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Membrane-Mediated Regulation of Sperm Fertilization Potential in Poultry

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Fertilization requires successful completion of molecular events taking place at different spatiotemporal scales. Transcriptionally and translationally inactive sperm need to rely on pre-assembled pathways modulated by extracellular signals that traverse the plasma membranes. However, species differences in how sperm respond to them delay the progress toward a comprehensive understanding of how activation of the signaling cascades is coordinated in poultry sperm. In chickens, recent studies have found that membrane rafts are present on the sperm surface and play important roles in regulating multistage fertilization. In this review, we focus on three steps in which membrane alteration plays a key role. The first is post-testicular maturation, in which bird sperm acquire fertilization functions through biochemical changes. The second part of this review concerns membrane regulation of sperm-egg binding and the acrosome reaction. Finally, we extend our discussion to the translation of membrane raft theory into a technical principle for the commercial production and genetic preservation of poultry.

Key words: acrosome reaction, membrane rafts, signaling pathway, sperm, sterol

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Introduction

The fertilization process is a net result of serial molecular events that enable ejaculated sperm to bind and penetrate the oocyte for gamete fusion. To ensure the success of fertilization, each step needs to proceed in a precise and ordered manner. However, mechanisms underlying the sequence of steps can, in part, be species-specific or consistent between taxa. One of the key and nearly universal steps in the animal kingdom is the acrosome reaction (AR), in which membrane fusion between the outer acrosomal and plasma membranes enables sperm to release proteolytic contents of the acrosome, facilitating penetration into oocyte investments, known as zona pellucida (ZP) in mammals (Hirohashi and Yanagimachi, 2018). After ejaculation, mammalian sperm must reside in the female reproductive tract for a prolonged period to undergo functional maturation, a process termed capacitation, before AR induction (Austin, 1951; Chang, 1951). In contrast to mammals, the capacitation process is not recognized in avian sperm because AR occurs immediately after exposure to the inner perivitelline layer (IPVL), which is analogous to mammalian ZP; this enables the formation of a hole on the IPVL for penetrating sperm (Horrocks *et al*., 2000; Lemoine *et al*., 2008). Another unique feature is that avian sperm sustain their fertilization ability for a much longer period than that in mammals (2 weeks in domestic fowl (Pierson *et al*., 1988) and 15 weeks in turkeys (Christensen and Bagley, 1989)) by remaining in sperm storage tubes (SSTs) of the female tract, in contrast to the rapid nature of AR. Previous pharmacological studies on chicken sperm have shown that AR induction is regulated by several signaling molecules that are similar to those that participate in mammalian sperm capacitation (Ashizawa *et al*., 2004, 2006; Lemoine *et al*., 2009). The commonality of signaling pathways is also observed in echinoderms, which can induce rapid AR soon after release from males (Neill and Vacquier, 2004). These findings suggest an evolutionary conservation of sperm signaling cascades in a wide range of animals, together with raising the question of how poultry sperm can maintain their ability to induce AR for a prolonged period.

Membrane rafts are specific membrane microdomains that

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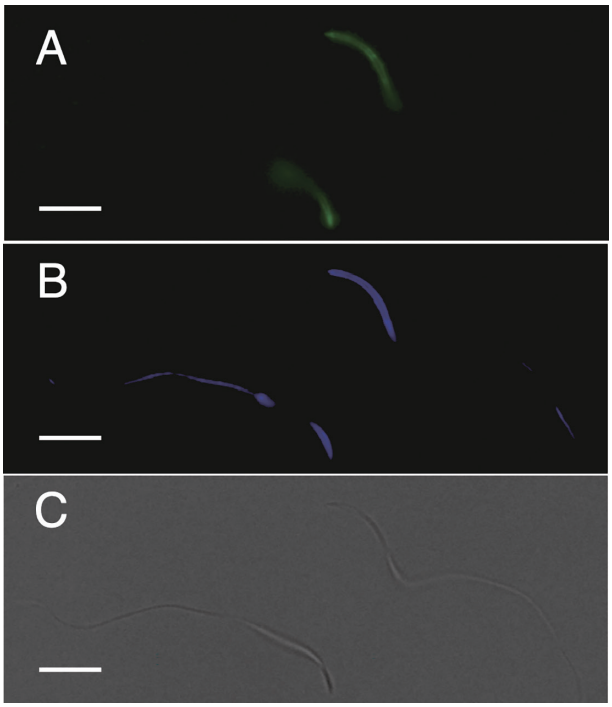

Fig. 1. **Organization of membrane rafts in chicken sperm.** G_{M1} and sterols were labeled with Alexa 488-conjugated cholera toxin B subunit (CTB) and filipin (A and B). The plasma membrane overlying sperm head has specific membrane domains. Bright field (C). Bars＝10 μm.

are enriched in sterols, ganglioside G_{M1}, and functional proteins relative to other regions (Simons and Toomre, 2000). Recently, these assemblies have received much attention from biologists because of their roles in the astonishing diversity of cellular processes (Jacobson *et al*., 2007). In the early 2000s, the functional roles of cellular membranes in sperm were well studied in echinoderms, which demonstrated the involvement of membrane rafts in sperm-egg binding and signaling pathways supporting AR and flagellar motility (Ohta *et al*., 1999; Maehashi *et al*., 2003). On the other hand, studies in mammals demonstrated that, in addition to these events, membrane rafts also play a role in post-testicular sperm maturation that occurs during transit in the male tract, a concept termed epididymal maturation (Girouard *et al*., 2008; Asano *et al*., 2010). These findings suggest the functional universality of membrane rafts beyond the cell types. Recently, research groups, including our laboratory, have investigated the regulatory mechanisms underlying avian sperm function by utilizing the concept of membrane rafts (Fig. 1).

In this review, we will provide a comprehensive understanding of membrane alteration and its function in post-testicular maturation, sperm-egg binding, and acrosome reaction, and then provide a perspective on targeting membrane rafts as a strategy for poultry reproductive technologies.

Membrane Function in Post-testicular Maturation

Epididymal maturation is a post-testicular sperm maturation that renders fertilization ability to specific subcellular compartments where they need to be fertilized (Cornwall and Hann, 1995). This process involves changes in the lipid and protein compositions of plasma membranes (Jones, 1998), and the development of flagellar motility (Morton *et al*., 1978) in mammals. However, there is controversy regarding the role of post-testicular maturation in avian sperm due to retrogression of the epididymis. In this context, numerous studies have shown that gradual elevation in sperm motility is coincident with the transit of the male genital tract (Howarth, 1983; Ahammad *et al*., 2011a ; Nixon *et al*., 2014), providing a concept that the avian male tract also contributes to the acquisition of sperm function.

Sperm are transcriptionally and translationally inactive and require the acquisition of functional proteins and lipids from the epididymal luminal fluid, which is compositionally controlled by epididymal secretion and absorption (Rankin *et al*., 1992; Sullivan, 2015). A similar phenomenon has been reported in chicken sperm, whereby a subset of secreted proteins in the male tract binds to the sperm surface and remains adherent even after prolonged preservation in the female tract (Esponda and Bedford, 1985; Morris *et al*., 1987). In addition, it has been shown that biochemical alteration of the plasma membrane during passage through the male genital tract also plays an important role in sperm binding capacity to the apical surface of epithelium in SSTs and in membrane toughness, leading to enhanced viability (Ahammad *et al*., 2011a, b). Taking together with the fact that a subset of epididymal luminal proteins is derived from the epithelial layer (Fujihara *et al*., 1983; Nixon *et al*., 2014), it is conceivable that the incorporation of male tract secretes to the sperm surface is required for avian sperm to ensure full fertilization ability in the female genital tract for a prolonged period.

Epididymosomes are small membrane vesicles released by the epididymal epithelium into the lumen, and are proposed to be the major pathway for transferring proteins and lipids to the sperm plasma membrane in mammals (Saez *et al*., 2003). Accumulated results from studies characterizing the functions of these exosomes suggest that selective transfer of these molecules occurs by biochemical interactions between membrane rafts present in epididymosomes and sperm (Girouard *et al*., 2008, 2009). Despite the lack of mechanistic studies on avian post-testicular maturation, these facts combined with the commonalities in sperm maturation between mammals and birds are suggestive of further studies on the involvement of membrane lipid regulation in functional changes during transit along the male tract in birds.

Membrane Regulation of Sperm-egg Binding and Acrosome Reaction

Sperm-egg binding is an initial step toward subsequent events such as AR, penetration into the egg extracellular envelope, and fusion with the egg plasma membrane. It is a shared feature between mammals and birds that ZP and IPVL components contain the receptor for respective sperm (Bleil and Wassarman, 1980; Han *et al*., 2010; Ichikawa *et al*., 2017). Sperm surfaces possess numerous molecules with a high binding affinity to ZP in mammals. Studies using knockout mouse models have suggested the involvement of multiple

binding events with a variety of receptor molecules in sperm (Ikawa *et al*., 2010). The mechanistic nature of sperm-egg binding is largely categorized into protein–protein and lipid–protein interactions, but the detailed mechanisms of these interactions remain unknown (Clark and Dell, 2006). Membrane rafts have been viewed as an integrative mechanistic module of ZP binding (Kondoh *et al*., 2005; van Gestel *et al*., 2007). Proteomic characterization of human and murine sperm rafts has revealed the relative abundance of proteins with a high binding affinity to ZP (Nixon *et al*., 2009; Asano *et al*., 2010; Nixon *et al*., 2011). In addition to proteinous molecules, sulfogalactosylglycerolipids (male gamete-specific glycolipids) are enriched in membrane microdomains and participate in ZP binding in porcine sperm (Khalil *et al*., 2006). In line with this, raft-enriched glycolipids contribute to binding the egg envelope in sea urchins (Maehashi *et al*., 2003) and rainbow trout sperm (Yu *et al*., 2002). Because our group found that the plasma membrane overlying the sperm head contains membrane rafts in chickens (Asano *et al*., 2016), we characterized the proteome associated with sperm rafts and successfully identified 82 proteins exclusively or relatively more enriched in the membrane rafts compared to non-rafts (Ushiyama *et al*., 2017a). Furthermore, an *in vitro* overlay assay showed a high binding affinity for membrane raft proteins with an abundance of a 60 kDa molecule. This is in agreement with a previous study showing the high affinity of ~ 60 kDa chicken sperm protein to recombinant IPVL protein responsible for sperm-binding (Bausek *et al*., 2004). Interestingly, our study found that the 45 kDa acrosin responsible for sperm binding to the IPVL in quails (Sasanami *et al*., 2011), is only so in non-rafts. Although the accumulation of further evidence is needed, our results suggest the roles of membrane rafts in sperm-egg interaction, providing a foundation for understanding redundancy, ensuring successful gamete recognition and adhesion in poultry.

AR is a regulated exocytosis that occurs only once in the life of sperm. In mammals, AR does not occur until capacitation occurs. Several mechanistic studies demonstrated that the initial trigger of this process is sterol efflux from the sperm plasma membranes (Travis and Kopf, 2002). This leads either directly or indirectly to several downstream events, such as cAMP-dependent PKA activation and protein tyrosine phosphorylation (Visconti *et al*., 1995a, b), increase in $[Ca^{2+}]i$ and hyperactivated motility (Ho and Suarez, 2001), and membrane hyperpolarization (Arnoult *et al*., 1999), which ultimately enhances acrosomal responsiveness. However, prolonged excitation towards completion of the capacitation process results in exceeding the threshold for exocytosis, and thereby the sperm undergoes a spontaneous AR with a premature loss of its fertilization ability (Kim and Gerton, 2003). Considering these factors, in conjunction with the functional heterogeneity of sperm, capacitation can be viewed as a process that simultaneously controls functions in the sperm population to increase fertilization success. On the other hand, capacitation is not a precondition for poultry sperm because of the nature of rapid AR (Fig. 2) (Howarth, 1970; Horrocks *et al*., 2000; Nixon *et al*., 2014). It remains unclear how AR is prohibited for such a long period until fertilization occurs. Recently, we found that Src family kinase (SFK) activation plays a role in inhibiting spontaneous AR by modulating membrane polarization potential in chickens (Priyadarshana *et al*., 2020), which is suggestive of the protective mechanisms of sperm undergoing spontaneous AR in poultry.

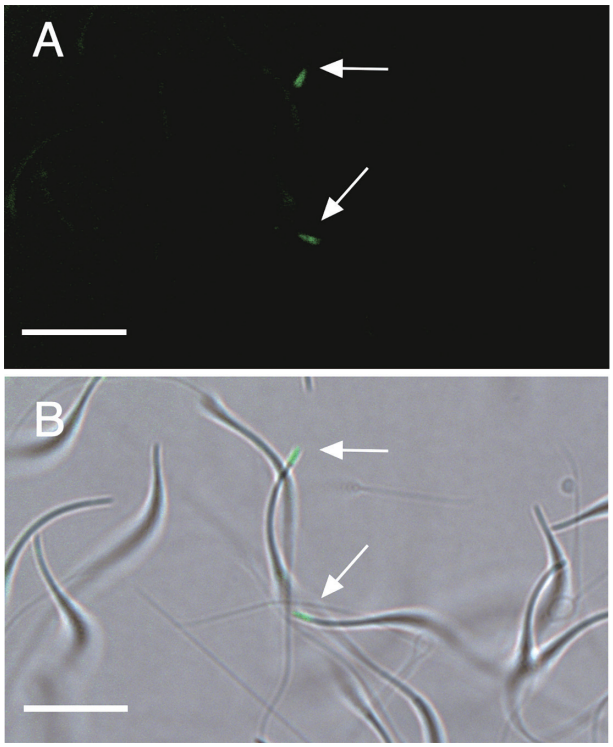

Fig. 2. **Detection of acrosome-reacted sperm.** Acrosome reaction in chicken sperm was visualized by labelling with FITC-conjugated peanut aggulutinin (A). White arrows indicate AR. Merged image (B). Bars＝10 µm.

Several signaling pathways have been reported to play a role in the regulation of acrosomal responsiveness in chickens. Ashizawa *et al*. (2006) found that protein kinase C (PKC) is involved in this process which is in agreement with the role of either Ca^{2+} supplementation or a calcium-dependent protein phosphatase inhibitor for in vitro AR (Ashizawa *et al*., 2004; Lemoine *et al*., 2008). Similarly, the cAMP/protein kinase A (PKA) pathway was also found to support AR ability and phosphorylation of effector molecules involved in separate signaling pathways (Lemoine *et al*., 2009). However, neither bicarbonate (soluble adenyl cyclase (AC) activator) or forskolin (transmembrane AC activator) potentiated AR in response to physiological stimulation (Lemoine *et al*., 2009; Priyadarshana *et al*., 2018), suggesting a specific mechanism behind the maintenance of AR in poultry. Our recent studies found that chicken sperm possess membrane rafts with the enrichment of several signaling molecules (Asano *et al*., 2016; Ushiyama *et al*., 2017a). Therefore, we performed the functional characterization of membrane rafts in chicken

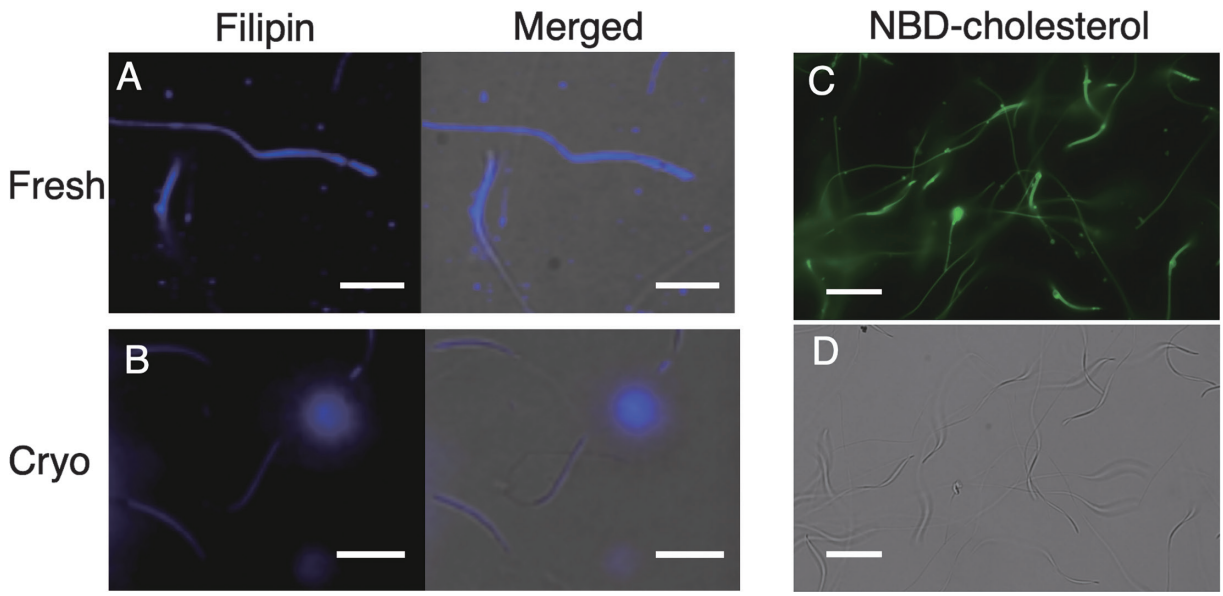

Fig. 3. **The distribution of sterols in chicken sperm.** Fresh and cryopreserved (Cryo) sperm were labelled with filipin (A and B). 22-(*N*(-7-nitrobenz-2-oxa-1,3-diazol-4-yl) amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) was loaded into the plasma membrane of fresh chicken sperm (C). Bright field (D). Bars＝10 μm.

sperm with a particular focus on a mechanism for AR, which indicated that these membrane domains are involved in the upstream mechanism of the cAMP/PKA pathway by regulating both AC activities (Priyadarshana *et al*., 2018). Notably, the depletion of membrane rafts by sterol removal stimulated spontaneous AR. Taken together with our recent finding that SFK is spatially and functionally regulated by membrane rafts (Priyadarshana *et al*., 2020), this suggests membrane regulation of AR ability in poultry sperm. More recently, it was reported that a PKA-dependent AR mechanism is mediated by AMP-activated protein kinase (AMPK) (Nguyen *et al*., 2019), a main intracellular energy sensor that has been known to stimulate AR and flagellar motility (Nguyen *et al*., 2014). This is in agreement with our result that the depletion of membrane rafts decreases AMPK activity and AR in chickens. (Ushiyama *et al*., 2019). Therefore, it is conceivable that membrane rafts act as critical platforms that are functionally and biochemically associated with PKA cascades.

Potential Translation of the Membrane Rafts Concept

During cryopreservation, avian sperm are subjected to cryodamage, which leads to a decline in fertilization ability, which is a persistent problem limiting the potential of utilization for commercial production or the preservation of genetic resources in cryobanks. Most attempts to improve the fertility rates of cryopreserved poultry semen have focused on empirical approaches, such as the types of cryoprotectants and extenders used, freezing rate, and cryopreservation methods (i.e., pellets and straws) (Donoghuea and Wishart, 2000).

Numerous studies have attempted to determine the primary cause of cryodamage, resulting in the hypothesis that membrane properties, such as phospholipid composition and sterol content, affect the freezing tolerance of both mammalian and poultry sperm (Giraud *et al*., 2000; Moore *et al*., 2005; Blesbois *et al*., 2008; Moce *et al*., 2010). Sterol is the predominant lipid in membranes, and it plays a role in stabilizing membrane permeability, regulating membrane fluidity, and preparing a suitable microenvironment for membrane-associated molecules (Elizabeth, 1998). However, studies on the evaluation of post-thaw semen quality in poultry have shown that cryodamage often appears in functions ensuring fertilization potential, while the sperm are viable and motile (Wishart, 1985; Tajima *et al*., 1989; Donoghuea and Wishart, 2000). This indicates complexity in the molecular and cellular cascades culminating in an impaired fertilization potential. In several mammalian species, it has been reported that sperm undergo sterol removal from plasma membranes during cryopreservation (Cerolini *et al*., 2001; Moore *et al*., 2005). Recently, we characterized biochemical changes in cryopreserved chicken sperm, demonstrating that sterol loss occurs following cryopreservation and causes an early apoptotic response via the depletion of membrane raft organization (Ushiyama *et al*., 2016). In fact, sterol loss from the membranes is known to activate apoptotic cascades in mammalian and fish sperm (Muller *et al*., 2008; Aitken, 2011). Furthermore, it was demonstrated that the redistribution of membrane rafts in pig sperm in response to capacitating stimuli was altered dramatically following cryopreservation, resulting in impaired functional ability (Vadnais and Althouse, 2011). This suggests that the compositional alteration of membrane rafts via the loss of sterol is a major factor that leads to the deterioration of fertilization ability. This view was corroborated by recent

results from our and other laboratories, where loading synthetic sterol into the sperm plasma membranes prior to cryopreservation enhanced post-thaw semen quality in several species, including chicken (Fig. 3) (Tomas *et al*., 2011; Blanch *et al*., 2012; Lee *et al*., 2015; Ushiyama *et al*., 2017a). Furthermore, excessive sterol loading to sperm has an adverse effect on cryosurvivability and AR induction (Oliveira *et al*., 2010; Spizziri *et al*., 2010; Ushiyama *et al*., 2017a), although the detailed mechanism remains unclear. Taken together, these results suggest that targeting membrane rafts is useful for improving the cryotolerance of poultry sperm and adding to our knowledge of membrane functionality responsible for fertilization potential in sperm.

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Conflicts of Interest

The authors declare no conflicts of interest.

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