

In Vitro Maturation and Embryo Production in Cattle

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Summary

When immature bovine oocytes are released from their follicles and are cultured in standard maturation medium, they resume the first meiotic division. The alteration of basic maturation conditions can affect oocyte competence significantly, as reflected by the morula and blastocyst yield after in vitro fertilization. The conditions used from the beginning of maturation up to the blastocyst stage have been shown to influence not only the developmental competence but also, potentially, the normal epigenetic make-up of the embryo. The methods described in this chapter outline the different steps of in vitro production of bovine embryos up to the blastocyst stage in semidefined conditions: (1) oocyte maturation, (2) in vitro fertilization, and (3) in vitro development. The first section explains procedures of ovary collection and oocyte aspiration and selection for in vitro maturation. The second section involves methods for the preparation of semen and oocytes for fertilization. The last section explains the best conditions to obtain blastocysts after 8 d of in vitro culture.

Key Words: Bovine; oocyte maturation; culture; developmental competence.

1. Introduction

When immature bovine oocytes are released from their follicles and are cultured in standard maturation medium, they resume the first meiotic division (**1**). Although it may appear difficult to influence the quality of an oocyte during the maturational period, the alteration of basic maturation conditions can affect oocyte competence significantly, as reflected by the morula and blastocyst yield after in vitro fertilization (IVF [**2**]). The conditions used from the beginning of maturation up to the blastocyst stage have been shown to influence not only the developmental competence but potentially the normal epigenetic make-up of the embryo (**3**).

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During in vitro maturation (IVM), oocytes undergo a series of cytoplasmic changes before the resumption of nuclear maturation, leading to variable competence of the resulting embryo (4,5). In addition, the synthesis and storage of certain forms of messenger RNA and protein during IVM and early embryonic development are thought to be necessary for further development (6,7).

The limited developmental competence of bovine oocytes after IVM can be used to understand the factors involved in the acquisition of such ability. For this reason and to understand the requirements for development of immature oocytes through IVM, all products with undefined components should be eliminated from culture conditions. Although serum or bovine serum albumin (BSA) typically are added to the medium as a protein supplement to improve culture efficiency (8), different lots of this protein with differing purity levels can produce highly variable effects during the period of culture in hamsters, ranging from highly stimulatory to highly inhibitory (9).

2. Materials

Unless otherwise stated, all chemicals used are purchased from Sigma-Aldrich (St. Louis, MO) and are cell culture tested or embryo tested. Every new lot of a chemical is tested individually before using. Every solution is prepared in Milli-Q water (18.2 m Ω .cm; total organic carbon < 5 ppb) produced by a Milli-Q Synthesis/A10 system (Millipore, Bedford, MA) and sterile filtered (0.22 μ m) with a MF-Millipore membrane filter GSWP (Millipore). To remove all soap solution residue, glassware is soaked in reverse osmosis water for 1 d and then rinsed two times with Milli-Q water. When filtering small volumes of solution (≤ 10 mL), the first 0.5 mL is rejected to avoid filter cytotoxicity.

1. Saline.
2. Antibiotic antimycotic powder.
3. HEPES-buffered tyrode's medium: 114 mM NaCl, 3.2 mM KCl, 2 mM NaHCO₃, 0.34 mM NaH₂PO₄, 10 mM Na lactate, 2.0 mM CaCl₂, 0.5 mM MgCl₂, 10 mM HEPES, and 10 mg/L phenol red in Milli-Q water. Adjust pH to 7.4. The osmolarity should be 255 to 265 mosmol. Keep at 4°C for up to 15 d. Can be frozen. Supplement appropriately (*see* medium compositions in **step 29**) on day of use.
4. BSA.
5. Sodium pyruvate: Prepare a 40-mM solution in saline and freeze at -20°C.
6. Gentamicin: Prepare a 50-mg/mL solution in saline and keep at 4°C for 1 mo. Protect from light. The solution can become cytotoxic if powder is too old. A dark, detached, and unexpanded cumulus after oocyte maturation is a sign of cytotoxicity.
7. Mineral oil: Oil is filtered on an analytical filter funnel of 0.45 μ m (Nalgene), blended with phosphate-buffered saline (PBS; 5:1 oil:PBS) to remove toxic hydrosoluble metallic ions (Fe²⁺, Cu²⁺), and left to stand for 24 h to separate the

PBS from oil before use. PBS can be replaced by Hank's or any other cell culture medium without phenol red. Possible contamination of oil would be visible at the interface of oil and PBS. Protect from light.

8. Synthetic oviduct fluid (SOF): 107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4 , 1.71 mM CaCl_2 , 0.49 mM MgCl_2 , 25.07 mM NaHCO_3 , 3.30 mM Na lactate, and 1.50 mM glucose (**10**) in Milli-Q water. The osmolarity should be 270 mosmol and the pH 7.55. Keep at 4°C for 15 d. Do not freeze. Supplement appropriately (see medium compositions in **steps 30, 33, and 34**), on day of use.
9. Modified Eagle's medium (MEM) nonessential amino acids (Invitrogen, Burlington, Ontario, Canada): Keep at 4°C and protect from light.
10. MEM essential amino acids (Invitrogen): Keep at 4°C and protect from light.
11. Glutamine: Prepare a 50 mM solution in SOF and use fresh.
12. Follicle-stimulating hormone (FSH; National hormone and peptide program): Dissolve in 0.5% BSA in Milli-Q water, aliquot, lyophilize, and freeze at -20°C.
13. Luteinizing hormone (LH; National hormone and peptide program): Dissolve in 0.5% BSA in Milli-Q water, aliquot, lyophilize, and freeze at -20°C.
14. Estradiol-17 β : Prepare a 1 mg/mL solution in 95% ethanol and keep at -20°C for 1 mo. Use and put back rapidly in freezer. Protect from light.
15. Modified Tyrode's solution: 114 mM NaCl, 3.2 mM KCl, 25 mM NaHCO_3 , 0.34 mM NaH_2PO_4 , 10 mM Na lactate, 2.0 mM CaCl_2 , 0.5 mM MgCl_2 and 10 mg/L phenol red (**11**) in Milli-Q water. The osmolarity should be 280–300 mosmol. Keep at 4°C for 15 d. Do not freeze. Supplement appropriately (see medium composition in **step 31**) on day of use.
16. Fatty acid-free BSA.
17. Heparin: dissolve in saline and freeze at -20°C.
18. 2 mM Penicillamine, 1 mM hypotaurine, 250 μM epinephrine (PHE): The PHE mixture is prepared using the procedure described by Miller et al. (**12**). A penicillamine stock solution is prepared by adding 3 mg of penicillamine to 10 mL physiological saline. A hypotaurine stock solution is prepared by adding 1.09 mg of hypotaurine to 10 mL of physiological saline. The epinephrine stock solution is prepared by adding 165 mg of sodium lactate (syrup, 60%) and 50 mg of sodium meta-bisulfite to 50 mL of Milli-Q water. After adjusting the pH to 4.0 with 1 N HCl, 1.83 mg of epinephrine is added to 40 mL of this solution. The PHE stock solution is prepared by adding 250 μL of penicillamine stock, 250 μL of hypotaurine stock, and 200 μL of epinephrine stock to 400 μL of physiological saline. The PHE stock solution is placed into vials, wrapped in aluminum fold and stored at -20°C.
19. Percoll.
20. Modified Tyrode's medium for sperm (Sp-TALP): 100 mM NaCl, 3.1 mM KCl, 25 mM NaHCO_3 , 0.29 mM NaH_2PO_4 , 21.6 mM Na lactate, 2.0 mM CaCl_2 , 1.5 mM MgCl_2 , 10 mM HEPES, and 10 mg/L phenol red (**13**) in Milli-Q water. Adjust pH to 7.4. The osmolarity should be 280–300 mosmol. Keep at 4°C for 15 d. The medium can be frozen. Supplement with 0.6% BSA, 1 mM sodium pyruvate, and 50 $\mu\text{g/mL}$ gentamicin.

21. PBS: 136.89 mM NaCl, 2.68 mM KCl, 8.03 mM Na₂HPO₄, 0.66 mM CaCl₂, 0.5 mM MgCl₂, 1.47 mM KH₂PO₄, 5.55 mM glucose, and 1 mM sodium pyruvate in Milli-Q water. Adjust pH to 7.1. Keep at 4°C for 15 d. Supplement appropriately (*see* medium compositions in **step 32**), on day of use.
22. Ethylene diamine tetraacetic acid: Prepare a 10 mM solution in SOF and keep at 4°C. Protect from light.
23. Propidium iodide.
24. Hoechst 33354.
25. Formalin solution (HT50-1-1). A general histological fixative for in vitro diagnostic.
26. Triton X-100.
27. Mowiol gelatin.
28. Transportation medium: 0.9% NaCl aqueous solution (saline) containing 100 000 IU/L penicillin, 100 mg/L streptomycin, and 250 µg/L amphotericin B (antibiotic antimycotic powder).
29. Oocyte wash medium: HEPES-buffered Tyrode's medium supplemented with 0.3% BSA, 0.2 mM sodium pyruvate, and 50 µg/mL gentamicin.
30. Maturation medium: SOF supplemented with 0.8% BSA, 0.33 mM sodium pyruvate, 1X MEM nonessential amino acids, 1X MEM essential amino acids, 1 mM glutamine, 0.5 µg/mL FSH, 5 µg/mL LH, 1 µg/mL estradiol-17β, and 50 µg/mL gentamicin.
31. Fertilization medium: Modified Tyrode's solution, supplemented with 0.6% fatty acid-free BSA, 0.2 mM sodium pyruvate, 2 µg/mL heparin, and 50 µg/mL gentamicin (*see* **Note 1**).
32. Decumulation medium: Modified PBS without Ca²⁺ and Mg²⁺ supplemented with 0.3% BSA and 50 µg/mL gentamicin.
33. Development medium 1 (used for the first 72 h of culture): SOF supplemented with 0.8% BSA, 0.33 mM sodium pyruvate, 1X MEM nonessential amino acids, 1 mM glutamine, 10 µM ethylene diamine tetraacetic acid, and 50 µg/mL gentamicin.
34. Development medium 2: SOF supplemented with 0.8% BSA, 0.33 mM sodium pyruvate, 1X MEM essential amino acids, 1X MEM nonessential amino acids, 1 mM glutamine, and 50 µg/mL gentamicin.

3. Methods

The methods described below outline (1) oocyte maturation in vitro, (2) in vitro fertilization, and (3) culture of embryo up to the blastocyst stage in semi-defined conditions (*see* **Note 2**).

3.1. Oocyte Maturation

1. Collect ovaries from cycling heifers or cows within 30 min after slaughter and transport to the laboratory at 30–35°C in transportation medium (*see* **Note 3**).
2. Wash the ovaries with transportation medium (*see* **Note 4**).

3. Aspirate follicular fluid from 3- to 6-mm follicles using an 18-gage needle (Becton-Dickinson) on a 10-mL syringe (Becton-Dickinson; *see* **Note 5**).
4. Stand tubes containing follicular fluid at room temperature for 10–15 min and aspirate precipitate (*see* **Note 6**). Transfer the precipitate into a Petri dish (Fisher, 100 × 15 mm).
5. Centrifuge supernatant at 3000g during 10 min at room temperature to obtain clear follicular fluid. Using a stereomicroscope, select oocytes with at least five layers of compact cumulus cells and belonged to classes 1–3 (**Table 1** and **ref. 14**), and wash them in follicular fluid.
6. Rapidly wash oocytes three times in oocyte wash medium in Petri dishes (Nunc, 35 × 10 mm).
7. Transfer selected oocytes into 50-μL droplets (10 oocytes/drop) of maturation medium overlaid with 9 mL of mineral oil in Nunc Petri dishes, 60 × 15 mm (*see* **Note 7**).
8. Culture oocytes for 24 h at 38.5°C under 5% CO₂ and 95% air atmosphere with saturated humidity.

3.2. In Vitro Fertilization

IVF is performed using frozen semen from selected bulls (*see* **Note 8**).

1. Prepare a discontinuous Percoll gradient by adding 2 mL of 45% Percoll over 2 mL of 90% Percoll in a 15-mL Falcon centrifuge tube (**15**).
2. Let the tube stand until the Percoll solution has reached room temperature.
3. Thaw one or two straws of spermatozoa (100×10^6 spermatozoa) in a 35°C water bath for 1 min.
4. Blend the semen sample and add it on top of the Percoll gradient.
5. Centrifuge at 700g for 30 min at 26°C.
6. Remove rapidly the supernatant and resuspend the pellet in 1 mL of Sp-TALP.
7. Centrifuge the resuspended pellet at 250g for 5 min at 26°C.
8. Remove rapidly the supernatant and blend the pellet.
9. Use aliquots of 5 μL of sperm pellet diluted in fertilization medium to evaluate the concentration and motility of spermatozoa with an hemocytometer chamber.
10. Resuspend the sperm pellet in fertilization medium to obtain a concentration of 25×10^6 cells/mL.
11. After maturation, wash cumulus–oocyte complexes (COCs) twice for 5 min in 2 mL of oocyte wash medium in Nunc Petri dishes (35 × 10 mm).
12. Transfer washed COCs in 48-μL droplets (5 COCs/drop) of fertilization medium overlaid with 9 mL of mineral oil (*see* **Note 9**).
13. Following the transfer of oocytes and just before adding spermatozoa, add 2 μL of PHE to each droplet.
14. Add 2 μL of sperm suspension into each fertilization droplet containing COCs (final concentration = 1×10^6 cells/mL). Coincubate COCs and spermatozoa for 15–18 h at 38.5°C under 5% CO₂ in air with saturated humidity.

Table 1
Classification of Bovine Oocytes^a

Class	No. of cumulus layers	Expansion of cumulus
1 and 2	≥5	Compact
3	≥5	Slight expansion in outer layers
4	≥5	Full expansion with dark clumps
5	1	Only corona radiata
6	0	No cumulus

^aAll oocytes, not including the cumulus, have diameters ≥120 μm.
(Adapted from Blondin and Sirard [14]).

3.3. In Vitro Development

1. Between 15 and 18 h after insemination, strip presumptive zygotes of all surrounding cumulus cells by repeated pipetting in decumulation medium and wash them three times in the same medium in order to avoid transfer of any cumulus cell to the development medium (development medium 1).
2. Transfer presumptive zygotes in 50-μL droplets (20–30 zygotes/drop) of development medium 1, overlaid with 9 mL of mineral oil and culture them at 38.5°C under 5% CO₂, 7% O₂, 88% N₂ with saturated humidity.
3. After 72 h of culture, assess cleavage stage and transfer all the embryos into development medium 2. Transfer embryos to new drops of medium every 72 h.
4. On day 8 of culture, assess the number and quality of embryos developing to the morula and blastocyst stage.

Determination of inner cell mass (ICM) and trophectoderm cell numbers can also be done in blastocysts (*see* **Note 10**).

4. Notes

1. Heparin concentration must be adjusted for each lot of semen used. A heparin concentration of 1, 2, 5, and 10 μg/mL should be tested and the penetration/polyspermy rate verified.
2. Multiple factors are critical to the outcome of IVM—in vitro production: exposure to cytotoxic materials (Petri dish, syringe, needle, surgical gloves), water quality, chemical quality, gas composition, temperature, pH, osmolality, light exposure, and assay techniques. For a review of the general topic of quality control during mammalian IVF and embryo culture, *see* **refs. 16 and 17**. Each new brand or lot of plastic material should be tested. Each new lot of a chemical should also be tested. Every protocol must contain a control treatment to assess the reproducibility of results obtained week after week.

3. The time interval that the oocytes remained in the postmortem ovarian follicles has a significant effect on the subsequent embryonic development. The optimal time for oocyte recovery after the ovaries are collected is 4 h (18).
4. To avoid contamination, ovary washing and aspiration are performed outside the culture room.
5. To remove toxicity of plastic syringe and needle, aspirate follicular fluid of 8- to 10-mm follicles to rinse the needle and syringe before to use them for aspiration of 3- to 6-mm follicles. The needle must be changed frequently to keep a sharp cutting tip.
6. The culture room can be maintained at 27°C or warmer.
7. The droplets, covered with mineral oil, should be preincubated under the culture conditions for a minimum of 2 h before transfer of oocytes or embryos.
8. Frozen bovine semen used can come from an individual bull or a pool of bulls (usually five). Final sperm concentration must be adjusted for every lot of semen used. Sometimes 500,000 spermatozoa/mL give better results than 1×10^6 cells/mL.
9. To limit the contact surface of droplets with Petri dish, droplets are made in two steps in Fisher Petri dishes (60×15 mm). Drops of 18 μ L of fertilization medium are overlaid with 9 mL of mineral oil and then another 30 μ L of fertilization medium is added to each drop.
10. Nuclei of ICM and trophectoderm are differentially labeled with propidium iodide and Hoescht, respectively, with the protocol described by Van Soom et al. (19) with two modifications. Oocytes are washed in HEPES-buffered Tyrode's medium instead of TCM-199 HEPES. For the coloration of the ICM by Hoescht, blastocysts are fixed in 100 μ L of formalin solution for 15 min, then rinsed in 200 μ L of 0.5% Triton X-100 before 100 μ L being mounted over a drop of Mowiol gelatin containing Hoescht 33354 (5 mg/mL [20]).

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