RhoA improves cryopreservation of rooster sperm through the Rho/RhoA-associated kinase/cofilin pathway

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ABSTRACT Cryopreservation of rooster sperm leads to relatively low semen quality due to cytoskeletal damage during the freeze-thawing process. This study aimed to explore how the addition of RhoA recombinant protein affected the viability and subcellular structure of rooster sperm after freeze-thawing and elucidated the molecular mechanisms of sperm cryopreservation. Semen quality and acrosome integrity testing revealed that the addition of 0.5 μg/mL RhoA recombinant protein to the cryoprotectant fluid significantly increased sperm motility, survival rate, linearity, straight-line velocity, and acrosome integrity after freeze-thawing ($P < 0.05$). Ultrastructure analysis of cryopreserved sperm showed structural damage to the sperm plasma membrane, nuclear membrane, and tail. However, compared to the control, these structural changes were reduced upon the addition of RhoA recombinant protein to the cryoprotective fluid ($P < 0.05$). Western blotting revealed that the expression of Rho/RhoA-associated kinase and p-cofilin was increased, and cofilin expression was decreased after sperm cryopreservation with recombinant RhoA protein. Treatment with Y-27632, a ROCK antagonist, suppressed ROCK and p-cofilin expression and decreased semen quality, acrosome integrity, and ultrastructure integrity. In summary, we have demonstrated a cryoprotective effect in spermatozoa involving the Rho/ROCK pathway during freeze-thawing. Furthermore, the addition of 0.5 μg/mL RhoA recombinant protein to the cryoprotective fluid improved rooster semen quality and subcellular structural homeostasis after freeze-thawing via the Rho/ROCK pathway. This pathway may regulate the dynamic reorganization of the actin cytoskeleton by regulating the cofilin phosphorylation.

Key words: RhoA, rooster sperm, semen quality, programmed freezing, ultrastructure, cryopreservation

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INTRODUCTION

The cryopreservation of rooster sperm is important for genetic resource conservation, genetic improvement, and improving productivity (Robles et al., 2019). Despite the advantages of sperm cryopreservation, the availability of high-quality frozen semen for artificial insemination (**AI**) is a global challenge due to poultry sperm structure (Duplaix and Sexton, 1983). It is crucial to develop effective techniques that provide high-quality semen after freeze-thawing. Due to the unique structure of rooster sperm, general cryoprotection methods fail to effectively protect poultry sperm (Clark and Shaffener, 1960). Compared to the structure of mammalian sperm, the long cylindrical structure of the poultry sperm head is not conductive to the uptake of cryoprotectant solutions by osmosis (Siudzinska and Lukaszewicz, 2008). As such, cryoprotectants do not effectively inhibit the formation of ice crystals and ice recrystallization during rooster sperm freeze-thawing. The rooster sperm flagellum is longer than the mammalian sperm flagellum and is more likely to break during freeze-thawing (Siudzinska and Lukaszewicz, 2008). To improve the efficacy of cryoprotectants in rooster sperm, the field has increasingly focused on novel additives for sperm preservation (Beirao et al., 2012; Zandiyeh et al., 2020; Correia et al., 2021).

RhoA is a small guanosine triphosphatase (**GTPase**) protein from the Rho family (Delgado-Buenrostro et al., 2016; Ard et al., 2021) that is involved in regulating the

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cytoskeleton, including cell motility, cell adhesion, and chromosome inheritance (Gu et al., 2017). It can be inserted into the phospholipid bilayer and is involved in actin assembly (Gu et al., 2017). RhoA activity can be catalyzed by guanine nucleotide exchange factors, which phosphorylate GDP to GTP (Jordan and Canman, 2012; Inaba et al., 2021; Lin et al., 2021). Our previous research showed that RhoA was involved in the cryoprotection of dormant embryos as a binary molecular "switch" (Jordan and Canman, 2012; Gu et al., 2017), but the effect of recombinant RhoA protein supplementation as a cryoprotectant during sperm freeze-thawing is unclear.

We previously found that the addition of exogenous recombinant RhoA protein could effectively improve the freezing resistance of dormant mouse embryos via the Rho/ROCK pathway and subsequently uncovered through high-throughput sequencing that *RHOA* was differentially expressed in rooster sperm before and after freeze-thawing (Gu et al., 2017; Qi, et al., 2020). Thus, we speculate that RhoA may function in rooster sperm to resist freezing damage. RhoA is widely distributed in the head and flagellum of animal sperm and plays an important role in capacitation (Delgado-Buenrostro et al., 2016; Reyes-Miguel et al., 2020). Furthermore, RhoA is involved in regulating a variety of biological functions by regulating the expression of Rho/RhoA-associated kinase (**ROCK**), primarily through the phosphorylation pathway (Gagné et al., 2020; Liu et al., 2020; Zhang et al., 2021). ROCK is a downstream targeting effector of RhoA that regulates cell contraction, migration, adhesion, and proliferation (Amano et al., 2010). ROCK consists of 2 isoforms, ROCK1 and ROCK2, with ROCK1 being highly expressed in non-neural tissues, such as liver, lung, and testis, and ROCK2 being highly expressed in brain, heart, and muscle tissues (Knipe et al., 2018). However, the effect of the Rho/ROCK pathway on sperm cryoprotection is unclear.

This work aimed to explore the effects of RhoA recombinant protein on the cryoprotection and subcellular structure of rooster sperm during freeze-thawing and to elucidate the potential cryoprotective mechanism of RhoA.

MATERIALS AND METHODS

Semen Collection

All experimental protocols were approved by the Animal Care and Use Committee of Beijing University of Agriculture (Beijing, China). In total, 30 Beijing You Chicken roosters were fed a balanced diet and provided with free access to water under a natural light cycle and natural temperature in the Beijing University of Agriculture. The roosters had a week to acclimate to new surroundings. All roosters had at least 1 d of sexual rest before semen collection. Semen samples were collected from 30 roosters by the abdominal massage method (Quinn and Burrows, 1936). Immediately after collection, the rooster semen samples were transported to the laboratory in a thermos flask at 37°C.

Assignment of Experimental Groups for Rooster Semen Freezing and Thawing

Before freezing, semen was assigned to 7 treatment groups, each with 3 replicates, with different levels of recombinant RhoA protein added (0, 0.1, 0.5, 1, 2, 4, or 8 μg/mL). Recombinant RhoA protein was purchased from Sino Biological lnc. (12441-H07B, source: Human), and the protein source was human. Then, the rooster semen was diluted (1:1) using the Beltsville Poultry Semen Extender (BPSE) (pH = 7.4) with a solution of sodium glutamate (0.867 g/100 mL), D-fructose (0.5 g/100 mL), potassium dihydrogen phosphate (0.065 g/100 mL), potassium hydrogen phosphate (1.27 g/100 mL), sodium acetate (0.26 g/100 mL), magnesium chloride hexahydrate (0.034 g/100 mL), citric acid potassium (0.064 g/100 mL), and water. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The semen was maintained at 5°C for 2 h, and diluted again at a final dilution ratio of 1:3 with the addition 6% of dimethylacetamide (final concentration) to the PBSE diluent. After incubating at 5°C for 1 h, the semen was frozen from 5°C to −35°C (at a ramp down rate of ×7°C/min) and from −35°C to −120°C (at a ramp down rate of −9°C/min) using a Control Rate Freezer (CL-8800i, BMT Scientific, Guangzhou, China). The frozen semen was stored in liquid nitrogen until further use. Frozen semen was thawed in 37°C water bath for 40 s.

Semen Quality Assays

The motility, linearity index (**LIN**, %), and linear velocity (**VSL**, μm/s) of fresh and frozen-thawed semen were analyzed using a computer-assisted sperm analyzer system (WLJX-9000 Weili Color Sperm Analysis System, Weili New Century Science, Beijing, China). The system settings were adjusted and avian sperm was detected (Qi et al., 2020). Five randomly selected fields of view were analyzed for each sample. Sperm viability was analyzed using the Eosin-Nigrosine method (G2581, Solarbio, Beijing, China). Sperm acrosome integrity was analyzed using the Sperm Cap-CTC Staining Kit (GMS14053, GenMed Scientifics Inc, America). Green fluorescence on the sperm acrosome indicated that the acrosome was intact. At least 200 sperm from each group were analyzed.

Transmission Electron Microscopy

To observe the cross-sectional ultrastructure of rooster sperm, samples were fixed with 2.5% glutaraldehyde, pH 7.3, for 4 h at 4°C. After fixation, samples were washed 3 times in phosphate buffered saline (**PBS**). Samples were then embedded in 3% liquid agarose. After

the agarose cooled, a scalpel was used to cut samples into 2 mm^3 pieces. The isolated samples were post-fixed in 1% osmium tetroxide for 2 h. Samples were then dehydrated in an ascending grade of ethanol series (30 −100%) and then replaced with 100% acetone. A mixture of 100% acetone and SUPRR resin was then added to the samples (3:1 for 8 h, 2:1 for 8 h) at 25°C. Then, a 1:1 mixture of 100% acetone and SUPRR resin was added to the samples for 48 h at 60°C. The blocks were further trimmed for ultramicrotomy (LEICA EM UC6, Wetzlar, Germany). Ultrathin sections were contrasted with uranyl acetate and lead citrate, and examined and photographed using a transmission electron microscope Hitachi HT-7650 electron microscope (Tokyo, Japan). To observe the overall structure of the rooster sperm, after fixation, samples were washed 3 times with PBS. The samples were then stained with 3% phosphotungstic acid for 30 s and washed with PBS 3 times. After the sample was dried, transmission electron microscopy (**TEM,** Hitachi HT-7650 electron microscope) was used to photograph the sample.

Western Blot Analysis

Sperm samples were lysed at 4°C in radioimmunoprecipitation assay buffer (Solarbio, R0010) mixed with protease and phosphatase inhibitors. Equal amounts of protein were boiled for 10 min and centrifuged at 16,000 RPM for 5 min. The supernatant was isolated and sodium dodecyl sulfate polyacrylamide gel electrophoresis (**SDS−PAGE**) loading buffer (P1015, Solarbio, China) was added and subjected to SDS-PAGE. Proteins were then transferred to Immun-Blot PVDF membranes (YA1701, Solarbio). The membranes were blocked for 2 h in PBS with 0.1% Tween20 (PBST) containing 5% milk and probed overnight with primary antibodies at 4°C. The following primary antibodies were used: Rabbit anti-GAPDH antibody (1:3,000 dilution, Bioss, Beijing, China); Rabbit anti-ROCK1 antibody (1:500, Bioss); Rabbit anti-cofilin antibody (1:1,000, Abcam, United Kingdom); Rabbit anti-phospho-cofilin antibody (1:1,000, Bioss); Rabbit anti-Arp 2/3 complex antibody (1:500, Bioss). After washing 3 times with PBST, the membranes were incubated for 1 h with a Goat anti-rabbit immunoglobulin (**IgG**) secondary antibody (1:3,000, Bioss). Proteins were detected using the Pierce ECL Plus Western blotting substrate kit (32,132 × 3, ThermoFisher Scientific, Beijing, China).

Evaluation the Effects of Y-27632 on Semen Quality After Cryopreservation

The inhibitor Y-27632 was purchased from Sigma-Aldrich (America). To determine the effects of Y-27632 on semen quality, different amounts of Y-27632 (0, 1, 10, 20, 40, 80 mM) diluted (1:1) in BPSE (pH = 7.4) were added and maintained at 5°C for 2 h. Then, the semen was diluted again, maintained at 5°C for 2 h and frozen using cryo-programming. It was determined that 10 mM Y-27632 was optimal upon testing the semen quality after freeze-thawing.

Statistical Analysis

All data were analyzed using SPSS 22.0 (IBM Corp., Armonk, NY). One-way analysis of variance followed by Tukey's multiple comparison test was used to determine statistically significant differences among groups. A student's t test was used to examine statistically significant differences between 2 groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Sperm Quality Before and After Cryopreservation

To determine the effect of RhoA on sperm cryopreservation, different amounts of RhoA recombinant protein were added in the cryoprotectant agents. We observed that RhoA did not affect sperm viability, motility, linearity index, and linear velocity before freezing. The effects of adding recombinant RhoA protein on sperm motility, viability, linearity index, and linear velocity before freezing are shown in Table 1. After sperm freeze-thawing, sperm viability, motility, linearity index, and linear velocity increased quadratically with increasing levels of recombinant RhoA protein (Table 2, $P < 0.05$). The addition of 0.5 and 1 μg/mL RhoA recombinant protein to the cryoprotectant agent led to significant increases in sperm viability, motility, linearity index, and linear velocity following sperm freeze-thawing ($P < 0.05$).

Sperm Acrosome Integrity Before and After Freeze-Thawing

The effect of adding recombinant RhoA protein on sperm acrosome integrity is shown in Figure 1. The addition of different levels of RhoA did not affect sperm

Table 1. Effect of adding RhoA on quality of sperm before freezing and thawing.

	Different levels of RhoA recombinant protein (μg/mL)								*P*-value		
Item	0	0.1	0.5	1	2	4	8	S.E.M.	ANOVA	Linear	Quadratic
Viability (%)	89.45	92.14	91.25	91.40	89.80	91.84	87.75	0.775	0.793	0.553	0.283
Motility (%)	76.82	75.39	76.97	79.17	77.83	75.86	77.01	0.913	0.970	0.866	0.653
Linearity index (%)	46.48	48.35	48.03	46.64	49.41	48.19	49.20	0.327	0.074	0.029	0.939
Linear velocity (μm/s)	51.74	49.97	48.84	48.86	49.75	50.54	50.65	0.637	0.928	0.906	0.253

Table 2. Effect of adding RhoA on quality of sperm after freezing and thawing.

Item	Different levels of RhoA recombinant protein (μg/mL)							S.E.M.	*P*-value		
	0	0.1	0.5	1	2	4	8		ANOVA	Linear	Quadratic
Viability (%)	52.27^{d}	59.97^{cd}	72.98^{ab}	74.89^{a}	65.07^{bc}	64.05^{bc}	52.04^{d}	1.976	< 0.001	0.968	< 0.001
Motility (%)	11.48^{c}	13.04^{c}	32.41^{a}	24.06^{b}	16.88^{c}	14.52^{c}	14.67^{c}	1.608	< 0.001	0.686	< 0.001
Linearity index (%)	24.00^{b}	34.20^{a}	38.79^{a}	36.52^{a}	34.18^{a}	32.57^{a}	34.30^{a}	1.091	0.001	0.018	< 0.001
Linear velocity (μm/s)	7.34^{b}	10.80^{ab}	12.41^{a}	12.67^{a}	10.83^{ab}	11.25^{ab}	10.15^{ab}	0.451	0.010	0.102	0.001

$^{a-d}$Mean values within a column without common superscripts differ significantly ($P < 0.05$).

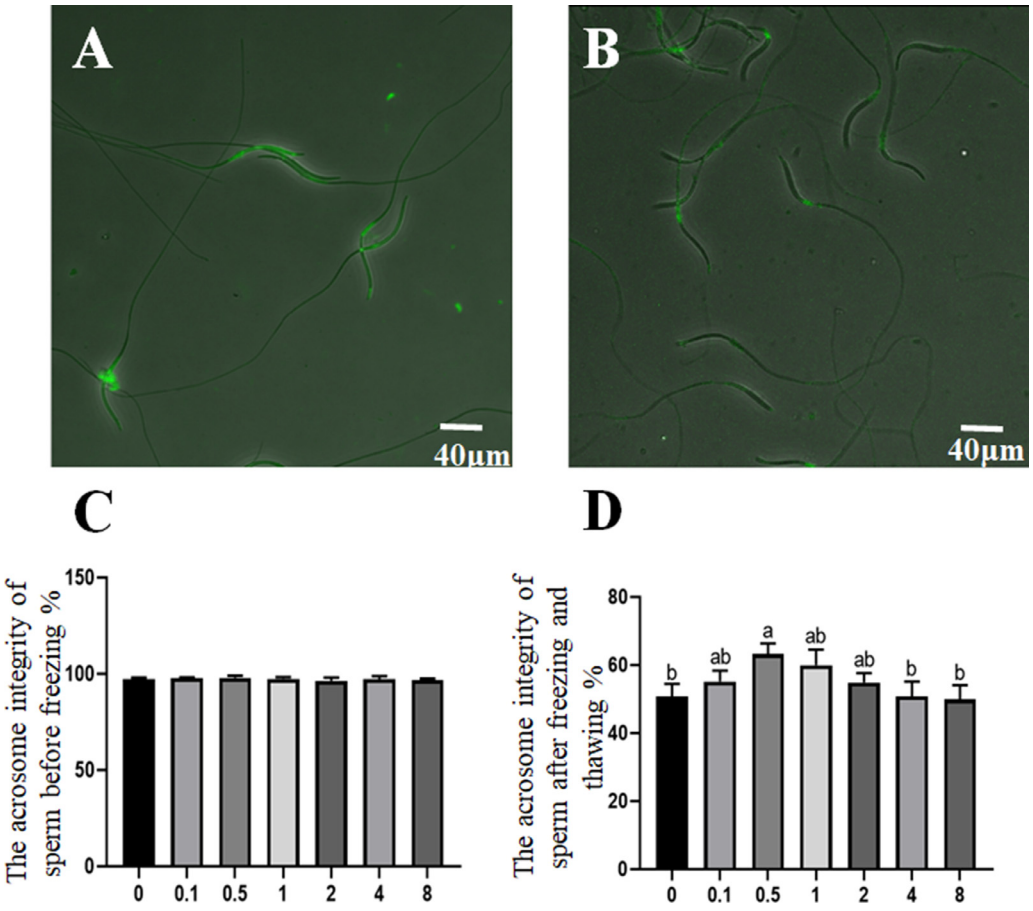

Figure 1. Effect of adding RhoA on sperm acrosome integrity after freezing and thawing. The effect of adding RhoA (0, 0.1, 0.5, 1, 2, 4, 8 μg/mL) in cryoprotectant agents on sperm acrosome integrity before and after sperm frozen-thawed, measured by Fluorescence microscope. Each group counts more than 200 sperms. (A) The acrosomes of sperm were intact. (B) The acrosomes of sperm were not intact. (C) The acrosome integrity of sperm before freezing. (D) The acrosome integrity of sperm after freezing and thawing.

acrosome integrity prior to freezing. After sperm freezing and thawing, sperm acrosome integrity increased quadratically with increasing levels of RhoA added to the cryoprotectant agents ($P < 0.05$). Adding 0.5 μg/mL RhoA recombinant protein to the cryoprotectant agents led to a significant increase (64.56%) of sperm acrosome integrity following freeze-thawing when compared to the control ($P < 0.05$).

Sperm Ultrastructure After Freeze-Thawing

We found that sperm exhibited cytoskeletal damage to different degrees in the rooster spermatozoa head, neck, and tail after freeze-thawing with the addition of exogenous RhoA. Sperm ultrastructure before and after adding different amounts of recombinant RhoA protein is shown in Figures 2 and 3. In terms of the overall subcellular structure of the spermatozoa, the structural damage included fracture and rupture in the head and tail of the spermatozoa, damage to the membrane structure, overflow of cytosol, and separation of the 9 doublet-microtubules in the tail. A cross-sectional view of cellular ultrastructure revealed damage to spermatozoa including cytoplasm spillage, mitochondrial sheath detachment, head plasma membrane swelling or loss, cell membrane breakage, and tail plasma membrane swelling.

To better understand the effect of adding different levels of RhoA recombinant protein on the subcellular structure of spermatozoa after freezing and thawing, we performed quantitative analyses of the structural damage in spermatozoa (Figure 4). Results showed that the spermatozoa tail and acrosomal plasma membrane

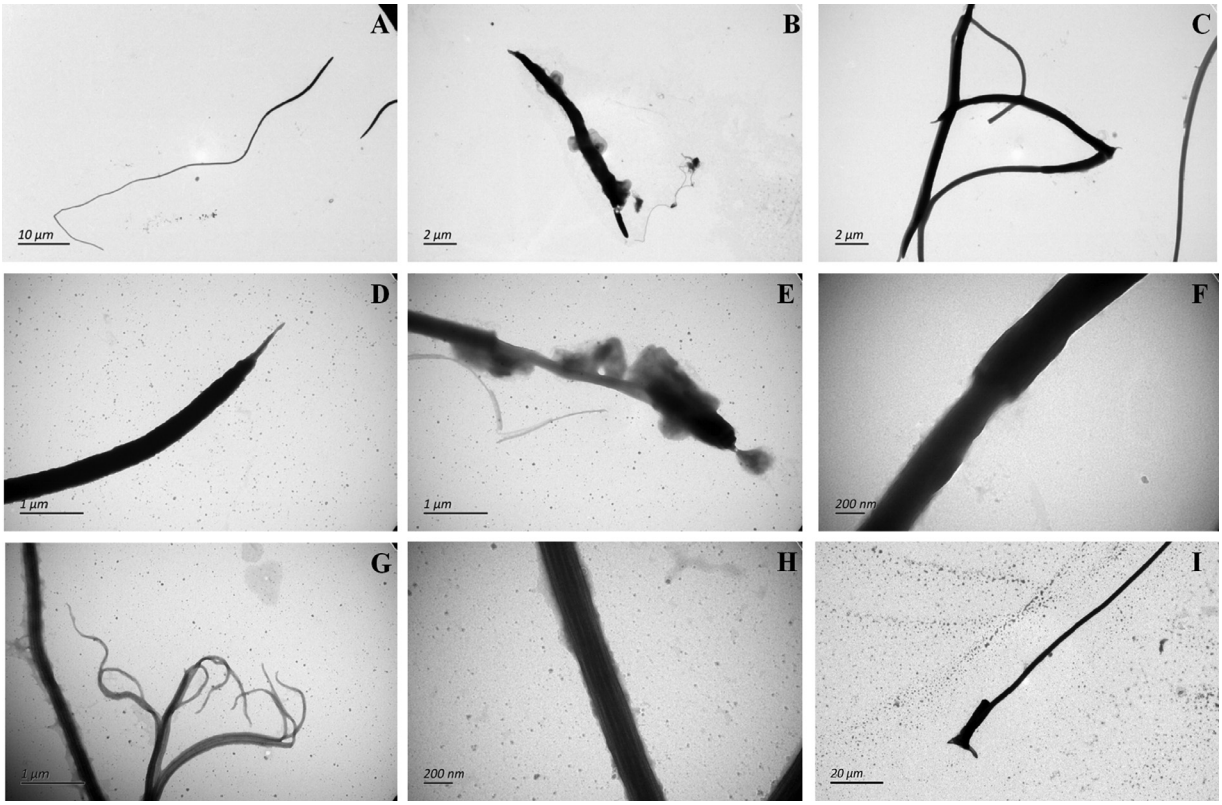

Figure 2. Changes in the overall ultrastructure of sperm after freezing and thawing. The effect of different levels of RhoA recombinant protein on the ultrastructure of overall sperm after frozen-thawed, measured by transmission electron microscopy (TEM). The method of staining samples was 3% phosphotungstic acid. (A) A cock sperm with the intact ultrastructure (Without the addition of RhoA recombinant protein). (B) The sperm head ruptured and the cytoplasm spilled out (Addition of 0.5 μg/mL of RhoA recombinant protein). (C) The sperm head was fractured (Addition of 0.1 μg/mL of RhoA recombinant protein). (D) A sperm head with intact ultrastructure (Without the addition of RhoA recombinant protein). (E) The membrane structure of the sperm acrosome is damaged (Without the addition of RhoA recombinant protein). (F) The integral ultrastructure in sperm connecting piece (Addition of 1 μg/mL of RhoA recombinant protein). (G) 9 doublet microtubules in sperm axoneme were separated (Without the addition of RhoA recombinant protein). (H) Sperm flagella without freezing damage (Without the addition of RhoA recombinant protein). (I) The sperm with a fracture head (Addition of 0.5 μg/mL of RhoA recombinant protein).

breakage rates were significantly decreased ($P < 0.05$) upon adding different levels of RhoA. The addition 0.5 μg/mL and 1 μg/mL RhoA significantly decreased the breakage rates of acrosomal nuclear membrane, plasma membrane, and sperm tail after freeze-thawing compared with the control group ($P < 0.05$). Adding different levels of RhoA protein had no significant effect on the integrity of the acrosomal plasma membrane after sperm freeze-thawing.

The Effect of Y-27632 on Sperm Cryopreservation

We observed that, when compared with the control, the addition of 0.5 μg/mL RhoA significantly increased the protein expression levels of ROCK1 and P-cofilin after freeze-thawing ($P < 0.05$), while that of cofilin was significantly decreased ($P < 0.05$; Figures 5A−5C). Y-27632 was added to the treatment and control groups and was found to significantly decrease the protein expression levels of ROCK1 and P-cofilin compared with the control ($P < 0.05$), while that of cofilin was significantly increased ($P < 0.05$). The effect of adding Y-27632 on sperm cryopreservation is summarized in Figure 5.

In the cellular level assays, we detected sperm motility, viability, and acrosome integrity under different treatment groups after freezing and thawing (Figures 5D−5F). Upon the addition of 0.5 μg/ml RhoA, sperm motility, viability, and acrosome integrity were significantly increased compared to those of the control group after freeze-thawing ($P < 0.05$). Adding Y-27632 to the different treatment groups significantly decreased sperm motility, viability, and acrosome integrity compared with the control ($P < 0.05$).

In parallel, we assayed the changes in sperm subcellular structure under different conditions (Figures 5G−5I). Compared to the control, adding 0.5 μg/mL RhoA significantly decreased damage in the sperm tail, acrosome plasma membrane, and nuclear membrane after freeze-thawing ($P < 0.05$). In addition, adding Y-27632 under different treatment conditions led to a marked increase in the damage in the sperm tail, acrosome plasma membrane, and nuclear membrane compared with the RhoA group ($P < 0.05$), and it was not significantly different compared to the control ($P < 0.05$).

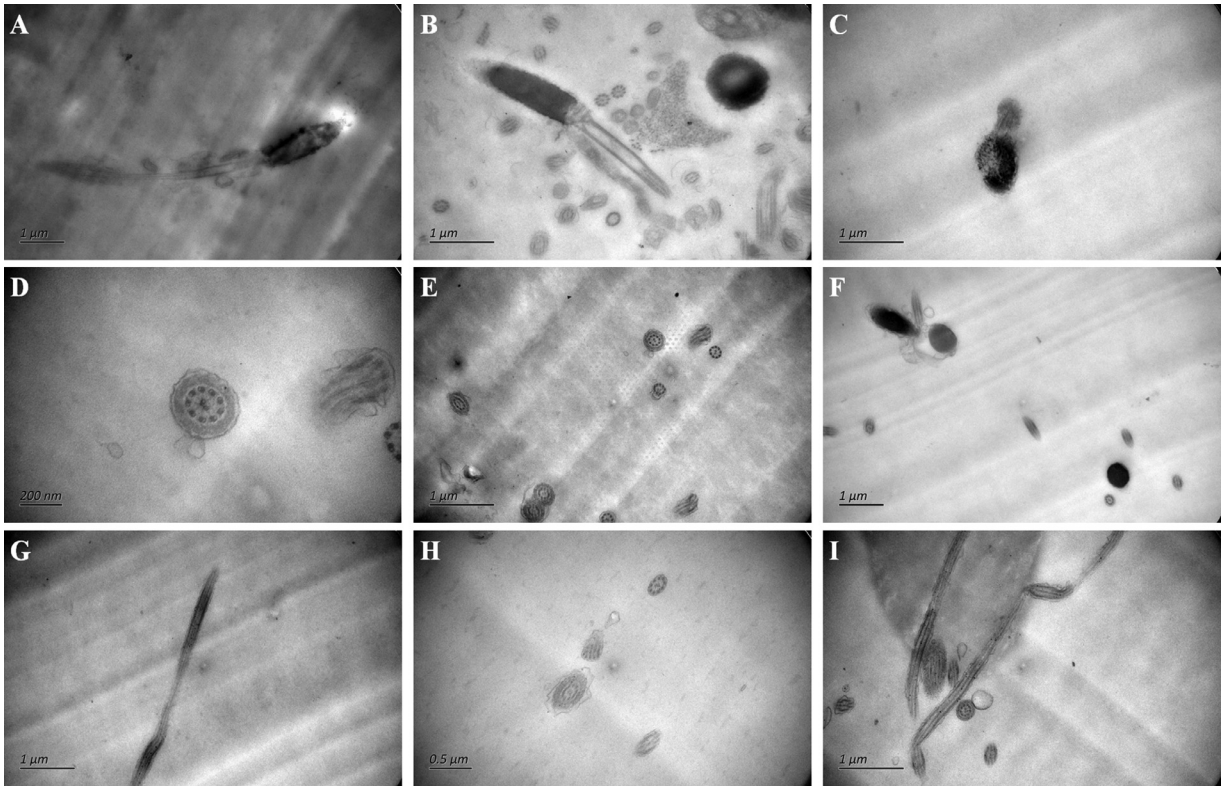

Figure 3. The ultrastructure of sperm in cross-section and longitudinal section after freezing-thawing. The effect of different levels of RhoA recombinant protein on the ultrastructure of cross section and vertical section of sperm after frozen-thawed, measured by transmission electron microscopy (TEM). (A) Frozen sperm with disruption acrosome and damage neck (Without the addition of RhoA recombinant protein). (B) The sperm with intact head and neck (Addition of 1 μg/mL of RhoA recombinant protein). (C) Frozen sperm with damage membrane (Addition of 4 μg/mL of RhoA recombinant protein). (D) Cross-section of intact sperm caudal mid-section (Without the addition of RhoA recombinant protein). (E) Sperm tail cross-section (Addition of 0.5 μg/mL of RhoA recombinant protein). (F) Swelling or loss of plasma membrane in the sperm head (Addition of 2 μg/mL of RhoA recombinant protein). (G) Plasma membrane of sperm tail is intact(Addition of 8 μg/mL of RhoA recombinant protein). (H) Swelling of the plasma membrane in the middle part of the sperm tail (Without the addition of RhoA recombinant protein). (I) Longitudinal section of the sperm tail and the tail is broken (Addition of 0.1 μg/mL of RhoA recombinant protein).

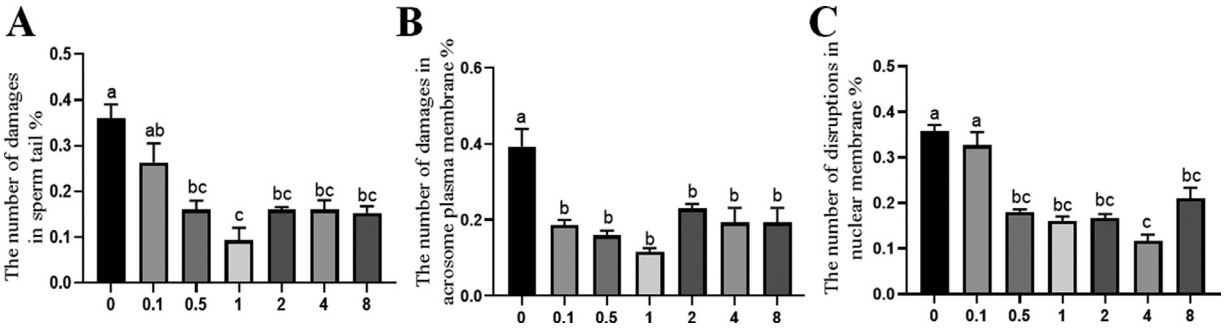

Figure 4. Effect of different levels of RhoA on the ultrastructure of frozen-thawed spermatozoa. The quantitative statistics on the effect of different levels of RhoA on ultrastructure of sperm after freezing and thawing. Each replicate group counts more than 200 sperm. (A) The number of damages in sperm tail %. (B) The number of damages in acrosome plasma membrane %. (C) The number of disruptions in nuclear membrane %.

DISCUSSION

The formation of ice crystals and ice recrystallization are key factors that cause sperm cryo-damage. Previous methods for reducing the physical damage caused by ice crystallization relied on the addition of hypertonic solutions, such as dimethyl sulfoxide, dimethyl acetamide, or glycerin, to properly dehydrate the sperm; however, due to the specific structure of rooster sperm this method failed to robustly improve semen quality. Our previous work demonstrated that RhoA is differentially expressed in rooster sperm after freeze-thawing (Qi et al., 2020). Here, we investigated whether RhoA is effective in improving semen quality after freeze-thawing and whether it is involved in improving the subcellular structure of sperm via the Rho/ROCK pathway.

Semen quality is an important parameter used to evaluate the reproductive performance of roosters and includes sperm motility and viability, semen volume, and pH (Ericsson et al., 1993; Zelante et al., 2006). This experiment primarily investigated the effects of the addition of different levels of recombinant RhoA protein on

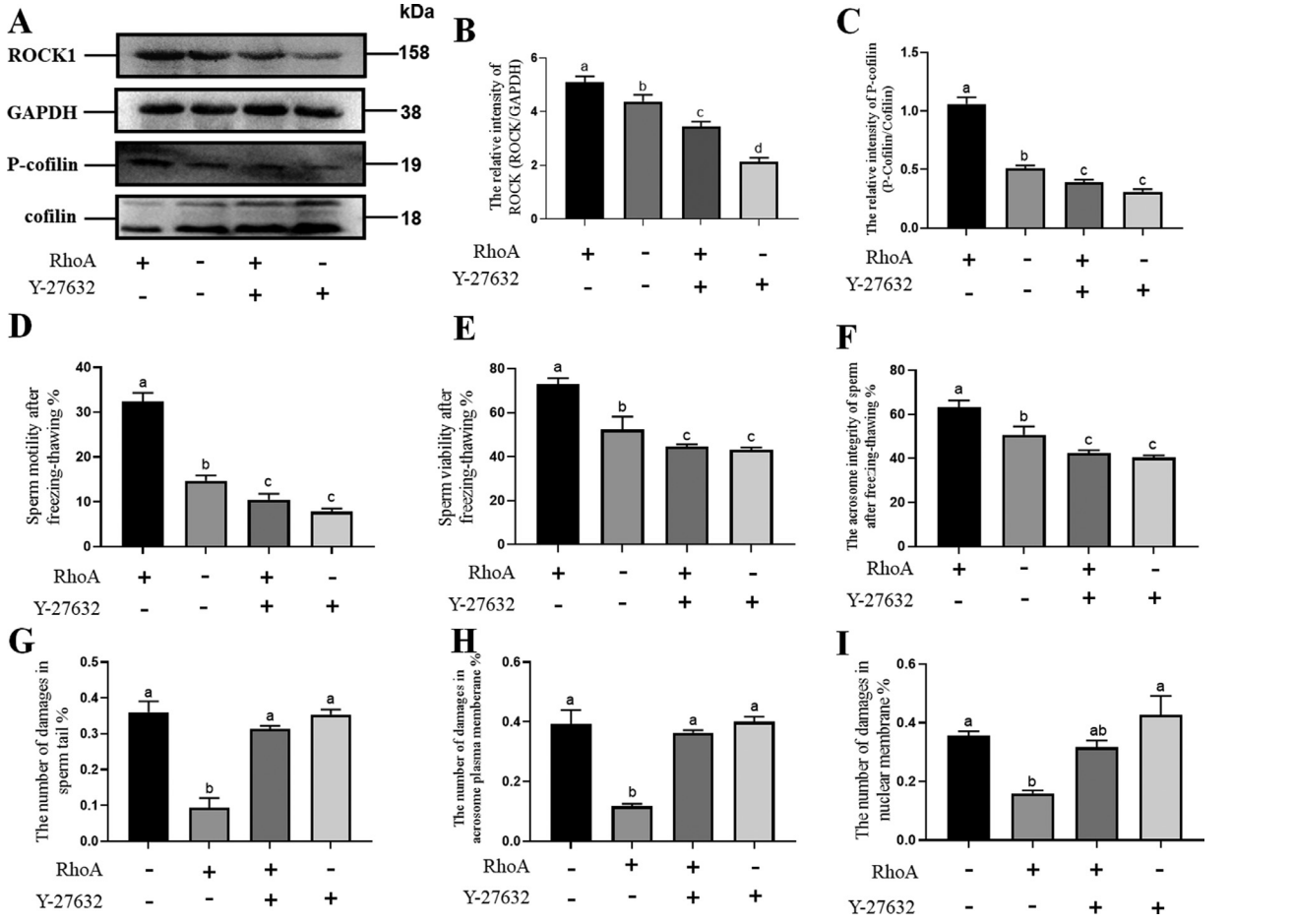

Figure 5. Y-27632 effects on sperm cryoprotection. Note: Y-27632 (Rho downstream effector kinase inhibitor, Abcam). "+" indicates that the corresponding additive was added in the treatment. "-" indicates that the corresponding additive was not added in the treatment. The level of RhoA added in the treatment groups was 0.5 μg/mL. The level of Y-27632 was 10 mM. (A) Effect of addition Y-27632 on the protein expression of RhoA downstream proteins ROCK1, cofilin, and P-cofilin. (B) Relative expression of ROCK1 (ROCK1/GAPDH). (C) Relative expression of P-cofilin (P-cofilin/cofilin). (D) Effect of Y-27632 on sperm viability after freezing and thawing. (E) Effect of Y-27632 on sperm survival rate after freezing and thawing. (F) Effect of Y-27632 on sperm acrosome integrity rate after freezing and thawing. (G–I) The quantitative statistics on the effect of Y-27632 on ultrastructure of sperm after freezing and thawing. Each replicate group counts more than 200 sperm. (G) The effect of Y-27632 on the number of damages in sperm tail after freezing and thawing. (H) The effect of Y-27632 on the number of damages in acrosome plasma membrane after freezing and thawing. (I) The effect of Y-27632 on the number of damages in nuclear membrane after freezing and thawing.

sperm motility, viability, linearity index, linear velocity, acrosome integrity, and subcellular ultrastructure changes of sperm after freezing and thawing. Results showed that the addition of different amounts of recombinant RhoA protein had no significant effect on semen quality prior to semen freezing and thawing. This indicates that the effect of RhoA recombinant protein on semen quality occurs during the process of freezing and thawing, rather than before.

Sperm motility (a+b) is a proportion of the number of straight moving sperm in semen, which is highly positively correlated with the fertilization rate (Bozkurt and Öğretmen, 2012). RhoA is located in the spermatozoa head and tail, where it can adhere to the membrane surface in spermatozoa and participate in the regulation of cytoskeleton cohesion and reorganization (Ducummon and Berger, 2006; Byrne et al., 2016). It was previously shown that RhoA can modulate actin polymerization and alter cell membrane stability, indicating RhoA can participate in the sperm capacitation process (Chen et al., 2014). We previously demonstrated that the addition of RhoA significantly increases the recovery rate of normally hatched murine embryos, and the RhoA/ROCK signaling pathway-mediated phosphorylation of RhoA facilitates improved freezing resistance in dormant mouse embryos (Gu et al., 2017). The present results revealed that the addition of 0.5 or 1 μg/mL recombinant RhoA protein significantly increased the motility and viability of sperm after freeze-thawing compared to the control, which may be due to RhoA phosphorylation mediated by the RhoA/ROCK signaling pathway. In addition, the adherence of RhoA to the cell membrane surface during freeze-thawing may reduce the aggregation of ice crystals within and outside the cell membrane and inhibit ice recrystallization, thus protecting the structural integrity of the sperm membrane (Kim et al., 2017). The increase in sperm linearity index and linear velocity after semen freeze-thawing is consistent with the results of sperm motility and viability and may be related to the role of RhoA in regulating sperm

actin to maintain the normal physiological state of spermatozoa. Under normal physiological conditions, excess RhoA inhibits the cellular heat shock response to reduce proteostasis and cellular adaptation (Meijering et al., 2015). Moreover, Rho is able to mediate excitotoxic cell death by calcium-dependent activation of p38α (Semenova et al., 2007). Here, the addition of high concentrations of RhoA recombinant protein (2, 4, and 8 μg/mL) caused a decline in sperm motility and viability after freeze-thawing, which may be related to a decrease in sperm homeostasis and excitotoxic death caused by high concentrations of RhoA during semen freeze-thawing (Semenova et al., 2007; Meijering et al., 2015).

Sperm acrosome integrity is an important indicator used to evaluate the reproductive performance of roosters (Takahashi et al., 2010; Chauychu-noo et al., 2021). Damage to the sperm acrosome leads to leakage of enzymes, such as acrosomal enzymes and hyaluronidase, resulting in reduced sperm viability, sperm capacitation, and fertilization rates (Schill and Wolff, 1974). Interestingly, we found that the addition of 0.5 μg/mL RhoA recombinant protein increased acrosome integrity after sperm freeze-thawing, suggesting that the addition of exogenous RhoA maintained the integrity of the sperm acrosome. It is probable that RhoA protein adhered to the acrosome and thus reduced damage from ice crystallization during freeze-thawing. Alternatively, RhoA may be involved in regulating cytoskeletal homeostasis to improve sperm acrosome integrity. To further verify this hypothesis, we determined the changes in sperm subcellular ultrastructure by transmission electron microscopy (**TEM**).

Changes in the morphological structure of spermatozoa are an important factor in semen quality (84). The ultrastructure of livestock and poultry sperm differs greatly, and the poor tolerance of cryopreservation in poultry sperm is related to its subcellular structure. Compared to mammalian spermatozoa, such as porcine and bovine, poultry spermatozoa have long cylindrical heads that are not conducive to the penetration of cryoprotectants (Kondracki et al., 2020) and also have a longer tail that is prone to breakage during freezing and thawing. We found that cryo-damage of the spermatozoa occurred in various parts, including the membrane structure (plasma and nuclear membranes), spermatozoa head, mitochondrial sheath, and doublet microtubules. Furthermore, each part was observed to exhibit different levels of damage under different treatments. These results suggest a potential dose-dependent protective effect of spermatozoa ultrastructure by RhoA recombinant protein. Quantitative analysis of sperm ultrastructural damage revealed that the addition of 0.5 and 1 μg/mL recombinant RhoA protein was able to maintain homeostasis of sperm subcellular structure after freeze-thawing. RhoA is a small GTPase protein that can be inserted into phospholipid bilayers to participate in actin assembly and cytoskeletal polymerization; therefore, the improvement of sperm ultrastructure after freezing and thawing presented here may be related to actin assembly and cytoskeletal polymerization (Li et al., 2020; Lu et al., 2020). In addition, the sperm flagellum consists of 2 central microtubules, 9 doublet microtubules, and a dynein arm, and provides motility for spermatozoa to maintain the linear mobility of sperm under normal physiological conditions (Touré et al., 2020). In the present results, the increase in sperm viability, linearity index, and linear velocity after freezing and thawing could be associated with improving sperm flagellum structure after freeze-thawing due to the presence of recombinant RhoA protein.

It is well known that the Rho/ROCK pathway is involved in regulating many cellular processes, such as cell motility, cell survival, and cytoskeletal polymerization, by activating downstream ROCK through the binding of Rho to GTP (Fujimura et al., 2015; Sullivan et al., 2016; Wakabayashi et al., 2019). However, there is a gap in understanding as to whether RhoA is involved in sperm cryopreservation by regulating the downstream effector ROCK1. Here, we found that the addition of recombinant RhoA protein increased ROCK1 protein expression after sperm freezing and thawing. When ROCK1 was inhibited, the addition of RhoA did not improve sperm quality and the subcellular structure of sperm was not affected after freeze-thawing, suggesting that RhoA improves the subcellular ultrastructure of sperm after freezing and thawing via the Rho/ROCK pathway, thereby improving semen quality. ROCK is a central regulator of the actin cytoskeleton (Ishizaki et al., 1996; Michelle et al., 2014) that regulates ROCK1-dependent actin cytoskeleton polymerization (Scotter et al., 2006), cell apoptosis (Igarashi et al., 2020), and adhesion (Pirone et al., 2006). Furthermore, several studies have shown that ROCK can activate downstream LIM kinase activity and subsequently regulate the phosphorylation level of cofilin, thus regulating microfilament dynamics and reorganization (Pritchard et al., 2004; Montani et al., 2009; Pak et al., 2016). Presently, we found that cofilin phosphorylation was consistent with ROCK1 expression levels, indicating that cofilin may be involved in sperm cryoprotection via phosphorylation (Figure 6). Cofilin is a member of the Actin Depolymerizing Factor/Cofilin (ADF/Cofilin) family, which are a type of actin binding proteins (Bernstein and Bamburg, 2010; Narita, 2020). Phosphorylation of cofilin is essential for the regulation of dynamic changes in actin and the cytoskeleton after cells receive external stimuli (Jin et al., 2020). P-cofilin can bind to actin with ADP, hydrolyze its own ATP to ADP, form actin with ADP/Pi, cut actin filaments, and generate new polymeric fragments for actin polymerization to regulate the dynamic reorganization of actin (Yeoh et al., 2002; Pollard and Borisy, 2003). Here, the improvement in subcellular ultrastructure after sperm freeze-thawing may be related to the involvement of Rho/ROCK pathway-mediated cofilin phosphorylation in regulating the dynamic reorganization of actin and cytoskeleton (Figure 6).

In summary, we have demonstrated a cryoprotective mechanism in spermatozoa which involves the Rho/ROCK pathway during spermatozoa freeze-thawing and

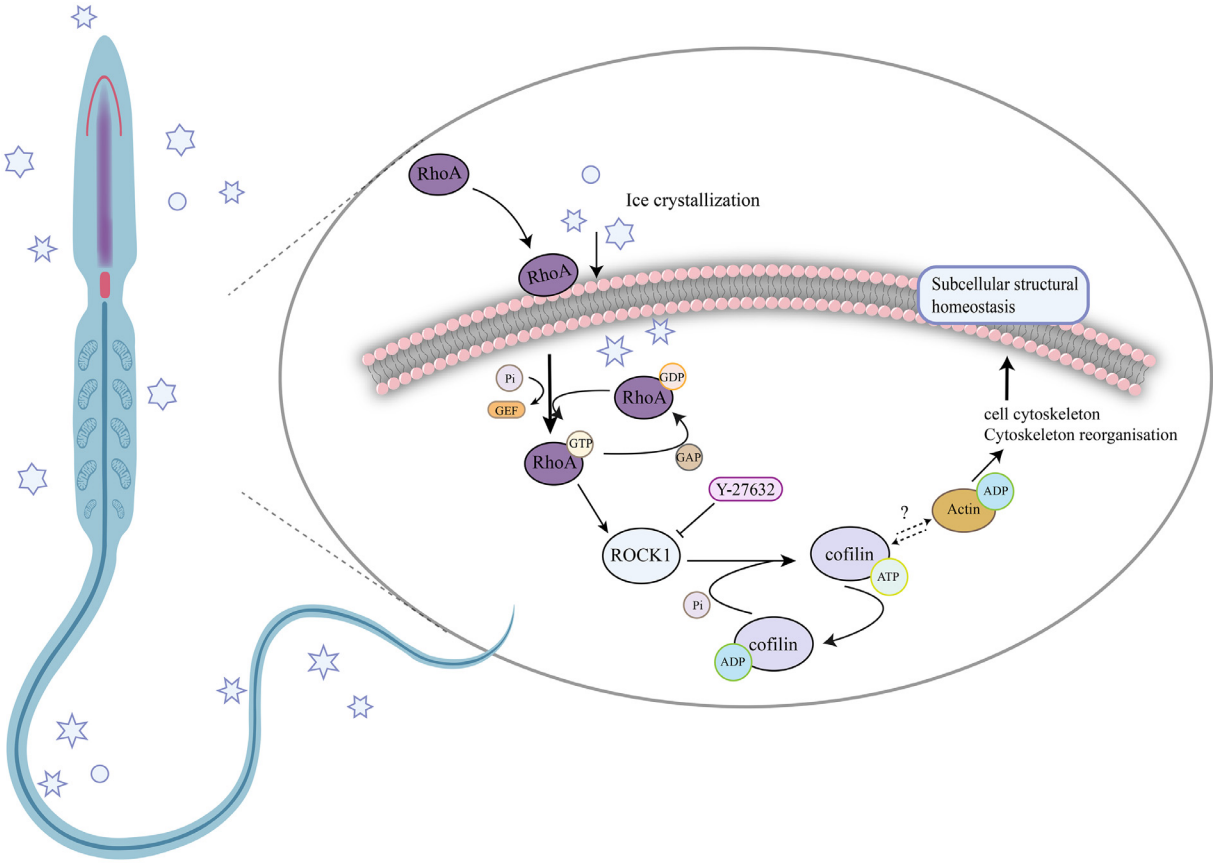

Figure 6. Schematic representation of the RhoA pathway in rooster spermatozoa cryoprotection.

found that the exogenous addition of recombinant RhoA protein can improve sperm motility, viability, acrosome integrity, and subcellular ultrastructure after freezing and thawing via this pathway. The Rho/ROCK pathway may influence the dynamic reorganization of actin and the cytoskeleton by regulating the phosphorylation level of cofilin. This approach, which starts with improving the sperm's own resistance to freezing (rather than reducing the physical damage caused by the external environment), offers a new insight into improving the quality of sperm cryopreservation.

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DISCLOSURES

The authors have no conflicts of interest to declare. We confirm that this manuscript has not been published elsewhere and is not under consideration in whole or in part by another journal. All authors have approved the manuscript and agree with submission to poultry science.

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