SEMEN DILUTION AND PRESERVATION AT DIFFERENT TEMPERATURES

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THERIOGENOLOGY LECTURES – A5

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- Extension of semen is one of the advantages of AI
  - In natural service, 1 ejaculate might settle 1 cow
- In AI, with the use of extenders, one collection may be used for **from 300 to more than 1000 cows and heifers**

Object of extending semen

- To increase the volume of the ejaculate
- To aid in preserving the viability of sperm
- A large number of females may be successfuly inseminated to a single collection

Initial Semen Processing

- Bull semen extenders provide substrates for sperm to generate the energy required for survival and motility, buffering capacity to control acidity, and macromolecules to provide protection during the cooling and freezing process.
- The macromolecules are typically provided by including yolk from hens' eggs or heat-treated whole milk.
- This is why frozen thawed semen appears either yellow or "milky."

Preservation of Semen

- The two primary techniques for semen storage are **chilling and cryopreservation.**
- For the chilling technique, **semen is stored at 4-5°C for 3 days for maximum and best results.**

Preservation of Semen

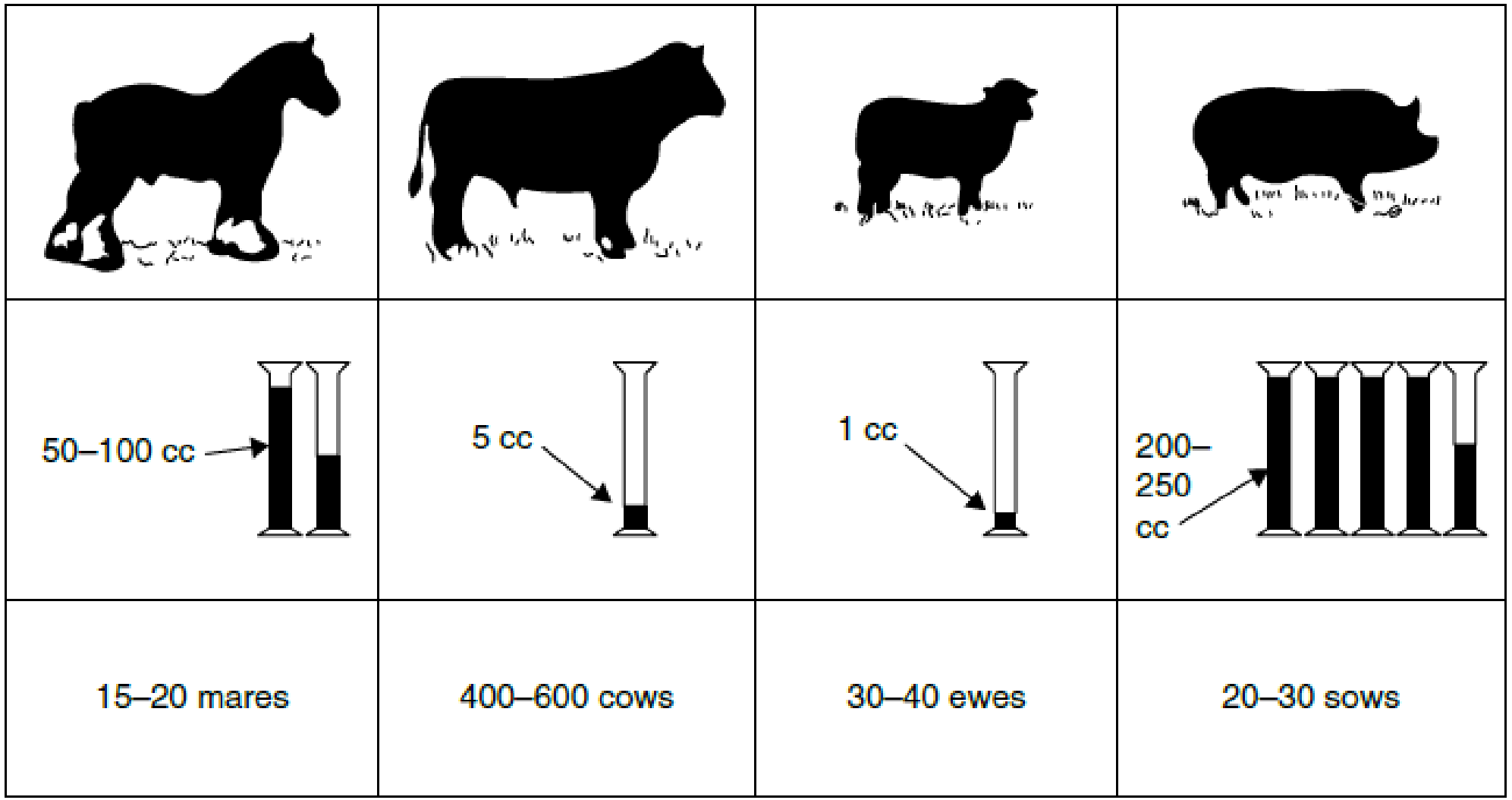
- In the cryopreservation technique, semen is exposed to freezing for 3 h at 4°C.
- Meanwhile, it is filled into 0.25-mL straws and finally preserved and stored in liquid nitrogen for years.
- The crucial factors for long-term semen preservation to retain its quality **include cooling for 2-3 h, adding a cryoprotectant, and freezing in liquid nitrogen**

Semen extenders

- Semen extenders are used as a **medium for pre serving sperm to enable fertilization.**
- Semen extenders can also **maintain and preserve sperm metabolic processes**, **control the pH of the medium during and after post-thawing**, **control bacterial transmission and contamination**, and **reduce cryogenic damage**.

Objectives of semen dilution/extension

- To **preserve the fertilizing potential of semen for a long period of time**.
- To **increase the number of services per ejaculate**.
- Ideally approximately **500 cows can be inseminated from diluted/ extended semen of a single ejaculate.**
- Extenders provide the spermatozoa with a medium in which they are able **to remain viable and fertile for a prolonged time period**.



Semen volumes produced by farm animals.
Stallions and boars give large-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 single semen dose is also indicated (After Hammond *et al*., 1983.)

In order to be an ideal extender !

- It should be able to provide conducive environment to prolong sperm survival and modify the sperm structure to prepare them for freezing

In order to be an ideal extender !

- It should be isotonic with blood and should maintain this during preservation
- It should have capacity of maintenance of pH (6.6-6.8) with high buffering capacity in that range
- It should ensure supply of metabolites for nutrition and survival of sperm cells

In order to be an ideal extender !

- It should contain lipoprotein and lecithin, minerals in adequate quantity and should contain substances to be used for aerobic metabolism by the spermatozoa
- It should provide protection against cryoinjuries
- It should prevent the formation of ice crystals

In order to be an ideal extender !

- It should not contain any material which may exert any adverse effect on spermatozoa, female genitalia, fertilization process or growth and development of the zygote
- It should contain antibiotic in sufficient quantity to check the microbial growth

In order to be an ideal extender !

- **Egg yolk semen extenders** are extensively used in the laboratory and field techniques because of their **reasonable price and satisfactory results**

Commonly Used Extenders for the Preservation of Semen

- **Preservation of Semen at Ambient Temperature (18-30°C) :**
  - A. Coconut Milk Extender
  - B. Milovanov's Extender
  - C. Illini Variable Temperature (IVT) Extender
  - D. Cornell University Extender (CUE)

A. Coconut Milk Extender

Buffer, Antibiotics and Catalase (Solution I)

Sodium Citrate dihydrate	2.2 g
Penicillin G. sodium	60.0 mg
Dihydrostreptomycin sulphate	135.0 mg
Sulphanilamide	300.0 mg
Polymyxin B sulfate	10.0 mg
Aqueous solution of catalase	15000 units
Mycostatin	1000 units
Triple glass distilled water	60-70 ml

B. Milovanov's Extender

Solution I	
Potassium dihydrogen phosphate	0.720 g
Triple glass disilled water upto	100 ml
Solution II	
Sodium citrate dihydrate	20.276 g
Glucose	5.7 g
Sodium bicarbonate	1.26 g
Sulphanilamide	3.0 g
Triple glass distilled water upto	900 ml

C. Illini Variable temperature (IVT) Extender

Sodium citrate dihydrate	2.0 g
Sodium bicarbonate	0.21 g
Potassium chloride	0.04 g
Glucose	0.3 g
Sulphanilamide	0.3 g
Triple glass distilled water upto	100 ml

Commonly Used Extenders for the Preservation of Semen

- **Extenders used for Preservation of Semen at Refrigeration - Temperature (4°C)**
  - For preservation at refrigeration temperature semen is diluted with suitable extender at 35°C.
  - Such semen samples are placed in a wide mouth glass tube with a stopper and the tube is placed in a beaker containing water at room temperature.
  - The beaker along with semen tube is stored in refrigerator maintained at 4°C.
  - Such preserved semen samples can be stored up to 3-4 days.

Commonly Used Extenders for the Preservation of Semen

- Extenders used for Preservation of Semen at Refrigeration

Temperature (4°C):

A. Egg yolk Citrate (EYC) extender

B. D2 Dilutor

C. Egg yolk Glucose Bicarbonate extender (EYGB)

Commonly Used Extenders for the Preservation of Semen

- **Extenders used for cryopreservation of Semen (-196°C)**
- As a principle all extenders which can be used for dilution and preservation of cattle and buffalo semen at refrigeration temperature can be used for cryopreservation of semen of corresponding species of animal on addition of a suitable **cryoprotectant**.

Addition of the Cryoprotective Agent - Glycerol

- Once semen is partially extended and cooled to 4°C, a final extension with medium containing glycerol is required.
- Glycerol is required to protect the sperm during the freezing process by a mechanism that remains unknown.

Addition of the Cryoprotective Agent - Glycerol

- It must be used **at relatively high concentrations** which can be **damaging** to the sperm **at higher temperatures**.
- **This is one reason that glycerol is added after cooling.**

Addition of the Cryoprotective Agent - Glycerol

- Addition of **trehalose** to bull semen extenders has provided a modest improvement in fertility when used in combination with glycerol, which remains the cryoprotective agent of choice in the AI industry.

Addition of the Cryoprotective Agent - Glycerol

- **The final extension is important for a second reason:** it determines **the final sperm concentration in the extended semen** and therefore **the number of sperm in each straw or breeding unit**.

Addition of the Cryoprotective Agent - Glycerol

- This number varies considerably depending upon the fertility and popularity of a sire, but typically ranges from **10 to 40 million sperm/straw.**

Commonly Used Extenders for the Preservation of Semen

- The following extenders are frequently used for cryopreservation of cattle and buffalo semen :

A. Commercial extenders

B. Tris citric acid egg yolk extender

C. LFYG (Lactose, Fructose, Egg yolk, Glycerol) extender

D. Egg yolk citrate extender (EYC)

E. Skim milk or whole milk extender

Equilibration, Packaging, and Freezing

- After glycerol is added, extended semen is equilibrated for a few to several hours at 4°C.
- During this period, semen is packaged in 0.25 ml plastic straws
- This package provides not only a convenient means for storing and inseminating semen, but a high surface to volume ratio which has helped to develop more effective freezing and thawing procedures.

Equilibration, Packaging, and Freezing

- Straws are then positioned in a freezer or liquid nitrogen tank and frozen in liquid nitrogen vapor at an optimal rate selected to minimize freezing damage to the sperm.
- Straws of frozen semen are then stored in liquid nitrogen until needed.

Transfer and Storage

- Frozen semen is **transported and stored in liquid nitrogen tanks** designed specifically for this purpose.
- As long as semen remains submerged in liquid nitrogen, the **condition** of the **sperm** and its **fertility** remains **unchanged**.

Transfer and Storage

- Problems can arise when straws are exposed to elevated temperatures before they are actually needed for AI.
  - If a tank is allowed to run out of liquid nitrogen,
  - If straws remain out of a tank too long when being transferred,
  - If too much time is taken when inspecting or selecting straws in the neck of the nitrogen tank.

Transfer and Storage

- Damage can occur even if the semen does not completely defrost or thaw, as physical changes in the frozen medium at sub-freezing temperatures will result in reduced sperm motility and fertility.

Transfer and Storage

- As with each of the required steps in freezing semen, the damaging effects of exposing straws to elevated temperatures are cumulative and care should be taken to avoid any unnecessary removal of straws from liquid nitrogen.

Successful freezing and thawing of bull semen

Factor important in cryopreservation	Commonly used conditions or substances
Processing semen promptly	Immediately following collection
Macromolecules to protect sperm against pre-freeze cold shock	Buffered egg yolk or heated milk
Cooling rate and pre-freeze time	Cool in 1–2 h; pre-freeze time varies with extender and species
Special cryoprotectant	Usually glycerol
Freezing rate	Varies: about 10 min from +5°C to –100°C
Storage temperature	–196°C in liquid nitrogen
Thawing rate	Usually thaw at 30–37°C