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Artificial Insemination

TIMOTHY J. PARKINSON AND JANE M. MORRELL

The successful use of artificial insemination (AI) as a means of animal breeding relies upon three major premises: first, that spermatozoa can survive outside the body; second, that they can be reintroduced into the female genital tract in a way that results in an acceptable conception rate; and third, that the fertile period of the female can be identified.

The degree to which these underlying premises can be fulfilled dictates the success with which AI can be applied to an animal species. For example, in cattle, the spermatozoa can be preserved outside the body almost indefinitely (after cryopreservation). A technically straightforward intrauterine insemination means that the number of spermatozoa needed for each insemination dose is low; hence, each ejaculate can be used for breeding many females. Conception rates with AI are identical to those of natural service, whilst the visible oestrous behaviour of cows means that detection of the fertile period is not difficult. Hence, in this species, in which all three premises are fulfilled, the use of AI is widespread. Conversely, in many other species, in which one or more of the premises are less adequately fulfilled, AI is less successful and therefore less widely used.

AI regimens have been developed for most domestic and many semidomestic species. It is routinely practiced in cattle, sheep, pigs, goats, fowl, turkeys, salmon, and trout and is used in dogs, domestic foxes, buffalo, horses, and even bees. Of these, cattle, pigs, and sheep/goats account for the vast majority of mammalian inseminations. The use of AI is essential in turkey breeding as natural mating is not possible in this species, so that very large numbers of inseminations are performed. AI in salmonid farming is also very widespread. The discussion of AI in this chapter will be limited to the major domestic mammals.

Advantages and Disadvantages of Artificial Insemination

Artificial insemination offers several potential advantages over natural service. Historically, the most important reason was the control of venereal diseases, particularly in cattle. Nowadays, the most commonly advocated reason is as a means of genetic improvement.

The potential for AI to be used to control venereal disease was a major impetus to the development of cattle AI in the UK during the 1940s. At that time, the venereal pathogens *Tritrichomonas foetus* and *Campylobacter fetus* subsp. *venerealis* were epizootic in most cattle populations, but in the UK, in common with most countries in which bovine AI was introduced in the face of trichomonosis and campylobacteriosis, these pathogens were virtually eliminated by its use (see Chapter 24). However, the converse is also true: uncontrolled use of sires in AI can disseminate disease. Many diseases are transmissible through semen, including not only the classic venereal diseases, but also other conditions that would not generally be regarded as primarily venereal (Roberts 1986). Rigorous monitoring of the health of AI donor sires is therefore regarded in many countries as an integral part of national disease control programmes.

The use of AI as a means of genetic improvement stems from the fact that, in most food-producing animals, each ejaculate can be divided into many insemination doses, such that each sire can potentially be used to breed a very large number of females. Hence, the total number of sires needed is reduced, with a consequential increase in the selection intensity that can be applied to the male side. In dairy cattle, only the best 1% of cows is selected as potential bull mothers and only about the best 1% to 3% of their male progeny eventually become sires of the next generation. In beef cattle and pigs, the selection intensity is not quite so great but, nevertheless, is very much more intense than can be achieved in natural breeding.

Direct genetic selection of sires is only one of the ways in which AI can be used for achieving genetic improvement, inasmuch as AI allows rapid dissemination and/or substitution of new breeds. In the UK, AI was one of the main means whereby the Friesian breed of cattle displaced the indigenous British dairy breeds. Subsequently, AI has also been the means by which the Friesian has been displaced by the Holstein. In such breed substitution programmes, AI can be used to change the gene pool of a national herd rapidly; a technique that is also used for upgrading unimproved cattle in remote areas. In this process, AI has the advantage of being both cheap and simple, inasmuch as local distribution of extended and cooled semen from a small number of imported sires is within the economic capabilities of even the poorest countries.

International trade in livestock is also facilitated by AI. Improved stock can be imported in the form of semen for AI rather than having to move animals themselves. By this means, many of the problems of acclimatisation, such as a lack of resistance to local diseases, can be eliminated. Importing semen also allows the importing country to exert a far greater level of effective control over the health status of the donor sires than if the livestock itself were imported.

The third major advantage of AI is the reduction in the number of sires that individual farmers need to maintain. The males of agricultural species generally require accommodation in which they can be segregated from the breeding females, so that breeding can be controlled; these buildings should also preclude, as far as

possible, injury to farm staff. The significant housing and labour costs involved in keeping such animals can be obviated by the use of AI. Moreover, farmers generally have access to genetic material through AI centres that would be far beyond their financial ability to buy outright.

Nevertheless, although AI carries many advantages over natural breeding, the technique is not without drawbacks. Detection of the fertile period in the female oestrous cycle is potentially the most problematic aspect of AI programmes. In cattle, the prominent behaviour of oestrous females in which they repeatedly mount each other allows relatively accurate human identification of the fertile period. Likewise in pigs, receptive females 'freeze' when pressure is applied to their back. However, in most other species, detection of the most fertile period is less easy. In such species, detection of oestrus therefore requires the presence of infertile (e.g., vasectomised) males, or the timing of oestrus must be controlled by pharmacological regimens (e.g., oestrus synchronisation/induction; see Chapter 8), or management procedures (e.g., timing of weaning in sows). Thus for ewes, which do not normally display any signs of oestrus in the absence of a male, AI requires either the presence of vasectomised rams to detect oestrus or pharmacological manipulation of oestrus to define the timing of the fertile period. Hence, detection of the fertile period of the ewe is, to a greater or lesser extent, a costly procedure, thereby detracting from the appeal of AI in this species. It may therefore be considered that an economic 'trade-off' exists in such species between the genetic advantages conferred by the use of superior AI sires on one hand and the costs of maintaining teaser males or pharmacological manipulation on the other.

Once oestrus has been identified, the female animal has to be restrained for insemination, which generally requires separation from the herd or flock and holding in specialised pens. The process of insemination also requires trained personnel with a certain degree of technical proficiency, as with insemination of sows; however, in some cases the procedure may be technically demanding, as in the case of laparoscopic intrauterine insemination of ewes.

It is also necessary to log insemination dates into an adequate recording system so that expected dates of return to oestrus are known and in order to allow birth dates to be calculated, thus allowing appropriate observations to be made. In addition, the identity of the sire needs to be recorded (and his pedigree known) to avoid inbreeding. Some form of positive pregnancy diagnosis is advantageous, especially when males are not present in the herd, to ensure that non-returns to oestrus signify pregnancy rather than anoestrus.

The value of AI as a rapid means of transmission of the genes of superior sires has already been identified. However, a corresponding disadvantage exists, inasmuch as genetic faults can also be widely disseminated if they are present in an AI sire. Dominant traits should rarely be transmitted in this way, but recessive traits may be very widely transmitted, especially if the recessive gene is present in the general population at such a low incidence that many individuals may have to be bred before the condition is expressed in a homozygous progeny. Hence, AI programmes should be underpinned by an efficient reporting system for monitoring abnormalities in the progeny, with clearly defined criteria for the withdrawal from use of sires that carry deleterious genes. For example, in cattle, achondroplasia is transmitted as a simple recessive gene (Jones et al. 1978) that, when present in the homozygous condition, causes failure of long bone development, resulting in the birth of so-called 'bulldog' (achondroplastic) calves (see Chapter 9). The incidence of this gene in the general cattle population is so low that the birth of one or two calves with this deformity is regarded as sufficient reason to slaughter the bull and withdraw all stocks of its semen. Spastic paresis, which is probably genetically transmitted, is dealt with in a similar manner (Keith 1981). More recently, a complex vertebral malformation was identified in Holstein cattle in many countries (Agerholm et al. 2001), but affected sires could be effectively removed from the breeding pool by rapid identification through breeding records and genomic analysis (Thomsen et al. 2006). Moreover, it is also possible to disseminate conformational faults which, although not initially undesirable, can still have a significant effect upon the progeny (e.g., poor hind leg/foot conformation or poor udder attachment in cattle).

Concern over the possibility of inbreeding and reduction of the effective population of a breed has been suggested as a consequence of the widespread use of AI from a small number of highly selected blood lines. There is evidence that this may be taking place, particularly within the Holstein breed of cattle. A number of studies have identified real or potential negative effects of inbreeding on traits such as dystocia and reproduction (Adamec et al. 2006, McParland et al. 2007a), culling rate (Sewalem et al. 2006), and production traits (Biffani et al. 2002, Croquet et al. 2006), but generally not on type/conformational traits. Estimates of coefficients of inbreeding in Holstein cattle have shown a period of rapid increase during the 1990s, whilst the numbers of effective contributors to the breed is declining (Sorensen et al. 2005, Koenig & Simianer 2006, Kim & Kirkpatrick 2009). The rate of increase of inbreeding may have reduced in recent years (Stachowicz et al. 2011). This trend is also seen in beef cattle such as the Hereford and Charolais breeds (McParland et al. 2007b). McParland et al. (2007a) noted that although these effects were statistically significant, they were small and were unlikely to cause great financial loss at present levels. However, others advocate a more active role in the management of inbreeding (Kearney et al. 2004, Colleau & Moureaux 2006, Haile-Mariam et al. 2007).

Preparation of Semen for Use in Artificial Insemination

The methods for collection of semen from domestic mammals are described in Chapter 35. In most AI regimens, semen evaluation is limited to measuring numbers of spermatozoa, motility, and, usually, morphology. More sophisticated analyses may be used in determining whether an individual sire produces semen of a sufficiently high quality for acceptance into an AI programme, but such evaluations are rarely carried out on day-to-day collections of semen. Unless the semen is to be directly inseminated without delay into a single female, it is then extended and either cooled or frozen. Direct inseminations are performed most commonly in the bitch, usually in response to some incapacity of the sire that precludes normal mating (Roberts 1986) or the mare with chronic endometritis (Asbury 1986). Even in these circumstances, it is more common to place the semen in an extender before insemination. The major properties of semen extenders (Watson 1979) are described here.

Extension

The ejaculates of most domestic animals contain more spermatozoa than are needed for achieving a pregnancy. Hence, by diluting the semen, it can potentially be used for several inseminations. In species such as the dog and the horse, the whole sperm-rich

fraction of the ejaculate is diluted and cooled, then used either for sequential inseminations of the same female over her extended oestrus period or after various determinations of the fertile period (Jeffcoate & Lindsay 1989, Brinsko & Varner 1993). In food animal species, the ejaculate is generally diluted so that it can be used to inseminate many females.

In either case, the maximum degree of dilution is determined from the minimum number of spermatozoa and the volume of inseminate that is deemed necessary to achieve acceptable pregnancy rates. These factors are themselves determined by the site of insemination, the survival of spermatozoa in extender, and the idiosyncrasies of individual species and sires. In general, when an intrauterine insemination can be achieved, the minimum numbers of spermatozoa are one or two orders of magnitude lower than for an intracervical insemination, which is itself one or two orders of magnitude lower than for an intravaginal insemination. Hence, when widespread use of sires is required, a great advantage exists in devising methods of achieving intrauterine insemination, even when this requires as complex a procedure as laparoscopic insemination, as in the ewe.

Addition of Volume

Insemination doses must be prepared in a volume that is a compromise between ease of handling and an appropriate volume for the site of insemination. Thus for ovine intracervical inseminations, minimising volume is important to reduce retrograde loss from the cervix (Evans & Maxwell 1987), whereas for porcine intrauterine inseminations, a minimum volume of 50 mL is required to spread the semen through the capacious uterus of the sow (Reed 1982).

Dilution of semen is not entirely straightforward, for mammalian spermatozoa placed in simple diluents exhibit an initial increase in motility, which is then rapidly followed by a loss of motility and increase in vital staining (Mann 1964). This phenomenon, known as the 'dilution effect', represents a loss of cell viability, probably through leaching of structural components of the cell membrane. Although it was of great concern among the early practitioners of AI, the use of extenders containing macromolecules such as proteins or polyvinyl alcohol was subsequently found to abrogate the dilution effect (Suter et al. 1979, Clay et al. 1984).

Buffering

Spermatozoa have a narrow range of tolerance to changes in pH, so provision of buffering capacity is necessary. Buffering is especially important when the semen is only to be cooled and not cryopreserved, as the metabolic activity of cooled spermatozoa remains appreciable (Salisbury et al. 1978). Although in many diluents the major volume component is also the major buffering agent, buffers are a minor constituent of some diluents. Simple buffers are effective, with citrate originally being widely used (Willett & Salisbury 1942). Phosphate-buffered saline is rather less suitable, as it predisposes to head-to-head agglutination of sperm. More recently, organic buffers have been used. Tris (tris(hydroxymethyl) aminomethane) is probably the most widely employed of such buffers, but the successful use of many similar materials (e.g., TES, HEPES, Tricene) has been described. The proteins contained in skimmed milk products also provide considerable buffering capacity to diluents. More recently, there has been a move away from using material of animal origin in semen extenders because of the risks of inadvertently transmitting disease. Lecithins from plants and liposomes have been used as substitutes for skimmed milk and egg yolk in semen extenders.

Maintenance of Osmotic Pressure

Seminal plasma has an osmotic pressure of 285 mOsmol, although spermatozoa can tolerate a moderate range of tonicity (Foote 1969). Some debate has centred on whether spermatozoa respond better to a slightly hyperosmotic (Foote 1970) or isosmotic diluent, with the former being generally favoured. Apart from the osmotic activity of the ionic component of diluents, a substantial contribution is made by proteins and, particularly, by sugars, which are added to provide nutrition for the spermatozoa or to contribute to the cryoprotective properties (Watson 1990) of the diluent.

Provision of an Energy Substrate

Most diluents make some provision of energy substrates for sperm. In general, simple sugars such as glucose, fructose, mannose, and arabinose are suitable substrates, although the rate at which these sugars are metabolised varies substantially between species (reviewed by Bedford & Hoskins 1990). Lactose, which is present in milk-based diluents, is not metabolised to any appreciable extent. However, egg yolk, also a component of many diluents, provides many substrates for the metabolism of spermatozoa (Salisbury et al. 1978). The provision of energy is relatively less important when semen is to be frozen, for the spermatozoa will only remain active for a few hours at most before freezing suspends metabolic activity. However, when the metabolism of spermatozoa has to be sustained for several days (e.g., if cooled semen is to be used) provision of energy is important.

Antimicrobial Activity

Antibiotics are added to most semen diluents as a prophylactic measure against the transmission of pathogenical bacteria and to reduce the load of nonpathogenical organisms that contaminate the semen. In cattle AI, benzylpenicillin and streptomycin (Melrose 1962) are the most widely used antibiotics, for these are efficacious against *C. fetus.* Most other antibiotics either fail to control this organism or are directly detrimental to sperm. Concern over the potential transmission of *Mycoplasma* and *Ureaplasma* species in bovine semen has led to the incorporation of lincomycin and spectinomycin (Almquist & Zaugg 1974) into semen diluents in an effort to control these organisms. There is evidence that the efficiency of antibiotics may be reduced in the presence of some components of diluents, notably egg yolk (Morgan et al. 1959); hence, the practice in some bovine AI centres is to preincubate the raw semen with antibiotic cocktails before the main dilution occurs. This procedure is commonplace in the United States but is rarely undertaken in Europe. Nowadays, there is increasing concern over the spread of antimicrobial resistance, which raises questions about whether the inclusion of antibiotics in semen extenders is justifiable because it has a prophylactic rather than a therapeutic purpose. Alternatives to antibiotics, such as the removal of contaminating bacteria during processing of the semen, would be preferable (Morrell & Wallgren 2014).

Extending Life Span

The life span of spermatozoa of most other species can be prolonged more conveniently by:

- Cooling to a temperature well below ambient;
- Freezing (cryopreservation); and
- Suspending the metabolic activity of the spermatozoa while maintaining it at ambient or cooled temperatures.

Cooling and Cold Shock

Semen can be stored in a liquid form if the metabolic activity of the spermatozoa is reduced by cooling. The process of cooling from body temperature to 5°C results in considerable damage to the cells unless they are protected from the effects of the 'cold shock'. The development of cold shock is exacerbated by rapid cooling rates but cannot be entirely prevented even by slow cooling.

Cold shock results in damage to cell membranes, causing leakage of intracellular potassium, enzymes, lipids, cholesterol, lipoprotein, and adenosine triphosphate (ATP) (Salisbury et al. 1978). How this affects spermatozoon function remains incompletely understood, although it is clear that most of the effects are mediated through changes in the properties of cell membranes. Lowering temperature causes membrane phospholipids to change from a fluid to a gel phase, which, because this occurs at different temperatures for different structural lipids, can lead to phase separation. As a consequence, membrane proteins become irreversibly clustered, leading to loss of function (De Leeuw et al. 1990). Thus cold-shocked spermatozoa are more permeable, especially to calcium (Robertson & Watson 1986). The membranes are also more fusogenic, leading to alterations in the capacitation process (Johnson et al. 2000). Together, these changes are referred to as 'cryocapacitation'. Species differences and probably differences between individual sires depend on the composition of the membranes: ratios of different phospholipids and the concentration of cholesterol in the membranes appear to be critical determinants of the response to cold shock.

The most effective way of protecting spermatozoa against the detrimental effects of cooling is by the inclusion of egg yolk or milk in the diluent. Diluents for cooled storage of semen therefore typically contain approximately 20% of egg yolk plus a buffer. Skimmed milk, whole milk, and coconut milk have also been used successfully, although egg yolk is by far the most commonly used additive (Vishwanath & Shannon 2000). Whole milk contains a protein, lactenin, which is spermicidal, so milk for use as a semen diluent must be heat-treated (e.g., in the skimming process) to inactivate this toxic factor (Flipsse et al. 1954). Chemically defined extenders containing phosphocaseinate have now been developed (Batellier et al. 1998) and are tending to replace skimmed milk extenders. Early diluents were based on phosphate buffer, but citrate soon proved to be better, probably because it improves the solubility of egg yolk proteins. More recently, zwitterion buffers have also been used; Tris has been the most widely used but good results have also been achieved with TES (N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid) or TEST (Tris titrated with TES) (Holt 2000).

Detailed understanding of the mechanisms by which egg yolk and other natural products prevent cold shock have been elusive, with the result that it has been difficult to produce chemically defined diluents. Early work identified that the key components of these materials were lecithins, proteins, lipoproteins, and similar complexes of large molecules (reviewed by Salisbury et al. 1978). Studies in the 1970s and 1980s (e.g., Watson 1976, 1981, Foulkes 1977) showed that the low density lipoprotein (LDL) fraction is the key component of egg yolk, with lipoproteins probably being the most important (Watson 1990). In the last 20 years it has been shown that lecithins from plants, e.g., soy lecithin, also have a protective effect and can be substituted for egg yolk (Hinsch et al. 1997) at least for some species. The evolution of soy-based extenders has been reviewed recently (Layek et al. 2016).

It has been suggested that LDL stabilises the spermatozoal membrane (Watson 1975), or protects the spermatozoon by forming a protective layer over its surface (Quinn et al. 1980) or by replacing membrane phospholipids that become lost or damaged (Foulkes et al. 1980). LDL, glycolipids, and cholesterol become incorporated into membranes, reducing their tendency to change from a liquid to a gel phase during cooling (Isachenko et al. 2004). Further studies focused upon the interaction of LDL with protein components in the seminal plasma (Vishwanath et al. 1992). Bergeron and Manjunath (2006) developed this idea further based upon their work on bovine seminal plasma proteins (BSP). They suggested that, *in vivo*, BSP bind to the membrane of the spermatozoa during ejaculation, facilitating the process of capacitation in the female tract. During storage, BSP binds to spermatozoal membranes and causes loss of phospholipid and cholesterol, thereby inducing one of the main mechanisms of cold shock. It is suggested that LDL, particularly phosphatidylcholine, binds BSP in the extender, preventing its interaction with the spermatozoa and hence protecting the membranes from damage.

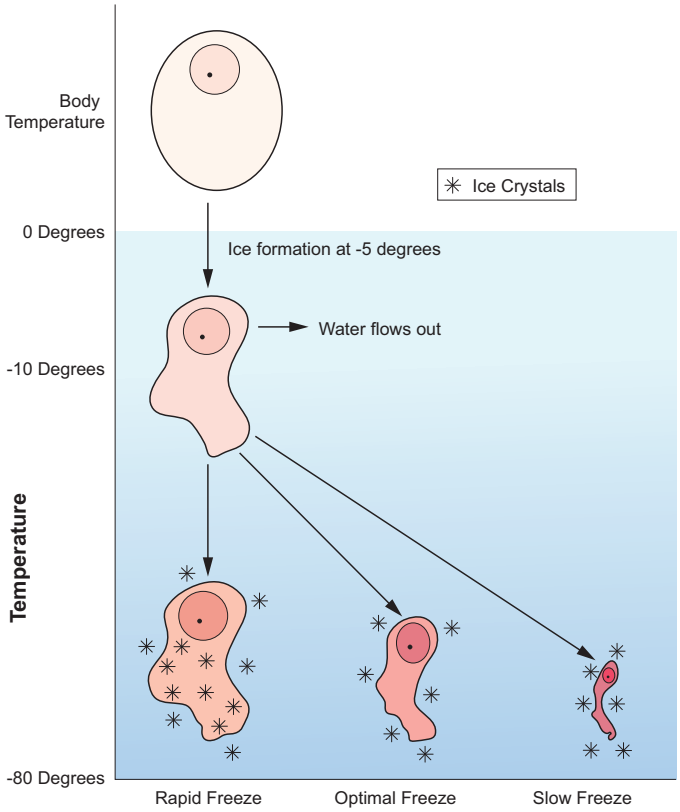
The possibility of including liposomes in extenders to stabilise spermatozoal membranes during cryopreservation is also attracting attention (Röpke et al. 2011). Phosphatidylcholine liposomes have been incorporated into commercial extenders and used successfully for several species, e.g., bull (Röpke et al. 2011), dog (Belala et al. 2016), buffalo (Kumar et al. 2015), and stallion (Pillet et al. 2012).

The fertility of bovine semen stored at 5°C in such diluents remains acceptable for 2 to 4 days (Foote et al. 1960), although that of ram semen only persists for 12 to 24 hours (Evans & Maxwell 1987). The decline in fertility that occurs after this time is initially due to decreased motility and survival in the female genital tract rather than to the death of spermatozoa per se. Short-term storage of semen by chilling to 5°C is, however, a very cheap and effective way of establishing an AI programme for cattle and is of value for on-farm collection and insemination of sheep, whereas the use of liquid boar semen at ambient temperatures remains, effectively, the basis of the technique in that species. Short-term 5°C storage is also widely used in the horse and the dog as it avoids the unpredictable response to freezing that characterises the semen of these species.

Cryopreservation and Cryoprotectants

Longer term storage of semen is achieved through cryopreservation, which facilitates dissemination of desirable genetic material to most parts of the world. Semen doses can be quarantined until the male has been shown to be free of disease at the time of semen collection. Cryopreservation maintains the fertile life of spermatozoa virtually indefinitely, although a large proportion of individual spermatozoa fail to survive the considerable stresses of freezing and thawing. To survive freezing, an extender is used that contains not only substances that protect against cold shock but also cryoprotectants such as glycerol (Polge et al. 1949), which protect them from the deleterious consequences of ice crystal formation.

The general responses of cells to freezing (reviewed by Farrant 1980, Watson 1990) were not understood until long after empirical methods of cryopreservation had become widely adopted. Initially, as the temperature of the external medium falls below its freezing point, crystals of pure water start to form. The concentration of solutes in the unfrozen part of the medium therefore rises as does its osmotic pressure. Ice crystals do not extend into the cell at this stage as they are excluded by the cell membrane. Thus the intracellular contents undergo a period of supercooling, during which the cell loses water to the unfrozen part of the extracellular



• **Fig. 43.1** Shrinkage of cells during cryopreservation. Extracellular freezing induces conditions that allow osmotically induced loss of water from cells during slow freezing. This correlates with survival on thawing. Rapidly cooled cells do not have time to shrink, form intracellular ice, and are dead on thawing.

medium by osmosis (Fig. 43.1). A variable degree of cell dehydration follows, which is terminated by the formation of intracellular ice crystals. Thus damage can occur to cells in one of two ways. When a substantial degree of cellular dehydration occurs, the high concentrations of solutes in the residual intracellular water can be damaging, whereas, if only slight dehydration occurs, large ice crystals can form within the cell, which cause physical damage to its internal and bounding membranes. The degree to which each affects the cell is determined by the size of the cell and the rate of cooling. Thus the larger the cell, the slower its inherent rate of dehydration; the slower the rate, the more dehydration; the faster the rate, the greater the damage by ice formation.

However, the effects of freezing and thawing also need to be explained in terms beyond those of osmotic effects and ice crystal damage. Spermatozoal membranes are unusual in terms of the lipids of which they are composed and of the arrangements of lipids within the membranes. Cooling and cryopreservation causes phase changes in membrane lipids, which results in loss of integrity, loss of control of calcium movement, and loss of membrane ATPase activity (Holt 2000). Many spermatozoa do not survive these processes – typically less than 50% of the viable spermatozoa that were present before cryopreservation. Moreover, even for the spermatozoa that do survive, capacitation-like changes to the head occur (Curry 2000, Thomas et al. 2006), which alter the duration of their survival in the female reproductive tract. It has been suggested that species-to-species variations in the ability of spermatozoa to survive cryopreservation, as well as some sire-to-sire variability within species, can be attributed to the ability of membranes to withstand or accommodate these changes.

Responses of spermatozoa to cryopreservation can be improved by the use of cryoprotectants, the composition of the extender and the rate at which spermatozoa are frozen and thawed. Cryoprotective agents are classified as those that enter the cell (permeating) or that are retained in the extracellular fluids (non-permeating). The actions of cryoprotectants were originally thought to be mediated solely through hydrogen-bonding of water, thereby reducing the availability of water either for dehydration or for ice crystal formation. Penetrating cryoprotectants (e.g., glycerol or dimethyl sulphoxide (DMSO)) reduce the loss of water from the cell, thereby reducing solute damage, and bind it in a form that renders it unavailable for ice crystal formation. Non-penetrating cryoprotectants, such as disaccharides or proteins, may hasten dehydration during very rapid cooling, thereby minimising intracellular ice formation.

However, other mechanisms are also involved. Polar molecules such as glycerol and sugars appear to form hydrogen bonds with the polar head groups of membrane lipids (Vishwanath & Shannon 2000), stabilising the membranes during transition through critical temperature zones (Woelders 1997). Likewise, insertion of glycerol into the membrane bilayer also appears to affect stability and water permeability of the membranes through altering the physical properties of the membrane lipids (Holt 2000). Sugars such as glycerol alter the mechanical properties of the diluent by increasing its viscosity. This prevents crystallisation of solutes and increases the glass-forming tendency of the medium (a property that is exploited in vitrification methods of freezing larger cells; Isachenko et al. 2004).

Unfortunately, permeating cryoprotectants are relatively toxic to spermatozoa. Glycerol, which is the main primary cryoprotectant used in freezing mammalian sperm, has direct toxic effects upon spermatozoa (Watson 1979, 1990). Concentrations of glycerol that can be tolerated by spermatozoa depend on the species and the other components of the diluent. For example, extenders for bovine semen that contain disaccharides can use lower percentages (3%–4%) of glycerol than diluents that lack such disaccharides, which have a final glycerol concentration of at least 7% (Unal et al. 1978). Bulls' spermatozoa seem to be relatively resistant to the toxic effects of glycerol, whereas boars' spermatozoa, for example, tolerate much lower concentrations before toxic effects start to occur.

Whether the toxic effects of glycerol are exacerbated at high temperatures has been a matter of debate. Early studies (Polge 1953) showed that the addition of glycerol at 28°C was more damaging to bovine spermatozoa than its addition at 4°C, although Salisbury et al. (1978), reviewing the (by then) copious literature, concluded that the effects of temperature were equivocal. It was subsequently suggested that multistep addition of glycerol avoids excessive changes in cell volume that can result in membrane rupture (Gao et al. 1993, 1995). Nevertheless, normal practice in commercial bovine AI centres is that, when the final concentration of glycerol is high (≈7%), a primary dilution of the semen is made with a diluent containing little or no glycerol, with glycerolisation being carried out after reducing the temperature to 4°C. However, diluents that use lower final concentrations (< 5%) are added in one step at 30°C. With boar semen, the toxicity of glycerol at high temperatures is much less equivocal, and low-temperature glycerolisation is desirable (Paquignon 1985). It is possible that the spermatozoa from individual sires may show less tolerance to glycerol than others.

The potential toxicity of glycerol has led to the search for other, less toxic, cryoprotectants. Whereas substances such as ethylene

glycol and propanediol can be used to cryopreserve embryos, they do not have any advantage over glycerol for freezing spermatozoa. Various amides, however, have been shown to be beneficial, at least for the semen of some individuals and depending on which cryoextender is used as the base (see review by Alvarenga et al. 2005). A combination of dimethylformamide and glycerol in INRA96 was a useful cryoprotectant for stallion semen (Alvarez et al. 2014). In contrast, dimethylacetamide did not improve the survival of boars' spermatozoa (Yang et al. 2016).

Oxidative damage also occurs to the membranes of spermatozoa during the freezing process. A number of studies have implicated membrane lipid peroxidation as a cause of defective spermatozoal function after cryopreservation (Salamon & Maxwell 1995). Attempts to overcome this problem have included the addition of antioxidants such as glutathione peroxidase (Slaweta & Laskowska 1987) or catalase (Shannon 1972), although it has also been suggested that the measurement of the degree of oxidation of membrane phospholipids may be a useful means of assessing the quality of frozen–thawed spermatozoa (Neild et al. 2005).

The first methods for freezing semen involved placing diluted semen in glass ampoules for freezing in a mixture of alcohol and solid carbon dioxide at −79°C, or drops of diluted semen were placed directly on to the surface of a block of solid carbon dioxide where they froze in pellet form (Salisbury et al. 1978). Long-term storage at −79°C was not satisfactory, however, as deterioration occurred at that temperature (Pickett et al. 1961, Stewart 1964). Storage in liquid nitrogen at −196°C has subsequently become established as the standard method for long-term preservation of semen and, over the 40 years for which it has been practised, has maintained semen fertility unscathed. At the present time, semen is frozen in the vapour of liquid nitrogen and then stored long-term in liquid nitrogen. Diluted semen is packed into thin, plastic tubes ('straws' or 'paillettes') of 0.25 or 0.5 mL capacity, then in the simpler techniques, these tubes are suspended in the vapour of liquid nitrogen, which is at about −120°C, for about 10 minutes (Cassou 1964, Jondet 1964). The straws are then plunged into the liquid nitrogen. Subsequently, it was found that a greater degree of control of freezing rate could be achieved by the use of microprocessor-controlled freezers. Some studies have suggested that improved survival of spermatozoa may be sufficient to justify the increased cost of the processing (e.g., Landa & Almquist 1979, Parkinson & Whitfield 1987). On the other hand, although the control of freezing rate is not as good in open tank methods as in programmable freezers, the latter are not as amenable to processing large batches of semen, or if large batchers are processed, there can be significant variation in cooling rate (and hence post-thaw survival) across the cooling chamber. Hence many organisations have chosen to continue to use the less well-controlled open tank methods (Vishwanath & Shannon 2000). Boar semen may be frozen in larger volumes of 5 mL in flat packs (Eriksson et al. 2002).

New Methods of Cryopreservation

Vitrification, a process in which a liquid turns into a solid without the formation of ice crystals, has been evaluated for cryopreservation of cell types that do not survive traditional freezing methods particularly well. Spermatozoa are particularly sensitive to ice crystal formation because their long tail is likely to span regions in the freezing diluent that contain both ice crystals and hyperosmotic diluent (Holt 2000). These random patterns of extracellular ice, together with the intracellular ice that can also be formed during the cooling process, are a significant source of cell damage. In freezing large cells, such as embryos or oocytes, vitrification processes normally require high concentrations of cryoprotectants, which themselves impede cell survival through their toxic effects (Vajta et al. 1997; Vajta 2000). However, recent studies have suggested that ultrafast freezing of spermatozoa can result in vitrification without the presence of a cryoprotectant (Isachenko et al. 2004) and, providing ice recrystallisation during thawing is prevented, results in acceptable post-thaw recovery rates. As yet, it has not been possible to recover motile spermatozoa post-vitrification. However, offspring have been produced after intracytoplasmic injection of reconstituted vitrified spermatozoa (Sánchez et al. 2011, Isachenko et al. 2012), leading to the hope that refinements in the procedure may eventually lead to the retention of spermatozoal motility.

A further new technique is the development of a 'multithermal gradient' as a means of controlling and optimising the formation of ice during the freezing process (Arav et al. 2002). Samples are moved at a constant velocity through a linear temperature gradient (Gacitua & Arav 2005), which controls the point at which ice forms and the morphological characteristics of the ice crystals. This method appears to result in good post-thaw recovery rates (ranging from 45% in the boar to 65% in the bull; Arav et al. 2002) and has the advantage that semen can be frozen in bulk, rather than necessitating the small volumes of pellets or paillettes (Gacitua & Arav 2005).

Thawing

Thawing of the semen needs to be rapid, since slow thawing allows recrystallisation of ice within the cells, causing membrane damage (Salisbury et al. 1978). In practice, the rate of thawing is rarely critical, although thawing at 30° to 37°C for 10 to 60 seconds is commonly recommended for bovine semen (see Fig. 43.3B). Much higher temperatures (e.g., 60°C) for a short period of time have sometimes been recommended as a means of improving recovery rates; however, the practical difficulties of using such methods in the field have meant that they have not been widely adopted. Of far greater importance is the temperature control of the thawed semen, which should not be allowed to cool lower than the final temperature achieved during thawing; otherwise, substantial losses of spermatozoa can occur. Rethawed spermatozoa are as sensitive to fluctuations in temperature as are their unfrozen counterparts (Roberts 1986) and cold shock can easily be induced if post-thaw cooling occurs (Nebel 2007). In addition, controlling the temperature of semen during withdrawal from storage flasks is important (see Fig. 43.3A): temperatures in the top third of the neck of the flask are high enough for ice recrystallisation to occur in the straws, resulting in markedly reduced post-thaw survival (Nebel 2007).

Ambient Temperature Preservation

For spermatozoa to be stored at ambient temperatures, alternatives to cooling have to be found to slow down their metabolic rate. There may also be advantages to ambient temperature preservation of semen because both cooling and cryopreservation are associated with a significant loss of both numbers and functionality of sperm. Moreover, some species do not tolerate cooling or cryopreservation well (e.g., the boar), so for these species, ambient temperature dilution is imperative. Strategies that have been used for ambient temperature diluents include:

- Decreasing the proportion of egg yolk to a level at which the beneficial effects on cold shock are retained but the toxic effects of other yolk components are avoided;

- Lowering pH to approximately 5.5, resulting in a reversible inhibition of spermatozoal motility (although reducing the pH below 5.5 is spermicidal);
- Saturating the diluent with carbon dioxide also causes a reversible inhibition of motility; diluents, such as the Illinois Variable Temperature (IVT) diluent, that were based on this principle were able to maintain the viability of spermatozoa for approximately 48 hours; longer term maintenance (up to 5 days) is achieved in diluents (e.g., the Cornell University Extender (CUE) or Caprogen diluent) that are self-carbonating from the reaction of citrate and bicarbonate (Vishwanath & Shannon 2000);
- Gassing with nitrogen; this reduces oxygen tension in the diluent, thereby inhibiting the motility of spermatozoa without the need to lower pH; this is used in the Caprogen diluent; and
- A combination of buffers, glucose, and antibiotics (Beltsville Thawing solution) sufficient to maintain boar spermatozoa for several days; longer term storage (up to 7 days) requires a more complex mixture of buffers and also additional antibiotics.

Other components of ambient temperature diluents (simple sugars, buffers, egg yolk/milk, antibiotics, catalase) are similar to those used in diluents for cooled or cryopreserved semen.

Microencapsulation

Encapsulation has also been investigated as a means of preserving spermatozoa without the need for recourse to cryopreservation. Sperm encapsulation is the process of enclosing spermatozoa within a semipermeable membrane (Nebel et al. 1985). Spermatozoa remain viable within the microcapsules because the semipermeable membrane permits the exchange of low molecular–weight nutrients and metabolites with the medium within which they are contained (Roca et al. 2006a), but prevents transfer of large molecules.

The process of microencapsulation has been described by Nebel et al. (1993). After extension in an ambient temperature diluent, spermatozoa are suspended in a sodium alginate solution. Spraying into a high calcium buffer solidifies droplets of diluted sperm, after which the semipermeable membrane is constructed on the surface of the droplet. Polyamines such as protamine sulphate, polyvinylamine, and poly L-lysine have been used successfully. Calcium is then chelated, which converts the gel core of the microcapsule back to a liquid phase. Barium has been used as an alternative to calcium, with the advantage that it does not precipitate premature capacitation (Torre et al. 2007).

Microencapsulation has been used for the ram (Maxwell et al. 1996), the boar (Faustini et al. 2004), and the bull (Vishwanath et al. 1997) semen. Spermatozoa are released from the capsules over a period of at least 24 hours, and in the case of the bull, this may be extended to 96 hours. Preliminary results show that acceptable conception rates can be achieved with encapsulated spermatozoa and that the timing of insemination in relation to the time of ovulation is less critical than with other forms of preserved semen. However, limited development of the technology has taken place for use in insemination, although it is more widely used for other forms of cell preservation (Torre et al. 2007). One such development is to use a solid gel network to immobilise the spermatozoa instead of enclosing them in gel microcapsules to mimic the conditions in the epididymis (Standerholen et al. 2015). Insemination trials in cattle indicate that 56-day non-return rates were similar between conventional frozen thawed semen doses (72.5%) and frozen–thawed, gel-immobilised doses (72.7%) (Standerholen et al. 2015).

The possibility of encapsulating sexed spermatozoa as an attempt to compensate for low spermatozoa numbers is of interest. Use of encapsulated sexed boar spermatozoa reduced the incidence of polyspermy in *in vitro* fertilisation (IVF) and did not produce any additional damage to the spermatozoa during storage for 72 hours (Spinaci et al. 2013).

Spermatozoa Selection

It is possible to improve semen quality by selecting functional spermatozoa from a semen sample. Various methods have been proposed over several decades, as reviewed by Morrell and Rodriguez-Martinez (2016), but only colloid centrifugation of stallion spermatozoa has proved to be practical in the field. Basically, these methods mimic the selection for 'normal' spermatozoa that is believed to occur in the female reproductive tract (Suarez 2007) and can therefore be described as 'biomimetic' (Morrell & Rodriguez-Martinez 2009). These methods rely on one or more physical attributes of functional spermatozoa, namely that they are motile and have normal morphology as well as intact plasma and acrosomal membranes. Thus migration techniques such as 'swim-up' rely on motile spermatozoa being able to move away from an area containing many spermatozoa to one containing relatively few. Passage through Sephadex gel, glass wool, or colloids impedes spermatozoa with reacted acrosomes, damaged membranes, or abnormal morphology. Colloid centrifugation also separates spermatozoa with mature chromatin from those with immature or damaged chromatin.

Colloid-Selected Spermatozoa

Colloids consist of nano-sized particles in suspension; both silica colloids and iodixanol are suitable for the preparation of spermatozoa. Initially, polyvinylpyrrolidone-coated silica was used in density gradients to prepare human spermatozoa for assisted reproduction (Bolton & Braude 1984), and it was also used in this form to prepare animal spermatozoa for IVF. There was some concern that this type of coated silica was toxic to bull spermatozoa (Avery & Greve 1995), but this issue has since been resolved by replacing the PVP-coated silica with silane-coated silica. From 2008, silane-coated silica has been used as a single layer instead of a density gradient (single layer centrifugation; SLC), thus facilitating scaling up of the method to process whole animal ejaculates (Morrell et al. 2009a), even from boars (Morrell et al. 2011). Such SLC-selected spermatozoa samples contain a higher proportion of morphologically normal spermatozoa, which have good motility, intact membranes and good chromatin integrity, than unselected controls (Morrell et al. 2009b). Selected stallion spermatozoa samples survive longer during cooled storage and also show enhanced cryosurvival (Hoogewijs et al. 2011, Martinez-Alborcia et al. 2012); pregnancy rates after insemination are better than controls (Morrell et al. 2014).

There has been some debate whether the apparently enhanced spermatozoa quality after colloid centrifugation is due to selection for a particular spermatozoa subpopulation, or merely to exclusion of immotile or damaged spermatozoa. There is some evidence that the spermatozoa that pass through the colloid produce less hydrogen peroxide as a by-product of metabolism than controls (Morrell et al. 2017), lending credence to the theory that there is selection for a particular spermatozoa subpopulation. However, further research is needed to verify this suggestion. Whatever the mechanism of action, the use of good quality spermatozoa samples for assisted reproduction is associated with enhanced fertility, and therefore this method of processing semen samples is worth pursuing.

Sexed Semen

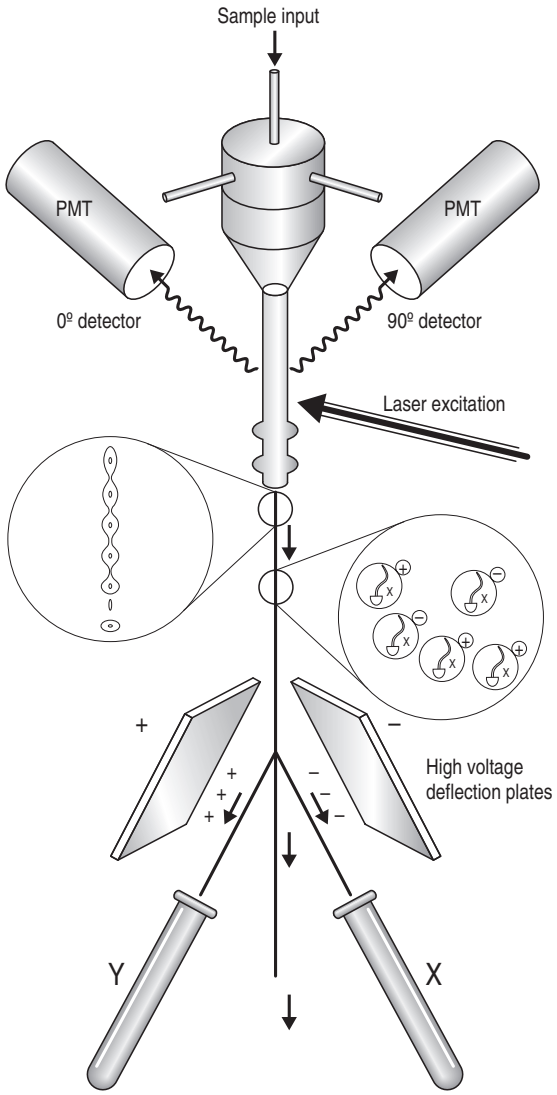
A long-standing goal of the AI industries has been to be able to control the sex of offspring, by separating X- and Y-bearing sperm. Many methods have been developed in attempts to achieve this goal. Most of these proved impractical or ineffective, but in recent years, flow cytometry has been developed to a point at which spermatozoa sexing is available as a commercial service. The technique of sexing semen has been reviewed by Seidel and Garner (2002), Garner (2006), and Seidel (2007).

Separation of spermatozoa on the basis of surface antigens is an attractive option, as it would allow bulk processing of semen. However, early attempts, based on the H-Y antigen, and more recent investigations, based on sex-specific proteins, have yet to yield practical sorting methods. Major problems with this approach are the limited transcription that takes place in the haploid cell and the cytoplasmic bridges that join spermatozoa until just before their release from the seminiferous epithelium. Electrophoresis methods based on the principle that X- and Y-bearing spermatozoa may have different surface charges have also only had limited success to date.

Hence most attention has been upon the using the different mass of DNA in the X- and Y-bearing spermatozoa as the basis for sorting and separation: X-bearing spermatozoa of domestic animals contain approximately 4% more DNA than Y-bearing sperm. The principle of sexing semen is to stain the DNA with a fluorescent dye that binds to the DNA but does not damage it. The difference in fluorescence between the X- and Y-bearing spermatozoa can then be measured and cells sorted accordingly (Keeler et al. 1983, Johnson et al. 1987). DNA is stained with the membrane-permeable agent bisbenzimidazole DNA-binding dye, Hoechst 33342 (Morrell et al. 1988, Garner 2006), which binds specifically to A-T pairs along the minor groove of the DNA (Seidel & Garner 2002). Spermatozoa are separated from live cells by further staining with a food dye, FD&C#40, which permeates dead cells and quenches the fluorescence of Hoechst 33342 but is excluded from living cells. The stained cells are then passed through a flow cytometer for sorting, based on fluorescence from the stained DNA.

After measuring the amount of DNA, the vibrating nozzle in the flow cytometer breaks the solution containing the spermatozoa into tiny droplets, most of which contain a single spermatozoon (some droplets contain no sperm, a few contain two sperm). A charge is placed on the droplet as it leaves the sorter such that droplets containing spermatozoa with Y chromosomes are given a negative charge and those containing spermatozoa with an X chromosome are given a positive charge. The droplets then pass through an electromagnetic field, which deflects droplets with a positive charge (X chromosomes) towards the negative side (and vice versa for negatively charged droplets). Somewhere in the region of 20% of spermatozoa are successfully sorted as X-bearing, 20% as Y-bearing, with the remainder unidentified or dead. This process is illustrated in Fig. 43.2.

The process is relatively slow, however. Currently, 14 insemination doses of 2×10^6 live spermatozoa of each sex can be produced per hour (Seidel 2014). Moreover, the spermatozoa have been subjected to a number of insults during the separation process (staining, laser, high pressure, electrical charge), which impair their subsequent function. There is evidence of more chromatin abnormalities in sorted spermatozoa (Bochenek et al. 2006), but H33342 does not appear to cause any mutagenic damage to the genome. Altering the conditions for the cell sorting process can reduce the overall level of spermatozoa losses (Schenk & Seidel 2007). Recent developments by the patent holders have apparently improved the sex-sorting conditions such that conception rates are now similar between nonsexed and sexed spermatozoa doses (Lenz et al. 2016). However, no details of the process were provided. It remains to be seen whether similar results can be obtained in field AI under conventional conditions.



• **Fig. 43.2** Flow cytometric sorting of X- and Y-bearing sperm. After staining, the sperm are passed by photomultiplier tubes (PMT). The electronic signals from the PMT are sent to a computer for analysis. Thereafter the fluid stream is broken into droplets and a positive charge is placed on droplets identified to contain a Y-bearing sperm and negative charge on droplets with X-bearing sperm. The drops then travel between high voltage fields, which direct them to their respective collection tubes. (Derived from Johnson & Welch 1999, Seidel & Garner 2002.)

Sorted spermatozoa can survive cryopreservation, but the low numbers of spermatozoa that are present generally require specialised insemination routes to achieve acceptable pregnancy rates (see later in this chapter). Alternatively, sexed semen can be used in *in vitro* fertilisation (cattle) or intracytoplasmic spermatozoa injection (horse) for the production of embryos of known sex (Wheeler et al. 2006). However, as noted by Garner (2006), the effective use of sexed semen in animal breeding industries requires further development in the speed and efficiency of sex sorting, as

TABLE 43.1 Diseases that may be transmitted through artificial insemination

	Cattle	Sheep	Pigs	Horse
Present and Spread Through Semen	Bovine herpesvirus 1 Bovine immunodeficiency-like virus Bovine venereal campylobacteriosis Brucellosis Contagious bovine pleuropneumonia Epizootic haemorrhagic disease Foot & mouth disease *Histophilus somni* Japanese encephalitis Johnes' disease Mycoplasma spp Trichomonosis Tuberculosis Vesicular stomatitis	Bluetongue Contagious caprine pleuropneumonia Foot & mouth disease Japanese encephalitis Johnes' disease Lentivirus (maedi-visna) Ovine enzootic abortion Ovine epididymitis (*Brucella ovis*) Ovine/caprine brucellosis Peste de petits ruminants Sheep pox/goat pox Vesicular stomatitis	African swine fever Aujeszky's disease Classical swine fever Foot & mouth disease Japanese encephalitis Leptospirosis Porcine brucellosis Reproductive and respiratory syndrome Swine vesicular disease	African horse sickness Dourine Equine viral arteritis Venezuelan equine encephalomyelitis Vesicular stomatitis
Low Risk of Spread	Akabane virus Bluetongue Bovine ephemeral fever Bovine virus diarrhoea Enzootic bovine leucosis Leptospirosis	Caprine arthritis/encephalitis Leptospirosis		Contagious equine metritis Equine herpesvirus 3 (coital exanthema) Piroplasmosis Equine infectious anaemia
Reference	Eaglesome & Garcia 1997, OIE 2016	Cseh et al. 2012, OIE 2016	Althouse & Rossow 2011, OIE 2016	Metcalf 2001, OIE 2016

well as development in the technology of AI in order to utilise such spermatozoa efficiently. The idea of combining encapsulation and sex-sorting, as previously mentioned, may represent one such development (Spinaci et al. 2013).

Diseases Transmissible in Semen

Many infectious agents can be transmitted through semen. Foot and mouth virus can be transmitted in the semen of all species that are susceptible to this infection (Callis & McKercher 1980, Radostits et al. 2007). Indeed, control of foot and mouth transmission has, until recently, underpinned the UK legislation controlling AI of cattle, with broadly similar regulations in force throughout much of the developed world. Diseases of cattle, small ruminants, and horses that are potentially transmissible in semen are listed in Table 43.1.

The World Organisation for Animal Health (OIE) therefore stipulates that sires that are to be used in AI centres for international trade should be tested for freedom from a number of diseases. Many importing countries also require animals to be free of additional diseases. When semen is diluted in egg yolk, there is generally a requirement that the eggs are derived from specific-pathogen-free (SPF) flocks. These regulations are also applied to use of semen in domestic trade within the European Union.

Artificial Insemination of Cattle

Collection and Storage of Semen

Semen is usually collected by an artificial vagina, although electroejaculation is occasionally used (see Chapter 35). After assessment for motility, the concentration of spermatozoa, and, occasionally, their morphology, the semen is extended into insemination doses. The degree of extension and the extenders used depend on its intended application. Most bull semen is cryopreserved, although some is used after simple extension and chilling to 4°C. The practice of using an ambient temperature extender for bull semen is virtually unique to New Zealand, where the practice is made possible by the low incidence of infectious disease, and is required for the highly seasonal pattern of reproduction in its dairy cows.

For cryopreservation or for use at 4°C, the semen is first extended with an extender based on either egg yolk or skimmed milk (Salisbury et al. 1978, Vishwanath & Shannon 2000); see Table 43.2. The semen is then cooled to the appropriate storage temperature. If it is destined for use cooled, spermatozoal motility will be reassessed before releasing the semen for use. If the semen is destined for cryopreservation, glycerol is also added; then the semen is packed into 0.25 or 0.5 mL paillettes (or, rarely, 0.5 or 1.0 mL glass ampoules). The semen is then 'equilibrated' for 1 to 4 hours. It was originally considered that this was the period in which glycerol penetrated the sperm, although subsequent observations indicate that the penetration of glycerol is very rapid and that most of the equilibration period is concerned with membrane stabilisation during exposure to low temperatures (Watson 1979). Recently, there has been a trend to equilibrating the semen in cryoprotectant overnight before freezing (Anzar et al. 2011), which may be more compatible with the routines on the bull station. The semen is then frozen in liquid nitrogen vapour or in a microprocessor-controlled freezer. The semen thereafter remains in liquid nitrogen until thawed for use. Freezing in alcohol and solid carbon dioxide or in pellets on blocks of solid carbon dioxide, although formerly used widely, has now virtually ceased.

The ability to perform an intrauterine insemination in cattle means that a relatively low dose of spermatozoa is required to achieve acceptable pregnancy rates. Typically, of the 10 to 25 × 10^6 spermatozoa that are required in each insemination dose, 6

TABLE 43.2 Diluents for use in cryopreservation of bovine semen

Constituent	Skimmed Milk	Egg Yolk–Citrate	Reading Diluent	Egg Yolk–Tris	Netherlands Extender
Egg yolk	100 mL	200 mL	200 mL	200 mL	200 mL
UHT skimmed milk	870 mL				
Fructose	12.5 g				
Lactose			82.8 g		
2.9% sodium citrate buffer[a]		770 mL			
Tris buffer				800 mL[b]	800 mL[c]
Citrate–HCO_3 buffer					753 mL
Glycerol			47 mL		47 mL
Stage 1:	30 mL	30 mL		Nil	
Stage 2:	110 mL	110 mL		140 mL	
Antibiotics	Typically, 1000 IU penicillin + 1000 µg streptomycin/mL				

[a]Trisodium citrate dihydride.
[b]30.28 g Tris, 17.30 g/litre citric acid monohydrate.
[c]31.5 g Tris, 12.3 g/litre citric acid monohydrate.
Stage 1 diluents are added at 30° to 37°C, after which the semen is cooled to 4°C. Stage 2 diluents are added at 4°C. Single stage diluents are added at 30° to 37°C, after which the semen is cooled to 4°C.
From: Salisbury et al. 1978, Parkinson & Whitfield 1987, Vishwanath & Shannon 2000, J. Wilmington, personal communication.

to 7×10^6 survive freezing, a figure that is generally regarded as the minimum dose compatible with acceptable fertility (Milk Marketing Board 1967, Sullivan & Elliott 1968). An exception is when sexed semen doses are used because the sexing procedure takes such a long time: typically 1.5 to 2.0×10^6 spermatozoa are inseminated, although pregnancy rates tend to be 80% of those obtained with unsexed spermatozoa (Garner 2006). Lower numbers of spermatozoa can be used for fresh semen (Salisbury & VanDemark 1961).

The use of ambient temperature extenders for bull semen is precluded in most countries by the risk of contamination of the semen with foot-and-mouth disease virus. However, it has many advantages over other methods of extension because many more spermatozoa survive in the absence of freezing, albeit for a limited time. Whereas cryopreserved bull semen requires about 10 to 25×10^6 spermatozoa per insemination, semen extended in an ambient temperature extender can achieve acceptable fertility with fewer than 2.5×10^6 spermatozoa per insemination (Shannon et al. 1984). Initial work on ambient temperature diluents for bovine semen was based upon the use of the IVT diluent (Salisbury & VanDemark 1961, Melrose 1962), but the development of ambient temperature dilution has subsequently been based upon the use of the CUE and Caprogen diluents (Shannon 1965; Table 43.3). With the subsequent modifications that have taken place, these diluents are now capable of maintaining spermatozoa viability and acceptable conception rates for up to 5 days, with an insemination dose of between 0.5 and 2.5×10^6 spermatozoa.

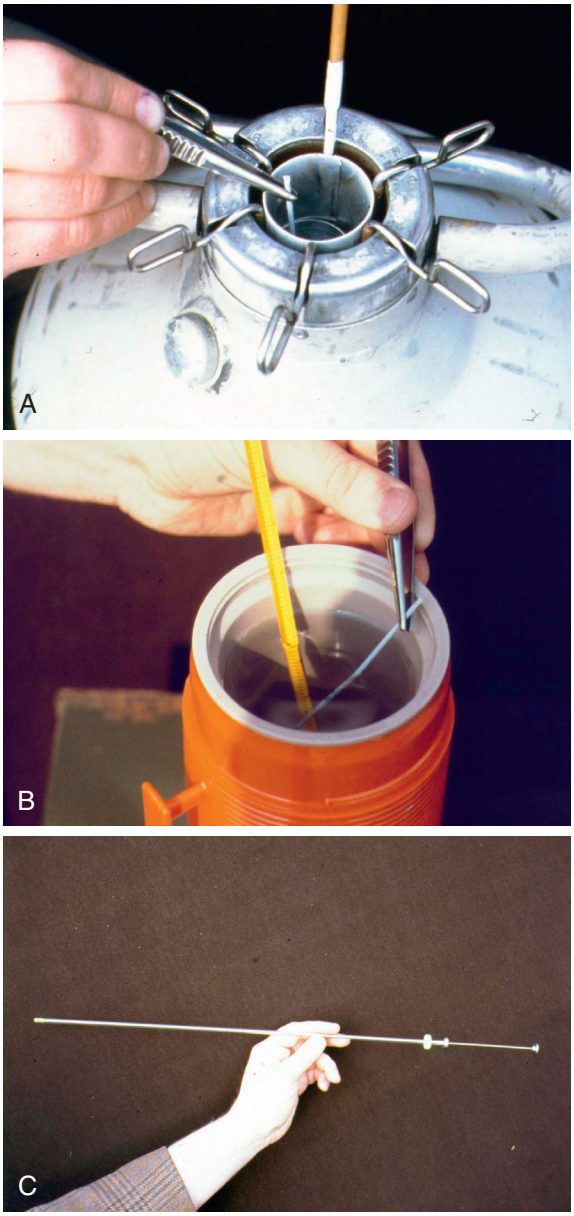
TABLE 43.3 Diluents for ambient temperature extension of bovine semen

Basic Diluent (g/Litre)	Cornell University Extender	Caprogen Diluent
Sodium citrate	14.5	20
Citric acid	0.87	
Sodium bicarbonate	2.1	
Glycine	20	10
Glycerol		12.5
Glucose	3	3
Potassium chloride	0.4	
Caproic acid		0.3125
Preparation of Diluent		
Basic diluent	80%	80%
Egg yolk	20%	20%
Nitrogen gas		Bubbled for 20 min at 5°C
Catalase		4.5 mg/L

Derived from: Shannon 1965, Shannon & Curson 1984, Vishwanath & Shannon 2000.

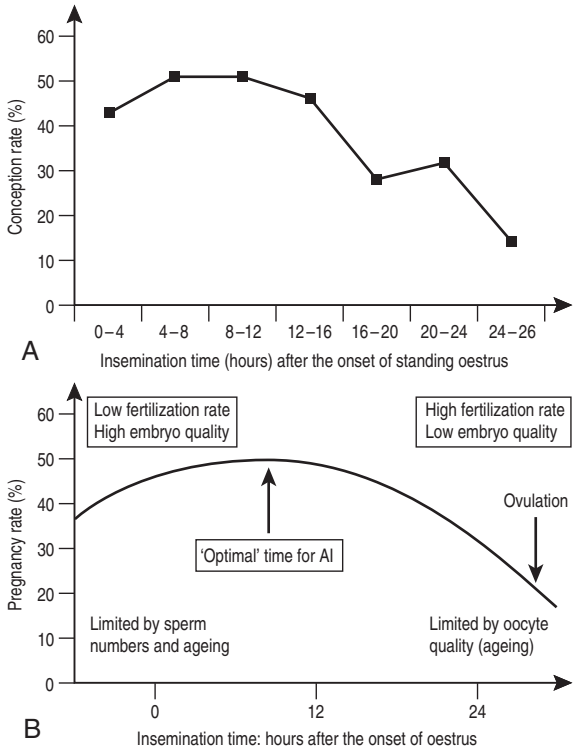
Insemination

Before insemination, straws of semen are withdrawn from liquid nitrogen storage, ensuring that the canister containing the frozen semen remains contained within the neck of the storage flask (Fig. 43.3A). After identification of the sire, excess liquid nitrogen



• **Fig. 43.3** Semen handling for bovine artificial insemination. (A) Withdrawing a straw of frozen semen from the liquid nitrogen flask. The canister containing the semen should not be lifted higher than the level of the top of the neck of the flask. (B) Thawing. After checking the identity of the sire, the straw is thawed. Water temperature is not really critical but placing the straw in water at 37°C for 10 seconds is a typical thawing regime. (C) The straw is placed in an insemination catheter, which is then covered with a plastic sheath. The catheter is then ready for use, but care must be exercised not to allow the semen to become chilled again before it is inseminated.

is flicked out of the end of the straw before it is deposited into thawing water (Fig. 43.3B). After thawing, the straw is dried and the identity of the sire is rechecked, before loading it into the insemination catheter (Fig. 43.3C). It is important that the temperature remains constant after thawing, as semen must not be allowed to cool lower than the temperature at which it was removed from the thawing water. Hence, it is generally preferable to thaw small numbers of straws at a time, depending on the proficiency of the inseminator and the temperature of the environment.



• **Fig. 43.4** Optimal timing of insemination in cattle (A) derived from observations of the onset of oestrus; (B) as a compromise between fertilisation rate and embryo survival rate. (Redrawn from Saacke et al. 2000.)

Timing

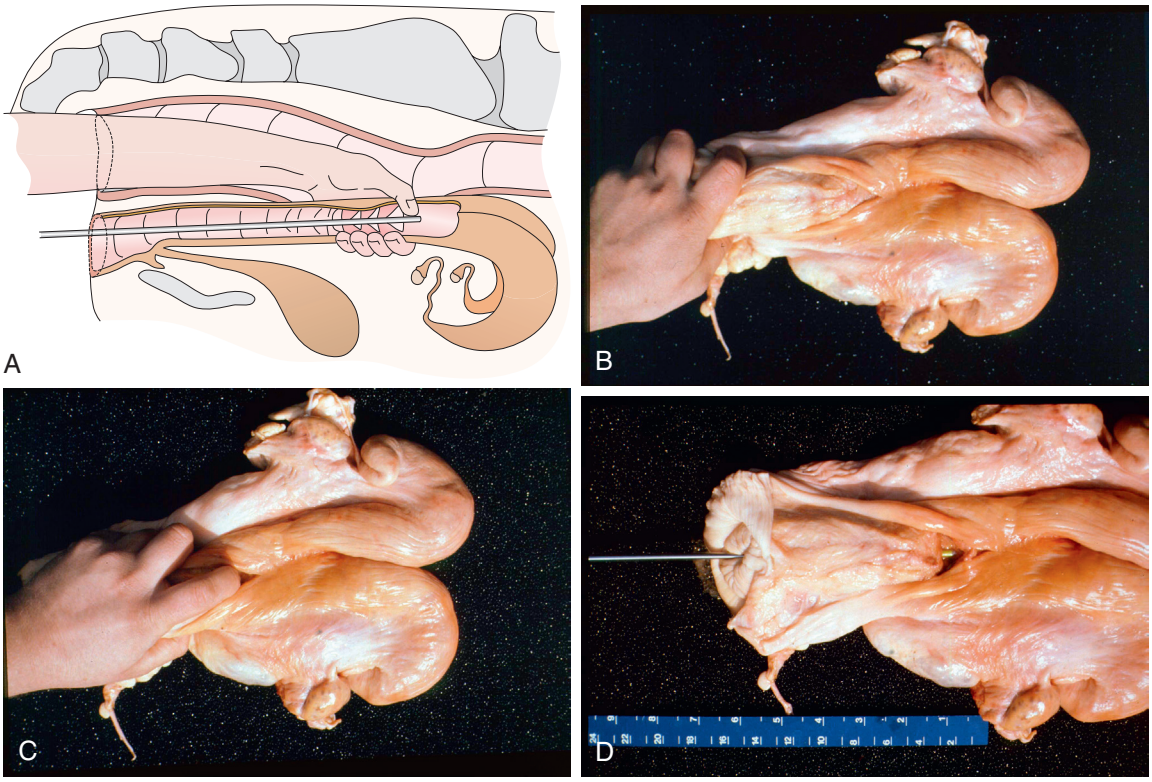
Cows ovulate approximately 12 hours after the end of oestrus. The ideal time for insemination is therefore 6 to 24 hours before ovulation (Roberts 1986). Observations of conception rates of animals that were inseminated at different times after oestrus (Fig. 43.4A) led to the development of the 'a.m.–p.m.' rule, by which cows first seen in heat in the morning were inseminated that afternoon, whereas those first seen in heat in the afternoon were inseminated the next morning (Olds & Sheath 1954, Foote 1979).

In fact the optimal timing of insemination is a compromise between many factors. Insemination early in oestrus is likely to achieve a low fertilisation rate because of limitations on spermatozoal viability, whereas oocytes that are successfully fertilised are likely to yield high quality embryos. Conversely, insemination late in oestrus is less likely to result in limitations on spermatozoa viability but is more likely to result in the fertilisation of an aged oocyte, resulting in impaired embryo development (Saacke et al. 2000). According to this proposal, the 'optimum' timing of insemination represents a compromise between the processes of aging of spermatozoon and oocyte (Fig. 43.4B). However, these processes are also affected by the duration of oestrus and the interval between the onset of oestrus and ovulation as well as the degree to which the spermatozoa have been affected by the preservation process. The latter can further involve both precapacitation changes and impairment of spermatozoa viability in the female tract.

Site

Standard Method of Insemination

Cows are inseminated at the internal cervical os or just inside the short uterine body. The standard technique of insemination is to grasp the cervix through the rectum with the left hand. A



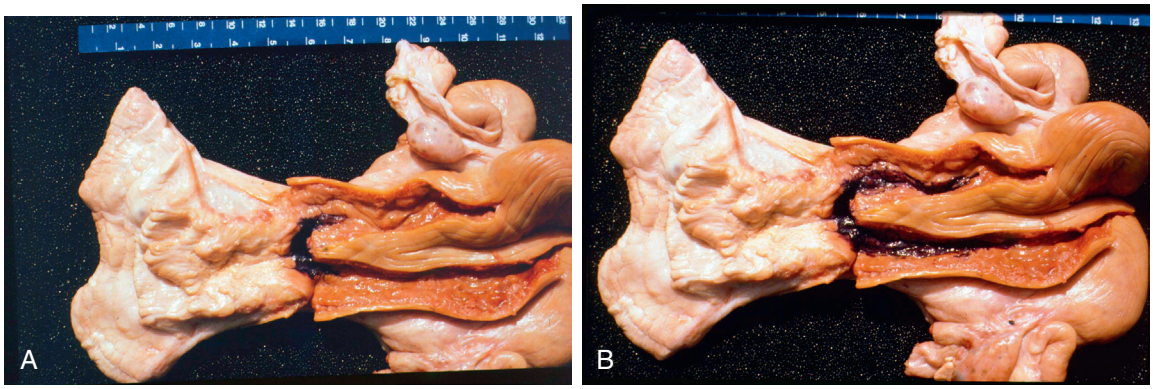
• **Fig. 43.5** Rectovaginal method of insemination. (A) General method. (B) Partially dissected bovine uterus showing grip on the cervix for the obliteration of the vaginal fornix to facilitate entry of the catheter into the external os of the cervix. (C) Second grip on the cervix to allow passage of the catheter through the tortuous cervical canal: the index finger is gently pressed over the internal os in order to feel the tip of the catheter as it emerges from the cervical canal. (D) Site of deposition of semen in the uterine body: the recommended site is no more than 0.5 cm deep to the internal cervical os.

catheter, into which a paillette of semen has been inserted (Figs 43.3C, 43.5A) is then passed into the vagina and manipulated into and through the cervix by the right hand. This technique, the rectovaginal method of insemination, requires considerable practice for success. The vulval lips are opened by downwards pressure from the arm in the rectum, while the circular folds of vaginal mucosa are obliterated by pushing the cervix forward. The catheter is initially inserted pointing upwards at an angle of about 30° to avoid entering the urethral meatus or fossa and is then moved horizontally until it engages in the external os of the cervix. The left hand squeezes the anterior vagina on to the caudally projecting external os of the cervix, thereby obliterating the fornix of the vagina (Fig. 43.5B) and facilitating entry of the catheter into the cervix. Entry into the external os is accompanied by a characteristic 'gritty' sensation. The catheter is then introduced through the convoluted cervical canal by manipulation of the cervix through the rectal wall. One finger is placed over the internal os of the cervix so that the tip of the catheter can be palpated as it emerges from the cervical canal (Fig. 43.5C). As soon as the catheter has emerged, deposition of semen into the uterus begins and the catheter is not advanced any deeper into the uterus. In this way, semen should be equally distributed between the two uterine horns (Figs 43.5D and 43.6A, B). Insemination deeper into the uterus runs the risks of either inseminating into the uterine horn contralateral to the ovulation site or scoring the endometrium with the tip of the insemination catheter. Reduced fertility is the consequence of these errors.

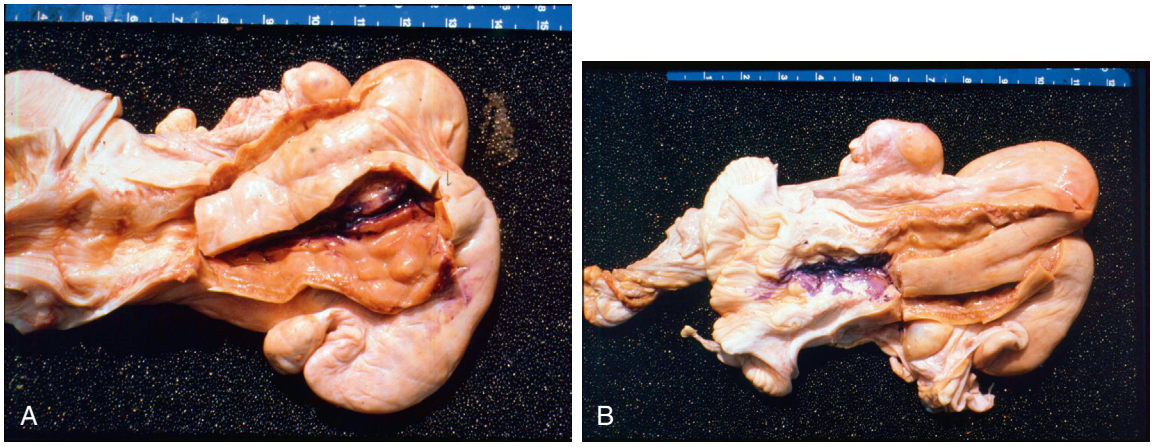
No forward pressure should be exerted on the catheter with the right hand because the uterine wall is friable and easily penetrated if the catheter moves suddenly. The most common fault of insemination (Fig. 43.7A) is twisting the cervix in the left hand, so that one uterine horn is partly occluded. Alternatively, the catheter may be partly withdrawn during the deposition of semen, resulting in a partial intracervical insemination (Fig. 43.7B). Penetration of the cervical canal of maiden cattle is difficult at oestrus and virtually impossible at other stages of the oestrous cycle. Such animals are therefore often beyond the capabilities of inexperienced inseminators; it is common to find that semen is deposited into the caudal part of the cervix. However, the cervix of parous cattle can be traversed relatively easily at most stages of the oestrous cycle and early pregnancy. It is therefore imperative to know whether an animal is likely to be pregnant before insemination is attempted, for abortion can be induced if an insemination catheter penetrates the fetal membranes or if infection is introduced into a pregnant uterus by poor insemination hygiene.

Alternative Sites for Insemination

Although traditional methods of insemination avoid deep uterine deposition of semen in cattle, this has been advocated as a means of improving conception rates (Senger et al. 1988, Dalton et al. 1999). However, this has not been achieved in all studies (e.g., McKenna et al. 1990) and has not been widely adopted in practice. Single horn insemination has also been advocated as a means of improving conception rates in cows. This technique requires



• **Fig. 43.6** Distribution of semen in the cow after artificial insemination. A dark-coloured dye has been placed into the uterine body (A) and runs equally into both uterine horns (B). As inseminators are trained not to palpate the uterus or ovaries, they should be unaware of the side on which ovulation will occur, so semen must have access to both uterine horns.



• **Fig. 43.7** Distribution of dye in the reproductive tract of the cow after faulty insemination. (A) Uterus twisted during insemination, occluding one uterine horn. The dye is present in only one horn, typically the right. (B) Catheter withdrawn into the cervix during semen deposition. Some dye is present in the uterine body, but most is within the cervical canal.

palpation of the ovaries to determine the site of ovulation and a careful straightening of the ipsilateral horn to allow the insemination catheter to be inserted to within approximately 2 cm of the uterotubal junction. Again, some authors have found that this technique results in improved conception rates (Pallares et al. 1986, Lopez-Gatius & Camon-Urgel 1988), whereas others have not (Hawk & Tanabe 1986, Momont et al. 1989). Disadvantages include premature rupture of the follicle, perforation of the uterine wall, risk of polyspermic fertilisations (Hunter 2003), and possibly also an increase in the time required to perform the insemination.

Despite the equivocal results in field trials, some continue to advocate the use of deep intrauterine insemination, especially in situations in which semen may be compromised, as for example, in bulls whose semen does not respond well to preservation or when sexed semen is used (Hunter 2000, Lopez-Gatius 2000).

Management of Insemination

Inseminations may be performed by technicians employed by AI organisations but increasingly is being done by farm staff.

Fertility after AI is generally monitored by recording the proportion of cows that return to oestrus after the initial service. The proportion of females that do not return to oestrus is sufficiently closely related to the proportion that actually have become and remained pregnant to be a useful monitor of fertility (reviewed by Salisbury et al. 1978). The figure thus obtained, the non-return rate (NRR), which is an overestimate of calving rate, is generally in a fixed ratio to the calving rate depending on the interval after initial insemination at which it is calculated. The NRR is useful for observing trends in performance and for identifying below average performance. The NRR can be used by AI centres to monitor both the fertility of their bulls and the results obtained by their technicians. Technicians whose performance is below average are normally required to undergo a period of retraining and/or limited licensure until their results improve. Bulls that produce consistently low figures are removed from the AI service. Some centres make use of an 'adjusted NRR', whereby a number of factors that may affect fertility, such as cow or heifer, age of female, number of parities, location of farm, and identity of inseminator, are taken into account to create a 'fertility index score'. This score is believed to give a more accurate picture of a bull's fertility than using the unadjusted NRR.

Farm staff can be trained to inseminate cattle, with many countries allowing 'farmer-inseminators' to breed their own cattle. There are two potential advantages that persuade farmers to undertake

their own inseminations: first, the avoidance of the costs associated with having an AI centre technician perform the insemination; and, second, the hope that improved timing of insemination to ovulation will raise conception rates. Morton (2000) undertook a widespread survey of the results achieved by farmer-inseminators in comparison to AI centre technicians. Overall, farmer-inseminators achieved a 3% lower conception rate than centre technicians (45% vs 48%). However, although 13% of farmer-inseminators achieved conception rates that were more than 5% higher than centre technicians, 45% had results that were more than 5% worse, and 12% had results that were more than 15% worse than centre technicians.

In practice, farmer-inseminators who are well trained, motivated, have enough time to perform inseminations, and have reasonably large herds tend to achieve good results, whereas those for whom it is 'another job' or who do not have the opportunity to develop a good insemination technique do not. Poor insemination technique can be associated with disastrously poor pregnancy rates.

Control of Infectious Diseases

Many diseases can be spread in bovine semen (Table 43.1); AI centre technicians, by travelling between farms, also pose a considerable risk as disease vectors. Consequently, there is legislative control over the health status of semen used in domestic trade, whereas international trade in bovine semen is subject to the requirements and recommendations of the OIE. There are also controls over the hygienic practices of inseminators. Diseases that are specifically regulated by the OIE are listed in Table 43.4.

Most of the serious viral diseases of cattle (particularly foot-and-mouth disease) can potentially be transmitted through AI. Legislative regulation of bovine AI has for many years been based upon the primary precept of preventing such transmission from occurring. Many other viral diseases, such as bovine herpesvirus-1, can also be transmitted in this manner (Chapman et al. 1979, Kahrs et al. 1980). Bovine viral diarrhoea (BVD) virus can be present in the semen of bulls (Barlow et al. 1986), potentially causing early embryonic death and abortions in inseminated cows (Grahn et al. 1984), as well as its better known ability to cause the birth of persistently infected progeny. Recently, it has become apparent that Schmallenberg virus can be transmitted in semen (Schulz et al. 2014). Other viral diseases that could be of potential concern to bovine AI include Akabane, ephemeral fever, and bovine immunodeficiency viruses. However, Thibier and Guerin (2000) reviewed literature suggesting that Akabane virus has not been detected in semen; moreover, although ephemeral fever and bovine immunodeficiency virus may be present in bull semen, transmission by AI has not been demonstrated. There is no evidence that the prion agent responsible for bovine spongiform encephalopathy (BSE) can be transmitted through semen (Wrathall 1997, Wrathall et al. 2008).

TABLE 43.4 World Organisation for Animal Health requirements for disease testing of sires use of semen in artificial insemination programmes

Cattle	Sheep and Goats	Pigs
Bluetongue	Bluetongue	African swine fever
Bovine herpesvirus-1	Brucellosis	Aujeszky's disease
Bovine viral diarrhoea	Caprine arthritis/encephalitis	Brucellosis
Brucellosis	Contagious agalactia	Classical swine fever
Campylobacter fetus subsp. *venerealis*	Contagious caprine pleuropneumonia	Enterovirus encephalomyelitis
Tritrichomonas foetus	Lentivirus (maedi-visna)	Porcine reproductive and respiratory syndrome
Tuberculosis	Ovine epididymitis	Transmissible gastroenteritis
	Paratuberculosis	Tuberculosis
	Peste des petits ruminants	
	Scrapie	
	Tuberculosis (goats)	

From: OIE 2016.

A number of bacterial diseases are transmissible in semen, including tuberculosis and brucellosis (Eaglesome & Garcia 1997). *Histophilus somni,* pathological species of *Mycoplasma* and *Ureaplasma diversum* may also be present in semen (Humphrey et al. 1982). Most importantly, the classic venereal pathogens of cattle, *T. foetus* and *C. fetus* subsp. *venerealis* are transmissible by AI, so control of these two organisms remains the second major precept upon which legislation governing cattle AI is based.

Three other conditions warrant specific mention. Firstly, although enzootic bovine leukosis (EBL) virus is considered not to be transmissible in semen (Radostits et al. 2007), infected bulls are excluded from AI studs. Secondly, bluetongue virus generally causes few or no clinical signs in infected cattle (Radostits et al. 2007), but cattle can act as sources of infection for other animals via arthropod hosts. Because the disease in sheep is severe, semen from potentially infected bulls (Bowen & Howard 1984) is carefully excluded from most countries in which sheep production is of economic importance. Finally, Johne's disease is also commonly regulated, as semen from clinical cases and subclinically infected animals is potentially contaminated with the organism.

In cattle, control of these diseases rests upon four major strategies:

i. Direct effects of freezing and thawing: *Leptospira* spp. may be killed by the freezing process.
ii. Antibiotics that are added to semen diluents in order to kill both pathogenical bacteria (including *C. fetus*) and the contaminant bacteria that originate from the penis and prepuce during semen. Antibiotics are also used to control mycoplasma and ureaplasma species.
iii. Serology: Diseases that can be detected by serology, such as brucellosis, IBR, EBL, Q fever, are controlled by exclusion of seropositive bulls from AI studs. Likewise, tuberculosis is controlled by exclusion of bulls that react to tuberculin testing. However, a negative serology test result only indicates that the animal has not reacted to an infection by producing antibodies; recent infections or congenital carriers may never exhibit an antibody response.
iv. Quarantine: The final and most potent means of control of disease is the quarantine of semen after its collection. After semen has been frozen, it is placed in a container where it remains untouched for 28 days. If, during that period, the donor bull develops any disease, the semen is destroyed. If not, it is released for use.

Artificial Insemination of Sheep

Artificial insemination of sheep has been comprehensively reviewed by Evans and Maxwell (1987), Chemineau et al. (1991), and Kukovics et al. (2011). The sheep is less amenable to artificial insemination than is the cow because oestrus cannot be detected readily

TABLE 43.5 Minimum numbers of motile spermatozoa for insemination of ewes at different sites

Route	Number of Motile Sperm (x 10^6)			Insemination Volume	Concentration of Inseminate (x 10^6/mL)
	Fresh	Liquid	Frozen		
Vaginal	300	400		0.3–0.5 mL	2000
Intracervical	100	150	180	0.05–0.2 mL	1000
Transcervical intrauterine		60		0.1–0.5 mL	200–400
Laparoscopic intrauterine	20	20	20	0.05–0.10 mL per horn	400–800

Derived from: Evans & Maxwell 1987, Usboko 1995, Shipley et al. 2007.

without the presence of rams, the technique of insemination is less straightforward than in cows, and ovine semen is less easy to freeze than bovine semen. In Eastern Europe, South America, and Australasia, AI is widely used in sheep breeding programmes, but its use is much less widespread in Western Europe and North America, mainly because of the high costs of handling and inseminating sheep compared with the costs of natural service. Fertility after AI depends on breed (whether dairy or meat breeds) and other factors such as parity, climate, farm, technique, and inseminator (Anel et al. 2005, Palacin et al. 2012).

In contrast to cows, ewes normally display oestrous behaviour only in the presence of a ram. In order to determine the time at which AI should be performed, either the timing of oestrus should be controlled or it should be detected with rams. In the former situation, pharmacological methods are used to induce and synchronise oestrus, so that the time of the fertile period is defined. In the latter situation, raddled, vasectomised rams are used to detect oestrus. The cost:benefit ratio for the use of AI in sheep has therefore to be considered carefully. When there are substantial costs associated with AI, such as either maintaining rams, or of buying and administering drugs for oestrus synchronisation, or of the procedures associated with insemination itself, these have to be set against the financial benefits gained from the superior carcass or wool characteristics of the progeny born to AI.

However, the most important limitation of the use of AI in sheep is in the method of insemination, as intrauterine insemination is difficult to achieve because the cervical canal of the ewe is so tortuous. Because intracervical AI results in both a lower conception rate and a lower number of lambs per litter than natural service, a number of methods of insemination have been devised that try to bypass the cervix. All of these methods have had varying success in achieving an economically viable compromise between fertility, technical difficulty, and numbers of spermatozoa needed for insemination. The methods currently in widespread use are via the intravaginal, intracervical, transcervical intrauterine, and laparoscopic intrauterine routes.

Collection and Storage of Semen

Semen is collected by an artificial vagina or by electroejaculation (see Chapter 35) and is subjected to routine examination for motility and spermatozoa concentration. Most inseminations of ewes are performed using semen that has been extended by the addition of simple extenders, because the small volume of the ovine ejaculate means that it is difficult to control spermatozoa numbers if used for direct insemination. Extender and the final number and concentrations of spermatozoa depend on the route of insemination, whether the insemination is undertaken during the natural breeding season or after induction of out-of-season breeding, and how the semen is to be stored between collection and insemination (i.e., direct insemination, cooled storage, cryopreservation). Recommendations for spermatozoa numbers and insemination volume are shown in Table 43.5.

TABLE 43.6 Diluents for ram semen for use at 5° to 15°C

Constituent (g/L)	Tris Diluent	Egg Yolk–Citrate	Skimmed Milk
Tris	36.3		
Fructose		5.0	
Glucose	5.0		10.0[a]
Citric acid	19.9		
Sodium citrate		23.7	
Skimmed milk powder			90.0
Egg yolk (mL/L)	140	150	50.0
Antibiotics	1000 IU penicillin and 1000 µg streptomycin/mL		

[a]Recommended by Feredean et al. 1967 for addition to the basic skimmed milk diluent.
Derived from: Evans & Maxwell 1987, Salamon & Maxwell 2000.

Liquid Semen

Extenders that are in routine use for unfrozen semen include buffers containing citrate or Tris, together with either egg yolk or cow's milk (Table 43.6). When milk is used, it should be heated first to destroy the spermicidal lactenin protein, so either skimmed or UHT-treated milk is used. After dilution, the semen is cooled and stored at either +15°C or +4°C until used. The semen should be used within 8 hours of collection, as fertility declines progressively after this time (Chemineau et al. 1991), with a 30% reduction in lambing rates after storage for 24 hours (Maxwell & Salamon 1993). More recently, Quan et al. (2016) suggested that the motility of spermatozoa was preserved for longer in Tris– or Tes–egg yolk extenders than in skimmed milk.

Cryopreservation

Although simple extenders are capable of supporting spermatozoal viability for the relatively brief periods demanded for direct insemination, cryopreservation is required for long-term storage of semen. Unfortunately, cryopreservation of ovine semen is not particularly straightforward because the process itself causes a significant level of damage to ovine spermatozoa. In consequence, frozen semen is not recommended for intravaginal insemination, and although it can be used for intracervical insemination, fertility results are relatively poor.

There are also a number of difficulties encountered when semen is frozen for intracervical insemination. These arise as a relatively large number of spermatozoa ($150–200 \times 10^6$ total sperm; Salamon & Robinson 1962, Langford & Marcus 1982) have to be contained within the limited volume of inseminate that can be placed within the ovine cervix. Because the anatomy of the cervical canal limits the insemination volume to below about 0.25 mL (Evans & Maxwell 1987), the dilution rates are limited to between 1 : 1 and 1 : 4. In consequence, insufficient protection can be afforded to the spermatozoa by the diluent against cold shock and freezing damage (Miller 1986), generally resulting in mediocre postfreezing survival of functional sperm.

For intrauterine insemination, in which lower numbers of spermatozoa are required, far more satisfactory dilution rates of semen can be achieved, so cryopreservation is more successful. Furthermore, because the insemination site does not require spermatozoa to traverse the cervix, they do not have to survive in such a robust state as is needed for intracervical insemination. Hence the conception rates that are achieved with the use of cryopreserved semen for intrauterine insemination are commensurate with those of natural service after oestrus synchronisation (see, for example, Davis et al. 1984).

Extenders

Early extenders for cryopreservation of ram semen were based on citrate, egg yolk, a monosaccharide such as glucose or fructose, and/or milk. Other cryoextenders have been based on disaccharides (e.g., lactose), trisaccharides (e.g., raffinose), complex polysaccharides (e.g., gum arabic), or other complex molecules (e.g., polyvinylpyrrolidone). Optimal glycerol concentrations seem to be in the range of 4% to 6% (Salamon & Maxwell 2000), although this concentration is affected by other components of the extender, such as egg yolk and sugars. Although early studies suggested that there might be advantages to adding glycerol at 5°C, or to its stepwise addition, current practice is to add the glycerol at approximately 30°C. Two diluents that are currently widely recommended for ram semen are Tris–glucose–egg yolk (Salamon & Visser 1972) and Tris–citrate–fructose–egg yolk (Shipley et al. 2007) (Table 43.7), although there are many other diluents that are used successfully in commercial or research practice.

The problem of low dilution rates for semen that is to be used for intracervical insemination was addressed by Salamon (see Evans & Maxwell 1987) by preparing a range of different extenders for freezing semen at ratios of 1 : 1 to 1 : 4, in which the constituents were present in higher concentrations. In this way, compensation is achieved for the effects of the ratio between extender and seminal plasma. Chemineau et al. (1991) dealt with the same problem by using two different extenders (an egg yolk–lactose diluent and a glycerolised skimmed milk diluent) in varying proportions depending on the final dilution rate to be achieved.

One of the possibilities for improving the response to cryopreservation was the inclusion of seminal plasma in the extender.

TABLE 43.7 Diluents for the cryopreservation of ram semen

Constituent	Tris–Glucose	Tris–Citrate–Fructose
Basic Diluent		
Tris (g/L)	36.3	24.4
Fructose (g/L)		10.0
Glucose (g/L)	5.0	
Citric acid (g/L)	19.9	13.6
Glycerol (mL/L)	50.0	64.0
Preparation of Diluent		
Basic diluent	85%	80%
Egg yolk	15%	20%

Derived from: Evans & Maxwell 1987, Shipley et al. 2007.

Evidence reviewed by Maxwell et al. (2007) suggests that it is generally beneficial in terms of post-thaw membrane integrity and motility. Low molecular weight proteins (15–25 kDa) appear to be most associated with these benefits, and it has been postulated that a spermadhesin protein may be the critical component. As yet, however, the inclusion of seminal plasma has not been incorporated into field practice.

More recently, attention has focused on the inclusion of specific lipids such as oleic acid (Hashem et al. 2017) and palmitoleic acid (Eslami et al. 2017) into extenders. Bashawat et al. (2016) found that the low density of lipoprotein gave better overall outcomes after freezing than did egg yolk. Fish oil, as a source of docosahexaenoic acid, improved postfreezing recovery (Abdi-Benemar et al. 2015), whereas supplementing the diet with fish oil also improved post-thaw spermatozoal quality, pregnancy rate, and lambing rate (Masoudi et al. 2016a). Soybean lecithin as a source of phospholipid (Paz et al. 2010), specifically phosphatidylcholine (Masoudi et al. 2016b), has been successfully used to replace egg yolk in diluents. Finally, cholesterol, in the form of cholesterol-loaded cyclodextrin, has been used to improve the motility and proportion of intact acrosomes in a skimmed milk diluent (Salmon et al. 2017).

Addition of various antioxidants to semen extenders in an attempt to reduce lipid peroxidation has been increasing in popularity. Thus cysteine (Toker et al. 2016), green tea extract (Mehdipour et al. 2016), and taurine (Banday et al. 2017) were found to have a beneficial effect on post-thaw spermatozoal quality.

Freezing Protocols

After dilution, ram semen should be cooled slowly from 30°C to 5°C, as rapid cooling below 5°C is associated with poor spermatozoal survival (Fiser & Fairfull 1986). Hence cooling to 5°C should take place over 1.5 to 2 hours: Shipley et al (2007) recommend that this is best managed in a water bath. During this period of cooling, equilibration with glycerol takes place. Thereafter, semen can be successfully frozen in pellet form on the surface of blocks of solid carbon dioxide (Salamon 1971) or in paillettes in the vapour of liquid nitrogen (Fiser & Fairfull 1984). There is, however, much variation in the ability of the semen of individual rams to survive cryopreservation and the semen of a proportion of rams does not survive freezing successfully.

Paillettes are thawed in water, typically at temperatures of 35° to 40°C. Pellets can be thawed in a dry tube in a water bath or can be placed in prewarmed thawing solution. It is suggested that pellets are thawed at 40°C, then maintained in a 30°C water bath until required for insemination (Shipley et al. 2007).

Insemination

Vaginal Route

Vaginal insemination deposits semen into the cranial part of the vagina without attempting to locate the cervix. The requirements of this method, in terms of both technical proficiency and handling facilities for the sheep, are minimal. However, the large numbers of spermatozoa that are required for each insemination means that the vaginal route is not really amenable for use with stored semen. Moreover, conception rates are also poor after pharmacological oestrus synchronisation, so intravaginal insemination is best suited to use after oestrus detection during the natural breeding season. The ideal timing of insemination is before ovulation, i.e., 12 to 18 hours after the onset of oestrus (Evans & Maxwell 1987). Highest conception rates are therefore achieved when the timing of insemination is optimised by drafting ewes for insemination twice per day.

Intracervical Route

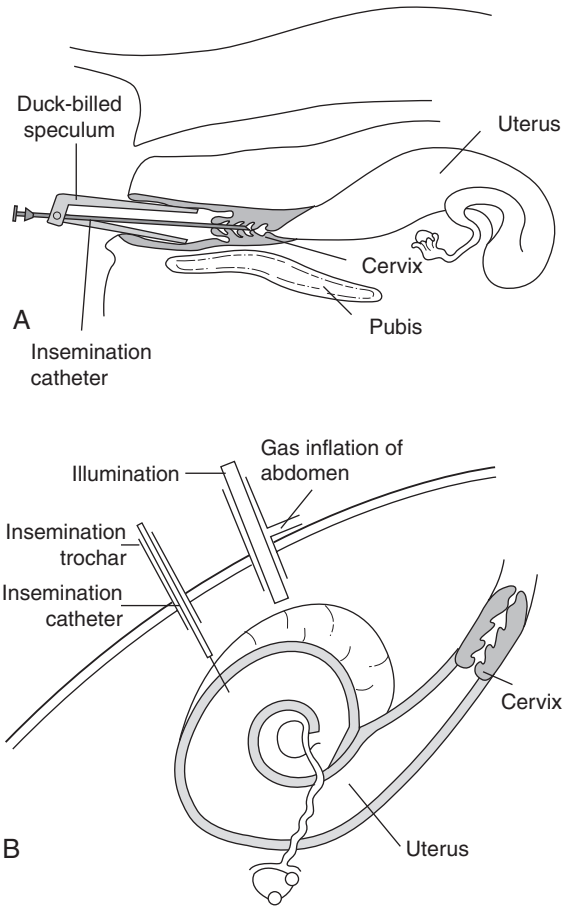
Intracervical insemination is best achieved with the hindquarters of the ewe elevated. After cleaning of the perineum, the vagina is opened with a duck-billed speculum, and the cervix is located (Fig. 43.8A). The insemination catheter is then inserted as far as possible into the cervix; penetration of the cervix is typically 0 to 2 cm.

The conception rate is highly correlated with the depth of penetration (Table 43.8) and hence the technical proficiency of the inseminator. Conception rates achieved with the use of unfrozen semen by this method are adequate after oestrus synchronisation (Chemineau et al. 1991). The ideal time for insemination is 55 ± 1 hours after removal of intravaginal progesterone inserts or 15 to 17 hours after the onset of detected oestrus. However, even when substantial numbers of cryopreserved spermatozoa are used for intracervical insemination, conception rates are below those of natural service or fresh semen insemination (Colas 1979). Conception rates of 65% to 80% are typical when this method is used in the ewe's breeding season, with a lower figure for out-of-season breeding regimens. According to Kukovics et al. (2011), pregnancy rates of 80% to 90% were achieved on Hungarian sheep farms using fresh semen inseminated via this route.

Laparoscopic Intrauterine Route

The method of direct intrauterine, laparoscopic insemination (Fig. 43.8B) was developed to overcome many of the difficulties of intravaginal and intracervical insemination. In this method, ewes are sedated and restrained in a cradle. Laparoscopy is performed close to the udder after administering local anaesthetic. The abdomen is insufflated with CO_2, the uterus is located and semen is injected into the uterine lumen via a small stab wound (Killeen & Caffery 1982). The semen can be introduced to the uterus via a simple pipette (Evans & Maxwell 1987) or by the use of specialised insemination equipment (Chemineau et al. 1991). With oestrus-synchronised ewes, the ideal timing of insemination is between 48 and 65 hours after withdrawal of progesterone sponges, with conception rates declining after 72 hours postwithdrawal (Salamon & Maxwell 2000). Conception rates to frozen semen inseminated by this method are higher than for intracervical insemination because of better cryopreservation of spermatozoa and a site of insemination that avoids spermatozoa having to traverse the cervix. Conversely, the laparoscopy is technically demanding, and there are far greater implications for the welfare of the ewes than with intracervical insemination.

The development of laparoscopic intrauterine insemination of ewes is undoubtedly the most significant development in sheep AI in recent years, for it circumvents many of the problems of traditional methods (Haresign et al. 1986). The number of spermatozoa required for each insemination is lower and the volume of inseminate is proportionally greater, allowing more appropriate



• **Fig. 43.8** Insemination of the ewe: (A) intracervical route. (B) Laparoscopic intrauterine insemination.

TABLE 43.8 Lambing rates (%) in relation to the depth of intracervical insemination

No. Spermatozoa Per Inseminate ($\times 10^6$)	Site of Insemination: On the Entrance Fold of the Cervix	Up to 1 cm into the Cervix	Deeper than 1 cm into the Cervix
400 (undiluted)	50	68.8	71.4
100 (diluted)	43.8	66.7	71.4
50 (diluted)	25.7	60.7	66.7

From: Evans & Maxwell 1987.

dilution rates and therefore better preservation of sperm. Hence conception rates are similar to those achieved by natural service, and embryonic mortality is reduced to an acceptable level. The method also allows for the possibility of genuine progeny testing of rams, as semen from an individual sire can be used in many flocks over prolonged periods of time. Consequently, laparoscopic AI has been adopted in most of the major sheep producing countries (Haresign 1992). In Israel, a pregnancy rate of 71.4% was achieved with this method (Alfaris et al. 2012).

Transcervical Route

Transcervical insemination is a new method that attempts to achieve an intrauterine insemination via the cervical route. In this method the cervix is fixed by being grasped with forceps, and an insemination needle is introduced as far as possible through its lumen. Although better conception rates can be achieved by this method than by conventional intracervical insemination (Souza et al. 1994), both the retraction of the cervix and the penetration of the cervical lumen are associated with significant levels of trauma (Usboko 1995, Campbell et al. 1996). It is probable that, with the development of expertise in the method, its use will become more widespread, but at present, it seems to have little to commend it over laparoscopic insemination. In fact, pregnancy rates may be lower than those achieved with other methods even when no cervical trauma is evident (Wulster-Radcliffe et al. 2004).

Conception Rates to AI

Conception rates depend on the route and timing of insemination, whether ewes are being bred at synchronised or spontaneous oestrus, and whether fresh or frozen semen is used (Table 43.9). Equally importantly, it also depends on the management and condition of the ewes and the care with which the AI programme is managed. Results may also be difficult to interpret due to confusion in the terminology used, e.g., whether transcervical is really intracervical or not.

Control of Infectious Diseases

Diseases of sheep and goats that are transmissible in semen and that are regulated by OIE are listed in Table 43.4. *Brucella melitensis* and *B. abortus* can both be transmitted in semen, and of the organisms responsible for ovine epididymitis, *B. ovis* can be transmitted in semen; the pleomorphic Gram-negative organisms are also excreted in semen. Other significant diseases of sheep and goats that are controlled in AI donor sires include maedi-visna, caprine arthritis encephalitis virus, sheep pox, and goat pox, although there is no clear evidence for their transmission through semen (Thibier & Guerin 2000). It does not appear likely that the prion responsible for scrapie can be transmitted through this route (Wrathal 1997).

TABLE 43.9 Typical lambing rates achieved via different routes of insemination using fresh or frozen semen

Site of Insemination	Semen	Number of Motile Sperm	Lambing Rate (%)
Intravaginal	Fresh	400 x 10^6	20–60
	Frozen		5–20
Intracervical	Fresh	200 x 10^6	40–80
Synchronised oestrus	Frozen		25–40
Spontaneous oestrus	Frozen		30–60
Laparoscopic intrauterine	Fresh	20 x 10^6	70–95
	Frozen		40–80
Transcervical	Fresh	200 x 10^6	40–80
	Frozen		30–70

Derived from: Shipley et al. 2007.

Artificial Insemination of Goats

Artificial insemination of goats is generally very similar to that of sheep; many of the factors affecting pregnancy rates in sheep are also relevant for goats (Abecia et al. 2016). However, it is much easier to achieve an intrauterine insemination via the cervix of the goat than that of the ewe. Second, although oestrus detection is more efficient in the presence of a buck (or after a synchronisation protocol), it is possible to detect it in the absence of a buck (Nuti 2007). Consequently, there has been a significant uptake of AI as a means of breeding goats in both the commercial and lifestyle sectors. In France, for example, the numbers of goat inseminations increased fourfold during the 1980s (Chemineau et al. 1991). On the other hand, there are difficulties in preserving buck semen, whether cooled or cryopreserved, because of toxic interactions between seminal plasma and milk/egg yolk-based extenders.

Collection and Storage of Semen

Semen is generally collected using an artificial vagina (AV). It can be collected by electroejaculation, but this is less well tolerated than in the ram. Because electroejaculation affects the composition of seminal plasma, it can reduce the ability of spermatozoa to survive preservation (Leboeuf et al. 2000). Bucks are seasonal breeders with a decrease in the quality and quantity of semen produced in the spring and summer. This decline can be mitigated if bucks continue to copulate regularly throughout the year (Corteel 1981).

Effects of Seminal Plasma upon Storage

The major problem with the preservation of goat semen is that there are components in seminal plasma that impair the viability of spermatozoa stored in media containing milk and egg yolk. The toxic interaction with egg yolk is due to an egg yolk–coagulating enzyme (EYCE) that is secreted by the bulbourethral gland. This enzyme coagulates egg yolk and hydrolyses lecithin to fatty acids and spermicidal lysolecithins (Iritani & Nishikawa 1964). It is considered that the primary activity of EYCE is via a phospholipase A, but it may also contain lipase (Leboeuf et al. 2000). Bulbourethral gland secretions also have a toxic interaction with milk. A 55 to 60 kDa glycoprotein lipase, originally named SBUIII (Nunes et al. 1982), now called BUSgp60 (Pellicer-Rubio et al. 1997), which is structurally related to pancreatic lipase PLRP2, releases oleic acid from milk triglyceride (Pellicer-Rubio & Combarnous 1998). Skimmed milk can therefore be used as a diluent, although its effectiveness depends on the extent to which it contains any residual fat.

As a consequence, extenders for goat semen have either been based on skimmed milk, in which spermatozoa survive adequately, or the seminal plasma has been removed before using egg yolk–based diluents (Corteel 1974). When yolk-based diluents are used, washing the spermatozoa is achieved by dilution in Krebs-Ringer phosphate solution and centrifugation (Corteel & Paquignon 1984). Spermatozoa survive for 12 to 24 h after semen collection.

Liquid Semen

Two main strategies have developed for the use of liquid semen in goat AI.

Cooled Semen in Simple Diluents

- Removal of seminal plasma by washing in a simple buffer, followed by dilution in egg yolk–citrate or egg yolk–tris–fructose diluent and use at 5°C (Haibel 1986, Evans & Maxwell 1987, Chemineau et al. 1991).
- Because removal of seminal plasma is time-consuming and leads to loss of total numbers of spermatozoa, skimmed milk extenders can be used as an alternative for storage at 5°C. Such extenders are widely used (Leboeuf et al. 2003), although the effective lifespan of spermatozoa in skimmed milk extenders is relatively short (around 12 hours).
- Low levels of inclusion of egg yolk (~2.5%) do not result in the toxic effects of high inclusion rates; glucose citrate or Tris fructose plus a low concentration of egg yolk have also been used effectively (Shamsuddin et al. 2000, Bispo et al. 2012, Jiménez-Rabadán et al. 2012).
- Extenders that do not contain material of animal origin, e.g., soy lecithin (Roof et al. 2012), have been found to be as effective, if not more so, than egg yolk extenders.

Ambient Temperature Diluents

- If spermatozoa are not cooled below 15°C, egg yolk is not needed to prevent cold shock (Leboeuf et al. 2000). Skimmed milk and the commercial, soya–lipid-based diluent Biociphos Plus (IMV, L'Aigle, France) can maintain the viability of spermatozoa at 15° to 22°C for approximately 24 hours (Paulenz et al. 2005).
- Defined diluents, based on the antioxidant TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl) (Mara et al. 2007) or phosphocaseinate (Leboeuf et al. 2003), have also proved effective.

Cryopreservation

For cryopreservation, egg yolk–based extenders are more widely used than skimmed milk–based ones, although acceptable results can be achieved with skimmed milk (Corteel 1974). The most widely used extenders (Leboeuf et al. 2000) are skimmed milk–glucose (Corteel 1974) and Tris–glucose–citric acid–egg yolk (Salamon & Ritar 1982), although soy lecithin extenders are gaining in popularity (Roof et al. 2012). When the final concentration of egg yolk is kept below 2.0% of the diluent (Ritar & Salamon 1982), egg yolk–based extenders can be used without removal of seminal plasma. Glycerol is the most effective cryoprotectant and can be added in one or two steps. For washed spermatozoa, Chemineau et al. (1991) recommend that primary dilution should be in glycerol-free, egg yolk–citrate diluent, followed by addition of extender containing 14% glycerol once the semen has reached 4°C. However, when unwashed spermatozoa are used, semen can be glycerolised in a single step at 30°C (Ritar & Salamon 1982, 1983).

For intracervical insemination, there are similar problems of numbers of spermatozoa in relation to the volume of inseminate to those that apply in the sheep. Hence, Salamon (Evans & Maxwell 1987) also suggested variable composition extenders (which also contain a final concentration of egg yolk of <2.0%) for one step dilution of goat semen for use in intracervical insemination at dilution rates of between 1 : 1 and 1 : 4.

Semen can be frozen either by the pellet method (i.e., on the surface of solid carbon dioxide) or in paillettes that are suspended over the vapour of liquid nitrogen or used in programmable freezers. Post-thaw recovery rates are better with pellet freezing than with other methods (Purdy 2006), but because acceptable recovery rates are also achieved with paillettes, the convenience of handling semen means that it is usually the preferred method of cryopreservation. Although much variation exists between individual animals, overall results with doses of 50×10^6 motile frozen–thawed spermatozoa inseminated into the uterus are comparable with those of natural service (Ritar & Salamon 1983). The relationship between numbers of spermatozoa and fertility is not known because few data from large scale surveys on the subject exist. Nevertheless, Haibel (1986) suggested that 100 to 125×10^6 spermatozoa are used per insemination, whereas Evans and Maxwell (1987) recommended similar numbers to those used in sheep. Semen is thawed using similar protocols to those for the ram.

Insemination

Similar routes of insemination can be used in the doe to those employed in the ewe. Intravaginal insemination is effective if fresh semen is directly inseminated (Nuti 2007) but gives poor results with extended or frozen semen. Hence the main means of insemination are intracervical insemination or laparoscopic intrauterine. Intracervical insemination is the most widely practised method, especially in 'lifestyle' herds. In a significant number of does, an intrauterine insemination can be achieved via the intracervical route (50%–60%; Cseh et al. 2012) because the caprine cervix is relatively easier to traverse than the ovine cervix. Interestingly, although intrauterine insemination can be achieved simply by direct pressure of the insemination catheter upon the cervix, many technicians prefer to deposit at least some of the semen into the cervical canal in case the intrauterine insemination had been entirely into a single uterine horn (Haibel 1986). Nuti (2007), however, suggested that the rates of insemination failure after intracervical insemination are relatively high, either because of failure to enter the cervix properly or because of damage to the cervix or uterus during the insemination process. Depth of insemination is related to pregnancy rate: intrauterine insemination results in the highest rate, with the chances of conception declining significantly as the site of deposition becomes further caudal in the cervix (Salvador et al. 2005). Operator proficiency is therefore a critical determinant of pregnancy success. Pregnancy rates as high as 80% are reported for fresh semen.

Laparoscopic intrauterine insemination is performed in the same way as in the ewe. It has the advantages of requiring a lower dose of spermatozoa (~20×10^6) and achieving a higher pregnancy rate than intracervical insemination but is more demanding to perform (Cseh et al. 2012). Sohnrey and Holtz (2005) suggested that deep corneal transcervical insemination produces results that are at least as good as that achieved by laparoscopy.

Artificial Insemination of Pigs

One of the main stimuli to the use of AI in pig breeding is the possibility it confers of maintaining a closed herd by obviating the necessity of introducing purchased boars. Furthermore, because the traits of economic importance in the pig (i.e., carcase conformation, growth rate, and feed conversion efficiency) have high heritability and can be evaluated in the sires themselves, genetic selection can be intense so that the potential value of AI boars in enhancing the genetic base of a breeding herd is considerable.

Traditionally, the use of AI in pig breeding has been greatest in Eastern Europe and in countries such as Holland and Denmark with very high densities of pigs. However, there has been a marked increase in the use of pig AI in many other countries, in parallel with the improvements in AI technology and the demand for high genetic–merit sires (Johnson et al. 2000). In the UK, only about 9% of the national herd was bred by AI in the survey of Iritani (1980), whereas the figure in 2004 was 60% to 70% (Goss 2004). The United States has seen a similar growth of AI in pigs over the same period (Johnson et al. 2000). In Europe, more than 90% of sows have been bred by AI in the last two decades (Maes et al. 2011). Most inseminations in Europe are performed by farm staff as the costs of a technician service are too great to be economically viable, although technician services are available in some countries.

Two main difficulties exist in pig AI. First, the period of maximum fertility is not particularly easy to detect in sows (Evans & McKenna 1986) so that animals have traditionally had to be inseminated two (or even three) times during oestrus to maximise conception rate and litter size. Second, pregnancy rates and litter size are reported to be lower after AI with frozen semen than with fresh semen (Alm et al. 2006). Hence, insemination of sows has been based upon multiple inseminations of semen that has been extended and stored at 16° to 18°C containing large numbers of spermatozoa (~2.3×10^9). This has meant that each ejaculate produces enough doses to inseminate only 15 to 25 sows (Maes et al. 2011), a smaller number of doses per ejaculate than in other species such as bulls. Reducing the insemination dose to 1.8×10^9 causes a decrease in litter size of 0.07 piglets (Feitsma 2009), which has a marked effect on the economics of pig breeding.

Collection and Storage of Semen

Semen is manually collected from boars in the presence of a dummy sow (see Chapter 35). The frequency of collection depends on the semen characteristics of individual boars, but it is usually twice per week (Vyt et al. 2007). The pre-ejaculatory fluid is discarded, and the postsperm-rich fraction may not be included either, depending on individual practice or whether a sealed collection device is used. Between 100 and 150 mL of sperm-rich fraction is produced, which is filtered to remove gel, and is extended for insemination after evaluation for spermatozoa concentration, motility, and possibly spermatozoa morphology. Previously, when using the 'gloved hand' technique, it was possible to move the collecting vessel into position once it was apparent that the clear spermatozoa-free prostatic portion was changing to the coloured, sperm-rich fraction of the ejaculate, but the availability of sealed collection containers and automatic collection devices means that the whole ejaculate is collected instead. The sealed collection devices are believed to reduce the number of bacteria that contaminate the ejaculate during semen collection.

Liquid Semen

Approximately 99% of pig inseminations are undertaken using liquid semen (Johnson et al. 2000), and because pig spermatozoa are highly sensitive to cold shock, nearly all of these use ambient temperature extenders. When semen is to be stored at ambient temperatures, the activity of spermatozoa has to be inhibited to ensure an adequate period of viability.

Extenders designed to be used at ambient temperature were based upon the IVT extender throughout the 1960s and 1970s (Paquignon 1984). These have more recently been superseded by diluents such as the Guelph (= Kiev) diluent, BTS (Beltsville Thawing Solution) diluent, and Zorlesco diluent (Haeger & Mackle 1971, Pursel & Johnson 1975, Gottardi et al. 1980). The Guelph diluent is simpler than the IVT diluent to prepare but maintains the fertility of semen for a similar length of time. The more complicated Zorlesco diluent may maintain semen fertility for up to 12 days, although field trials indicated that the effective period is probably shorter than that. Newer diluents, such as the Zorpva (Cheng 1988), Reading (Revell & Glossop 1989), and Androhep (Weitze 1990) diluents, have reliably maintained the fertility of spermatozoa over longer periods of up to 5 days and are, consequently, in increasingly widespread use (Almond et al. 1998), although the most widely used diluent in the United States is the BTS (Johnson et al. 2000). Addition of antioxidants, buffering agents, and antibiotics extend the shelf life of the semen doses (Knox et al. 2016). Examples of short- and long-term diluents are given in Table 43.10.

Insemination doses are variously recommended to contain a minimum of 1×10^9 sperm, with increased numbers when semen is not used on the day of collection. Althouse (2007) suggests that, as a rule of thumb, spermatozoa numbers are increased by 1×10^9 for every day of delay between collection and insemination; hence a common dose rate for commercial boar AI services is 2.3×10^9 spermatozoa (Maes et al. 2011). If semen is collected in situations in which spermatozoa concentration cannot be determined accurately or if it is for local use within a breeding establishment, conservative dilution at 1:4-1:7 is recommended (Althouse 2007) to result in 2 to 4.5×10^9 spermatozoa per dose (Almond et al. 1998). The volume of material that is inseminated is important, because low volume insemination fails to achieve acceptable conception rates. Hence, a total insemination volume of 70 to 100 mL (typically 80 mL) is used. The necessity for using such a large volume is probably to stimulate uterine motility in the sow, thereby ensuring that adequate numbers of spermatozoa reach the site of fertilisation. Semen is typically packaged into plastic tubes, bags, flatpacks, or bottles and is maintained above 15°C before insemination.

For AI in commercial piggeries, it is common practice to mix the semen of different boars in insemination doses. This reduces the risk associated with the responses of an individual boar's semen to extension and dilution, making the outcomes of insemination more predictable (Godet et al. 1996). However, it does make the tracking of an individual boar's performance for the elimination of poor performers more difficult.

Cryopreservation

The semen of boars does not generally respond well to cryopreservation. Recovery after thawing is poor and highly variable between individual sires, and fertility of semen is invariably substantially lower than that of extended semen. Typical results for frozen semen are, at best, 70% of sows conceiving to a double insemination, compared with 80% to 90% for liquid semen insemination. Litter size is also lower after frozen than after liquid semen insemination (Roca et al. 2006b). Consequently, only approximately 1% of pig inseminations use frozen semen, which is largely limited to semen that has been traded internationally.

There are two major limitations to success in freezing boar semen. First, it is highly sensitive to cold shock, and neither egg yolk nor skimmed milk provides anything like the degree of protection against cold shock that it provides to the spermatozoa of most other species (Watson 1979). Second, although glycerol is probably the best cryoprotectant for the boar spermatozoa, its toxic effects are more pronounced in this species than in most others (Wilmut & Polge 1974). In consequence, the numbers of spermatozoa that are

TABLE 43.10 Diluents for ambient temperature preservation of boar semen

	Short Term (Up to 3 Days)		Long Term (3–5 Days)		
Constituent (g/L)	Guelph (Kiev)	BTS	Zorlesco	Zorpva	Androhep
D glucose	60.0	37.0	11.5	11.5	26.0
Disodium EDTA	3.7	1.25	2.3	2.35	2.4
Sodium hydrogen carbonate	1.2	1.25	1.25	1.75	1.2
Trisodium citrate	3.7	6.0	11.7	11.65	8.0
Citric acid			4.1	4.1	
Tris			5.5	5.5	
Hepes					9.0
Polyvinyl alcohol (type II)				1.0	
Bovine serum albumin					2.5
Cysteine			0.1	0.07	
Antibiotics	Lincomycin, spectinomycin, gentamicin, neomycin, and/or benzyl penicillin plus streptomycin are commonly added to suppress mycoplasmas and *Leptospira* spp.				

Derived from: Haeger & Mackle 1971, Evans & McKenna 1986, Cheng 1988, Weitze 1990, Almond et al. 1998, Johnson et al. 2000.

required to obtain even the mediocre conception rates that follow the use of cryopreserved semen are very high indeed: typically 5 to 6×10^9 spermatozoa per insemination dose (Paquignon 1984). Hence, when frozen semen is used, only about five animals can be inseminated per ejaculate. This low extension rate effectively limits the use of frozen semen to international traffic in pig genetics and to the preservation of gene stocks for future use.

A great deal of work has been undertaken to improve the results of cryopreservation of porcine semen, which, despite the unpromising appearance of early studies, has resulted in better and more consistent survival of freezing. Progress has been made in a number of areas (reviewed by Johnson et al. 2000, Roca et al. 2006b).

Improvement in Extenders

The protective effect of egg yolk is greatly improved when detergent (OrvusEs Paste (OEP)) is added to the extender, probably as it breaks down the fat globules and allows better interaction of the egg phospholipids with the membranes.

Addition of antioxidants, such as vitamin E, glutathione, or superoxide dismutase, prevents lipid peroxidation during freezing and thawing. Addition of resveratrol from grapes or epigallocatechin-3-gallate from green tea to the extender had a beneficial effect on post-thaw penetration rates in IVF, even though no effect on spermatozoa viability or acrosome integrity was observed (Gadani et al. 2017). It has been suggested that adding seminal plasma to thawed boar spermatozoa may counteract lipid disorder arising during the cryopreservation process (Torres et al. 2016).

Improvement in Control of Freezing

Temperature control is a critical aspect of the success with which the spermatozoa of any species can be frozen. The large volume straws (5–6 mL) in which pig semen has traditionally been frozen do not provide good control of temperature, either during freezing or during thawing, especially in the centre of the straw. Much better responses are achieved when semen is packaged into 0.5 mL straws in aliquots of approximately 2×10^9 sperm, then diluted after thawing to an appropriate volume for insemination. Alternative 'flat pack' systems of freezing have also been developed, which package up to 5×10^9 spermatozoa in 5 mL of diluent but ensure even cooling throughout the pack. Saravia et al. (2010) reported good cryosurvival in a simplified procedure, freezing spermatozoa from only the first 10 mL of the sperm-rich fraction in 'mini flat-packs', with a considerable shortening of the procedure. As the authors indicate, the remainder of the ejaculate could be used for processing conventional semen doses (Saravia et al. 2010).

Selection of Sires

Boars can be selected for response to the freezing of their semen; selection for 'good' freezers results in more consistent quality of frozen–thawed semen and fewer ejaculates that fail to survive the freezing process. Roca et al. (2006a) even suggested that boar semen should be test frozen for identification of 'good' and 'bad' freezers. In this context, it is interesting to note that, in the early days of bovine AI, a similar selection process was practised, with an effective elimination of 'bad' freezers (at least of dairy breeds) from studs.

Management of Insemination

Spermatozoa that survive freezing have undergone significant capacitation-like changes that reduce the duration of their survival in the female tract. Re-examination of the insemination process with regard to the site of deposition of semen and the timing of insemination (see later in this chapter) is allowing significant improvements in pregnancy rates and litter sizes to cryopreserved semen.

Current Practice

Saravia et al. (2005) summarise the processing of semen for freezing, based on methods described by Eriksson and Rodriguez-Martinez (2000a, b). After holding at room temperature for approximately 1 hour, the semen is extended 1 : 1 in BTS. The extended semen is

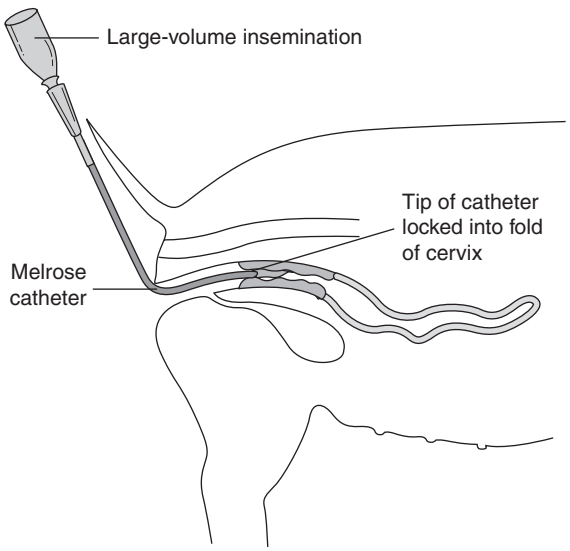
held at 16°C for 3 hours, after which it is centrifuged at 800 g for 10 minutes. After centrifugation, the supernatant is discarded, and the pellets are resuspended in a lactose–egg yolk diluent. The semen is then cooled to 5°C over 2 hours in the centrifuge. Thereafter, semen is mixed with a further lactose–egg yolk extender, which also contains OEP and glycerol (to give a final concentration of 3% glycerol).

After packaging into appropriate containers, the semen is frozen in static liquid nitrogen vapour or in a programmable freezer. The optimal cooling rate over the range 0° to 50°C is generally accepted as being −30°C/min (Johnson et al. 2000). This cooling rate has to be matched by a very rapid thawing rate.

Insemination

The optimum time for AI is during a 24-hour period in the middle of the 50 to 60 hours of oestrus, so that capacitated spermatozoa are present at the time of ovulation. The highest fertility results are achieved when insemination is undertaken 10 to 12 hours before ovulation (Evans & McKenna 1986). However, the interval between the onset of oestrus and ovulation is variable, and even twice daily oestrus detection may not accurately pinpoint the onset of oestrous behaviour. Hence it is recommended that optimal conception rates and litter sizes are achieved by delaying insemination until 12 to 18 hours after the first detection of oestrus, with a second insemination 12 hours (Evans & McKenna 1986) or 18 to 24 hours (Almond et al. 1998) later. The most widely used means of inseminating sows is the method developed by Melrose and O'Hagen (1959) and Rowson (1962), which involves passing a spiral rubber catheter into the cervix of the sow (Fig. 43.9). The catheter is rotated into the cervix until its spiral groove becomes locked into the cervical canal. The cervical lock prevents the catheter from becoming dislodged if the sow moves during insemination and prevents loss of the large volume of fluid that has to be inseminated.

However, there are very substantial losses of spermatozoa after insemination (reviewed by Roca et al. 2006a). Some 30% to 40% of the semen flows backwards out of the cervix, and of the spermatozoa that remain, many become trapped in the cervix or are eliminated by the immune system of the uterus. Hence, 90% of the spermatozoa are eliminated within 2 to 3 hours of insemination, and of the approximately 2.3×10^9 spermatozoa that are inseminated, only 1×10^5 become established at the uterotubal junction. This loss of spermatozoa is exacerbated when they are functionally impaired (e.g., when they have suffered damage through cryopreservation), with numbers at the uterotubal junction being tenfold lower than after insemination with liquid semen.



• **Fig. 43.9** Insemination of the sow, using a Melrose-type catheter.

In order to make the process of pig AI more efficient, two new approaches are being developed: more precise synchronisation of the timing of insemination with that of ovulations and deep intrauterine insemination.

Fertility is maximal when insemination is performed up to 12 to 18 hours before, or just after, ovulation (Nissen et al. 1997). Hence, if the timing of ovulation can be identified more accurately using signs other than those of behavioural oestrus, acceptable fertility might be achieved with a single insemination. Ultrasonography has been used, but scanning has to be undertaken frequently to give a useful prediction of the timing of ovulation (Bortolozzo et al. 2005, Sumransap et al. 2007). Hence ultrasound-based prediction of ovulation has yet to be refined to a point at which it is useful in commercial practice (Soede et al. 1998, Serret et al. 2005). Controlling the time of ovulation with gonadotrophins (GnRH, hCG, eCG) (de Rensis et al. 2003, Baer & Bilkei 2004, Kauffold et al. 2007), whether applied after the onset of observed oestrus or given at a fixed point after weaning, has been successfully used to allow adequate fertility to single inseminations. However, when single inseminations are used, the timing in relation to ovulation is critical (Garcia et al. 2007).

Intrauterine insemination has been performed in the caudal part of the uterus (postcervical insemination: PCI or deep within the uterine horns (deep uterine insemination; DUI)). Both are achieved by passing an insemination device through a conventional AI catheter; the device for PCI is rigid; that for DUI is longer, finer, and more flexible. PCI insemination requires more than 1×10^9 spermatozoa to obtain an acceptable litter size, although pregnancy rates are good even with lower numbers of sperm. In insemination trials with 1.5×10^9 spermatozoa in two doses, a farrowing rate of 84% with a litter size of 13.9 piglets was achieved (Hernández-Caravaca et al. 2012). However, success rates vary considerably with small numbers of spermatozoa (i.e., $0.5–1 \times 10^9$; Knox 2016). DUI with 600×10^6 spermatozoa is expected to give similar results in terms of pregnancy rate and litter size to conventional intracervical insemination with $2–3 \times 10^9$ spermatozoa (Roca et al. 2006b). Over 80% pregnancy rate has been achieved with 1×10^9 thawed spermatozoa deposited by DUI (Bathgate et al. 2008). Laparoscopic methods have been developed in which small numbers of spermatozoa are deposited into the ampullary part of the uterine tube very close to the time of ovulation (Vazquez et al. 2006). This has been used experimentally for sexed-semen inseminations, with good results (Roca et al. 2006b). However, the methods do not yet appear to be feasible for commercial use because they require general anaesthesia of the sow.

Insemination by Farm Staff

A semen delivery service was introduced into the UK in the mid-1960s (Melrose et al. 1968), and since then it has undergone steady expansion. Processed semen is dispatched from the processing centre to farms, where the farm staff, using conventional intracervical insemination catheters, inseminates the sows. This system allows double inseminations to be performed, and the risk of disease transmission is minimal. It requires some technical proficiency on the part of the farm staff in terms of oestrus detection as well as in the insemination procedure itself.

The use of AI with delivered semen has also received considerable impetus from increased use of batch management of pig herds (see also Chapter 32). Thus, large groups of sows are weaned simultaneously in the knowledge that most of them will be in oestrus at a predictable interval after weaning. Standing or advance orders are placed for the semen to inseminate these sows, with small numbers of boars kept to mate the small proportion of sows that return to oestrus outside the predicted time period and to detect and mate animals that fail to conceive.

On-farm collection and insemination programmes can be used when large numbers of sows have to be inseminated as cheaply as possible. In this situation, inseminations are performed with raw or diluted semen immediately after collection. In a number of countries, AI centres have provided producers with semen extender for on-farm insemination programmes. This method minimises the risk of disease transmission but limits each farm to using the boars it has available. Access to performance/progeny-tested sires is therefore limited.

Inseminator Service

Technicians employed by AI centres transport the semen from the centre to the farm, where they inseminate the sows. This was the main form of insemination service in Western Europe when pig AI first started, but it has gradually lost favour, except in regions of exceptionally high pig density. Inseminator services generally provide only a single insemination of the sow in a given oestrus period, resulting in significantly lower conception rates and lower numbers of piglets per litter than are achieved by natural service (Reed 1982). Also, the cost of a technician service is high in relation to the value of the progeny, especially as litter sizes are lower than with natural service. Furthermore, itinerant inseminators are potential vectors for disease transmission between farms. Therefore the trends towards closed herds and 'minimal disease' precautions on most pig farms have mitigated against the use of such technicians.

Control of Infectious Diseases

A large number of pathogens can be transmitted through boar semen. The diseases that are specifically controlled by OIE are listed in Table 43.4.

Bacterial pathogens include *Brucella suis, Staphylococcus aureus, Streptococcus* spp., and *Mycoplasma* spp., which are transmitted by the venereal route and have significant effects upon sow fertility. *Leptospira* serovars and *Erysipelothrix rhusiopathiae,* although not directly spread through semen, are spread as urinary contaminants, so contamination of the semen through contact with urine or the presence of the organisms within the prepuce is likely (Cutler 1986).

More seriously, classical swine fever (hog cholera), African swine fever, and Aujeszky's disease can also be spread through semen, as can foot-and-mouth virus (Cutler 1986, Mengeling 1986, Almond et al. 1998). Porcine reproductive and respiratory syndrome (PPRS) virus can be shed in the semen and can be transmitted by insemination, although it is not specifically controlled by OIE (Yaeger et al. 1993). Likewise, parvovirus and Japanese encephalitis can also potentially be transmitted by semen (Thibier & Guerin 2000).

Routine serological testing of boars is undertaken by AI centres to ensure that they do not carry either venereally transmitted diseases or other conditions that might jeopardise the health status of the recipient herd. Antibiotics are added to semen to remove nonspecific and pathogenical bacteria. However, the most valuable means of controlling disease transmission is to quarantine incoming boars for a month, during which time observations for signs of clinical disease and/or further serological testing are undertaken.

Artificial Insemination of Horses

Artificial insemination of horses has been practised for many years. Initially, most of the breeding by AI took place in Eastern Europe, the former USSR (Tischner 1992), and China. Conversely, development of AI in horses in western countries was hampered for a long time by the refusal of key registration authorities to allow the registration of foals bred by AI. However, the recent acceptance of such registrations by the American Quarter Horse and American Paint Horse associations (Loomis 2001) has provided a significant impetus to the development of equine AI. Thus, although the Thoroughbred Breeders Association remains unwilling to allow registration of foals conceived by AI, most other breed societies allow restricted or unrestricted registrations of AI-bred foals. In most parts of Europe, up to 90% of Warmblood mares are now bred by AI.

There are a number of technical difficulties that have to be overcome in the development of AI programmes for horses:

- Semen collection: Coitus in horses is relatively protracted, which makes semen collection rather more difficult than in other large herbivores. In some countries, most of the seminal plasma is removed by centrifugation to enhance spermatozoa survival (Brinsko & Varner 1993).
- Storage of semen: Stallion semen is relatively susceptible to damage due to cold shock and cryopreservation (Amann 1984).
- Insemination: Synchronising insemination with ovulation has to be accurate. However, because of the variability and duration of oestrus in the mare, predicting the time of ovulation is difficult without regular and frequent examination. A commonly used practice is to inseminate every 48 hours once a dominant follicle has been detected until ovulation occurs. On the other hand, insemination of the mare is much easier than insemination of the cow because the equine cervix is soft and does not have a convoluted luminal canal.

Collection and Storage of Semen

Semen is normally collected by AV (see Chapter 35). Some people prefer to use an open-ended AV to allow collection of the separate fractions of the ejaculate. Alternatively, the gel fraction can be separated by use of a nylon mesh filter placed in the AV or semen bottle at the time of collection or by filtration through gauze after collection. The latter is associated with a greater loss of spermatozoa than other methods (Blanchard et al. 2003). In commercial AI programmes, it may be advantageous to train stallions to mount a 'breeding phantom' dummy-mounting device, in preference to the use of mares. Ground collection is also possible; this method is thought to place less strain on the back and hind legs than mounting a phantom.

Liquid Semen

After collection, equine semen requires careful temperature control to prevent damage to the spermatozoa by cold shock (Brinsko & Varner 1993). If the mares that are to be inseminated are close by, insemination can be performed directly using raw semen, but it is generally preferable to place the semen in an extender even if it is for direct insemination straight away. Simple extenders (Table 43.11), such as skimmed milk plus antibiotics used at a ratio of

TABLE 43.11 Diluents for short-term storage of equine semen at 4°C

Component (g/L)	Skimmed Milk Diluent (Kenney)	Modified Kenney Extender	Egg Yolk–Glucose
Fat-free dried milk powder	24	24	
Glucose	49	26.5	70
Sucrose		40	
Sodium hydrogen carbonate	1.5		
Egg yolk			70 mL
Antibiotics	Penicillin G, streptomycin, gentamicin, polymyxin B, and/or amikacin are commonly added		

Derived from: Kenney et al. 1975, Tischner 1992, Brinsko & Varner 1993, Blanchard et al. 2003.

1 : 1 or 1 : 2, with semen maintained at ambient temperatures are adequate if the semen is to be used within 6 hours of collection (Samper 2007).

Equine semen can also be stored for longer periods of time before insemination if it is cooled. Most studies (see Batellier et al. 2001) have concluded that storage at 4° to 6°C is the ideal temperature (although there are some reports of better results at 15° or even 20°C). Extenders for cooled storage are mostly based upon milk, using either skimmed milk, cream-gel ('half-and-half' cream partially set with gelatin), or fat-free dried milk powder. Kenney's diluent (Kenney et al. 1975) and its very close derivative (Francl et al. 1987) EZ Mixin (Animal Reproduction Systems, Chino, CA) are probably the most widely used diluents in the United States because of their simplicity of use and the ease with which spermatozoa can be visualised after dilution (Blanchard et al. 2003) (Table 43.11).

Other extenders that are used for cooled storage of equine semen are based on milk plus egg yolk or are chemically defined (e.g., INRA-96; Batellier et al. 1998). Extenders based on egg yolk appear to provide best protection when seminal plasma is removed (Jasko et al. 1992), whereas milk-based diluents support cooled storage less well when seminal plasma is completely removed, the effect of which can be overcome if the extender is supplemented with salts such as Tyrode's (Rigby et al. 2001). However, milk-based diluents that are supplemented with Tyrode's salts require the removal of seminal plasma for maximal survival of spermatozoa (Katila et al. 2005). The defined diluent INRA-96, which is based on Hank's salts supplemented with 67 mmol/L glucose, 126 mmol/L lactose, and 27 g/L native phosphocaseinate (Batellier et al. 1998, Pagl et al. 2006), gives pregnancy rates that are better than simple skimmed milk diluents, especially for stallions whose semen does not respond well to simple extenders (Batellier et al. 2001). Webb and Humes (2006) showed that further improvements to the performance of semen in INRA-96 can be obtained by removal of the seminal plasma by centrifugation. However, although motility of spermatozoa may be improved by removal of most of the seminal plasma, there may be an increase in chromatin damage (Morrell et al. 2010). Masuda et al. (2004) suggested that an extender based on 2% milk casein plus 5% egg yolk also gave good results.

With these diluents, the rate of cooling from body temperature to 19°C is not critical, but between 19° and 8°C, slow cooling (ideally 0.1°–0.3°/min; no more than 0.5°C/minute) is necessary to avoid causing cold shock (Douglas-Hamilton et al. 1984, Katila 1997). Below 8°C, rate of cooling is not critical. Controlling the rate of cooling semen in the field has been greatly facilitated by cooled storage units such as the Equitainer (Hamilton Research, Inc., South Hamilton, MA). In this system, diluted semen is placed in an insulated container with prechilled cooling packs that reduce the temperature in the semen at an appropriate rate to prevent cold shock developing. Such devices can maintain semen fertility at acceptable levels for approximately 48 hours. Air should be excluded from storage vessels as the survival of spermatozoa is significantly greater when no air is present (Katila 1997, Batellier et al. 2001) because of the effects of aerobic respiration and lipid peroxidation. Addition of antioxidants, such as ascorbic acid, pyruvate, and, most recently, N-acetyl cysteine (Pagl et al. 2006), is therefore advocated as a means of increasing the longevity of spermatozoa in cooled storage.

Cryopreservation

Stallion semen has been successfully cryopreserved (Pickett & Amann 1993) using low concentrations of glycerol in diluents containing sugars, egg yolk, and/or skimmed milk. Before freezing, most of the seminal plasma is removed by centrifugation, usually after temporary extension of the semen in a diluent that will maintain its viability at ambient temperatures. Various diluents have been used for this step, including UHT skimmed milk, Kenney's diluent, lactose-EDTA, INRA-82 (glucose, lactose, rafinose, citrate, Hepes, plus egg yolk (Vidament 2005)), and EDTA–citrate. To minimise damage to spermatozoa during the centrifugation process, semen may be 'cushioned', by layering it on to a high viscosity solution before centrifugation. High concentrations of glucose or dense, inert, isotonic substances (e.g., Cushion-Fluid, Minitube; Landshut, Germany) (Sieme et al. 2006) have been used as the basis for cushioning media for centrifugation. After discarding the supernatant, the semen is resuspended in the freezing extender. It is then slowly cooled from ambient temperatures to 4°C because slow cooling over this phase is preferable to rapid cooling.

Freezing extenders are typically based on sugars, buffers, and egg yolk; many are available commercially and many others have been described in the literature. Among these, Kenney's plus 4% clarified egg yolk, INRA-82 plus 20% egg yolk, or lactose–EDTA plus 20% egg yolk (Pickett & Amman 1993, Loomis 2006) have been widely used as freezing media. Subsequent studies have suggested that defined media containing egg- or soy-derived phosphatidylcholine (Ricker et al. 2006) maintain spermatozoal survival during freezing at least as well as extenders containing whole egg yolk. Glycerol is the best tolerated cryoprotectant and is typically used at a final concentration of 2.5%, although some diluents use concentrations of up to 4% (Cochran et al. 1984, Heitland et al. 1996). More recently, there has been a move towards substituting methyl formamide and other amides for some of the glycerol in cryoextenders for stallion semen (Alvarenga et al. 2005). This substitution seems to aid cryosurvival for some individuals that display poor cryosurvival when only glycerol is used.

The use of 0.5 mL paillettes has superseded older, larger volume containers for freezing equine semen: little semen is now frozen in other containers. The insemination dose is commonly loaded into four to eight straws (Samper 2007). Freezing may be over the vapour of liquid nitrogen or as pellets on the surface of blocks of solid carbon dioxide. When programmable freezers are used,

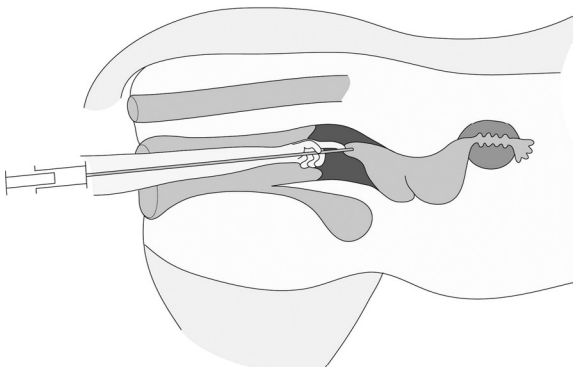
cooling rates of 40°C/min to −60°C then 60°C/min until −140°C is reached (thereafter plunged into liquid nitrogen) have given good results (Vidament 2005).

Thawing rates are more critical for cryopreserved equine sperm than for many other species. Loomis and Squires (2005) suggest that semen frozen in 0.5 mL straws should be thawed at 37°C for a minimum of 20 seconds. It is important that the temperature of the thawing water is accurately controlled because spermatozoa die rapidly if the temperature is raised to 39°C or 40°C. Higher thawing temperatures (e.g., 75°C for 7 seconds) give better post-thaw results, provided the straws are removed at the correct time; however, it is difficult to ensure that this occurs in field use. The contents of four to eight straws are pooled before insemination. When semen is supplied in 4 to 5 mL tubes, thawing should be at 50°C for 45 seconds.

Evaluating the fertility of cryopreserved equine semen is not a straightforward process. Post-thaw motility is a poor predictor of fertility in all species; it identifies the worst ejaculates but fails to discriminate between levels of fertility. In the stallion, it is particularly unreliable (Pickett & Amann 1993) given the minimal amount of reliable data on conception rates in mares. However, attempts have been made to find objective measurements that have high levels of correlation with fertility. Christensen et al. (1996) used *in vitro* induction of acrosome reactions as a predictor of fertility, whilst Samper (1992) and Hellander (1992) have used combinations of glass–wool/Sephadex filtration and motility estimates to predict fertility. Nevertheless, despite the progress that these methods represent, it remains difficult to predict the fertility of a stallion's semen after cryopreservation.

Insemination

The standard method of insemination with cooled or frozen semen is to deposit it into the caudal part of the uterine body via a catheter passed through the cervix (Fig. 43.10). Insemination is best performed with the mare restrained in stocks. After applying a tail bandage and cleaning the perineal area, a hand is inserted into the vagina and the cervix is located. The index finger is inserted into the cervix, and an insemination catheter is passed through the vagina, then alongside the index finger, and so into the uterus. A vaginal speculum can be used to aid locating the cervix. Semen is deposited slowly, in volumes ranging from 0.5 to 80 mL. Most inseminations use a low volume of fluid as there is little benefit from higher volumes upon uterine contractility, and there is a greater risk of retrograde flow from the cervix with larger volumes.



• **Fig. 43.10** Insemination of the mare.

Timing of Insemination

Insemination with cooled semen should take place somewhere between 48 hours before and approximately 6 hours after ovulation. Insemination later than 6 hours after ovulation may result in conception but is more likely to result in embryonic death. The optimal timing depends, however, upon the success with which the spermatozoa have survived the cooling process: the semen of some stallions needs to be inseminated within a few hours of ovulation if it is to achieve an acceptable result. Samper (2007) suggests that a fertile mare can be inseminated once or twice (12–24 hours apart) with good quality semen, but if the semen is of poorer quality or the fertility of the mare questionable, a single insemination as close as possible to the time of ovulation is preferable.

Insemination with frozen–thawed semen needs to be performed between 12 hours before and 6 hours after ovulation. Regular examination of the mare is required to ensure that this timing is achieved. In practice, most mares are inseminated once within 6 hours after ovulation or twice, once before and once after ovulation (Loomis & Squires 2005). Mares are often given human chorionic gonadotrophin (hCG) or a gonadotrophin-releasing hormone (GnRH) analogue to induce ovulation and thus control the timing of insemination more precisely. This has the added advantage of reducing the number of occasions on which the mare has to be examined. Samper (2001) suggested giving 2500 IU hCG once a follicle larger than 35 mm is present, then examining the mare's ovaries every 6 to 8 hours to pinpoint the time of ovulation, which will occur 36 ± 17 hours after treatment. The GnRH analogue deslorelin (Ovuplant) can be given as a short-term implant, resulting in a much closer regulation of the time of ovulation (Hemberg et al. 2006), with ovulation occurring at 38 ± 2 hours post-treatment, so that it is only necessary to examine mares at 36 and 41 hours post-implantation to determine the point of ovulation.

Spermatozoa Numbers and Conception Rates

Cooled Semen

Cooled semen inseminations are typically made with 250 to 500 × 10^9 progressively motile spermatozoa (Brinsko 2006), commonly doubled to 1 × 10^9 motile sperm when insemination is to be delayed by more than 24 hours. Conception rates of 73% to 75% have been recorded for this method. However, this number of spermatozoa almost certainly represents a wastage, at least in fertile stallions, as 100 × 10^6 motile spermatozoa have been used for insemination without any loss of fertility (Pace & Sullivan 1975, Pickett 1980), although lower numbers (e.g., 50 × 10^6) give more variable results (Householder et al. 1981). On the other hand, the use of higher numbers of spermatozoa is said to eliminate much of the variation between stallions.

There is, however, a great deal of variability in the quality of cooled semen that is supplied to horse breeders. Metcalf (1998) showed that semen rated as excellent or good on the basis of percentage of motile and morphologically normal sperm had higher (87% and 62%, respectively) pregnancy rates than semen rated as fair or poor (33% and 11%). Loomis (2001) reported a wide range of numbers of progressively motile sperm in cooled semen (mean: 598 × 10^6; SD: 604 × 10^6; range: 8–4257 × 10^6). Moreover, although controlled trials have shown that it is possible to reduce sperm numbers without loss of fertility, Brinsko (2006) showed evidence from use in the field that doses of less than 500 × 10^6 sperm are associated with much lower pregnancy rates (20%) than doses of more than 500 × 10^6 spermatozoa.

Frozen Semen

Frozen semen inseminations typically contain between 150 and 600×10^6 motile sperm. With most stallions having post-thaw recovery rates of 40% to 70% (Vidament 2005), this necessitates a total insemination dose of around 800×10^6 spermatozoa. The fertility of frozen semen in commercial programmes has been reported to range between 32% and 73% per cycle and 56% to 89% over the breeding season (Loomis 2001). Various combinations of spermatozoal numbers and insemination timings have been reported: Vidament (2005) suggested that best results are achieved with up to two daily inseminations with 300 or 400×10^6 spermatozoa from the time of hCG administration or a single postovulation insemination, figures with which Newcombe et al. (2005) concurred.

New Insemination Methods for Compromised Spermatozoa

Losses of spermatozoa in traversing the uterus from the site of insemination to the uterotubal junction are high, especially when the functional integrity of the spermatozoa has been compromised by freezing or by sex sorting. Deep intrauterine insemination has been investigated as a means of reducing the numbers of spermatozoa and improving the pregnancy rates. In this method, a semirigid or flexible insemination catheter is guided to the cranial part of the uterine horn, and 20 to 200×10^6 spermatozoa are deposited. The catheter can be guided per rectum or using ultrasonography (Sieme et al. 2003). Early attempts at this method resulted in high embryonic loss rates due to traumatic injury to the uterus by the catheter (Morris 2006a), although this problem now appears to have been resolved (Sanchez et al. 2005). Whether an improvement in conception rates is achieved over conventional methods remains unclear: neither Squires et al. (2003) nor Sanchez et al. (2005) saw improved pregnancy rates, although sperm numbers to achieve conceptions were successfully reduced. A reduction in the incidence of postbreeding endometritis may be obtained if fewer spermatozoa are inseminated (Samper & Plough 2010).

Direct insemination on to the uterotubal junction has been investigated as an alternative to deep intrauterine insemination. Hysteroscopic insemination at the uterotubal junction allows reduction of sperm numbers to 10 to 25×10^6 (Sieme et al. 2004, Ball 2006) or even as low as 1 to 5×10^6 (Morris et al. 2003, Sieme et al. 2003, Morris 2006b), which allows acceptable pregnancy rates to be achieved even with sex-sorted sperm (Lindsey et al. 2001). Finally, direct insemination into the uterine tube has also been successfully used for insemination of compromised sperm (Lindsey et al. 2001, Morris 2004). Although these techniques have significant benefits in terms of improved usage of spermatozoa, especially when only small numbers are available (e.g., with sexed spermatozoa), the costs are substantial and training is required. Thus they are not in widespread use in the field.

Control of Infectious Diseases

A considerable number of pathogens are transmissible through equine semen (Table 43.1). Most importantly, this includes equine viral arteritis, which can be shed in the semen of stallions for long periods of time after infection. Because of the difficulties of identifying shedding stallions, the use of methods for processing semen that remove the virus has been recommended (Morrell & Geraghty 2006, Morrell et al. 2014). Many other viruses can be transmitted through semen, including EHV-III, equine infectious anaemia, and, possibly, EHV-II and vesicular stomatitis. The case with EHV-I is less clear-cut for, although its transmission through semen appears likely, no recorded cases have occurred (Klug & Sieme 1992). Many nonspecific bacterial contaminants of semen may cause infertility in inseminated mares. The presence of β-haemolytic streptococci in semen is associated with reduced fertility of mares (Klug & Sieme 1992), as are haemolytic *E. coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa. Klebsiella* spp. are of more clear-cut significance as a cause of infertility, whereas *Taylorella equigenitalis,* the causal organism of contagious equine metritis, is an important venereal pathogen, although not controlled by OIE. Within its geographical area of distribution, *Trypanosoma equiperdum,* the protozoon responsible for dourine, is venereally transmitted. Legislative control exists in many countries to control the spread of equine viral arteritis, equine infectious anaemia, contagious equine metritis, and dourine, at least in the bloodstock industries, but control of venereal diseases in ponies and riding horses is often minimal. The simple precautions of serological examination of stallions for the presence of viral infection and serial bacteriological examination of their external genitalia before using the animals as AI (or, indeed, natural service) sires are all that is required to control most venereal pathogens, yet, all too often, this does not occur until after the onset of a disease outbreak. However, a negative serology result only indicates the absence of an antibody response to the virus, not the absence of infection.

Artificial Insemination of Dogs

It was upon the dog that the earliest recorded studies of AI were undertaken, by the Italian natural philosopher Spallanzani. Despite such an impeccable pedigree, demand for AI of dogs has been slow to develop, as there has historically been a significant degree of resistance to it by many breed societies. This position has changed in more recent years (UK Kennel Club 2017), so the use of AI in dogs has increased markedly. In parallel, methods have been developed for breeding nondomestic Canidae for conservation or commercial purposes.

Collection and Storage of Semen

Semen is usually collected from the dog by digital manipulation (see also Chapter 35). The use of electroejaculation under general anaesthesia has also been attempted (Kutzler 2005). The preejaculatory fluid, sperm-rich fraction and a little of the postejaculatory (prostatic) fluid are collected. The whole ejaculate may be immediately inseminated into the bitch's vagina, but it is more common to dilute the semen so that multiple inseminations can be performed. Where the whole ejaculate is used, only a few millilitres of prostatic fluid should be collected.

Liquid Semen

Dog semen can be stored in a cooled condition for 24 to 72 hours at 4°C, after dilution in simple diluents (e.g., skimmed milk) (Harrop 1962). This is generally long enough for at least two inseminations to be performed on the bitch, or for air freight transport to most international destinations. Hence, this method is very widely used in dog AI (Pena et al. 2006). Better longevity of semen can be achieved with the use of diluents such as Kenney's diluent for equine semen (Bouchard et al. 1990; Table 43.11) or Tris–glucose–egg yolk (Iguer-Ouada & Verstegen 2001; Table 43.12). Removal of the prostatic fluid by centrifugation improves

TABLE 43.12 Diluents for cooled storage of canine semen

Component	Tris–Glucose/ Tris–Fructose	Skimmed Milk
Tris (g/L)	30.25	
Sodium citrate monohydrate (g/L)	17	
Glucose or fructose (g/L)	12.5	
Skimmed milk		80%
Buffer	80%	
Egg yolk	20%	20%
Antibiotics	Commonly penicillin, streptomycin	

Derived from: Iguer-Ouada & Verstegen 2001, Romagnoli 2002.

storage and viability, especially when samples are dilute (Romagnoli 2002), although semen can be preserved successfully without its removal (England & Ponzio 1996). Cooling from body temperature to 4°C should be slow to prevent cold shock: Bouchard et al. (1990) recommended cooling at 0.3° to 1.0°C/min. This rate can be achieved in practice by placing the diluted semen in a container filled with water at room temperature, which is then placed at 4°C overnight (Pena et al. 2006). The use of the Equitainer as a means of cooling/transporting canine semen has also been recommended. Dilution rates have to take into account the volume that can be inseminated into the bitch as well as the density of the semen but should aim to achieve at least 150×10^6 progressive motile sperm.

Cryopreservation

Cryopreservation of dog semen is more difficult than for many species, but success has been improving over recent years. Many different diluents have been tried, largely on an empirical basis or because they work for other species. Egg yolk appears to be an important component of most diluents. Likewise, many different glycerolisation regimens have been employed in an attempt to achieve a satisfactory compromise between its toxic effects upon sperm and the concentrations needed to give effective cryoprotection (England 1993). Although the optimal glycerol concentration appears to be about 6%, its addition in one or multiple steps does not have a major effect upon post-thaw survival (Silva et al. 2003).

Consequently, there are many regimens for freezing semen. Two methods are described as examples of those in current use. Thomassen et al. (2006) diluted semen in a Tris–fructose–citric acid extender, with 8% glycerol and 20% egg yolk, in a single step at 35°C. It was then cooled slowly to 5°C before packaging into 0.5 mL paillettes for freezing. Pena et al (2006) diluted semen 1:1 in a Tris–glucose extender before centrifugation to separate the seminal plasma. After that the spermatozoa were resuspended in further Tris–glucose–egg yolk diluent, with a two-step glycerolisation (3% at room temperature, followed by slow cooling to 5°C, then diluted further with a similar diluent with a higher content of glycerol, to provide a final glycerol concentration of 7%). Freezing is also in 0.5 mL paillettes.

Early methods for freezing relied on freezing in pellets on blocks of solid carbon dioxide or in paillettes suspended in the vapour of liquid nitrogen. The results achieved by these methods are remarkably similar: in 2001, Nianski et al. found no difference between the post-thaw recovery of semen frozen as pellets or in 0.25 or 0.5 mL paillettes. The use of programmable freezers has the potential to result in significant improvements in post-thaw recovery rates, although, as the optimal cooling rate is determined by the concentration of glycerol and the other components of the diluent, it is difficult to generalise about optimal cooling rates. Thus, Rota et al. (1998) found no difference in post-thaw recovery between cooling at 10°C/min and 50°C/min between −10°C and −40°C; however, a cooling rate of 30°C/min is commonly recommended over this period. Thawing at 70°C in water for 8 seconds provides good post-thaw recoveries, although 37°C for 30 seconds is also used (Thomassen et al. 2006).

Insemination

Liquid Semen

When the reason for AI is failure of copulation, semen is collected from the dog by digital manipulation, and the whole ejaculate may be inseminated into the vagina of the bitch immediately after collection. It may, however, be preferable to extend the semen, inseminating one portion immediately and the remainder 48 hours later.

Similarly, when cooled semen has been transported from the sire to the bitch, insemination may be performed upon receipt of the semen and repeated 48 hours later. It is therefore imperative to ensure that the recipient bitch is approaching the fertile period of oestrus before semen is collected.

When fresh or cooled semen is used, intravaginal insemination is undertaken, with the semen deposited as close as possible to the external os of the cervix (Burke 1986). The semen may be deposited through a shortened bovine insemination catheter, which may require the use of a speculum to be guided into the correct site. Some authors recommend inserting one or two fingers into the vulva after insemination, in order to promote the motility of the female genital tract that normally occurs during the copulatory tie. Alternatively, an Osiris catheter, which consists of an outer sheath with a Foley bulb and an inner catheter, can be used (Nianski 2006). The inflatable balloon is considered to both simulate the copulatory tie and prevent retrograde flow of semen. However, there is no unequivocal evidence that this type of catheter improves conception rates beyond those achieved with simple catheters. Whichever method is used, the hindquarters of the bitch should be raised for a few minutes after insemination to prevent retrograde loss of semen. Pregnancy rates of approximately 80% can be expected with fresh semen (England & Miller 2008).

Frozen Semen

Conception rates using frozen semen are poorer with intravaginal than intrauterine insemination. Hence direct intrauterine insemination, either via the cervix or directly via surgery or laparoscopy, is currently recommended. Linde-Forsberg et al. (1999) reported whelping rates of nearly 85% with intrauterine insemination compared with approximately 60% with the intravaginal route. However, higher pregnancy rates were achieved when multiple intravaginal inseminations were performed, although there was no benefit from multiple inseminations by the intrauterine route. The Osiris catheter has been used to try to improve conception rates to intravaginal insemination (Nianski 2006); however, whelping rates by this method were also approximately 60%.

Passing a catheter through the cervix is difficult because of the length of the vagina, the length of the cervix, and the site of its external os (England & Lofstedt 2000). However, the Norwegian

catheter, which consists of a 2 mm catheter inside a Teflon sheath, has been used successfully, although considerable practice is required to master the technique (Romagnoli 2002). Thomassen et al. (2006) describe the method of intrauterine insemination as follows: Insemination is performed on standing bitches, which normally do not require sedation. The cervix is fixed through the abdominal wall with one hand as the steel catheter is manipulated through the cervix into the uterus with the other. After deposition of the semen, care is taken to avoid backflow by ensuring that the plastic sheath remains in close contact with the catheter and by elevating the hindquarters of the bitch (as described previously). As an alternative to transabdominal fixation of the cervix, an endoscope can be used to locate the cervical os. Insemination is easier in bitches of moderate size than in giant and miniature breeds, and is more difficult in animals that are excessively fat. However, pregnancy rates are reported to be similar to the Scandinavian method described previously (Payan-Carreira et al. 2011).

Insemination via a surgical laparotomy or via laparoscopy can also be used for insemination of frozen semen, especially when practitioners are not confident with the transcervical method.

Timing of Insemination

The bitch has a prolonged period of receptivity to the male but a relatively short fertilisation period (see Figs. 33.13–33.15). Thus the fertility achieved in canine AI primarily depends on achieving correct timing of insemination in relation to that of ovulation. When natural service is used, many breeders allow bitches only a single mating, which typically occurs 12 days after the onset of prooestral bleeding. For successful AI, much closer attention to the time of the fertilisation period is needed, especially when cryopreserved semen is used, although more latitude exists when cooled semen is used (Jeffcoate & Lindsay 1989, Linde-Forsberg & Forsberg 1989, 1993, Morton & Bruce 1989). The inherent fertility of the dog is also of importance, with the longevity of his spermatozoa in the female tract being a critical determinant of fertility. Dogs with long-lived spermatozoa can achieve pregnancies even if the timing of insemination is not optimal, whereas with dogs whose sperm have poor survival, sperm death is more likely to have occurred before ovulation under such circumstances (see Figs. 33.13–33.15).

The timing of ovulation can be predicted from the preovulatory rise in circulating progesterone concentrations or measurement of LH concentrations. Alternatively, the ovaries can be examined by ultrasonography. Vaginal cytology, although useful as an indicator of the approach of ovulation, is too imprecise for frozen semen inseminations. Measuring serum progesterone concentrations daily after the onset of oestrus (or after the appearance of a high percentage of cornified vaginal epithelial cells) provides a reasonably good predictor of the timing of ovulation. Progesterone concentrations rise from baseline values to 2.0 to 3.0 ng/mL on the day of the LH surge, thereafter increasing to 4.0 to 8.0 ng/mL on the day of ovulation, and 10 to 25 ng/mL during the 2 days after ovulation (Romagnoli 2002). Hence the first rise in progesterone concentrations can be taken as an indicator of the LH surge. Alternatively, LH can be measured directly using an 'animal-side' assay method (e.g., the semiquantitative Witness-LH, Zoetis, Parsippany, NJ); the assay is intended for use with serum, although it has also been used to detect LH in urine (Durrant et al. 2006). Insemination should ideally be performed 2 to 3 days after ovulation. Thomassen et al. (2006) showed that insemination at the optimal time results in a higher whelping rate and litter size (78% and 5.8 ± 0.2 pups, respectively) than late insemination (56% and 4.5 ± 0.5 pups). Another suggestion for fresh or cooled semen is to inseminate on the day of ovulation and again 2 days later, whereas with frozen semen, the first insemination is done 2 days after ovulation with the second 48 hours later (Payan-Carreira et al. 2011).

Artificial Insemination of Cats

Insemination of domestic cats is undertaken relatively rarely because of the small volumes of semen obtained (0.05–0.25 mL) and the need for anaesthesia for collection and insemination. However, it is used in conserving rare breeds or for international trade and especially for the conservation of wild Felidae, many of these species being endangered. Recent reviews of feline AI have been published by Rijsselaere and van Soom (2010) and England and Russo (2014).

Semen is collected by electroejaculation (Zambelli & Cunto 2006) under general anaesthesia or by urethral catherisation under sedation with medetomidine, which stimulates the α_2-adrenergic receptors (Rijsselaere & van Soom 2010). Semen can also be harvested postcastration from the epididymides, vasa deferentia, or even from minced testis tissue.

Cooled storage of semen requires prevention of cold shock. Egg yolk is less effective at protecting cat semen against cold shock than it is in most other domestic species and, although simple Test (Tes+Tris)-yolk will maintain sperm viability at 5°C, the low density lipoprotein fraction is far more effective than whole yolk (Glover & Watson 1987). Such diluents can maintain semen quality for approximately 24 hours, with occasional reports of longer periods of storage.

Similar extenders are used for freezing as those for cooled storage or with extenders based on Tris, citrate, glucose, fructose, or lactose plus egg yolk. Glycerol is the best cryoprotectant for cat semen, but because of sensitivity to its toxic effects, it is usually added at concentrations of up to 4%. Antioxidants, such as taurine, appear important in the maintenance of post-thaw viability. After loading into paillettes, semen can be frozen in liquid nitrogen vapour, in programmable freezers (at a cooling rate of 10°C/min between +5° and −80°C) or, less successfully, in pellets on dry ice.

Ovulation has to be induced before insemination. The most commonly used hormone for this purpose is hCG: one or two doses are given and insemination performed 15 to 30 hours later. Opinions vary concerning whether it is better to inseminate before or after ovulation has occurred because, with natural mating, ovulation would occur approximately 24 to 48 hours after coitus (England & Russo 2014). Intravaginal insemination has been reported, with pregnancy rates varying from 11% to 78% (reviewed by Rijsselaere & van Soom 2010). In domestic cats, intrauterine insemination via a laparotomy is more convenient, yielding pregnancy rates of 80% for fresh semen and 0% to 20% for frozen semen. However, in some countries this procedure is considered to be invasive and unethical and is prohibited for normal breeding. Sperm doses are 50 to 80 $\times 10^6$ for vaginal insemination and 20 to 50 $\times 10^6$ for intrauterine insemination (England & Russo 2014).

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