

Comparison of Membrane Characteristics between Freshly Ejaculated and Cryopreserved Sperm in the Chicken

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Cryopreserved sperm undergoes serious damage which affects its fertilizing ability. Despite progress in understanding the nature of functional deterioration in mammalian sperm, little is known about the mechanism involved in the induction of functional damage in avian sperm. Cellular membranes are considered the primary site of cryo-damage to sperm. Membrane rafts are specific membrane regions enriched in sterols, ganglioside G_{M1}, and functional proteins and they play important roles in the regulation of diverse functions exerted in mammalian sperm during fertilization. Several reports investigating cryopreservation-induced membrane changes in mammalian sperm have suggested that cryopreservation induces a compositional alteration of membrane rafts via a loss of membrane sterols, leading to impaired fertilizing ability. Recently, we demonstrated that membrane rafts are present in chicken sperm. Therefore, we investigated a possible mechanism for the induction of functional damage in cryopreserved chicken sperm, with particular attention to cryopreservation-induced compositional changes in membrane rafts. Sterol quantification showed that loss of sterols from sperm membranes occurred following cryopreservation. Biochemical analyses of detergent-insoluble membranes showed that the lipid and protein compositions of membrane rafts were altered dramatically by cryopreservation. To determine the physiological role of these changes, we examined external translocation of phosphatidylserine (PS), representing an early apoptotic change, and found that cryopreservation induced apoptotic changes in chicken sperm. Furthermore, methyl- β -cyclodextrin-induced loss of sterols from the plasma membranes stimulated PS translocation that was not accompanied with caspase-3 activation, which plays an important role downstream of the apoptotic cascade. Based on the results obtained in this study, we discuss a new mechanism for reduction of the fertilizing ability in avian sperm after cryopreservation.

Key words: apoptosis, cryopreservation, membrane rafts, sperm, sterol

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Introduction

To date, many efforts have been made to develop suitable protocols for the cryopreservation of avian sperm. However, fertility rates of cryopreserved poultry semen remain highly variable and are not reliable for use in commercial production or the preservation of genetic resources in cryobanks. Most attempts to improve the deteriorated fertility rates of cryopreserved poultry semen have focused on empirical approaches, such as the types of cryoprotectants and extenders used, freezing rate, and methods of cryopreservation (i.e., pellets and straws) (Donoghuea and Wishart, 2000). However, limited attention has been given to understand why poultry sperm lose their fertilizing ability after cryopreserva-

tion.

The success of sperm cryopreservation is generally evaluated based on motility or plasma membrane integrity; the survivability of sperm is usually 40–50% of the initial population (Donoghuea and Wishart, 2000). However, a previous study demonstrated that the *in vivo* fertilizing ability of cryopreserved chicken sperm decreased to 1.6% of that of fresh sperm (Wishart, 1985). The severe loss of fertilizing ability was also demonstrated by another study using competitive insemination of fresh and frozen-thawed chicken sperm (Tajima *et al.*, 1989). The difference between the survivability and the fertilizing ability of cryopreserved sperm suggests that the cryopreservation procedure causes serious damage to functions necessary for fertilization, even though the sperm are viable and motile. The susceptibility of sperm to damage during cryopreservation differs among species; poultry sperm freezability is often poor compared to mammalian sperm (Long, 2006). Several reports on attempts to improve the survivability of cryopreserved mam-

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malian sperm have shown that an increase in sterol content relative to phospholipid content in plasma membranes is critical to avoid deterioration of survivability and fertilizing ability (Darin-Bennett and White, 1977; Purdy and Graham, 2004; Moce *et al.*, 2010). This is mostly because mammalian sperm lose much of the sterols from the plasma membranes after cryopreservation (Cerolini *et al.*, 2001; Moore *et al.*, 2005). Sterol loss is believed to be an initial trigger which stimulates premature activation of signaling cascades involved in the acrosome reaction, resulting in an early loss of functionality in cryopreserved mammalian sperm (Vadnais and Althouse, 2011; Naresh and Atreja, 2015). Despite progress in understanding the processes resulting in the impaired fertilizing ability of mammalian sperm, little is known about how poultry sperm undergo functional damage by cryopreservation.

Membranes are considered the primary site of cryodamage to sperm. Membrane rafts are specific membrane microdomains enriched in sterols, ganglioside $G_{M1}(G_{M1})$, and functional proteins and they play important roles in the regulation of cellular processes in diverse cell types (Simons and Toomre, 2000). Recently, results from other groups and our group demonstrated that membrane rafts are present in sperm of several mammalian species, and are involved in sperm-egg binding (Khalil *et al.*, 2006; Nixon *et al.*, 2009), capacitation (Thaler *et al.*, 2006), and the acrosome reaction (Asano *et al.*, 2013). Importantly, it was demonstrated that 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). Considering that the loss of membrane sterols occurs in cryopreserved mammalian sperm, these results suggest that cryopreservation induces a compositional alteration of membrane rafts via a loss of sterols, leading to deterioration of fertilizing ability. Recently, we reported that membrane rafts are also present in chicken sperm (Asano *et al.*, 2016). Therefore, we investigated a mechanism by which chicken sperm undergo functional damage following cryopreservation by determining compositional changes in membrane rafts. In this study, we found that cryopreservation induced a loss of membrane sterols and a compositional alteration of membrane rafts in chicken sperm. Furthermore, the loss of sterols stimulated apoptotic changes characterized by phosphatidylserine (PS) translocation in chicken sperm, consistent with phenomenon seen in other systems (Li *et al.*, 2006; Motoyama *et al.*, 2009; Onodera *et al.*, 2013). Our findings in chicken sperm suggest a possibility that cryopreservation cause an alteration of membrane rafts via a loss of sterols resulting in apoptotic changes, and provide a foundation to investigate the mechanism involved in functional impairment of cryopreserved poultry sperm.

Materials and Methods

Reagents and Animals

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Cholera toxin subunit b con-

jugated to horseradish peroxidase (CTB-HRP) was purchased from Life Technologies (Grand Island, NY, USA). A Cholesterol E Kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). The Annexin V-FITC Apoptosis Detection Kit and CaspGLOW Fluorescein Active Caspase-3 Staining Kit were obtained from BioVision (Milpitas, CA, USA). Fertile Rhode Island Red chickens (RIR), raised at the Agricultural and Forestry Research Center, University of Tsukuba, Japan, were utilized for semen collection using the dorsal-abdominal massage method (Burrows, 1937). All animal work was performed following approval of the University of Tsukuba's Institutional Animal Care and Use Committee (Approval number 15-018).

Sperm Cryopreservation

Cryopreservation of RIR sperm was performed as described previously (Tajima *et al.*, 1989). In brief, clean semen samples collected from multiple males were pooled and diluted three times with Minnesota Avian buffer (MnA) containing 8% (v/v) glycerol. Samples were snap-frozen in liquid nitrogen for storage after loading into a 0.25 ml straw. For thawing, frozen semen were thawed at 5°C for 10 min. Semen were rediluted, centrifuged and then resuspended in MnA as described previously (Tajima *et al.*, 1989).

Evaluation of Characteristics of Fresh and Cryopreserved Sperm

Fresh and cryopreserved chicken sperm were utilized for evaluation of viability, motility and acrosome-intactness. For viability assay, sperm were treated with 12 μ M propidium iodide (PI) for 5 min. For motility assay, sperm were categorized into motile and immotile sperm under a microscope equipped with a warm plate. For assay of acrosome-intactness, sperm were treated with 40 μ g/ml FITC-peanut agglutinin for 15 min. At least 100 sperm for each sample were utilized for the evaluations.

Sterol Quantification

Isolation of sperm membranes was performed as described previously (Asano *et al.*, 2010). In brief, fresh and frozen-thawed sperm (1×10^8 cells) were subjected to dounce homogenization and sonication using a BH-200P (TOMY SEIKO, Tokyo, Japan). Lysates were centrifuged at $10,000 \times g$ for 10 min, and the supernatants containing membranes were utilized for sterol quantification using the Cholesterol E Kit (Wako Pure Chemical Industries).

Isolation of Membrane Rafts

Fresh and frozen-thawed sperm (1×10^8 cells) were subjected to dounce homogenization and sonication in phosphate-buffered saline (PBS) containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and then centrifuged at $10,000 \times g$ for 10 min. The supernatants were further centrifuged at $20,000 \times g$ for 2 h to obtain the membrane pellet. Membrane pellets were resuspended in PBS and protein content was measured by a micro BCA assay (Thermo Scientific, Rockford, IL, USA). Membrane rafts are biochemically defined as insoluble membranes when subjected to Triton X-100 (TX-100) treatment at low temperatures (Brown and Rose, 1992). Utilizing this definition, sperm membranes with equivalent amounts of protein were

Table 1. Characteristics of fresh and cryopreserved chicken sperm

Sperm	Percentage (mean±SEM) of sperm		
	viable	motile	acrosome-intact
Fresh	82.3±0.7*	87.3±0.9*	96.3±0.9*
Cryopreserved	50.0±0.6	41.8±4.7	74.7±2.2

* Significant difference when compared to cryopreserved sperm ($P < 0.05$).

extracted with 0.5% TX-100 at 4°C for 15 min as described previously (Travis *et al.*, 2001) and centrifuged at 20,000×*g* for 2 h to obtain the detergent-insoluble membrane (DIM) fraction.

Protein and Lipid Analyses

To compare protein compositions in membrane rafts isolated from fresh and frozen-thawed sperm, proteins from DIM fractions were separated by SDS-PAGE and visualized with SYPRO Ruby (Life Technologies) gel stain using a ChemiDoc XRS+ imaging system (Bio-Rad, Hercules, CA, USA).

To determine the G_{M1} amount in DIM fractions, a slot blot assay was performed as described previously (Asano *et al.*, 2009). In brief, DIM fractions obtained from fresh and frozen-thawed sperm were blotted using a slot blot manifold (Hoefer, San Francisco, CA, USA) by drawing the sample with gentle vacuum pressure onto a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% bovine serum albumin (BSA) in PBS, the membrane was incubated with 0.5 μg/ml CTB-HRP in PBS containing 1% BSA for 1 h. G_{M1} expression was detected by chemiluminescence and quantified by densitometry using Image J v1.49 software (<http://imagej.nih.gov/ij/index.html>).

Apoptosis Assay

Apoptotic changes were examined in fresh and frozen-thawed sperm using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's instructions. In brief, sperm were fixed with 2% paraformaldehyde and incubated with annexin V-FITC and PI for 5 min. Sperm were centrifuged at 500×*g* for 5 min and resuspended in PBS. At least 100 sperm cells from each sample were categorized as live (annexin V-FITC⁻, PI⁻), apoptotic (annexin V-FITC⁺, PI[±]), or dead (annexin V-FITC⁻, PI⁺) under a fluorescence microscope. This evaluation was performed within 24 h post-sample preparation. The same experiment was performed with fresh sperm depleted of membrane sterols by exposure to 1 mM methyl-β-cyclodextrin (MBCD) at 39°C for 15 min.

Detection of Active Caspase-3

Caspase-3 is the main executor present downstream in the apoptotic cascade (Fuentes-Prior and Salvesen, 2004). The detection of active caspase-3 in sperm was performed with a colorimetric assay and a fluorescent microscopy.

Colorimetric assay was performed using the ApoAlert Caspase-3 Colorimetric Assay Kit according to manufacturer's instruction. In brief, fresh, frozen-thawed and MBCD-

treated fresh sperm (5×10^7 cells) were lysed and centrifuged at 20,000×*g* to remove sperm debris and membranes. Soluble proteins in the supernatants were incubated with the caspase-3 inhibitor, DEVD-FMK, or DMSO for 30 min on ice and then incubated with caspase-3 substrate, DEVD-pNA, for 1 h at 37°C. Samples were subjected to spectrophotometry with a wavelength of 400 nm.

Fluorescent microscopy was performed using the Casp GLOW Fluorescein Active Caspase-3 Staining Kit according to the manufacturer's instructions. In brief, MBCD-treated or untreated fresh sperm were incubated with the fluorescent caspase-3 inhibitor, FITC-DEVD-FMK, for 1 h at 39°C. Sperm were centrifuged at 1,000×*g* for 5 min and resuspended in wash buffer. Samples were evaluated under a fluorescence microscope within 24 h post-sample preparation.

Statistical Analysis

After preliminary trials, data for lipid analyses were gathered from 4–6 replications. A comparison of viability, motility, acrosome-intactness, total sterol contents, and G_{M1} contents between fresh and frozen-thawed sperm was carried out with paired *t*-tests. A comparison of apoptotic profiles between fresh and frozen-thawed sperm was carried out with the Mann-Whitney U test. Results are expressed as the mean ± standard error of the mean (SEM). Probability values lower than 0.05 were considered significant.

Results

Characteristics of Fresh and Cryopreserved Sperm

Viability, motility and acrosome-intactness were evaluated in fresh and cryopreserved sperm, showing all of their percentages were significantly higher in fresh sperm (82.3±0.7%, 87.3±0.9%, and 96.3±0.9%) than those of cryopreserved sperm (50.0±0.6%, 41.8±4.7%, and 74.7±2.2%; $P < 0.05$).

Sterol Quantification in Chicken Sperm

The sterol content of fresh sperm was 6.8±0.8 μg (Fig. 1). However, a significant decrease in cholesterol content was observed in frozen-thawed sperm (3.5±0.5 μg, $P < 0.05$). These results demonstrate that a loss of membrane sterols occurs in chicken sperm following cryopreservation.

Compositional Changes of Membrane Rafts

The protein profile of membrane rafts was changed by cryopreservation (Fig. 2A). In particular, protein bands between 53–72 kDa prominently disappeared in frozen-thawed sperm. Consistent with this, the G_{M1} content of

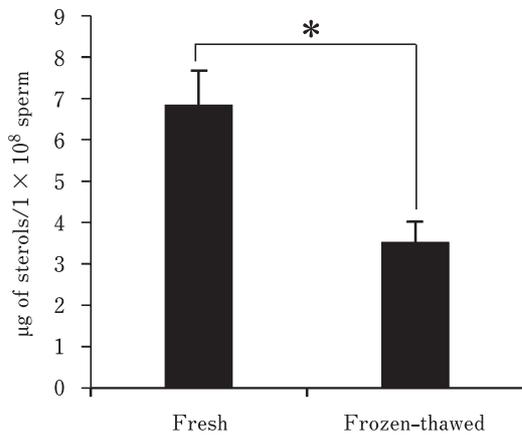


Fig. 1. **Quantification of membrane sterols in fresh and frozen-thawed chicken sperm.** Membranes were isolated by sonication from fresh and frozen-thawed sperm and subjected to sterol quantification. Sterol content was significantly lower in membranes isolated from frozen-thawed sperm than fresh sperm. * $P < 0.05$.

membrane rafts isolated from frozen-thawed sperm (23.0 ± 14.6 a.u.) was significantly lower than that of fresh sperm (125.5 ± 14.9 a.u.) (Fig. 2B). Together, these results demonstrate that cryopreservation induces alterations in the lipid and protein compositions of membrane rafts in chicken sperm.

Apoptotic Changes in Chicken Sperm

We evaluated apoptosis based on the binding of annexin V to PS translocated to the external sperm surface. The percentage of apoptotic sperm increased significantly in frozen-thawed sperm ($21.7 \pm 3.0\%$) compared with fresh sperm ($6.7 \pm 0.4\%$) (Fig. 3A, $P < 0.05$). The percentage of dead sperm increased concomitantly in frozen-thawed sperm ($28.6 \pm 4.1\%$) compared with fresh sperm ($4.0 \pm 0.8\%$, Fig. 3A). To determine whether sterol loss induces PS translocation in chicken sperm, fresh sperm were treated with MBCD prior to the apoptosis assay. The percentage of apoptotic sperm increased significantly in MBCD-treated sperm ($16.0 \pm 1.7\%$) compared with non-treated sperm ($5.0 \pm 0.7\%$) (Fig. 3B, $P < 0.05$). Localization of annexin V was mostly detected in all of the regions in both cryopreserved (Fig. 3C) and MBCD-treated sperm (data not shown). Together, these results demonstrate that cryopreservation and loss of sterols from the plasma membranes induces the apoptotic change in chicken sperm. To identify the downstream pathway of apoptosis induced by cryopreservation, we evaluated caspase-3 activity in fresh, frozen-thawed, and MBCD-treated fresh sperm using a colorimetric assay. However, there was no difference in caspase-3 activity among fresh, frozen-thawed and MBCD-treated fresh sperm (data not shown). We also performed a fluorescent microscopy to detect active caspase-3 using a specific inhibitor conjugated to a fluorophore. However, a fluorescent signal representing active caspase-3 was not observed in either MBCD-treated or untreated fresh

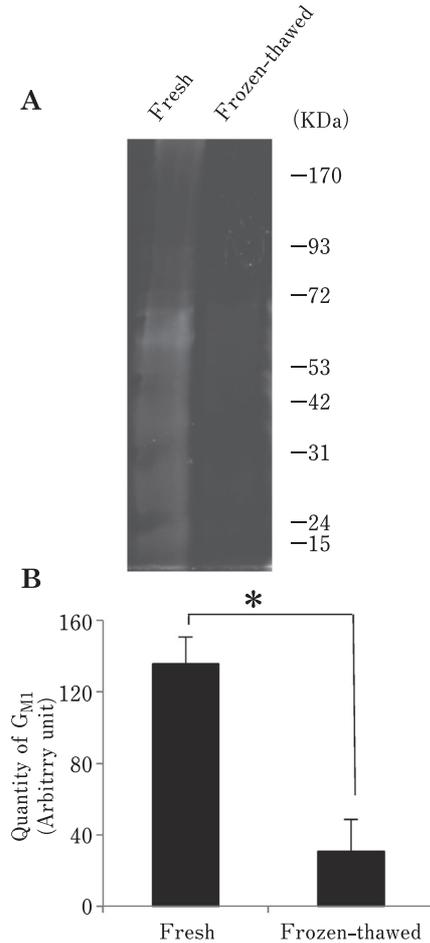


Fig. 2. **Protein and lipid composition analyses in detergent-insoluble membranes (DIM) isolated from fresh and frozen-thawed chicken sperm.** Sperm membranes were isolated by sonication and utilized for protein quantification. Samples with equivalent amounts of protein were extracted with 0.5% TX-100 and centrifuged to obtain DIM fractions. Proteins were processed for SDS-PAGE and visualized with SYPRO Ruby gel stain. The G_{M1} content of DIM fractions was analyzed using a slot blot. Protein bands detected in the DIM fraction from fresh sperm disappeared following cryopreservation (A). The G_{M1} content in the DIM fraction was lower in frozen-thawed sperm compared to fresh sperm (B). * $P < 0.05$.

sperm (Fig. 3D). Together, these results suggest that apoptotic changes induced by cryopreservation in chicken sperm are not accompanied with caspase-3 activation.

Discussion

Previously, it was demonstrated in mammalian sperm that cryopreservation induces a loss of sterols from the membrane (Chakrabarty *et al.*, 2007). This alteration of membrane composition has been suggested to result in functional damage to mammalian sperm (Morales *et al.*, 2010). This view agrees with evidence showing that pre-loading of

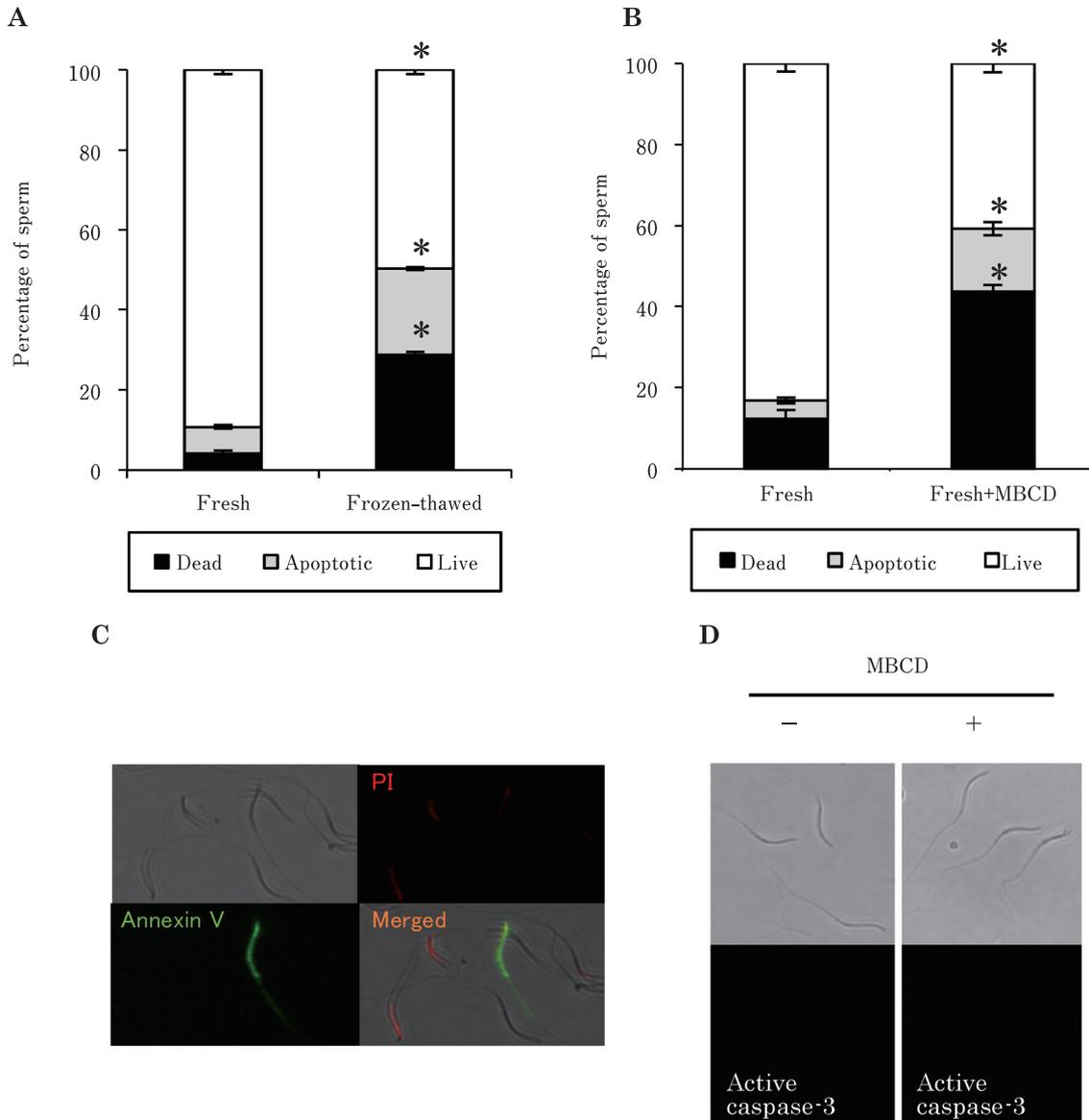


Fig. 3. Apoptosis assays of fresh and frozen-thawed chicken sperm. Fresh, frozen-thawed, or 1 mM MBCD-treated fresh sperm was fixed and treated with annexin V-FITC and propidium iodide (PI). Sperm were counted and categorized into the following: live (annexin V-FITC⁻, PI⁻), apoptotic (annexin-V-FITC⁺, PI[±]), or dead (annexin-V-FITC⁻, PI⁺). Detection of active caspase-3 was performed in fresh sperm treated with 1 mM MBCD using a fluorescent marker. The number of apoptotic and dead sperm was increased by cryopreservation (A). Loss of membrane sterols by MBCD treatment increased the number of apoptotic and dead sperm (B). Localization of annexin V was mostly observed in all of the regions (C). Expression of active caspase-3 was not detected in fresh sperm with or without MBCD treatment (D). * $P < 0.05$.

cholesterol, one type of sterol, into plasma membranes prior to cryopreservation enhanced the functionality of sperm in several mammalian species (Pamornsakda *et al.*, 2011; Tomas *et al.*, 2011; Hussain *et al.*, 2013). Despite the direct link between membrane sterol content and the impaired function of mammalian sperm, there has been no report

exploring the change in sterol content in cryopreserved avian sperm. Therefore, we performed a quantification of membrane sterol content in fresh and cryopreserved sperm, which demonstrated for the first time that cryopreservation induced the loss of sterols from membranes in chicken sperm. A previous study showed that the membrane sterol:phospholi-

pid ratio did not differ between fresh and cryopreserved chicken sperm (Blesbois *et al.*, 2005). Together with these data, our results suggest that in addition to sterols, phospholipids might also be lost from membranes in chicken sperm after cryopreservation. Consistent with this, multiple studies have demonstrated that cryopreservation causes the loss of phospholipids from membranes in the sperm of several mammalian species, probably leading to reduced fertility (Darin-Bennett *et al.*, 1973; Chakrabarty *et al.*, 2007). Therefore, preservation of membrane lipids during cryopreservation would be useful to improve the impaired fertilizing ability of cryopreserved avian sperm.

Membrane rafts are functional membrane domains enriched in specific lipids such as sterols and G_{MI} as well as proteins, and regulate several processes involved in fertilization in mammalian sperm. Recently, our cell biology and biochemical experiments demonstrated that membrane rafts are present in chicken sperm (Asano *et al.*, 2016), suggesting the functional involvement of membrane domains in mechanisms necessary to achieve fertilization in chicken sperm. Multiple studies have demonstrated in mammalian sperm that the organization and functionality of membrane rafts are regulated by the loss of sterols from plasma membranes (Shadan *et al.*, 2004; Bou Khalil *et al.*, 2006; Asano *et al.*, 2013). Therefore, we examined the possibility that the cryopreservation-induced loss of sterols alters the nature of membrane rafts in chicken sperm. Our results showed that proteins and G_{MI} were lost from membrane rafts after cryopreservation, suggesting that the cryopreservation-induced loss of sterols results in a depletion of membrane rafts. Although the exact functions of membrane rafts in chicken sperm remain unclear, previous studies using other cell types have demonstrated that the depletion of membrane rafts caused by sterol loss induced apoptotic changes characterized by translocation of PS to the cell surface (Li *et al.*, 2006; Motoyama *et al.*, 2009; Onodera *et al.*, 2013). Therefore, we examined whether PS translocation occurs in chicken sperm following cryopreservation and demonstrated that cryopreservation induced the apoptotic change in chicken sperm. This is consistent with previous studies performed on cryopreserved sperm of several mammalian species (Kim *et al.*, 2010; Said *et al.*, 2010; Kadirvel *et al.*, 2012). However, the initial trigger that induces apoptotic changes following cryopreservation remains unknown in the sperm of all species. MBCD is a sterol acceptor that is widely used to disrupt the integrity of membrane rafts. Using this agent in this study, we found that the loss of sterols from plasma membranes induces PS translocation to the surface of chicken sperm. Together with these, our results suggest a possibility that cryopreservation induces compositional alteration of membrane rafts via the loss of sterols from the plasma membranes, leading to the apoptotic change in chicken sperm.

Previous studies on human and bull sperm demonstrated that PS translocation induced by cryopreservation is accompanied by the activation of caspases (Martin *et al.*, 2004; Paasch *et al.*, 2004) known as cysteinyl-aspartate-specific

proteases, which cleave with high specificity at the aspartate residue leading to proteolytic degradation (Fuentes-Prior and Salvesen, 2004). Of all the known caspases, caspase-3 plays an important role downstream of the apoptotic cascade (Fuentes-Prior and Salvesen, 2004), and its activation has been reported in the cryopreserved sperm of several mammalian species (Paasch *et al.*, 2004; Zribi *et al.*, 2012). Therefore, we examined whether treatment of sperm with MBCD activates caspase-3 using 2 different methodologies. The results showed that the loss of sterols does not activate caspase-3 in chicken sperm, suggesting that caspase-3 activation is not involved in cryopreservation-induced apoptosis, at least in chicken sperm. A previous study evaluating several caspase activities in bull sperm demonstrated that cryopreservation induced the activation of caspase-9 but not caspase-3 or -8 (Martin *et al.*, 2007). Furthermore, a study using human sperm demonstrated that caspase-1, -8, and -9 were activated by cryopreservation (Grunewald *et al.*, 2005). Therefore, it is likely that there is a species-dependent difference in the manner of caspase activation following cryopreservation, although the procedures utilized for cryopreservation differ between species. Further investigation is required to determine what caspases are involved in the downstream apoptotic cascade in chicken sperm.

In this study, we found that many annexin V+ sperm were also stained with PI. Previous study exploring correlation of PS translocation with DNA fragmentation suggested that these double positive sperm represent to be at late apoptotic stage and were originally derived from population of annexin V+ and PI- sperm at early apoptotic stage (Shen *et al.*, 2002). Together with this, our result suggests that cryopreservation facilitates chicken sperm to proceed toward the late apoptotic stage. In chicken sperm, physiological role of PS translocation has not been understood yet. In other systems, it has been demonstrated that the translocation of PS is a central signal to attract macrophages for phagocytosis of apoptotic cells (Wu *et al.*, 2006). Macrophages are one of the dominant leukocyte cell types in uterine epithelium of pig (Kaeoket *et al.*, 2002). Previous *in vitro* study demonstrated in human that macrophages specifically phagocytized sperm that already completed with capacitation process (Oren-Benaroya *et al.*, 2007). Considering the fact that capacitation increases PS translocation in human sperm (Schuffner *et al.*, 2002), these suggest that translocation of PS is crucial to attract macrophages to ingest apoptotic sperm in the genital tract. This is consistent with previous study showing that the occurrence of apoptosis was positively correlated to a decline in fertilizing ability of cryopreserved bull sperm after artificial insemination (Anzar *et al.*, 2002). Together with these, it is probable that the increase in PS translocation after cryopreservation reduces number of sperm able to fertilize an egg in chicken. Of note, our experiments to determine an initial trigger for PS translocation demonstrated the involvement of sterol loss from the plasma membranes. Previously, it has been hypothesized in mammalian sperm that loss of sterols from the plasma membranes is a one of triggers for induction of apoptotic changes (Aitken, 2011).

Together with these, our results suggest a possibility that a decline in fertilizing ability in cryopreserved chicken sperm is, at least in part, a result of the increase in the apoptotic changes induced by sterol loss.

In summary, we demonstrated in chicken sperm that cryopreservation induced a loss of sterols from membranes and a depletion of membrane rafts. Cryopreservation induced PS translocation to the surface of sperm possibly via sterol loss and this response was not accompanied with caspase-3 activation. Our results provide a possible mechanism involved in the decline of the fertilizing ability of cryopreserved avian sperm.

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