Cattle embryo frontiers

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Conventionally, the criteria for selecting bovine in vitro fertilized embryos for transfer are based on morphological quality at the time of transfer. However, this approach is widely considered extremely subjective and inadequate. Furthermore, the pregnancy outcomes of blastocysts estimated by evaluators to be morphologically good to excellent remain low (37–52%). Therefore, novel criteria allowing objective and reliable selection of embryos for transfer are required to advance bovine IVF technology.

Following ovum pick-up (OPU), which collects a limited number of oocytes, culture system for undergoing in vitro oocyte maturation (IVM), in vitro fertilization (IVF) and embryo culture (IVC) individually appear to be practical. Recently, we developed a micro-well culture dish based on the well-of-the-well system, which allows tracking of individual embryo with time-lapse cinematography (TLC) observation. TLC is an effective method for continuous imaging of embryo development in vitro, allowing analysis of the developmental kinetics, blastomere number, symmetry of cell division, and the extent of cytoplasmic fragmentation, which have been used to select the best embryos for transfer in human assisted reproductive technologies. In cattle, these criteria are possible predictors for developmental competence to the blastocyst stage, but rarely used to selecting embryos for transfer.

In addition to the studies using TLC, measurement of embryo metabolism parameters such as oxygen consumption has attracted attention in predicting embryonic development. Modified scanning electrochemical microscopy (SECM) appears to be a reliable, noninvasive, and highly sensitive method for measuring oxygen consumption in individual embryos.

Here we sought to identify various prognostic factors that allow prediction of bovine blastocyst qualities such as embryonic cell number, apoptosis incidence, hatchability, chromosome abnormalities, and gene expression using a combination of TLC imaging with micro-well culture dish and oxygen consumption measurement. Subsequently, to examine the practical advantage of the identified prognostic factors for predicting post-transfer viability, OPU-IVF–derived blastocysts that were assessed by individual or multiple factors were transferred to recipient cows.

Food and Fertilizer Technology Center
(FFTC)- for Asian and Pacific Region-

What is FFTC for?

- FFTC IS A REGIONAL INFORMATION CENTER

to collect and disseminate practical and technical information in agriculture, toward contributing to the mission:

Achievement of increased productivity and incomes for small-scale farmers

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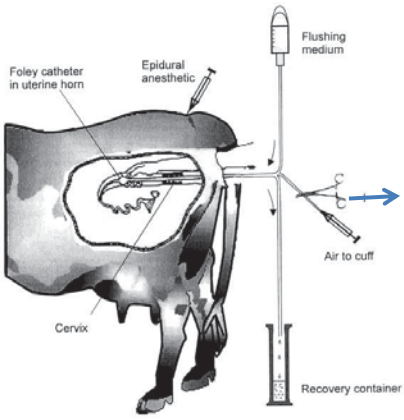
Cattle embryo frontiers

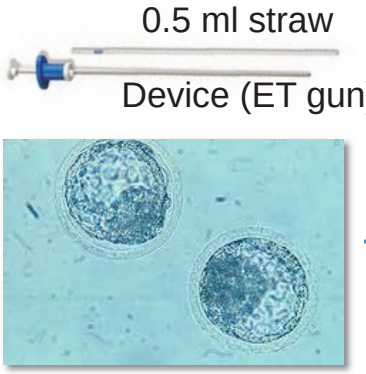
- In vitro embryo production (IVEP)
- Ovum Pick-Up (OPU)
- Selection of in vitro produced embryos using Real-time culture cell monitoring system

Embryo transfer (ET) in cattle

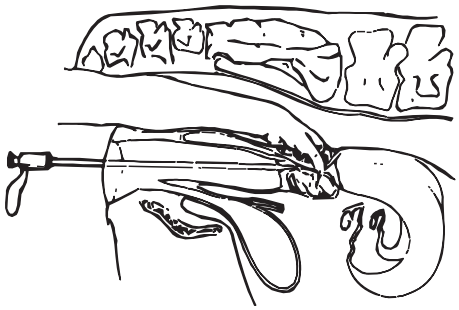
Embryo transfer: removing (flushing) embryos from tract (non-surgically) and transfer to surrogate mothers



Flushing



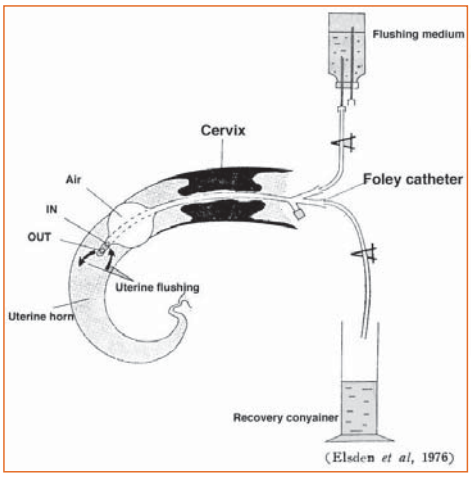
Embryos



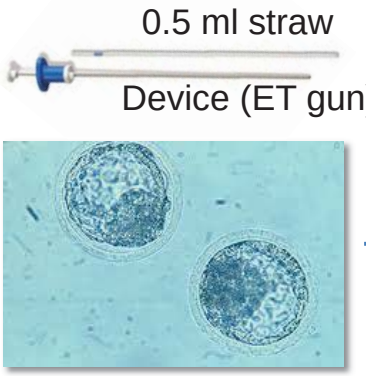
Transfer

Embryo transfer (ET) in cattle

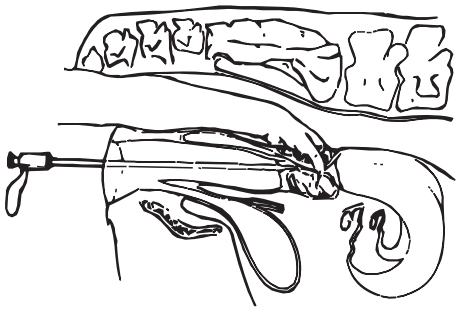
Embryo transfer: removing (flushing) embryos from tract (non-surgically) and transfer to surrogate mothers



Flushing



Embryos



Transfer

体外　　胚　　生産

In vitro embryo production (IVEP)

"In glass"
(under laboratory conditions)

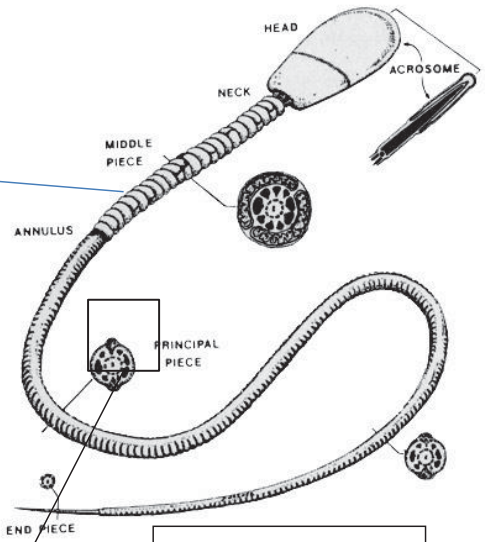
Oocyte **maturation**, sperm capacitation, **fertilization** and **embryo development** (to a level that allows transfer of resultant embryo)

体内

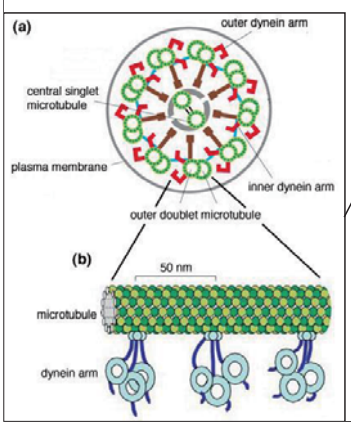
In vivo= "within living (organism)"

Structure of a spermatozoon

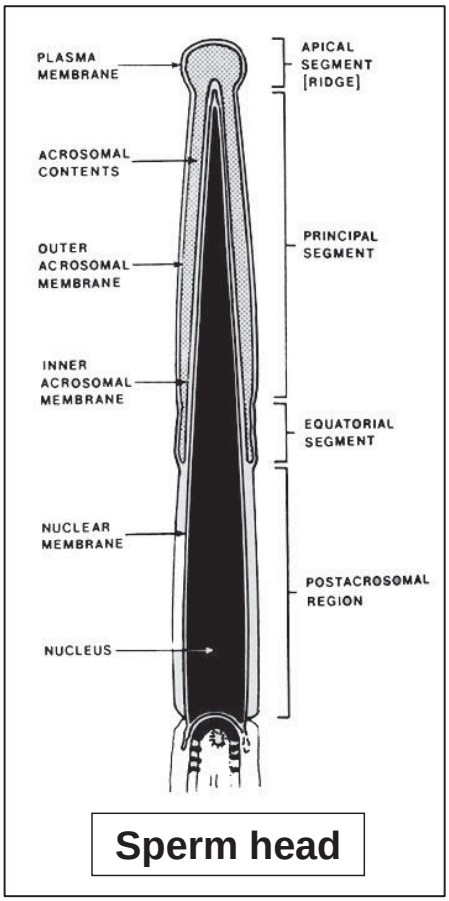
Energy production:
Mitochondria



60μm long

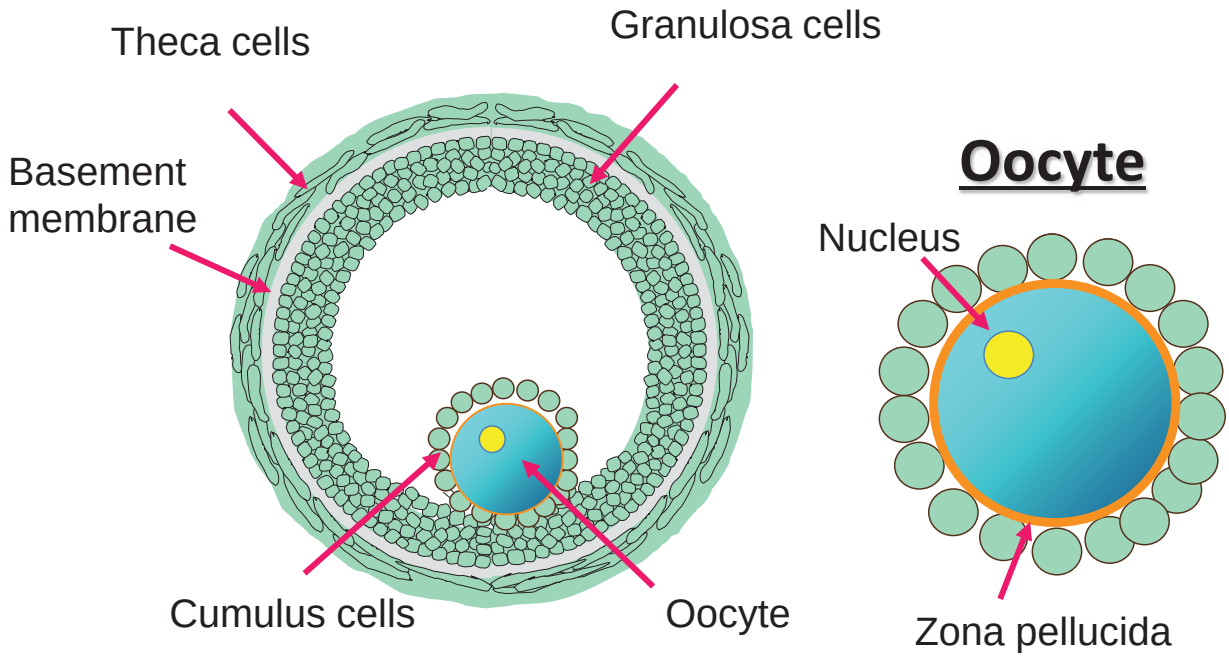


Tail movement:
1 central + 9 outer pairs of microtubules



Sperm head

Follicle



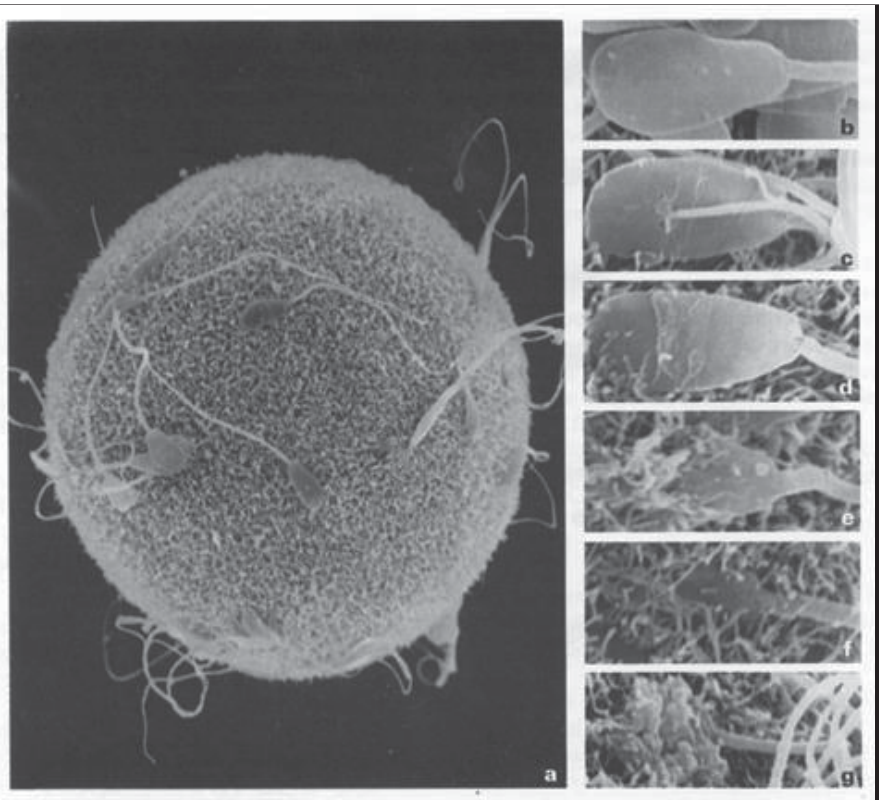


Fig. 3. Scanning electron micrographs of phases of the acrosome reaction and the internalization of the sperm head into the ooplasm in cattle. (**a**) Bovine ovum 0·5 h after insemination *in vitro*. Before insemination the cumulus investment and the zona pellucida were enzymically removed. ×1100. (**b**) Sperm head with intact acrosome. ×4200. (**c**) Sperm head, in which the acrosome reaction is initiated by fenestration of the membrane coats in front of the equatorial segment. ×4200. (**d**) Sperm head, in which the acrosome reaction is completed. The acrosomal region is now covered with the inner acrosomal membrane. ×4500. (**e**) The internalization of the sperm head is initiated and the ovum microvilli appear to contact the equatorial segment and the postacrosomal region. ×5000. (**f**) The sperm head gradually disappears from the ovum surface. ×5000. (**g**) The microvilli covering the site of internalization become more bulbous. ×3300.

Sperm capacitation

- 1951 Austin – rat /Chang – rabbit:

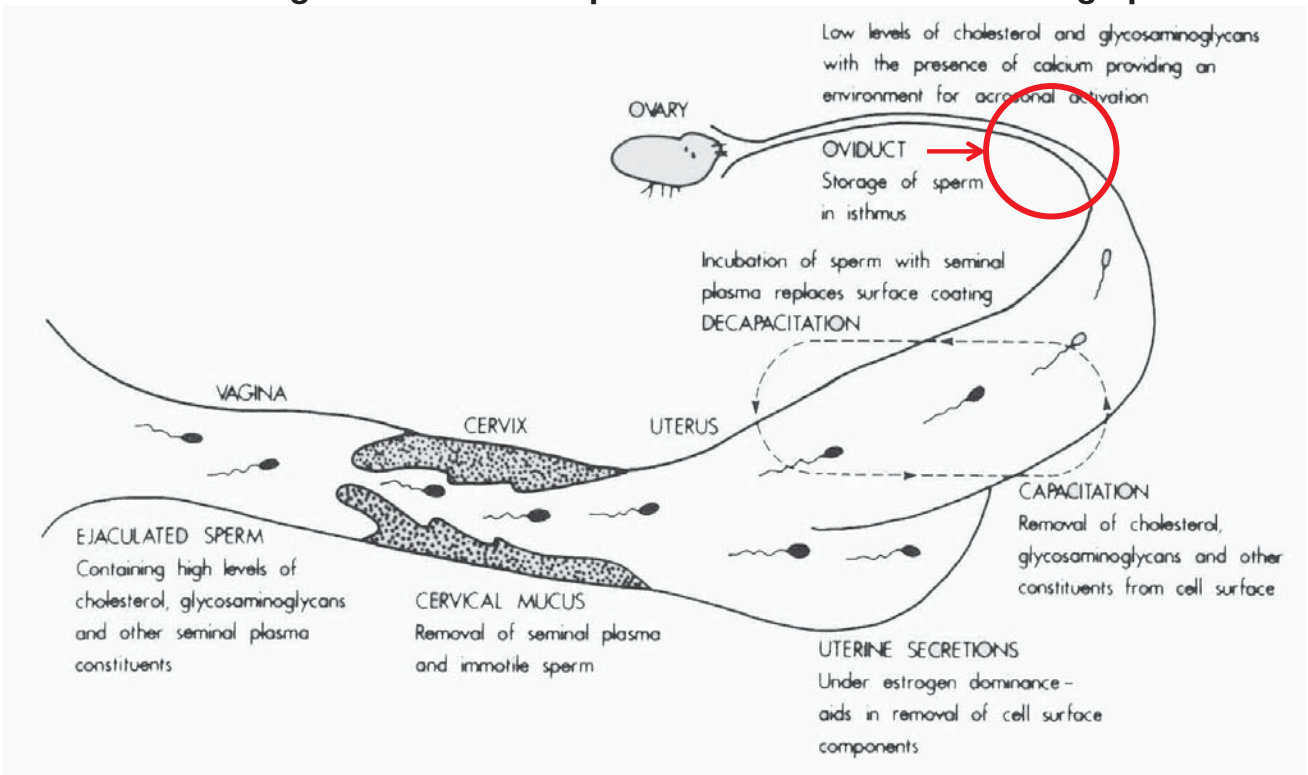
  Fertilization did not occur until several hours after sperm are placed in the uterus or oviduct

Ejaculated sperm must acquire the ability to penetrate and activate the oocyte = **capacitation**!

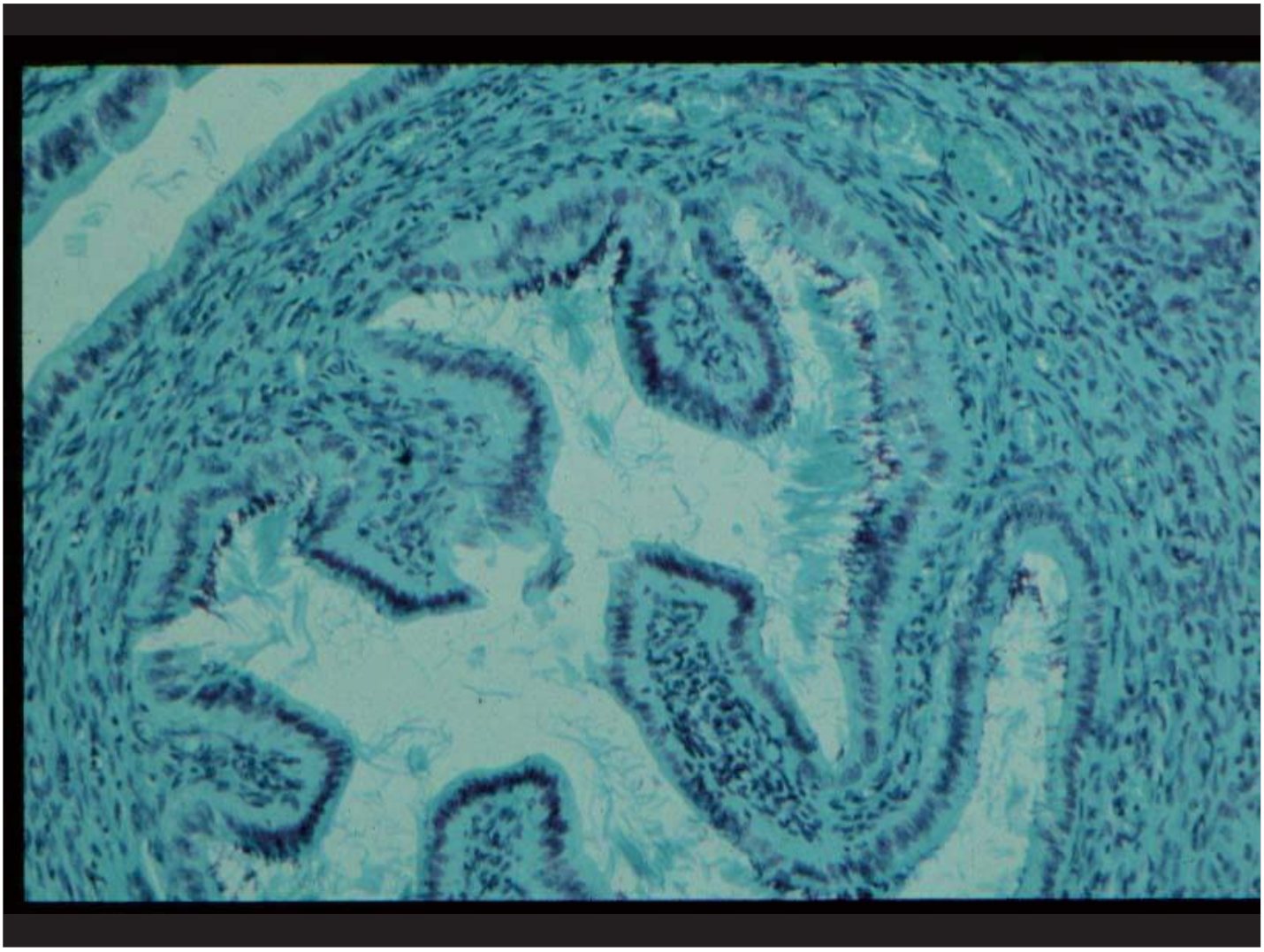
It occurs under exposition of sperm to cervical/uterine environment

Sperm transport

- **Movement of sperm from the site of ejaculation to oviduct (fertilization site)**
- **Functional linkage between the reproductive tract and fertilizing sperm**

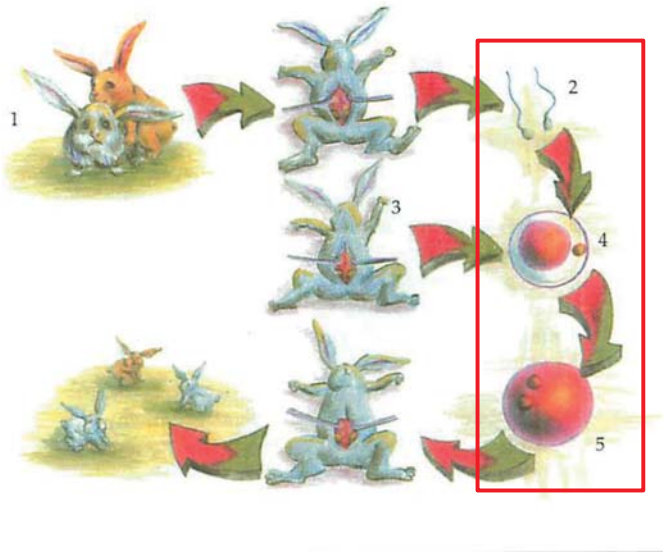


Events in sperm capacitation during transport through the female genital tract.



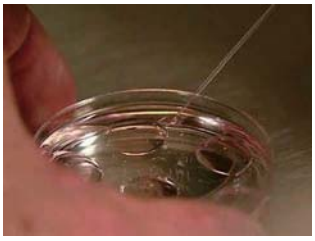
In Vitro Fertilization (IVF)

- 1959 - *Chang* – first successful IVF (rabbit)



IVF= fertilization outside the body (“in glass”)

In Vitro (= in glass)



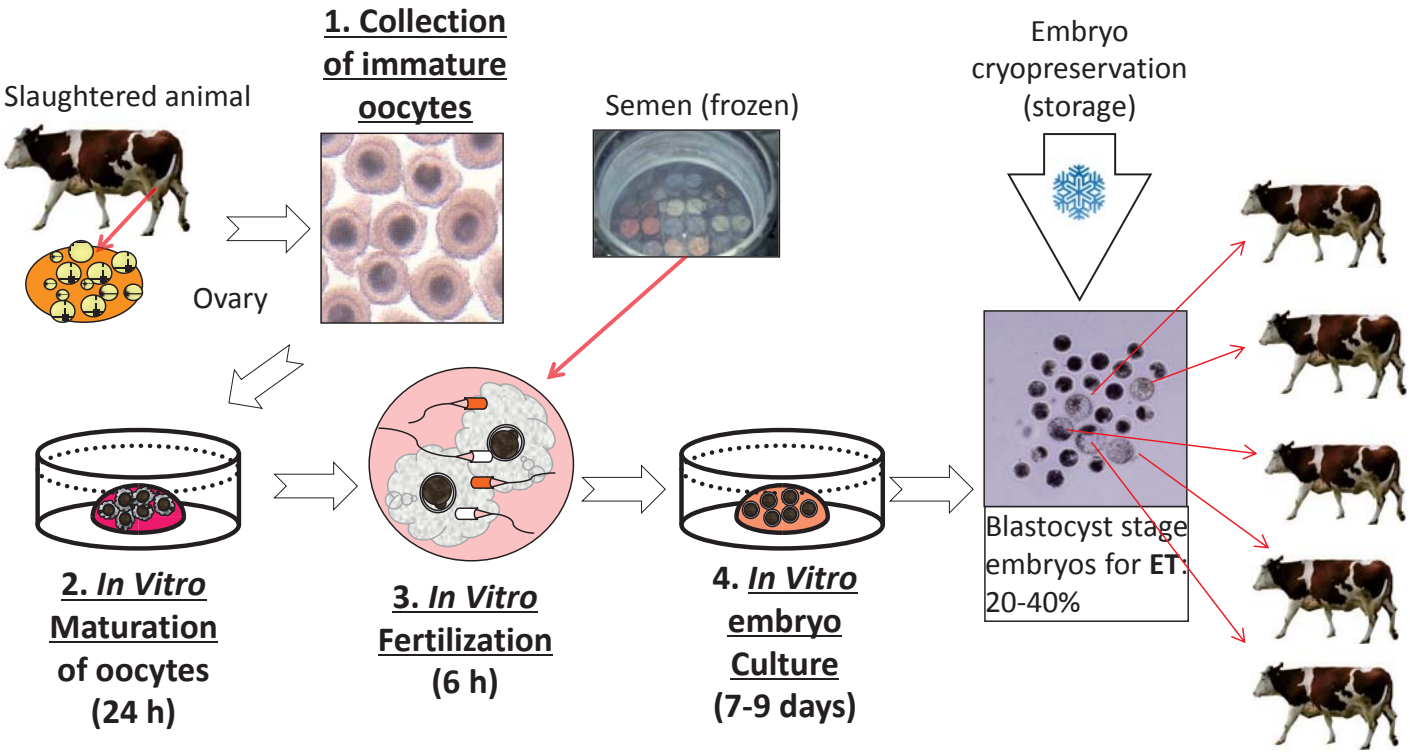
Petri dish (plastic) with culture medium

Source: Egy klonozo vallomasai, (Vajta G.) 2004 Noran

Dr. M.C. Chang visited Kyoto Univ in 1983

Dr. C.R. Austin visited NILGS in 1987

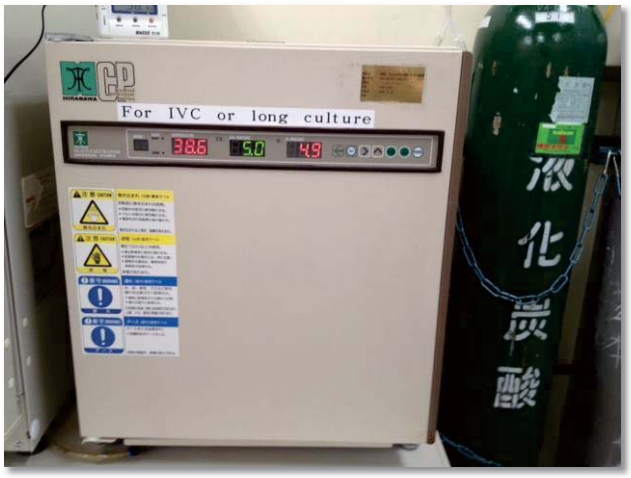
The overview of IVEP protocol (cattle)



The IVEP lab

Incubators

- high humidity
- 38-39 °C
- 5% CO_2
- 5% O_2 (for embryo culture)



The point of IVEP

General aim (human)

➢ To overcome barriers of natural fertilization (such as low sperm concentration or obturated oviduct) > **infertility treatment**

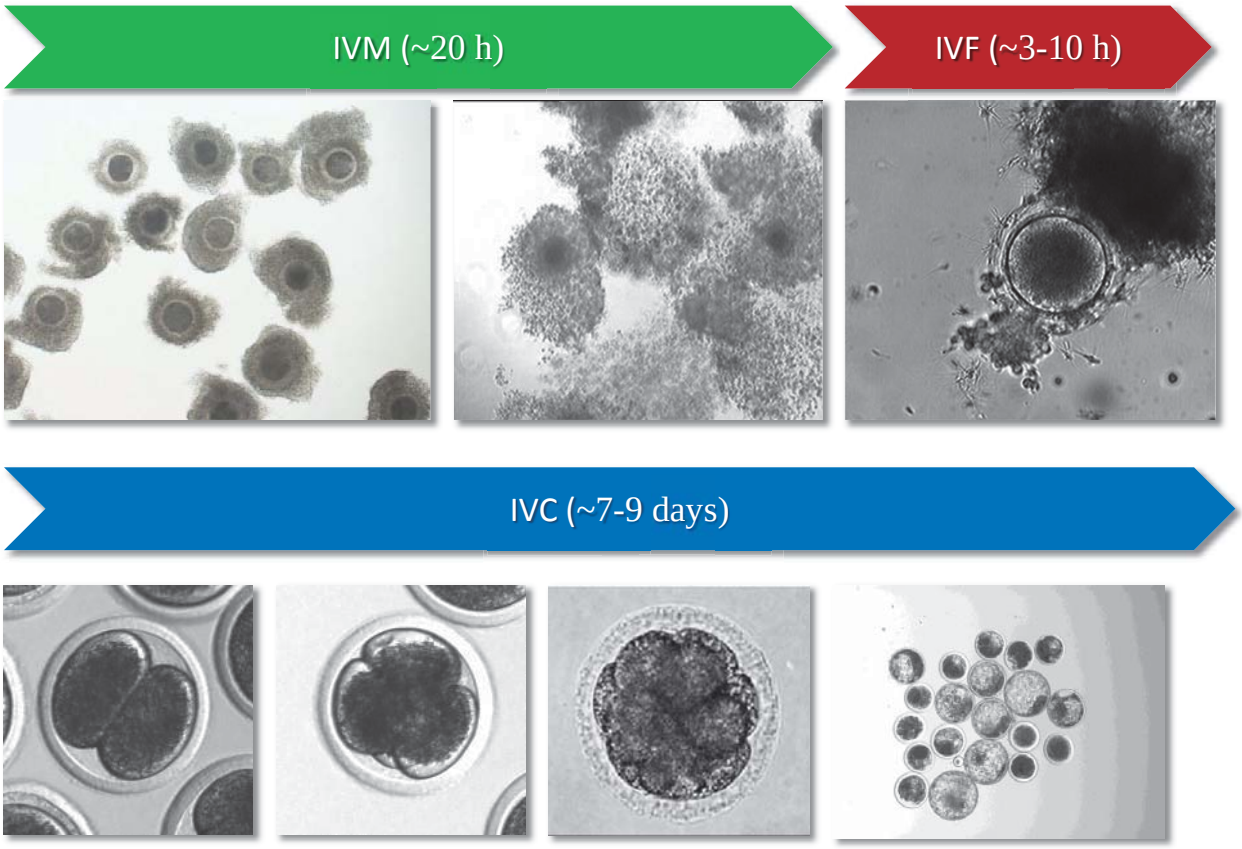
Animal Industry

➢ Utilization of abandoned oocytes in high performing females

➢ Slaughterhouse waste (ovaries) can be used as oocyte donors
• No need for oocyte donor animals
• Large numbers of embryos at low price > good base material for embryo research (animal models for humans).

➢ An efficient way to use frozen and sex/determined sperm (which are not so applicable for AI)

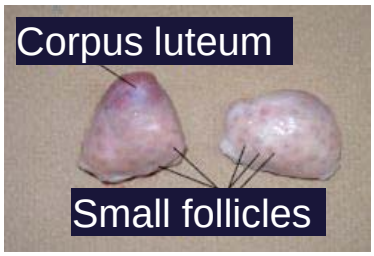
Time intervals of IVEP procedures in cattle

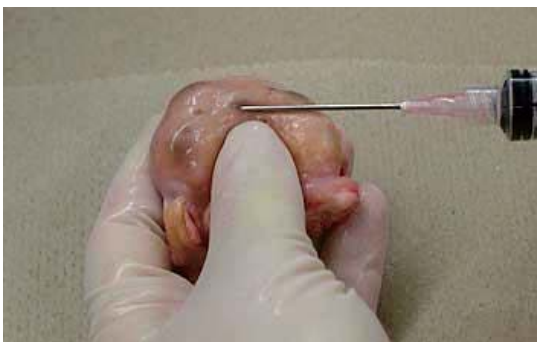


1. Oocyte collection

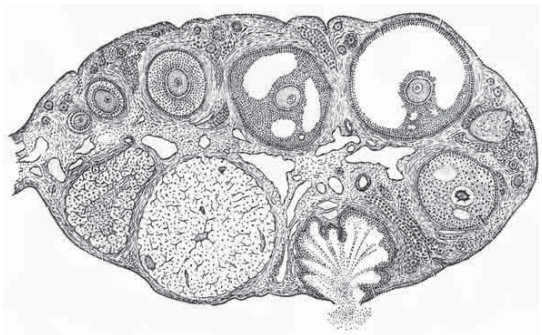
1.1 Slaughterhouse ovaries

- Cheap source for large quantities of oocytes
- Cow: ~10 oocytes/ovary
- Methods: aspiration or slicing

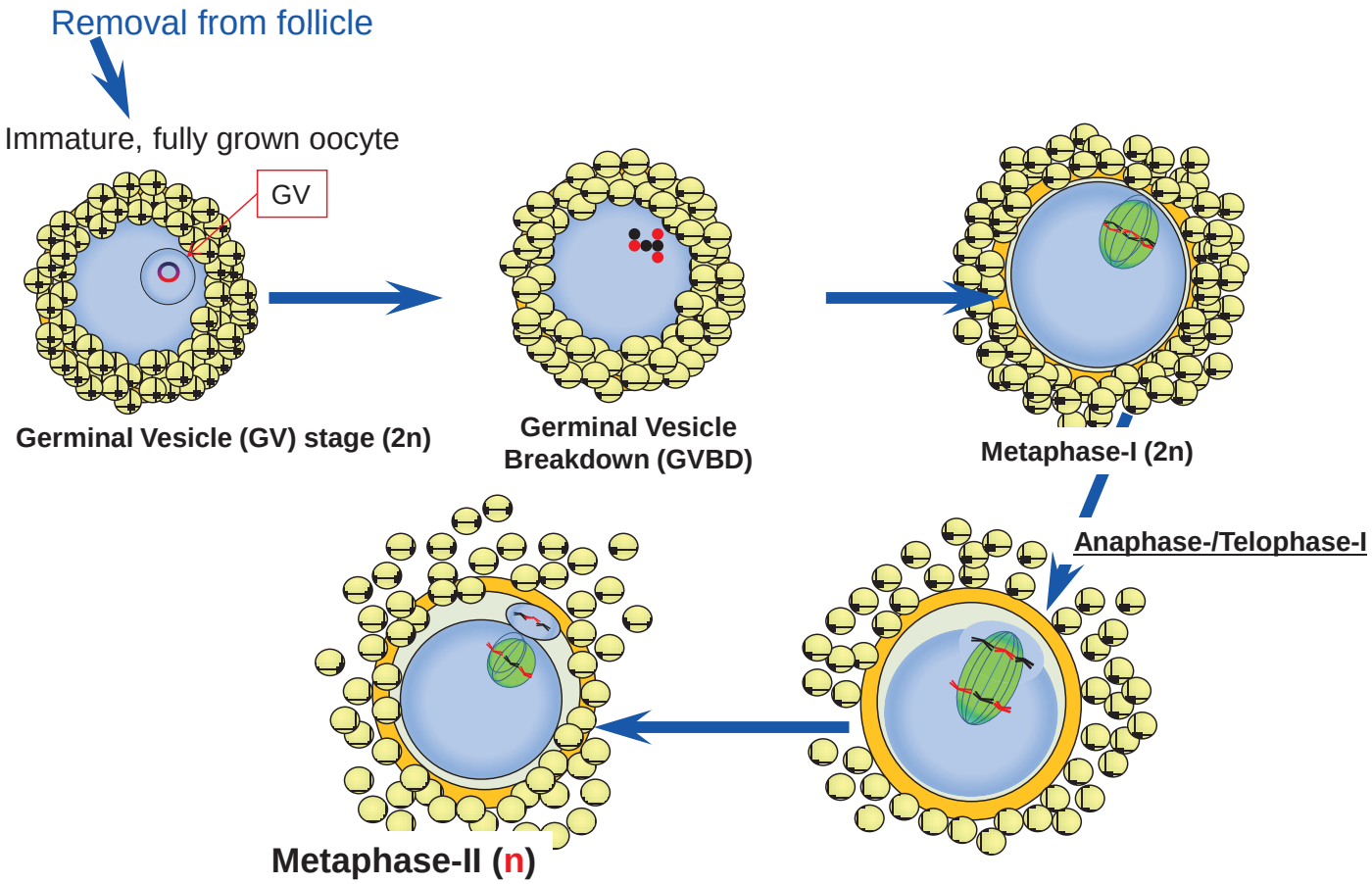




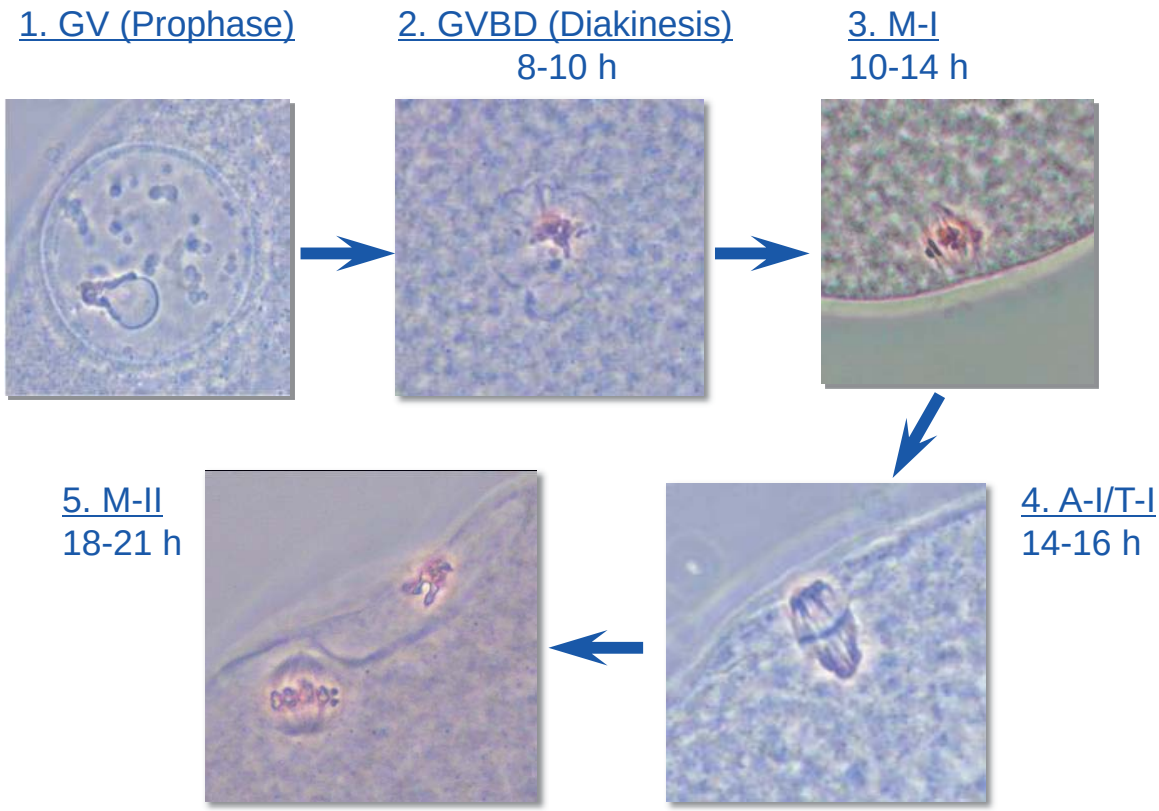
Method A) Aspiration by needle/syringe



2. In Vitro Maturation (IVM) of oocytes

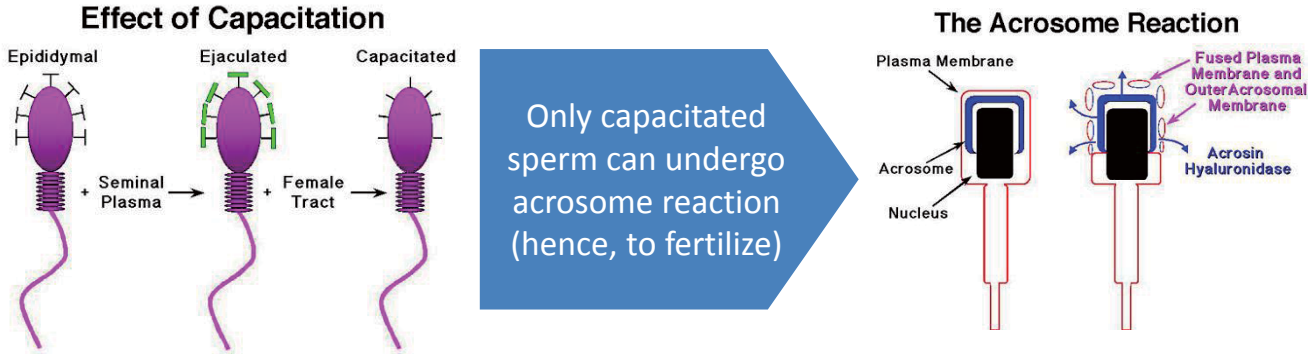


Nuclear events in oocytes during IVM



3. In Vitro Fertilization (IVF)

- Possible by fresh, frozen, ejaculated or epididymal sperm as well
- 1959 - *Chang* – successful *In Vitro* Fertilization requires sperm capacitation > **Basis of IVF**



Artificial induction of sperm capacitation during IVF

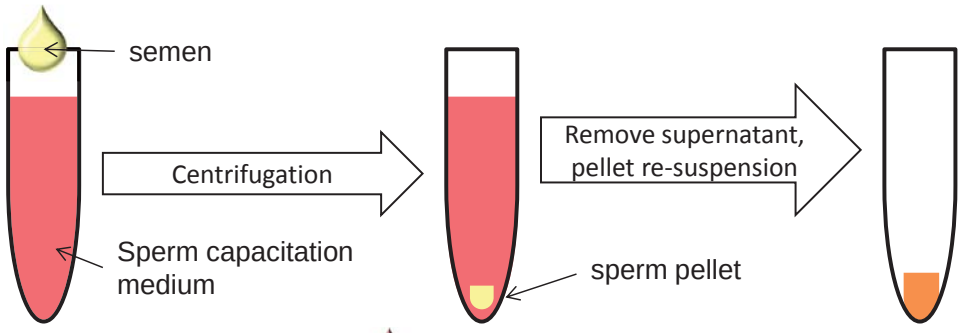
Species	Sperm capacitation
Cattle	Heparin, Caffeine, Taurine, Ca^{2+} ionophore
Buffalo	Heparin
Sheep	Heparin, Caffeine, Ca^{2+} ionophore
Goat	Heparin
Pig	Caffeine, Theophlylline

Sperm treatment methods before IVF

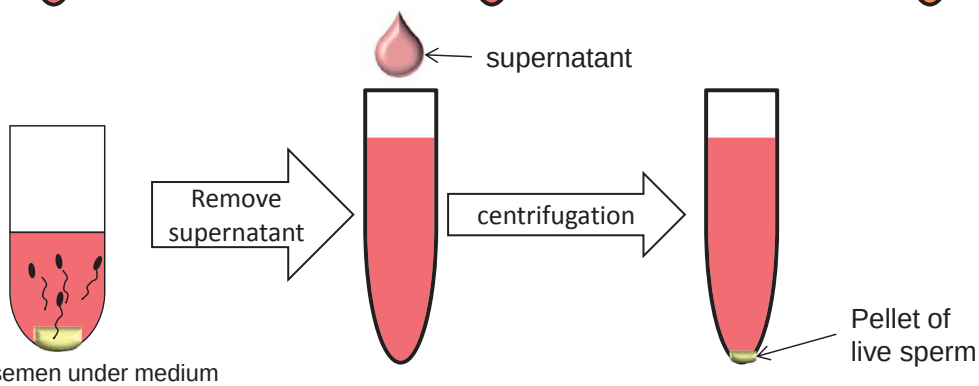
Aims

1. Remove extender, seminal plasma
2. Enhance capacitation
3. Selection of live spermatozoa

A) simple washing

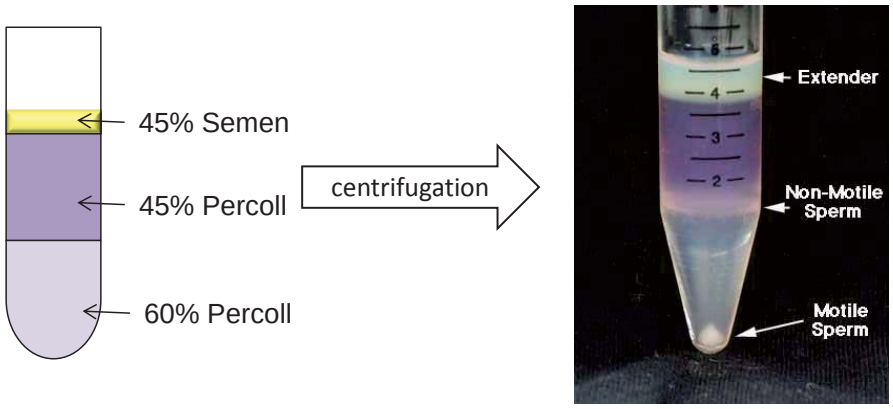


B) "Swim up"



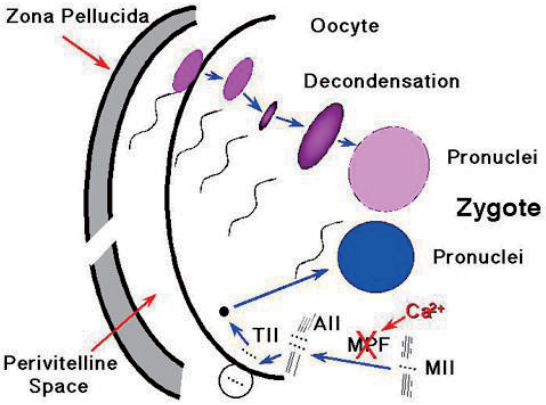
1. Put semen under medium
2. Incubate for 30 min

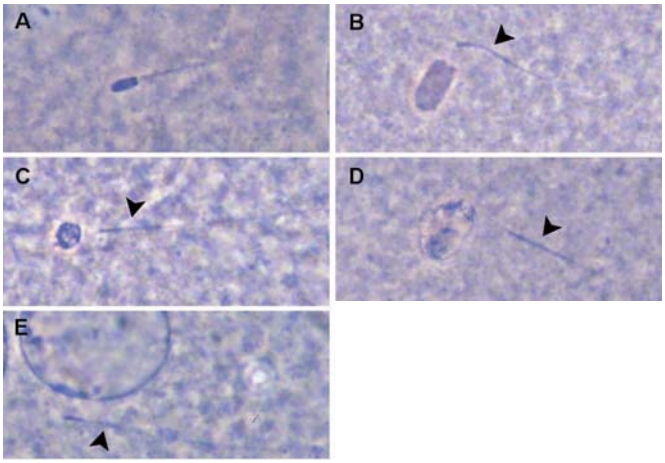
C) Density gradient centrifugation



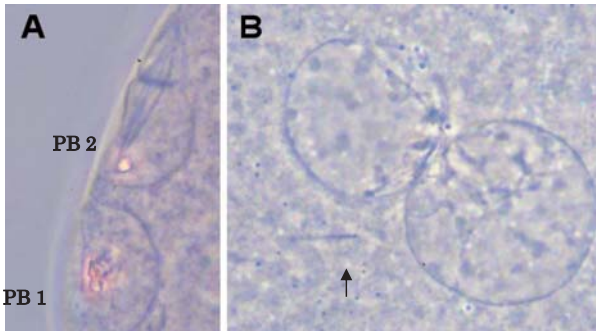
Percoll: silica colloid (15-30 mm) coated with polyvinylpyrrolidone

Nuclear events during IVF





1. Male pronucleus formation



2. Extrusion of the second polar body **3. Female pronucleus > syngamy**

4. In Vitro Culture (IVC) of embryos

Following IVF, the zygotes can be cultured for further development before they are transferred into the uterus (or cryopreserved).

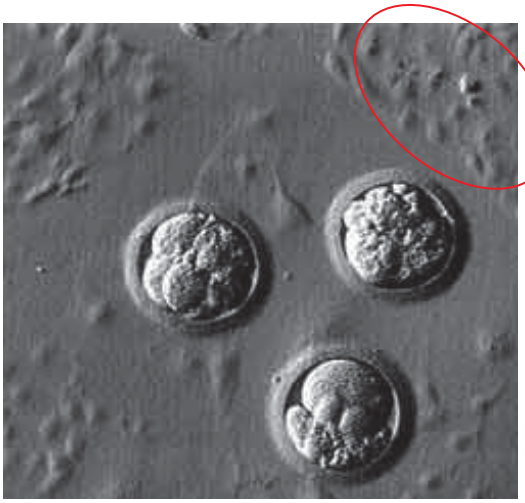
Systems of in vitro culture of embryos

1) Transfer to the ligated oviduct of a temporary recipient (sheep, rabbit) for 4-5 days.
   Advantage: perfect environment in the oviduct; Disadvantage: difficult + not defined

2) Co-culture in vitro with somatic cells (oviductal epithelial cells, granurosa cells ctc. in culture medium for 7-8 days.
   Advantage: supply of growth factors from co-culture cells; Disadvantage: not defined

3) Culture in the simple medium without somatic cell support.
   Advantage: defined, easy to use; Disadvantage: not perfect…

Co-culture of embryos with other cells



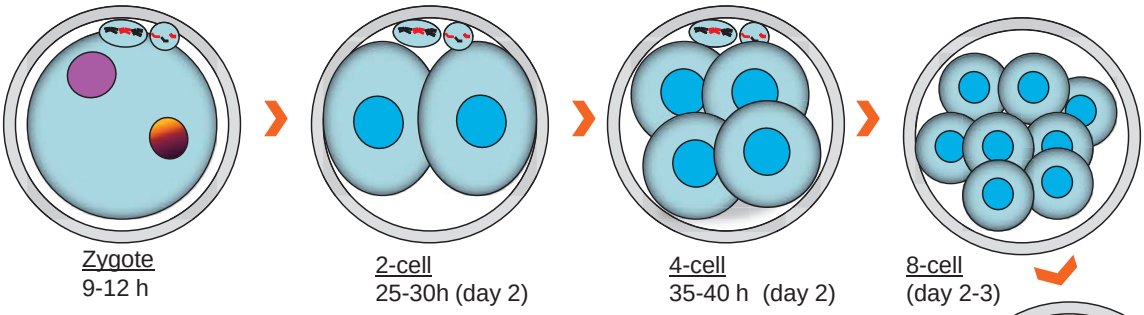
Oviduct epithelium cells
- Consume O_2 > reduce oxidative stress
- Produce growth factors for embryos

Disadvantage: production of undefined factors > not expedient for scientific purposes…

Due to its disadvantage and recent advances in the development of culture media, co-culture is rarely used these days…

http://www.advancedfertility.com/coculture.htm

Developmental stages of embryos during IVC



Zygote
9-12 h

2-cell
25-30h (day 2)

4-cell
35-40 h (day 2)

8-cell
(day 2-3)

16-cell
(day 4)

Morula (compaction)
Day 5-6

Blastocyst (blastulation)
Day 6-7

Blastocyst (Expanded)
Day 7-8

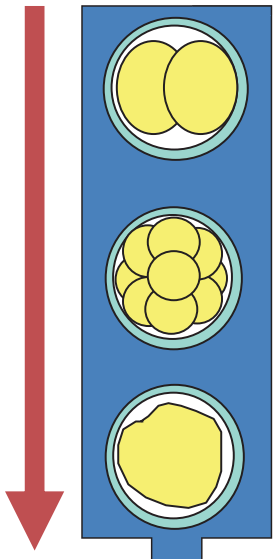
Blastocyst (Hatching)
Day 8-10

Stages for ET (cow)!

Culture stress: a cause of developmental arrest in cultured embryos (in most species)

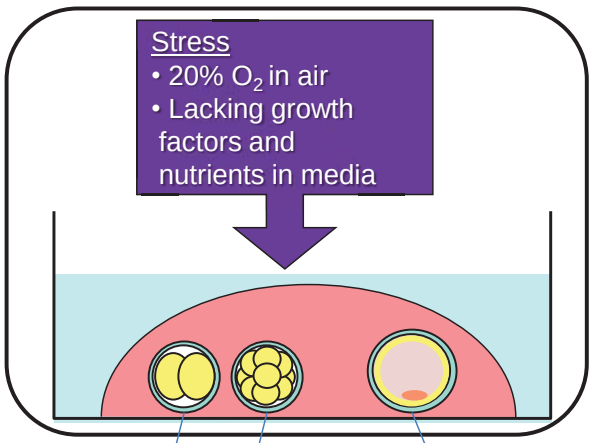
In vivo development

In vitro development (defined)



Oviduct
- Nutrients (known and unknown)
- Proteins, growth factors (known
- and unknown)
- Low O_2 tension (5~7%)
- High CO_2 tension (5%)

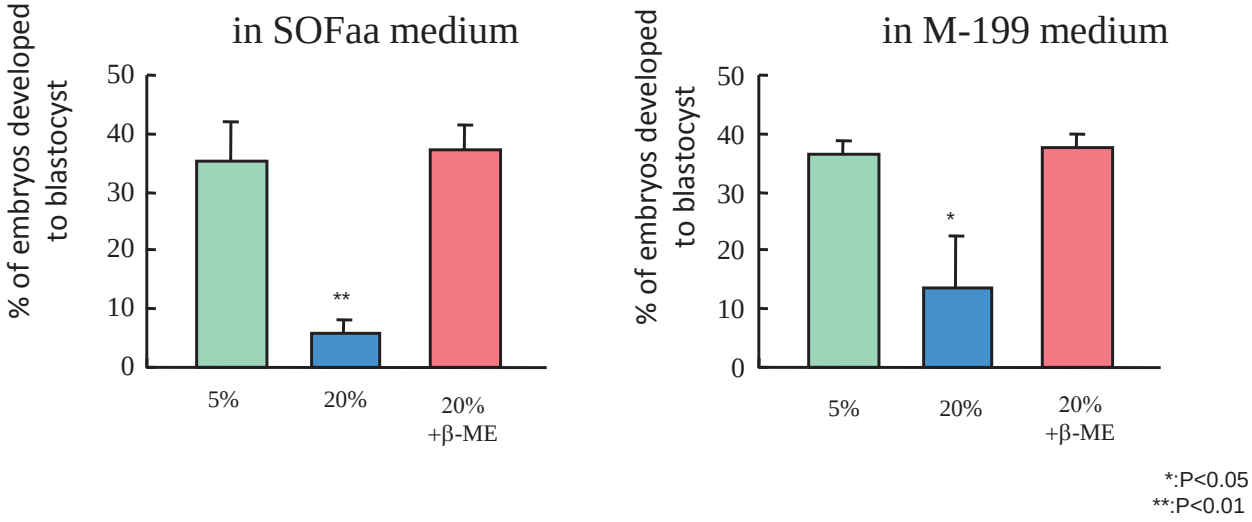
Blastocyst



Developmental arrest!

Blastocyst with low cell number!

Reducing agents promote embryo development under oxidative stress

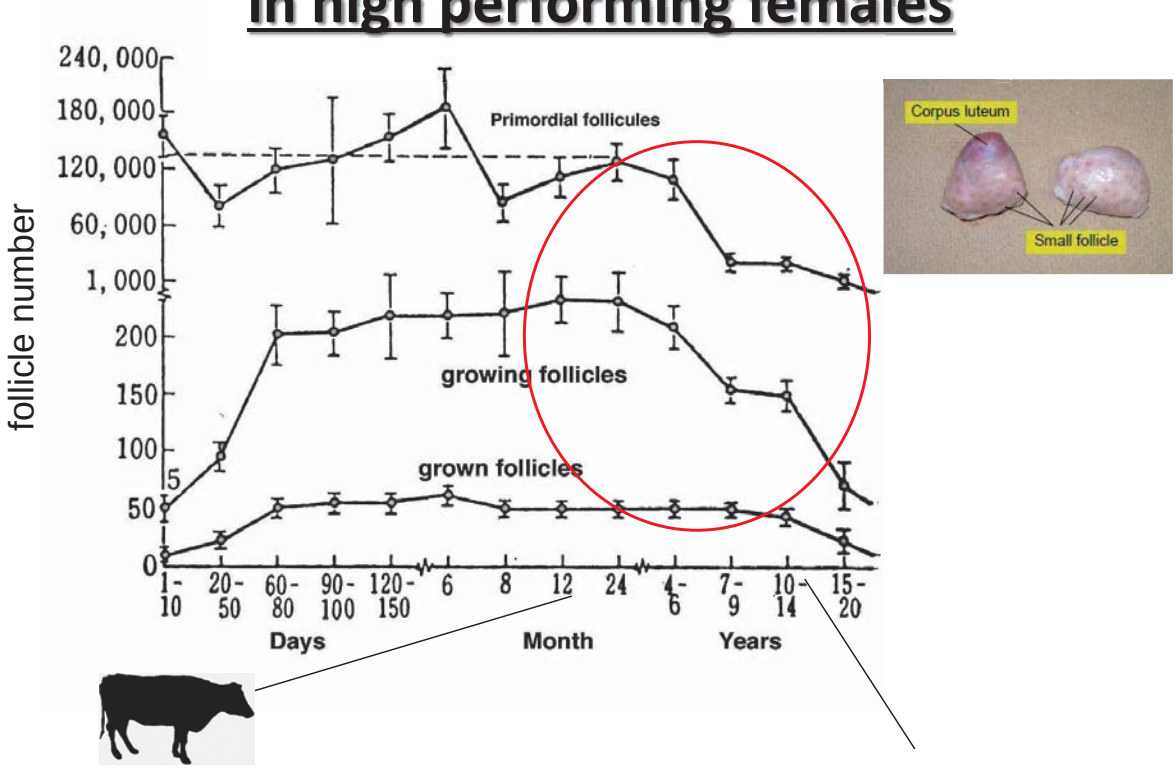


*:P<0.05
**:P<0.01

Cattle embryo frontiers

- In vitro embryo production (IVEP)
- Ovum Pick-Up (OPU)
- Selection of in vitro produced embryos using Real-time culture cell monitoring system

The point of OPU: utilization of oocytes in high performing females



1 oocyte/21 days ⟶ 17.4 oocytes/year ⟶ 174 oocytes/10years

Only a small proportion of total oocytes is ovulated during the lifetime of a cow!

Comparison of embryo production between MOET and OPU-IVF

Embryo production system	No. of OPU/MOET sessions	No. of follicles/CL/ session	No. of collected oocytes/session	No. of blastocysts produced / session
OPU-IVF	60	43.4 ± 16.4	36.7 ± 18.3	11.8 ± 7.6[a]
MOET	36	14.8 ± 9.8	9.3 ± 8.5	6.4 ± 6.3[b]

Values (means ± SD) within the same column with different superscripts differ (P < 0.01).

Data from more than 10 days OPU interval were collected.

Imai et al, J Reprod Dev 56(Suppl): S19-29, 2006

Embryo production by OPU-IVF in various donors

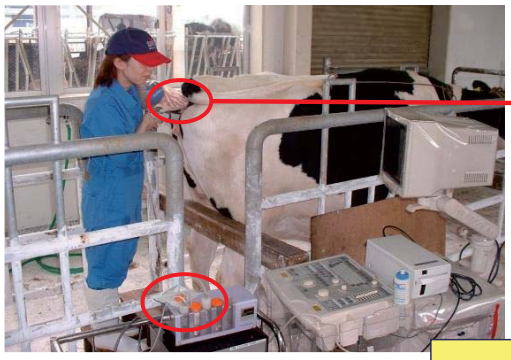
Donors	No. OPU sessions (intervals)	No. follicles	No. oocytes collected	No. blastocysts produced	% blastocyst
Dry cows	60 (>10)	43.4	36.7	11.8	41.6
Pregnant cows	16 (7)	43.1	39.3	12.3	40.7
Reproductive disorder	17 (>10)	36.2	30.0	7.8	29.3
Pretreated FGT	8 (11)	29.3	19.0	12.8	68.1
Pretreated SOV	8 (>10)	45.6	25.5	13.9	57.2
9 months calves	28 (7)	29.8	22.4	5.0	26.0
6 months calves	2 (7)	45.0	32.5	5.0	19.5

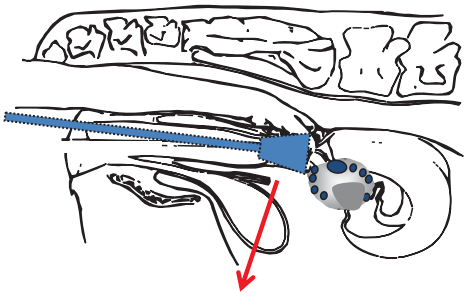
Embryo production by OPU-IVF

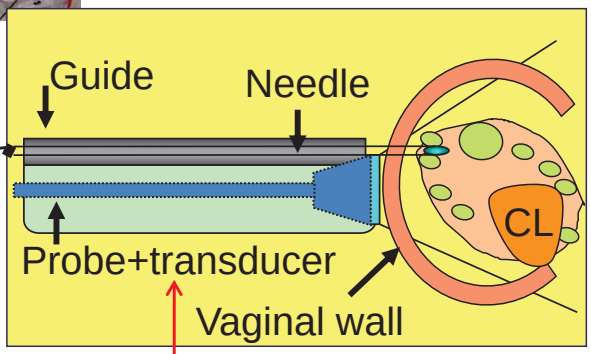
Country	Year	No. OPU sessions	No. collected oocytes	No. produced blastocysts
Japan	2011*	1,748	28,855	3,256
USA	2012	13,950	266,869	66,901
Canada	2012	1,018	9,549	4,453
Brazil	2012	72,743	872,927	349,171
Panama	2012	757	14,333	3,726
Mexico	2012	569	13,681	2,888
Argentine	2012	461	7,940	2,308
Netherland	2012	3,543	28,587	3,238
Germany	2012	1,268	5,562	3,900
France	2012	338	2,414	639

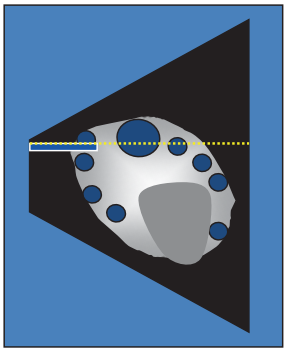
IETS Statistics and Data Retrieval Committee Report 2013
*2011.4 to 2012.3

1.2 Oocyte collection from live animals by ultrasound-guided vaginal Ovum Pick-Up (OPU)

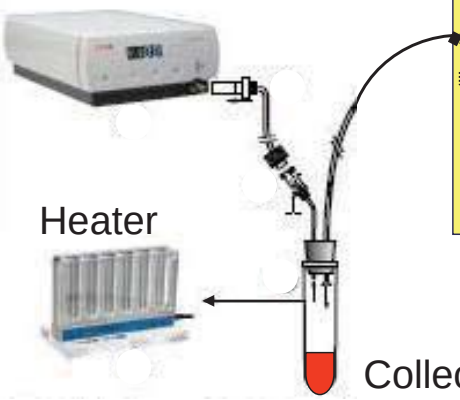






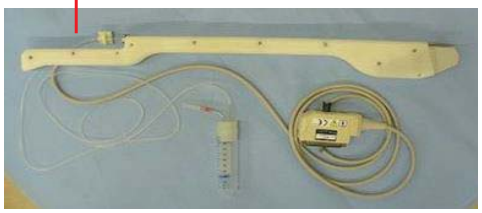


Vacuum



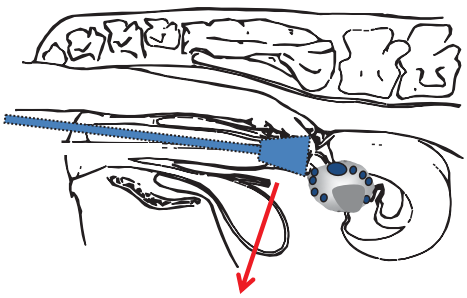
Heater

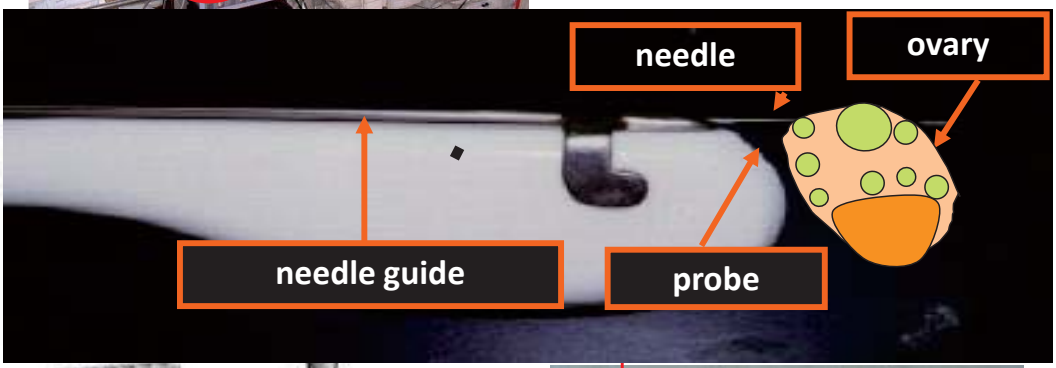
Collecting tube

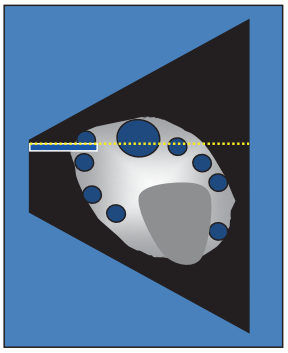


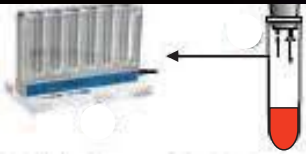
Ultrasound guide

1.2 Oocyte collection from live animals by ultrasound-guided vaginal Ovum Pick-Up (OPU)

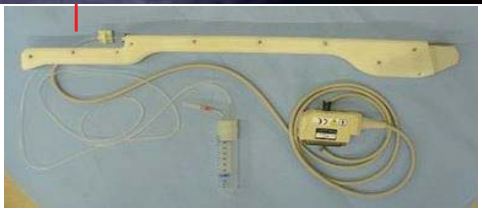






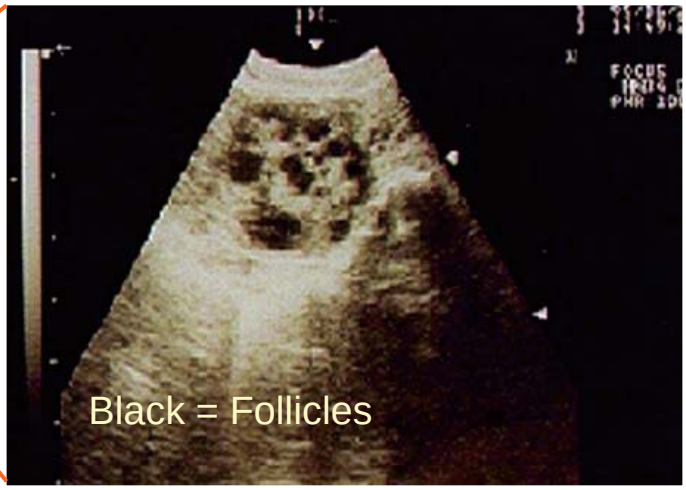


Collecting tube

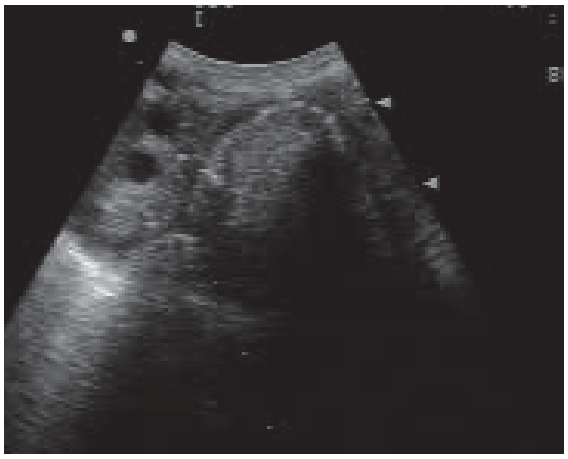


Ultrasound guide

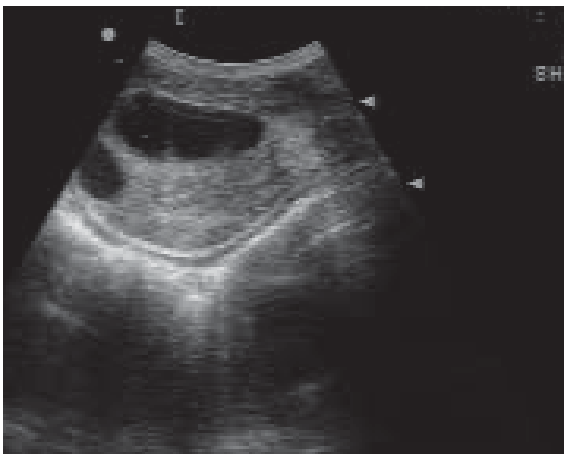
Display of follicles visualized by ultrasonography on monitor



Oocyte aspiration from follicles



2-4 mm in diameter
No-stimulation



10-15 mm in diameter
30AU FSH

Cattle embryo frontiers

- In vitro embryo production (IVEP)
- Ovum Pick-Up (OPU)
- Selection of in vitro produced embryos using Real-time culture cell monitoring system

Time Lapse Cinematography (TLC)

Real-time culture cell monitoring system (Astec, Fukuoka, Japan)

TLC: Every 15min it takes a photo; 673 photos will be taken until the end of IVC for 168h after insemination

Time Lapse Cinematography (TLC)

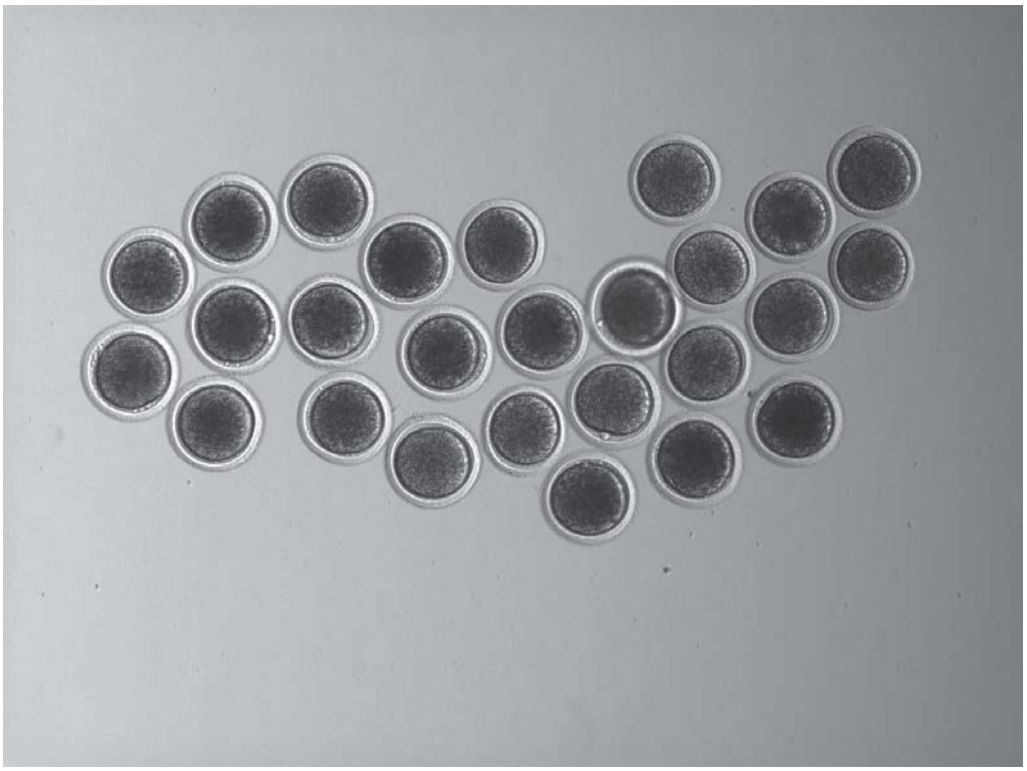
Built-in microscope and video camera

Real-time culture cell monitoring system (Astec, Fukuoka, Japan)

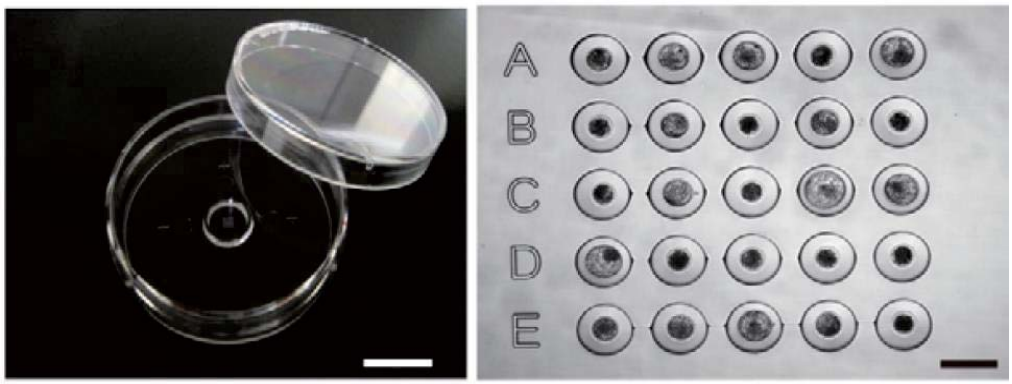
TLC: Every 15min it takes a photo; 673 photos will be taken until the end of IVC for 168h after insemination

Tracking in vitro embryo development

using TLC for 1 week development in 20 minutes



Individual culture in IVF embryo

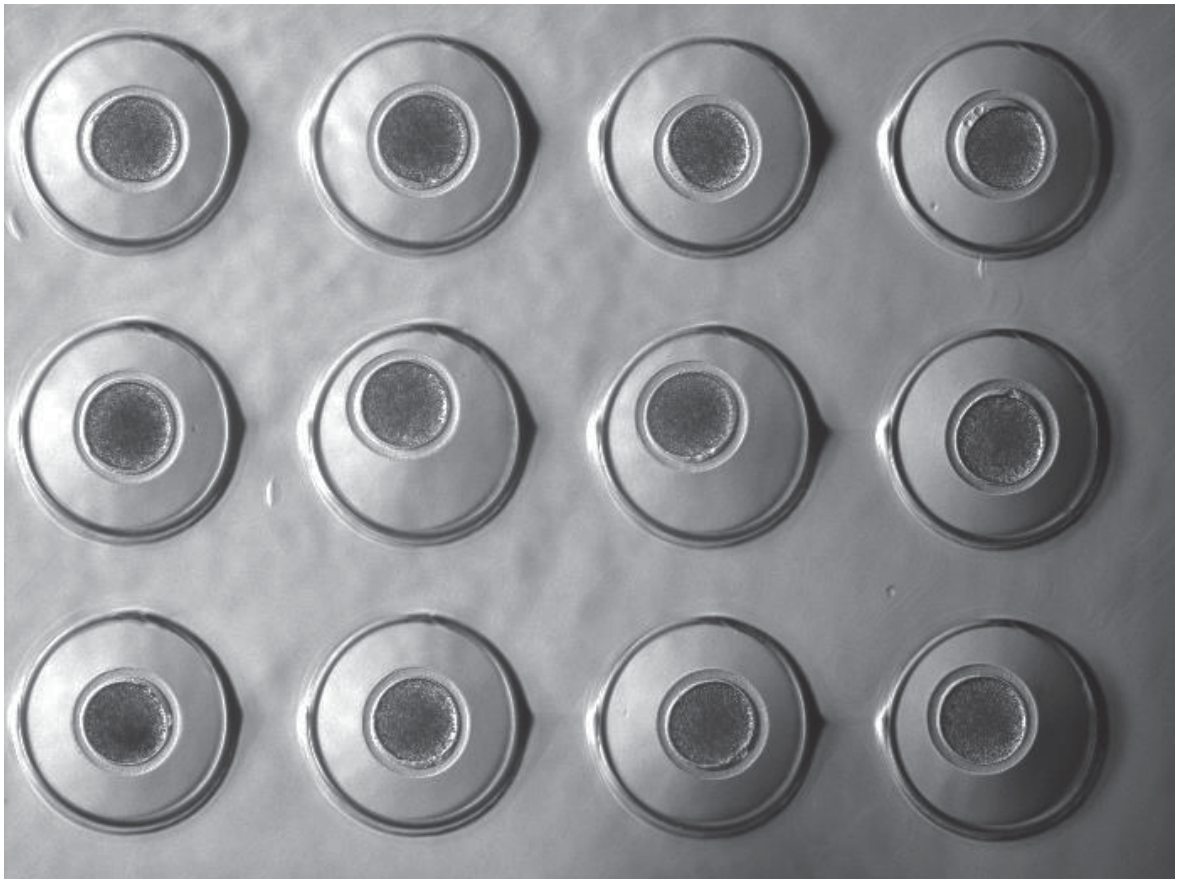


WOW dish : 35mm culture dish
well : 7mm in diameter at the center of dish
25 microwells (280μm in diameter and 160μm in depth, taper=7°)

Culture : 125μl of medium, 25 embryos
5%O_2, 5%CO_2, 90%N_2 and saturated humidity

Sugimura et al, Biol Reprod, 2010

Observation of embryo development by WOW-TLC



Five prognostic factors for embryo quality

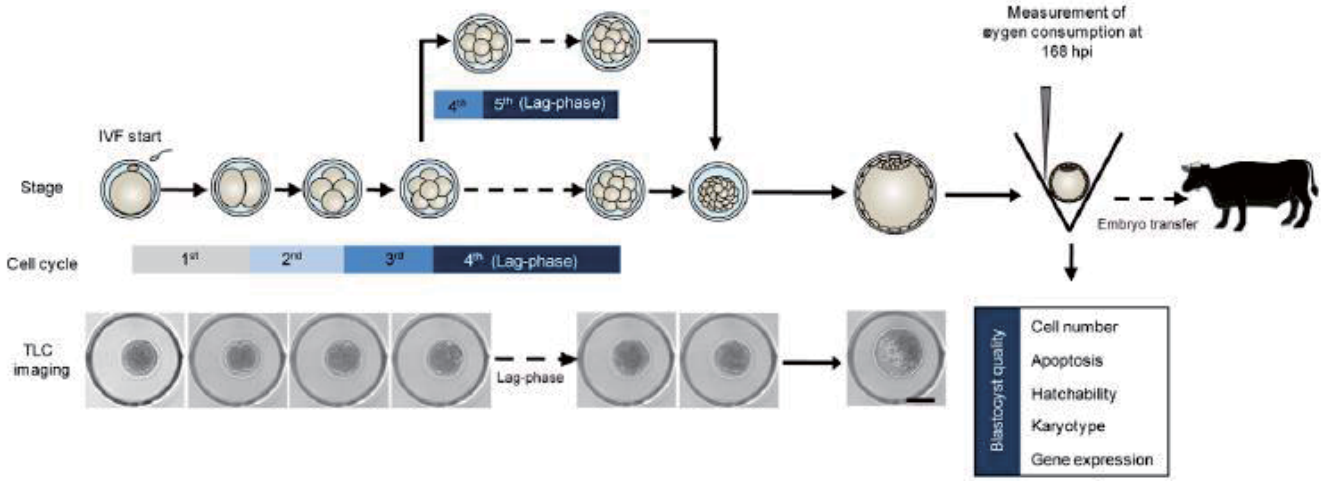
PF1: Timing at 1st cell cycle

PF2: Number of blastomeres after 1st cell cycle

PF3: Existence of fragments

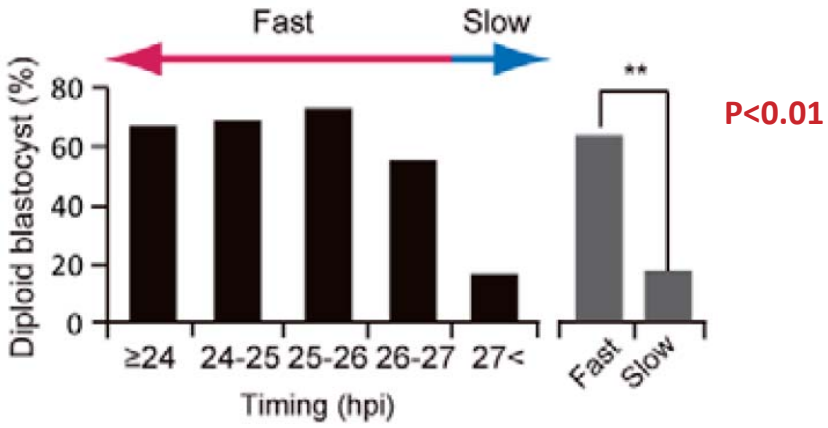
PF4: Number of blastomeres at lag-phase

PF5: Oxygen consumption



Sugimura et al, PLoS ONE (2012)

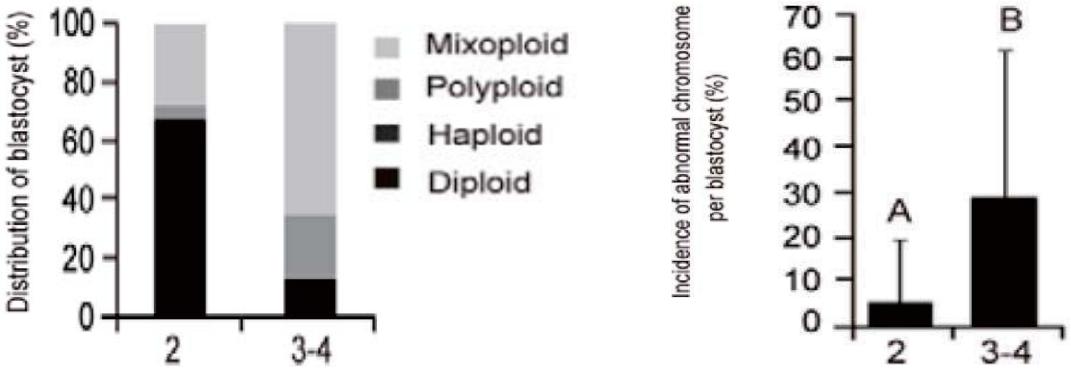
PF1: Timing at 1st cell cycle



P<0.01

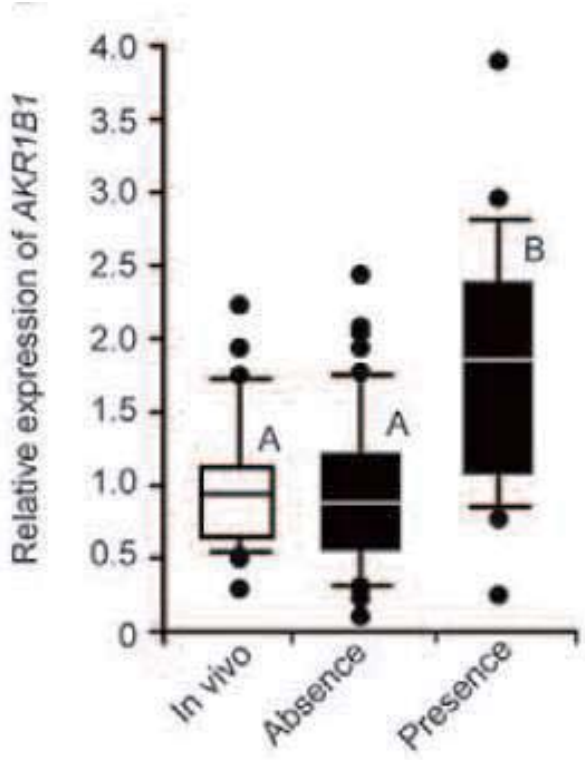
Sugimura et al, PLoS ONE (2012)

PF2: Number of blastomeres after 1st cell cycle



Sugimura et al, PLoS ONE (2012)

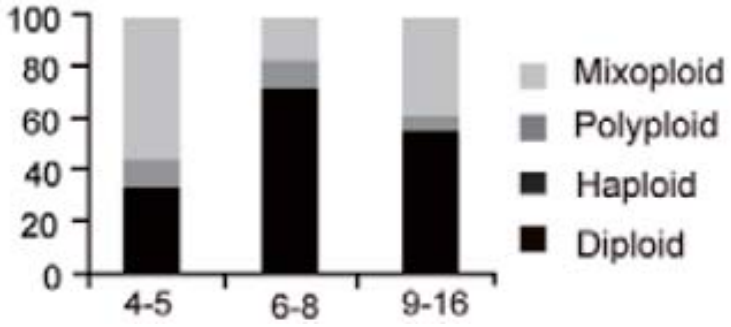
PF3: Existence of fragments



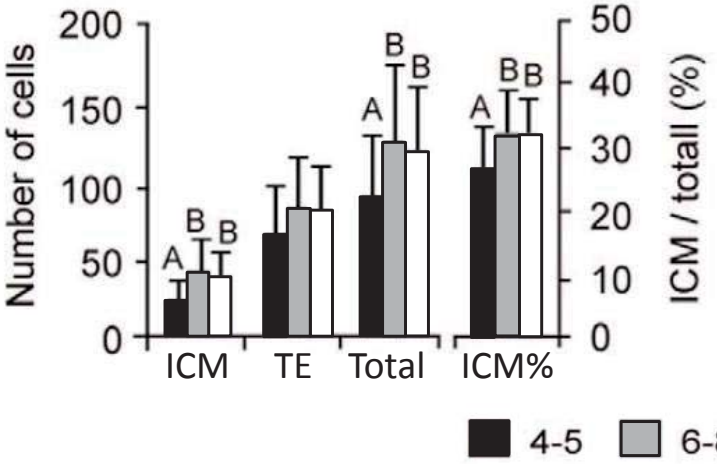
Upregulation of AKR1B1 has been associated with embryos that fail to establish pregnancies.

Sugimura et al, PLoS ONE (2012)

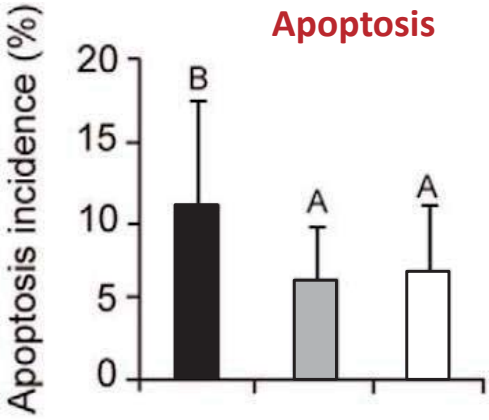
PF4: Number of blastomeres at lag-phase



Number of cells per blastocyst

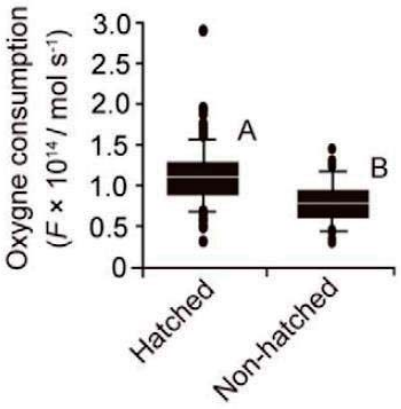


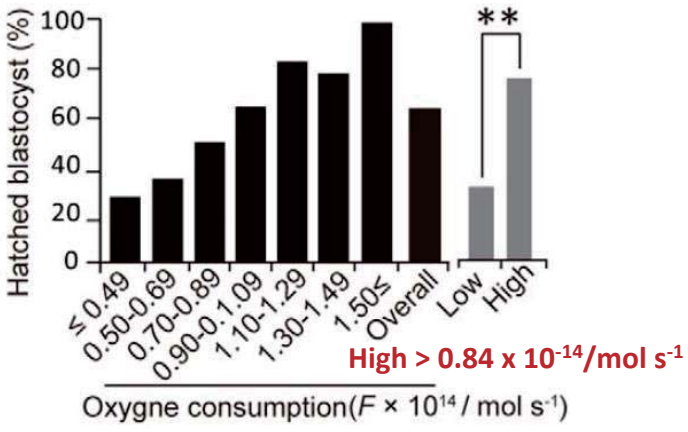
Apoptosis



Sugimura et al, 2012

PF5: Oxygen consumption of blastocysts





High > 0.84 x 10^{-14}/mol s^{-1}

Sugimura et al, PLoS ONE (2012)

Conception rates in IVP embryos selected by prognostic factors

Combinations	No. of transferred	No. of conceptions	% of conception
Conventional method	52	21	40.4
PF1 and PF2	27	18	66.7*
PF1, PF2, and PF3	24	17	70.8*
PF1, PF2, PF3, and PF4	22	16	72.7*
PF1, PF2, PF3, PF4, and PF5	19	15	78.9**

Compared with conventional method: * $p<0.05$, ** $p<0.01$

The birth weight was 29.2 ± 3.3 kg that was close to that derived from AI embryos (28.7 ± 4.2 kg) and we observed no neonatal overgrowth or death.

Sugimura et al, PLoS ONE (2012)

Future developments of in vitro embryo culture systems

- Improve media and culture system to resemble *in vivo* environment (+ study *in vivo* development)
- Research to understand the mechanisms of altered development and gene expression in IVP embryos
- Reduce culture shocks and (causes of) abnormal gene expression during IVEP > improve embryo quality

The Nagai family and a IVF piglet (1993)

SPERM CAPACITATION AND IN VITRO FERTILIZATION IN CATTLE

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ABSTRACT

The slaughtered cows or heifers have a large number of immature oocytes within primordial follicles or developing follicles. Therefore, it is useful to aspirate immature oocytes, which would be wasted if left unprocessed, from ovaries; subsequently they are matured in vitro (IVM), fertilized in vitro (IVF), and cultured in vitro (IVC). Also most recently it has been shown that immature oocytes can be collected by repeated Ovum pick-up (OPU) from living cows or heifers with high performances and used for IVF. A large number of normal embryos can be produced in vitro by performing IVM-IVF-IVC. So the improvements of genetics and propagation of livestock are considered to be more efficient by combining IVF technique to OPU and embryo transfer techniques. Thus, the research and development were perused. Currently, there have been considerable improvements in IVM-IVF-IVC and the technique has reached the level of practical use. Moreover, obtaining oocytes as experimental materials from slaughterhouses is easy compared to the collection from experimental animals which require breeding facilities. Therefore, from this regard, oocytes collected from ovaries in slaughtered animals should be used to a great extent as the material for studying mechanisms of maturation, fertilization, and subsequent development. Furthermore, the importance of the role played by IVF as a basic technique for the recent production of cloned cattle is needless to mention. Consequently, in the following, IVF in cattle is described here.

Keywords: Cattle, In vitro fertilization, Oocytes, Embryo transfer, Ovum pick-up

INTRODUCTION

In the field of animal breeding, the artificial insemination (AI) technique using frozen semen has come into practical use for cattle breeding (Smith and Polge 1950; Stewart 1951). With this technique, it has become possible to produce a large number of offspring with superior genetic traits from sire line at a time. On the other hand, so-called embryo transfer (ET) technique, which is to collect embryos from superior donors and transfer them into recipients, was developed to propagate superior genetic traits from

maternal line. This ET technique was experimentally successful for a long time, and there were reports for cattle (Willett et al. 1952), sheep (Warwick and Berry 1949), swine (Kvasnickii 1951), horse (Oguri and Tsutsumi 1972), and rabbit (Heape 1890). This technique has been widely applied in improving genetics and breeding of cattle in the world. The most popular method used today is the superovulation program in which gonadotropic hormone is administered to donor cows to induce more ovulations than normal numbers, and followed by AI or natural mating with bull, and collecting embryos non-surgically from uterus via vagina. This method is named as MOET (Nicholas and Smith 1983); multiple ovulation and embryo transfer. However, there is a limit to the number of embryos collected from a donor cow. Moreover, there are large individual differences in the responsiveness to superovulation regimen such as the number of ovulated eggs. Consequently, the use of follicular oocytes in ovaries had drawn attention because the ovaries of cows or heifers have a large number of immature oocytes which can be *in vitro* matured (IVM) and fertilized (IVF) and developed to the stage of blastocyst by *in vitro* culture (IVC) for ET.

On the other hand, for IVF technique, human IVF was established before cattle IVF and recognized for its technical development achievement by R.G Edwards (Steptoe and Edwards 1978) in the world, and he won the Nobel (Prize in medicine) in 2010. In the world out of 40 newborn one is currently born through IVF; therefore, it is a good example showing that technology development and its commercialization have progressed so well. This human IVF is the research as a part of fertility treatment, and for example, parents of Louise, the world's first IVF child, tried natural pregnancy for nine years without success and committed desire to perform IVF using ovulated (*in vivo* matured) oocytes. Since then studies of human IVF have focused mainly on *in vivo* matured oocytes. However, in the case of cattle, considering that the number of immature oocytes, which can be used for IVF after IVM, in 2 ovaries per a cow is 20, a huge number of immature oocytes can be collected from slaughtered Japanese Black breed cattle (Wagyu); approximately 200,000~250,000 Wagyu cows or heifers are slaughtered per year in Japan. Therefore, for IVF in cattle emphasis has been placed on IVF of IVM oocytes and IVC of them to embryos applicable for ET; research and development have been conducted for IVM-IVF-IVC of immature oocytes. The main reason why Wagyu embryos have been targeted by IVF is that *in vitro* produced Wagyu embryos are welcomed to dairy farmers who try to get profit by selling Wagyu calves obtained after ET of them to dairy cows.

Pieterse et al. (1988) developed transvaginal ultrasound-guided ovum pick-up (OPU) as a minimal invasive method, and it has become possible to collect oocytes many times from the same donor animal. Most recently OPU combined with IVF/IVC (OPU/IVF/IVC) has so advanced that we can produce and select embryos with a higher quality than *in vivo* produced embryos in cattle (Sugimura et al. 2012). Thus, the importance of IVF in dairy cattle is growing, because a higher pregnancy rate may be obtained for OPU/IVF/IVC embryos than *in vivo* produced ones.

HISTORY OF IVF

The research on mammalian IVF made rapid progress since the rabbit experiment conducted by Chang (1951) and rat experiment by Austin (1951) that discovered that spermatozoa introduced into uterus or oviduct of females are unable to fertilize the ova immediately, and while they stay for several hours in the female reproductive tract, they undergo physiological and functional changes, then finally become able to penetrate into oocytes. Austin (1952) named such physiological changes in spermatozoa as “capacitation”, and the study of capacitation was proceeding side by side with the study of IVF, ever since.

1) Capacitation of Spermatozoa by Female Reproductive Tract

In early researches of IVF, the method to collect spermatozoa from female reproductive tract, several hours after mating, was used for inducing the capacitation of spermatozoa. Dauzier et al. (1954) tried rabbit IVF by adding spermatozoa, collected from female reproductive tract 12 hours after mating, to ovulated eggs in incubator; later, Thibault and Dauzier (1960) had shown pronucleus stage and first cleavage embryos as the proof of successful rabbit IVF. However, Chang (1959) completed the IVF system of Dauzier et al. (1954), and confirmed the normal characteristics of IVF by producing the offspring for the first time by transferring rabbit IVF oocytes into recipients. The first successful IVM-IVF using follicular oocytes in cattle was reported by Iritani and Niwa (1977). This study collected follicular oocytes from ovaries obtained at the slaughterhouses and matured them in vitro, and then IVM oocytes were fertilized with ejaculated spermatozoa pre-cultured in resected female reproductive tract; spermatozoa were confirmed of penetrating oocytes.

2) Sperm Capacitation Using Only Culture Solution

The researches on the induction of sperm capacitation by using defined culture medium with known components suitable for the analyses of various factors, were mainly conducted on rodents. Yamaguchi and Chang (1963) successfully induced capacitation of epididymal spermatozoa in culture and conducted subsequent IVF by the capacitated spermatozoa in hamster. Furthermore, since Barros and Austin (1967) succeeded in IVF of follicular oocytes in hamster, it was elucidated that oocytes are able to complete the final maturation process and spermatozoa are able to be capacitated without the influence of female reproductive tract. Later, pregnancy or production of offspring by IVF of ovulated oocytes was reported in rat (Toyoda and Chang 1974), mouse (Pavlok 1967), and rabbit (Bedford and Chang 1962).

However, in cattle it is time consuming to establish the method to induce capacitation of spermatozoa by culture solution only, without the use of resected female reproductive tract. It was as late as in 1982 the first IVF calf was born from ovulated oocytes (Brackett et al. 1982) by using high osmotic pressure [380 mOsm/kg] synthetic culture solution (BO solution) developed as IVF solution for rabbit (Brackett and

Oliphant 1975). Following this report, Lenz et al. (1983) reported that capacitation of epididymal spermatozoa was induced by pre-culture at a high temperature (at 39°C), and capacitated spermatozoa were introduced to fertilize IVM oocytes at 39°C by accident resulting in the high rate of sperm penetration into the oocytes (Table 2). Moreover, Iritani et al. (1984) kept ejaculated bovine spermatozoa in a room temperature for a long time to induce capacitation of spermatozoa for conducting IVF of IVM oocytes, and then spermatozoa penetrated into oocytes at high rates. However, those succeeded in calf production by IVM-IVF, which is truly the proof of sperm capacitation were Hanada et al. (1986) (Figure 1), and they used frozen-thawed spermatozoa treated by ionophore A23178 or caffeine for the induction of sperm capacitation.

3) Production of Offspring Derived from IVM-IVF-IVC Oocytes

This is the major topic of this review on IVF. There were reports made in early 1970's on productions of offspring derived from IVM-IVF (Cross and Brinster 1970; Mukherjee 1972) in mice. However, to achieve successful offspring production from IVM-IVF-IVC oocytes in cattle required more time. In 1985, Hanada et al. (1986) successfully produced calves derived from IVM-IVF-IVC oocytes using conventional static culture. Since then improvements of IVM-IVF techniques have progressed globally in cattle and achieved the practical use level. Moreover, in order to determine the developmental capacity of IVM oocytes to an offspring, *in vivo* fertilization of IVM oocytes was conducted. That is, IVM oocytes were transferred to the oviducts after mating, in order to fertilize *in vivo* by using rabbit (Thibault et al. 1975), sheep (Moor and Trounson 1977), swine (Nagai et al. 1990), and cattle (Hunter et al. 1972; Sreenan 1970), development of IVM oocytes to offspring was identified; in cattle, only up to the early developmental stage was confirmed.

IVM OF IMMATURE FOLLICULAR OOCYTES

1) IVM of Oocytes Collected from Ovaries Resected at Slaughterhouses

Oogenesis starts in a fetal period and completed soon after birth. Then, for the long period of time until puberty, many of the oocytes undergo degeneration by atresia. Out of remaining oocytes, some resume the meiosis and are ovulated in response to the gonadotropic hormone released from pituitary gland.

On the other hand, in 1935 oocytes from tertiary follicles were reported to resume the meiosis when cultured in physiological solution (Pincus and Enzmann 1935). Edwards (1965) was the first to culture oocytes form many animals including pigs and cows in vitro, and reported that the process of the meiosis was the same in in vitro and in vivo matured oocytes. Subsequently many reports were made on IVM oocytes in cattle (Shioya et al. 1988; Sirard and First 1988). Table 3 shows the number of oocytes collected from one ovary and the rate of usable oocytes for IVM when ova were collected in various

methods. When considering the large number of livestock processed at slaughterhouses as mentioned above and the large number of oocytes possible to collect, vast number of livestock' oocytes can be collected.

Currently, as a basic culture medium for cattle, mainly TCM199 is used. Additionally, supplements such as serum (Leibfried-Rutledge et al. 1986), hormone (Sirard et al. 1988) (gonadotropic hormone, luteinizing hormone, and estradiol), pyruvate (Geshi et al. 2000), and growth factors such as EGF (Coskun et al. 1991; Harper and Brackett 1993; Kobayashi et al. 1994; Lonergan et al. 1996; Rieger et al. 1998) and IGF (Herrler et al. 1992) are added. However, the large variation of IVF rate occurred depending on the added hormone or serum. Consequently, the IVM system using no-serum (defined) medium was developed (Saeki et al. 1991), and the production of calves derived from IVM-IVF oocytes has been reported (Nagao et al. 1994). Furthermore, it was reported that the addition of cysteamine increases the percentage of oocytes develop to the blastocyst stage and their cryoresistance to freezing process (de Matos et al. 1995; de Matos et al. 1996; de Matos et al. 1997). The addition of a particularly low-concentration of adenylate cyclase (Luciano et al. 2004) to collection medium when collecting oocytes from ovary was reported to increase the developmental rate of IVM-IVF oocytes.

The first production of a calf from IVM-IVF-IVC oocytes at Cambridge, so called "the Mecca of livestock breeding technology", was achieved by non-static culture of oocytes. Therefore, from now on, for the purpose of obtaining the high quality (strong) IVM oocytes that are essential for advanced (not simple) breeding technique such as production of cloned animal from somatic cells, we may need to reconsider this non-static culture, besides establishing the complete IVM system using the defined medium.

2) Repeated Transvaginal Oocyte Collection: Ovum Pick-Up (OPU)

A conventional method of embryo production using superovulation program has disadvantage, which requires 2 to 3 months period till the next embryo collection once the superovulation procedure was performed on a donor cow. As the method for solving this problem, a method of embryo production by repeated collections of oocytes from ovaries of donor cows and perform IVM-IVF was developed (Pieterse et al. 1991). This method was originally developed in 1986 for collecting mature oocytes for human IVF (Feichtinger and Kemeter 1986); then, applied to repeated transvaginal oocytes collection of cattle in 1988 (Pieterse et al. 1988). In 1990, being combined with IVF and embryonic transfer technique, the calf was produced by this method (Kruip et al. 1990). This is a method of oocyte pick-up guided by ultrasound diagnostic (OPU); while monitoring the image of ovaries in cow on the screen, oocytes are repeatedly aspirated from follicles transvaginally, and then the oocytes are subjected to IVM-IVF. Kruip et al. (1993) used 10 cows for 214 times of aspiration and fertilization experiments lasting 5 months. They

aspirated 3,035 follicles and collected 1,678 oocytes. Among them, 1,198 oocytes underwent IVM-IVF that resulted in 219 (18.8%) blastocysts. Thus, 8.8 blastocysts were obtained monthly, and 20 blastocysts were transferred to 20 recipient cows, and 8 resulted in pregnancy. As shown by this result, the method was equivalent to the conventional method of embryo production by superovulation program to a donor cow. Moreover, the method has advantages other than of being able to repeatedly collect the oocytes as described above. Among them are: (1) By IVM-IVF of collected immature oocytes, production of calves with known maternal line is possible. (2) Fertilized eggs can be produced from previously impossible cases such as prepubertal heifer (Rick et al. 1996) and pregnant (before 90 days of gestation) cows (Reiders and van Wagendonk-de Leeuw 1996). (3) Time-course observation on oocyte maturation is possible; therefore, the basic research on oocyte maturation progresses.

CURRENT METHODS OF IVF

In cattle, artificial insemination (AI) by using frozen spermatozoa has already being common method and the access to the frozen spermatozoa has been widely established. Due to such background, the cattle IVF characteristically developed using frozen semen. In December 1985, experiment resulted in the world first production of calf from IVM-IVF (Figure 1) at the research farm of National Institute of Animal Industry, Ministry of Agriculture, Forestry, and Fisheries. Successful application of complete defined culture medium (Hanada 1985) using BO solution (Brackett and Oliphant 1975), with ionophore or caffeine, to treat frozen spermatozoa for capacitation was reported, and this method became the fundamental model of Japanese cattle IVF system. Later, method using heparin added TALP (39°C) as a culture solution was developed by the laboratory led by N.L First of University of Wisconsin in U. S., and on February 1986, they produced a calf (the first outside of Japan) by IVF, using this method (Parrish et al. 1986). This is another base of current IVF systems. Subsequently, they clarified that glucose inhibits the fertilization promoting effect of heparin and the method was modified (Parrish et al. 1989). Furthermore, Niwa and Ohgoda (1988) reported that synergistic effect of heparin and caffeine on sperm capacitation, and combined the two systems. This is the standard method of current IVF in Japan. Moreover, development of capacitation method that is effective on any spermatozoa was tried, and collection of motile spermatozoa by centrifugation using a Percoll density gradient method was shown to be effective on the spermatozoa with low fertility rates (Parrish et al. 1995). Addition of casein phosphopeptide (CPP), purified from milk casein, to BO solution, and further increasing calcium concentration were also reported to improve fertilization rates (Nagai et al. 1996). Since BO solution contains a high concentration of phosphoric acid, added calcium tends to precipitate as calcium phosphate. That is the disadvantage of BO solution, in which the spermatozoa are unable to fully take in the calcium necessary for capacitation and acrosomal reaction, and addition of CPP which has a high affinity to calcium solve

this problem (by enabling the addition of high concentration calcium.) On the other hand, most recently the addition of D-penicillamine, hypotaurine, and epinephrine (PHE) mixture to BO solution was reported to maintain motility and longevity of bovine sperm and enhance stable production of blastocysts in vitro (Kang et al. 2015).

At this moment, no method effective for all spermatozoa was established yet. Therefore, its establishment and practical application are urgently needed.

IVC OF IVM-IVF OOCYTES

The first IVF calf was produced by surgically transferring 4-cell stage embryo into the oviduct of recipient cow (Brackett et al. 1982). However, in order to non-surgically transfer the embryo, it is necessary to develop the embryo to the blastocyst stage that is possible to transfer to uterus. In an early research, technique of culturing the embryo in rabbit (Hanada et al. 1986) or sheep (Lu et al. 1987) oviduct, to the blastocyst stage was established, but the surgical procedure was time consuming and 100% collections of embryos were not possible, research of developing by culturing in vitro (IVC) of IVM-IVF oocytes was conducted. Kajiwara et al. (1987) developed the method of obtaining the blastocysts in a high rate by co-culturing IVM-IVF oocytes with cumulus cells. Subsequently, IVF calves were produced by transfer of fertilized oocytes using oocytes obtained by the similar method (Fukuda et al. 1990; Goto et al. 1988). Likewise, a method using other cells for co-culture was developed, which includes epithelial cells of oviduct (Eystone and First 1989), rat liver cells (Voelkel et al. 1992), and trophoblast cells (Heyman et al. 1987). On the other hand, in southern hemisphere (New Zealand), an original culturing method was developed in IVC of ovine IVM-IVF oocytes, using a low-oxygen concentration culture system without co-culturingany cells (Tervit et al. 1972), and the technique has been applied to cattle (Fukui et al. 1991; Takahashi and First 1992). Moreover, non-co-culturing system using 95% air, without lowered oxygen concentration, was being developed. Table 4 shows the major reports of non-co-culturing systems by a synthetic culture medium (Rosenkrans and First 1991). Nagao et al. (1994) studied on the effect of co-culture or non-co-culture and atmosphere (5% O_2 or 20% O_2) on the culture of IVF oocytes. And they reported that in case of co-culture, the high oxygen concentration, and of non-co-culture, the low oxygen concentration resulted in significantly high rates of developing to blastocysts than the reverse of each combination. Also, Takahashi et al. (1993) used TCM199 with addition of thiol compound (β-mercaptoethanol and cysteamine) for culture of IVM-IVF oocytes and reported that glutathione concentration of embryo and its development rate to the blastocyst stage were significantly increased compared to the case with no additives. And they proved that reducing state of fertilized oocytes is advantageous in IVC. Considering these effects of reducing state in non-co-culture systems, it is unlikely

for lipid hyperoxidation to occur in a low oxygen or reducing state; and, therefore, it is considered that the development rate to blastocysts may increase.

Moreover, it is notable that Nagao et al. (1994) succeeded in non-protein maturation culture of immature oocytes and non-protein culture of IVM-IVF oocytes. Such elucidation of factors involved in oocyte maturation and development of fertilized oocytes using completely defined culture system is awaited. Up to now, proteins excreted by granulosa cell (Satoh et al. 1994) and epithelial cells of oviduct (Eystone and First 1991) are shown to promote the development of IVM-IVF embryos, by using similar method. Also, by using biochemical method, glucose and glutamine metabolism of IVM-IVF embryo was studied. As the result, it was found that the early stage embryo (8-16 cell stage) was unable to metabolize glucose, and on contrary, the glutamine metabolism was high in 2-4 cell stage embryos (Rieger et al. 1992). Moreover, glucose concentration in a conventional cell culture (5.56 mM) was shown to inhibit the development of early embryo, but pyruvate and lactic acid promote the development (Takahashi and First 1992).

At this moment, developing rate to blastocyst stage embryo from IVM-IVF oocytes are approximately 20~40% to subjected oocytes; however, there are large variations in rates among conducted facilities even by the same method. The establishment of completely defined culture system and application of biochemical and molecular biological techniques are urgently needed to identify the cause of this problem as well.

Though pregnancy rates from ET of IVM-IVF-IVC blastocysts vary by the facilities, according to the recent record of Livestock Biotechnology Center of Livestock Industry Association of Japan, Inc. which produces the largest number of cattle embryos in Japan has pregnancy rate of over 50%, in case of using recipient with confirmed synchronized estrous and fresh IVM-IVF-IVC embryos. However, when they were frozen, the rate decreases; therefore, improvement of the quality of IVF embryos obtained from IVM-IVF-IVC are expected in the future.

ISSUES AND ANSWERS FOR CATTLE IVF

(1) Calves produced from bovine IVM-IVF oocytes have higher rates of being over-weighted than in vivo produced embryos. Refer to reviews (Young et al. 1998; Walker et al. 1998) on this issue.

(2) Quality evaluations are difficult in IVM-IVF-IVC embryos. There is a need to establish the IVF system to produce calves in high reliability, not to merely raise the development rates to blastocyst stage embryos. Although relatively large number of mature oocytes can be collected by current

method, in case of the cows from commercial farms, high pregnancy rates are required. Thus, quality over quantity of IVM-IVF embryos is expected.

(3) Ensure the collection of fertilized oocytes with the known maternal line. In order to achieve this, it is necessary to collect ovaries individually or collect oocytes (in vivo) directly from cows (OPU). Moreover, it is considered necessary to especially facilitate the paternity test at the time of registration of the resulting calves.

(4) Transfer of the techniques to actual use is delayed. There are following factors besides technological developments: (a) Lack of understanding at the level of commercial farms. (b) Low evaluation of in vitro derived calves at a calf market; now this is changing. (c) Delays in acting on the farms specializing in certain registered cows (which sells at a high price for certain cows.) Especially on (a) and (b), strategies such as clarifying the production effects of in vitro derived calves in integrated operation should be emphasized to large-scale beef cattle farms.

OUTLOOK

The technical improvements of the last decades in flow cytometry allow the large scale production of sex-sorted bull semen which has led to its use on the commercial level (Garner and Seidel 2008). When combined with ovum pick up (OPU) and IVF this sex-sorting allows the production of high quality blastocysts with traceable genetic background (Matoba et al. 2014). Also with time-lapse monitoring of IVP embryos the effective selection of high quality embryos having high implantation potential to go to term has been reported to be greatly improved: minimal stress during culture (Sugimura et al. 2012). This technique allows us to trace all the steps of early stage development of IVP embryos under microscope and makes it possible to select embryos having better potential to go to term by using check points characteristic for high quality embryos than in vivo produced embryos of which background is unknown. These technologies will contribute to improvement of cattle breeding in near future. Beside these technologies, by (1) control of embryo productions by methods such as cloning and phenotype transformations with introductions of foreign genes, (2) mass production and storage of embryos through the developmental methods of growing immature small oocytes in culture and IVM of in vitro grown oocytes (Hirao et al. 2004), (3) preservation of genetic resources, and (4) understanding the fertilization and developments of embryos from the point of reproductive physiology (fertilization and developmental biology), we should create the possibility of developing new reproductive techniques.

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Table 1. The Number of Ovarian Follicles per the Cow

Animal species	No. of primordial follicles	No. of developing follicles	No. of ovulations
Cattle	120,000	300	1

Data cited from Erickson (1966)

Table 2. The Effect of temperatures of IVF culture medium on fertilization rates in cattle

Temparature (°C)	Fertilization rate (%)
37	7
39	53

Data cited from Lentz et al. (1983)

Table 3. Numbers and Qualities of Oocytes collected from Ovaries

Method	Researchers	No. of oocytes collected from one ovary	Rate of usable oocytes (%)
Collection by Aspiration[a)]	(Katsuka and Smorag 1984)	10.2	45.0
	(Iwasaki et al. 1987)	9.4	56.4
	(Lonergan et al. 1992)	9.7	31.3
Ovary resection[b)]	(Lonergan et al. 1992)	17.2	60.7
Slicing[c)]	(Xu et al. 1992)	55.5	70.3
	(Hamano and Kuwayama 1993)	31.6	

[a)] Aspiration of follicular fluid, including oocytes using a vacuum aspirator or a syringe.

[b)] Collection of oocytes from follicles after resection.

[c)] Slicing of ovaries using razor blades (commonly, layering of several blades).

Table 4. IVC System of IVM-IVF Oocytes Using Synthetic and Non-co-culture Medium

Culture Medium	Researchers	Atmospheric Condition	Addition of Serums
CR1aa	(Rosenkrans and First 1991)	5% CO2, 95% Air	None
SOF	(Fukui et al. 1991)	5% CO2, 5% O2, 90%N2	FCS
	(Takahashi and First 1992)	5% CO2, 95% Air	BS
TCM-199	(Nagao et al. 1994)	5% CO2, 5% O2, 90%N2	None
TCM-199 + Thiol Com.	(Takahashi et al. 1993)	5% CO2, 95% Air	FCS

FCS: fetal bovine serum

BSA: bovine serum albumin

BS: bovine serum

Thiol Com.: β-mercaptoethanol, cysteamine

Figure 1. The first calf produced from IVM-IVF oocyte and a recipient cow (Hanada et al. 1986).