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REVIEW ARTICLE

Cryoprotectant in semen extender: From egg yolk to low-density lipoprotein (LDL)

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Abstract

Egg yolk is an important component of semen extender. It is used as cryoprotentant to prevent the membrane damage of sperm cell during freezing process. Egg yolk (EY) of hen is frequently used in semen extender due to its easy availability. But with advancement in knowledge regarding the chemical composition of egg yolk from different avian species and the role of egg yolk components in membrane protection, the egg yolk from different avian species are now being utilized as cryoprotectants as semen extenders during freezing. It is reported that egg yolks from different avian species have beneficial effect on the semen cryopreservation and post thaw semen quality as compared to hen egg yolk. LDL is thought to be the component in egg yolk that has cryoprotective action. Hence the LDL is looked upon as an alternative to replace egg yolk in semen extenders. Cryopreservation performed using LDL in place of egg yolk at 8-9% in different farm animals has shown better results. As the other components in egg yolk play role in membrane protection, and their role cannot be undermined. Therefore there is need to study and formulate optimum concentration of egg yolk and LDL combination for better cryopreserved semen quality.

Keywords: Avian species, cryopreservation, egg yolk, low density lipoprotein.

Introduction

Semen cryopreservation a technique for storage of semen has allowed specific opportunities for the conservation and widespread dissemination of valuable genetic resources through sperm banks, guarantee of a constant commercial supply of semen, and collaboration in breed improvement programs by means of artificial insemination (Holt *et al*., 1994). Cryopreservation involves several steps, such as dilution, cooling, freezing and thawing (Luvoni), during which, spermatozoa are exposed to cold shock, which increases their susceptibility to lipid peroxidation leading to membrane damage (Bucak *et al*., 2008). Spermatozoa subjected to cryopreservation are most sensitive to a rapid reduction in temperature i.e. cooling rate especially from 25 to 5 ^{0}C (Watson, 1981; White, 1993), this produces cold shock, a membrane transition phase behavior is exhibited by biological membranes (Morris *et al.*, 1987). Cold shock results in a loss of selective permeability and integrity of the plasma membrane (Ortman *et al*., 1994), release of intracellular enzymes (Harrison and White, 1972) and lipids (Darin-bennett *et al*., 1973; Pickett *et al*., 1967), redistribution of ions (Quinn and White, 1968), change in acrosome membranes (Jones and Martin, 1973) and mitochondria (Watson, 1995), loss of motility and diminished metabolism. Other factors that affecting the proportion of normal sperms during cryopreservation are diluents composition and osmotic stress and factors influencing functional status of survivors e.g., membrane stability, oxidative damage, membrane receptor integrity, nuclear structure (Watson, 2000). When cells are frozen, they are subjected to stresses resulting from water–solute interaction that arises through ice crystallization. Exposure to the hyperosmotic, unfrozen solution causes an efflux of intracellular water, cell shrinkage, and potentially and influx of ions (Mazur, 1984). Thawing results in a reversal of these effects; the consequent influx of water may cause membrane disruption. Therefore, cryoprotective agents in semen extender play a crucial role during semen cryopreservation (Schneider and Mazur, 1984). They resist sudden temperature change, protect sperm

against cold and hot shock damage (Watson, 1999), as well as prevent ice formation during freezing and dissolution in the thawing process. Egg yolk (EY) is frequently used as a cryoprotective agent in mammalian semen diluents, and showed to be highly effective for the maintenance of sperm fertility in different species (Sansone *et al*., 2000; Garde *et al*., 2003).

Egg Yolk in semen Extender

During the process of cryopreservation, extenders are routinely used to dilute and create multiple insemination doses from a single ejaculate and also contain buffers and nutrients that provide spermatozoa with an environment that maintains viability post-collection (Kuster, 1999). Few decades before successful freezing protocols solely depended on suppressing extracellular ice formation and were aided by both the addition of chemical agents (Luyet, 1938) and by cooling. But the discovery of cryoprotective agents added a new era in semen cryopreservation. The cryoprotective agents in freezing extender are the most important constituents and have a large influence on post-thaw sperm survival. EY is the most effective cryoprotective agent of freezing extender that protects sperm against cold shock (Bergeron and Manjunath, 2006). 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). In fact, it is a component of semen freezing extenders used for majority of the live-stock species, including the buffalo (*Bubalus bubalis*) (Sansone *et al*., 2000; Bathgate *et al*., 2006). It is generally used at a concentration of 20% (vol/vol) in semen extender for bovine (Hafez, 2000) and in different concentrations in buck depending on cryopreservation technique followed (Bispo, 2011). Egg yolk during sperm cryopreservation act as protectant of the plasma membrane and acrosome against temperature-related injury, in association with the other components (Amirat *et al*., 2004). It is believed that the beneficial role of EY in sperm cryopreservation can be attributed to phospholipids (Lanz *et al*., 1965), cholesterol (Darin-Bennett and White, 1973) and low density lipoproteins (LDL) content which afford successful protection to the sperm against cold shock and the lipid-phase transition effect during the freeze–thaw process (Moussa *et al*., 2002). These findings have opened up new opportunity to improve cyyopreservation efficiency by replacing hen egg yolk with egg yolk from different avian species (domestic chicken, goose, turkey, duck, Japanese quail and chucker) in the extender and to study its effect on semen cryopreservation.

Egg Yolk from Different Avian Species

Taking into account the fact that exact mechanism by which EY preserves the bull spermatozoa during freeze-thaw process is unknown (Bathgate, 2006) and with an objective to further improve the technique of semen cryopreservation, researchers evaluated the chemical composition from egg yolk of different avian species and reports that egg yolk from avian species such as the duck, quail, pigeon or chicken have different combinations of fatty acids, phospholipids and cholesterol, which could result in different cryopreservation effects on the sperm. (Trimeche *et al*., 1997; Choi *et al*., 2001; Andrabi *et al*., 2007; Bahtgate *et al*., 2006; Humes and Webb, 2006; Clulow *et al*., 2007; Moreno *et al*., 2008; Su *et al*., 2008). Similarly highest amount of cholesterol was observed in ostrich compared to partridge, duck and chicken EYs (16.29), (13.93), (10.81) and (13.91) mg g^{-1} of yolk, respectively (Kazmierska *et al*., 2005; Krawczyk, 2009). Whereas, duck EY has more monounsaturated fatty acid than chicken EY (Bathgate *et al*., 2006), and both duck and chicken EYs have a different ratio of the fatty acids comprising the total yolk lipids (Surai *et al*., 1999). Sinanoglou *et al*. (2011) observed that the total EY lipids was decreased in the following order, ostrich, duck, turkey, and fat from ostrich EYs was characterized by the highest content of α-linolenic and low level of linoleic acid and palmitoleic acid compared with other sources of yolk.

Later it is suggested that the improvement or decline in post-thaw quality of mammalian spermatozoa with EY of different avian species in freezing extender is attributed to the differences in biochemical composition of the yolks (Trimeche *et al*., 1997; Bathgate *et al*., 2006). Considering the above fact, trials were conducted to replace Hen egg yolk by egg yolk of different avian species. Humes and Webb (2006) observed that substitution of chukar egg yolk for hen yolk improved total motility, progressive motility and straight-line velocity in frozen-thawed stallion semen. Andrabi *et al. (*2008) reported that duck egg yolk supplementation in semen extender improved freezability of buffalo bull spermatozoa. Similarly, Clulow *et al*. (2004) reported that cryopreservation of stallion semen with duck EY (DEY), compared with CHEY resulted in significantly higher forward motility and livability post-thaw.

LDL (Low Density Lipoproteins)

Numerous researchers (Polge *et al*., 1970; Evans *et al*., 1973; Watson and Martin, 1975) proposed that low-density fraction of egg yolk, mainly composed of low density lipoproteins (LDL), could be largely responsible for the resistance against cold shock and for the improvement of sperm motility, acrosome and plasma membrane integrity after freezing and thawing process. Graham and Foote (1987) suggested that LDL could adhere to cell membranes during the freeze-thaw process, thus preserving spermatozoa membranes. Later, Demianowicz and Strezek (1996) and Moussa *et al*. (2002) confirmed that LDL have a cryoprotective action in the egg yolk. Though the exact mechanism of LDL action is still unclear, it is suggested that LDL adhere to sperm membrane and provide protection to sperm by stabilizing the membrane. A second hypothesis suggests that phospholipids present in LDL protect sperm by forming a protective film on the sperm surface or by replacing sperm membrane phospholipids that are lost or damaged during the cryopreservation process (Foulkes *et al*., 1980; Quinn *et al*., 1980; Graham and Foote, 1987). A third mechanism of protection suggests that LDL seizes the deleterious proteins present in seminal plasma thus improving the freezability of spermatozoa (Manjunath *et al*., 2002; Bergeron and Manjunath, 2006). Fourth hypothesis i.e. another study suggests that egg yolk lipoproteins compete with detrimental seminal plasma cationic peptides in binding to the sperm membrane and thus protect the sperm (Vishwanath *et al.,* 1992). Manjunath (2012) giving new insights into the understanding of the mechanism of sperm protection by egg yolk concluded that the LDL and caseins decrease binding of Binder of Sperm (BSP) proteins to sperm and prevent lipid loss from the sperm membrane. BSP is a family of closely related major proteins from bull seminal plasma and is secreted by seminal vesicles and represent up to 60% of total proteins of bull seminal plasma. 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). 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. 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). But at the same time BSP proteins are detrimental to sperm in the context of sperm storage. Seminal plasma stimulate cholesterol and choline phospholipid efflux from the 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). Since, the sperms are to be stored for long time through process of cryopreservation are affected most by these BSP proteins present in seminal plasma that intrinsically remove sperm membrane lipids (Manjunath, 2012) leading to detoriated post thaw semen quality and affecting success rate of A.I. Since BSP proteins are choline phospholipid binding proteins and that LDL contains choline phospholipids, LDL in the egg yolk interact with BSP proteins in the seminal plasma factors and is responsible for sperm protection (Manjunath, 2012). So several lines of evidence 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). Hence this LDL is now being looked upon as an alternative to replace with egg yolk during process of semen cryopreservation.

Based on this knowledge, with the impetus to develop extenders chemically better defined than those containing whole egg yolk, several recently studies have tested replacing whole egg yolk with LDL for cryopreservation of bovine (Manjunath *et al*., 2002, Bergeron *et al*., 2004; Amirat *et al*., 2005), caprine (Al Ahmad *et al*., 2008) porcine (Jiang *et al*., 2007) and canine (Neves, 2007; Varela *et al*., 2009; Bencharif *et al*., 2010) sperm. However, the use of LDL for ovine sperm cryopreservation has apparently not been reported. Moussa *et al*. (2002) obtained better results in terms of motility and movement characteristics when replacing whole egg yolk by 8% (w/v) LDL in bull semen freezing. Hu *et al*. (2011) also reported that sperm cryopreserved in the extender containing 8% LDL in place of egg yolk exhibited the greatest percentages of post-thaw sperm motility, acrosome integrity and membrane integrity and favored the highest anti-oxidant activities of CAT, GSH-Px and GSH in comparison with other groups. Hu *et al*. (2006) found out that the extender with 9% LDL concentration significantly enhanced motility, acrosome and plasma membrane integrity of boar sperm after freezing and thawing. Al-Ahmed *et al*. (2008) reported that extender with 8% LDL significantly improved seminal attributes in buck.

Conclusion

It can be concluded that egg yolk is essential component for semen cryopreservation that has a cryoprotective role. But the egg yolk from different avian species utilized as cryoprotectent has shown improved the quality of cryopreserved semen. It has been well reported that the action preformed by egg

yolk is mainly through LDL. So considering the importance of LDL in egg yolk and its role in protecting sperm membrane during freezing, is looked upon as an alternative to replace with egg yolk during process of semen cryopreservation. But the research conducted with objective to replace egg yolk by LDL as cryprotectant are directed for its complete replacement. So taking into account the fact that exact mechanism of egg yolk as cryoprotectant is still to understood and other components in egg yolk i.e protein, fatty acids, phospholipids and cholesterol are also thought to play role in successful cryopreservation, there absence cannot be denied. Hence, there is need to study the combined effect of LDL and egg yolk on the cypyopreservation of semen for better results.

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