**Semen preservation**

1- **Above freezing point**
2- **Below freezing point**
3- **Dry freezing point**

Semen is used either immediately after collection ("**fresh**") for example turkeys, human beings; after storage at a reduced temperature ("stored") for example horses, pigs, dogs; or after freezing and thawing ("cryopreservation") for example, bulls.

**Fresh semen**

In contrast to animal species, human semen is not extended prior to processing (see previous section) and is not usually kept for more than a few hours before use. Goat semen **cannot be kept at 37°C** because an enzymatic component of the bulbo-urethral gland secretion hydrolyses milk triglycerides into free fatty acids, which adversely affects the motility and membrane integrity of buck spermatozoa. For liquid preservation, goat semen can be stored at 4°C although fertility is retained for only 12-24h. The rate of extension used for stallion semen varies between countries but rates of 1:2, 1:3 or even 1:4 (v/v) semen: extender are common. The standard practice in some countries is to have 500 million or one billion progressively motile stallion spermatozoa for fresh or cooled semen doses respectively. Boar semen doses contain three billion progressively motile spermatozoa.

**Stored semen**

Storing extended semen at reduced temperature helps to extend sperm life by slowing their metabolism as well as by inhibiting bacterial growth. Bacteria grow by utilizing the nutrients in semen extenders, thus competing with spermatozoa for these limited resources, and release metabolic byproducts, thus creating an environment that is not conducive to maintaining viable spermatozoa. Furthermore, as bacteria die, they may release endotoxins that are toxic to spermatozoa. However, cooled stored semen is the method of choice for breeding horses and pigs, enabling the semen dose to be transported to different locations for

insemination. Stallion semen is stored at approximately 6°C while boar semen is stored between 16 and 18°C. Most boar semen doses are sold as cooled doses. In contrast, some stallions produce spermatozoa that do not tolerate cooling, rapidly losing progressive motility. In such cases, the only option currently is to use fresh semen doses for AI immediately after semen collection, although a new method of processing, centrifugation through a single layer of colloid, has been shown to solve the problem, as discussed later.

**Cryopreservation**

Semen is most useful for AI if it can be cryopreserved, since this method of preservation ideally enables the semen to be stored for an unlimited period without loss of quality until needed for AI. Since the frozen semen does not deteriorate, it can be quarantined until the male has been shown to be free from disease at the time of semen collection. However, the spermatozoa of various species differ in their ability to withstand cryopreservation: ruminant spermatozoa survive well whereas poultry spermatozoa do not, with less than 2% retaining their fertilizing ability on thawing (Wishart, 1985). For farm animal breeding, the cost of cryopreservation and the likelihood of a successful outcome following AI must be considered when deciding whether to use fresh, cooled or frozen sperm doses. The spermatozoa are mixed with a protective solution containing lipoproteins, sugars and a cryoprotectant such as glycerol. These constituents help to preserve membrane integrity during the processes of cooling and re-warming. However, sperm motility must also be maintained, so that the thawed spermatozoa can reach the oocytes after insemination and fertilize them. In most species, the seminal plasma is removed by centrifugation before mixing with the cryoextender, for example, stallion, boar, goat and human semen. The extended semen is packed in straws and frozen in liquid nitrogen vapour before plunging into liquid nitrogen for long-term storage. There is considerable variation in the success of sperm cryopreservation between different species, despite intensive research into the constituents of cryoextenders and the rates of cooling and re-warming. Human spermatozoa can be frozen relatively successfully using commercially available cryoextenders and programmable freezing machines.

**Storage of Frozen Semen**

Maintenance of low temperatures is important to the successful storage of frozen semen. In 1957, Van Demark and coworkers1 compared the motility of semen frozen to and stored at −79° C, −65° C, −51° C, −37° C, and −23° C. After 1 day of storage, the motility of semen stored at −37°C and −23°C was basically zero. Semen stored for 1 day at 79°C had 38% motility, compared with 29% and 14% motility for 65°C and 51° C storage, respectively. In studies in which −79°C storage was compared with −96°C2 or −92°C and −196°C 79° C was inferior to the lower temperatures when judged by maintenance of sperm motility as measured on thawing. The reasons that subzero temperatures of −79°C and above cannot adequately preserve sperm are unclear and probably are quite complex. Rapatz,2 however, has proposed that rearrangement in the crystalline structure of frozen semen (recrystallization) may be one cause. This phenomenon has been observed to occur in frozen semen down to a temperature of −80° C. Below −80° C the structure of ice is more stable, and below −100° C it is very stable. Although no direct evidence exists for recrystallization, a correlation has been found between storage injury of spermatozoa and recrystallization. In addition to the obvious error of permitting a liquid nitrogen storage tank to go dry, stored semen also may be exposed to adverse high temperatures when straws are being removed for thawing. Figure 34-1 shows the temperature gradient that exists in the neck of a typical liquid nitrogen storage tank. As may be noted, dangerously high temperatures prevail in the upper third of the neck of the tank where canes and goblets are raised for removal. When straws are exposed to these temperatures the semen temperature rises quickly. The thermal response of semen in 0.5-ml straws exposed to temperatures of −22° C (2 inches from the top of the tank) and 5° C (1 inch from the top of the tank) is shown in Figure 34-2. The time required to reach −100°C to −80°C, which is the beginning of ice recrystallization, is approximately 10 to 12 seconds for both temperatures. Thermal injury to sperm is permanent and cannot be corrected by returning semen to the liquid nitrogen. For optimal maintenance of sperm viability, canisters and canes containing semen should be raised into the neck of the tank only for the time required to retrieve a single straw. This time should not exceed 5 to 8 seconds.

**Processing semen and freezing rates**

Semen freezing-thawing survival is greatly influenced by the semen dilution rate. Semen is extended for protecting spermatozoa during cooling, freezing and thawing. Some authors use a dilution rate of 2–5 folds, with the extender composition adjusted to the dilution rate (Evans and Maxwell 1987). After dilution, semen is cooled to a temperature close to +4_C. During this period of refrigeration, there is an adaptation of sperm cells to a reduced metabolism. Equilibration means the time interval the sperm cell remains in contact with glycerol (before freezing), to allow cryoprotectant to penetrate the cells, enabling equilibrium between intra and extracellular concentrations of glycerol and other osmotically active extender elements (Evans and Maxwell 1987). During equilibration, glycerol may participate in some harmful events, as alterations in the structure and biochemical integrity of spermatozoa and the increase of acrosome reaction that will reduce fertility. In our laboratory (EZN, Santarem) we join the semen extender on a ratio 1:2 or 1:3, depending on ejaculate concentration. As other workers (Evans and Maxwell 1987; Salamon and Maxwell 2000) we joined the extender in a single step at 30_C to give a sperm concentration of 1,200 or 800 million/ml, respectively for ram and buck. Our extender containsTris, citric acid, glucose, egg yolk, glycerol and antibiotics, and has been adapted from the extender of Evans and Maxwell (1987). After dilution, semen is packed in 0.25 ml French mini-straws that are sealed with dry PVC (polyvinyl chloride). Cooling and equilibration from 30 to 4_C are done in a refrigeration chamber during 4 h. Rapid cooling of extended semen from 30 to 15_C may not affect sperm survival (Lebouef et al. 2000). By the contrary fast cooling from 30 to 10, 5 or 0_C, decreases post thaw motility and sperm fertility (Fisher et al. 1987; Evans and Maxwell 1987). Afterwards mini straws are horizontally placed in a rack inside a metallic box containing nitrogen (LN), 4–6 cm above the liquid nitrogen surface, undergoing freezing in LN vapours during 20 min. Freezing rate is regulated by the distance between the straws and the level of liquid nitrogen. Ram and buck spermatozoa packed in straws tolerate a variation in the freezing rates. Placing straws over liquid nitrogen vapours, at temperatures between -75 and -125_C has no effect on sperm quality. By the contrary, temperatures of -55_C reduce sperm survival

(Evans and Maxwell 1987). Freezing rates from 50 to 100_C/ min are usually selected for freezing ram and buck semen. However, from -10 to -60_C, we must use cooling rates faster than 50_C/min. Afterwards we can slow freezing rate (20–30_C/min) until freezing is complete (Byrne et al. 2000; Anel et al. 2003). It is advised to freeze semen according to a parabolashaped curve, which is possible putting the straws 4–6 cm above liquid nitrogen as we do in our laboratory. The most adequate freezing rate is the fastest one that allows extracellular water freezing without intracellular ice formation. Optimal freezing rates for sperm vary according to species from 1 to 10_C/min for humans and 50 to 100_C/min for bulls (Woelders 1997).

**Thawing semen**

In the freeze-thawing method, warming phase is also important for the survival of sperm as well as cooling phase (Fisher et al. 1987). During thawing, frozen semen will also cross the critical temperature between -15 and -60C. Thawing rate depends whether cooling rate has been sufficiently high to induce intracellular freezing or low enough to produce cell dehydration. In the first situation, fast thawing is required to prevent recrystallisation of any intracellular ice present in the spermatozoa. Spermatozoa thawed at a fast rate are exposed during less time to the concentrated solute and cryoprotectant, and the restoration of the intra and extracellular equilibrium is more rapid than with slower thawing (Fisher et al. 1987). Ram and buck semen is generally thawed at 38–42_C during 30 s, but thawing at higher temperatures (60–75_C), produces similar post-thaw motility, acrosome integrity and fertility of spermatozoa (Evans and Maxwell 1987). Semen may be thawed in physiological serum or in a variable thawing solution, depending on the composition of the freezing extender (without glycerol). Some researchers found a relationship between the composition of the freezing extender and that of the thawing solution when Tris-based freezing media were used (Evans and Maxwell 1987; Salamon and Maxwell 2000).