Acta vet. scand. 1991, *32,* 431–453.

Cryopreservation of Boar Semen
I: A literature review

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Bwanga, C. O.: Cryopreservation of boar semen. I: A literature review. Acta vet. scand. 1991, 32, 431–453. – The present review summarizes information concerning the methods available to cryopreserve boar semen, covering the historical background, cryobiology and cryoprotecting considerations, technological developments and recent advances in cryopreservation methodologies. Successful methods for cryopreservation of boar semen have not been achieved despite numerous efforts world wide. Improvements in semen preservation technologies have been deterred by lack of in vitro methods that can accurately predict in vivo fertilizing capacity of frozen boar semen. The cell membrane is of crucial importance with regard to freeze-thaw survival of spermatozoa. It is important to optimize the survival of the plasma membrane as this is a non homogenous entity both in structure and function. The boar sperm membrane exhibits extreme sensitivity to freezing treatment. Freezing and thawing results in considerable changes in electrolyte dynamics and damages have mainly been associated with alterations in the head membranes especially at thawing. To date fruitless efforts have been carried out to find a cryoprotectant for the spermatozoa membranes and glycerol still continues to be used despite its harmful effects to the membranes.

deep freezing; thawing; membrane.

Introduction
At present, deep frozen boars semen is used on a very limited scale owing mainly to its lower fertility results when compared with fresh semen. Therefore, use of deep frozen boar semen has been mainly confined to the introduction and/or improvement of superior genetics in pigs. It is of considerable importance to appreciate the future role that deep frozen boar semen would play such as in breeding programmes with progeny testing, a proper timing of insemination times with semen available at the time of need and the use of semen in areas that are geographically far apart from Artificial Insemination (A.I.) centres. This is however, hampered by the unsatisfactory attempts towards the development of a reliable technology for cryopreservation – which has ultimately resulted in a limited positive impact on the swine industry.

The present review aims to highlight a range of aspects embracing the historical background, cryobiology and cryoprotectant considerations, technological developments and recent advances in cryopreservation methodologies of boar semen.

Historical background
The first recorded observation regarding semen preservation (*Spallanzani* 1976), noted that when human, stallion, and frog spermatozoa were cooled in snow for up to 30 min they became inactive but could be revived; and thus from the first experiment, temperature reduction was employed to depress me-

tabolic activity and prolong the active life of spermatozoa. A century later, *Mantegazza* (1866), observed that human spermatozoa survived in semen frozen to −17° C, and this ranks as one of the earliest reports of the recovery of mammalian cells after exposure to a temperature below their freezing point.

The magnificent discovery of the cryoprotective action of glycerol (*Polge et al.* 1949) opened up an era of successful cryopreservation of not only gametes from various species, but also other cells and tissues. Fertility reports with deep frozen bull spermatozoa (*Stewart* 1951) led to intensive development, in the ensuing decade, of cryopreservation methods that would be applicable for practical insemination purposes. Also research efforts aimed at developing a method for deep freezing of boar semen. Consequently, successful viability of frozen-thawed boar spermatozoa was reported (*Polge* 1956, *Hoffman* 1959, *Hess et al.* 1960, *Dukelow & Graham* 1962, *Bader* 1964, *Iida & Adachi* 1966, *King & MacPherson* 1966, *Kojima et al.* 1967, *Rohloff* 1967, *Bamba et al.* 1968).

Fertility trials were then carried out with frozen-thawed boar spermatozoa (*Settergren* 1958, *King & MacPherson* 1967, *Dalrumple & MacPherson* 1969) but with discouraging results. Nonetheless, although as a rule, fertility was very low, sporadic pregnancies were obtained (*Hoffman* 1959, *Baier* 1962, *Waide et al.* 1969).

Polge et al. (1970) explicitly demonstrated the fertility of frozen-thawed boar spermatozoa by inseminating surgically into the oviducts of gilts. However, no fertility was reported when thawed spermatozoa were inseminated intracervically.

Shortly afterwards, 3 methods for deep freezing boar spermatozoa – with maintained fertility – after intracervical insemination were first presented by *Crabo & Einarsson* 1971, *Graham et al.* 1971a,b and *Pursel & Johnson* 1971. Further, and in succession, other modified successful procedures were reported by *Crabo et al.* (1972b), *Richter & Liedicke* (1972), *Salamon & Visser* (1972), *Vincente* (1972), and *Wilmut & Polge* (1972). The composition of the diluents and semen processing procedures varied widely, but all had in common – low levels of glycerol in the final dilution, a short equilibration time and freezing in pellets on dry ice (*Nagase & Niwa* 1963). A concise and comprehensive review of the early freezing methods and their practical application was presented by *Einarsson* (1973). The diluents incorporated buffers with low or medium ionic strength (*Graham et al.* 1978) based on earlier in vitro studies that demonstrated the great sensitivity of boar spermatozoa to buffers with high ionic compositions (*Crabo et al.* 1972a). In addition, the peculiar sensitivity of boar spermatozoa to osmotic changes prompted the adjustment of osmotic pressure and pH to values found in boar semen (*Einarsson* 1971).

Thawing procedures, similarly varied with the techniques. Some workers, however, thawed pellets in preheated solutions, firstly introduced by *Richter & Liedicke* (1972). The solutions among others were: seminal plasma (*Crabo & Einarsson* 1971, *Crabo et al.* 1972b, *Einarsson et al.* 1973), skim milk (*Einarsson et al.* 1972), Hulsenberg diluter (*Richter & Liedicke* 1972), *Paquignon & du Mesnil du Boisson* 1973) and TES-NaK-glucose-extender (*Einarsson et al.* 1972). Insemination experiments, provided evidence that thawing in seminal plasma comparatively yielded better pregnancy rates with normal litter sizes (for review, see *Einarsson* 1973, *Larsson & Einarsson* 1975). The practical application of seminal plasma was however, prohibitive.

None of these early methods was adapted to field use in pig A.I. It is of utmost import-

ance that both farrowing rate and litter size are directly comparable with the results obtained with fresh boar semen (*Einarsson* 1973). In that respect, only 2 methods were close to satisfying that notion (*Crabo & Einarsson* 1971, *Crabo et al.* 1972b).

Cryobiology

Advances in cryopreservation procedures of spermatozoa have played an outstanding role in the development of the present knowledge in cryobiology. Many evaluations of the influence of low temperatures have been based entirely upon some measurement of survival (*Polge* 1980); and this has enriched understanding of the factors and variables which govern whether a biological system either withstands decrease in temperature or succumbs to injury. The exact mechanism of cryoinjury or cryoprotection at the molecular and macromolecular levels is still ill-understood (*Taylor* 1987). Noteworthy, knowledge of the physico-chemical aspects of low temperatures is fundamental to the understanding of the nature of resistance to low temperature or cryoinjury and cell death (*Taylor* 1987).

Freezing and thawing undoubtedly results in cell membrane damage (*Healey* 1969, *Pace & Graham* 1970, *Crabo et al.* 1972a, *Graham et al.* 1972), and current research efforts aim at minimization and prevention of these damages (*Watson* 1979). The challenge to cells in cryopreservation is not the ability to remain viable at –196° C but rather the lethality of an intermediate zone of temperatures, about –15 to –60° C that a cell must traverse twice, once during cooling and again during warming (*Mazur* 1985). The major effect of temperatures on any system is the reduction of molecular motion (*Taylor* 1987) and these inbibitory effects of low temperatures provide the basic means for achieving long-term preservation of cells, tissues and organs (*Taylor* 1987). Temperature reduction to absolute Zero (–273.16° C) inhibits all reactions and molecular motion, while thermally driven reactions do not occur in aqueous systems at –196° C (*Mazur* 1985), and no changes of biological importance occur at temperatures below –150° C.

Cold shock

This phenomenon first described for bull semen (*Milovanov* 1934) and later for boar semen (*Lasley & Bogart* 1944) entails susceptibility of the cells to rapid cooling to a few degrees above 0° C. Cold shock not only occurs in spermatozoa, but it is recognised also in most cells exposed to low temperatures (*Amann & Pickett* 1987). The susceptibility of mammalian spermatozoa to cold shock varies with species (*Watson* 1981).

Cold shock is evidenced by the presence of many spermatozoa swimming in a circular motion, premature and irreversible loss of motility, decreased energy production, increased membrane permeability and loss of intracellular molecules and ions (*White & Wales* 1960, *Watson* 1981, *Watson & Plummer* 1985). Survival after cold shock is positively correlated with fertility after freezing and thawing (*Moroz et al.* 1980).

The peculiar sensitivity of boar spermatozoa to cooling (*Polge* 1956) presents a scientific and practical problem to be overcome when cryopreserving boar semen. The cellular damage emanating from rapid cooling to temperatures in the range of 0–10° or 15° C is remarkably widespread (*Morris & Watson* 1984), and subsequently an understanding of the stress associated with cooling and the responses of spermatozoa is of utmost importance in the development of protective methods (*Watson* 1985).

Polge (1956) found that the sperm rich fraction og boar semen tolerated slow cooling and was more resistant to cold shock than

the whole ejaculate. Boar spermatozoa are extremely sensitive immediately after ejaculation and will not survive slow cooling to below 15° C but they gain resistance during incubation (*Benson et al.* 1967, *Pursel et al.* 1970, 1972a,b, 1973a). Resistance to cold shock is an inherent characteristic of spermatozoa (*Pursel et al.* 1973). By 4–7 h post ejaculation 50–80 % of the cells have required resistance as judged by their motility and morphology (*Butler & Roberts* 1975, *Pursel et al.* 1972a,b, 1973a). The development of resistance during incubation is influenced by pH, dilution and extender composition (*Butler & Roberts* 1975, *Pursel et al.* 1972a,b, 1973a).

Cold shock particularly affects the acrosome, with loss of its contents, but it has also been associated with breakage or loss of the plasma membrane (*Pursel et al.* 1972a). *Pursel et al.* (1970) reported significant increase of both lactate dehydrogenase (LDH) and glutamate oxaloacetate transaminase (GOT) in the extracellular medium. Furthermore, on examination of leakage of glycolytic enzymes, *Harrison & White* (1972) found the release of glucose phosphate isomerase and LDH from boar spermatozoa to be comparable to that found in the bull and ram. Acrosomal changes were further reflected in a loss of acrosomal proteinase (*Johnson & Pursel* 1974). As a whole the loss of enzymes is linked to the decline in metabolic activity (*Watson* 1981). Cold shock also results in release of lipids, predominantly phospholipids, from boar spermatozoae (*Pickett & Komarek* 1967). The specific phospholipids released by boar and ram spermatozoa originate from acrosomal membranes (*Darin-Bennett et al.* 1973) but plasma membranes may also be involved. Profound cation disturbances occur in cold shocked boar spermatozoa (*Hood et al.* 1970) and the uptake or loss of ions is consistent with a breakdown in the barrier characteristics of membranes (*Watson* 1981).

Variation in susceptibility to cold shock is affected by several factors. Epidydimal boar spermatozoa are less susceptible to cold shock than ejaculated spermatozoa (*Lasley & Bogart* 1944) as is true for bull and ram spermatozoa. This is due to maturational changes that occur along the epidydimis. The lipid content of spermatozoa decreases markedly from the testis to the cauda epidydimidis (*Watson* 1981). A reduction in total phospholipid content occurs during epidydimal passage (*Pickett et al.* 1967, *Johnson et al.* 1972) and retention of choline plasmalogen (*Grogan et al.* 1966). The development of cold shock sensitivity is linked with the changing lipid composition of the spermatozoa membranes.

Seminal plasma has been associated with susceptibility to cold shock. *Moore et al.* (1976) showed that contact with seminal vesicular secretions increased the susceptibility to cold shock, probably due to the binding of basic proteins to spermatozoa cell membranes. Both protective and sensitising factors in seminal plasma are incriminated. These are derived from different accessory glands and hence present in different seminal fractions. *Pursel et al.* (1973a) found boar spermatozoa to be more susceptible to cold shock after dilution or washing and resuspension in a tris lactose diluent. On the contrary, lactose has been found to be protective (*Wilmut & Polge* 1977b, *Zorn* 1987). This suggested that removal of seminal plasma renders the cells more susceptible. Furthermore, additives like casein, and others protective for bull and ram spermatozoa, particularly egg yolk offered no beneficial effect upon boar spermatozoa (*Pursel et al.* 1973).

In conclusion, the importance of the final temperature to develop cold shock suggests

that lipid phase transitions are involved; and the extent to which membrane damage occurs during cooling and freezing depends on plasma membrane composition (*de Leeuw et al.* 1990). The lower the temperature the greater the proportion of lipids which have undergone a phase transition (*Watson* 1981, *Watson & Plummer* 1985).

Freezing and its effects

When a spermatozoa suspension is cooled below 0° C the first event is the formation of extracellular ice crystals resulting in an increased concentration of the solutes in the remaining liquid water (*Mazur* 1965, *Watson* 1979). At about –5° C the cells and the surrounding medium remain unfrozen due to supercooling and depression of the freezing point by added protective solutes. At a point between –5° and –15° C, depending on the cooling rate and extender, ice crystals begin to form in the extender. The cells however, remain supercooled, presumably because the plasma membrane blocks the growth of ice crystals from the medium into the cytoplasm (*Mazur* 1985). As extracellular water freezes, salts excluded from the ice crystals accumulate at increasing concentrations in the remaining unfrozen water. This increases the osmotic gradient across the plasma membrane. There occurs therefore a differential vapour pressure across the cell membrane with a tendency for water to move out of the cell (*Mazur* 1965). Therefore water inside the spermatozoa flows out and freezes externally.

A number of factors govern the ease with which water may move across the cell; its membrane permeability at a given temperature, the surface to volume ratio of the cell and the freezing or cooling rate. As permeability of cells is temperature dependent, the rate of cooling affects the time during which any water movement can occur in the cells. Hence, the need to define the optimum cooling rate for cell survival (*Mazur* 1963, 1977). Furthermore, a difference in water permeability coefficients occurs between cell types and between species (*Dick* 1966). If the cooling rate is sufficiently slow or the membrane is sufficiently permeable to water, the vapour pressure for water remains small. The cell then loses water rapidly by exosmosis in order to concentrate the intracellular solutes to maintain the chemical potential of intracellular water in equilibrium with that of extracellular water. Increased intracellular solute concentrations have been thought to be detrimental to spermatozoa membranes (*Farrant* 1980). Dehydration then occurs, water freezes extracellularly but the cell does not freeze intracellularly (*Mazur* 1965). If the cooling is very rapid the cells are rendered relatively impermeable to water, intracellular water cannot leave fast enough to maintain equilibrium, the vapour pressure increases and the cell is remarkably supercooled and intracellular ice formation occurs (*Mazur* 1977). When the cell contents are supercooled to about –10° C intracellular ice formation may occur (*Mazur* 1965). If the cooling rate is moderate or rapid, fairly large crystals of ice form and these may be injurious to the cells (*Mazur* 1985). However, if the cooling rate is extremely rapid, intracellular ice forms as microcrystals and damage from intracellular ice is greatly reduced (*Courtens & Paquignon* 1985, *Koehler* 1985). Cells that have dehydrated to reduce their internal water content to approximate that which will occur in the extracellular fluid after formation of ice, have either a low or no probability of undergoing intracellular ice formation (*Watson* 1979). Cells that are extensively supercooled when ice formation occurs, but contain a concentration of water considerably above the equilibrium of water in extracellular ice,

will have a high probability of forming intracellular ice leading to considerable cell damage (*Watson* 1979). If the residual water content of a cell is about 10 %, the residual water cannot freeze at any temperature (*Mazur* 1984). Supercooling of cells in itself is not thought to be detrimental as long as crystallization does not occur (*Pursel & Park* 1985). However, following ice crystal formation the temperature of the sample rises to the freezing point, as latent heat of fusion is released, and water is removed from solution so rapidly that cells are exposed to considerable osmotic stress, lethal to them.

Intracellular ice appears to be uniformly lethal (*Meryman* 1971), and large intracellular ice crystals cause damage and cell death. Contrary to this, ice microcrystals in spermatozoa have evidently not been found detrimental (*Courtens & Paquignon* 1985, *Koehler* 1985). Conversely, cells containing ice microcrystals must be warmed very rapidly to preclude recrystallization into large intracellular crystals that would damage cells (*Mazur* 1965). Hence, if cooling rate is rapid – as is generally accepted for boar spermatoza – the warming rate must equally be rapid (*Polge* 1976). Cooling of spermatozoa suspensions to about 5° C prior to initiation of freezing significantly promotes freeze-thaw survival in comparison to cooling to temperatures between 22 and 8° C (*Graham & Crabo* 1972, *Konanov* 1980, *Westendorf et al.* 1975). Generally, a cooling interval of 4 h between semen collection and cooling to 5° C has proved to be adequate (*Graham et al.* 1971a, *Crabo et al.* 1972b, *Pursel & Johnson* 1975).

The critical temperature zone for boar spermatozoa has been reported to be –15 to –25° C (*Hess et al.* 1960). Later studies have documented that the greatest proportion of cellular damage occurs at temperatures of –3 to –10° C, and substantially similar damage exists in temperatur range 0 to –3° C (after crystallization) and –10 to –20° C, during which ice crystal formation is noted (*Niwa & Taguchi* 1981, *Pursel & Park* 1985). However, only 15–25 % of the spermatozoa damage occurs between –20 and –80° C (*Pursel & Park* 1985).

It is agreed that supraoptimal rates lead to a drop in survival (*Mazur* 1977), but suboptimal rates have also been associated with substantial injury to cells as shown on mouse ova (*Leibo et al.* 1978), human lymphocytes (*Thorpe et al.* 1976) and on human red cells (*Farrant* 1972, *Rapatz & Luyet* 1978).

Regardless of the freezing rate, extracellular ice will form and this is associated with increase in the concentration of solutes in the extracellular medium. High concentrations of solutes in the extracellular medium was for a long time thought to be the primary cause of damage to cells (*Mazur* 1985) but this is probably not true. It has been observed that exclusively hypertonic solutions induce considerably less damage to suspensions of cells than slow freezing, suggesting that survival may be entirely dependent on the fraction of unfrozen water (with high survivals at about 15 %) in the extracellular environment (*Mazur et al.* 1981, *Mazur* 1984). It has further been postulated (*Mazur* 1985) that during slow freezing, cells are sequested in narrow channels of unfrozen solution between ice crystals and that these channels become progressively narrower as temperature is lowered. The expanding ice crystals reduce space for cells which results in damaging deformations as confirmed in frozen boar spermatozoa (*Courtens & Paquignon* 1985). Cell survival is thought to be correlated more with the fraction of unfrozen water than with other factors (*Mazur et al.* 1981). In conclusion, a correlation exists between cooling rates that result in

intracellular ice and cooling rates that are lethal to cells and survival of cells during freezing is maximum at a given critical value (*Mazur* 1970, *McGann et al.* 1981). Furthermore, since membrane damage plays a crucial role in cooling and freezing injury, agents that prevent intracellular ice formation and/or stabilize the membrane should be beneficial in preservation of spermatozoa (*de Leeuw et al.* 1990).

Cryoprotective agents

Cryoprotection denotes the preservation of cell structure and metabolism against injury associated with freezing events either within or around the cell. In the absence of cryoprotectants, very few spermatozoa of any species so far studied have been found to survive freezing to very low temperatures (*Polge* 1980). Cryoprotective agents greatly extend the tolerance of spermatozoa to freezing – and optimal cooling rate depends upon the nature and concentration of the cryoprotectant that is used (*Polge* 1980).

A wide spectrum of compounds have been shown to exert a cryoprotective effect (*Doebbler* 1966, *Meryman* 1971). Among them only glycerol, erythritol, xylitol, adonitol, acetamide, di-methyl sulphoxide (DMSO) in concentrations from 0.5 to 1M, improved the post thaw motility of boar spermatozoa but the percentage of normal acrosomes decreased (*Paquignon* 1985). Various investigations on cryoprotective agents have been carried out with limited success.

Cryoprotectants have been divided into those that penetrate the cell and those which remain extracellular. Glycerol-like substances (e.g. DMSO, other polyols) are generally considered as penetrating agents and together with other non-penetrating agents (e.g. various sugars, polyvinyl pyrollidone (PVP), have been evaluated for cryoprotective effect in boar spermatozoa (*Wilmut & Polge* 1977a,b,c). Limited benefits accrued from combinations of glycerol with other agents, and thus consistently acceptable results were only obtained with glycerol (*Salamon et al.* 1973, *Visser & Salamon* 1974). Attempts to substitute glycerol with polyvinyl-alcohol resulted in the death of spermatozoa (*Okolski & Jamer* 1988).

Since the demonstration of glycerol's cryoprotective efficacy (*Polge* 1949), attempts for direct adoption of its benefits as achieved in bull spermatozoa, have met with limited success in boar spermatozoa (*Polge* 1956, *Watson* 1979). However, most trials, have achieved maximal motility and fertility with glycerol (*Wilmut & Polge* 1972, 1977c, *Salamon et al.* 1973). These notwithstanding, earlier studies demonstrated its depressive effect upon fertility even in unfrozen boar spermatozoa though with minimal effects on motility (*Polge* 1956, *King & MacPherson* 1966).

Glycerol in high concentrations affets fertilizing capacity in both unfrozen (*Wilmut & Polge* 1974, *Neville et al.* 1970) and frozen boar spermatozoa (*Graham et al.* 1971a, *Wilmut & Polge* 1974, *Wilmut & Polge* 1977c). The optimal concentration of glycerol has not been established but relatively low values (1 to 3 %) give maximum post-thaw viability (*Pursel & Johson* 1975, *Westendorf et al.* 1975, *Wilmut & Polge* 1977b, *Scheid et al.* 1980). *Almlid & Johnson* (1988) found maximum post thaw viability with 3 or 4 % glycerol and on further study using *in vitro* finfings to predict fertilizing capacity, *Almlid et al.* (1989) suggested that 4 % rather than 2 % may be close to optimum. The reduction of glycerol's concentration – probably the major developmental step – has been utilized in various techniques of spermatozoa cryopreservation (*Westendorf et al.* 1975, *Pursel & Johnson* 1975,

Paquignon & Courot 1976, *Larsson et al.* 1977). However, *Polge* (1976) concluded that glycerol is not indispensable.
Dose-dependent studies have demonstrated that increased glycerol levels negatively affect acrosomal integrity, while the opposite is true for motility in the range of 0 to 7 % (*Graham & Crabo* 1972, *Pursel et al.* 1978a, *Scheid et al.* 1980). Moreover, comparing sperm frozen in 0, 1, 2, or 5 % both post-thaw motility and acrosome integrity decreased at 5 % glycerol concentration (*Graham & Crabo* 1972). Furthermore, glycerol alters the membrane permeability (*Bower et al.* 1973) oxygen comsumption is lowered (*Sanford et al.* 1972) survival is reduced after incubation at 37° C (*Paquignon & Courot* 1975) and the proportion of fertilized ova decreases even after surgical transfer (*Wilmut & Polge* 1977c).
Glycerol and other penetrating agents presumably act by their colligative or water-binding properties (*Mazur et al.* 1970, *Meryman* 1971), thus lowered the freezing point and improving cell survival (*Mazur* 1984). It is thought to be related more directly to membrane preservation of the head than the midpiece (*Murdoch & Jones* 1978). The mechanism by which glycerol exerts its cryoprotective action is yet unclear (*Mazur* 1985). This may be either intracellular or extracellular or both, but strong suggestions of a possible extracellular role have been presented (*Almlid & Johnson* 1988, *Almlid et al.* 1989).

Technological developments in the cryopreservation of boar semen

The freezing diluents

Various research groups have developed their own diluents for cryopreservation of boar semen (*Polge et al.* 1970, *Graham et al.* 1971b, *Crabo & Einarsson* 1971, *Visser & Salamon* 1974, *Pursel & Johnson* 1971b, 1975, *Westendorf et al.* 1975, *Larsson & Einarsson* 1976, *Paquignon & Courot* 1976). Diluents with buffers of varying composition have been tried with only a few proving usable in subsequent fertility tests (*Einarsson* 1973). The composition of extenders differs widely and only few systematic studies have been performed to determine the respective roles of various components (*Salamon et al.* 1973, *Visser & Salamon* 1974, *Wilmut & Polge* 1977a,b). The diluents have been developed for different deep freezing technologies and are technically not transferable between methods (*Osinowo & Salamon* 1976).
The freezing extenders may be divided into 2 different groups. Firstly, those that are without buffers: egg-yolk, glucose (*Polge et al.* 1970); egg-yolk, lactose, Orvus Es Paste (*Westendorf et al.* 1975). Secondly, those that comprise an organic tris (hydroxymethyl) amino methane buffer: Beltsville F3 (*Pursel & Johnson* 1971a); tris, fructose, E.D.T.A. (*Visser & Salamon* 1974); tris, glycine, glucose (*Obando et al.* 1984); tris, glucose, E.D.T.A. (*Park et al.* 1977); and others contain a Zwitterionic Tes-N-Tris (hydroxymethyl) methyl 2 amino ethane sufonic acid buffer: TEST (*Graham et al.* 1971a); Beltsville F5 (*Pursel & Johnson* 1975), TES NaK (*Crabo & Einarsson* 1971, *Larsson et al.* 1977).
Cellular damage appears to be dependent upon ionic strength of the diluents and maximum efficiency has been obtained from diluents with a low concentration of electrolytes (*Crabo et al.* 1972a, *Senegacnik et al.* 1980). Most of them are either isotonic or hypertonic with respect to seminal plasma, as *King & MacPherson* (1966) documented that spermatozoa can tolerate a wide range of osmotic pressures (280 to 420 mOsm/Kg H_2O). Sugars have been added as osmotic factors. The osmotic pressure and pH values

of the buffers have been adjusted to correspond as close as possible to normal parameters as found in the seminal plasma (*Einarsson* 1971). In unbuffered media egg-yolk provides the buffering capacity. In general, the diluents comprise the following majoı components: sugars, proteins or lipoproteins, buffers, cryoprotectant (mainly glycerol) and additives. In some cases seminal plasma has been included in the final medium (*Salamon & Visser* 1974).

Conflicting reports concerning the temperature of addition of glycerol to spermatozoa have been presented. *Wilmut & Polge* (1974) reported no adverse effects of similar concentrations (5 and 10 %) of glycerol stored for 6 h at 5° C or for 30 min at 20° C, but 6 h at 20° C reduced fertilization rate. *Maxwell & Salamon* (1979) using pellets found no difference in farrowing results when glycerol was added at either 30 or 5° C. *Westendorf et al.* (1975), using straws, detected no difference in post thaw motility whether glycerol was added at either 15 or 5° C; while *Almlid & Johnson* (1988), also using straws, documented that addition of glycerol at 0° C compared with 5° C had a negative effect upon post thaw sperm viability.

Information on the degree and speed of glycerol penetration of boar semen is limited. Shortterm pre-freeze exposure (*Wilmut et al.* 1973). *Cöster* (1978) reported that 5 min equilibration time at 5° C gave better sperm viability than 90 min equilibration. *Almlid & Johnson* (1988) however, detected neither a difference in .5, 2, 5, 15, or 75 min equilibration times nor an interaction with glycerol concentrations. These observations indicate that glycerol penetrates the spermatozoa within seconds and that equilibrium is reached within a short time (< 30 seconds) as earlier reported for boar spermatozoa (*Wilmut & Polge* 1974). This rapid permeation of glycerol is the basis of its short term exposure in current standard procedures. Glycerol's deleterious effects appear to be independent of the freezing method (*Scheid et al.* 1980). The harmful effects have been alleviated by partial removal by centrifugation prior to freezing (*Pursel et al.* 1978b).

Diluent additives

Sugars. They provide extracellular cryoprotection, due to their osmotic properties. Their combination with glycerol has been found indispensable for protection during freezing (*Salamon et al.* 1973). Glucose, fructose and lactose make up a large proportion of the diluent's ingredients. In several studies the efficacy of these sugars along with other mono-, di-, and trisaccharides have been compared but with inconclusive results (*Salamon et al.* 1973, *Visser & Salamon* 1974, *Wilmut & Polge* 1977b) since the properties of the added sugars appear to depend on the interactions with other components of the diluents. Sugars do not penetrate plasma membranes and probably act by either raising the percentage of unfrozen water at a given temperature or by reducing the concentration of salts in the unfrozen extracellular water (*Mazur* 1984). In the presence of glycerol (2–3 %), diluents with glucose (2–4 %) gave maximum motility (*Salamon et al.* 1973, *Senegacnik* 1976). However, in a diluent with a Tris buffer, fructose was more suitable than glucose or lactose (*Visser & Salamon* 1974). The protective affected accruing from a particular sugar depends on its concentration and the criteria of spermatozoa evaluation. High concentrations of glucose and fructose improve the proportion of spermatozoa with high motility and normal acrosomes (*Salamon et al.* 1973, *Wilmut & Polge* 1977b). However, high levels of lactose, maltose, sucrose, raffinose and glycine exert a negative effect upon motility but a positive effect

on the proportion of normal acrosomes (*Salamon et al.* 1973, *Wilmut & Polge* 1977b, *Visser & Salamon* 1974). Lactose provided the best protective effect on acrosomes (*Wilmut & Polge* 1977b). Deep freezing without glycerol has been achieved but with low post thaw viability, with fructose providing maximum motility (*Wilmut & Polge* 1977c). However, glucose and lactose are most often used in the current freezing techniques (*Pursel & Johnson* 1975, *Westendorf et al.* 1975, *Paquignon & Courot* 1976).

Proteins and lipoproteins. These are provided in the form of egg-yolk, milk or casein but egg-yolk is regarded as an almost indispensable ingredient. Whole egg yolk is vividly protective for bull and ram spermatozoa (*Watson* 1981) but provides little or no protection for boar spermatozoa (*Benson et al.* 1967, *Pursel et al.* 1970, 1972b, 1973a) in spite of earlier claims (*Polge* 1956). Its cryoprotective effect is lower than that of glycerol (*Salamon et al.* 1973). Its inclusion in the diluent improves the post thaw motility and the preservation of acrosomes (*Visser & Salamon* 1974). Few studies have compared the effect of different egg yolk concentrations. Increasing its concentration (7.5 upto 22.5 %) in both fructose and tris diluents improved post thaw motility although this had a depressive effect upon viability during incubation (*Visser & Salamon* 1974). *Watson & Plummer* (1985) confirmed that 10 % egg yolk has no beneficial effect in protecting both plasma and acrosomal membranes of boar spermatozoa.

These studies support earlier observations (*Pursel et al.* 1973a) that a lipoprotein fraction of egg yolk protects boar sperm acrosomes during cold shock. This led to the use of only the supernatant fraction post centrifugation (*Graham et al.* 1971a, *Pursel & Johnson* 1975). The active component in species where egg yolk is protective is probably lecithin (*Quinn et al.* 1980) but lecithin apparently had no effect upon boar spermatozoa (*Pursel et al.* 1973a). Phosphatidyl serine protects boar spermatozoa (*Butler & Roberts* 1975, *Foulkes* 1977); whereas *Simpson et al.* (1987) reported protection against ultra structural damage, maintenance of motility and respiration to caudal spermatozoa with 2.0 mg/ml of phosphatidyl choline. However, washing eliminates the protective effect suggesting the existence of a fairly loose easily disruptable interaction of the phospholipid with boar spermatozoa membranes (*Simpson et al.* 1987). Protection has been claimed to be due to both phospholipids and the low density lipoprotein fraction (*Gebauer et al.* 1970, *Foulkes* 1977). The mechanism of action is unclear (*Paquignon* 1985) but could be mediated by either a less intense cellular dehydration (*Courtens & Paquignon* 1985) or by stabilization of the spermatozoa plasma membrane (*Watson* 1975).

Protection offered by a combination of phophatidylserine and cholesterol is the same as that of egg yolk (*Paquignon* 1985). *Moore & Hibbitt* (1977), found casein to be less effective. In studies with other polymeric additives; *Graham & Crabo* (1972) found a more beneficial effect in the presence of egg yolk and/or glycerol.

Other additives. Orvus Es Paste (O.E.P.), a synthetic detergent and wetting agent with an organic binder has been incorporated in TEST, BF5, and Hulsenberg diluents. It was introduced by *Graham et al.* (1971a) who found that it decreased freeze thaw damage to boar spermatozoa, after screening of 78 different surfactants on their effectiveness in membrane integrity preservation. O.E.P. has been associated with increased post thaw motility and normal acrosomes (*Graham et*

al. 1971a, *Graham & Crabo* 1972, *Romeny et al.* 1974, *Westendorf et al.* 1975, *Pursel et al.* 1978b) and significantly enhanced fertility capacity (*Pursel et al.* 1978b). Its effect is independent of cooling rate and maximum benefit is derived in extenders with glycerol (*Pursel et al.* 1978b). The optimum concentration has varied from 0.5 to 2 % (*Westendorf et al.* 1975, *Pursel et al.* 1978b). *Pursel et al.* (1978b) obtained a quadratic response in acrosome integrity and motility in the range 0.5 to 2 % and found the optimum concentration to be 1 to 1.5 % for spermatozoa acrosomes and 0.5 to 1 % for post thaw motility. In earlier studies *Graham & Crabo* (1972), reported highest post thaw motility with 0.25 % and concommitantly increased leakage of glutamic-oxaloacetic transaminase with higher concentrations. The mechanism of action of O.E.P. has not been elucidated (*Paquignon* 1985) but it appears that it acts by altering components of egg yolk rather than by directly affecting cellular membranes (*Pursel et al.* 1978b, *Strzezek et al.* 1984, *Zorn* 1987).

Cryoprotective roles of some antioxidants has been demonstrated (*Kononov & Narizhnyi* 1982). Addition of baclanoside, with prevention of lipid peroxidation had beneficial effects to spermatozoa at freezing (*Nauck* 1988). Sialic acid present in seminal plasma may be protective at freezing; and addition of sialic acid to semen, at a concentration of 0.042 mg/ml, improved sperm motility and qyality after freezing (*Wemheuer* 1988). This indicates that some cryoprotective substances may be present in seminal plasma contrary to negative findings of *Fazano* (1986). Other additives like chloroquine, catalase, caffeine, vitamin E, selenium and glyceryphosphorylcholine did not improve post thaw motility (*Wemheuer* 1988). Unsuccessful cryoprotection with other non-permeating agents such as Celacol M_{20} dextrin peptone, Polyvinylpyrolidone (P.V.P.) and Ethylene diamine tetra acetic acid (E.D.T.A.) has been reported (*Salamon et al.* 1973).

Seminal plasma. Controversial reports concerning its role in cryopreserving media exists in the literature. The removal of seminal plasma before freezing and thawing was claimed to be beneficial in early studies (*Polge* 1956, *Roy* 1955, *Hofman* 1959). The total exclusion of seminal plasma prior to freezing raises the proportion of normal acrosomes post thaw (*Pursel & Johnson* 1971b). Further investigations (*Pursel et al.* 1973a: showed that its presence during equilibration promotes the resistance of spermatozoa to freezing and thawing damage. Adsorption of boar seminal proteins to spermatozoa post ejaculation occurs (*Moore et al.* 1976, *Pavelko & Crabo* 1976). *Moore et al.* (1976) showed that the binding of basic proteins from the seminal plasma on the spermatozoa membrane is detrimental to spermatozoa during cooling. However, the elimination of seminal plasma by centrifugation or vesiculectomy affects neither the freezability nor the fertility capacity of spermatozoa (*Salamon* 1973, *Moore & Hibbitt* 1977). Despite the conflicting results, it appears advantageous to maintain as much of the peripheral proteins as possible during freezing and thawing for maintenance of spermatozoa in the female genital tract (*Crabo* 1985). This may in part be supported on seminal plasma by *Zorn* (1987) and *Wemheuer* (1988).

Freezing process

To date 4 main techniques for deep freezing boar semen have been developed and tested under field conditions (*Pursel & Johnson* 1975, *Westendorf et al.* 1975, Paquignon & Courot 1976, *Larsson et al.* 1977). They

have all involved freezing in pellets but one, *Westendorf et al* (1975). They have common features including; equilibration in seminal plasma prior to centrifugation, concentration of semen, freezing of spermatozoa in a concentrated form and addition of low concentrations of glycerol.

1. Equilibration is performed in seminal plasma prior to centrifugation (*Pursel & Johnson* 1975, *Larsson et al.* 1977) or in an extender with or without seminal plasma during cooling to 15° C (*Westendorf et al.* 1975) or holding at 15° C (*Paquignon & Courot* 1975). The equilibration time promotes the capacity of spermatozoa to withstand freezing and thawing. The development of resistance is probably due to interaction of spermatozoa membranes with seminal proteins (*Pavelko & Crabo* 1976). The freezing techniques, so far with satisfactory results recommend equilibration time of at least 4 1/2 h. In the method of *Pursel & Johnson* (1975), the 2 h holding period is used for the development of this resistance, but in the other procedures this probably occurs during cooling and holding at 15° C.

2. The 2nd principle is the concentration of semen by centrifugation and removal of seminal plasma before spermatozoa are cooled below 15° C. The centrifugation is done either at the time of collection (*Paquignon & Courot* 1976) or after incubation (*Pursel & Johnson* 1975, *Westendorf et al.* 1975, *Larsson et al.* 1977). This allows for resuspension of spermatozoa at a high sperm concentration, advantageous at cooling (*Pursel et al.* 1973b) and a lower volume for freezing and storage.

3. The 3rd principle is the freezing of spermatozoa in a concentrated form to preserve semen quality (*Pursel & Johnson* 1975). A low concentration (250 × 10^6spermatozoa/ml) raises the level of motile spermatozoa whereas a high concentration (1 000 × 10^6spermatozoa/ml) lowers it (*Graham & Crabo* 1972). *Westendorf et al.* (1975) recommemded a concentration of 900 × 10^6 while *Paquignon & Courot* (1975) found a concentration of 400 to 800 × 10^6spermatozoa/ml most suitable. Concentrations of between 450 and 1000 × 10^6spermatozoa/ml have been commonly used.

4. The addition of low concentrations of glycerol requires that spermatozoa should be frozen rapidly (*Mazur* 1977). This high speed freezing requirement can be achieved by freezing semen in pellets on dry ice (*Nagase & Niwa* 1963). This has been adapted in 3 of the methods (*Pursel & Johnson* 1975, *Paquignon & Courot* 1976, *Larsson et al.* 1977).

In early studies, boar spermatozoa was frozen in ampules using low cooling rates and resultant sperm motility was highest when glycerol concentrations exceeded 5 %. Later documentation (*Polge* 1976) lends support to higher rates of cooling in combination with low rates of glycerol. In comparative studies, *Schorner* (1974) reported little difference in post thaw motility between pellet and ampule frozen at –1° C/min in extenders with 5 % glycerol. *Waide* (1975) reported that sperm frozen in thin walled aluminium packages at rapid cooling rate in liquid nitrogen vapour (+5 to –100° C in 3 min) gave superior post thaw motility in comparison to spermatozoa cooled at 1–2° C/min from +5 to –20° and 3° C/min from –20 to –70° C in dry ice-alcohol. Variations in cooling rates among different regions of the pellets frozen in dry ice, assesed by post thaw motility, have been reported (*Larsson & Graham* 1973, *Pursel & Johnson* 1976, *Kozumplik* 1978, *Pursel & Park* 1985) but *Salamon* (1973) found no difference.

Inexhaustive information is available regarding the cooling character of maxi straws in the procedure of *Westendorf et al.* (1975).

Schrader (1976) reported a lower cooling rate for maxi straws compared with pellets. *Park & Pursel* (1985) compared different cooling rates for 5 ml maxi straws and found significant differences in post thaw viability. Major differences have been reported in controlled comparative studies between maxi and mini straws with registration of different freezing curves, in the centre and periphery of the straws (*Baron* 1986) however, *Weitze et al.* (1987) in similar studies did not get major differences. Nevertheless, *Weitze et al.* (1988) compared and related packaging in flattened straws (1.7 ml volume) with round maxi ones and mini straws and as a function of a difference in surface area to volume ratio reported apparent benefit of freezing in flattened straws. The poor post thaw viability in maxi straws has been attributed to sub optimal freezing and thawing rates in the centrally located spermatozoa (*Baron* 1986, *Fazano* 1986, *Weitze et al.* 1987, *Weitze et al.* 1988).

It is desirable to freeze boar spermatozoa in large volumes for practical reasons. Some encouraging results, in 6 ml straws (*Westendorf et al.* 1975) have been obtained but larger volumes were not as good (*Larsson et al.* 1976). Most freezing is currently done in 5 ml plastic maximum straws (*Aumüller* 1982). Packaging in early studies was done in either ampules (see *Polge* 1956) glass tubes (e.g. *Settergren* 1958) before the introduction of pellets (*Nagase & Niwa* 1963). Developmental procedures have embraced the use of maxi straws (6 or 5 ml volumes), minitubes (with volumes of either 0.25 ml, 0.5 ml, or 1 ml); and flattened straws by German workers (*Weitze et al.* 1988).

Thawing process

Thawing procedures. For acceptable boar spermatozoa survival, the thawing method must differ from the procedures used in other species because of the large volume of frozen material per insemination dose. The warming phase of the freeze thaw process is as important as the cooling phase for spermatozoa survival (*Mazur* 1985).

Different thawing methods have been described. The thawing of pellets is superior at higher than at lower warming rates (*Salamon et al.* 1973) and when done at 37° C compared with lower temperatures, in dry test tubes, since some portion of the pellet is frozen at suboptimal rate (*Larson & Graham* 1973). Similarly only slight improvement was registered when thawing was done at 50° C or 60° C in dry test tubes. At thawing, the pellet frozen semen is simultaneously diluted in thawing diluents preheated to 50° C and thus causing rapid warming (Richter & Liedicke 1972). Due to the better heat conduction on the entire surface of the pellet, thawing in heated solutions accelerates the thawing rate and increases the survival of spermatozoa (*Richter & Liedicke* 1972). Comparative studies between thawing in solutions and in dry aluminium pans found solutions to be more superior in enhancing post thaw percentage of NAR acrosomes (*Crabo et al.* 1972c, *Pursel & Johnson* 1976) and improved pregnancy rates (*Crabo et al.* 1972c). Fast warming rates for pellets are probably essential to spermatozoa survival as they prevent damage that would result from recrystallization of intracellular ice (*Mazur* 1977). *Waide et al.* (1977) compared thawing semen in aluminium packages in water bath temperatures ranging from 0 to 80° C; and obtained maximum post thaw motility with temperatures between 40 to 50° C. The thawing period was not disclosed.

Maxi straws have been thawed in temperatures varying from 35 to 90° C. *Westendorf et al.* (1975) reported that the percentage motility and NAR acrosomes of thawed

straws (6 mm inside diameter, 8 mm outside diameter) were higher when thawing was done at 90° C than at 50° C in a water bath; and the temperature of the thawed semen ranged between 20 to 25° C at the time of removal from the bath. *Perezcanto-Fernandez* (1978) compared thawing temperatures of 50° C for 50 sec with 35° C for 60, 90, 120 sec for maxi straws and found no advantage of 35° C at any of the durations. *Schuler et al.* (1979) used water bath temperatures of 52° C for 52 sec to thaw maxi (6 ml) straws. Flattened straws have been thawed in water baths at temperatures of 39° C for 15 sec, 50° C for either 13 or 10 sec (*Weitze et al.* 1988). In later advances, in comparative thawing of both flat and maxi straws in microwaves and a water bath, sperm motility and incidence of spermatozoa with intact acrosomes was lower for thawing in microwaves than water baths (*Ewert* 1988).

Mini straws have been subjected to various thawing temperatures 39° C for 15 sec (*Baron* 1986, *Weitze et al.* 1987) and 20° C for 45 sec, and 70° C for 8 sec (*Baron* 1986) and 50° C for 12 sec which has been considered optimal.

Damages resulting from rapid cooling are dependent on the rate of cooling and final temperature to which spermatozoa are cooled (*Fiser & Fairful* 1986). In contrast, rapid warming has been considered to be rarely detrimental (*Watson* 1981, *Amann & Pickett* 1987). However, *Bamba & Cran* (1985) have shown that boar spermatozoa are severely damaged by warm shock. Further studies in the boar (*Bamba & Cran* 1988a), bull and rabbit (*Bamba & Cran* 1988b) seem to confirm the undesirable effects of rapid warming.

Thawing diluents. The thawing diluents so far used can be divided into 2 broad categories; protein containing and saline based thawing solutions. The protein containing are mainly seminal plasma (*Crabo & Einarsson* 1971) and skim milk (*Einarsson et al.* 1972). The saline thawing solutions are currently the most widely used and include Beltsville thawing solution (BTS) (*Pursel & Johnson* 1975), Hulsenberg diluter (*Westendorf et al.* 1975), OLEP (*Larsson & Einarsson* 1976, INRA-ITP (*Paquignon & Courot* 1976). The main sugar component in these solutions is glucose, and lactose is additionally incorporated in Hulsenberg diluent, and fructose and pyruvate replaces glucose in OLEP. Ethylene diamine tetraacetic acid (E.D.T.A.) a constituent in Hulsenberg, BTS and INRA-ITP diluents improves the survival of spermatozoa after incubation (*Visser & Salamon* 1974, *Westendorf et al.* 1975) as did catalase (*Visser & Salamon* 1974).

The thawing solutions contain numerous electrolytes compared to freezing diluents. The optimum values of osmotic pressures in diluents for boar spermatozoa have been pointed out (*Pursel & Johnson* 1975). Glucose solution appeared less efficient in some studies (*Crabo et al.* 1972c, *Larsson & Einarsson* 1976) but *Salamon et al.* (1973) obtained optimum motility with increasing glucose concentrations. The physiological levels of osmotic pressure and pH in the thawing diluent are not enough to maintain the fertilizing ability of frozen thawed spermatozoa (*Larsson & Einarsson* **1976).** *Senegacnik et al.* (1980) found that thawing media were best when the concentration of electrolytes was several times higher than that of freezing media. Among the diluents only Hulsenberg is hypertonic, while BTS, OLEP and INRA-ITP are isotonic. In yet another study (*Vasques & Graham* 1980) the inclusion of glycerol in the thawing medium enhanced the rate of post thaw motile spermatozoa.

Limited studies have compared the efficacy of thawing solutions. While comparing Hulsenberg, BL1, seminal plasma, skim milk and BTS (*Pursel & Johnson* 1976), BTS offered the most normal acrosomes and motile spermatozoa. However, according to *Paquignon et al.* (1977) INRA-ITP was superior to BTS in terms of the survival of spermatozoa during incubation, pregnancy rate and embryo survival.

Conclusions

Successful methods for ryopreservation of boar semen have not been achieved despite numerous efforts world wide. Improvements in semen preservation technologies have been deterred by lack of *in vitro* methods that can accurately predict *in vivo* fertilizing capacity of frozen boar semen.

The cell membrane is of crucial importance with regard to freeze-thaw survival of spermatozoa. It is important to optimize the survival of the plasma membrane as this is a non homogenous entity both in structure and function. The non homogeneity of the membranes may be responsible for the differences observed in responses of different parts of the spermatozoon to similar treatments. The boar sperm membrane exhibits extreme sensitivity to freezing treatment. Freezing and thawing results in considerable changes in electrolyte dynamics and damages have mainly been associated with alterations in the head membranes especially at thawing. Freeze-fracture studies have elaborated the sensitivity of boar spermatozoa to different temperatures and this has been reflected in the alteration of plasma membrane composition. Lipid phase transitions occur at cooling and freeze-thaw processes of spermatozoa.

To date fruitless efforts have been carried out to find a cryoprotectant for the spermatozoa membranes and glycerol still continues to be used despite its harmful effects to the membranes. Low concentrations of glycerol are currently accepted in freezing methodologies. However, low concentrations of glycerol are associated with a decrease in the amount of unfrozen water around the spermatozoa suspensions and subsequently an understanding of the correlation between the unfrozen water vis a vis glycerol concentration, and the lipid phase transitions is of paramount importance. This should also be done to explain the response to various diluents. A clear elucidation of such correlations might aid in the discovery of a suitable cryoprotectant or a supplement to glycerol for better membrane preservence. This must be the goal for future research work.

Acknowledgements

I am very grateful to Professor Heriberto Rodriguez-Martinez and Professor Stig Einarsson for valuable discussions and critical readings of the manuscript.

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Sammanfattning

Djupfrysning av galtsperma.
I. En litteraturöversikt.

Denna översiktsartikel sammanfattar och diskuterar de tillgängliga djupfrysningsmetoderna av galtsperma. Den ger en sammanfattning av den historiska bakgrunden, grundläggande kunskaper i kryobiologi, frysskyddande medel samt se senaste framstegen i djupfrysningstekniken. Tillräckligt goda djupfrysningsmetoder har inte presenterats trots stora ansträngningar i olika länder. In vitro metoderna att värdera kvaliteten på den djupfrysta sperman har inte varit tillräckligt säkre för att samtidigt värdera befruktningsförmågan. Cellmembranen är av utomordentligt stor betydelse för infrysning-upptiningsmomenten. Spermiecellmembranen är inte en enhetlig struktur utan varierar både i morfologi och funktion och är mycket känslig för låga temperaturer. Frysning och upptining medför förändringar i elektrolyttransporten över membranen speciellt i den del som täcker spermiehuvudet. Fortfarande är glycerol den frysskuddande substans som används trots dess skadliga inverkan på cellmembranen hos galtspermier.

(Received October 31, 1990; accepted December 20, 1990)

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