

A Lecture on
In Vitro Fertilization & Culture of Embryos
Reproductive Biotechnology (VGO 606)
M.V.Sc. Course (Veterinary Gynae. & Obst.)

Submitted By
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In vitro embryo production

Uses

- **Multiplication and conservation of superior germplasm**
- **Production of cloned or transgenic animals**
- **Production of embryonic stem cells**

Steps

1. **Collection of oocytes**
2. **In vitro maturation (IVM)**
3. **In vitro fertilization (IVF)**
4. **In vitro culture (IVC) of fertilized oocytes**

Source of oocytes

Slaughterhouse ovaries

Advantage: Immature oocytes available in large numbers

Limitation: No information about pedigree of dam

From live animals through Transvaginal Oocyte retrieval (TVOR) or Ovum Pick-UP (OPU)

Advantage: Known pedigree of offspring

Limitation: Complicated

Limited availability of oocytes

Slaughterhouse ovaries

Follicle dissection

- Intact follicles (2-8 mm diameter) dissected out
- Examined under a microscope for identification and Selection of non-atretic follicles
- Follicles ruptured in a Petri dish for getting oocytes

Advantages

Enables collection of oocytes from healthy non-atretic follicles

Minimal damage to the cumulus mass

Limitation

Slow speed of operation

Aspiration

Surface follicles aspirated using an 18- or 20-gauge needle attached to a 5-ml syringe.

Follicular contents pooled in a Petri dish

Advantage Fast and easy to perform

Limitations Inability to distinguish between atretic and
non-atretic follicles

Some damage to cumulus mass and the resultant
loss of oocyte quality

Recovery of oocytes from only 40-70% of
follicles

Slicing

Preferred over aspiration when ovaries are in a short supply or if oocyte yield is low

Ovary sliced into small pieces to ensure the rupture of most of follicles

Can be applied either directly to the ovary or after aspiration of follicles

Advantage **Up to 3 times higher oocyte yields**

Limitation **More time consuming**

Species	Method	Total oocytes/ ovary	Usable quality (%)
Cattle	Aspiration	9-12	46-80
	Slicing	4-55	34-70
	Dissection	17	72
Buffalo	Puncture	2-3	50
	Aspiration	0.7-1.7	51-73
	Slicing	5.7-6.2	46

Reasons for low oocyte recovery in buffalo

- 1. Lower number of primordial follicles**
- 2. Lower population of antral follicles**
- 3. High incidence of deep atresia**
- 4. Slaughter of buffaloes in a subfertile and unproductive state**
- 5. Presence of CL**
- 6. Stressful summer season**

Morphological criteria for classification of follicles

Non atretic

Uniformly bright appearance, Firm vascularization;
Has regular granulosa layers, No free-floating particles in FF

Intermediate or light atretic

Loss of translucency, Slightly greyish appearance
May have a few small free-floating particles in FF

Atretic

Dull grey appearance, Has blood vessels either empty or filled
irregularly with clotted blood
Partial detachment of granulosa layers
Has free-floating bodies in FF

Incidence of atresia

Method	Proportion of follicles found to be atretic (%)	Species
E_2/P_4 molar ratio	70	Cattle
E_2/P_4 molar ratio	95	Buffalo
Histology	82	Buffalo
Flow cytometry	16-38	Cattle

Grading of oocytes

Grade I

Compact COCs with homogenous ooplasm and an unexpanded cumulus mass having ≥ 5 layers of cumulus cells.

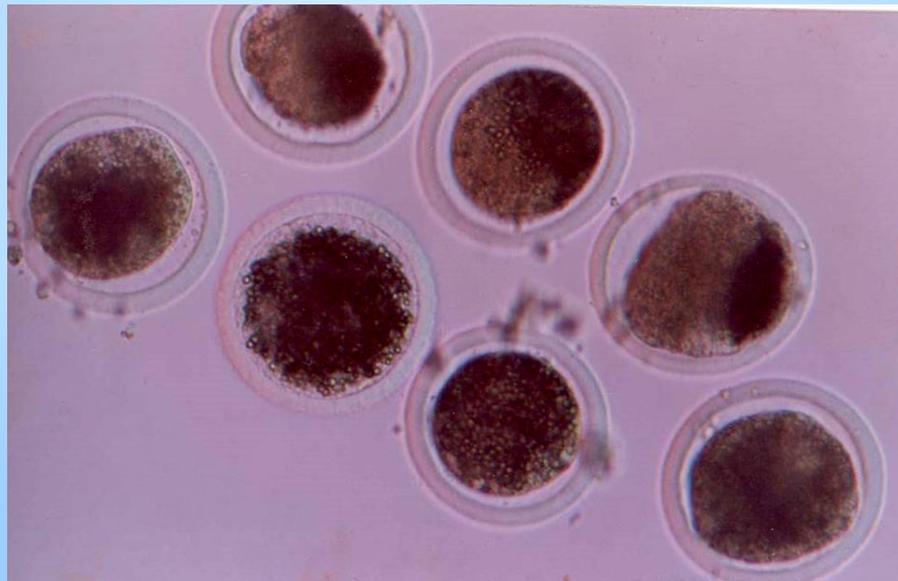
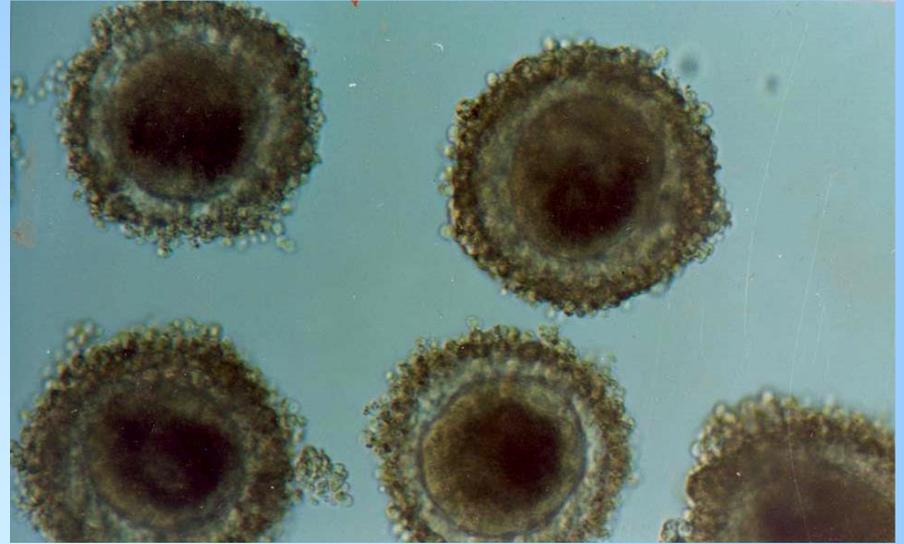
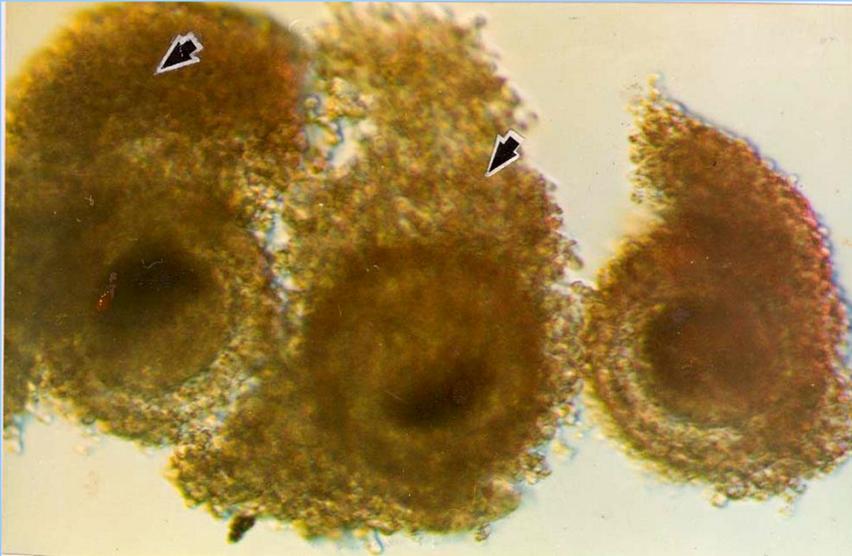
Grade II

COCs with homogenous ooplasm, but with 1-4 layers of cumulus cells

Grade III

Oocytes without cumulus cells and/or with shrunken cytoplasm.

Buffalo oocytes



In vitro maturation

Nuclear maturation (24 h): Chromosomes in MII phase

Inductive phase (6-8 h)

Oocyte undergoes reprogramming by follicular cumulus cells, few changes in structure or synthetic activity.
Culminates in GVBD

Post-inductive phase (18 h)

Oocyte components undergo reorganization.

Cytoplasmic maturation

Capacity of embryos to develop to blastocyst stage

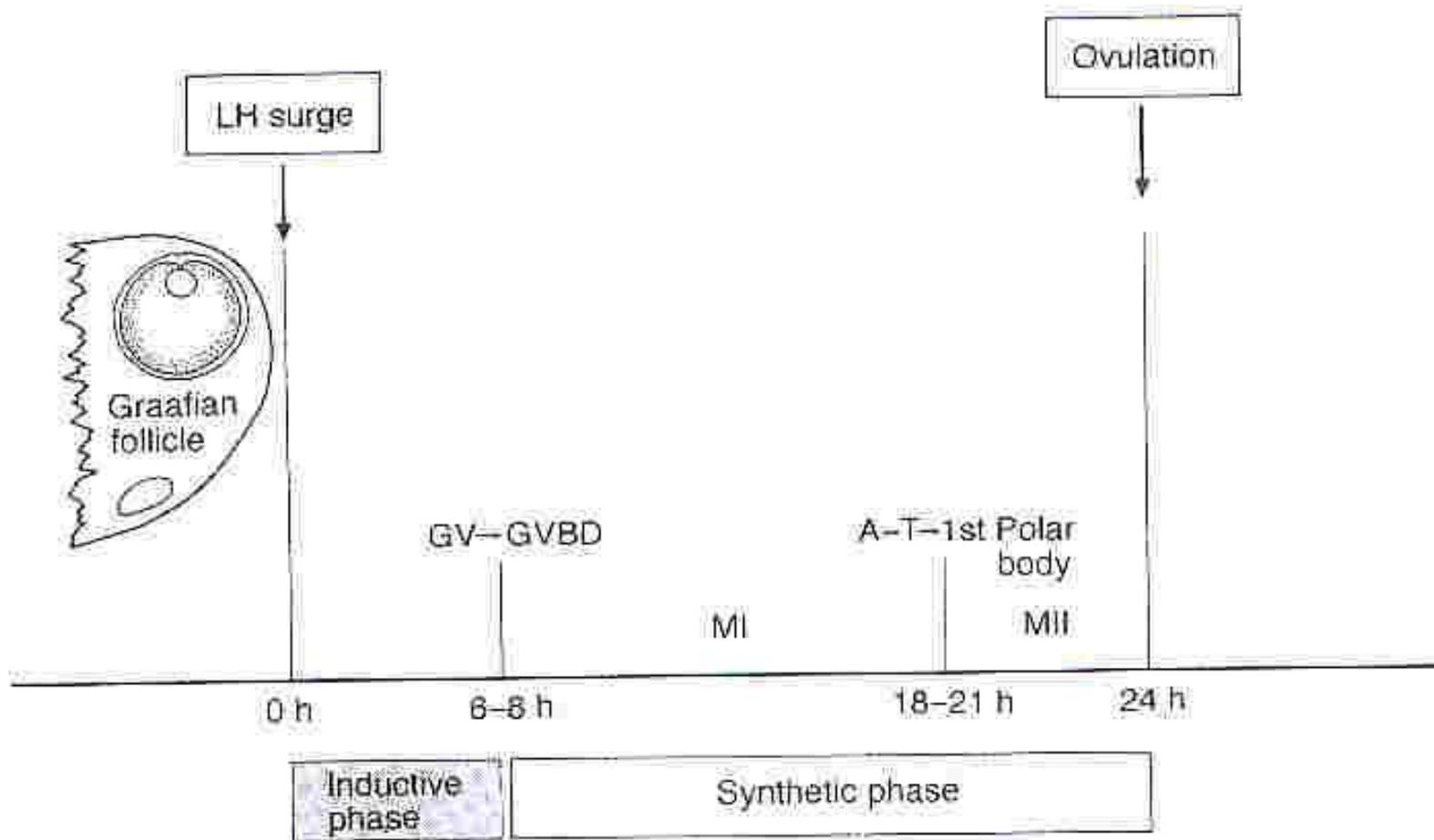


Fig. 4.4. Chronology of events during oocyte maturation in the cow. GV, germinal-vesicle oocyte; GVBD, germinal-vesicle breakdown; MI, metaphase I; A-T, anaphase-telophase; MII, metaphase II.

IVM media

Basic media	Hormones	Serum source	Nuclear maturation rate (%)
<p data-bbox="67 489 426 539">Complex media</p> <p data-bbox="67 644 568 815">TCM-199 (HEPES buffered, with Earle's salts)</p> <p data-bbox="67 925 336 968">Ham's F-10</p> <p data-bbox="67 1075 378 1125">Simple media</p> <p data-bbox="67 1225 170 1268">SOF</p> <p data-bbox="67 1303 146 1346">CR</p>	<p data-bbox="627 489 915 596">LH + FSH + Estradiol</p>	<p data-bbox="1029 489 1132 532">FBS</p> <p data-bbox="1029 568 1132 611">EBS</p> <p data-bbox="1029 646 1132 689">SBS</p> <p data-bbox="1029 725 1093 768">SS</p>	<p data-bbox="1304 489 1431 532">55-80</p>

Culture conditions

Water: **Ultrapure water**

Osmolality: **275-285 mOsm/kg**

Antibiotics: **Gentamycin sulphate (25-50 µg/ml)**
or Penicillin (50-100 IU/ml) +
Streptomycin (50-100 µg/ml)

Temperature: **38-39°C (Core temperature of bovines)**

Gas phase: Generally in 5% CO₂ in air
Preferably in low oxygen tension

Humidity: With 90-95% relative humidity

Time: 24 h

Volume: Groups of 10-15 in 50-100 μ l droplets of IVM
medium (5 μ l/COC)

Cover: Under sterile paraffin oil

Other supplements

Essential

Hormones (LH, FSH, Estradiol)

BSA

Serum

Supplementary

Follicular fluid

Epidermal growth factor

Insulin-like growth factor-I

Insulin-like growth factor-II

Evaluation of nuclear maturation

Staining with Giemsa or Aceto-orcein

Advantage Gives an accurate nuclear maturation rate

Disadvantage Oocyte gets killed

Cumulus expansion

Advantage Not very accurate

Disadvantage Oocyte does not get killed

Classification based on cumulus expansion

Grade I

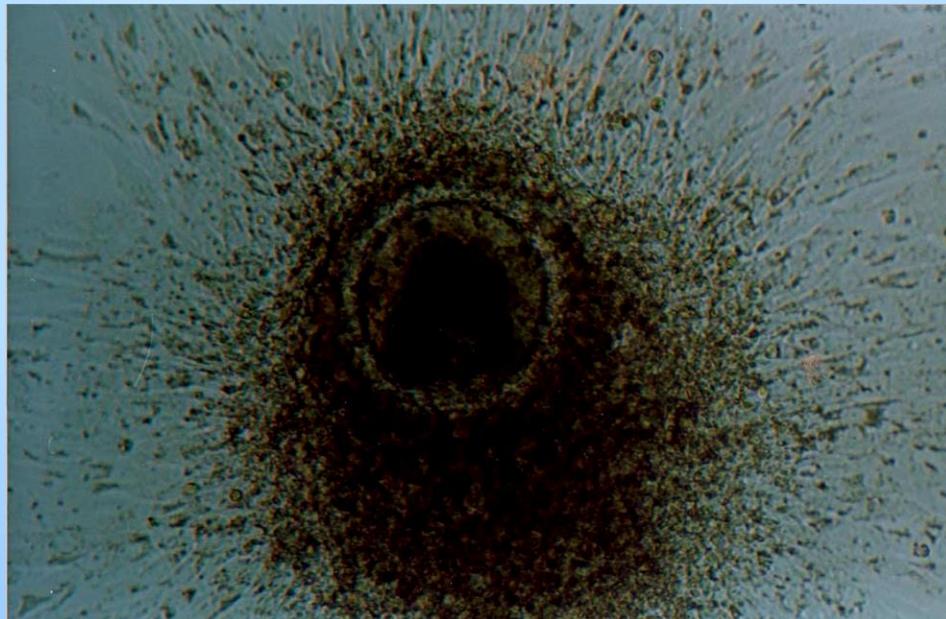
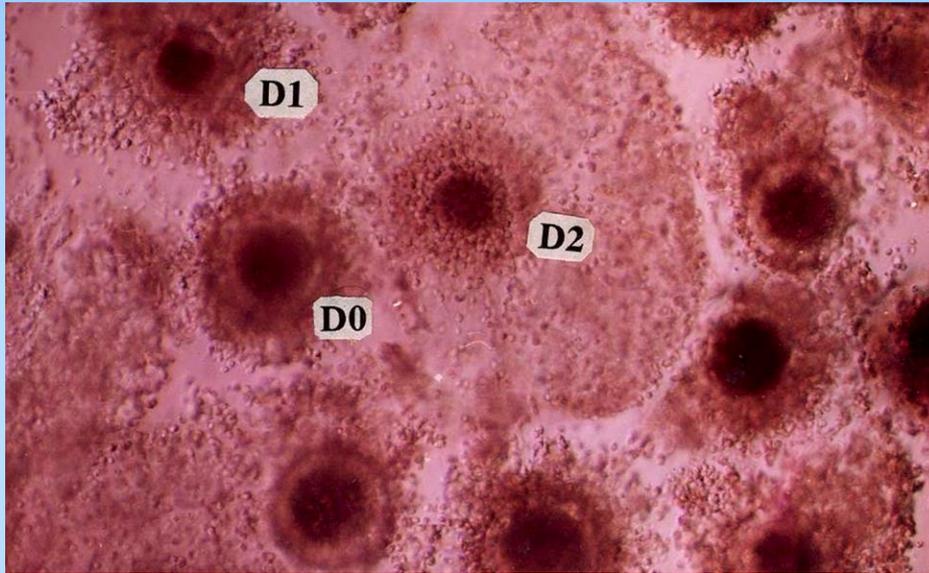
Enlargement of cumulus mass to at least x 3 diameters away from the ZP

Grade II

Enlargement of cumulus mass to at least x 2 diameters away from the ZP

Grade III

Cumulus cells remain tightly adhered to the ZP



Processing of spermatozoa

1. Artificial capacitation

Heparin (10-100 $\mu\text{g/ml}$)

BSA

Follicular fluid

Calcium ionophore A23187

Platelet-activating factor

2. Increasing motility

Caffeine (5-10 nM/ml)

Theophylline

3. Screening the bulls

4. Sperm-oocyte incubation

Sperm-oocyte incubation

Medium

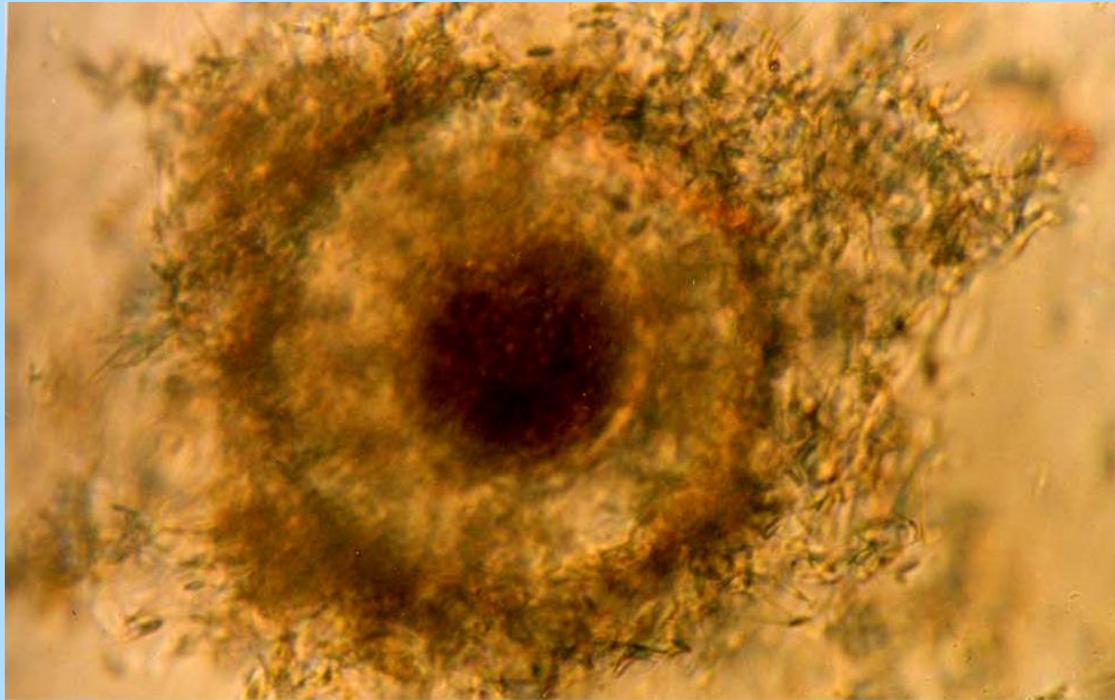
Brackett and Oliphant medium or Tyrodes medium supplemented with albumin, lactate and pyruvate (TALP)

Sperm concentration

1-12 million sperm/ml

Incubation time

6-24 h



Evaluation of fertilization rate

Staining with Giemsa or Aceto-orcein

Advantage

Enables checking the incidence of polyspermy

Disadvantage

Oocyte gets killed

Cleavage rate

Advantage

Oocyte does not get killed

In vitro culture (IVC) of cleaved embryos

Culture Conditions: Same as those for IVM

Time: Up to 9 days post insemination (psi)

Media: Generally TCM-199
Preferably simple media

Serum: 10 % FBS

Simple media

1. **Synthetic oviductal fluid (SOF)** mSOF, mSOFaa
2. **Charle's Rosenkrans (CR) medium** CR1, CR2, CR1aa, CR2aa, mCR2aa
3. **Chatot Ziomek Bavister (CZB) medium**
4. **Potassium Simplex Optimization medium (KSOM)**
5. **Hamster embryo culture medium (HECM)**

Composition of mCR2aa

S. No.	Chemical	Molarity (mM)
1	Water	-
2	NaCl	108.3
3	NaHCO ₃	24.9
4	NEAA	1.0 ml/100 ml
5	EAA	2.0 ml/100 ml
6	Glutamine	1
7	KCl	2.9
8.	Hemicalcium lactate	2.5
9	Na Pyruvate	0.5
10	Glycine	0.5
11	Alanine	0.5
12	Glucose	1
13	Phenol red	5 µg/ml
14	Gentamycin	50 µg/ml
15	BSA	0.6%

Composition of mSOFaa

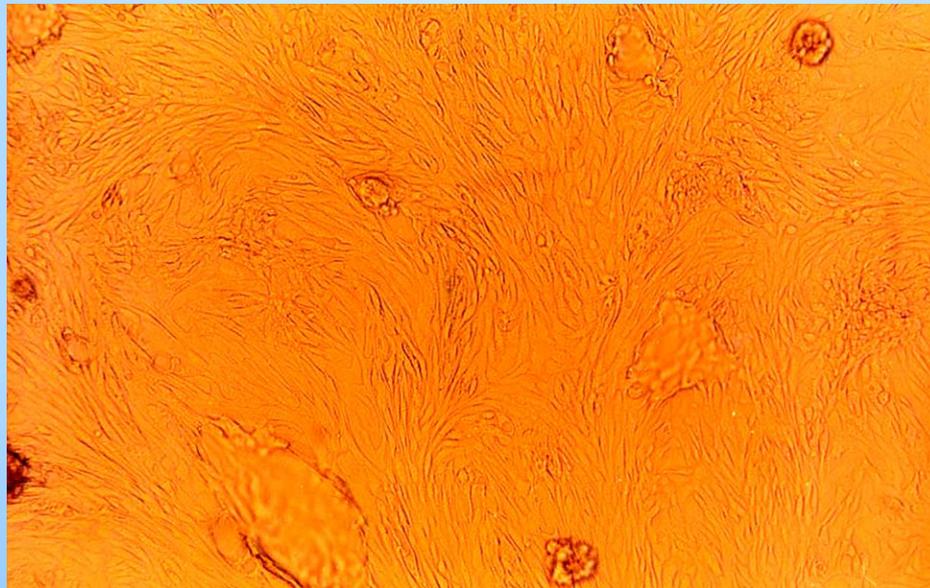
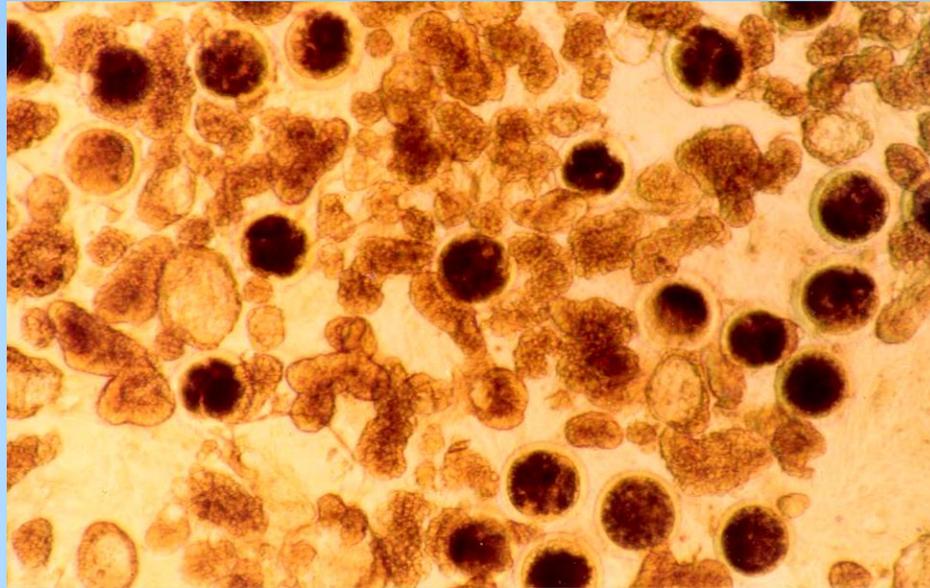
S. No.	Chemical	Molarity (mM)
1	Water	-
2	NaCl	107.70
3	NaHCO₃	25.07
4	NEAA	1.0 ml/100ml
5	EAA	2.0 ml/100 ml
6	Glutamine	1
7	KCl	7.16
8	CaCl₂	1.71
9	Glucose	1.50
10	KH₂PO₄	1.19
11	Phenol red	5 µg/ml
12	Gentamycin	50 µg/ml
13	Na Pyruvate	0.33
14	MgCl₂-6H₂O	0.49
15	Na Lactate	3.30
16	BSA	0.8%

Overcoming developmental block

Co-culture with oviductal epithelial cells

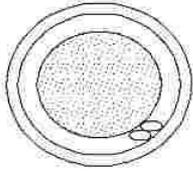
Co-culture with BRL, Vero cells

IVC in simple media



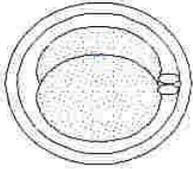
Days after oestrus

0-2



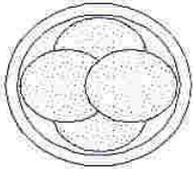
1 Cell

1-3



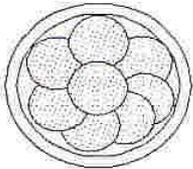
2 Cell

2-3



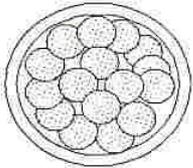
4 Cell

3-5



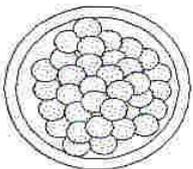
8 Cell

4-5



16 Cell

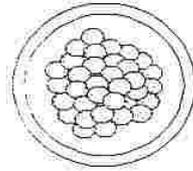
5-6



Morula

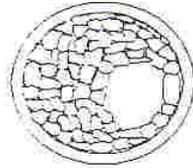
Days after oestrus

5-7



Tight morula

7-8



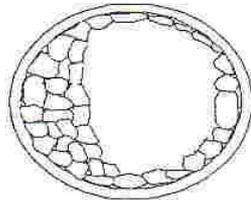
Early blastocyst

7-9



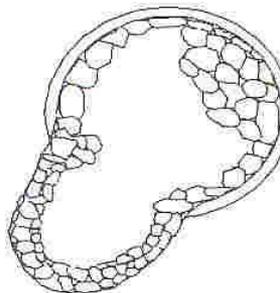
Blastocyst

8-10



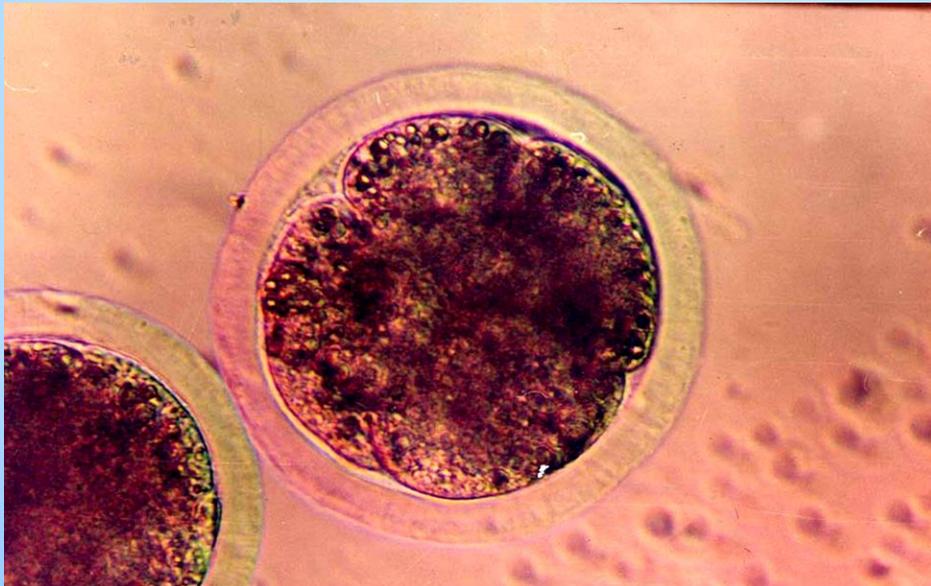
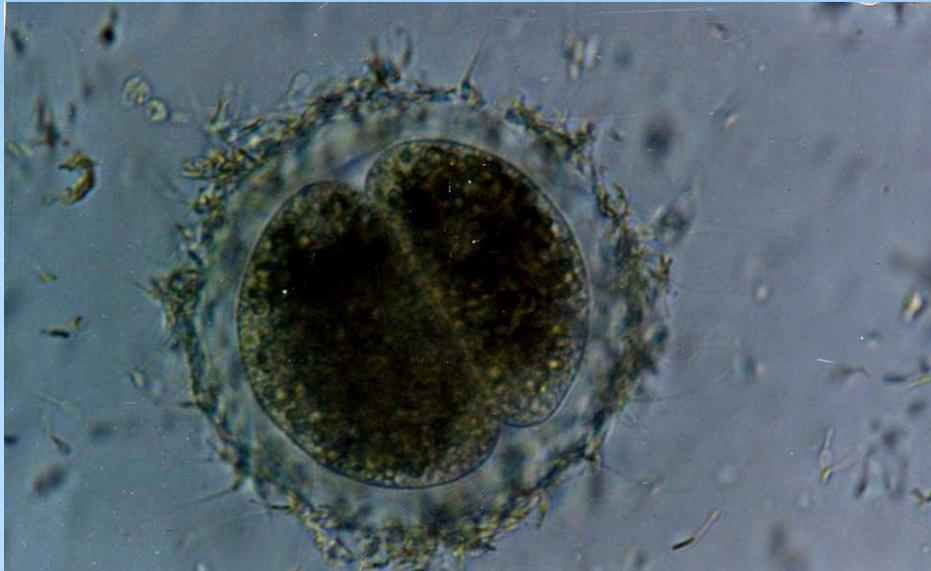
Expanded blastocyst

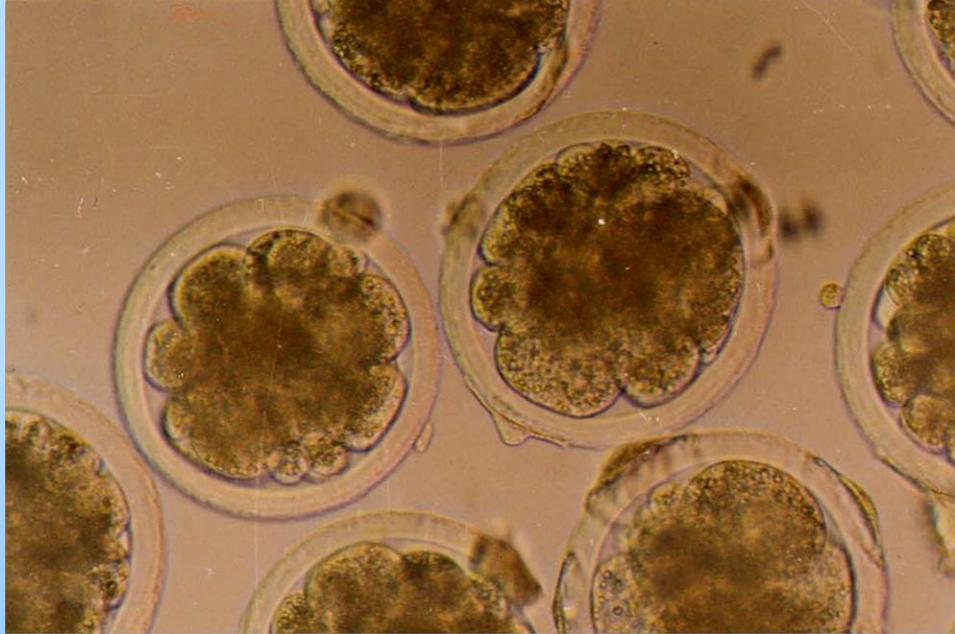
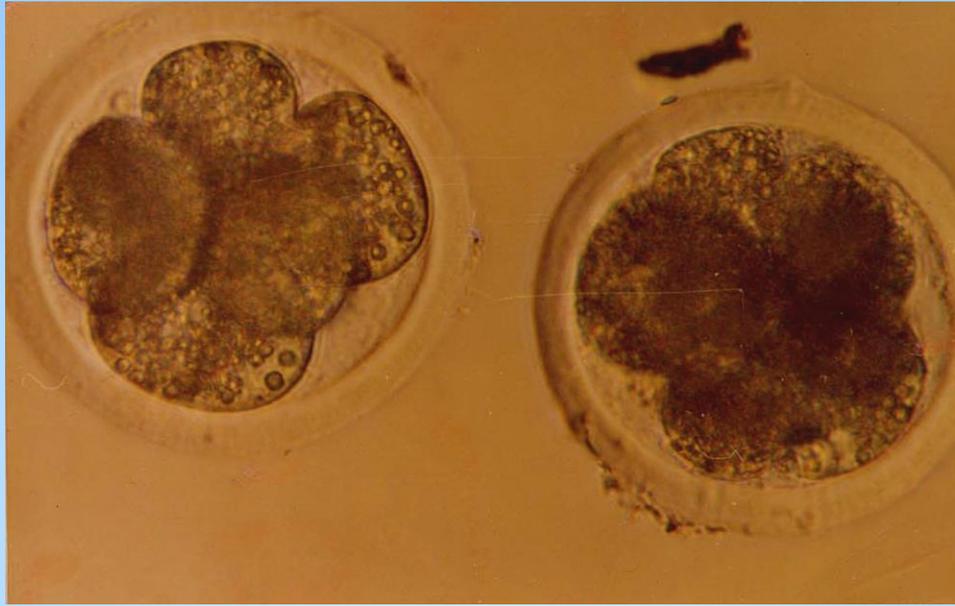
9-11



Hatching blastocyst

Stages in the development of bovine embryos





Developmental stages of buffalo embryos

Morula

Embryo with >32 cells, individual blastomeres are difficult to discern, cellular mass occupies most of the perivitelline space

Compact morula

Morula with compaction of blastomeres clearly visible, individual blastomeres coalesced.

Early blastocyst

Formation of blastocoele just started, visual differentiation between trophoblast and the ICM may be possible

Blastocyst

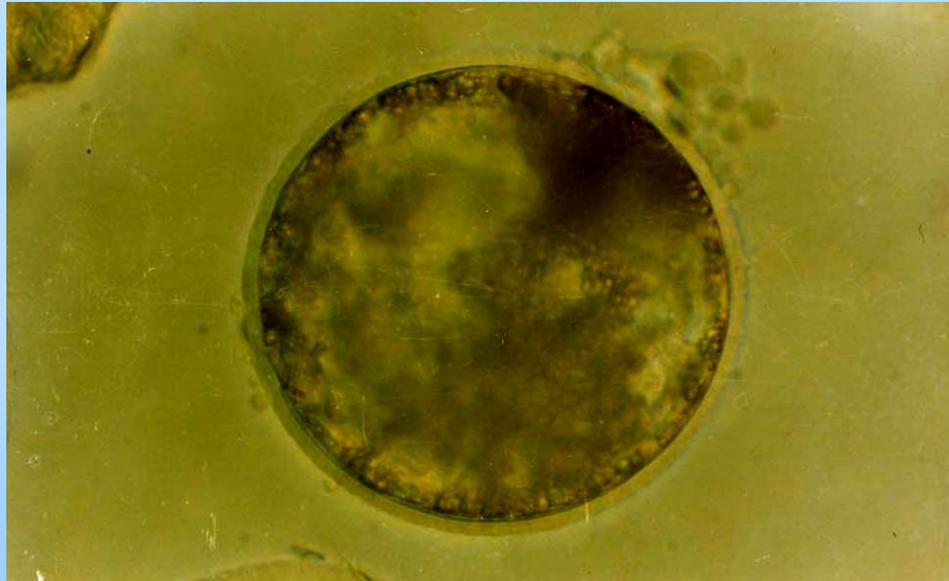
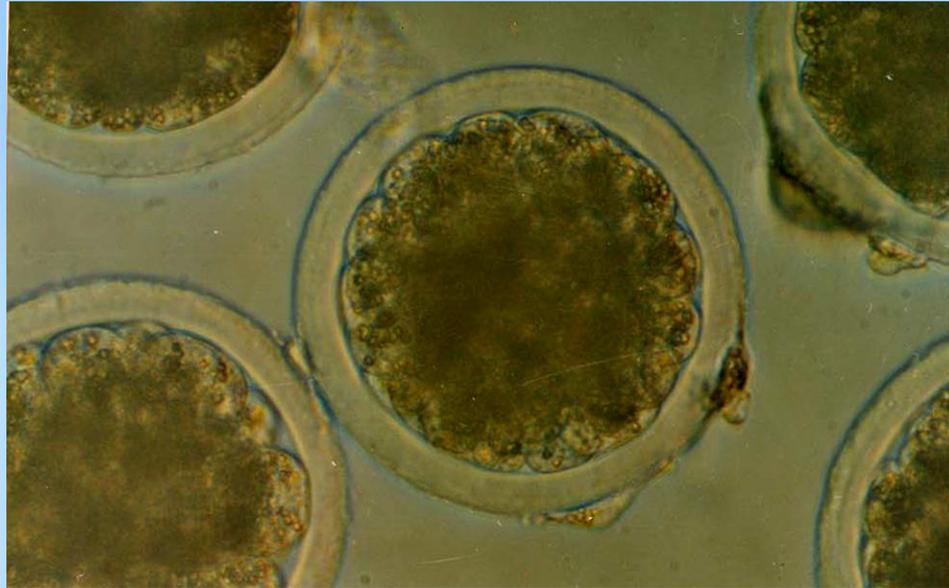
Well defined blastocoele. The trophoblast layer and ICM can be clearly differentiated

Expanded blastocyst

Zona thinning clearly discernable

Hatched blastocyst

Blastocyst partly or completely out of the ruptured zona





Evaluation of embryo quality

- 1. Development in relation to time**
- 2. Total cell number**
- 3. Hatching rate**