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Recent development in freezing strategies of pig semen—A review

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ABSTRACT

Of late studies on frozen thawed boar semen have dramatically improved boar semen cryopreservation technique, albeit the commercial application of cryopreserved boar semen has not yet been popular. Some studies claimed successful fertility/ fertilization with frozen boar semen. Multiple researches are being carried out to evolve a suitable freezing protocol for cryopreservation of boar semen. In general, freezing protocol adopts freezing rates of either 20°, 40° or 60°C/min in lactose egg yolk extender with 2–3% glycerol using medium straw (0.5 ml) for freezing of boar semen. The supplementation of vitamin E or its analogues Trolox, butylated hydroxytoluene, reduced glutathione, catalase, superoxide dismutase, ascorbic acid, and alpha-lipoic acid to the freezing media of boar semen increase the cryosurvival of frozen-thawed boar spermatozoa. Treating sperm with cholesterol-loaded methyl-β-cyclodextrin increases sperm cryosurvival rates and sperm quality after thawing by partly decreasing membrane damage induced during phase transition from fluid to the crystalline-gel state. High fertility rates with cooled, frozen-thawed or sex-sorted boar semen are feasible to achieve by using appropriate insemination procedures. Post-cervical intra-uterine insemination allowed a three-fold reduction of spermatozoa to be inseminated, whereas deep uterine insemination allowed a substantial reduction in the number of cooled (5–20 folds) or frozen-thawed (6-folds) spermatozoa. With combination of different approaches, acceptable fertility with cryopreserved boar semen can be achieved facilitating its use in routine and commercial application. This review depicts best ways possible to adopt suitable freezing strategies for cryopreservation of boar semen.

Keywords: Cryopreservation, Freezing strategies, Pig, Semen

The first successful attempt of boar semen cryopreservation was reported in 1956 (Polge 1956). Although fertility with frozen boar semen appeared in the literature before 1970, the results could not be repeated. Polge *et al.* (1970) first described a method for successful fertilization with frozen-thawed boar semen through oviductal insemination. Fertility with frozen-thawed boar semen by cervical insemination was reported in 1971 separately by Crabo and Einarsson, Graham *et al.* and Pursel and Johnson. In 1975, two different boar sperm cryopreservation methods were developed: the German or Hulsenberger method (Westerndorf *et al*. 1975) and the American or Beltsville method (Pursel and Johnson 1975). In Hulsenberger method, freezing was done in straws over vapour of liquid nitrogen while in Beltsville method freezing was performed in pellet form in carbonic ice. Both the methods were based on the use of freezing extenders containing egg yolk and glycerol as cryoprotectant. However, the two methods differed in the type of sugars used in the freezing extender. While Beltsville method involved the addition of glucose, Hulsenberger method utilized lactose. These two methods or their modifications are commonly used hitherto (Eriksson 2000). Research efforts in recent times have been directed toward optimization of cryopreservation protocols and artificial insemination (AI) methods using frozen boar semen. Cryopreservation success has increased after introduction of programmable freezing machine and controlled cooling and freezing rate (Hammit and Martin 1989).

Frozen boar semen provides many benefits to the swine industry, namely long-range distribution of high quality genetic material, establishing genetic resource banks for conservation of genetic diversity, improvement in biosecurity and even for reserve supplies in response to sudden disease outbreak. Up-gradation of indigenous pigs through artificial insemination (AI) with semen of improved breed of boar is one of the handful biotechnological tools as it yields most often farrowing rates and litter sizes close to natural mating. The mere advantages of AI could be maximized with accomplishment of cryopreservation of boar spermatozoa having optimum fertility. However, the practice of cryopreservation of boar semen in commercial fields is sparse due to lower freezability of boar sperm as compared to other animals (Johnson *et al*. 2000, Mazur *et al*. 2008) and less than 1% of all inseminations in pig are

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done using frozen–thawed semen (Rodriguez-Gil and Estrada 2013), most often after export from one country to another and primarily for the purpose of upgrading the genetic base of the herd. Several factors constrain commercial application of boar semen frozen in liquid nitrogen especially lower sperm survival post-thaw (Thurston *et al*. 2001, Roca *et al.* 2006a, Hernandez *et al.* 2007a), which yields low farrowing rates and litter sizes (Roca *et al*. 2006b, Baishya *et al.* 2016) because of impaired fertilizing ability and reproductive performance after AI (Johnson *et al*. 2000) thus making such technology not a commercially viable option for pig producers, despite its breeding relevance (Martinez *et al*. 2005).

In India, very few attempts (Nath 1992, Khan 2009, Baishya *et al*. 2014a, b; Baishya *et al*. 2015, 2016; Baishya *et al*. 2018a, b, c) have been reported to develop and assess freezing protocols for boar semen cryopreservation. This review depicts the recent developments in freezing strategies for cryopreservation of boar semen.

Freezing strategies for cryopreservation of boar semen

Current research on cryopreservation of boar semen lays emphasis on various strategies to improve the quality of post thawed spermatozoa, increase the number of spermatozoa available for fertilization and reduce the sperm damage. Various strategies adopted to improve the post thaw quality and availability of boar sperm are discussed below.

Optimum freezing protocol: An optimum freezing protocol depends mainly on freezing medium, concentration of the cryoprotectant, freezing packages, cooling, freezing and thawing rates for maximum sperm cryosurvival.

Diluent and cryoprotectant for freezing of boar semen: During the past few decades, many diluents have been evolved by different researchers for cryopreservation of semen with varying degree of success. Effective freezing media offer membrane protection during cooling, while proper concentrations of cryoprotectant reduce intracellular ice crystal formations and osmotic stress during freezing. Furthermore, cooling and freezing rates allow spermatozoa to adjust to temperature changes while limiting exposure to cryoprotectant and formation of large ice crystals (Fiser *et al.* 1993).

Many diluents such as glucose-egg yolk, lactose-egg yolk EDTA-Tris diluent glucose + sodium citrate + egg yolk + glycerol, Lactose-egg yolk-glycerol, Tris-egg yolk-dextrose-EDTA-citric acid-glycerol, Tes-tris-glucose-egg yolk-glycerol, lactose-egg yolk-glycerol with or without detergents and emulsifying agents have been tested by various scientists (Nath 1992, Errikson *et al*. 2002, Gadea *et al*. 2004, Maldjian *et al*. 2005, Roca *et al.* 2006a, Corcuera *et al*. 2007, Flores *et al.* 2008, Hu *et al*. 2008, Roca *et al*. 2008, Casa *et al.* 2009, Jeong *et al.* 2009, Khan 2009, Zhang *et al.* 2009, Saravia *et al*. 2010) and reported varying success rate. Cryopreservation extenders contain hen egg yolk (EY) because it protects the cell against cold shock. As the protective effect is mainly due to the presence of low-density lipoproteins (LDL) in the EY plasma, beneficial effects have been reported (Jiang *et al*. 2007a) by replacing the EY plasma with LDL fraction on sperm quality and DNA integrity following freeze-thawing of boar semen. It was demonstrated that LDL isolated from ostrich EY rather than from hen rendered acceptable fertility rates and litter sizes after AI (Fraser *et al*. 2007) and sperm quality parameters including DNA integrity (Fraser and Strzezek 2007). The optimal concentration of LDL fraction was found to be 9% of the freezing extender (Jian *et al*. 2007b) and the highest cryoprotective effects were conferred by the extract obtained from pigeon EY (Wang *et al*. 2014). The effect of different types of sugars used in egg yolk based extender on the post-thawed boar semen quality revealed that the lactose-based extender resulted in a higher percentage of post-thawed sperm motility, viability, intact acrosome and functional plasma membrane than sorbitol-based extender and fructose-based extender yielded a higher post-thawed sperm motility and viability than sorbitol-based extender (Chanapiwat *et al.* 2012). Lactose-egg yolk was found suitable for cryopreservation of boar semen and hence it is used extensively for boar semen freezing protocol. The addition of 5% seminal plasma from good freezability ejaculates to the freezing extender improved the sperm motility and membrane integrity at post-thawing and *in vitro* penetration rates after *in vitro* fertilization (Hernandez *et al*. 2007). This improvement could be mediated through inhibition of *in vitro* capacitation and cooling-induced capacitation-like changes via heparin-binding seminal plasma proteins (Vadnais and Roberts 2010).

Cryoprotectant is added to the freezing medium to reduce and control intracellular ice crystal formation. Cryoprotectant aids in the freezing process by causing a hypertonic cellular environment and by modifying the sperm membrane to encourage water to leave the cell (Corcuera *et al.* 2007). Many cyroprotectants have been tested while freezing swine sperm including Dimethyl Sulfoxide (DMSO), Polyvinylpyrrolidone (PVP), and Ethylenediaminetetraacetic acid (EDTA), however none provided the same level of protection as glycerol (Johnson *et al.* 2000). A number of studies utilizing glycerol, the most common cryoprotectant for swine, determined that boar sperm had the highest percentage of motile (52.5%) and acrosome intact sperm (56%) when concentrations of 2 to 4% were used in the final volume of freezing medium (Corcuera *et al*. 2007, Hernandez *et al*. 2007b). However, concentrations greater than this caused decreased survivability post-thaw and compromised acrosomes, resulting in reduced fertility (Holt 2000). Improved post-thaw sperm motility was reported (De Mercado *et al*. 2009) on using 2% glycerol with 80 mM L-glutamine. A study indicated that replacing lactose and glycerol in extender by powdered coconut water plus dimethylformamide yielded higher post-thaw sperm quality, however fertility test was required for confirmation (Silva *et al.* 2015). The effects of different concentrations of glycerol (0, 1, 2, 3 and 5%) and dimethylacetamide (DMA: 0, 1, 3 and 5%) on post-sperm quality characteristics following semen freezing in dry ice

or liquid nitrogen indicated that the freezing extender containing 3% glycerol in combination with the straw-freezing method using dry ice produced the best post-thaw quality parameters of boar semen and combinations of glycerol and DMA did not enhance the cryosurvival of boar spermatozoa (Yang *et al*. 2016). Hitherto, glycerol is the main cryoprotectant, as no cryoprotectant has been found to yield better results than its optimal concentration ranging from 2 to 3%.

Semen packages for freezing: Semen packaging system has a great influence on the cryosurvival of boar spermatozoa. Researchers used different freezing packaging materials for freezing of boar semen, viz. 5 ml Maxi straws (Westerndorf *et al*. 1975), 0.50 ml medium straws (Baron 1986 and Fazano 1986), 1.70–2.00 ml flattened Maxi straws (Leps 1988 and Simmet 1993), and 0.25 ml and 5 ml plastic bags (Larsson and Einarsson 1976b and Rodriguez-Martinez *et al.* 1996). All the packaging systems have their advantages and disadvantages. Small and medium straw offer large surface to volume ratio and provide cryobiologically suitable shape, these require thawing 10–20 straws per insemination. Weitze *et al*. (1987) reported that though 5 ml maxi straw contains one insemination dose, it has a relatively small surface to volume ratio, which constrains optimum freezing and thawing. However, Cordova *et al*. (2001) found no differences between 0.5 and 5 ml straws in terms of post-thaw sperm quality, total penetration, monospermy and polyspermy rates. Flat packs (5 ml) are considered cryobiologically convenient (very thin and with a large surface to dissipate heat during cooling and warm rapidly during thawing) with high post-thaw sperm motility (Eriksson *et al*. 2001). Flat packs that allowed more homogenous dehydration than 0.5 ml straws during boar sperm cryopreservation on electron microscopic cryoscanning (Ekwall *et al*. 2007), proved successful when fertility was tested, with acceptable farrowing rates and litter sizes (Eriksson *et al*. 2002) and can obviate pooling of several small or medium straws for a single dose of AI. However, doses with such large sperm numbers conspire against the best use of the ejaculates, but with the introduction of intrauterine deposition of semen, a new vista was opened for the use of smaller containers with high numbers of spermatozoa to contain a single AI-dose (Rodriguez-Martinez and Wallgren 2011).

Boar spermatozoa were frozen in small volumes (0.5–0.7 ml) with high concentration in novel containers, the so-called 'MiniFlatPack' (MFP) that contained 1–2 billion spermatozoa/ml (Bwanga *et al.* 1991). The freezing was more homogeneous with more cryosurvivability MFP than in medium straws (Ekwall *et al.* 2007) and fertility using deep-intrauterine AI was equal or higher than that for 0.5 ml plastic straws (Saravia *et al.* 2005, Wongtawan *et al.* 2006). However, there is no consensus among the scientists about the freezing packaging system suitable for commercial application of frozen boar semen.

Cooling and freezing rate: Sperm damages emanated during cryopreservation procedures could be mitigated to some extent through the improvement in freezing strategies developed by various scientists.

The optimum cooling/freezing rate for cryopreservation of boar semen should be generally in the range of 30–50°C as adopted by various workers (Bwanga *et al.* 1991, Johnson *et al.* 2000, Roca *et al*. 2006, Saravia *et al*. 2010) for cryopreservation of boar semen. Cooling down of boar semen from 15–17°C to 4°C was also conducted at –0.1°C/min (Casa *et al*. 2009), however, higher cooling rates (0.4 and –1.5°C /min) did not cause higher cryodamage to boar semen and could shorten the whole protocol (Juarez *et al*. 2011). Uniform cooling and freezing in the range of 20°C to 60°C/min from –6 to –140°C with controlled rate of freezing method resulted in substantial improvement of post-thaw sperm qualities as compared to that of uncontrolled and rapid freezing with conventional method (Baishya *et al*. 2014). Freezing of boar semen in medium straw between the cooling rates of 10, 40 and 60°C/min showed no significant difference in post-thaw sperm motility (Hernandez *et al*. 2007). Eriksson and Rodriguez-Martinez (2000) while studying the effect of three different freezing rates (20, 50 and 80°C/min) using Maxi-straws and flat packs recorded higher sperm motility (54.6%) post thaw at freezing rate of 50°C/min than at 20°C/min (51.5%) or at 80°C/min (50%).

Saravia *et al.* (2010) carried out the freezing of sperm-rich fraction (SRF) of boar semen by a simplified shorter procedure in mini flat packaging using cooling rate of 3°C/min from 5 to –5°C, 1 min holding for crystallisation, and thereafter 50°C/min from –5°C to –140°C in lactose-egg yolk-glycerol-Equex STM extender and recorded the mean percentage of post-thaw sperm motility as 70.60±2.60. Siqueira *et al.* (2011) conducted freezing of boar semen by dividing each semen ejaculates into two portions, i.e. sperm peak portion (first 10 ml of ejaculate) and the remaining spermatozoa of the sperm-rich fraction of the ejaculate using the cooling rate of 3°C/min from 5 to –5°C; 1 min holding for crystallization, and thereafter froze at 50°C/min from –5°C to –140°C in mini flat pack. The post-thaw sperm motility was recorded to be 50% for both the portions, and plasma membrane and acrosome integrity did not differ between the two fractions.

It could be inferred from the above studies that the optimum freezing procedure could consist of a cooling rate 3°C/min from 5 to –6°C, 1 min holding at –6°C and then freezing at the rate of 20°C to 60°C/min from –6°C to –140°C with or without Equex-STM.

Thawing rate: Thawing rate is one of the important aspects of a freezing protocol and response of spermatozoa to thawing depends on the cooling rate used. Many researches were conducted to find out a suitable thawing rate. It is widely accepted that thawing rate at 37°C for 20 sec is the best. However, Tomas *et al.* (2014) showed that faster thawing rates at 70°C for 8 sec yielded better post-thaw boar sperm motility and membrane integrity. For improvement of post-thaw boar sperm quality thawing medium Beltsville Thawing Solution was supplemented

with seminal plasma for its beneficial effect on sperm motility and membrane integrity as it could prevent the destabilization of plasma membrane and other capacitation-like changes (Vadnais and Althouse 2011, Gomez-Fernandez *et al*. 2012). While, some authors reported no effects on sow fertility after supplementing the thawing extenders with seminal plasma (Abad *et al.* 2007), others observed an increase in the reproductive performance not only when added to frozen-thawed ejaculated but also when added to frozen-thawed epididymal sperm (Okazaki *et al.* 2009, 2012; Garcia *et al.* 2010). Certain additives were also included only in the thawing extender with positive effects. The combined addition of Ca^{2+}- chelating agents, 6 mM EDTA and 6 mM EGTA to the thawing extender increased sperm motility, acrosomal status, IVF outcomes and pregnancy rates following AI (Okazaki *et al*. 2011). However, supplementing thawing solution with caffeine and β-mercaptoethanol increased reproductive performance indirectly because caffeine inhibited the migration of polymorphonuclear leucocytes into the uterine lumen (Yamaguchi *et al.* 2009) and β-mercaptoethanol prevented sperm capacitation and degenerative acrosome exocytosis (Yamaguchi and Funahashi 2012).

Addition of antioxidants to freezing medium: Antioxidants have been used to protect spermatozoa from the deleterious effects of cryopreservation. During cryopreservation, reactive oxygen species (ROS) are produced due to oxidative stresses resulting from cooling, freezing and thawing processes. ROS exerts influence on different sperm functions, viz. hyperactivation, capacitation, acrosome reaction, and zona binding (Kodama *et al.* 1996, Lamirande *et al*.1997). When there is imbalance between ROS production and detoxification by antioxidants, an excess of ROS creates oxidative stress. ROS such as hydrogen peroxide (H_2O_2) is known to arrest motility and block oxidative metabolism in spermatozoa (Tosic 1947). ROS molecules are formed from the incomplete reduction of molecular oxygen and are toxic to cells because of their high reactivity. The ROS toxicity is related to the ionization and subsequent inactivation of proteins and peroxidation of unsaturated lipids (Medeiros *et al*. 2002). It also causes sperm DNA damage and consequently could affect embryo development (Aitken *et al*. 1998). Boar spermatozoa are particularly susceptible to lipid peroxidation because of high level of polyunsaturated phospholipids in the plasma membrane since unsaturated fatty acids readily undergo peroxidation (Halliwell and Gutterridge 1984). Relatively low antioxidant capacity of boar seminal plasma (Brezezinska-Slevbodzinska *et al.* 1995) also favours the excess production of ROS.

Numerous studies have been conducted on different antioxidants to diminish the toxic effect of ROS in boar semen preservation and to improve the post-thaw survival of boar spermatozoa (Gadea *et al*. 2004, Roca *et al.* 2004, Kaeoket 2008, Jeong *et al.* 2009,Trzcinska *et al*. 2015, Varu-Ghiuru *et al*. 2015, Trzcinska and Bryla 2016, Baishya *et al*. 2018a). Pena *et al.* (2003) also observed that, during cryopreservation vitamin E analogoue Trolox at 200 µg/ml had positive effects on sperm motility, mitochondrial membrane potential and membrane integrity of cryopreserved porcine spermatozoa. It was found that addition of 200 µg/ml alpha-tocopherol to the freezing extender could protect the sperm membrane from oxidative damage (Breininger *et al*. 2005), and reduce both tyrosine phophorylation and capacitation-like status of sperm in boar (Satorre *et al*. 2007). Jeong *et al.* (2009) evaluated the influence of α-tocopherol supplementation at various concentrations in Lactose Egg yolk Glycerol extender during boar semen cryopreservation. They found the highest post-thaw sperm motility (45.4%) with supplementation of 200 µM α-tocopherol in extender and the lowest (29.5%) in extender containing no α-tocopherol. They also observed that α-tocopherol supplementation at 200 µg/ml might protect boar sperm against excessive ROS generation by reducing lipid peroxidation and lowering the expression of apoptosis genes by reducing DNA fragmentation. Satorre *et al.* (2012) also observed that quality of frozen boar semen was improved with addition of α-tocopherol in both freezing and thawing media. Kaeoket *et al.* (2008) recorded that the supplementation of *L*-cysteine (5 mM), glutathione (1 mM), and water soluble Vitamin E (200 µM) to the freezing media resulted in an improvement of boar sperm motility, viability, and acrosome integrity of thawed spermatozoa. L-cysteine plays a role in the intracellular protective mechanisms against oxidative stress, and as a membrane stabilizer and inhibitor of sperm capacitation (Zhang *et al*. 2012). Chanapiwat *et al.* (2009) observed that the supplementation of anti-oxidant L-cysteine alone or in combination with docosahexaenoic acid-enriched hen egg yolk significantly improved the post-thaw progressive motility and acrosome activity. The addition of 5 or 10 mM L-cysteine in lactose-egg yolk extender was optimal for improving the quality of frozen-thawed boar spermatozoa (Kaeoket *et al*. 2010). Gadea *et al.* (2004) reported that the addition of reduced glutathione (GSH) that protected spermatozoa against oxidative damage (Alvarez and Storey1989), to the freezing media resulted in an improvement in per cent motility of boar sperm and motion parameters of thawed spermatozoa and a decrease in the number of spermatozoa with changes in the sulfhydryl groups in membrane proteins. The addition of GSH to both freezing and thawing extenders was found to increase boar sperm quality at post-thawing, stabilize the nucleoprotein structure (Gadea *et al*. 2005a) and improve fertilizing ability (Estrada *et al*. 2014) both *in vivo* and *in vitro*. When added to the freezing extender, the protective effect of GSH on sperm function was dose-dependent and it was more pronounced with 1 mM GSH than with 5 mM GSH (Gadea *et al*. 2005b) and exogenous supplementation of GSH was effective only when its endogenous level was low in semen. The highest improvement in post-thaw boar sperm quality was recorded when both freezing and thawing media were supplemented with 5 mM of GSH plus 100 µM of L-ascorbic acid (Giaretta *et al*. 2015). Bailey *et al.* (2000) recorded that when 0.2,

0.4 or 0.8 mM butylated hydroxytoluene (BHT) was added to the freezing media, post-thaw boar sperm survival was higher, lipid oxidation was reduced and embryo development after IVF was improved. Roca *et al.* (2004) examined the potential protective effect of BHT on cryopreservation injuries to boar spermatozoa and recorded the post-thaw sperm motility as 32.16, 45.44, 44.07, 44.52 and 37.73% in extender containing 0, 0.2, 0.4, 0.8 and 1.6 mM BHT respectively. The addition of 1.0 mM BHT to the freezing extender efficiently improved the fertilizing ability of post-thaw boar spermatozoa (Trzcinska *et al*. 2015). The other antioxidants namely catalase and superoxide dismutase (SOD) (Pursel *et al.* 1978), L-glutamine (De Mercado *et al.* 2009) were used in the freezing media to enhance the quality of post-thaw boar spermatozoa. The addition of SOD or catalase to boar spermatozoa freezing extender reduced post-thaw ROS generation and improved sperm motility, viability and *in vitro* fertilizing potential of thawed spermatozoa (Roca *et al*. 2005). De Mercado *et al*. (2009) reported that L-glutamine had the ability to cryo-protect boar sperm based on enhancement of post-thaw sperm motility and it could be used as a partial glycerol substitute in the freezing extender. Addition of catalase, pyruvate and mer-captoethanol to cooling and freezing media significantly improved boar sperm motility after thawing without affecting sperm morphology or viability (Thurston *et al*. 2002). Better boar sperm motility after thawing could be obtained with the supplementation of 10 µM lutein, 200 µM Trolox, 200 µM ascorbic acid and 200–400 µM Trolox and ascorbic acid in lactose-egg yolk extender containing a final concentration of 3% glycerol and 0.5% Equex-STM (Varo-Ghiuru *et al*. 2015). It was reported that addition of ascorbic acid had a protective effect on metabolic activity and viability of cryopreserved porcine spermatozoa (Pena *et al*. 2003, 2004). Alpha-lipoic acid (ALA) a natural antioxidant, conferred a cryoprotective capacity to the extender used for boar semen during the process of freezing-thawing, and the optimal concentration of ALA for the frozen extender is 6 mg/ml (Shen *et al*. 2016). Baishya *et al.* (2018b) revealed that supplementation of GSH (1 mM), vitamin E (0.2 mM), and BHT (0.2 mM) in freezing medium resulted in an improvement of sperm motility, acrosomal status and plasma membrane integrity along with reduction of lipid peroxidation after freezing. The magnitude of these protective effects exhibited by GSH, vitamin E and BHT on cryopreserved boar spermatozoa were similar, and thus any one of them could be incorporated in porcine semen for better freezability. The highest improvement in post-thaw boar sperm quality was recorded when both freezing and thawing media were supplemented with 5 mM of GSH + 100 µM of L-ascorbic acid (Giaretta *et al*. 2015).

It could be concluded that the supplementation of vitamin E (or its analogue Trolox), BHT, GSH, catalase, SOD, ascorbic acid, and ALA to the freezing media of boar semen could increase the cryosurvival of frozen-thawed boar spermatozoa.

Addition of cholesterol loaded cyclodextrin in holding medium: Supplementation of cholesterol to the holding medium is a new technique to improve the quality of frozen semen. Cholesterol has multiple effects on sperm membrane, which stabilize the membrane during cooling and freezing processes, reduce membrane permeability, facilitate morphological membrane characteristics and serves as a membrane antioxidant (Crockett 1998). The cholesterol to phospholipid ratio is an important determinant of membrane fluidity and stability at low temperature. Rapid depletion of sperm cholesterol content during freezing process could cause lowering of cholesterol to phospholipid ratio making the sperm more vulnerable to cold shock (Watson 1981). Incubation of spermatozoa with cholesterol was found to increase the cholesterol content in sperm plasma membrane by two to three folds as compared to control in bull (Purdy and Graham 2004), stallion (Moore *et al*. 2005) and ram (Moce *et al*. 2010) which could increase the cholesterol:phospholipid ratio as found in cold-shock resistant sperm. Watson (1981) reported that treating sperm with cholesterol before cryopreservation could reduce the sensitivity of sperm membranes to cooling damage, by eliminating or at least minimizing the lateral phase separation of the lipids. Cholesterol modulates the fluidity of membranes by interacting with the fatty acyl chains of the phospholipids. Attempts have been made to make cholesterol soluble by incorporating it into liposomes and adding these liposome-cholesterol complexes to sperm. Results have been variable using this technique (from no response to moderate improvement after cooling at 4°C or after cryopreservation), depending on species (Wilhelm *et al.* 1996).

Cyclodextrins have been used to insert or remove cholesterol from cell membranes. Cyclodextrins, cyclic oligomers of glucose that form water soluble complexes with other organic molecules that may not be water soluble, can be used to deliver cholesterol to the sperm plasma membrane (Purdy and Graham 2004). It has been demonstrated (Tomas *et al.* 2011, Blanch *et al.* 2012, Lee *et al.* 2015) that the supplementation of cyclodextrin preloaded with cholesterol to the cooling media could improve the resistance of sperm to cryodamage due to cold shock and freezing damage. Methyl-β-cyclodextrin is the most commonly utilized cyclodextrin to preload cholesterol for treating sperm, although 2-hydroxypropyl-β-cyclodextrin was effective for treating ram sperm (Moce *et al*. 2010). Addition of cholesterol-loaded methyl-β-cyclodextrin (CLC) to semen modifies membrane permeability to water and cryoprotectants (Li *et al*. 2006). CLC molecules could improve post-thaw sperm quality and *in vitro* fertilizing ability without affecting capacitation status and DNA integrity (Tomas *et al.* 2011, 2013, Blanch *et al.* 2012). Further, CLC could efficiently eliminate p32 tyrosine phosphoprotein, a quantifiable bio-chemical marker of cold shock damage, reduce spontaneous acrosome reactions and improve viability in cold-shocked sperm (Galantino-Homer *et al*. 2006). However, adding

CLC to cryopreservation extenders might need to increase glycerol concentration from 3 to 5% (Blanch *et al.* 2014) which could have detrimental effects on the perinuclear theca in boar sperm (Arenas-Nunez *et al*. 2013). Bailey *et al*. (2008) reported that treating of boar sperm in cholesterol loaded Methyl-β cyclodextrin improved the resistance of sperm to cryodamage. They also reported that higher level of cholesterol loaded methyl-cyclodextrin was not more effective than 5 mg/ml. Cryosurvival of boar spermatozoa is enhanced by exposure to methyl beta cyclodextrin (MBCD) before freezing due to its protective effect (Zeng and Terada 2001). Bailey *et al*. (2008) could not find appreciable difference in the efficacy of 2-hydroxypropyl-β-cyclodextrin when compared with commercial methyl-β-cyclodextrin loaded with cholesterol. Methyl-β-cyclodextrin without cholesterol did not alter the response of sperm to cold shock, indicating that the beneficial effect was mediated via cholesterol. Also, cholesterol alone was not as effective as the CLC demonstrating the utility of cyclodextrin as a delivery molecule (Bailey *et al*. 2008). Baishya *et al.* (2018b) found that treating sperm with BHT (0.2 mM), CLC (5 mg/ 200–240 × 10^6 sperm), and BHT (0.2 mM) + CLC (5 mg/ 200–240 × 10^6 sperm) before freezing resulted in an improvement of sperm motility, enhancement of plasma membrane integrity, increment of acrosomal integrity and reduction of spermatozoa susceptible to lipid peroxidation after freeze-thawing, however, had no influence on the post-thaw mitochondrial membrane potential and DNA integrity. The beneficial effects on post-thaw sperm quality were more pronounced with BHT plus CLC than with BHT or CLC alone and thus BHT in combination with CLC could be a superior additive for cryopreservation of boar semen.

Finally it could be stated that treating sperm with CLC increased sperm cryosurvival rates and sperm quality after thawing by partly decreasing membrane damage induced during phase transition from fluid to the crystalline-gel state.

Additional factors

Selection of season: Ejaculates of boar, irrespective of breed, collected during winter and spring were reported to have higher freezability than those obtained during autumn and summer (Barranco *et al*. 2013) indicating the relevancy of temperature and photoperiod on the production of sperm cell, i.e. spermatogenesis (Sancho *et al.* 2004, Yeste *et al*. 2010).

Fraction of ejaculate: The first 10 ml of the sperm-rich fraction (SRF) of ejaculate that contained about 25% spermatozoa of SRF was found to be more resilient to handling and freezing as compared to rest of the ejaculate (Saravia *et al*. 2009). This could be due to beneficial effect to the spermatozoa of the SRF portion because of higher contents of cauda epididymal fluid and specific proteins or lower amounts of seminal plasma sperm adhesins, bicarbonate or zine levels when compared with other fractions of the ejaculate (Rodriguez-Martinez *et al*. 2009, Saravia *et al*. 2010). Freezing of the first part of SRF could be done in a relatively shorter time (about 3.5 h) without compromising freezability as compared to conventional freezing (about 8 h). The portion of the SRF was found to contain fertility-associated proteins (Rodriguez-Martinez *et al*. 2009) and could be frozen using mini flat packs and used for intra uterine insemination while the rest of the ejaculate might be utilized for conventional liquid semen doses for AI.

Holding time: Holding time, i.e. the period of storage at 15–18°C from semen collection to initiation of freezing was found to have beneficial influence in boar semen cryopreservation, and allowing 10–24 h is better than 3 h (Eriksson *et al*. 2001, Juarez *et al*. 2011, Alkmin *et al*. 2014) while other worker (Gale *et al*. 2014) reported no effect of holding time on post-thaw sperm quality. Holding time could increase sperm cryotolerance by maintaining the lipid architecture of plasma membrane (Casa and Althouse 2013) through a process involving phosphorylation of some proteins, such as HPSYO (Yeste *et al*. 2014).

Centrifugation: Centrifugation of boar semen before freezing removed seminal plasma and increased sperm concentration which could be re-diluted using extenders. Carvajal *et al*. (2004) observed that short-time centrifugation at a relatively high g-force (2400×g for 3 min) minimized damage to boar spermatozoa and yielded higher percentage of viable sperm with an intact acrosome having higher cryosurvivability relative to the initial sperm population after centrifugation. Use of iodixanol, a non-ionic, polysucrose-based gradient @ 60% W/V iodixanol at the bottom of the centrifuge tubes during centrifugation as a cushion for recovery of sperm from sperm-rich fraction could increase the percentage of viable spermatozoa with lower lipid membrane disorder after freezing (Matas *et al*. 2007). Use of single-layer centrifugation through porcine-specific colloids such as Androcoll-P (Martinez-Alborcia *et al*. 2012a) would allow removal of cholesterol and seminal plasma proteins from sperm surface without affecting sperm membrane integrity that could improve post-thaw sperm quality and *in vitro* fertilizing ability (Kruse *et al*. 2011). The process resulted in removal of non-functional, dead boar spermatozoa as their presence before and during cryopreservation was detrimental for sperm freezability (Martinez-Alborcia *et al*. 2012b, Roca *et al*. 2013).

Boar semen freezability and fertility markers: There was considerable variation in cryopreservation of semen among boars (Medrano 1998). Though there was variability among ejaculates from the same boar, the inter-ejaculate difference was considerably lower than the inter-boar difference. Roca *et al.* (2006a) observed that 70% of total variability in cryosurvival of boar semen could be due to boar effects. Thus pre-selection of boars for freezability using standard protocols could be a reliable way of identifying potential candidates for production of frozen/thawed semen with an acceptable quality. Modifications of the cryopreservation conditions for individual boars could further improve the cryosurvival of semen of sub-optimal freezers. Thurston *et*

al. (2002) used Amplified Fragment Length Polymorphism (AFLP) technique to compare 'bad' and 'good' freezer Landrace boars. This technique detects and evaluates differences in DNA sequences that correspond with phenotypic variability such as coat colour and also semen freezability. They found distinct differences in AFLP profiles, indicating the existence of markers for semen freezability. It was concluded that these markers might be related to variations in seminal plasma composition or accessory sexual gland functions rather than to diversity in sperm bio-chemistry and physiology.

The differences in freezability of boar ejaculates could be due to variations in the expression of genes involved in cell resistance of the ejaculates against damages by freeze-thawing procedures (Yeste 2015). Sperm proteins, viz. heat shock protein 90, acrosin-binding protein, triosophosphate isomerise, voltage-dependent anion channel 2 and seminal plasma protein fibronectin 1 that are involved in the response to osmotic and thermal stress, could predict ejaculates freezability in extended semen and serve as fertility markers (Casas *et al*. 2010, Vilagran *et al*. 2015).

Bacteria in ejaculate: The bacteria in collected boar ejaculates are present owing to contamination released lipopolysaccharide (LPS), a component of bacterial cell walls, on bacteriolysis due to conventional antibiotic treatment which stimulated Toll-Like-Receptor (TLR) that led to expression and secretion of cytokines or chemokines or induced apoptotic pathway (Kenny and O'Neill 2008). Thus activation of TLRs induced sperm membrane abnormality and apoptosis which resulted in a reduction of both sperm motility and fertilizing ability. Hence high population of gram-positive and gram-negative bacteria in boar ejaculates could impair sperm functionality before the cooling or freezing process.

Insemination strategy: In conjugation with evolving improved freezing methodologies, efforts have been made for consolidation of the procedures of breeding with frozen boar sperm since the post thaw quality of boar sperm is very poor with shorter life span (2–8 h) than fresh liquid spermatozoa. Works have been conducted to determine the proper sperm number required in an AI dose for frozen boar semen, number of inseminations needed, timing relative to ovulation, and method of semen deposition.

Boar deposits more than 50×10^9 spermatozoa in the cervical canal during natural mating, which helps to overcome the female's hostile uterine environment where only the strongest, fastest and lucky spermatozoa are able to reach the site of fertilization. The AI procedure in pigs deposits the semen dose within the posterior portion of the cervical canal (approximately 15 cm deep into the cervix) by means of a catheter that engages the folds of the cervix. The standard protocol recommends the deposition of a high number of spermatozoa (generally more than $2{,}500\times10^6$ cells) in a large volume of extender (80–100 ml) two or three times during oestrus. This protocol limits the number of doses that can be prepared from one ejaculate to approximately 20–25 (Vazquez *et al.* 2008). Various workers reported that by using AI doses of nearly 5×10^9 cells, the highest pregnancy rate in gilts at day 30 was 65%, while pregnancy rate in sows at day 30 was 79%, and the highest farrowing rates in sows ranged from 76 to 82% (Eriksson and Rodriguez-Martinez 2000, Eriksson *et al*. 2002, Okazaki *et al.* 2009). Well-managed herds, optimum timing of insemination and less backflow of semen during deposition resulted in good fertility using standard AI with only $1000–2000\times10^6$ spermatozoa per insemination (Steverink *et al*. 1997, Watson and Behan 2002). Various studies indicated that double insemination resulted in better farrowing rate and litter size as compared to single insemination (Kemp *et al.* 1996). Breeding method with $5–6\times10^9$ frozen-thawed sperm in spontaneously ovulating females that yielded the best pregnancy rates in gilts (65%) and farrowing rates in sows (>72%) utilized two inseminations, one at 24 h and the other at 36 h following oestrus (Eriksson and Rodriguez-Martinez 2000, Eriksson *et al*. 2002). This system seems to allow the best chance for fertilization to occur despite the decreased lifespan of frozen-thawed sperm. This along with the damage that occur to sperm during the cooling and freezing phases of cryopreservation and the capacitated-like state that results might accentuate the critical importance of interval from AI to ovulation for frozen boar semen than liquid semen (Waberski *et al*. 1994, Bailey *et al*. 2008). Bolarin *et al.* (2006) found that insemination close to the time of ovulation was the most important factor to achieve high fertility with low numbers of spermatozoa. Using frozen sperm doses of 1×10^9 twice per estrus, they obtained higher pregnancy rate and litter size in sows inseminated peri-ovulatory compared with inseminations pre-or post-ovulation (84.0% and 9.45±0.23, 48% and 8.67±0.54, and 48.8% and 7.54±0.62, respectively). Spencer *et al*. (2010) found that a longer interval between insemination with frozen boar semen and ovulation reduced pregnancy rates, number of normal foetuses and embryo survival.

New sperm technologies which include frozen-thawed semen, sex-sorted semen, sperm mediated gene transfer, encapsulated sperm necessitate the development of new strategies to achieve successful fertilization using lesser number of motile spermatozoa. New insemination procedures have been evolved based on the deposition site of the insemination to reduce the number of spermatozoa. The new procedures deposit the spermatozoa in the uterine body (post cervical insemination or intrauterine insemination, IUI), or deep in the uterine horn (deep intrauterine insemination, DUI), or in the oviduct (laparoscopic intra-oviductal insemination). Transcervical insemination allowed the reduction of a fresh semen dose by one third without significantly compromising the fertility of weaned sows (Watson and Behan 2002). Sumransap *et al.* (2007) reported good fertility with $1{,}000\times10^6$ spermatozoa directly deposited into the uterine body (IUI). Bathgate *et al.* (2008) used a double DUI 24 h and 36 h after detection of oestrus with 1×10^9 frozen-thawed boar spermatozoa, achieving results that were low but not significantly different

than the control group with 1×10^9 fresh spermatozoa inseminated cervically (farrowing rates 34.7±6.8 vs 43.6±7.3%, respectively). Wongtawan *et al.* (2006) did DUI in sows with 1×10^9 spermatozoa frozen in 0.7 ml Flat Pack or medium straws that were not extended after thawing. Spermatozoa frozen in 0.7 ml Flat Packs achieved a pregnancy rate of 31.8%, compared with 40% after AI of sperm frozen in medium straws. Application of IUI and DUI resulted in higher fertility using decreased sperm numbers or damaged sperm cells with both fresh-chilled and frozen-thawed sperm (Rozeboom *et al*. 2004, Vazquez *et al*. 2005). Doses of 5×10^9 total frozen-thawed sperm have resulted in 70% pregnancy rates utilizing IUI (Bielas *et al*. 2008), while a DUI dose of 1×10^9 sperm yielded pregnancy rates of ~78% and litter sizes of >9 (Roca *et al*. 2003). The technology of IUI and DUI could improve the usefulness of frozen-thawed sperm, despite the difficulty during the breeding process as it allowed for reduction in sperm number per dose and thus decreased the expenses.

Didion *et al*. (2013) reported high rates of approximately 75 to 80% following AI with frozen-thawed sperm. In India, an incipient attempt was made by Baishya *et al.* (2016) to record the fertility status of frozen boar semen on cervical AI. They reckoned the farrowing rate as 44% with mean litter size at birth 5.91±0.69 numbers. Although the fertility rate was moderate, yet it would help in further improvement and utilization of frozen boar semen for AI in India.

Therefore high fertility rates with cooled, frozen-thawed or sex-sorted boar semen were feasible to achieve by using appropriate insemination procedures. Post-cervical IUI allowed a threefold reduction of spermatozoa to be inseminated, whereas DUI allowed a substantial reduction in the number of cooled (5–20 folds) or frozen-thawed (6-folds) spermatozoa.

Future strategy for improvement of cryopreservation technique of boar semen

Although cryopreserved boar semen has been available since 1975, still there is a continuous need for further improvement of cryopreservation strategy to obtain optimum fertility outcome of frozen boar semen. Some factors like freezing medium, cryoprotectant concentration, cooling, freezing and thawing rates, and freezing package which could influence the success of cryopreservation of boar semen should be worked out for maximum sperm cryosurvival. Supplementation of antioxidant and additive to the pre-freezing and freezing media of boar semen can reduce sperm damage and increase the cryosurvival of frozen-thawed boar spermatozoa. Future study would be required to improve the fertility parameters of frozen boar semen involving larger number of females using varying sperm concentrations, performing AI closer to the time of ovulation and adopting new insemination technique.

Conclusion

To conclude, it may be stated that vigorous research works have been conducted by many researchers to establish a commercially viable freezing protocol for boar semen with optimum fertility and the goal has been achieved to some extent as revealed by the consequence of findings of many scientist. Today the niche for cryopreserved boar sperm is limited only to semen bank; however, days are not too far when it will be used extensively in AI like that in cattle.

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