Storage of boar semen

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Abstract

The problems, aspects and methods of liquid storage and freeze–thawing of boar semen are discussed and a review is given on examination of spermatozoa by the recent fluorescent staining methods. Published by Elsevier Science B.V.

Keywords: Liquid and frozen semen; Cold shock; Diluent; Cryoprotectant; Thawing of semen; Staining of sperm

1. Introduction

The first attempts on artificial insemination (AI) of pigs were made in 1926–1927 by Ivanov, which were continued between 1930 and 1936 by Milovanov et al. (cited by Serdiuk, 1970). The early diluents (glucose–sulphate and glucose–tartrate) for boar semen were proposed by Milovanov in the years of 1931–1933 (Milovanov, 1962). Since these earliest studies of semen storage in the pig, it has been understood that only a portion of spermatozoa in the original semen sample survives preservation of any type. It is also well known that survival of cells is much greater after liquid than frozen storage of semen. As temperature declines, there is an inevitable reduction in the proportion of spermatozoa that maintain normal membrane integrity, ultrastructure and biochemical components.

Utilisation of preserved semen for AI in pigs has increased approximately threefold in the past 15 years. More than 99% of the estimated 19 million inseminations conducted worldwide are made with semen that has been extended in the liquid state and used on

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the same day, or stored at 15–20°C for 1 to 5 days. Eighty-five percent of all inseminations are conducted on the day of collection or on the following day. Virtually all AI with liquid-stored semen is used for market hog production. Frozen boar semen has been available commercially since 1975 both in pellet form and in straws. However, less than 1% of all inseminations are made using frozen–thawed semen, most often after export from one country to another and primarily for the purpose of upgrading the genetic base in a particular country or herd. Countries differ in the percentage of sows and gilts that are inseminated: in some only 5% of their breeding females, while in others, mainly in Europe, nearly 90% of the females are inseminated in a particular country.

From 1975 through 1990, increases in the use of liquid-stored boar semen grew with astounding proportions in Europe. Since 1990, however, the growth has been primarily in North and South America where it is still continuing at the time of writing. This increase in pig AI, particularly in the Western Hemisphere, is due to improved technology of semen processing, increased demand for higher quality pork, increased profitability from high quality carcasses and improved transportation systems, making the liquid-stored semen available on a same or next day basis, virtually anywhere in the world. It should also be noted that a greater proportion of pig producers are innovative and willing to try new technology. The United States has experienced substantial growth in pig AI in the last 5 years, moving from about 5% of the sow population inseminated in 1993 to an estimated 45–50% in 1998–1999.

Three international conferences over the past 15 years have been devoted to improvement of methods, providing information and stimulation of research on storage of boar semen. For more complete details on various aspects related to storage of boar semen and AI, readers are advised to consult the proceedings from the First, Second and Third International Conferences on Boar Semen Preservation (Johnson and Larsson, 1985; Johnson and Rath, 1991; Rath et al., 1996). This chapter will not be devoted to an extensive and detailed examination of the various critical aspects of liquid and frozen storage of boar semen. Rather it will cover highlights of the advances made in the two semen storage methods and also give an update on the in vitro evaluation of semen and refer the reader to relevant protocols available in the literature.

## 2. Storage of boar semen in liquid state

Two main factors influence sperm cell function after ejaculation and during in vitro storage: the temperature at which the semen is collected and stored after dilution, and the conditions of the suspension medium.

### 2.1. Cold shock

It is known that boar spermatozoa are very susceptible to cold shock. This occurs when freshly ejaculated boar spermatozoa are cooled quickly from body temperature to temperatures below 15°C, which results in a loss of viability of an increasing number of spermatozoa, especially when cooling is quickly continued to 1–2°C.
When prediluted semen samples are held above 15°C for several hours, the spermatozoa acquire a gradual resistance to cold shock (Pursel et al., 1973). Rapid cooling from 35°C to 15°C of freshly diluted semen leads to a significant loss of motility, whereas cooling down large volumes (100 ml) slowly in 10°C steps enhances cooling resistance. Preincubation for at least 24 h before a possible decrease of temperature below 15°C enhances cold shock resistance, an effect which deserves consideration in routine work (Weber, 1989).

The changes within the incubated cells that render them resistant to cold shock are not entirely known. As recently reviewed by Watson (1996), it is believed that cold shock may be related to the lipid composition of the membrane bilayer affecting the fluidity of the plasma membrane. As the temperature is lowered, restriction of lateral movement of membrane phospholipids could result in a transition from a fluid to a gel phase. Due to the different transition temperatures for different membrane lipids, phase separations may occur, whereby proteins become irreversibly clustered (De Leeuw et al., 1990). These authors concluded that the response of a particular cell membrane to low temperature is strongly related to the detailed membrane composition of that cell. Observed differences in the cold-induced structural changes between cold-resistant bull and cold-sensitive boar spermatozoa could be explained by the differences in membrane composition. Since the fatty acid composition of phospholipid determines phase behaviour, the most striking differences in the type of phospholipids between boar and bull spermatozoa, that is a lower percentage of phosphatidylcholine and the higher percentage of phosphatidylethanolamine and sphingomyelin in boar, make it difficult to assess the stability of boar sperm membranes on the basis of differences in phospholipid composition.

Another factor which influences the thermotrophic behaviour of membranes is the percentage of cholesterol. As the cholesterol/phospholipid ratio in boar spermatozoa is very low, and as cholesterol is distributed asymmetrically, it is present more in the outer than the inner monolayer of the membrane. This combination could render the inner monolayer especially vulnerable to cold shock. Cold-induced reorganization of membrane particles, although partially reversible, could influence membrane function in a number of ways. These include an increase of permeability (leakage of cations and enzymes), a reduction in enzyme activity and diffusion controlled membrane processes, and changes in lateral motion in channels (De Leeuw et al., 1990).

Dilution and cooling render the boar sperm membrane more permeable (Ortman and Rodriguez-Martinez, 1994). Watson (1996) emphasized that phase events and concomitant entrance of free calcium ions from the environment into the cell could stimulate calcium dependant processes associated with capacitation and consequently render the cell membrane more fusagenic, so that the usual mechanism of capacitation may be by-passed. More insight is needed into the mechanisms responsible for cold-induced membrane changes and the consequences to the preservational state of the spermatozoa.

Important in a practical sense is that cold shock sensitivity of the boar spermatozoa seems to change in a time and temperature-dependent manner. Factors which are believed to play a role are maturation state, seminal plasma, dilution and ageing during incubation. As the maturation of spermatozoa cannot be influenced when ejaculated semen is used, and seminal plasma seems not to play an essential role (Kotzias-Bandeira
et al., 1997), research on temperature dependent medium-membrane interactions during incubation and storage could increase knowledge on how to prevent or reduce cooling changes (Petrounkina et al., 1997).

2.2. Dilution effect

Spermatozoa are diluted with seminal fluids from the accessory glands at ejaculation and their motility is retained for a few hours. To extend their survival in vitro, it is necessary to reduce the metabolic activity by chemical inhibitors or by lowering the temperature, which also requires dilution.

In addition to a prolonged lifespan, mammalian spermatozoa respond to dilution by an initial increase of activity, followed by a loss of motility and an increase in membrane damage. With excessive dilution, especially when using pure electrolyte media, there is considerable loss of cell viability. No definite explanations have been offered for this so-called dilution effect, but Watson (1995) considers it to indicate cell injury, as a consequence of loss of intracellular components and/or dilution of a protective agent in seminal fluid. A reduction in the concentration of important seminal plasma components may contribute to the effect, which can be eliminated by the addition of albumin. Furthermore, minimal additions of millimolar concentrations of $K^+$ may also assist to maintain the motility of spermatozoa, as a high concentration of $K^+$ in the seminal fluid is essential for cell viability (Harrison et al., 1978).

Harrison et al. (1982) proposed that the dilution effect is due to the absence of proteinaceous motility stimulants from seminal plasma and showed that serum albumin could stimulate motility in a reversible manner. When spermatozoa were washed in the absence of albumin, an increased tendency to stick to glass surfaces indicated surface membrane changes. Waberski et al. (1989) found that bovine serum albumin (BSA) stimulated the motility of spermatozoa during a six day storage test. Buhr (1990), on the other hand, used BSA in an attempt to overcome the fluidity of the membrane, and therefore the deleterious effect of lysophospholipids and free fatty acids, produced by cooling. It was assumed that changes in membrane lipid fluidity could affect trans-membrane movement of $Ca^{2+}$, which is essential in the capacitation process but deleterious during storage. However, BSA induced only a temporary decrease in membrane fluidity which was difficult to interpret. Nevertheless, BSA improved fertility when the semen was stored between 3 and 5 days (Waberski et al., 1994a).

2.3. Diluents

The requirements of a storage medium have been established on several occasions and were reviewed by Watson (1990). The important factors are the pH, ionic strength, type of ions and osmotic pressure of the medium. Anti-microbial substances are also commonly included in diluents.

In freshly ejaculated boar semen the pH varies between 7.2 and 7.5, and below this the motility and metabolism of spermatozoa are reduced gradually. The high glucose content of most diluents for boar semen causes a considerable reduction of intracellular pH below 6.0. This intracellular acidosis obviously enables the cells to survive storage.
of some days at ambient temperature. The glycolytic metabolism of boar spermatozoa is very weak in comparison to other species, and since diluted boar semen is placed routinely in plastic tubes with little or no access to oxygen, this situation causes a reduction of motility even at relatively high temperatures.

The ionic strength of the diluent seems not to be of primary importance in diluents for boar semen where the osmolality is maintained by non-ionic components, such as glucose. This may explain the importance of surface-bound proteins which are more readily solubilized in high ionic strength media (Watson, 1995). The ions in media for fresh semen are introduced as sodium bicarbonate and/or sodium citrate and, in certain diluents, potassium chloride. The former compounds are used as buffers, the latter in low concentration (minimum 4 mM K\(^+\)) to maintain the Na\(^-\)–K\(^+\) pump of the cells in preventing intracellular K\(^-\)-exhaustion and a loss of motility (Alvarez and Storey, 1982). Bicarbonate, on the other hand, is known to cause changes in membrane lipid architecture within a few minutes of exposure, which initiates final membrane destabilization as an important step towards capacitation (Harrison, 1996).

Newer diluents are based on zwitterionic organic buffer(s), especially TES and HEPES (Crabo et al., 1972, Weitze, 1990), which capture heavy metals and control pH. Boar spermatozoa tolerate a relative wide range of osmolality between 240 and 380 mosM, but it seems that isotonic or slightly hypertonic media offer better preservation of fertilising capacity than hypertonic diluents (Weitze, 1990). One of the extenders in this group, Androhep is used extensively (Table 1).

As boar semen has to be stored in a liquid state at ambient temperature above 15–18\(^\circ\)C, a number of extenders have been developed which decrease the metabolic activity of spermatozoa using an environment high in CO\(_2\). Different modifications of glucose–sodium citrate media were mainly used. The Illinois Variable Temperature (IVT) diluent, developed in the 1950s for storage of bull semen at ambient temperature, contains a glucose–citrate–bicarbonate–egg yolk solution gassed with CO\(_2\) and was used in a modified form for boar semen by du Mesnil du Buisson and Dauzier (1959). The medium, supplemented with potassium chloride, is still used as a ‘‘post-diluter’’ in the two step extension system (Stähr and Nehring, 1997). The composition of several extenders is provided in Table 1.

Ethylenediamine-tetra-acetic acid (EDTA), as a chelating substance, captures divalent metal ions, especially Ca\(^{++}\), and is believed to limit their movement across the plasma membrane (Watson, 1990), preventing the initiation of capacitation and the acrosome reaction. The inclusion of EDTA in diluents was an important step towards widespread use of liquid-stored semen in pig AI (Kiev extender, Plisko, 1965). Several modifications have been made to the Kiev extender in an attempt to increase storage time and fertility, but the results have been unequivocal.

At the present time one of the most widely used extenders is the Beltsville-TS (BTS), developed by Pursel and Johnson (1975) for thawing boar spermatozoa frozen in the pellet form, and later adapted for liquid storage (Johnson et al., 1988). This extender contains a low concentration of potassium and is believed to play a role in maintaining the intracellular concentration of this ion at physiological levels during storage.

The Zorlesco diluent is a relatively complex medium (containing tris buffer, citric acid, BSA and cysteine, in addition to glucose and EDTA) (Gottardi et al. 1980) and the
Table 1
Composition of extenders used commercially for liquid storage of boar semen (all extenders are also supplemented with antibiotics)

<table>
<thead>
<tr>
<th>Ingredient (g/l)</th>
<th>Extenders</th>
<th>ZORLESCO*</th>
<th>ANDROHEPb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BTSc</td>
<td>KIEVD</td>
<td>IVTe</td>
</tr>
<tr>
<td>Glucose</td>
<td>37.0</td>
<td>60.0</td>
<td>3.0</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.25</td>
<td>3.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Sodium–citrate</td>
<td>6.0</td>
<td>3.7</td>
<td>24.3</td>
</tr>
<tr>
<td>Sodium–bicarb</td>
<td>1.25</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.75</td>
<td>1.2</td>
<td>2.4</td>
</tr>
<tr>
<td>TRIS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Citric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td>0.05f</td>
</tr>
<tr>
<td>BSA</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Gottardi et al. (1980).


d. Plisko (1965).


Androhep diluent (Weitze, 1990), contains HEPES and BSA. The latter extender is used for up to 5 days of semen storage in routine AI and has therefore been called a long-term extender. Liquid semen extension is straightforward in that ingredients can be weighed out and diluted with high quality water.

The use of sulfhydral-group bearing substances (Schönow I, Zorlesco) is believed to stabilize sperm membranes and inhibit capacitation. Dilution with the Schönow I medium gives better viability of spermatozoa after storage, but is more laborious as the diluted semen should be further extended with the Schönow II diluent portion before insemination. Thus, the simple one step-methods are favoured commercially, especially when the semen is stored for less than 50 h (Stahr and Nehring, 1997).

To prevent the growth of microflora in the stored semen, the diluents are supplemented with antibiotics. Currently, up to 300 mg gentamycin or 1.0 g neomycin sulfate are added to 1 l of diluent, replacing penicillin and streptomycin which still have some importance in suppressing leptospira. The legislative ruling in the European Community prescribes the use of an effective combination of antibiotics, in particular against leptospira and mycoplasma, which must produce an effect at least equivalent to combinations of 500 IU/ml penicillin, 500 IU/ml streptomycin, 150 mg/ml lincomycin and 300 mg/ml spectinomycin.

2.4. Duration of storage

Structural and functional changes to spermatozoa connected with liquid storage of boar semen resemble a natural ageing process and may be determined by the conditions
and length of storage. Ageing of spermatozoa occurs during storage in vitro and after insemination in vivo when the fertile sperm population awaits ovulation to be released from the lower part of the isthmus. This means that the success of fertilisation is influenced by two periods of ageing: the in vitro storage for a period of up to 5 days and in vivo ageing, which increases when the interval between AI and ovulation is extended over 12 to 24 h (Waberski et al., 1994b; Soede et al., 1995a).

From a practical standpoint, a decrease in fertilising ability during storage cannot be prevented, even when so-called “long-term” media are used. Nevertheless, special media components, such as BSA and type of buffer, seem to reduce storage-dependent ageing processes and, therefore, a decrease in fertilising capacity. Increased number of spermatozoa per dose, the presence of seminal plasma and a low dilution rate seem to reduce the loss of fertility associated with storage. In this context, clear differences in fertility between boars must be considered, which can only partly be related to differences in the quality of spermatozoa such as motility and morphology (Weitze, 1990).

The ageing-related functional changes within different compartments of the spermatozoon, such as the mitochondria, flagellum, acrosome and plasma membrane, are poorly understood. The reduction of motility which occurs during storage has long been the main parameter used to judge the decrease of fertilising ability. The loss of ATP and cAMP, as well as reduced calcium uptake, are characteristic of decreased motility. An important indicator of storage-related membrane damage is a change in membrane permeability, such as increased permeability to stains and a release of intracellular substances. In relation to this permeability, a distinction must be made between the acrosomal and plasma membranes of spermatozoa. A loss of net negative charge also gives an indication of the ageing processes. This could be caused by lipid peroxidation, due to the relatively high content of unsaturated fatty acids in the phospholipids of the boar sperm membrane, through which the lipid phase of the membrane could be damaged. These changes are balanced by the protective action of the seminal antioxidants superoxide dismutase and glutathione peroxidase (Petzoldt and Nehring, 1988).

As revealed in AI experiments monitored by sonographic ovulation detection, inseminations with liquid semen performed within 2 days after collection did not reduce fertility levels with increasing intervals between AI and ovulation (Weitze et al., 1989). However, when semen stored for 48 to 87 h was used, the fertilisation rate decreased even when ovulation occurred between 12 and 24 h after insemination. The dependence of fertility results on time of ovulation became more striking when inseminations were performed with semen stored for longer than 87 h, suggesting a combined effect of in vitro and in vivo ageing of spermatozoa (Waberski et al., 1994b). From the results of further insemination experiments in which ovulation was monitored, the authors concluded that apart from slight differences between media for storage of boar semen, the decrease in fertilising capacity due to in vitro ageing cannot be prevented, even during the first day of storage. Improvement of the AI procedure, with special emphasis on the time of insemination relative to the time of ovulation, as well as the selection of boars with high quality semen, are still essential to obtain satisfactory results with liquid stored boar semen. In conclusion, ageing of spermatozoa after ejaculation and liquid storage seems to be a physiological process which cannot be avoided completely by preservation.
measures, so additional care must be taken on timing of insemination relative to ovulation to maintain satisfactory fertility.

2.5. Physiological relationships

On the basis that epididymal spermatozoa can be successfully used for insemination in a number of species, including pigs, it could be assumed that seminal plasma from the accessory glands is of minor importance. However, today there is no doubt that seminal plasma has important regulatory functions in the processes before penetration of oocytes by the spermatozoa. These functions are based on direct interactions between seminal plasma and the spermatozoa and refer to nutrition, protection, regulation of motility, capacitation, gamete recognition and binding. Indirect effects influencing the physiology of spermatozoa are directed to the female genital tract, as seminal plasma is known to enhance uterine contraction, relax the tubal isthmus and modulate immune responses in the uterus. It is believed that these effects together ensure the survival, maturation and transport of the spermatozoa in the female genital tract and are involved in the binding of spermatozoa to the zona pellucida (Waberski, 1996).

Extension of semen for AI results in a quick and considerable dilution of the seminal plasma shortly after ejaculation, so that only a small proportion of the original seminal fluid is still present in an insemination dose. Normally, dilution does not surpass a 1:10 ratio. Either this low concentration of seminal plasma is sufficient to ensure the survival, maturation and transport of the spermatozoa, or the initial ejaculatory contact between seminal plasma and spermatozoa before dilution enables them to survive and arrive at the place of fertilisation with retained fertilising capacity. When seminal plasma or extender was introduced into the uterus before the semen, significant differences in egg fertilisation rates and numbers of accessory spermatozoa in the zona pellucida were obtained (Willmen et al., 1989). Using an inseminate dose of $2 \times 10^9$ spermatozoa, a pre-infusion of seminal plasma increased the percentage of embryos from 84.7% to 95.8% and resulted in an increase of the number of accessory spermatozoa from 8.0 to 74.7 per zona. This beneficial effect of seminal plasma on fertilisation rate and accessory sperm number was attributed to enhanced cell transport to the site of fertilisation. In addition, other mechanisms aimed directly at the spermatozoa, which increase their fertilising competence, could be responsible for the higher numbers of spermatozoa in the zona pellucida of the day 3–5 embryos (Rath et al., 1989, Waberski et al., 1994b).

2.6. Assessment of quality and quantity of spermatozoa

The fertilising competence of the inseminate is often estimated in terms of the quality (motility, morphology, functional integrity, microbial contamination) and quantity of spermatozoa (the number of high quality spermatozoa per dose). The effects of motility, morphology and number of spermatozoa are discussed here.

2.6.1. Motility of spermatozoa

Progressive motility of spermatozoa is an indicator of both unimpaired metabolism and intactness of membranes. Estimation of motility, therefore, has fundamental importance in the daily quality control of the semen. The percentage of motile spermatozoa,
furthermore, is used to calculate the required degree of dilution and to estimate the number of "intact" spermatozoa per insemination dose. Regular motility checks after dilution and during the holding period give information concerning the preservability of the semen of each boar and of its individual peculiarities. As boar spermatozoa show a higher percentage of circular movement than those from other species, it is recommended to estimate the different forms of motility, including proportions of progressive spermatozoa. Such estimates, undertaken by phase contrast microscopy within 20–30 min after dilution, cannot be integrated easily into the production processes. Stored semen should be examined daily, with motility values above 60% considered satisfactory.

2.6.2. Morphology of spermatozoa

Boars used for commercial AI are selected to a certain degree on the basis of a low incidence of morphologically abnormal spermatozoa, so that statistical calculations concerning their correlation with fertility are not very informative. Nevertheless, the variance between the percentages of proximal and distal cytoplasmic droplets on spermatozoa in the ejaculates is sufficiently large to establish a statistical relationship with fertility. A number of studies have also shown a significant negative correlation between the percentage of spermatozoa with cytoplasmic droplets and farrowing rate and number of live born piglets (Zeuner, 1992). Since cytoplasmic droplets generally represent the most frequent morphological alteration, Waberski et al. (1994a) investigated the effect of proximal and distal droplets on fertility in a field experiment. They found a negative correlation between the proportion of distal droplets and fertility, which contradicts the assumption that these droplets depress fertility less than proximal droplets (Hurtgen, 1984). The retention of cytoplasmic droplets is probably a primary defect, originating in the testis and hindering the physiological migration of the droplet in the epididymis. Waberski et al. (1994a) concluded that the presence of cytoplasmic droplets is a serious morphological defect, which is of particular importance when semen stored for long-term is used for AI and might be associated with lowered resistance of spermatozoa against in vitro ageing. They recommend that the total percentage of cytoplasmic droplets in ejaculates used for AI should not exceed 15%, especially when stored semen is used.

A complete morphological examination is recommended when boars are introduced into the AI centre and during subsequent regular routine examinations. In addition to the incidence of cytoplasmic droplets, the percentage of other morphological alterations should not exceed 20%. The use of a phase-contrast microscope and glutaraldehyde–BTS or formol–citrate fixed wet preparations is recommended, since they avoid the artifacts associated with dried smears (Pursel et al., 1972).

2.6.3. Number of spermatozoa

The number of spermatozoa plays an important role in fertilisation, because fertility improves with increasing number of spermatozoa delivered up to a certain threshold. The threshold varies with semen quality (Saacke et al., 1991), and differences in fertility between semen treatments (i.e. extender, age of semen) can only be revealed when numbers of spermatozoa in the inseminate are below threshold values (Saacke et al., 1994).
In the commercial situation, the aim is to maintain spermatozoa in a viable state for a variable period of time in order to fertilise a high proportion of ova with a minimum sperm dose (Reed, 1985). Fulfilling all of these requirements has not been achieved by any available boar semen extender when storage time exceeds 3 days. A number of field trials using semen stored for longer periods in various diluents have yielded reduced fertility (reviewed by Weitze, 1990). However, doubling of the sperm dose to \(6 \times 10^9\) on the 4th day of storage (Johnson et al., 1988), as well as the use of boars with good semen quality (Martin Rillo, 1984) can prevent the fertility decline of stored semen. This option usually is not practicable for commercial use, but gives valuable insight into the interaction of the quality and quantity of spermatozoa. When the number of spermatozoa in the inseminate was reduced from 2 to \(0.5 \times 10^9\), a significant reduction of fertility from 90.2% to 58.3% was reported (Willmen et al., 1991). Moreover, pretreatment of the uterus with seminal plasma compared with saline before insemination of \(0.5 \times 10^9\) spermatozoa per gilt gave no improvement in fertility. Considering the low number of spermatozoa used, both accessory spermatozoa counts and fertilisation rates were surprisingly high in the two groups. This was attributed to optimum AI conditions, such as winter season, close boar contact, skillful animal handling and detection of oestrus, and to high quality of ejaculated semen. A reduction in the inseminate dose to \(0.3 \times 10^9\) spermatozoa resulted in a decrease of normal embryo development rate to 60%. This was due to a higher percentage of degenerated embryos, and confirms the assumption of Saacke et al. (1994) that low numbers of spermatozoa at the site of fertilisation reduces competition among them and thereby allows fertilisation by less competent spermatozoa. Thus, less competent spermatozoa are capable of fertilisation, but are unable to sustain normal embryonic development. The observation that normal and degenerated embryos had similar numbers of accessory spermatozoa supports the hypothesis that less competent spermatozoa have normal binding and penetration ability (Waberski et al., 1996). Thus, quantitative and qualitative deficiencies in semen might lead to different degrees of fertilising incompetence of the sperm population in the female genital tract, both resulting in low fertility.

2.7. Fertility results after insemination of liquid-stored semen

As mentioned earlier, there are a number of extenders used for dilution of boar semen. Some have been in use at one or more locations for 30 years. Quite frequently the results of fertility trials that initiated the use of the extender in the first place are not applicable in current practice. However, as a general rule one can expect farrowing rates ranging from 65% to 70% if the semen is used in the first 2 days after collection and a reduction down to about 50% with 5-day-old semen. The litter size may also be affected by longer storage times. Several references covering various extenders over the past 10 years can be consulted for specific information on fertility (Johnson et al., 1988; Johnson and Rath, 1991; Rath et al., 1996; Johnson, 1998).

2.7.1. Commercial use of liquid-stored boar semen

Pig producers have adapted the AI technology to their specific needs, some by collecting semen and inseminating females on their own farms, which is frequently
referred to as do-it-yourself AI (DIYAI). Other countries adopted extensive networks of AI Centres with services set up to deliver the semen. The delivery systems have worked well to establish AI. However, as AI has grown in use in the last 10 years there has been a decrease in the use of outside inseminators and a preponderance of DIYAI. The latter has been encouraged by the increased use of AI for crossbred pig production. High animal health and disease prevention standards also encourage the use of DIYAI, as producers are able to close their farms to outsiders. For example, increased DIYAI was used for insemination in the Netherlands during the 1997 swine fever outbreak. Individual collection facilities have been used in the United States for many years, where many production units are large and can easily manage their own collection and insemination. However, the use of semen delivered from local AI Centers is also increasing in the USA. There is an increasing number of regional AI centers, where technical help can provide a consistent semen quality standards and levels of expertise in collection and dilution of semen. Many of these AI centres are organized along a cooperative structure.

Little variability exists worldwide in the methods of packaging liquid semen. The standard procedure involves collection of the whole ejaculate, a modified whole ejaculate or the sperm rich fraction. Dilution varies considerably in practice. However, dilution to about $3 \times 10^9$ in 80 ml is recommended. Where collection is done on the farm, producers will frequently inseminate higher doses of spermatozoa, since they tend to use all the semen collected on any given day, rather than store it. Furthermore, there is considerable mixing of semen heterosperm from different boars, which encourages the use of more semen than is actually needed.

2.7.2. Life span of gametes in the female genital tract

The fertile life span of oocytes and spermatozoa in the female genital tract is limited. Therefore, the timing of insemination relative to ovulation is crucial for achieving high fertility. Sub-optimum insemination time before ovulation leads to low conception rates and/or litter sizes, either by a failure of senescent spermatozoa to fertilise all released eggs, or by fertilisation of oocytes with less competent spermatozoa, resulting in increased embryonic mortality.

The fertile life span of gametes in the female genital tract should be viewed as the period of time during which the ability to undergo normal fertilisation and give rise to a viable embryo is retained (Hunter, 1988). This information can be reliably obtained only from insemination trials where the time of ovulation is known, which can be assessed now by sonographic monitoring of the ovaries (Weitze et al., 1989). Recent studies using sonographic detection of ovulation in spontaneously cycling and ovulating sows show differing 'optimum' insemination times relative to ovulation where fertilisation rates were at a maximum (Table 2).

The viable life span of spermatozoa in the female genital tract is not regarded as a constant figure. Variations are due to heterogeneity of the sperm population and the condition of the female genital tract, for example the composition of the fluids in the tract (Hunter, 1988). Variations found in insemination trials depend on season, farm managment, skill of the inseminator and quality and number of spermatozoa. Improvement of conditions can result in increased fertilisation even with low numbers of
spermatozoa at extended AI to ovulation intervals (Waberski and Weitze, 1996). However, in commercial practice, where many factors cannot be controlled, the fertile life span of spermatozoa in the female genital tract should not be overestimated. The time interval between two inseminations and between the last insemination and ovulation, respectively, should not exceed 12 to 18 h. In vitro storage of semen for a considerable length of time before insemination reduces the viability of spermatozoa in vivo and requires, therefore, a more proper timing of AI compared to that with fresh semen. The relationship of in vitro ageing and subsequent in vivo ageing of spermatozoa with fertilisation rates and numbers of accessory spermatozoa in the zona pellucida has been described by Waberski et al. (1994b).

2.7.3. Insemination in relation to ovulation

Ovulated oocytes have a limited life span in the female genital tract. Although successful fertilisation of pig oocytes has been observed with inseminations performed 4 h (Waberski et al., 1994a; Nissen, 1995) or even 8 h (Hunter, 1967; Soede et al., 1995a) after ovulation, there is evidence that farrowing rates are reduced due to ageing of oocytes compared with insemination before or close to ovulation. It is therefore recommended that insemination should be performed not later than 2 h after ovulation in order to obtain the maximum number of normally developed oocytes (Hunter, 1967).

In current AI practice, the majority of sows receive the first insemination before ovulation which occurs late in oestrus (Table 3). In a recent study, in which sows were first inseminated 24 to 0 h before ovulation, a second insemination 0 to 5 h after ovulation did not influence the percentage of normally developed embryos (Soede et al., 1995b). This confirms that shortly after fertilisation an effective block against polyspermy is established even when high numbers of spermatozoa subsequently reach the site of fertilisation. However, insemination in late oestrus, after ovulation, when blood progesterone levels rise up to 10 ng/ml, might affect fertilisation rates due to a decreased resistance of the endometrium to infectious agents (De Winter et al., 1996). Thus, the effect of multiple inseminations during oestrus on fertilisation remains questionable (Waberski and Weitze, 1996).
Table 3
Oestrus and ovulation in sows after weaning in different herds

Significant correlation between weaning–onset of oestrus and onset of oestrus–ovulation intervals
Herd 1: Hülsenberg Research Station, Germany; \( n = 483 \) sows (Weitze et al., 1994)
Herd 2: Wageningen University, Netherlands; \( n = 151 \) sows (Soede et al., 1995a)
Herd 3: Commercial Farm, Denmark; \( n = 143 \) sows (Nissen et al., 1997)

| Oestrus intervals | Herd 1 | | | Herd 2 | | | Herd 3 | | |
|-------------------|六 | | | 六 | | | 六 | | |
| Weaning–oestrus (h) | 124 ± 94 | 67–240 | | 93 ± 18 | 65–153 | | 92 ± 13 | 64–134 | |
| Duration of oestrus (h) | 60 ± 15 | 33–153 | | 50 ± 13 | 24–88 | | 60 ± 14 | 30–89 | |
| Ovulation intervals | | | | | | | | | |
| Oestrus onset–ovulation (h) | 45 ± 13 | 19–120 | | 35 ± 8 | 10–58 | | 42 ± 11 | 17–68 | |
| Time of ovulation in oestrus (%) | 71 | 35–100 | | 72 ± 15 | 39–133 | | 71 ± 14 | | |

*Median time of ovulation for herd from beginning of oestrus, where 0% = beginning of oestrus and 100% = end of oestrus. Determined by ultrasound.

2.7.4. Insemination strategy adapted to oestrus behaviour

The interval from onset of oestrus to ovulation in the sow may vary from 19 to 120 h (Weitze et al., 1994). However, a significant relationship has been reported between the onset of oestrus after weaning, the duration of oestrus and the time of ovulation (Table 3), which suggests that an adaptation of the insemination strategy to oestrous behaviour of individual animals may be necessary. Sows can have early, regular and late oestrus after weaning. Those showing early oestrus after weaning usually have a long oestrous period, in contrast to sows having late oestrus after weaning which is of short duration. Sows with early oestrus should be inseminated later than those showing late oestrus, which should receive insemination immediately after detection of oestrus (Weitze et al., 1994).

Further knowledge on the influence of factors, like farm management, season and breed on the oestrous pattern and ovulation time of individual sows would allow the use of a more precise strategy for AI (Waberski and Weitze, 1996).

3. Storage of boar semen in frozen state

Boar semen differs in several aspects from the semen of other domestic animals. It is produced in large volumes and is extremely vulnerable to cold shock or sudden cooling immediately after collection. These and other characteristics of boar semen require special consideration in the design of freezing protocols (Pursel and Johnson, 1971, 1975; Paquignon and Courot, 1976; Larsson et al., 1977; Westendorf et al., 1975; Larsson, 1978).

It is not the intention of this chapter to describe in detail the freezing procedures developed in past decades and used with varying success. Reviews on the subject have been published by Einarsson (1973), Graham et al. (1978), Watson (1979), Johnson (1985) and Bwanga (1991). Here the freezing methods will be discussed in general, focusing on the factors in the freezing protocol which could be readily modified and
thus influence directly the success of frozen storage. In addition to the reviews mentioned above, the proceedings of the international conferences on boar semen preservation provide further detailed information on the subject.

Successful freezing of boar semen depends on an understanding of the factors and their interactions which influence the capacity of spermatozoa to survive freezing and thawing. The factors may be classified into two categories: (1) internal or fixed factors, such as the inherent characteristics of spermatozoa, and differences between boars and ejaculates, and (2) external factors, such as composition of diluents, type and concentration of cryoprotective agents, rates of dilution and of cooling, equilibration, and method of freezing and thawing of semen.

The external, unlike the internal, factors can be manipulated or modified in order to “optimize” the freezing protocol. In any protocol, rapid cooling of spermatozoa from body temperature to temperatures close to freezing irreversibly reduces viability of spermatozoa. Under such conditions, spermatozoa lose their membrane integrity and motility with irreversible reduction of carbohydrate metabolism (Watson, 1981; Watson and Morris, 1987). Rapid cooling also results in release of intracellular enzymes (Pursel et al., 1970; Pursel and Johnson, 1974) and lipids (Darin-Bennett et al., 1973) and a redistribution of ions (Hood et al., 1970). The magnitude of the cold shock depends on the rate of cooling and on the final temperature to which semen is cooled (Morris and Watson, 1984). For more details on cold shock, see the section on liquid storage.

Boar spermatozoa may acquire resistance to cooling simply by incubation at ambient temperature for several hours (Pursel et al., 1973). The effect is temperature dependant and the environment of seminal plasma is not essential, but the viability of spermatozoa is improved when incubated in its seminal plasma. After 2–7 h incubation most spermatozoa develop resistance to cooling (Pursel et al., 1973; Butler and Roberts, 1975).

Egg yolk, which provides protection against cold shock to spermatozoa of different domestic animals, does not give the same level of protection to boar spermatozoa (Benson et al., 1967). However, its protective effect can be improved by adding Orvus Es Paste (OEP) to the extender (Graham et al., 1971; Pursel et al., 1978). OEP (now known as Equex Stm; Nova Chemical, Scituate, MA), a synthetic detergent based on sodium and triethanolamine lauryl sulphate, has been included in most egg yolk-containing diluents for freezing of boar semen (Pursel and Johnson, 1975; Westendorf et al., 1975). It has been suggested that OEP gives protection by modifying the egg yolk constituents of the diluent (Pursel et al., 1978; Strzezek et al., 1984), as it has protective qualities only when used in combination with egg yolk.

Some other compounds reported to alleviate cooling injury are the antioxidants ditret-butyl-kresol (DTBK), echinochrome (an extract of sea urchins) (Golyshev, 1985) and butylated hydroxytoluene (BHT; Graham and Hammerstedt, 1992). The longevity of boar spermatozoa was increased by the presence of low concentrations of BHT (Bamba and Cran, 1992). However, BHT was effective only when spermatozoa were subjected to a slower rate of cooling to 5°C.

Based on the results of other species (Fiser and Fairfull, 1984) and also reported for cold shocked pig spermatozoa, the damage by cooling seems to be independent of the freezing damage, that is the spermatozoa which survive cold shock regardless of its
severity, have the same chance to withstand freezing and thawing as those not subjected to cold shock. However, it should be born in mind that the proportion of spermatozoa surviving a severe cold shock is low.

### 3.1. Diluents and cryoprotectants

During the past three decades, many diluents have been elaborated, most for a particular processing protocol, so that generally diluents are not transferable from one method to another (Osinowo and Salamon, 1976a,b). In general the diluents are composed of sugars, proteins and lipoproteins, buffers, additives and cryoprotective agents, and may be divided, based on their constituents, into two categories: (1) diluents without buffers, such as egg yolk–glucose (Baier, 1962; Polge et al., 1970), egg yolk–lactose, (Richter et al., 1975; Westendorf et al., 1975), egg yolk–saccharose–EDTA, Mg and Ca salts (Milovanov et al., 1974), and (2) diluents with buffers such as glycine–phosphate and glucose–phosphate (Iida and Adachi, 1966), egg yolk–glucose–citrate (Serdiuk, 1970), egg yolk–glucose–citrate–EDTA–potassium–unitol–urea (Shapiev et al., 1976), Beltsville F3 (BF3; Pursel and Johnson, 1971), Beltsville F5 (BF5; Pursel and Johnson, 1975), Tes-tris-fructose–citrate–egg yolk (TEST; Graham et al., 1971), Tes-NaK–glucose–egg yolk (Crabo and Einarsson, 1971; Larsson et al., 1977), Tris-fructose–EDTA–egg yolk (Salamon and Visser, 1973) Tris-glucose–EDTA–egg yolk (Park et al., 1977).

Glycerolated diluent portions are usually added when the partially diluted semen has been cooled to 5°C (Almlid and Johnson, 1988). Although the composition of diluents varies, most are characterised by a low concentration of glycerol and short equilibration time (Wilmut et al., 1973; Pursel and Johnson, 1975; Westendorf et al., 1975). Almlid and Johnson (1988) exposed diluted semen to glycerol from 0.5 to 75 min and observed no difference in the respective cryosurvival. The semen–glycerol equilibration time was re-visited recently (Fiser and Fairfull, 1996) for semen packaged in 0.5 ml straws and protected with various glycerol levels (0–6%), with surprising results. Longer times of exposure at 5°C were beneficial, with 4 h equilibration producing the highest percentage of motile spermatozoa with a normal apical ridge. As semen with no glycerol benefitted from the exposure to 5°C similar to glycerolated semen, it seems likely that the holding time at 5°C is solely responsible for changes in sperm membranes and may render them less susceptible to freezing damage. It seems unnecessary for boar semen to be equilibrated with glycerol, but only exposed to a temperature of 5°C. In this case, glycerol can be added any time before freezing, as glycerol penetrates rapidly into spermatozoa (Wilmut and Polge, 1974; Almlid and Johnson, 1988).

Many cryoprotective agents have been tested, but none have proved better for preserving boar spermatozoa than glycerol (Watson, 1995). From the other compounds tested, only exytritohol, xylitol, adonitol, acetamide and DMSO, in relatively low concentrations, improved the post-thawing motility of boar spermatozoa, but the proportion of sperm with intact acrosomes decreased (Paquignon, 1985). Thus, glycerol has been used extensively for protection of boar spermatozoa, and in general it is the cryopreservative of choice for the semen preservation of most mammals. However, boar spermatozoa show greater sensitivity than spermatozoa of other domestic animals to glycerol levels adequate for optimum cryopreservation (Almlid and Johnson, 1988).
The concentration of glycerol required for maximum survival of spermatozoa is determined by several considerations. One of them is cooling velocity (freezing rate). When static liquid nitrogen vapour and dry ice were used for freezing, the volume and the geometry of the semen sample influenced the rate of freezing and the glycerol concentration used. However, it is generally accepted that only relatively low concentrations of glycerol may be used in order to obtain satisfactory post-thaw survival (Pursel and Johnson, 1975; Westendorf et al., 1975; Paquignon and Courot, 1975; Scheid et al., 1980). Levels of glycerol necessary for adequate cryoprotection (Mazur, 1985) were reported to be detrimental to fertilisation by boar spermatozoa (Crabo and Einarsson, 1971; Wilmut and Polge, 1974) and resulted in damaged acrosomal membranes and altered membrane permeability (Watson, 1981).

3.2. Rate of cooling and its interaction with the concentration of glycerol

At present, two freezing methods developed in the mid-1970s, are commercially used for boar semen: the Beltsville and Westendorf methods. In the former method, Pursel and Johnson, 1975 the sperm-rich fractions of the ejaculates are held for 2 h after collection in the presence of seminal plasma and then, after centrifugation, cooled about 3 h, the semen is pelleted on dry ice (0.15–0.20 ml/pellet).

In the Westendorf method (Westendorf et al., 1975), which has been modified for commercial use (Almlid and Johnson, 1988; Almlid and Hofmo, 1996) the semen is diluted (1:2, semen:diluent and cooled) in Hülsenberg 8 diluent and subsequently frozen in maxi-straws.

The method of Visser and Salamon (1979), modified by Maxwell and Salamon (1979), also merits attention. The sperm rich fraction of the ejaculate is not held prior to processing over a several hour period; after processing and cooling, the semen is pelleted on dry ice. The method has not been used commercially.

The above packaging systems (pellets and maxi-straws) allowed studies on the effect of glycerol concentration on cryosurvival of boar spermatozoa, but the investigations of the effect of freezing and thawing rates were limited. The geometry of the maxi-straw, with its large gradient of temperatures, did not allow objective assessment of fast cooling rates, while the pellet allows only slight modification of freezing rates by changing its volume to surface ratio, as the surface of dry ice is of constant temperature (Fiser and Langford, 1980). Weitze et al. (1987) reported that the boar semen packaged in maxi-straws froze 3.75 times faster at the periphery than in the centre, resulting in significantly lower survival compared to semen in straws of smaller diameter.

The control of cooling and warming rates can be improved by using packages with a larger surface to volume ratio (Weitze et al., 1988), which will allow faster rates and minimise the temperature range within the package (Fiser and Fairfull, 1990). Therefore, new designs of semen packaging have been developed, such as 2 ml “flat straws” (Weitze et al., 1988; Ewert, 1988) or 5 ml plastic bags (Rodriguez-Martinez et al., 1996). Neither of these systems are currently in use.

The optimum concentration of glycerol required for best protection of spermatozoa is determined by several factors. One of them is cooling velocity. When a sperm suspension is cooled below the freezing point of the solution, the spermatozoa and the
diluent became increasingly supercooled until ice starts to form. The contents of the spermatozoa remain unfrozen and supercooled as the membrane system blocks the expansion of the ice. As the temperature decreases, supercooled cellular water, in response to the increased differential osmotic pressure, diffuses out of the cell and freezes. The rate of exosmosis is influenced by fixed cellular factors, in particular by the permeability of the cell membrane to water and glycerol at specific temperatures, the cell volume and the volume to surface ratio. The external factors that can be manipulated to regulate exosmosis are freezing rate and the concentration of the cryoprotectant. If the freezing rate is slow enough, the spermatozoa will be capable of losing their freezable water by exosmosis and thereby avoid supercooling; they dehydrate and no internal ice forms. If the spermatozoa are frozen rapidly, the intracellular water does not leave the cell before it freezes. According to the two factor theory of Mazur et al. (1972), damage to the cell can result from two principal causes: dehydration at suboptimum rates, also known as the solution effect, involving exposures of cells to high concentrations of solutes, pH changes and solute precipitation (Lovelock, 1953), and by intracellular ice formation when the semen is frozen too rapidly. The optimum freezing rate usually represents a compromise between these two extremes (Mazur, 1970). However, most studies on freezing of boar semen have used a fixed freezing protocol and thus only the effect of glycerol concentration was evident (Pursel et al., 1972; Scheid et al., 1980; Almlid and Johnson, 1988).

The importance of the interaction between glycerol concentration (Almlid and Johnson, 1988) and cooling velocity has been recognised in the case of boar spermatozoa by Fiser and Fairfull (1990). The authors demonstrated an inverted U-shape survival curve for boar spermatozoa frozen over a range of cooling rates from 1°C to 1500°C/min. The interaction between glycerol concentration and cooling velocity in relation to the percentage of motile spermatozoa indicates that the optimum cooling velocities shift to higher values with the decreasing glycerol concentration. At each glycerol level, the spermatozoa tolerate a range of cooling velocities without appreciable changes in survival. It was also shown that optimum glycerol concentration varied with the parameter investigated. Thus for motility, the best glycerol concentrations were 3–4%, whereas for acrosomal integrity (NAR), the optimum concentration was 0–1%. Contrary to glycerol concentration, the best cooling velocity for both parameters (motility and NAR) was 30°C/min. Therefore, for preservation of both components of spermatozoa function the authors recommended a compromise with 3% glycerol and cooling rate of 30°C/min. These conditions are similar to those recommended by Almlid and Johnson (1988) in a study on the effect of glycerol concentration on the viability of spermatozoa after freezing of semen packaged in 1.3 ml straws at 20°C/min. The latter authors also reported acrosomes damaged by concentrations of glycerol as low as 1–2%. These observations contribute to the growing body of evidence that the two factor theory of cryoinjury applies also to boar spermatozoa (Watson, 1990, 1995).

A relationship between concentration of cryoprotectant and cooling rate exists for most cell types (Mazur, 1985). The gradual decrease in motility and acrosome integrity of boar spermatozoa as the glycerol concentration exceeds 6% may be due to the chemical toxicity of glycerol, or to osmotic shock by rapid dilution of the glycerol at thawing (Bamba and Cran, 1988). Most studies on membrane permeability have been
conducted with simple models, such as red blood cells (Leibo, 1976). However, the spermatozoon has a more complicated structure than the erythrocyte, comprising a several geometries and different regions. Therefore, the optimum conditions for one part of the cell may be unsuitable for other regions, or damage to some parts may have greater consequences than to others.

The lipid composition and the organisation of sperm membranes changes during rapid cooling (Buhr et al., 1994) resulting in increased membrane permeability (Ortman and Rodriguez-Martinez, 1994). There has always been a suspicion, and now there is growing evidence, that frozen–thawed spermatozoa display characteristics of capacitated spermatozoa (Watson, 1996). This has also been noted when spermatozoa have been subjected to other types of insult, such as cell sorting in the presence of a fluorescent stain (Johnson, 1991). Maxwell et al. (1997) have shown that there are significantly more acrosome reacted spermatozoa after, than before the sorting procedure.

3.3. Induced ice nucleation and its effect on boar spermatozoa

The lack of induced ice nucleation (seeding) in semen processing procedures often has been criticised by cryobiologists. This technique could eliminate the damage from the excessive supercooling, crystallisation of cellular water and growth of ice crystals by aggregation and fusion after spontaneous nucleation, particularly in straws cooled slowly. Without external seeding the semen sample will supercool well below its freezing point to $-15^\circ$C (Watson, 1995). Nevertheless, the elimination or minimization of supercooling by induced nucleation at temperatures slightly below the freezing point of the sperm suspension did not improve the cryosurvival of boar spermatozoa (Fiser et al., 1991). The induced nucleation had no effect on post-thaw survival of spermatozoa frozen at optimum rates and only slight improvement in acrosomal maintenance was observed in boar spermatozoa frozen at suboptimum rates. This slight improvement is similar to that reported by Pursel and Park (1985) for boar semen frozen in maxi-straws which, due to their geometry, cannot accommodate optimum (higher) cooling rates. There is no practical value in this improvement, because of the overriding effect of suboptimum cooling rate which decreases the motility and acrosome integrity of spermatozoa to approximately half of the respective values for semen cooled at the optimum rate, regardless of induced nucleation. The reasons that the survival of boar spermatozoa in particular, and of mammalian spermatozoa in general, does not appear to be affected by seeding to the degree observed for mammalian embryos (Whittingham, 1980) may be due to differences in their sensitivity to glycerol and cooling rate. Moreover, glycerol levels used for freezing of spermatozoa (Watson, 1995) are considerable lower than those adopted for embryos (Whittingham, 1980; Wilmut, 1986). Thus, embryos in 0.25 ml straws would be supercooled to lower subzero temperatures than would spermatozoa. The increased rate of cooling after spontaneous nucleation is tolerated quite well by spermatozoa which require higher cooling rates (Fiser and Fairfull, 1990) for best survival, but it is lethal to embryos (Leibo et al., 1978) in which it causes intracellular freezing due to the insufficient removal of intracellular water by exosmosis (Mazur, 1985). Nevertheless, it should be kept in mind that “live spermatozoa” at $-196^\circ$C can be easily killed or damaged by the incorrect thawing (warming) procedure.
3.4. Thawing of semen

The rate of thawing through the critical temperature range is an important factor affecting survival of spermatozoa. Various methods of thawing boar semen have been described and are summarised in the review by Bwanga (1991). Boar semen has been frozen mostly as pellets on dry ice, but more recently in maxi-straws using static liquid nitrogen vapour. Survival of spermatozoa after rapid thawing of pellets in preheated diluent was superior to that obtained with spermatozoa packaged in maxi-straws in which, because of their diameter, a fast thawing rate could not be achieved effectively (Salamon et al. 1973; Weitze et al., 1987). The effectiveness of thawing rate depends on the original rate of freezing (Mazur, 1985). This interaction, which greatly influences the survival of any mammalian spermatozoa, was assessed for boar semen by Fiser et al. (1993), who recognised the limitations of packaging in pellets or maxi-staws, and employed 0.5 ml plastic straws cooled by a flow of liquid nitrogen vapour, using a programmable freezing system, to control freezing rate. The rate of thawing resulted from exposure of straws to air or water of various temperatures for a specific time. In boar semen frozen at the optimum rate (Fiser and Fairfull, 1990), the motility and acrosome integrity of spermatozoa improved with increasing thawing rate; similarly, faster thawing rates were beneficial for pellets (Salamon et al., 1973; Pursel and Johnson, 1976). This is consistent with the idea that cryoinjury after rapid freezing is caused predominantly by the regrowth of minute ice crystals during slow thawing and that a high warming velocity is essential for cryosurvival (Mazur, 1985). The effect of thawing rate on the acrosome is influenced by the glycerol concentration, higher concentrations being more damaging to acrosome integrity (Almlid and Johnson, 1988). It should be noted that only a small proportion of spermatozoa frozen at suboptimum rates survive after slow or rapid thawing. This suggests that boar spermatozoa are damaged critically by slow cooling and only a limited rescue can be achieved by the “optimum” thawing rate. Effective freezing requires the optimum combination of all three variables (glycerol concentration, cooling and warming velocities). Considering the maintenance of motility and acrosome integrity, the optimum represents a compromise: 3% glycerol, freezing rate (in the range between 0°C and −50°C) of 30°C/min, and a thawing rate of 1200°C/min.

The final temperature of rapid thawing seems to be of importance for boar spermatozoa. Bamba and Cran (1985) reported that fast warming of boar semen in the temperature range between 5°C and 37°C damaged acrosome membranes, resulting in undulation of the outer membrane with its invagination and the development of tubular networks within the acrosome. However, no release of enzymes or decrease of motility was observed. The magnitude of this so-called “warm shock” is influenced by the temperature range, suggesting that a phase change in the membrane lipids may occur (Bamba and Cran, 1988).

3.5. Fertility results of frozen–thawed boar semen

Attempts to obtain pregnancies with frozen–thawed semen before 1970 generally failed. Polge et al. (1970), using surgical insemination into the oviducts, explicitly demonstrated the fertilising capacity of frozen–thawed boar spermatozoa. In 1971,
Farrowings were reported after cervical insemination with thawed semen (Crabo and Einarsson, 1971; Graham et al., 1971; Pursel and Johnson, 1975). At the present time, the indications are that frozen semen may give up to 50% farrowing rate and about seven pigs per litter. This average was borne out by a study conducted on 36 farms in the Netherlands in 1978, where the average farrowing rate and litter size from single frozen–thawed (pellet method) and fresh semen inseminations respectively, were: 47% and 7.4 pigs per litter and 79% and 10.6 pigs per litter (Johnson et al., 1981). The study also showed a significant breed effect, with better “freezing” results produced by Dutch Large White than Dutch Landrace semen. In a review of nearly all the published fertility results conducted with frozen semen from 1970 to 1985, the average farrowing rates (litter sizes) were 55% (8.3) and 58% (9.0) for the pellet and straw methods, respectively (Johnson, 1985). Results of commercial use of frozen boar semen over a five year period in Norway indicate a 48% farrowing rate with 10.4 pigs per litter (Almlid and Hofmo, 1996), using the modified maxi-straw method of freezing and double inseminations. In order to improve the farrowing rates and litter sizes with frozen semen, it is necessary to inseminate as close to ovulation as possible. Current commercial practice utilises double inseminations: the first about 30 h after detection of oestrus and the second 10 to 12 h later.

Frozen semen is not used generally for production of pigs sold for slaughter because of the lower farrowing rate and litter size obtained after insemination with frozen–thawed compared with fresh semen (Johnson et al., 1981; Johnson, 1985). This limits the progress toward more intensive use of AI with frozen–thawed semen worldwide. Nevertheless, frozen semen is the vehicle for international transport of genetic material. The world’s pig population is being consistently upgraded through the utilisation of frozen semen, while slaughter pig production is improved locally through the use of liquid-stored semen. However, the usefulness of frozen-stored semen from boars of high genetic merit for repopulation after natural disasters, such as serious disease outbreaks, cannot be underestimated.

4. Examination of spermatozoa after fluorescent staining methods

Fertilising potential is directly related to the functional capacity of spermatozoa. Researchers have sought for generations to use in vitro technologies to measure the fertilising ability of spermatozoa. Examination of motility and acrosome integrity are still the current standards for assessment of boar spermatozoa in vitro, notwithstanding the knowledge that boar spermatozoa that survive freezing can be motile but do not necessarily fertilise (Polge, 1956). However, new technologies have come to the fore which utilise fluorescent dyes that bind to various regions of the cell to demonstrate particular functional characteristics of spermatozoa. The technologies that could give greater reliability to the estimation of the fertilising capacity of spermatozoa are described below.

4.1. Assessment by microscopy after staining with fluorescent stains

Fluorescent staining of boar spermatozoa to determine viability of large numbers of cells was adapted to flow cytometry by several laboratories in the 1980s (Resli et al.,
1983; Johnson and Garner, 1984; Garner et al., 1986). Resli used fluorescein diacetate (FDA), others used 6-carboxy fluorescein diacetate (CFDA) or more recently 6-carboxy methyl fluorescein diacetate (CMFDA; Molecular Probes, Eugene, OR). The latter two stains tend to be more stable than the original FDA. In this system, CFDA is converted by esterase enzymes in the live spermatozoa to a non-permeant fluorescent compound that is retained in the plasma membrane of living spermatozoa. When used along with propidium iodide (PI), CFDA or CMFDA is an effective indicator of viability of fresh and/or for frozen–thawed spermatozoa. Almlid and Johnson (1988) found these combined stains with flow cytometry useful for monitoring membrane damage in frozen–thawed boar spermatozoa during evaluation of various freezing rates. There are normally three populations: live-green positive, dead-red positive and a third population which are stained with both and represent dying spermatozoa. This process can be used effectively with flow cytometry where 10,000 spermatozoa may easily be evaluated in less than a minute. It can be used also for microscope examination. This staining system has the disadvantage that it is time dependent when samples must be run at a set time.

4.2. Assessment of viability by flow cytometry after staining with SYBR-14 and PI

A newly developed membrane-permeant nuclear stain was released for testing in 1993 by Molecular Probes. The stain, known as SYBR-14 and sold in kit form with PI by the developer as Fertilight, stains the DNA of living cells. The PI stain is used as a counterstain since it penetrates the plasma membranes of dead spermatozoa in several species (Garner and Johnson, 1995). SYBR-14/PI works effectively as a live/dead stain for either fluorescence microscopy or flow cytometry. Flow cytometry is the preferred method since large numbers of spermatozoa can be analysed. The usefulness of the stain has been demonstrated for several species (Garner et al., 1994; Garner and Johnson, 1995). Fig. 1 illustrates the strong correlation between spermatozoa stained for SYBR-14 (living) and those spermatozoa stained by PI (dead). In boars, the live stained cells represents 80 to 90% of the sperm population. Fresh ram spermatozoa, on the other hand, tend to have a lower percentage of SYBR-14 staining (49%) than fresh boar spermatozoa. SYBR-14 staining provides a new tool for measuring the viability of spermatozoa based on the condition of the DNA within the nucleus and the apparent membrane potential of the spermatozoa. Its use in combination with PI is useful for determining within a population those spermatozoa that are live as well as dead. The advantages of the method are its relative rapidity and that the levels of both dyes (SYBR-14 and PI) can be measured in the same sample.

4.3. Assessment by microscopy after staining with exclusion dye

Fixing and subsequent staining of spermatozoa with fluorescent dye has proven effective according to the report of De Leeuw et al. (1991). The report described the use of Hoechst 33258 to stain spermatozoa after they had been fixed with glutaraldehyde. Hoechst 33258 stains dead or damaged spermatozoa, since the stain will not penetrate the membranes of living cells. A count of the proportion of dead cells can be obtained after treatment of spermatozoa with fixative (generally 2% glutaraldehyde) and stain (10
Fig. 1.

μg per ml Hoechst 33258 final concentration) as a wet mount and then counting about 200 cells per slide under a phase contrast microscope equipped with epi-fluorescence. This method can also be adapted to flow cytometry. However, flow cytometric analysis requires a laser that operates in the ultraviolet light range which is not a standard feature on the smaller analytical machines.

4.4. Assessment by microscopy or flow cytometry after staining with CTC or FITC-PSA

The fluorescent antibiotic chlortetracycline (CTC) and the fluorescent lectin (FITC-conjugated agglutinin derived from Pisum sativum, FITC-PSA) have been utilised in studies to identify the degree of destabilisation of the sperm membrane (Maxwell and Johnson, 1997a,b). The CTC assay, first described for mouse spermatozoa by Saling and Storey (1979), is based on the transfer of neutral and uncomplexed CTC across the cell membranes. The CTC enters intracellular compartments containing high levels of free calcium, ionizes to an anion and binds to the calcium becoming more fluorescent as a result (Tsien, 1989). This CTC–Ca\(^2^+\) complex preferentially binds to hydrophobic regions of the cell membrane resulting in a pattern of membrane staining characteristic of various transitional phases of destabilisation, indicated in the literature by the letters F (uncapacitated, acrosome intact), B (capacitated, acrosome intact) and AR (acrosome reacted). A flow-cytometric assay for capacitation has also been developed based on CTC-fluorescence (Maxwell and Johnson, 1997a). The CTC assay based on fluorescence microscopy requires the manual counting of relatively low numbers of cells, and their subjective classification according to the F, B and AR patterns. This has potential disadvantages such as lack of precision due to the number of cells assessed, and the subjective nature of the assessment. The flow cytometric approach allows rapid counting.
of large numbers of cells (10,000) based upon the intensity of their fluorescence alone. Although this lacks the precise delineation of specific fluorescence categories, it does allow for a progressive increase in fluorescence, which might be associated with membrane destabilisation in the process of capacitation.

The integrity of sperm membranes can be assessed also by using FITC-PSA (Maxwell and Johnson, 1997b), which is reported to bind specifically to the acrosomal contents (Cross et al., 1986). Graham et al. (1990) established that fluorescently labelled PSA could be used in flow cytometry to assess the percentage of cells with or without intact acrosomes based on comparisons with naphthol yellow/erythrosin B, and an assessment of the percentage of cells with intact acrosomes when the acrosome reaction was induced with lysophosphatidylcholine. In order to detect changes taking place specifically in the live sperm population, Maxwell and Johnson (1997b) stained the spermatozoa with PI, and the PI-positive cells were excluded from the estimate of acrosome intact (low) and reacted (high FITC fluorescence). The proportion of acrosome reacted spermatozoa estimated by FITC-PSA fluorescence tended to be higher than by the CTC method (compare Fig. 2a and c), but as yet the acrosome status has not been confirmed in these spermatozoa by microscopy. Fig. 2 shows the membrane integrity of fresh spermatozoa after incubation (38°C, 4 h) and after cooling to 5°C at 0.25°C/min as assessed by (a) microscopy after CTC staining, (b) by flow cytometry after staining with CTC or (c) with FITC-PSA. These treatments increased the percentage of B pattern (capacitated) cells and of spermatozoa with high CTC fluorescence in comparison with fresh, uncapacitated (F pattern) cells. Freezing and thawing and flow cytometric sorting of boar spermatozoa into X and Y populations also causes a capacitation type response (Johnson, 1991; Maxwell and Johnson, 1997b). In other experiments, Maxwell and Johnson (1997a) observed percentages of F (uncapacitated) and B (capacitated) pattern cells of 84.0 vs. 37.6% and 8.0 vs. 44.8% for fresh vs. flow-sorted spermatozoa, respectively. Whether these membrane changes after flow cytometry may be considered akin to normal capacitation still remains to be proven. Satisfactory cleavage has been achieved after in vitro fertilisation of in vivo matured oocytes without further incubation of boar spermatozoa sorted for sex containing a significant proportion (55%) of B- and AR-pattern spermatozoa as assessed after staining with CTC (Maxwell et al., 1999).
The tests of membrane integrity described above may provide valuable information in the assessment of commercial semen processing methods.

4.5. Concluding remarks on semen evaluation

Assessment of the functional capacity of spermatozoa in vitro is of particular interest to the AI industry in order to select sires with good fertility. Evaluation of semen generally has been based on microscopic examination of motility and morphology of spermatozoa. Frequently, eosin stain has been used to determine the proportions of live and dead spermatozoa, while giemsa stain has often been used for morphological evaluation. These methods, along with visual inspection of the semen, are useful but their repeatability is questionable. Fluorescent staining improves microscopical evaluation of spermatozoa, but its major drawback is that only 100 or 200 spermatozoa can be evaluated per sample. The advent of flow cytometry for spermatozoa analysis in the 1980s, and the development of new stains in recent years, has led to more accurate means for assessing the viability of spermatozoa. However, finding one indirect test to evaluate the fertilising potential of spermatozoa continues to be elusive. The use of several tests, in combination, such as motility, acrosome integrity and a fluorescent assessment of membrane integrity of spermatozoa, would add significant credibility to evaluation of semen in the laboratory.

References


dilution and flow cytometric sorting in the presence or absence of seminal plasma. Reprod. Fertil. Dev. 8, 1165–1178.


