Reproductive biotechnologies and Artificial Insemination

Artificial Insemination (AI) Technology

- Definition; AI is a method of breeding in which semen is obtained from the male, processed and introduced into the female reproductive tract by means of instruments with no direct contact between the male and female
- Application of AI is predicated on three major premises
    1. The spermatozoa can survive outside the body.
    2. They can be reintroduced into the female genital tract in a way that results in an acceptable conception rate.
    3. The fertile period of the female can be identified.
- AI is routinely practiced in cattle, sheep, pigs, goats, fowl, turkeys, dogs, horses and even bees.

Cont …

- **Advantages of AI**

- Speeds up genetic improvement by enhancing use of superior sires for cross breeding and selective breeding programs (30-40 calves/ year natural mating vs. thousands of calves/ year AI) (1 bull can breed 500,000 cows in a lifetime. After death, semen can be used (Oldest frozen semen 40 - 45 years old)
- Facilitates progeny test under a range of environmental and managerial conditions, thereby further improving accuracy of selection
- Reduce large number of males kept for breeding – reducing management costs & risk of injury from aggressive males
- Control of venereal disease (trichomoniasis & complyobacteriosis)
- With the development of semen cryopreservation, AI has facilitated international trade in animals

Cont …

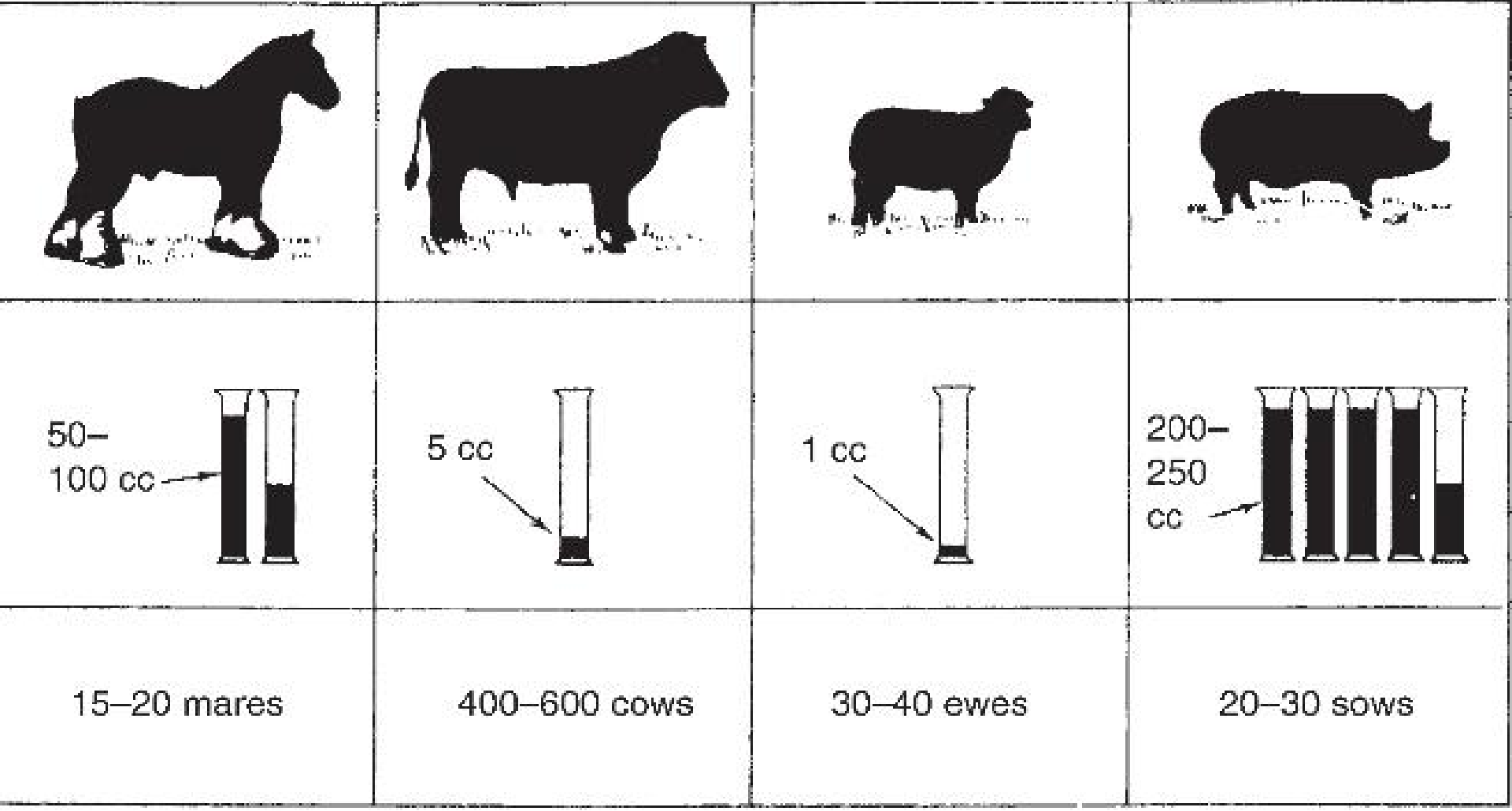
- Facilitates research on male reproductive physiology
- Others; better reproductive management

- **Disadvantages of AI**

- AI programs entail additional costs for estrus detection, semen collection and processing and insemination (labor, hormones, record keeping system, specialized facilities, equipments and supplies)
- AI requires training of specialized technicians (farmers if do it yourself AI)
- Genetic faults can be widely disseminated if they are present in an AI sire.
- Uncontrolled use of sires in AI can disseminate venereal disease.
- Fertility after AI is heavily dependent on efficiency of estrus detection system and competency of

Cont …

- **Collection and Handling of semen:**
- Semen Collection methods described in BSE
- Ejaculate semen should be promptly transported to the laboratory for evaluation and processing – as described in BSE
- Upon arrival, semen should be placed in water bath at 30˚C for rams and bucks, and 35 ˚C for the bulls and buffaloes or 37-38˚C for stallions and camels.
- Once semen is evaluated, it can be used in four ways:
  - Used immediately undiluted to inseminate one or two females.
  - Diluted and used immediately for insemination of several females.
  - Diluted and refrigerated for use over the next 3 days (cooled and chilled semen).



Semen volumes produced by farm animals
Stallions and boars give high-volume and low-density semen; bulls and rams give low-volume and high-density semen.
The number of females that might be inseminated with a

Cont …

- **Semen Dilution/Extension**
- Single ejaculate contains more sperm (hundred million to billions) than actually needed to achieve pregnancy
- Hence, by diluting the semen, it can potentially be used for several inseminations instead of one
- Dilution rate depends on minimum number of spermatozoa and volume of semen required to achieve acceptable pregnancy rates –
- These factors are determined by the site of insemination and the survival of sperm in diluent - variable across species
- **Semen extender functions**

⎫ Add volume

⎫ Provide Energy substrate (glucose, fructose and mannose)

⎫ Provide a PH Buffer (Phosphate, citrate , TRIS} - cooled > frozen

⎫ Maintenance of osmotic pressure - cooled < frozen

⎫ Provide protective antimicrobial activity (penicillin and streptomycin additives)

⎫ Protect the sperm against cold shock and injuries during freezing and

Cont …

- **The dilution process involves**:

- Both semen and extenders are placed in a water bath at 30-35 ˚C.
- Semen is diluted by gradually adding the extender to the semen **(!)**
- Semen to extender dilution ratios are 1:1 to 1:10 depending on the species, semen volume and sperm-cell concentration
- The recommended antibiotics are add and allowed to stand (holding time) for 5 minutes with raw semen or 2 hours diluted semen to increase the antibiotic action on microorganism

- **Short term sperm preservation – Cooling/Chilling**
- The life span of spermatozoa at ambient temperature is generally short, but can be extended by inhibiting their metabolism and motility with carbon dioxide (by cooling/refirgration). Cooling sperm, result in considerable damage to the cells, with leakage of intracellular potassium, enzymes, lipoprotein and ATP occurring due to membrane changes (**cold shock**) such as;

- Membrane phospholipids change from fluid to a gel phase.
- Membrane proteins become irreversibly clustered, leading to loss of function

Cont …

- **Semen Cooling Extender should contain;** A buffer, an Energy substrate, Antibiotic additives and Cold shock protecting agent
- Common cold shock protecting agent include proteins, lecithins, and lipoproteins (source egg yolk (not for goat) and heat treated skimmed milk).

Cont …

- **Long term preservation - cryopreservation**
- This is critical element for extensive use of AI in genetic improvement programs
- Long term storage of semen is achieved through cryopreservation i.e. frozen in Liquid N at (-196 ^{o}C)
- Freezing will cause cellular damage by inducing dehydration (water and electrolyte loss) or intracellular ice crystal formation in Spermatozoa
- Therefore in addition to the constituents of a cooling extender, freezing extenders need to have a cryoprotective agent (commonly Glycerol)

Milk yolk glycerol (MYG)	
Milk	75 mL
Egg yolk	20 mL
Glycerol	5 mL
Penicillin G	1000 I.U. mL^{-1}
Streptomycin	1.00 mg mL^{-1}
Lactose yolk glycerol (LYG)	
11% lactose solution	75 mL
Egg yolk	20 mL
Glycerol	5 mL
Penicillin G	1000 I.U. mL^{-1}
Streptomycin	1.00 mg mL^{-1}
Lactose fructose yolk glycerol (LFGY) 3:1	
11% lactose sol.	56.25 mL
6% fructose sol.	18.75 mL
Egg yolk	20 mL
Glycerol	5 mL
Penicillin G	1000 I.U. mL^{-1}
Streptomycin	1.00 mg mL^{-1}
Glucose yolk citrate glycerol (GYCG)	
Dist. Water	100 mL
Glucose	58 mg
Sod. Citrate	5 gm
Egg yolk	20 mL
Glycerol	7 mL
Penicillin G	1000 I.U. mL^{-1}
Streptomycin	1.00 mg mL^{-1}
Tris citrate fructose	
Tris (hydroxymethyl-amino-methane	3.1 mg 100 mL^{-1}
Citric acid	1.97 mg 100 mL^{-1}
Citric acid	1.55 mg 100 mL^{-1}
D (-) fructose	20 mL
Egg yolk	20 mL
Glycerol	7 mL
Penicillin	1000 I.U. mL^{-1}
Streptomycin	1.00 mg mL^{-1}

Composition of diluters used for preservation of bull semen

Dilution and Freezing

- Dilution rate adjusted to desired AI dose (25 – 30 million sperm Cells for bull)
- Concentration 1.25 billion/ml & 5 ml ejaculate = 6.25 billion total sperm
- 6.25 billion total / AI dose of 25 – 30 million = 250 AI doses
- One AI dose of bull semen is packed into thin plastic French straws (0.25 ml capacity).
- 0.25 ml x 250 = 62.5 ml Final dilution volume i.e. 57.5 ml Extender
- Semen and Extender are kept at30 -34 ^{o}C (Water bath or Incubator)
- Half of the total extender volume is gradually added to

Cont …

- The basic unit for semen storage and shipment is an individual **plastic French straws (0.25 – 0.5 ml)**, containing enough semen for a single insemination
- Straws are filled and sealed by using an automatic filling and sealing machine or manually. Semen is sucked into the straws and on meeting the polyvinyl alcohol powder plug at one end and impervious seal is made. The other end is pinched together and cold sealed.
- Individual straws are identified with the sire's name and registration number, breed, code number, and identification as to the specific collection.
- After filling, sealing, and freezing, the straws are placed into plastic cups (goblets). Each goblet holds five straws. Two **goblets** are clipped to an aluminum holding device called a **cane**. This provision for vertical storage makes it possible to store many straws of semen in a compact space

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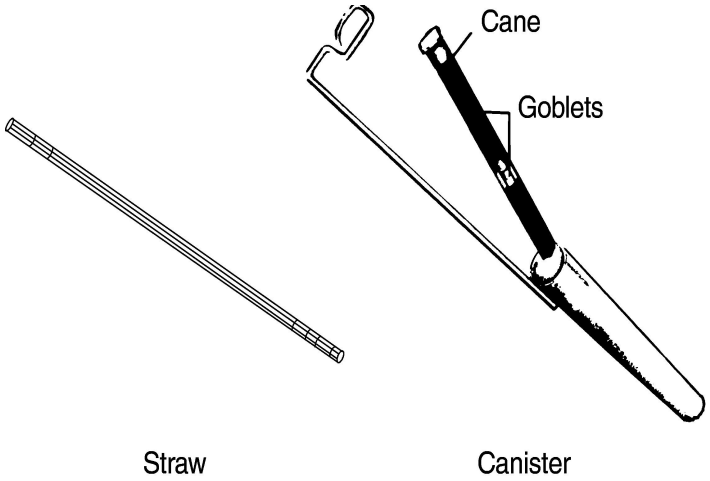

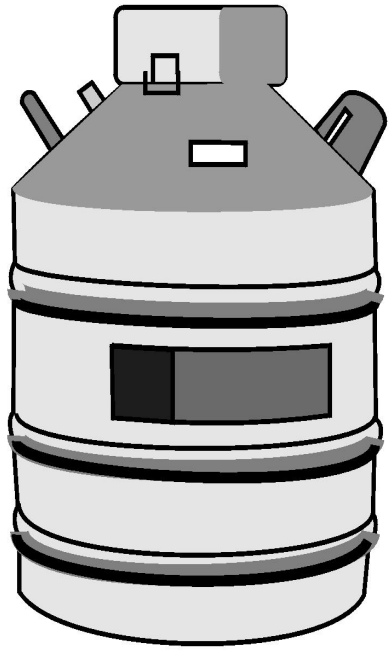

Liquid Nitrogen Storage Container

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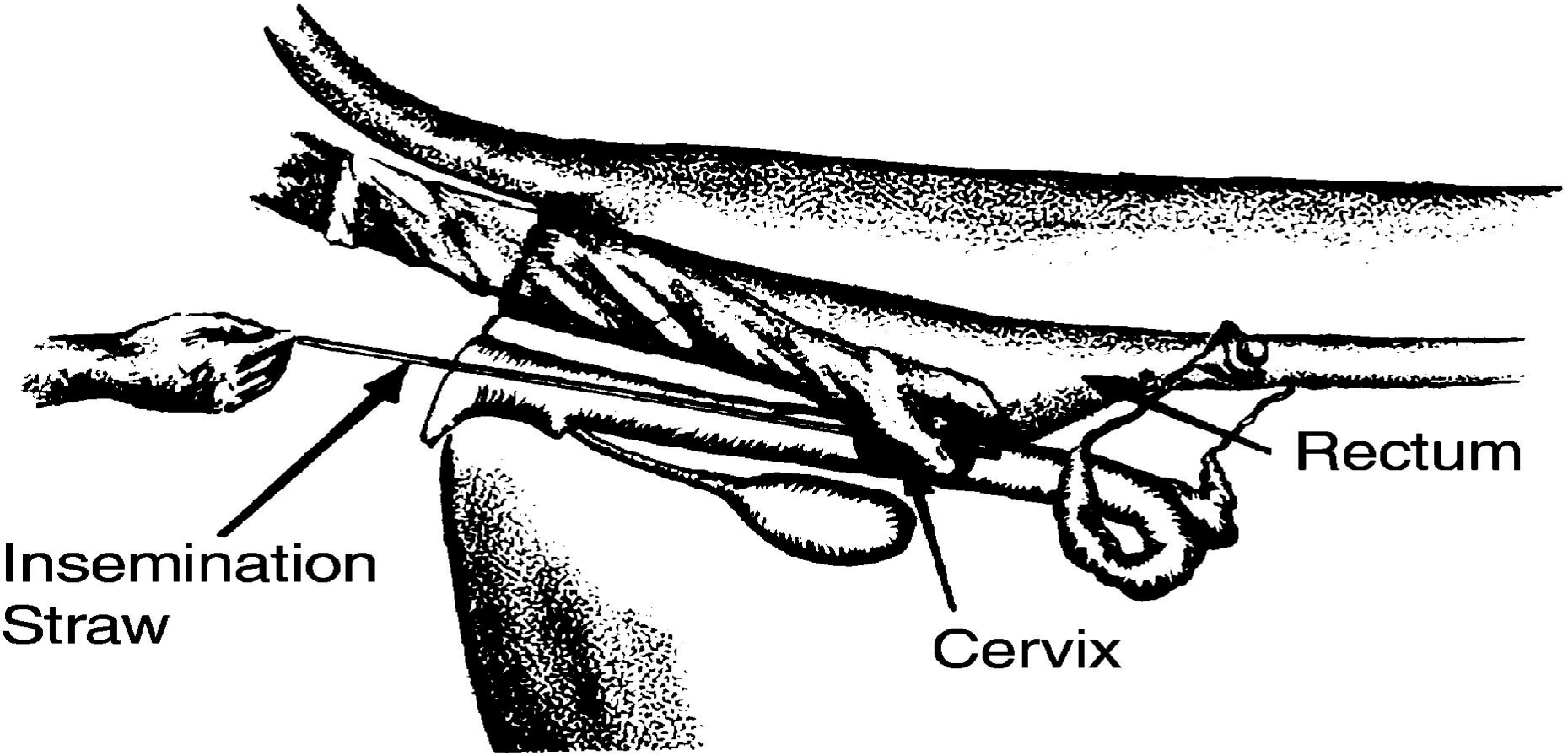
- **Thawing of frozen semen:**
- Thawing of the semen needs to be rapid; slow thawing allows recrystallisation of ice within the cells, causing membrane damage.
- The process of thawing is simply immersing the straws in water at +35 ˚C for only 7 seconds.
- **Artificial Insemination**
- **Equipments**
- Catheter – Stainless steel (Gun for Trans-cervical), Plastic (Cervical and Vaginal), protective materials and containers (Boar and Stallion)

AI in Cows

- Cows ovulate at about 12 hours after the end of the oestrus period. The ideal time for insemination is therefore 6 – 24 hours prior to ovulation.
- Practically, if the cow first seen in heat in the morning, she should be inseminated in the afternoon of the same day, or the cow first seen in heat in the afternoon, she should be inseminated on the next day morning (AM – PM Rule)
- Cows are insemination just into the short uterine body.
- Insemination into the cervix produce a lower fertilization rate.
- The standard technique of insemination - Trans-cervical intrauterine method;
- Grasp the cervix through the rectum with the left hand.
- A catheter (AI Gun) into the tip of which a straw of semen has been inserted, is then passed in to the vagina (30-45^{o} Inclination)
- After locating external cevical os – protective sheath is broken and AI gun is manipulated into and through the cervix by the right hand


Rectum
Insemination
Straw
Cervix

Estrus Synchronization

- The history of estrous cycle synchronization and the use of artificial insemination in cattle is a testament to how discoveries in basic science can be apply to advance the techniques used for livestock breeding and management.
- Synchronization of estrus involves manipulating or controlling the estrous cycle of the females, so that they can be breed at approximately the same time.
- Most estrous synchronization systems employ a method for controlling follicular wave development, promoting ovulation in anestrous cows, regressing the corpus luteum in cyclic cows, and synchronizing estrus and (or) ovulation at the end of treatment.
- Synchronization of the estrous cycle has the potential to:

What are the advantages?

- Allows an organized and efficient approach for AI
- Synchronizes estrus and thus reduce time required for estrus detection
- Improves record keeping and group cow management
- Labor saving tool for utilizing superior genetics, increase longevity and productivity of dairy cows
- Better management tool for maintaining proper calving interval (12-13 month)
- Facilitate adoption of artificial insemination

What are disadvantages of estrus synchronization include:

- Drug expense and labor
- An existing high level of management is required
- Good handling facilities are required
- Cows must be cycling and in good body condition

Principles of Synchronization

- Synchronization of estrus in cows is feasible by either curtailing or extending the length of estrus cycle, which can be maintained based on two principles
    - ¬ One is using of in-situ luteolytic agent (prostaglandin) that induces luteolysis of corpus luteum (CL) and exogenous administration of such agents mimics premature luteolysis and hence results in to shortening of left over diestrus phase of estrus cycle
    - ¬ The second principles is lengthening of diestrus phase through maintenance of CL in terms of progesterone production which determines the length of diestrus phase. Hence, with the administration of progesterone

Estrus synchronization Hormones

1. Prostaglandin: PGF2α

- Regression of the CL and decrease in progesterone synthesis and secretion
  - Lutalyse - Natural compound 25 mg dose I.M.
  - Estrumate - Analogue 500 mg dose I.M.
  - Prostomate - Analogue to Lutalyse

*Mode of action for **PGF2α***

- **Regress active corpus luteum**
- **Regresses Day 5-17 corpus luteum**

Response:

- **estrus (heat): 2-5 days after injection •**
- **heifers ~50 hours cows ~72 hours**

  ~60-65% of herd should respond to injection

2. Gonadotropin Releasing Hormone (GnRH)

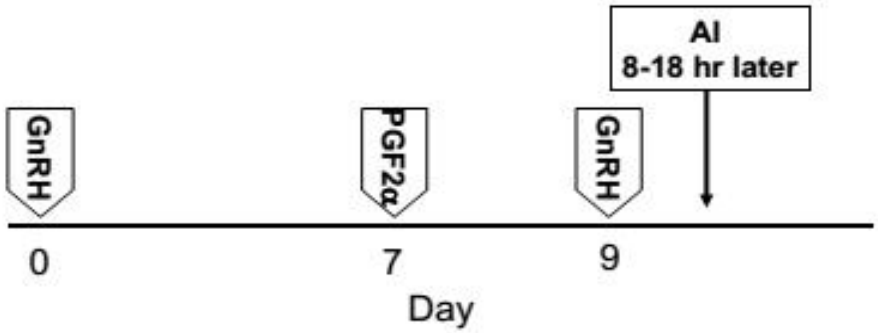
ϖ Ovulation and/or luteiniziation of the growing follicle

¬ Cystorelin - GnRH Analogue

3. Control Intra-vaginal Drug Release (CIDR®)

- Known as EZ-Breed TM
- Progesterone release device
- Controlling the estrus cycle by preventing cows coming to estrus.

4. OvSynch® or Timed Artificial Insemination



Advantages:

– No need for heat detection

– Increased AI submission rate

• OvSynch can be beneficial in Hot and humid condition where heat expression is usually low

– A tighter synchrony of ovulation

Disadvantages:

– Cost of Drugs

• a reduced dose of GnRH can be as effective

– Labor cost for animal handling

– Low conception rate compared to other breeding program

Embryo Transfer

Embryo Transfer

ϖ Embryo transfer is a technique by which embryos are collected from a donor female and are transferred to recipient females, which serve as surrogate mother for the remainder of pregnancy

- Two major genetic improvement tools exist:

1. artificial insemination
   - allows genetically superior males to produce thousands of offspring
2. embryo transfer
   - allows genetically superior females to produce hundreds of offspring

- AI and ET are designed to increase genetic selection intensity
- Use only the genetically best males as AI sires
- Use only the genetically best females as ET donors

Requirement for Embryo Transfer

1. Source of good quality embryos/EGGS
2. Proper uterine environment in the recipient at the time of ET
3. A reliable methods of transferring embryos .

Limitations of Embryo Transfer

1. Variability in embryo production of donors - 20 % do not produce any embryo
2. Low pregnancy rates after transfer of embryos to recipient.
3. Limitation in superovulation due to poor response to repeated treatment with fertility drugs –GnRH and eCG → Antibodies .

- Poor technique for collecting and transferring embryos in farm animals yet this problems is greatly improved

Production of embryos

In vivo production

- The mammalian ovary contains thousands of Oocytes
- However, domestic ruminants shed only one of two eggs per estrus cycle
- This number of eggs is increased by super-ovulation methods

Induction of Superovulation

Induction of ovulation is needed for :

- Treatment of anoestrus
- Lactation animals
- Post-pubertal animals
- Under nutrition cases
- Advancing Breeding season
- Oestrus Synchronization for time AI

Super ovulation

→Induction of multiple ovulations by ovarian super stimulation using fertility drugs

- It increases supply of embryos from animals of superior genetic merit
- Superovulation is induced using FSH, eCG or GnRH
- Superovalation can be done in conjunction with estrus synchronization
- Individual response to superovulation is variable (0 - 10)
- Superovulation is induced with exogenous FSH or eCG injected IM or SC.
- FSH has a shorter half-life . Thus, the total dose should be divided and injected at 12 hrs interval for 3-4 days

- For optimum response, gonadotrophin treatment is initiated on days 9–14 (oestrus = day 0) of a normal oestrous cycle, coinciding with the emergence of the second follicular wave.
- Prostaglandin is administered 48–72 hours later to cause regression of the mid-cycle corpus luteum and induce oestrus, which usually occurs 40–56 hours later.
- Behavioural manifestations of oestrus are usually normal, and it is common practice to inseminate donors on at least two occasions 12–18 hours apart when using frozen semen as ovulations may occur over a prolonged period of time .
- Single embryos can be recovered and transferred to other

Production of Embryos

1. **Insemination of super-ovulated donors:**

- Needs more sperm per insemination
- Poor fertilization rate in some eggs

Reasons:

- Poor sperm transport
- Ovulation over a period of time
- Defective ova

Production of Embryos

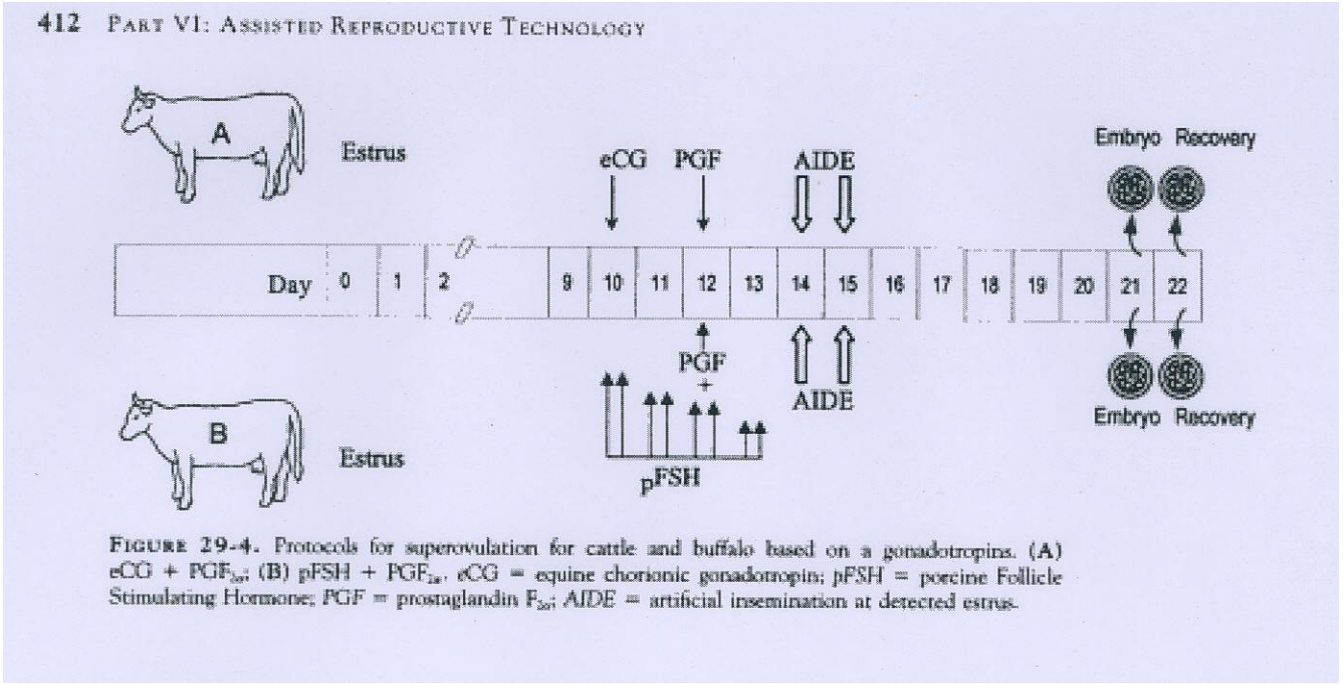


FIGURE 29-4. Protocols for superovulation for cattle and buffalo based on a gonadotropins. (A) eCG + $PGF_{2\alpha}$; (B) pFSH + $PGF_{2\alpha}$. *eCG* = equine chorionic gonadotropin; *pFSH* = porcine Follicle Stimulating Hormone; *PGF* = prostaglandin $F_{2\alpha}$; *AIDE* = artificial insemination at detected estrus.

Production of Embryos

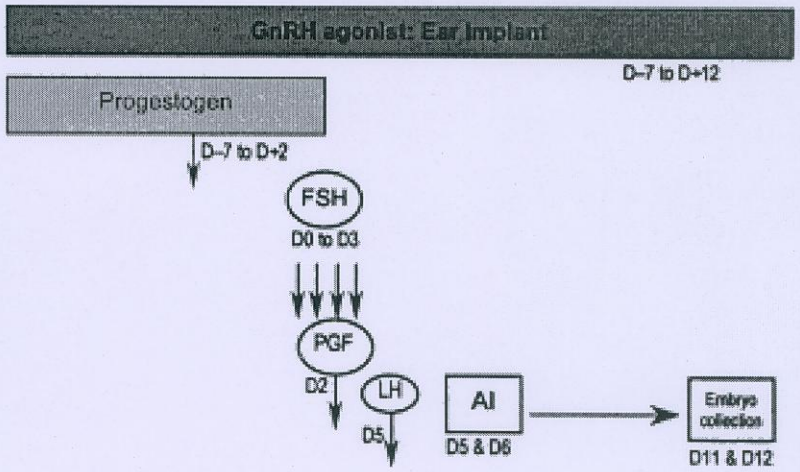


FIGURE 29-6. A novel protocol for superovulation in cattle using GnRH agonist + FSH + $PGF_{2\alpha}$ + LH (adapted from D'Occio MJ, Sudha G, Jillella D, et al. Use of a GnRH agonist to prevent endogenous LH surge and injection of exogenous LH to induce ovulation in heifers stimulated with FSH: A new model for superovulation. Theriogenology 1997; 47:601.). The GnRH agonist bioimplant, placed subcutaneously in the ear, releases about 20 μg deslorein/24 h. Day 0 = Day of first FSH. Treated cows are inseminated at time of the LH injection.

Two types of embryo collection

1. Non –Surgical methods
   1.1. Transcervical
   1.2. laparoscopy
2. Surgical methods

1.1. Trans-cervical method

- In this approach a 3- way Foley catheter is used for collecting embryos in cattle and mare
- The catheter with stelette is guided through cervix by rectal manipulation
- Can be positioned is uterine body or horns and the balloon is inflated –most prefer horns in cattle and cervix is mare .
- The uterine horn is filled with 30-60 ml warm BPS (warm) then stop irrigation open the out-let
- Then the fluid is allowed to flow into the

- This procedure is repeated until 300-800 ml of PBS medium is used
- The Foley catheter is inserted into the other horn and the same thing is repeated
- Transcervical route can be used is sheep and goats after Injection of PGF_2 + Oxytocin facilitate introduction of the catheter.

1.2. Laproscopy

- The basic difference between surgical technique and laparoscopy is that instruments for flushing are inserted through stab wounds rather than by mid-ventral incision
- A Laparoscope is inserted though one stab wound
- While visualizing the uterus a 2- way Foley catheter is inserted through another stab wound and guided into one horn and balloon is inflated.

- Next an IV catherer is inserted into the uterine lumen close to uteri-tubal junction
- About 30-40 me PBS is injected through IV catheter and flushing collected through Foley catheter
- Repeat the procedure until 150-200 ml PBS is finished
- The other horn is flushed similarly
- Consists of exposing the reproductive tracts by mid-ventral incisions under general anesthesia.
- Embryos collected from uterine horns 5 days after estrus
- Fluid introduced into the base of uterine horn and flushed towards utero- tubal

Selection of embryo for transfer

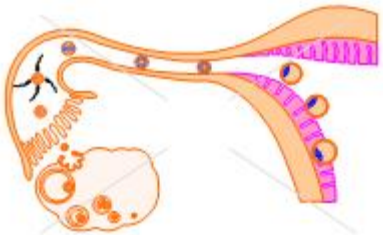
- The flushing for in-vivo cultured /matured embryos or in-vitro cultured/conceived (IVC) are examined directly under stereomicroscope –morphologically normal ones selected (blastomeres equal sized, uniform cytoplamic granules and complete cumulus enclosure)
- The embryos are kept in a container that prevents evaporation of the culture medium.

→paraffin are is used to cover the medium to prevent evaporation and contamination

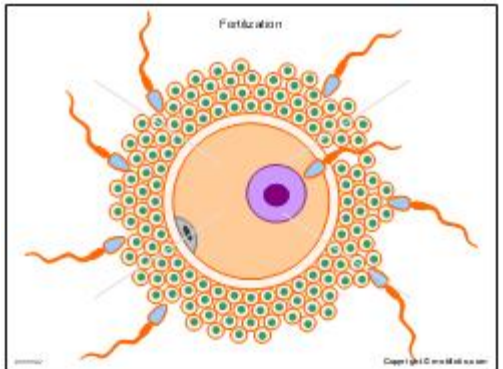
- Embryos from 8- cells to blastocyst stage result in good pregnancy rate

Ovary

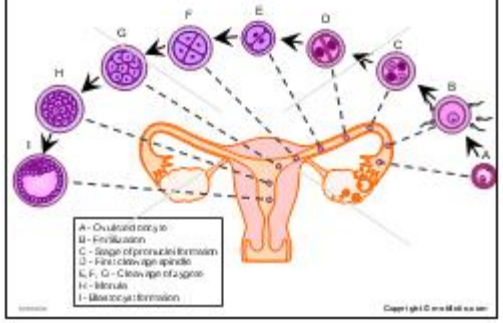
Fertilization and implantation



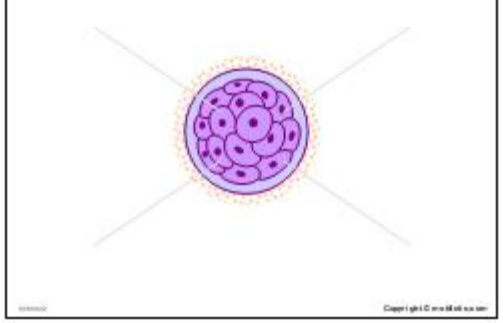
Fertilization



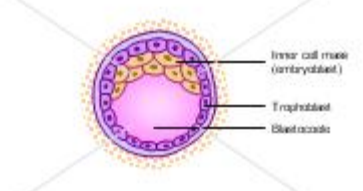
Stages of pre-embryonic development during the first week



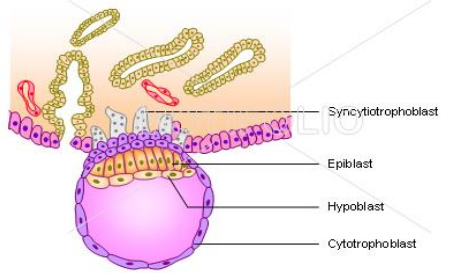
Early stages of implantation – 3 days, morula



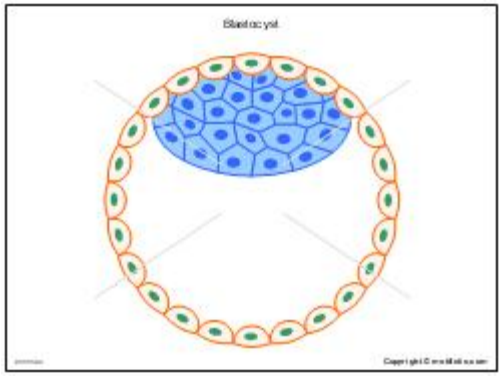
Early stages of implantation – 5 days, section of blastocyst



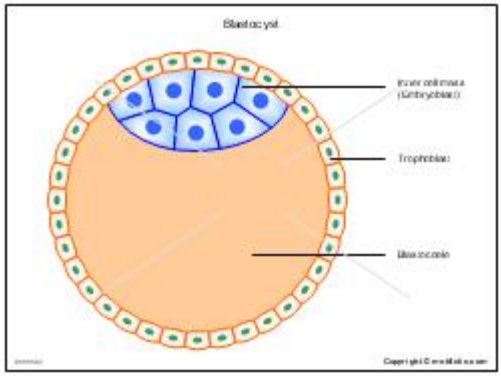
Implantation of blastocyst – 7 days blastocyst, begins to implant



Blastocyst



Blastocyst



ET Techniques

- ET→ Transfer of a good quality embryo obtained from a donor female into the reproductive tracts of a recipient females'.
- A recipient female should in the same reproductive stage of female from which the embryos are obtained
- ET to many female at a time requires estrus synchronization

- Synchronization of estrus donors and recipients
- Recipient that are in estrus at the same time as the donor are required.
- Pregnancy rate after ET is influenced by:

→ conditions (Fit breeders reproductive tract, no disease, good BCS)

→Preparation of the recipient (in estrus 7 days earlier, optimum period with 12 hrs)

Thawing of Embryos

- Embryos in 0.5ml straw are thawed in air for 20 seconds –Exposure to air reduces damage to Zona pellucida
- Then in water bath at 37^{o}C for 20 seconds
- Removal of cryoprotectant (ex: glycerol in conventional methods)

→Glycerol is diluted with PBS in 4-6 steps, each step takes 5-6 min

→In transferring embryos between steps a microscope is needed

ET Technique

- To overcame the stop wise dilation of the cryoprotectant a method of dilating glycerol within the straw by incorporating of diluted sucrose (a rehydrating solution) between 2 air bubbles before freezing has taken place
- After thawing the cryoprotectant and the sucrose solution are mixed by shaking the straw
- This avoid the need to unload embryos for rehydration and reloading a new