Chapter 1
Biotechnology and Animal Reproduction

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Biotechnology has great impact on breed improvement, reproductive rate, and animal production.

The most common reproductive applications that are integrated with biotechnology are artificial insemination (AI), semen preservation, fertilization capacity of sperms, sperm sexing, synchronization and fixed-time insemination, superovulation, embryo transfer (ET), and in vitro embryo production (IVEP).

1.1 Artificial Insemination

Artificial insemination has been practiced on many domestic animals for hundreds of years. It is one of the earliest reproductive biotechnologies and permits the use of superior males for breeding purposes. This technique involves semen collection from superior males, its dilution, freezing, and deposition in the female reproductive tract. The first successful artificial insemination (AI) was reported in a water spaniel bitch in 1780 by the Italian scientist, Spallanzani and got three puppies. Spallanzani's work was confirmed 2 years later by another scientist Rossi (Roberts 1971). After initial work on bitches, AI was done in mares by Pearson. The AI technique in different farm animals is based on AI techniques of horses developed by Ivanow (1907).

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1.1.1 Semen Collection Methods

There are many methods of semen collection in domestic animals. Primitive methods involved semen collection directly from the vagina of an estrum female with the help of a spoon or syringe with a long nozzle. But the semen was contaminated, contained mucus, and the quantity recovered was minute. It had also a limitation of estrus female. Rowson in 1947 devised a method of making fistula in the urethral opening leading toward the vaginal fornix. Rowson's method involved semen aspiration directly from vaginal fornix by using catheter after natural breeding but it resulted in fibrosis, urinary tract infection, and also had the same limitation as the primitive method. After that, massage method and electro-ejaculator methods were developed.

1.1.1.1 Massage Method

This method was devised in the bull for the first time by Case in 1925 and modified by Miller, Evans, and Goodwin in 1934 (Roberts 1971). This method involves the massage of ampulla and seminal vesicle per rectum.

Indications

- Used in the case of aged bulls unable to mount.
- Used for crippled bulls.
- Used for bulls with decreased libido or with the problem of impotency.
- Used for bulls that are unwilling or unable to copulate.

Procedure

- Restrain the bull properly, handle it quietly, and keep it relaxed.
- Wash, rinse, and dry with a brush and cotton pledgets or with a clean towel the prepuce and the preputial hairs and the region around the preputial opening with warm physiological saline.
- During washing, stroking of sheath should be done to induce urination, which will not cause any contamination during collection.
- Operator, wearing a glove gently inserts a lubricated hand and forearm into the bull's rectum emptying it of feces.
- Massage the vesicular gland a few times in backward and downward fashion toward urethra. This will result in release of cloudy fluid containing few spermatozoa.
- The ampulla is then massaged in the same fashion. The semen is stripped with pressure against floor of pelvis.
- Sometimes pelvic urethra is also massaged.
- Another person holds a rubber cone attached to a collection vial placed in a plastic bag attached to a metallic ring about 7.5 cm in diameter with a long handle.

Limitations of Massage Method

- Skill and experience is needed for massage.
- Semen samples collected are not usually clean and contain many bacteria, as semen dribbles through the prepuce and drips from preputial hairs.
- More secretions of accessory sex glands and low sperm concentration.
- Sometimes sample may also be contaminated with urine.

1.1.1.2 Electro Ejaculator Method

This technique was first described and used in rams by Gunn in 1936. The probes used for this purpose are of different sizes and shapes depending on the species. This technique is painful for bulls and so was criticized by the Animal welfare personnel (Roberts 1971). That is why, it is only used in animals as in the below indications.

Indications

- Used in the case of aged bulls unable to mount.
- Used for crippled bulls.
- Used for bulls with decreased libido or with the problem of impotency.
- Used for bulls that are unwilling or unable to copulate.

Procedure

- Restrain the bull properly, handle it quietly, and keep it relaxed.
- Wash, rinse, and dry with a brush and cotton pledgets or with a clean towel the prepuce and the preputial hairs and the region around the preputial opening with warm physiological saline.
- The preputial hairs should be clipped.
- Operator, wearing a glove, gently inserts a lubricated hand and forearm into the bull's rectum emptying it of feces.
- Now the probe is inserted into the rectum placing it in the midline against the floor of rectum. Probe should also be lubricated with a noninsulating material like "K.Y" jelly.
- After the proper placement of probe, 3–5 V of current is applied for 3–5 s. It will result in erection and dripping of seminal fluids.
- After 3–5 s of current application, animal is given rest for 3–5 s.
- After resting, again the same amount of current is applied for the same time, then again rest is given. This process is repeated at least 5 times.
- Now current is increased up to 10–15 V. This current is applied for 3–5 s and after that the animal is given rest for 3–5 s as done with the low voltage. This will result in semen ejaculation.
- High voltage of 10–15 V is applied for 5 times with intervals as applied in low voltage.

- Another person holds a rubber cone attached to a collection vial placed in a plastic bag attached to a metallic ring about 7.5 cm in diameter with a long handle.

Limitations of Electro Ejaculator Method

- Skill and experience is needed for massage.
- Semen samples collected are not usually clean and contain many bacteria, as semen dribbles through the prepuce and drips from preputial hairs.
- More secretions of accessory sex glands and low sperm concentration.
- All bulls stiffen and show arching of back due to pain. Sometimes, bull may lean to one side or raise and extend one or both the rear limbs.
- This may lead to ataxia.
- Sometimes, choking may lead to death of animal due to pressure exerted by rear limb extension.

1.1.1.3 Artificial Vagina Method

This is the most widely used method. The early models made by Russians consisted of a bag like artificial vagina placed inside the vagina of a cow or the dummy. The AV used these days were developed in England, Perry, and Maule (Stephen). It is preferred over the other methods because the semen collected by this method is clean; complete ejaculate is obtained which is closer to natural ejaculation. There are two types of artificial vagina for bulls: a triple layer type or winter-type AV and a double layer type.

The artificial vagina of a *triple layer type* consists of three major layers, an outer casing made of plastic, a rectangular inner sleeve A made of rubber and a triangular inner sleeve B made of rubber. The inner sleeve A is fitted to the outer casing, and the inner sleeve B is fitted to the inner sleeve A. At one end of the inner sleeve B, there is a collection vial. Semen flows into the collection vial. This arrangement protects semen from temperature shock due to changing of temperature. But the main disadvantage of the triple layer AV is that it is longer and heavier compared to the double layer type.

The artificial vagina of a double layer type is shorter and lighter than the triple layer AV. It is used in tropical and warm regions. Due to shorter size it is easier to use. It consists of an outer casing, inner rubber liner, a cone, a collection vial, and an insulating jacket. About half to two-third of the chamber formed between inner rubber liner and the hard casing is filled with warm water. The water should be 125–180 °F, 50–70 °C. At the time of collection, the temperature of AV should be between 40 and 50 °C (MacMillan et al. 1966). Only small amounts of lubricant should be used for lubricating the inner liner. More quantity of lubricant will contaminate the semen. For this purpose, white sterilized Vaseline, K.Y. jelly, or pure white mineral oil are used (Fig. 1.1).

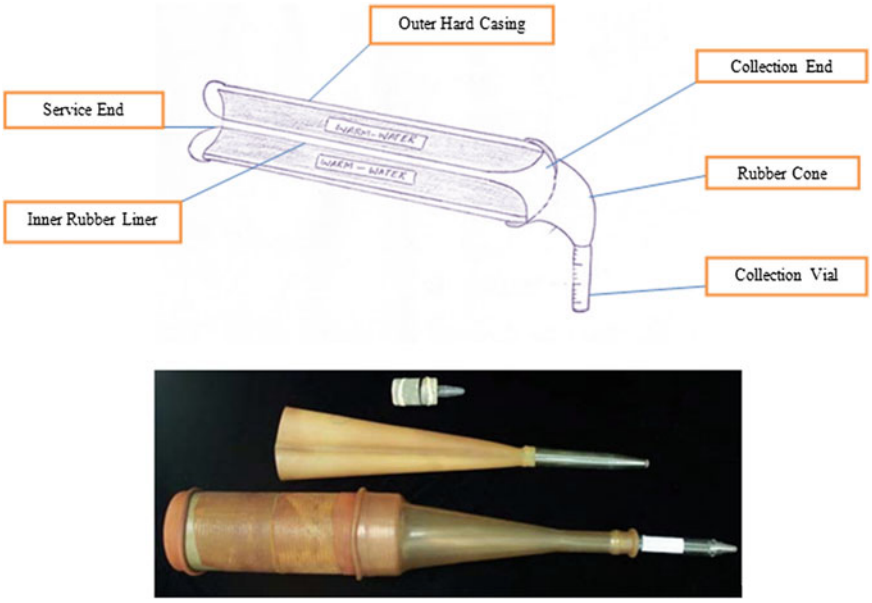

Fig. 1.1 Double layered artificial vagina

1.1.2 Advantages of Artificial Insemination

1. Artificial insemination not only increases the use of superior male animals but also makes their use more efficient. More people can be benefited from superior male. Use of the proven sires in dairy herds markedly increases milk production up to 30 % compared to natural breeding, Van Vleck.
2. Artificial insemination helps in great genetic improvement of farm animals. The selection and efficient use of superior bulls improves production.
3. Artificial insemination helps in controlling different venereal and other diseases like trichomoniasis, Vibriosis, brucellosis, etc.
4. The danger and expenses of keeping and handling bulls that prove to be inferior males can be eliminated.
5. It is easier to transport semen doses over long distances than to transport male animals.
6. Artificial insemination makes it possible to use the semen even after the death of a male.
7. Widespread use of artificial insemination in the dairy industry helps in proper breeding records.
8. Artificial insemination makes possible breeding of animals with size differences without injuries.

9. Artificial insemination made the use of those sires that are not capable of copulating, like aged or crippled sires.
10. It is a pre-requisite for embryo transfer.

1.1.3 Disadvantages of Artificial Insemination

1. Artificial insemination is an advanced and sophisticated technique, so well-trained personnel are required to supervise semen collection, examination, extension, freezing, shipping, and insemination of females.
2. Widespread use of artificial insemination increases the possibility of transmission of genetic abnormalities, for example COD, spastic syndrome, poor conformation especially of feet and limbs, and lack of libido.
3. Artificial insemination uses a limited number of elite bulls. This limited gene pool may improve milk production but it has a reverse effect due to increased inbreeding, which results in genetic abnormalities because of expression of recessive genes.

1.2 Sex Sorted Semen

Sex of the fetus is determined by the sperm because the sperm may carry either X or Y sex chromosome. Sperm having X sex chromosome when fertilizes an oocyte will result in a female and a sperm having Y sex chromosome when fertilizes an oocytes will result in a male offspring. The desire to separate X and Y bearing sperms is driven by the fact that one sex has more economic importance than the other for certain species. As in the dairy industry, the female calves are more important than the males because of maximum utilization of AI. As the major income of a dairy farm comes from milk, so it is advantageous to have more female calves that will become future producers (Senger 1999).

1.2.1 Procedure

The technique used for separation of X-bearing sperms from Y-bearing sperm is known as "Flow cytometry or cell sorting". Experiments have resulted in 80–90 % of successful separations of sperms in rabbit, cattle, and swine. It is well known that the X and Y chromosomes have different quantities of DNA. It is said that the X-bearing sperm has 2.8–4.2 % more DNA compared to Y-bearing chromosome depending on the species (Senger 1999). On the basis of difference of DNA, we can separate the X-bearing sperms from Y-bearing sperms. For this purpose, a DNA

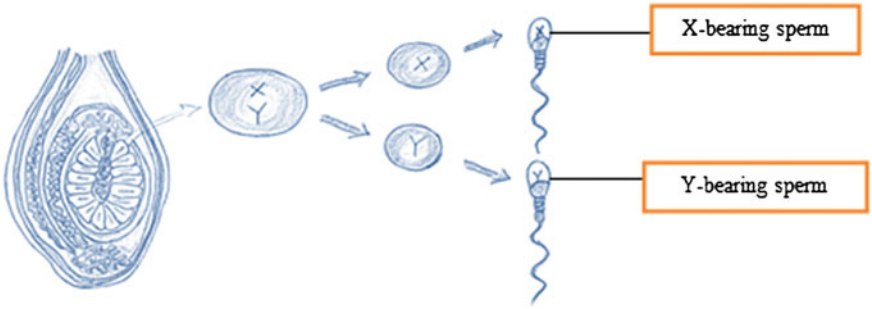

Fig. 1.2 X and Y bearing sperms produced by testes

stain or dye is used called Fluorochrome. The X-bearing sperm will take more DNA dye compared to the Y-bearing sperm. Vital stains used for sperm staining have a property of emission of light of specific wavelength when excited or activated by a light of specific wavelength.

Step 1: Collection of the semen from the male by using different methods as explained earlier (Fig. 1.2).

Step 2: Treatment of semen with Fluorochrome (Sperm staining dye). X-bearing sperms will take on more stain compared to Y-bearing sperms (Fig. 1.3).

Step 3: Once the spermatozoa enter the flow cytometer chamber, they pass single file through a small nozzle. After staining, the stained spermatozoa are excited by a laser beam. As a result of excitation, the X-bearing sperms emit more light compared to Y-bearing sperms. Sperms will emit light of different wavelength depending on their liveability and DNA contents. Dead sperms emit a very low beam of light when excited by laser beam, so they are easily differentiated (Fig. 1.4).

Step 4: After being excited, sperms pass through a light sensing device that is coupled with a computer. This device will determine the amount of light emitted by sperms and also order the passage of each sperm through a column below the nozzle.

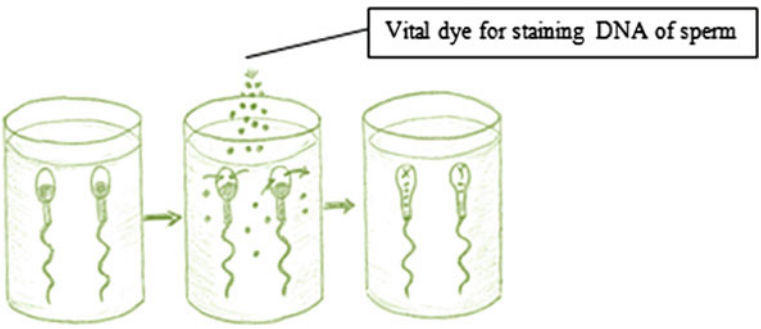

Fig. 1.3 Ejaculated sperm treatment by DNA dye Flurochrome

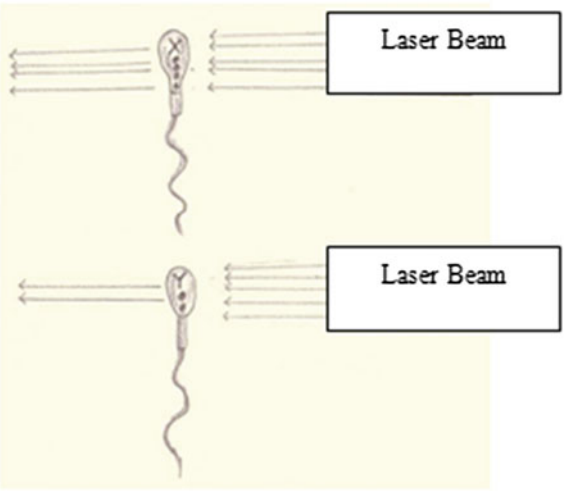

Fig. 1.4 X and Y bearing spermatozoa emitting different intensity of light when excited by a laser beam

Step 5: After that, sperms are charged on the basis of their DNA contents as they pass through the charged plates. Sperms are assigned either positive or negative charges depending on their DNA content (Fig. 1.5).

Step 6: Then the micro droplet containing a single sperm is passed through an electromagnetic field; the computer applies an appropriate charge and directs the droplet to one side or the other. Dead sperms are directed into the central tube. There are three vessels for sperm collection, one having higher proportion of X-bearing sperms, one having higher proportion of Y-bearing sperms, and one vessel having low proportion of dead sperms.

1.2.2 Limitations

Regardless of the problems associated with the separation of X-bearing sperm from Y- bearing sperm, the main limitation of this procedure is the cost of equipment. Also, the separation rate of this flow cytometer is slow.

1.3 Controlled Breeding and Synchronization

Early detection of estrus is becoming a major concern with the extension of the number of cows reared, the improvement of milking cows with high milk production, and the changes in the circumstances of feeding and management of cows.

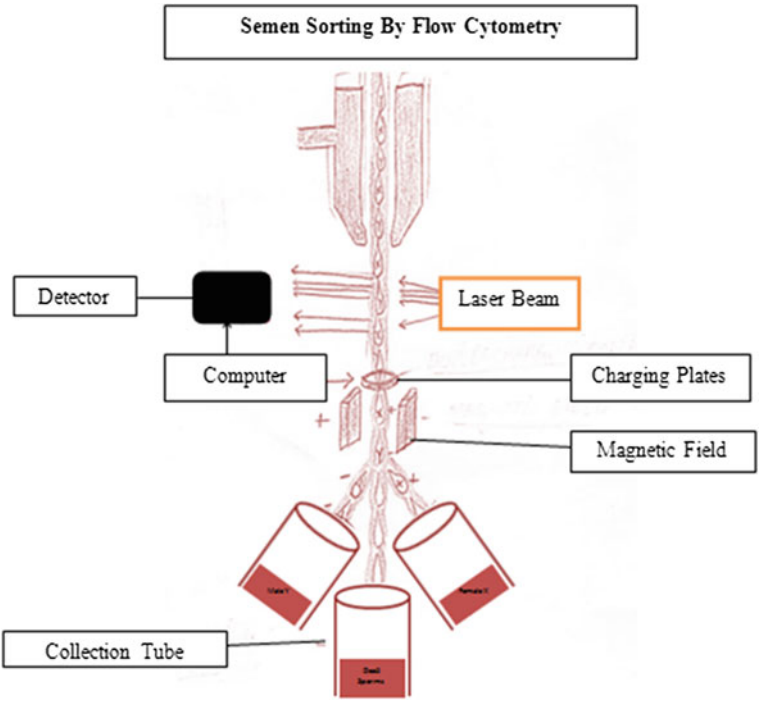

Fig. 1.5 Separation of X- and Y-bearing spermatozoa by flow cytometry

Estrus synchronization or controlled breeding is grouping of females for parturition at the same time. It is used at commercial dairy farms for uniform milk production throughout the year. It is closely linked with AI and is also a pre-requisite for embryo transfer, or is the first step of embryo transfer. Estrus detection is a major problem but by use of synchronization, we can reduce the time required for estrus detection with timed insemination (Sa'Filho et al. 2009).

1.3.1 Principle

- **Prolonging the luteal phase**
  - with P_4 for 9–14–21 days, on withdrawal follicular growth ensue, estrus, and ovulation occur within 2–8 days.
- **Shortening the luteal phase**

  $PGF_{2\alpha,}$ also E_2 regress CL within 1–3 days and estrus and ovulation occur.

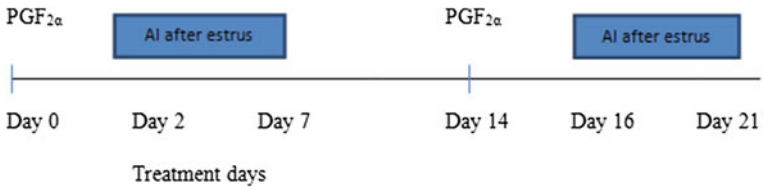

Fig. 1.6 Double PG protocol

1.3.2 Estrus Synchronization Protocols

1.3.2.1 Double PG Protocol

The main objective of double PG protocol is to have a high percentage of animals in Diestrus at the time of second injection. Double PG protocol involves an injection of prostaglandin (PGF2α) at random stage of the cycle. Animals with a mature corpus luteum (CL) will undergo regression after PGF2α injection and will come in heat within 2–5 days (Morrow 1986). A second shot of PGF2α is given after an interval of 11 or 14 days. The second shot will bring the remaining animals in heat. Artificial insemination is done after 3 days of the second shot (Fig. 1.6).

1.3.2.2 Ov-synch Protocol

Ovsynch protocol involves the use of $PGF_{2\alpha}$ and GnRH to synchronize ovulation in dairy animals. This protocol involvs the use of time AI (TAI), resulting in conception rates similar to that of AI after a detected estrus.

Procedure
Day 0: Inject GnRH to ovulate follicle and start a new follicular wave
Day 7: Inject $PGF_{2\alpha}$ to regress CL
Day 9: Inject GnRH to ovulate follicle
Day 10: Timed AI 16–20 h after second GnRH (Fig. 1.7)

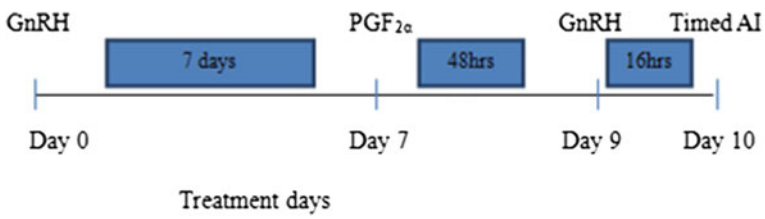

Fig. 1.7 Ovsynch protocol

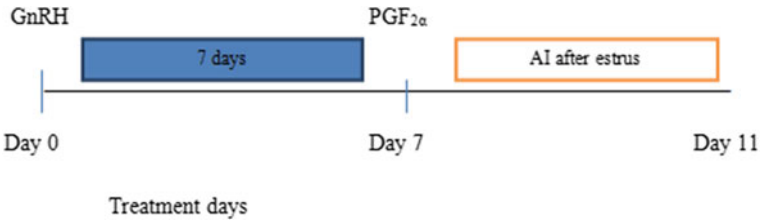

Fig. 1.8 Select synch protocol

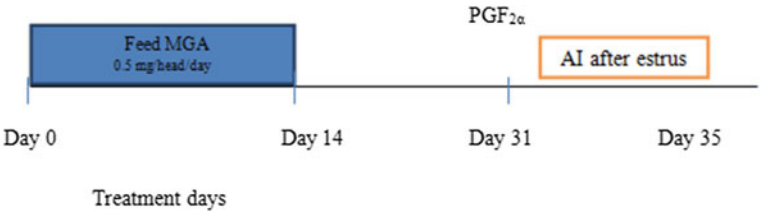

Fig. 1.9 MGA protocol

1.3.2.3 Co-synch Protocol

It is a modified method of Ovsynch. In this protocol fixed timed insemination is conducted at the same time of the second GnRH injection. Conception rate is low compared to Ovsynch, but labor is reduced in this method.

1.3.2.4 Select Synch

In the select synch program, the second GnRH is not administered. $PGF_{2\alpha}$ is injected 7 days after the first GnRH injection. After $PGF_{2\alpha}$ injection, the animal is observed for heat and is inseminated at the proper time. For the cow in which estrus is not seen, the fixed timed insemination should be conducted 3 days later. Select synch can be used in conjunction with TAI in a program called hybrid synch, by inseminating cows detected in heat after $PGF_{2\alpha}$ and conducting TAI+GnRH injection for the animals not detected in estrus by 84 h after $PGF_{2\alpha}$ (Fig. 1.8).

1.3.2.5 MGA Protocol

Malengesterol acetate is used only in the case of heifers, and not recommended in the case of heifers. It is generally fed in a grain carrier and either top dressed onto

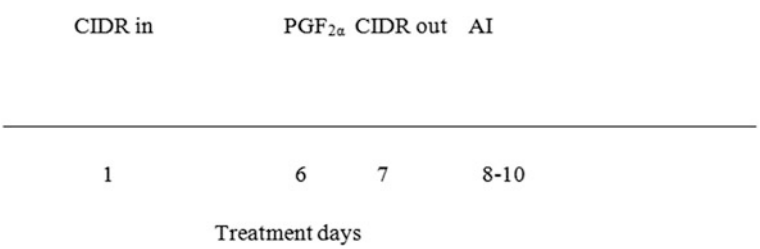

Fig. 1.10 CIDR protocol (designed by authors)

feed. MGA is given in feed (0.5 mg/head/day) for 14 days and $PGF_{2\alpha}$ is administered on either the 17th day or after the conclusion of MGA supplementation. Insemination is done 12 h after estrus (Larson et al. 1995) (Fig. 1.9).

1.3.2.6 CIDR Protocol

In this protocol, a progesterone-based intra-vaginal device CIDR is placed in the vagina of the dairy animals for 7 days. $PGF_{2\alpha}$ is injected on the 6th day of CIDR placement. CIDR is removed on the 7th day. Insemination is done after 12–24 h of estrus detection (Naseer et al. 2011) (Fig. 1.10).

1.4 Embryo Transfer

Embryo transfer is a technique in which embryo before implantation stage is collected from a donor female and is transferred to a recipient female that will serve as a surrogate mother for the remaining pregnancy. This technique has been applied to a large number of domestic and wild animals.

1.4.1 History of ET

The first successful transfer of rabbit embryos was done in 1890 and in bovines, embryos were collected successfully in 1930. The technique was developed in the 1970s and 1980s on a commercial basis. Initially, this technique utilized surgical procedures and is the main reason for early rejection in early days. ET grew in

popularity with the development of nonsurgical method. In buffalo, birth of calf by embryo transfer was done by Drost et al. in 1983. Birth of a buffalo calf from a frozen embryo was done by Nemat Ullah in 1987.

1.4.2 Basic Steps for Embryo Transfer

- Synchronization of donor and recipients
- Superovulation (FSH, luteolysis with PGF)
- Insemination at estrus (Day 0)
- Collection of embryos using flushing media (PBS) (Day 5)
  - Surgical (laparotomy) or nonsurgical (trans cervical)
- Grading of embryos-Cryopreserved
- Transfer (Fresh or Frozen) in recipients (Day 5)
  - Surgical or nonsurgical
- Pregnancy test.

1.4.3 Selection of Donor Cow

The selection criteria for donor depend on the species and also on the demands of breeder that will be more on the basis of economic issue than genetic issues. In bovine, the donor is selected on the basis of the following criteria.

- Most important is the performance records of the animal, show ring appeal, or both.
- The reproductive tract of the donor should be normal. For that purpose, properly palpate it through rectal palpation.
- Embryo transfer should be done after 60 days of previous parturition.
- Animal should have a regular estrous cycle beginning at a younger age and normal estrus interval.
- It should have a history of no more than 2 breeding pre-conception.
- First three calves born within two calendar years.
- No conformational or detectable genetic defects.
- No calving difficulties or reproductive irregularities.
- From 3 to 10 years of age. In older cows, muscles in the perineal region have less tone and tend to suck air into the uterus and rectum after the administration of epidural anesthesia. This is reduced by completely full rumen or by lifting the fore limbs resulting in positive pressure.

Table 1.1 Super ovulation treatments in cow

Day	Time	Treatment 1	Treatment 2	Treatment 3
10	AM PM	2500 IU PMSG	5 mg FSH 5 mg FSH	5 mg FSH 5 mg FSH
11	AM PM	Recipient receives $PGF_{2\alpha}$	4 mg FSH 4 mg FSH	5 mg FSH 5 mg FSH
12	AM PM	Donor receives $PGF_{2\alpha}$	3 mg FSH 3 mg FSH	5 mg FSH 5 mg FSH
13	AM PM		2 mg FSH 2 mg FSH	5 mg FSH 5 mg FSH
14	AM PM	AI	AI	AI
15	AM PM	AI AI	AI AI	AI AI

1.4.4 Superovulation of Donor

The most favorable and optimal time for superovulation treatments is between the 8th and 14th day of the cycle (Morrow 1986). Superovulation treatment will result in release of multiple eggs at a single estrus. Cows or heifers that are properly treated can release 10 or more viable eggs. Nearly 85 % of the donors respond to superovulation treatment protocols.

Treatments. Superovulation treatment protocol involves the use of single injection of 1500–3000 IU of equine chorionic gonadotropin (eCG) or previously known as pregnant mare serum gonadotropin (PMSG) in cow (Eldsen et al. 1978). However, response was better in terms of ovulations, embryos recovered, and pregnancies reported after superovulation treatment protocol using follicle stimulating hormone (FSH) twice daily in decreasing doses for 5 days or 5 mg FSH twice daily for 5 days (Eldsen et al. 1978) (Table 1.1).

1.4.5 Insemination of Animals

Most of the superovulated females are inseminated multiple times. One example: Inseminate the superovulated cow at 12, 24, and 36 h after onset of standing heat. Site for semen deposition: body of uterus or into the entrance of each uterine horn.

1.4.6 Selection and Preparation of Recipient

It is a critical step for embryo transfer success. Recipient must have the following characteristics.

- Recipient must have proper nutrition (BCS 6).
- Recipient should have gone through herd health program.
- Synchronization of estrus (within 1 day) between the donor and recipient cow.
- Recipient must not have any problem in reproductive tract.
- Recipient should not have weak CL.

1.4.7 Embryo Collection

In the early days till 1975, surgical techniques were used for ET. Surgical methods resulted in adhesions of the reproductive tract which in turn reduces the fertility of a very expensive elite animal. In addition, requirement of different facilities and recovery of embryos at farm level was impossible by surgical method. As non-surgical method was developed which was preferred over the surgical method as it was not damaging for the reproductive tract, repeatable, and could be performed at farm level. Nonsurgical method involves the recovery of embryos from uterus by penetrating the cervix during early diestrus.

1.4.8 Preparation of Donor

- Restrain the donor by placing in a squeeze chute. Rectum is evacuated of feces and air.
- Check the number of CLs on both the ovaries.
- Now administer the epidural anesthesia.
- After epidural anesthesia, wash the perineal area, and tie the tail.
- After epidural anesthesia, ballooning of rectum may occur. So remove the excess of air by using stomach tube or pump.

1.4.9 Catheters

There are basically three types of catheters used in the nonsurgical method of embryo collection. Most commonly, two-way and three-way Foley catheters are used. Foley catheter is mostly used because it is low cost and easily available. However, it is soft and difficult to thread into the uterine horns.

Sometimes we may also use “Modell Neustadt” two-way catheter. This is stiff and long compared to the Foley catheter. It is more useful in those animals with long uterine horns.

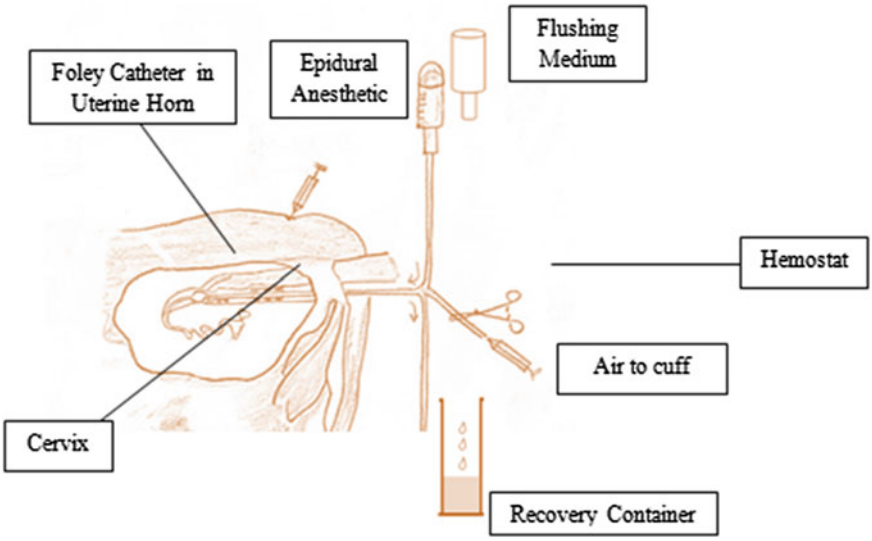

Fig. 1.11 Embryo collection by three-way Foley Catheter

1.4.10 Techniques

There are two methods of collection: the continuous way, closed-circuit system and the interrupted-syringe system. Closed system helps in maintaining sterility. It also prevents the loss of media and embryos. But extra tubing provides extra potential for contamination from either bacteria or chemicals. Interrupted method allows for the use of completely disposable equipment with the exception of catheter.

1.4.10.1 Interrupted Syringe Technique

The lubricated catheter with the stilette is passed through the vagina and the cervix. Sometimes cervical dilators are used before passing the catheter. The catheter is directed into the right uterine horn and stilette is gradually removed as the catheter is threaded down the horn. The catheter is so placed that the inflated cuff is present approximately half way between the uterine body and the tip of the horn. Inflate the cuff with 5 ml of sterile saline, then palpate with additional saline until the cuff completely fills the uterine lumen.

Collection is done with a disposable syringe using 25–35 ml of media per flush. The embryo is usually found in one to the first four flushes. Each horn is flushed at least eight flushes placed in 500 ml graduated cylinder. Normally, 85 % embryos are found in first four flushes. Do not force the media with pressure into the horn as it may lead to injury to uterus. In this method, first, inflate the uterine with media,

agitate the horn, and then remove this fluid. After repeating this procedure with both horns, each horn is infused with 30–40 ml of an antibiotic solution. $PGF_{2\alpha}$ may be given at this time or 1 week later to prevent pregnancy (Fig. 1.11).

1.4.10.2 Continuous-Flow Technique

Embryo collection is usually done using the three-way Foley catheter. The lubricated catheter with the stilette is passed through the vagina and the cervix. Sometimes cervical dilators are used before passing the catheter. Catheter is manipulated into the selected horn so that the inflated cuff is situated at the bifurcation of the horns. Inflate the cuff with 15 ml of the air or fluid, more can be added according to need. Approximately 500–800 ml of the media having temperature of 37 °C is placed in an Erlenmeyer flask fitted for infusion with tygon tubing. The tubing from the flask is attached with the inflow tube of Foley catheter. Another tube will be connected with the outflow of catheter carrying the flushed media into a 1000 ml graduated cylinder.

First, 20–30 ml of media is allowed to freely move in the system to check for blockages or to clear mucous or blood clots. Outflow tubing is then clamped and horn is filled with the media from inflow tubing. Massage the uterus gently and agitate the horn to dislodge the embryo from endometrial folds. Now outflow clamp is removed and inflow is clamped. Fluid is drained out of uterus into horn. After flushing wash the endometrium three times with media. $PGF_{2\alpha}$ may be given at this time or 1 week later to prevent pregnancy.

1.4.11 Searching of Embryo

Media after flushing is allowed to settle down for 35 min in a straight-sided cylinder. A siphon of sterile silastic tubing may then be set up to remove all but bottom 50 ml of medium. Remaining media is agitated, swirled, and aspirated into a syringe with a uterine infusion pipette. Place the media in a sterile, disposable petri dish. Observe the aspirated media under a stereo microscope (Fig. 1.12 and Table 1.2).

1.4.12 Transfer of Bovine Embryo

There are two methods of embryo transfer in bovines.

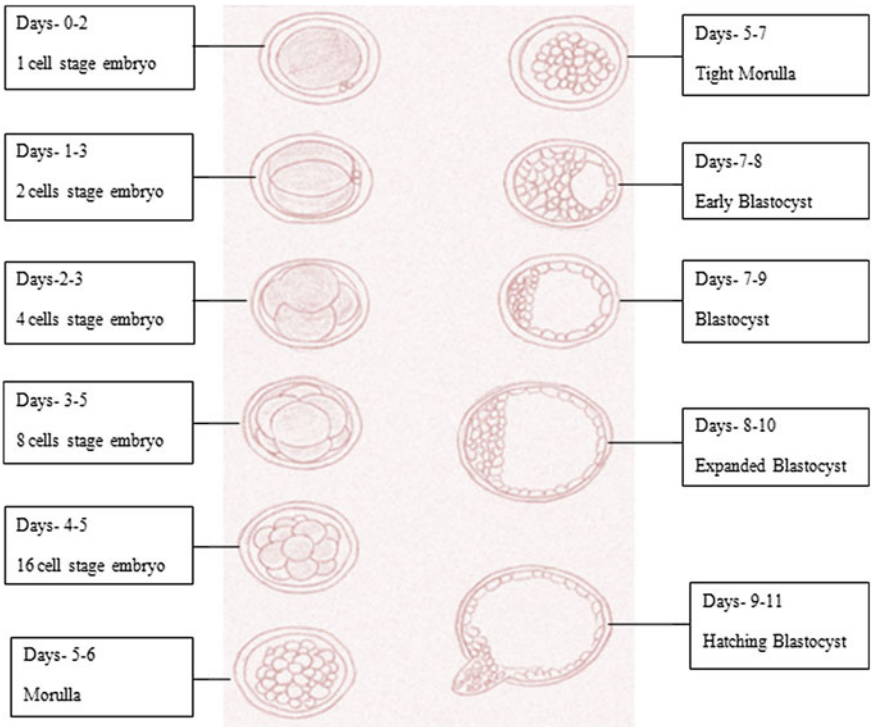

Fig. 1.12 Stages of development in pre-attached embryos

Table 1.2 Grading of embryo

Grade	Quality	Typical characteristics of embryo
1	Excellent	Embryo perfectly symmetrical, showing even granulation, and with a well-defined, distinct outline; no blastomere extrusion. The embryo should be at the expected stage of development for its age
2	Good	Embryo showing even granulation with a well-defined distinct outline; some blastomere extrusion and some minor blastomere degeneration; occasionally somewhat asymmetric in shape
3	Fair	Embryo intact but with a hazy outline in parts; obvious defects apparent such as extruded cells, vesiculation, and some degenerate blastomeres
4	Poor	Embryo showing uneven granulation with a hazy outline; much blastomere extrusion and degeneration evident; sometimes shaped abnormally
5	Degenerate	Degeneration so pronounced that it may not be possible to determine the exact developmental stage; sometimes shaped abnormally

1.4.12.1 Surgical Transfer Technique

Embryos are transferred by surgical method either by flank approach or by mid-ventral technique. This generally involves use of general anesthesia of the recipient, placing her in dorsal recumbency, surgical preparation of the mid-line just anterior to the mammary gland, and surgical invasion of the abdominal cavity. Once the uterus has been located and a suitable CL has been confirmed in one of the ovaries, a small puncture is made into the lumen of the uterine horn ipsilateral to the CL. The pipette loaded with the embryo is introduced through the puncture and the embryo is deposited. Routine closure, extubation, and post-operative recovery follow. To facilitate the procedure, most firms withhold feed (24–48 h) and water (12–24 h) from the recipient prior to midventral surgery.

1.4.12.2 Nonsurgical Transfer Technique

Many methods had been used in the past for transfer of embryo through nonsurgical technique. These include the use of telescoping stainless steel rods with flexible polyethylene tubing to deposit embryo in the cranial portion of uterine horn, transvaginal technique using stainless steel needles to circumvent the cervix and polyethylene tubes for embryo deposition. However, the most commonly used equipment for nonsurgical embryo transfers in the bovine is the Cassou AI gun and either 0.5 or 0.25 ml French straws. The recipient is examined for the presence of an appropriate CL and a normal uterus, and an epidural anesthesia is given to eliminate rectal contractions. The straw is loaded into the AI gun and the sheath is placed over it. The vulvar area is cleaned and wiped dry. The Cassou gun is inserted to the cervix and crossed through the cervix into the horn that is ipsilateral to CL.

1.5 In Vitro-Fertilization (IVF)

In vitro-fertilization is the collection of oocytes from a donor female that are matured in the lab and fertilization of that matured oocyte in a laboratory dish. The embryo resulting from that fertilization is cultured in a specific media for a few days and ultimately transferred into a female recipient. The eggs after collection are placed in CO_2 incubators in the IVF laboratory. Most viable spermatozoa are recovered after processing for inseminating the eggs. Because of the thick layer of zona pellucida and thousands of follicular cells around the ova, embryologists usually add approximately 100,000 spermatozoa for an egg. The addition of large number of viable spermatozoa to each ova will disperse the follicular cells and also

ensure fertilization of egg by one spermatozoa. IVF has been used to treat many infertility issues, i.e., when both fallopian tubes are blocked, fertilization of the egg cell has to take place outside the body.

1.5.1 History of IVF

For many years. The first cattle calf was born by using IVF in 1982, Brackett et al. In buffalo the birth of a live calf by using IVF technique in 1992, Totey et al. The first foal was born in 1991 by using this technique by Palmer and his associates. In goat, the first kid born through this technique was by Hanada.

1.5.2 Advantages of In Vitro Embryo Production

- Circumvention of the problem of timing ovulation for AI.
- Potential for producing more embryos.
- Make possible the use of animals suffering from certain issues of infertility such as tubal obstruction, Endometritis, etc.
- A reduced number of viable sperm needed for IVF compared to AI or natural breeding.
- By using sperm microinjection techniques, the potential of using nonviable sperm (Goto et al. 1991) and testicular or epididymal-derived sperm (Uehara and Yanagimachi 1976) for assisted fertilization.
- Potential of salvaging genetic material from female animals after death.

1.5.3 Embryo Production

In vitro production of embryo requires the recovery of oocytes and completion of three biological phases: maturation of oocytes, fertilization and developing the fertilized zygotes to the blastocyst stage, when they can be successfully frozen and subsequently transferred to recipients.

1.5.3.1 Sources of Oocytes or Donor

Any female after puberty is a potential donor of oocytes with a single requirement of presence of antral follicles on the ovaries with greater variations in the development competence of oocytes amongst donors (Lazzari and Galli 1993). Oocytes may be collected from slaughtered donor ovaries by aspirating; dissecting or slicing

(Caroan et al. 1994). Dissection technique allows the isolation of individual follicles. Sectioning of ovarian tissues gives the highest number of oocytes. Aspiration is the most efficient technique in terms of the time to obtain oocytes. Ovum pickup (OPU) by puncture of ovarian follicles in the live donor by laparoscopy or ultrasonography. OPU can provide four–eight oocytes per collection and may be an alternative to superovulation in the future. Oocytes recovery from live donors is accomplished by transvaginal ultrasound guided aspiration of follicles. This is done once or twice a week and can be repeated for many weeks (Kruip et al. 1994); resulting in average four to eight oocytes per session per donor.

1.5.3.2 Fertilization

Commercial frozen semen is used for fertilizing the oocytes. For fertilization, the motile sperms may be separated from the extender by several means. Direct washing can be used when a high percentage of sperms are progressively motile. Swim-up is also a common technique; however, it is not used with very viscous extenders. Centrifugation on Percoll gradients (45–90 %) ensures the highest recovery of motile sperms. The required number of sperms is diluted in bicarbonate buffered TALP IVF containing heparin (Parrish et al. 1989). The sperm-containing medium is dispensed in micro drops under paraffin oil or simply in wells without oil. At the same time, oocytes are matured for 22–24 h, after removal of cumulus cells. Oocytes are then placed in fertilization media and coincubated with sperm for 18–24 h at 38.5 °C in CO_2 incubator. The fertilization rate can be assessed indirectly by examination of cleavage rate 40–42 h after fertilization, but for more accurate analysis it is necessary to fix some oocytes 18–22 h after fertilization. Oocyte fixation is done in 3:1 ethanol:acetic acid for 24 h stained with lacmoid and observed under phase contrast microscope.

1.5.3.3 Embryo Culture

There are three systems for culturing the zygotes. In the first system, fertilized oocytes or cleaved embryo is transferred to ligated oviduct of recipient (sheep or rabbit). Five or 4 days later (i.e., 6 days post insemination), the embryos are recovered, graded, and frozen or transferred (Eyestone et al. 1987). In the second system, zygotes are co-cultured in vitro with somatic cells (oviductal epithelial cells, granulosal cells, buffalo liver cells, etc.) in medium TCM 199 or B_2 Menezo with 10 % serum or 1 % bovine serum albumin (BSA) at 38.5 °C in 5 % CO_2 or in medium conditioned by somatic cells (Vansteenbrugge et al. 1994). In the third system, zygotes are cultured in simple medium without any somatic cell support. A common medium for this purpose is synthetic oviductal fluid (SOF) (Tervit et al. 1972) in which serum is added (Walker et al. 1992) or BSA and amino acid addition in SOF (Gardner et al. 1994); incubation is performed at 38.5 °C in 5 % CO_2 and 5 % O_2.

1.5.3.4 Embryo Transfer

Embryo is transferred to the recipient as such or it is first cryopreserved using 10 % glycerol in phosphate buffered saline or using ethylene glycol (Voelkel and Hu 1992). Embryos that are frozen using ethylene glycol can be directly transferred after thawing without the washing procedure required to remove the cryoprotectant (Galli and Lazzari 1996).

1.5.4 Micro-Assisted Fertilization

In vitro fertilization (IVF) is mostly used while dealing with issues of male infertility but success rates are reduced either due to failure of sperm passage through zona pellucida (ZP) or due to inhibition of sperm-oocyte membrane interactions (Rogers et al. 1979; Overstreet 1980; Cohen et al. 1985). Microsurgical manipulation could assist spermatozoa which are otherwise unable to fertilize. Conventional IVF is very helpful in treating long-term and rological infertility; however, when mammalian sperms lose their ability to penetrate ova both in vivo and in vitro conditions, the capability of spermatozoa to govern the embryonic development can be assessed by the use of different micromanipulation techniques (Kyung and Koji 2006), a last option for most of the diagnosed cases of infertility of males involves the use of micromanipulators for penetration of sperm into ova.

1.5.5 Micromanipulation Techniques

Oocytes are held and micromanipulated in culture medium droplet while monitoring under an inverted microscope. The ova is kept still by the help of a holding pipette applying a gentle suction and several spermatozoa are deposited either into the perivitelline space or a single spermatozoa is directly injected into the cytoplasm of oocyte by using injection pipette. Depending on the site of sperm injections, these techniques are categorized as zonal, subzonal, and intracytoplasmic inseminations.

1.5.5.1 Partial Zona Dissection

Partial zona dissection (PZD) is technically the simplest and least traumatic to the oocyte. Partial Zona Dissection was first introduced by Gordon and Talansky (1986) in mice using digestive effect of acid Tyrode's (AT) medium on the ZP (Cohen et al. 1989). During PZD, an opening is created for ensuring sperm penetration (Malter and Cohen 1989). This procedure is performed in sucrose to induce

limited shrinkage that will help in minimizing the occyte damage. The micropipette is injected through ZP into the perivitelline space and moved out through the opposite end of ZP without disturbing the cytoplasm of ova. Then oocyte is released from holding pipette and an opening is made in the ZP overlying the micropipette by rubbing against holing pipette. Some researchers have also employed laser for PZD as it is a simple and precise technique.

Cumulus Oophorus cells of all oocytes are removed with 0.1 % hyaluronidase for 1–2 min at 37 °C, 3–9 h following egg collection. The corona radiate of oocyte is assessed for cell expansion and grouped as expanded, tight, or very tight. The oocytes are rinsed three or four times and corona cells are removed manually with hypodermic needles. When the cells are too tightly adhered to the ZP, a window is cleared for visualization of the ooplasm. The cytoplasm is reduced to facilitate needle piercing using a 0.1 M solution of sucrose in Earle's medium. The sucrose is added in one step and completely removed after micromanipulation in three or four steps. After sucrose exposure, ~50 % of the oocytes of each patient are randomly chosen for micromanipulation. All of these oocytes are checked at regular intervals 12–16 h following insemination.

1.5.5.2 Subzonal Insemination

Subzonal Insemination (SUZI) involves direct injection of spermatozoa into the perivitelline space by penetrating Zona pellucida. On the basis of morphology, 3–6 spermatozoa are aspirated into a microneedle and injected into perivitelline space. As spermatozoa are directly injected into the perivitelline space resulting in bypassing of acrosomal reaction, the techniques to artificially induce acrosomal reaction have been developed as the use of reacted sperm will decrease number of sperms required for the process, ultimately decreasing the risk of polyspermic fertilization. Two methods employed for this purpose include: Incubate the sperms in Tyrode's media+follicular fluid (50 %) and for 24 h after washing. Then electroporation is done and again incubated in 3.5 mmol/L of pentoxifylline. Although this method resulted in 54 % of sperms without acrosome, the efficacy was questionable.

1.5.5.3 Intracytoplasmic Sperm Insemination

Intracytoplasmic sperm injection (ICSI) has been described as beneficial in alleviating infertility in couples that could not be helped by standard in vitro fertilization (IVF) treatment or by subzonal insemination (SUZI) of the oocytes (Palmero et al. 1992, 1993; Van Steirteghem et al. 1993). Most of these issues of infertility are due to severe male-factor infertility (Van Steirteghem et al. 1993). The first successful use of ICSI demonstrated that freeze-dried human spermatozoa could develop into pronuclei when injected into hamster oocytes (Uehara and Yanagimachi 1976). Later, birth of normal calves after transfer of blastocysts was obtained following ICSI using bovine spermatozoa (Goto et al. 1990).

Microinjection of isolated sperm heads into oocytes is performed on a microwarm plate at 37 °C at 200X magnification using a piezomicromanipulator controller. Injection and holding pipettes used are mostly made of borosilicate glass capillary tubes. Sperm heads are aspirated into the injection pipette in a minimal amount of medium. Tip of pipette is brought in contact with zona pellucida of ova that is held by holding pipette. Zona is drilled by applying two to three pulses. Once the tip has reached the perivitelline space, it is forced onto oolemma and then into cytoplasm. After confirming that the tip is inside oocyte, a small amount of cytoplasm is withdrawn; a sperm head in a minimal amount of medium is expelled into the oocyte and pipette is withdrawn gently from oocyte. The injection procedure should be completed within 50 min after oocytes preparation. Before culturing, the ICSI oocytes are treated with ionomycin in combination with 6-dimethylaminopurine (DMAP) for activation of oocyte (Roh et al. 1998). All oocytes used in ICSI are washed thrice with TALP having 3 mg/ml BSA and cultured for 1 h in the same medium (50 ul) covered with paraffin oil in a culture dish in CO_2 incubator at 39 °C. After culture, oocytes are treated with 10 uM ionomycin in TALP containing 1 mg/ml BSA for 5 min at room temperature and then ionomycin-free TALP having 30 mg/ml BSA for 5 min to stop the activation process. The treated oocytes are washed thrice with culture medium and cultured for 3 h in the same medium at 39 °C under 5 % CO_2. Then transfer the oocytes to culture medium having 1.9 mM DMAP and culture for further 3 h. Finally, after washing thrice with culture medium, oocytes are cultured in the same medium at 39 °C under 5 % CO_2 (Kyung-Bon and Koji 2006).

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