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New insights into the understanding of the mechanism of sperm protection by extender components

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Abstract

Over the past 65 yr egg-yolk and milk have been routinely used in both liquid semen extenders and those used to cryopreserve sperm. However, the mechanism by which egg yolk and milk protect sperm during liquid storage or from freezing damage is poorly understood. Seminal plasma from many mammalian ungulates contains a family of proteins called Binder of SPerm (BSP). These proteins are secretory products of seminal vesicles that bind to sperm at ejaculation and modify the sperm membrane by removing cholesterol and phospholipids, which may adversely affect the ability of sperm to be preserved. Interestingly, BSP proteins bind to the low-density lipoproteins (LDL) present in egg yolk extender. The binding is rapid, specific and stable even after freeze-thawing of semen. LDL has a very high capacity for BSP protein binding. Furthermore, LDL prevents lipid efflux from the sperm membrane and maintains sperm motility during sperm storage. Recent, studies also indicate that casein micelles, α-lactalbumin and β-lactoglobulin, the major proteins in milk extenders also interact with BSP proteins present in the seminal plasma. Additionally, casein micelles reduced lipid loss from the sperm membrane and maintained sperm function during storage. These data indicate that the interaction between BSP proteins of seminal plasma and LDL or milk proteins could be the basis sperm protection.

Keywords: α-lactalbumin and β-lactoglobulin, Binder of SPerm (BSP) proteins, semen extenders, lipoproteins, seminal plasma.

Sperm preservation

The cryopreservation of semen and artificial insemination has enabled the worldwide distribution and use of desired genetic lines at a reasonable cost. Over the past 65 yr, the cryoprotective media for sperm storage have been continuously revised but the basic ingredients of the media remain unchanged. Egg yolk and/or milk and glycerol represent the indispensable compounds of practically all media used for bull sperm preservation in the liquid or frozen states. The role of glycerol in cryopreservation is that it allows sperm survival during freezing by lowering the freezing point of extended semen (reviewed in Bergeron and Manjunath, 2006). However, the protection afforded by egg yolk and milk during sperm storage or cryopreservation is more complex and is poorly understood.

Sperm protection by egg yolk

Egg yolk has been shown to increase sperm fertilizing ability when present in extenders for semen storage at ambient temperature (Dunn *et al*., 1950; Shannon and Curson, 1983; Barak *et al*., 1992) and appears to prevent sperm cell damage during cooling and freezing (Phillips and Lardy, 1940; De Leeuw *et al*., 1993). Egg yolk is generally used at a concentration of 20% (v/v) in extender and various components of egg yolk have been investigated to identify the most active component(s) responsible for the protective effect (Kampschmidt *et al*., 1953; Watson, 1976; Foulkes, 1977). Evidence indicates that low-density lipoproteins (LDL) are the egg yolk component showing the highest protective ability (Pace and Graham, 1974; Watson, 1981). In addition, some studies report that LDL is better than whole egg yolk to preserve sperm motility after freezing (Moussa *et al*., 2002; Amirat *et al*., 2004). However, the mechanism by which this protection is provided to sperm remains elusive. It is speculated that LDL associate with sperm membranes and provide protection to sperm by stabilizing the membrane, but there is contradictory evidence concerning the stability of this association (Watson, 1975; Foulkes, 1977; MacDonald and Foulkes, 1981). A second hypothesis suggests that it is the phospholipid fraction present in LDL that protects sperm by forming a protective film on the sperm surface (Quinn *et al*., 1980) or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process (Foulkes *et al*., 1980; Graham and Foote, 1987). Another study suggests that egg yolk lipoproteins compete with detrimental seminal plasma cationic peptides (<5 kDa) in binding to the sperm membrane and thus protect the sperm (Vishwanath *et al*., 1992). Several lines of evidence now indicate that LDL interacts with the major proteins of bull seminal plasma and this interaction appears to be crucial for sperm protection (Manjunath *et al*., 2002).

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Sperm protection by milk

For sperm preservation, skimmed milk or whole milk is used as buffers in which semen is directly diluted and can be stored at 4ºC or frozen in the presence of glycerol (reviewed in Kakar and Ganguli, 1978). Milk based extenders are as efficient as egg yolk based extender to protect sperm (Chen *et al*., 1993). Whole milk contains 5 major constituents: water (87.5%), proteins (3.2%), sugars (mainly lactose, 4.6%), lipids (3.7%) and minerals (0.8%; Amiot *et al*., 2002). Skimmed milk has the same composition as whole milk but contains <0.1% lipids (mostly triglycerides). The major proteins of milk are the caseins (80% of total milk proteins). They exist in large colloidal aggregates termed micelles. The other proteins of milk are collectively called whey proteins (20% of total milk proteins). α-lactalbumin and β-lactoglobulin are the major whey proteins (22 and 55%, respectively; Amiot *et al*., 2002).

Since skimmed milk is as efficient as whole milk to protect sperm during semen storage at 4ºC or cryopreservation (Almquist *et al*., 1954; Chen *et al*., 1993; Foote *et al*., 2002), lipid do not seem to be the constituent responsible for the protection afforded by milk. The most protective constituent of milk seems to be casein micelles (the major proteins of milk). In fact, it has been shown that casein micelles isolated from milk can protect stallion (Batellier *et al*., 1997), goat (Leboeuf *et al*., 2003), ram (Choong and Wales, 1962; O'Shea and Wales, 1966) and bull (O'Shea and Wales, 1966) sperm during storage at 4-5ºC. In addition, casein micelles can protect bull sperm during freezing in the presence of glycerol (Choong and Wales, 1963). However, how the casein micelles protect sperm during storage is not known. Furthermore, the addition of lactose to extender containing caseins improved the extender efficiency during bull sperm freezing (Choong and Wales, 1963). Thus, lactose also appears to be involved in sperm protection by milk. However, milk filtrate (milk depleted of lipids and proteins), which contains only lactose and minerals, is not sufficient to protect equine sperm during storage (Batellier *et al*., 1997). In the same manner, skimmed milk dialysate was not sufficient to protect bull sperm during cryopreservation (Garcia and Graham, 1987). In fact, the presence of the non-dialyzable fraction of skimmed milk was required to protect sperm (Garcia and Graham, 1987). Lactose is also a common component of egg yolk based extender used to freeze boar (Yi *et al*., 2002) and stallion (Loomis *et al*., 1983) sperm. Thus, lactose seems to improve extender's efficiency but is not sufficient to protect sperm by itself. Lactose has a low cellular permeability and it is believed to protect sperm by acting extracellularly (reviewed in Watson, 1990). In a recent study, we report that the BSP proteins, the major component of bull seminal plasma interact with milk proteins and this interaction appears to be critical for sperm protection during storage (Lusignan *et al*., 2011a).

Seminal plasma

Seminal plasma, which is a secretory product from testes, epididymides and accessory gland fluids serves as the carrier of sperm to the female genital tract and has been described as both beneficial and detrimental to sperm (for a review see Bergeron and Manjunath, 2006). More precisely, several workers have described seminal plasma factors that support sperm functions such as motility, viability and fertility and that facilitate capacitation. However, seminal plasma is also known to contain factors that are detrimental to sperm fertilizing ability, such as decapacitation factor and motility inhibiting factor. In addition, seminal plasma is detrimental to sperm storage because it contains factors that negatively affect sperm viability. However, the factors responsible for these activities have not been characterized precisely.

Binder of SPerm (BSP) proteins

Our laboratory has extensively investigated a family of closely related major proteins from bull seminal plasma named Binder of SPerm (BSP) proteins (for reviews see Manjunath *et al*., 2007, 2009). The BSP family comprises BSP1, BSP3 and BSP5 (previously called BSP-A1/-A2, BSP-A3 and BSP-30-kDa, respectively). They are secreted by seminal vesicles and represent up to 60% of total proteins of bull seminal plasma. BSP1 and BSP3 have apparent molecular masses ranging from 15 to 17 kDa and the BSP5 has a molecular mass of 28 kDa. BSP1 and BSP5 are glycosylated but not BSP3 (Manjunath *et al*., 1988). In general BSP proteins contain a variable amino-terminal end and two fibronectin type 2 (Fn2) domains (reviewed in Fan *et al*., 2006). They can bind to denatured collagen (gelatin), choline phospholipids, glycosaminoglycans and high-density lipoproteins (for a review see Manjunath *et al*., 2007).

Homologs of BSP proteins have been isolated and characterized in other mammalian seminal plasma or seminal vesicle secretions (reviewed in Bergeron and Manjunath, 2006). In most species BSPs exist in iso-forms and/or glycoforms. Bull, bison and stallion seminal plasma contain three forms; ram and goat seminal plasma contain four forms, whereas boar seminal plasma has one form. All BSP homologs from different species in general bind to gelatin and most of them also bind to heparin. In addition, BSP homologs bind to choline phospholipids (reviewed in Manjunath *et al*., 2007).

The concentration of BSP homologs in seminal plasma varies from species to species. In bull, the concentration of BSP proteins in seminal plasma is very

high (30-50 mg/ml or ~60% of total proteins). In boar, BSP homologs represent ~1% of the total seminal plasma proteins (0.3-0.5 mg/ml), while in ram, goat and stallion seminal plasma, the BSP homolog concentration is ~15, 6 and 2 mg/ml respectively, representing ~20-30% of total proteins present in seminal plasma. This difference in the amount of BSP protein homologs in ejaculates may have an implication in sperm storage as described later.

BSP proteins are detrimental to sperm

BSP proteins are beneficial for fertility; more specifically, BSP proteins participate in the sperm membrane lipid modifications that occur during sperm capacitation, which is a prerequisite for fertilization. Based on a series of studies, a molecular model depicting the role of BSP proteins in capacitation has been described (Manjunath and Thérien, 2002; Manjunath *et al*., 2007). Additionally, sperm-bound BSP proteins appear to mediate the formation of sperm reservoir by interacting with oviductal epithelial cell surface and also implicated in the maintenance of sperm motility in the oviduct (Gwathmey *et al*., 2003, 2006).

BSP proteins are detrimental to sperm in the context of sperm storage. Seminal plasma stimulate cholesterol and choline phospholipid efflux from the epididymal sperm membrane in a concentration and time dependent manner and this effect is essentially due to the BSP proteins present in seminal plasma (Thérien *et al*., 1998, 1999). In the same manner, when undiluted bovine ejaculates were stored at 37°C for 24 h, sperm lost ~40% of their cholesterol and ~40% of their choline phospholipids (Bergeron *et al*., 2004). Similar loss of cholesterol and phospholipids was also observed when semen was diluted 10 times or more with an extender devoid of egg yolk (Bergeron *et al*., 2004). It is known that a low level of cholesterol in the sperm membrane is correlated with a decreased tolerance to cold shock (Darin-Bennett and White, 1977) and that incorporation of cholesterol into bull sperm membrane increased the survival of sperm after cryopreservation (Purdy and Graham, 2004). Therefore, continuous exposure of sperm to seminal plasma that contains BSP proteins may damage the sperm membrane by removing lipids and rendering the membrane very sensitive to storage in the liquid or frozen states.

BSP proteins interact with extender components (LDL and milk proteins)

Since BSP proteins are choline phospholipid-binding proteins and that LDL contains choline phospholipids, we investigated whether or not BSP proteins, the seminal plasma factors detrimental to sperm storage, interact with LDL, the egg yolk component responsible for sperm protection. LDL was isolated by ultracentrifugation, incubated with seminal plasma and reisolated by ultracentrifugation (Manjunath *et al*., 2002). The SDS-PAGE analysis of reisolated LDL demonstrated the presence of all the BSP proteins, indicating that BSP proteins bind to LDL. The LDL-BSP protein interaction is specific since no other proteins present in bull seminal plasma bind LDL. The interaction between BSP proteins and LDL could also be demonstrated by gel filtration chromatography and agarose gel electrophoresis (Manjunath *et al*., 2002). Additionally, it is shown that LDL has a high capacity to bind BSP proteins (Manjunath *et al*., 2002; Lusignan *et al*., 2011b). Furthermore, the interaction between the LDL and BSP protein is stable after freeze-thawing of semen (Manjunath *et al*., 2002).

Since milk caseins have been shown to protect sperm during storage, we investigated whether or not BSP proteins interact with caseins and other milk proteins (α-lactalbumin and β-lactoglobulin). Results indicate that all these components interact with BSP proteins although with different affinities (Lusignan *et al*., 2011a, b). In addition, the interaction between caseins or whey proteins with BSP proteins is stable after freeze thawing.

LDL and caseins decrease binding of BSP proteins to sperm and prevent lipid loss from the sperm membrane

We observed that in the presence of LDL or egg yolk in the extender, there are about 50-80% less BSP proteins bound to sperm as compared to extender without egg yolk or LDL (Bergeron *et al*., 2004). Furthermore, in the absence of egg yolk or LDL in the extender, there is a continuous efflux of cholesterol and phospholipid from the sperm membrane during 24 h storage at 4°C (Bergeron *et al*., 2004). However, when LDL or egg yolk was present in the extender, no loss of cholesterol and phospholipid was observed. Interestingly, the presence of egg yolk or LDL in the extender preserved sperm motility. The presence of LDL mimicked the effect of whole egg yolk in the extender used to dilute semen. Therefore, LDL is the factor in egg yolk that protects sperm against the detrimental effect of BSP proteins during storage (Bergeron *et al*., 2004).

In a similar manner, the presence of caseins in the extender decreased the binding of BSP proteins to sperm and prevented sperm lipid loss while maintaining sperm motility and viability during storage (Bergeron *et al*., 2007). These observations indicated that milk caseins prevent the detrimental effect of BSP proteins on the sperm membrane during sperm preservation.

Novel concept of sperm protection by egg yolk and milk components

In view of the discovery that BSP proteins

specifically bind to LDL and form a stable complex, we suggest a new concept of sperm protection by egg yolk lipoproteins (Fig. 1). Upon ejaculation, BSP proteins secreted by seminal vesicles are added to sperm (Manjunath *et al*., 1988) and coat the sperm membrane (Desnoyers *et al*., 1992; Manjunath *et al*., 1994). Sperm bound BSP proteins induce sperm membrane cholesterol (Thérien *et al*., 1998) and phospholipid efflux (Thérien *et al*., 1999). If semen is not diluted, sperm are continuously exposed to a high concentration of BSP proteins and the lipid efflux continues, resulting in decreased sperm resistance to cold shock and freezing. Since ejaculates are diluted with egg yolk extenders within minutes after collection, LDL may sequestrate most of the BSP proteins present in semen. This could result in a minimal modification of the sperm plasma membrane and allow better sperm storage. Thus, egg yolk lipoproteins may offer protection to sperm by reducing the deleterious effect of seminal plasma proteins on sperm membranes.

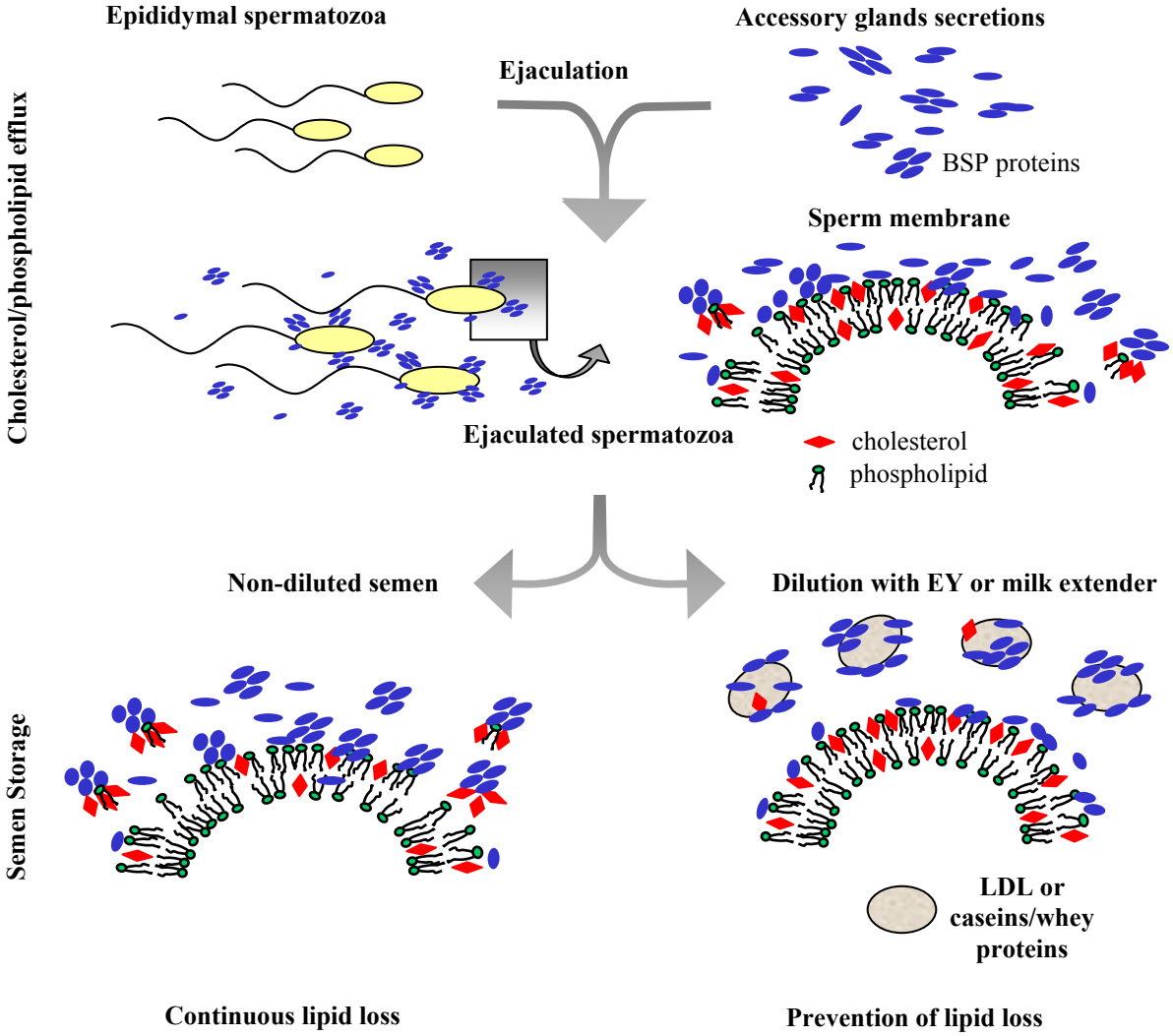

Figure1. Mechanism of sperm protection by egg yolk and milk. Modified from Manjunath *et al.*(2002).

The protective action of milk on sperm is analogous to the protective action of egg yolk. As egg yolk, skimmed milk prevents the binding of BSP proteins to sperm and sperm lipid loss while maintaining sperm motility and viability during sperm storage (Bergeron *et al*., 2007). Interestingly, while sperm protection by egg yolk involves the sequestration of BSP proteins by the LDL present in egg yolk (BSP proteins:lipoprotein interaction), the protection afforded by skimmed milk does not involve the participation of lipids or lipoproteins. It is the casein micelles and whey proteins present in skimmed milk, which sequestrate BSP proteins. Thus, sperm protection by milk involves a BSP protein:casein micelles or whey proteins (protein:protein) interaction.

During natural mating, a mechanism may also exist to eliminate the detrimental effect of BSP proteins on sperm. After being ejaculated into the vagina, sperm

swim through the cervical mucous and enter the uterus within minutes, leaving behind the seminal plasma. In the artificial insemination industry, BSP proteins are not removed from semen, but their effect is eliminated by the formation of rapid and stable complex with the extender components (egg yolk lipoproteins or milk proteins).

It is interesting to note that extenders containing egg yolk and/or skimmed milk are also used to store semen from other mammals such as boar, ram, goat, stallion, buffalo, dog, monkey and gazelle (reviewed in Bergeron *et al*., 2007). Furthermore, BSP proteins seem to be ubiquitous among mammals and all homologs from other species also bind to egg yolk-LDL (reviewed in Bergeron *et al*., 2007). It is most likely that BSP proteins from these species also interact with milk proteins and therefore, the mechanism of sperm protection by egg yolk (or milk) may be similar for all mammals.

Conclusions and perspectives

Our studies indicate that BSP proteins present in seminal plasma intrinsically remove sperm membrane lipids. This is detrimental to sperm storage in the liquid or frozen states. Fascinatingly, BSP proteins associate with LDL present in egg yolk extender as well as caseins and whey proteins present in milk extender. This association prevents or minimizes lipid loss from the sperm membrane thereby protecting sperm during storage. Sperm protection by egg yolk involves a protein:lipoprotein interaction, whereas sperm protection by milk involves a protein:protein interaction. We hope that our studies will help in finding new strategies to improve the efficiency of the mammalian sperm storage and in developing novel sperm extenders devoid of animal products for commercial applications, which is becoming an urgent need.

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