# Semen Dilution

## IMPORTANCE AND PROPERTIES OF SEMEN DILUTERS

The success of AI, particularly in cattle, goats, and sheep, depended greatly on the development of satisfactory semen diluters. The AI industry has adopted the term "extender" to replace diluter. When AI was first being adopted, some felt that the use of the term "diluted semen" created a stigma, something that was not to be done, such as adding water to milk, so a switch to "extended semen" was made, but the authors prefer the original term, "diluter".

A 6-ml ejaculate of semen from the bull may contain a sufficient number of motile sperm to inseminate 200 to 300 cows, but it would be virtually impossible to divide 6 ml into that many units. The AI pioneers also found that sperm in undiluted semen lived for only short periods of time and that cooling undiluted semen very slowly to 5°C caused the death of many sperm. It became obvious that a satisfactory semen diluter would have to do more than increase the volume of an ejaculate. The diluter would have to protect the sperm during cooling and extend the life of the sperm.

**The following properties of a good semen diluter have been delineated along with examples of materials that satisfy these properties.**

1- A diluter must be isotonic with semen (have the same free ion concentration) 2.9% sodium citrate dihydrate or 0.2 molar Tris solution.
2- Buffering capacity must be provided (prevent pH change by neutralizing acid produced by sperm metabolism) isotonic sodium citrate or Tris solution or milk.
3- Diluters must protect the sperm from cold shock injury during the cooling from body temperature to 5°C, lecithin and lipoproteins from egg yolk or milk.

4- Nutrients must be provided for sperm metabolism, egg yolk. milk, and some simple sugars.
5- Microbial contaminants must be controlled, antibiotics such as Gentamicin. Tylosin, and Linco-Spectin.
6- Sperm must be protected from injury during freezing and thawing, glycerol.

**BUFFER SOLUTIONS USED IN SEMEN DILUTERS**

The buffer solutions, which make up the major portion of semen diluters, serve a dual role:

1- By neutralizing the lactic acid produced by the metabolic activity of the sperm, minute changes in pH are prevented.
2- The proper concentration of the buffer salt provides an isotonic environment for the sperm.

In addition, the salt used must not be toxic to the sperm at the level required for isotonicity. Of the many compounds and combinations of compounds available that satisfy at least one of the three criteria listed, only four buffer solutions have been found to be satisfactory for use in semen diluters.

1- **Phosphate Buffer Solution**:

The-phosphate buffer was a component of the first satisfactory semen diluter reported in 1939.

It was composed of 2.0 g of $Na_2HPO_4 .12 H_2O$ and 0.2 g of $KH_2PO_4$ in sufficient distilled water to make 100 ml of solution.

While just as satisfactory as other buffet solutions, phosphate buffer has not been used because it produces an opaque mixture when added to egg yolk, resulting in poor sperm visibility.

2- **Citrate Buffer Solution:**

The suitability of sodium citrate dihydrate solution as a buffer for semen was discovered in 1941.

It is composed of 2.9 g of sodium citrate dihydrate in sufficient distilled water to make 100 ml of solution. An alternate sodium citrate buffer can be made by mixing 2.12 g of sodium citrate dihydrate and 0.183 g citric acid monohydrate with sufficient distilled water to make 100 ml of buffer.

The sodium citrate buffer soon replaced the phosphate buffer in preparing semen diluters. When mixed with egg yolk it leaves the mixture sufficiently transparent to give good visibility of the individual sperm.

3- **Tris Buffer Solution**:

Tris (hydroxymethyl) aminomethane has been used as a buffered medium for bull and boar sperm since 1963. The tris buffer seems to have value in prolonging sperm life at ambient temperature, 5°C and 196°C. Various molarities and pH levels have been tested.

A 0.2-M concentration and a pH of 6.5 plus 1% fructose gives best results for bull semen.

To prepare the buffer, 3.028 g tris and 1.0 g fructose are placed in a 200-m1 beaker and about 75 ml of distilled water is added. With the aid of a pH meter, enough 10% citric acid is added to lower the pH to 6.5.

4- **Milk**:

Both whole milk and skim milk meet all three of the criteria for a satisfactory buffer when heated to 90°C to 95°C for 10 minutes, and they meet all other requirements of a satisfactory semen diluter .

## ANTIMICROBIAL AGENTS FOR SEMEN DILUTERS

Attention was called to the problem of microbial contaminants in ejaculated bull semen as early as 1941. The number of organisms per ml can range from no detectable organisms to several million. The number of organisms can be reduced by properly cleaning the under-line of the male prior to collection. All equipment

used in semen collection, processing, and storing should be sterile and not contributors of other contaminants.

A wide variety of organisms has been isolated from semen. Many of these organisms are not pathogenic but 1- do compete with the sperm for nutrients and 2- do produce metabolic by-products that have an adverse effect on livability of the sperm.

For approximately 40 years, beginning in the late 1940s, penicillin (1,000 IU per ml of diluter) and streptomycin (1,000p4 per ml of diluter) were used to control both pathogenic and nonpathogenic bacteria in semen.

## EFFECTIVE DILUTERS FOR BULL SEMEN

1- **Yolk-Phosphate** :

The yolk-phosphate diluter is prepared by mixing equal parts of the phosphate buffer solution and fresh egg yolk.

The reaction of the phosphate ions on the fat globules of the egg yolk results in an opaque mixture, making it impossible to observe individual sperm in the mixture. Even though this diluter maintains good motility and fertility of bull sperm, it is not used .

2- **Yolk-Citrate** :

The yolk-citrate diluter is prepared by adding fresh egg yolk to the citrate buffer solution.

When the semen is to be frozen, 20% yolk and 80% buffer solution gives best results. Antibiotics should be added to the nonglycerol fraction of diluter.

The yolk-citrate diluter has become the standard against which all new and modified diluters have been compared.

3- **Yolk-Tris** :

The yolk-Tris diluter is prepared by adding 20% fresh egg yolk to the Tris buffered solution. Antibiotics are added at the recommended level. Some

processors have added 7% glycerol to the yolk-Tris diluter prior to semen dilution but the recommended procedure is to glycerolate only half of the diluter. Equilibration time may not be as critical with the yolk-Tris diluter as with other diluters.

4- **Whole Homogenized Milk and Skim Milk**:

Whole homogenized milk and skim milk satisfy the requirements of a good semen diluter. Milk heated to normal pasteurization temperature contains a material, lactenin, which is spermicidal. Heating the milk to 90°C to 95'C for 10 minutes inactivates lactenin. The only additions needed are the antibiotics to control microbial contaminants and glycerol if the semen is to be frozen. Whole milk has the disadvantage of poor sperm visibility under the microscope. This problem is apparently caused by light refraction by the fat globules contained in the whole homogenized milk.

**PROCESSING BULL SEMEN**

The processing of semen starts with diluter preparation and involves semen collection, dilution, microbial control, cooling, packaging, freezing, and storage .

1- Diluter Preparation should be prepared on the day preceding collection.
2- The total quantity can then be divided into two equal parts and designated part A and part B.
3- To part A, add 500µ Gentamicin; 100mg Tylosin, and 300/600 mg Linco-Spectin for each ml of diluter.
4- To part B, add 14% glycerol for yolk-citrate, yolk-Tris, and whole milk diluters.
5- Both parts should be cooled and stored at 5°C until the day of collection .
6- Semen Collection: In the day of collection the part A dilution must be placed in warming water 37 c before semen collection.

7- After collection the semen must be placed in water path 37 c during the evaluation made to prevent cold shock occurs.

8- Semen Dilution: The dilution of semen is carried out in two steps. The first step involves a predilution of the warm semen with three to four volumes of warm part A diluter for each volume of semen. The diluter used for predilution should contain no glycerol and be tempered in the 35°C water bath used to maintain the temperature of the semen.

9- The diluter used in this manner provides lecithin and lipoproteins to protect the sperm from cold shock during the cooling process. These materials apparently prevent changes in cell wall permeability during cooling. The cooling process should take a minimum of 2 hours. This time interval also meets the requirement that sperm must be in contact with the antibiotics in the part A diluter for a minimum of 2 hours in order for the antibiotics to act on any microorganisms present before any glycerol is introduced.

10- Dilution Rate :

The main objective in deciding what the dilution rate should be is to provide the optimum number of motile sperm per breeding unit that will be available at the time of insemination. It is generally accepted that 10 million motile sperm at the time of insemination will provide optimum conception rate. One needed to know the **initial motility** and **sperm concentration** of the ejaculate. The number of breeding units was determined by dividing the total number of motile sperm by 10 million.

The following is an example for calculating the number of breeding units that can be processed from an ejaculate of semen, including the amount of diluters and each antibiotic needed.

**Given**: 9 ml ejaculate, 60% motility, 1.25 x $10^9$ sperm concentration (SC) concentration, and a goal to provide 10 x $10^6$ motile sperm (MS) after thawing. Calculate: Number of 0.5 ml straws that can be filled, volume of semen diluter needed.

**Solution:**

TS = 1.25 x $10^9$ x 9 ml = 11.25 x $10^9$

Total motile Sperms = TS x 0.60 = 6.75 x $10^9$ Motile Sperms

$$\text{Number of 0.5 ml straws} = \frac{\text{Total motile Sperms}}{15x\ 106} = 450 \text{ straws}$$

$$\text{Total volume of diluter} = \frac{\text{Number of straws}}{2} = 225 \text{ ml total volume}$$

الحجم :9 مل

التركيز : 1.25 x $10^9$ نطفة/مل

الحركة الفردية: 60%

تركيز النطف في كل وحدة تلقيحية : 10 x $10^6$ نطفة متحركة بعد الاذابة

حجم الوحدة التلقيحية : 0.5 مل

الحل :

1- عدد النطف الكلي في السائل المنوي المجموع = 1.25 x $10^9$ نطفة/مل * 9 = 11.25 *$10^9$

2- عدد النطف المتحركة الكلي = 11.25 *$10^9$ * 0.60 = 6.75 x $10^9$

3- عدد الوحدات التلقيحية = 6.75 x $10^9$ / 15 * $10^6$=450 قصبة

4- حجم المخفف الواجب تحضيره = 450 / 2 =225 مل

11- Cooling Semen:

Cooling is accomplished by placing the diluted semen 35°C container in a container of water at the same temperature. These are placed in a refrigerator and cooled to 5°C.

12- Glycerolation and Equilibration :

Glycerol must be added to semen to protect it during freezing and thawing. Damage results from the selective freezing of free H2O both inside and outside the cells. Glycerol binds water and decreases the freezing point of solutions. Less ice is formed in its presence at any temperature.

The level of glycerol varies somewhat with the diluter ingredients. Yolk-citrate, whole milk, and yolk-tris diluters should contain 7% glycerol after final dilution. Skim milk diluter performs better with 10% glycerol. The part B fraction of diluter which has been cooled to 5°C is added to semen which has also been cooled to 5'C and diluted to one-half the final volume with part A diluter.