabolites and secreted waste accumulate in the culture environment; however, various growth-promoting factors are secreted by embryos. Appropriate balance between having sufficient nutrients for embryos, reducing concentrations of toxic metabolites, and having the appropriate dilution and uptake to benefit from secreted growth-promoting factors remains elusive.[18]

Practically, in many cases number of ova that can be grouped together is dependent on the recovery from the female donor or sire selection. It is challenging when embryos need to maintain their identity and therefore cannot be pooled together with other embryos. In order to improve 1 embryo culture development, a better understanding of embryo interactions should be evaluated. Four experiments were conducted to further elucidate how group culture and supplements benefit embryo development. Specific aspects of group culture were spent embryo media, embryo density, and timing of the helper effect. At present, there is limited research on reutilization of spent media in the new media for embryo culture. Furthermore, previous culture density studies had mixed results. Objectives were to: 1. determine the optimal number of presumptive embryos per culture environment, 2. evaluate effects of conditioned media on blastocyst development, 3. investigate the stage of development at which the effect impacts blastocyst development and determine the effect of noncleaved embryos on developing embryos, and 4. examine the effect of embryo density on embryo cleavage and development.

Materials and methods

Oocyte collection and maturation

All wash, maturation, fertilization, and culture media were supplied by IVF Limited T/A IVF Bioscience (Cornwall, UK). Bovine ovaries were obtained from abattoir (Caviness Beef Packers, Hereford, TX) and transported within 1 hour to 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 (18-20 ml/minute) at a pressure of 100 mm Hg. Selected oocytes with compact cumulus cells and homogeneous cytoplasm were washed twice in wash media. Groups of 10 cumulus-oocyte-complexes were placed in 50 µl drops of maturation media and incubated for 18-20 hours under a 10 ml oil overlay at 38.5 °C in a fully humidified (5% CO_2, 5% O_2, and 90% nitrogen) environment.

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 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 fully characterized for motility, morphology, and concentration for in vitro fertilization. BoviPure (BP-100) and BoviDilute (BD-100) were obtained from Nicadon Laboratory (Nidacon, Mölndal, Sweden). BoviPure solution was diluted with BoviDilute solution to obtain a bottom layer medium at 80% concentration and top layer medium at 40%. BoviPure density gradient centrifugation columns were prepared in 15 ml conical tubes with 2 ml of 40% media layered over 2 ml of 80% media. Frozen semen straws were placed in a water bath at 37 °C for 30 seconds and semen was gently layered on top of BoviPure top media. Columns were centrifuged at 1,507 x g for 13 minutes. After centrifugation, fluid above the sperm pellet was carefully removed and 7 µl sperm pellet was added to each fertilization well (~ 500,000 sperm/ml final concentration). Oocytes were co-incubated with semen in fertilizations well for 18-20 hours at 38.5 °C in a fully humidified (5% CO2, 5% O2, 90% nitrogen) environment.

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. Presumptive zygotes were randomly allocated to each group and placed in culture drops on a 60 x 15 mm cell culture dish under a 10 ml oil overlay. In Experiment 1, to examine the effect of number of embryos per 50 µl drop of culture media, presumptive zygotes were randomly allocated to groups of 1, 5, 10, 20, or 50 presumptive zygotes per 50 µl drop of culture media. Experiment 1 was replicated thrice using oocytes matured on the same day to remove any batch effect of oocytes. In Experiment 2, to examine the effect of conditioned media, 25 µl of conditioned media was removed from previous replicates and added to 25 µl of fresh culture media to create a 50% concentrated 50 µl drop of conditioned media. Conditioned media was pipetted directly out of a previous 7-day old culture drop and directly deposited into a drop of fresh culture media. Presumptive zygotes were than randomly allocated to control or conditioned culture media drops in groups of 20 and 50 embryos per drop. Experiment 2 was replicated twice using oocytes matured on the same day for each replicate. In Experiment 3, to examine the effect of cleaved and noncleaved embryos in groups, presumptive zygotes were placed in culture in groups of 10. After 24 hours, embryos were evaluated and embryos that had cleaved were amalgamated into new groups of 10 or 1. In Experiment 4, to create a high-density culture environment, embryos were place in 10 µl culture drops in groups of 2, 5, 10, and 25 embryos per drop. A Control group of 10 embryos in 50 µl drops were also included. Experiment 4 was replicated 6 times using oocytes matured on the same day for each replicate.

Data analyses

Contingency tables were constructed for cleavage outcomes for Experiment 1 and 4 and blastocyst development outcomes

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for Experiments 1-4. Cleavage and development to blastocysts were evaluated on the 7th or 8th day of culture. Embryo grown alone was the observational unit with a binary outcome recorded (achieved developmental stage or not). Data were analyzed via Chi-square. Pairwise comparison was performed using a 2-proportion z-tests. Significance level was set at $p < 0.05$. In order to confirm that there were no differences in development from batches of oocytes on different days, cleavage and blastocyst development outcomes were regressed as a function of culture group and batch of ovaries using a logistical regression model. Batches were not statistically different at predicting development of cleavage or blastocysts in the logistic regression model. Because no batch effect was detected, a Chi-square was performed to assess treatment differences. Sample size calculations were performed for medium effect size and all sample sizes were adequate for 80% power and 0.05 significance level. All analyses were performed on R software, Version 3.1.

Results

In Experiment 1 (Table 1), although cleavage rates were similar among groups, embryos in groups of at least 5 had higher ($p < 0.05$) blastocyst development than 1 embryo cultured alone but there was no developmental benefit of more ova (5, 10, 20, or 50/culture).

In Experiment 2 (Table 2), there was no significant difference in blastocyst development among groups that were cultured in conditioned media compared to original culture media.

In Experiment 3 (Table 3), there was no significant difference in development rates among selected, cleaved embryos, and controls. Again, control and original embryos in groups of 10 had higher ($p < 0.05$) developmental rates than 1 embryo cultured alone.

In Experiment 4 (Table 4), cleavage rates were similar among groups. The group of 5 embryos per 10 µl drop had significantly lower blastocyst development than the groups of 2, 10, 25, and control.

In all 4 experiments, there were no significant differences among batches of oocytes.

Discussion

In Experiment 1, embryos grouped in 5 or more had significantly higher blastocyst development but similar cleavage compared to embryos cultured alone that provided a baseline to initiate further exploration of group culture and helper effect concept. In groups of 5 or higher, various aspects (secreted factors in spent media, embryo density, and timing of group interaction) might have contributed to increased development of blastocysts.

Embryo density influences substances in the media and the quantity of factors that may be secreted or depleted by the embryos.[19] Similar results were reported when number of embryos were compared on development.[20] Frequencies of

Table 1. Effects of number of embryos per 50 µl in vitro culture drop on cleavage and blastocyst development

Number of embryos per 50 µl drop	Total number of embryos cultured	Percent cleaved ± SEM	Percent blastocysts ± SEM
1	125	56.8 ± 4.4	7.2^{a} ± 2.3
5	150	61.3 ± 3.9	19.3^{b} ± 3.2
10	170	56.5 ± 3.8	20.0^{b} ± 3.1
20	160	58.2 ± 3.9	19.4^{b} ± 3.1
50	150	66.7 ± 3.9	21.3^{b} ± 3.4

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

Table 2. Effects of conditioned media on blastocyst development

Number of embryos per 50 µl drop	Media	Total number of embryos cultured	Percent blastocysts ± SEM
20	Control	40	20.0 ± 6.4
20	Conditioned	40	22.5 ± 6.7
50	Control	50	20.0 ± 5.7
50	Conditioned	50	20.0 ± 5.7

Table 3. Effects of grouping cleaved embryos on blastocyst development

Number of embryos per 50 µl drop	Treatment group	Total number of embryos cultured	Percent blastocysts ± SEM
10	Control	40	27.5^{a} ± 7.2
10	Cleaved	30	30.0^{a} ± 8.5
1	Cleaved	32	3.1^{b} ± 3.1
1	Control	65	7.7^{b} ± 3.3

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

Table 4. Effects of number of embryos per 10 µl in vitro culture drop on cleavage and blastocyst development compared to control (10 embryos per 50 µl drop)

Number of embryos per drop	Size of drop (µl)	Total number of embryos cultured	Percent cleaved ± SEM	Percent blastocysts ± SEM
Control	50	110	59.1 ± 4.7	20.0[a] ± 3.8
2	10	88	73.9 ± 4.7	15.9[a] ± 3.9
5	10	90	70.0 ± 4.8	4.4[b] ± 2.2
10	10	110	68.2 ± 4.4	16.4[a] ± 3.5
25	10	75	69.3 ± 5.3	16.0[a] ± 4.2

[a, a,b]Within a column, means without a common superscript differed ($p < 0.05$)

blastocyst development in groups with 25, 50, and 100 embryos per 50 µl drop were higher than groups of 1, 5, 10, and 250.[20] Still, groups of 5 and 10 embryos were significantly higher than embryos cultured alone.[20] A negative effect was also observed once embryo density reached 250 embryos per 50 µl drop.[20] However, when embryos were cultured embryos in groups of 5, 10, 20, and 30 embryos per 100 µl drop, it was reported that the group of 30 embryos had significantly lower cleavage and blastocyst development rates than groups of 5, 10, and 20, suggesting a negative effect of high density.[21] In contrast, Experiment 4 results had no apparent negative effect of culturing 25 embryos in 10 µl drops. The group of 25 embryos had similar blastocyst development rates to groups of 2, 10, and control and the group of 5 embryos had significantly lower blastocyst development than other groups. Interestingly, the group of 2 embryos performed similar to groups of 10, 25, and control. Apparently, only a single other occupant in the cohort restored blastocyst development to that of the controls (10 embryos per 50 µl drop). Further, it may not matter if the second occupant was not exposed to sperm.[22]

Size of the culture environment appears to be a factor of embryo density that impacts development. Some studies have reported that a reduced volume of culture media improved embryo quality and development.[21,23] However, there was an increase in development with decreased volume in mice embryos.[7] Nine murine embryos cultured in 50 µl drops had significantly higher blastocyst development compared to 9 embryos cultured in 25, 12.5, or 6.25 µl drops.[7] Similarly, there was an increase in embryo density negatively impacted developmental competence and expression of heat shock protein 70.1 gene in bovine embryos.[21] Conversely, when microfluidic platforms were used to achieve submicroliter culture systems for murine embryos, embryos placed in groups of 2 per 100 nl culture chamber achieved 81.8% blastocyst development, whereas embryos grouped in 2 embryos per 5 µl conventional culture drop had a 3.3% blastocyst rate.[11] This study suggested that surface area to volume ratios of the culture environment might be important.

In our study, adding conditioned media from previous replicates in Experiment 2 had no beneficial effect on blastocyst development compared to controls. When epidermal growth factor and transforming growth factor-131 were added to their culture media for bovine embryos, there was an improvement in blastocyst development.[4] Overall, there was a significant difference in embryo development between 1 embryo and embryos cultured in groups of 5.[4] Addition of bovine oviductal epithelial or other cells to produce an undefined media positively influenced development.[2] Bovine oviductal epithelial cells secrete many beneficial nutritional factors and their composition evolves throughout the estrous cycle.[1] Oviduct fluid has a crucial role in protecting the developing embryo against oxidative stress by secreting free radical scavenging proteins and modulating antioxidant enzyme levels. DNA fragmentation that is a major contributor to cell death and the formation of low-quality embryos, is primarily caused by an imbalanced redox state and inadequate levels of antioxidants.[2] Despite the cells' positive influence on development, the protocol is inefficient, costly, and unsanitary for commercial production.[24] Similar pitfalls, combined with the potential for large offspring syndrome, are observed in a popular strategy of adding fetal serum to culture media. Although it may improve development, there is concern for its potential to spread pathogens and there are regulatory restrictions placed on embryos cultured in animal-based sera.[1]

Although metabolic waste is excreted into culture media, beneficial factors were also identified.[25] Spent media from embryo culture was used for noninvasive preimplantation genetic testing for aneuploidy.[25] The quantity of cell-free DNA obtained from spent DNA can vary depending on laboratory procedure. Depending on whether a single-step media, or sequential media that is changed once or twice between fertilization and blastulation is utilized, impact components are left in the spent media. Through the ability to capture the beneficial factors secreted by embryos themselves and using the conditioned media to enable or embryos to develop, costly and unsanitary addition of sera and oviductal cells could be avoided. Growing evidence supports various paracrine factors (e.g. epidermal growth factor,[26] platelet-activating factors,[27] and insulin-like growth factors [IGFs]) are secreted into spent media by preimplantation embryos.[28] Various cytokines positively influenced embryo viability.[29] A combination of fibroblast growth factor-2, leukemia inhibitory factor, and IGF1 supplemented in culture media of bovine embryos improved development and quality.[29] MicroRNAs and DNA are other factors that may be secreted by embryos in the culture media.[16] Small RNA sequencing and live embryo imaging demonstrated that embryonic miRNAs were essential for bovine embryos transitioning from morula to blastocyst.[16] Exosomes may also have an important role in the communication among embryos in the same culture environment. Exosomes are tetraspanin CD9-positive membranous microvesicles that package and transport these paracrine factors.[28] Exosomes mediated cell to cell communication by selectively delivering a wide range of proteins, lipids, mRNA, DNA fragments, and nucleic acid that modify cellular function of the recipient cells.[2,30] Exosomes also establish communication between embryo and maternal interactions.[2] Exosomes and conditioned media from bovine oviduct epithelial cells increased blastocyst production of bovine embryos.[2] Recently, embryos that developed to the blastocyst stage had substantially

elevated levels of fatty acid binding protein 3 compared to embryos that stopped developing at the 8-16 cell stage in the same culture environment, suggesting lipid management may impact embryo development (unpublished data).

A multi-step culture embryo culture process and a continuous culture media system was evaluated. Nutritional requirements of embryo's change as they progress from zygote to morula to blastocyst that likely mimics in vivo environment as embryo progresses from oviduct to uterus.[31] Morulae's prefer energy substrates (e.g. pyruvate, lactate, and amino acids). Bovine embryos have limited utilization of glucose until after compaction. Embryo's utilization and uptake of glucose increases and by the time a blastocyst is established, glucose is utilized more than any other available nutrient.[31] In a continuous culture system, maintaining the appropriate balance between sufficient nutrients to meet embryonic demand and breakdown of embryo metabolic waste products. Ammonium is a factor of concern. Amino acids in culture medium are easily broken down into ammonium at 37°C.[32] A build-up of ammonium has negative effects on physiology, viability, and fetal development of mouse and human embryos.[32] Embryos may secrete beneficial factors that are important for embryos around them or they may remove detrimental factors from embryos of marginal quality. In order to maintain identity of 1 embryo, while sharing culture media, certain well of well systems were developed.[7] In these types of dishes, 1 embryo is cultured in a microwell and culture drop covered multiple microwells thus embryos shared culture media. Embryos grown alone in microwells with shared culture media had no improvement in blastocyst development.[7] This study suggested that shared media alone was not able to overcome the problem of 1 embryo culture or possibly ineffective sharing of factors.[7]

In Experiment 3, no difference in development was detected between embryos selected out after cleavage and controls. Cleavage rate was evaluated 45 hours after insemination and noncleaved embryos were either removed or left in the dish as controls.[6] Cleaved embryos were either left in their original drop or combined into a new group. These researchers reported results similar to Experiment 3; noncleaved embryos had no negative effect on the potential of embryos in the same group to become blastocysts.[6] Human embryos were grouped after day 3 in groups of 2-5 embryos per drop based on embryo quality grades and they were also either grouped randomly regardless of quality or grouped together based on quality.[19] Selecting embryos on day 3 based on quality, significantly promoted higher blastocyst development compared to controls.[19] Further research is needed to determine whether poor quality or undeveloped embryos have a positive or negative impact on development of neighboring embryos.

In our experiments, a decision was made to utilize 1 bull, to control potential variations that might arise among different bulls. It is important to recognize that sires exhibit distinct capacities in producing blastocysts, potentially influencing experiments' outcome.[33] This variability among bulls may be attributed to differences in sperm capacitation during fertilization process, wherein the kinetics of sperm penetration has a pivotal role.[34] Sires with varying capacities for sperm capacitation result in differences in fertilizing capacity, impacting sperm penetration timing.[34] This consideration underscores the importance of controlling for bull-specific effects.

In summary, our results demonstrated that culturing bovine embryos in groups significantly improved development to blastocyst stage. In all situations, 1 embryo cultured alone performed significantly lower than embryos cultured in groups of > 5. High-density culture environments had no negative effect on blastocyst development. Conditioned media from previous replicates had not enhanced development of new embryos in culture. Lastly, grouping cleaved embryos together had not improved development. Further research is required to identify differences in chemical composition of group culture media and individual culture media to better understand the exchange among grouped embryos.

Conflict of interest

None to report.

References

1. Ferré LB, Kjelland ME, Strøbech LB, et al: Review: recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. Animal 2020;14:991-1004. doi: 10.1017/S1751731119002775
2. Sidrat T, Khan AA, Joo MD, et al: Bovine oviduct epithelial cell-derived culture media and exosomes improve mitochondrial health by restoring metabolic flux during pre-implantation development. Int J Biochem Mol Biol 2020;21:7589. doi: 10.3390/ijms21207589
3. Ferry L, Mermillod P, Massip A, et al: Bovine embryos cultured in serum-poor oviduct-conditioned medium need cooperation to reach the blastocyst stage. Theriogenology 1994;42:445-453. doi: 10.1016/0093-691X(94)90682-9
4. Keefer CL, Stice SL, Paprocki AM, et al: In vitro culture of bovine IVM-IVF embryos: cooperative interaction among embryos and the role of growth factors. Theriogenology 1994;41:1323-1331. doi: 10.1016/0093-691X(94)90491-Z
5. O'Doherty EM, Wade MG, Hill JL, et al: Effects of culturing bovine oocytes either singly or in groups on development to blastocysts. Theriogenology 1997;48:161-169. doi: 10.1016/S0093-691X(97)00199-4
6. Wydooghe E, Vandaele L, Piepers S, et al: Individual commitment to a group effect: strengths and weaknesses of bovine embryo group culture. Reproduction 2014;148:519-529. doi: 10.1530/REP-14-0213.
7. Dai SJ, Xu CL, Wang J, et al: Effect of culture medium volume and embryo density on early mouse embryonic development: tracking the development of the individual embryo. J Assist Reprod Genet 2012;29:617-623. doi: 10.1007/s10815-012-9744-8
8. Paria BC, Dey SK: Preimplantation embryo development in vitro: cooperative interactions among embryos and role of growth factors. Proc Natl Acad Sci USA 1990;87:4756-4760. doi: 10.1073/pnas.87.12.4756
9. Lane M, Gardner K: Effects of incubation volume and embryo density on the development and viability of mouse embryos in vitro. Hum Reprod 1992;17:558-562. doi: 10.1093/oxfordjournals.humrep.a137690
10. Kato Y, Tusnoda Y: Effects of the culture density of mouse zygotes on the development in vitro and in vivo. Theriogenology 1994;41:1315-1322. doi: 10.1016/0093-691X(94)90490-A

11. Melin J, Lee A, Foygel K, et al: In vitro embryo culture in defined, sub-microliter volumes. Dev Dyn 2009;238:950-955. doi: 10.1002/dvdy.21918
12. Moessner J, Dodson WC: The quality of human embryo growth is improved when embryos are cultured in groups rather than separately. Fertil Steril 1995;64:1034-1035. doi: 10.1016/S0015-0282(16)57925-4
13. Almagor M, Bejar C, Kafka I, et al: Pregnancy rates after communal growth of preimplantation human embryos in vitro. Fertil Steril 1996;66:394-397. doi: 10.1016/S0015-0282(16)58507-0
14. Rijnders PM, Jansen CAM: Influence of group culture and culture volume on the formation of human blastocysts: a prospective randomized study. Hum Reprod 1999;14:2333-2337. doi: 10.1093/humrep/14.9.2333
15. Fujita T, Umeki H, Shimura H, et al: Effect of group culture and embryo-culture conditioned medium on development of bovine embryos. J Reprod Dev 2006;52:137-142. doi: 10.1262/jrd.16084
16. Paulson EE, Fishman EL, Schultz RM, et al: Embryonic microRNAs are essential for bovine preimplantation embryo development. Proc Natl Acad Sci USA 2022;119:e2212942119. doi: 10.1073/pnas.2212942119
17. Reed ML, Woodward BJ, Swain JE: Single or group culture of mammalian embryos: the verdict of the literature. J Reprod Stem Cell Biotechnol 2011;2:77-87.
18. Shi C, Sun TC, Chen SW, et al: Effects of embryo density on cell number of day 3 embryos cultured in a 30-µl drop: a retrospective cohort study. Zygote 2022;30:487-494. doi: 10.1017/S0967199421000812
19. Tao T, Robichaud A, Mercier J, et al: Influence of group embryo culture strategies on the blastocyst development and pregnancy outcome. J Assist Reprod Genet 2013;30:63-68. doi: 10.1007/s10815-012-9892-x
20. Nagao Y, Iijima R, Saeki K: Interaction between embryos and culture conditions during in vitro development of bovine early embryos. Zygote 2008;16:127-133. doi: 10.1017/S0967199408004644
21. de Oliveira ATD, Lopes RFF, Rodrigues JL: Gene expression and developmental competence of bovine embryos produced in vitro under varying embryo density conditions. Theriogenology 2005;64:1559-1572. doi: 10.1016/j.theriogenology.2005.03.019
22. Looman J, Hickerson S, Gibbons J: Effects of unfertilized oocytes and blastocysts on bovine in vitro embryo development. In 2023 AETA and CETA/ACTE Joint Convention Proc Am Em Tran Assoc 2023.
23. Gil MA, Abeydeera LR, Day BN, et al: Effect of the volume of medium and number of oocytes during in vitro fertilization on embryo development in pigs. Theriogenology 2003;60:767-776. doi: 10.1016/S0093-691X(03)00051-7
24. Carvalho AV, Canon E, Jouneau L, et al: Different co-culture systems have the same impact on bovine embryo transcriptome. Reproduction 2017;154:695-710. doi: 10.1530/REP-17-0449
25. Belandres D, Shamonki M, Arrach N: Current status of spent embryo media research for preimplantation genetic testing. J Assist Reprod Genet 2019;36:819-826. doi: 10.1007/s10815-019-01437-6
26. Thongkittidilok C, Tharasanit T, Songsasen N, et al: Epidermal growth factor improves developmental competence and embryonic quality of singly cultured domestic cat embryos. J Reprod Dev 2015;61:269-276. doi: 10.1262/jrd.2014-167
27. Roudebush WE, Wininger JD, Jones AE, et al: Embryonic platelet-activating factor: an indicator of embryo viability. Hum Reprod 2002;17:1306-1310. doi: 10.1093/humrep/17.5.1306
28. Qu P, Qing S, Liu R, et al: Effects of embryo-derived exosomes on the development of bovine cloned embryos. PLoS One 2017;12:e0174535. doi: 10.1371/journal.pone.0174535
29. Stoecklein KS, Ortega MS, Spate LD, et al: Improved cryopreservation of in vitro produced bovine embryos using FGF2, LIF, and IGF1. PLoS One 2021;16:e0243727. doi: 10.1371/journal.pone.0243727
30. Vyas P, Balakier H, Librach CL: Ultrastructural identification of CD9 positive extracellular vesicles released from human embryos and transported through the zona pellucida. Syst Biol Reprod Med 2019;65:273-280. doi: 10.1080/19396368.2019.1619858
31. Gardner DK: Changes in requirements and utilization of nutrients during mammalian preimplantation embryo development and their significance in embryo culture. Theriogenology 1998;49:83-102. doi: 10.1016/S0093-691X(97)00404-4
32. Gardner DK, Hamilton R, McCallie B, et al: Human and mouse embryonic development, metabolism and gene expression are altered by an ammonium gradient in vitro. Reproduction 2013;146:49-61. doi: 10.1530/REP-12-0348
33. Lockhart KN, Drum JN, Balboula AZ, et al: Sire modulates developmental kinetics and transcriptome of the bovine embryo. Reproduction 2023;166:337-348. doi: 10.1530/REP-23-0030
34. Barceló-Fimbres M, Campos-Chillón L, Seidel Jr G: In vitro fertilization using non-sexed and sexed bovine sperm: sperm concentration, sorter pressure, and bull effects. Reprod Domest Anim 2011;46:495-502. doi: 10.1111/j.1439-0531.2010.01696.x