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Exploring how sucrose-colloid selection improves the fertilizing ability of chicken sperm after cryopreservation with glycerol

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ABSTRACT Currently, glycerol is the most effective cryoprotectant when combined with straw packaging for preserving chicken sperm. Glycerol, however, has toxic effects on sperm cells, which can reduce fertility when present in inseminated semen. Historically, the serial dilution (**SD**) method was developed to eliminate glycerol and mitigate its adverse effects. We have recently developed a new method for removing glycerol called sucrose-Percoll (**SP**), that can be performed at either 4°C (4°C-SP) or 20°C (20°C-SP). This SP protocol has been found to be simpler and faster to improve fertility compared to the traditional SD method. Nevertheless, the reasons for such effectiveness differences between glycerol removal procedures remained unclear and required more comprehensive understandings for future protocol developments. Here, we examined the effects of SP and SD protocols on the fertility duration. We also investigated the potential causes of varying effects of these methods by analyzing sperm quality parameters and sperm storage in the hen's reproductive tract. The fertility was significantly higher in 4°C-SP than 20°C-SP during the first 6 d after insemination, and also higher than sperm processed using SD. No difference was observed between 20°C-SP and SD between 7 and 13 d. However, a 2.7-time higher fertility was shown with 4°C-SP. In addition, the SP method demonstrated a 2-fold greater ability to remove glycerol than the SD method. Sperm centrifuged at 4°C-SP exhibited higher sperm storage compared to 20°C-SP and were higher than sperm treated with SD. Overall, our findings revealed that the differences in efficiencies between SP and SD methods were not related to in vitro sperm quality but resulted from a higher ability to remove glycerol, a higher storage capacity in the female reproductive tract, and a longer fertility ability. Since no impacts were observed in sperm cellular characteristics, further experiments are necessary to investigate the influences of glycerol removal treatments at the molecular level.

Key words: cryopreservation, fertility, glycerol, sperm storage tubules, toxicity

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INTRODUCTION

Cryopreservation of avian sperm is currently the only method available for conserving the genetic background of poultry on a large scale (Santiago-Moreno and Blesbois, 2022; Boes et al., 2023). Despite the potential use of several cryoprotectants, glycerol was the first one reported to be effective for preserving chicken sperm against cryoinjuries (Polge, 1951) and it continues to be the most efficient cryoprotectant when coupled with straw packaging (Thélie et al., 2019a; Lin et al., 2021). However, when leaving glycerolized semen samples in a warm environment at body temperature, glycerol may induce cytotoxicity in sperm cells (Macías García et al., 2012; Lin et al., 2023b). The study conducted by Tselutin et al. (1999) determined that the optimal concentration of glycerol for protecting chicken sperm during the freezing-thawing process was 11%. However, studies have shown that the presence of only 6% glycerol in inseminated semen can result in infertility in hens (Neville et al., 1971; Lin et al., 2023b).

Several studies, including ours, have attempted to understand the negative impacts of glycerol on sperm cells (Morrier et al., 2002; Zeng et al., 2014; Lin et al., 2023b). In summary, at physiological temperature, glycerol reduces sperm motility, induces cell death by causing membrane breakage, increases apoptosis, and also disrupts normal cell functions such as the acrosome reaction and energy metabolism. These alterations in sperm cell biology may lead to a defect in sperm storage within the sperm storage tubules (**SST**) in the female

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reproductive tract, which can explain the reduced fertility observed (Lin et al., 2023b). Currently, the only approach to avoid the negative effects of glycerol in chicken frozen-thawed semen is to eliminate it just after thawing. The first method introduced to remove glycerol from cryopreserved sperm was serial dilution (**SD**) combined with centrifugation (Lake and Stewart, 1978; Seigneurin and Blesbois, 1995). Despite being considered a promising opportunity to improve assisted reproductive techniques in poultry, the SD method relies on specialized equipment to maintain semen at 4°C, involves complex operations and is time-consuming (Lin et al., 2021), which explains its limited utilization in the poultry industry.

Recently, we described a new glycerol removal protocol called sucrose-Percoll (**SP**) centrifugation (Lin et al., 2023a). This SP solution is designed to stabilize sperm against osmotic shock by adding sucrose and to select motile sperm by combining it with Percoll. This technique is based on the passage of glycerolized sperm through the SP solution using 15 min of centrifugation to remove glycerol from cryopreserved chicken semen (Lin et al., 2023a). To facilitate easy handling in a farm environment, we have developed this SP method for use at 20°C, which is the typical temperature in chicken coops, and at 4°C, if the equipment for maintaining semen temperature is available. We have proven that the fertility obtained through SP treatment is higher than the SD method, suggesting its potential use in the poultry industry. However, the cellular mechanisms underlying these efficiency differences were unknown, and their identifications may lead to future protocol improvements.

To identify the causes of differential efficiencies between glycerol removal approaches (i.e., SP methods at 4°C or 20°C and SD centrifugation), we first compared the persistence of fertility by examining the fertility rates immediately after insemination (early fertility) and 1 wk after insemination (late fertility). Subsequently, we evaluated glycerol remaining concentration and in vitro sperm quality parameters after glycerol removal treatments. Finally, to evaluate the effects of different protocols on sperm storage in female reproductive tract, we inseminated hens with cryopreserved sperm obtained by SP or SD centrifugation and then observed the distribution of sperm in the SST.

MATERIALS AND METHODS

Animal Management

All experiments were conducted in accordance with the legislation governing animal treatment and were approved by the French Ministry of Higher Education, Research and Innovation, and the Val-de-Loire Animal Ethics Committee (authorization numbers: N° APAFIS #4026-2016021015509521 and APAFIS #34415-202112141205965). A total of 18 adult T44 roosters (31−45 wk old, Gallus gallus domesticus, Sasso, France), housed in individual cages, were used as semen donors. A herd of 45 adult Lohmann hens (35−85 wk old), housed in groups of 4 in cages, were used for fertility tests and SST experiments. All animals were subjected to a lighting regimen of 14 h of light and 10 h of darkness. The temperature was controlled at 20°C, and they were fed a standard diet with water available ad libitum at the INRAe Poultry Experimental Unit UE-PEAT (2018). https://doi.org/10.15454/1.5572326250887292E12).

Semen Collection, Freezing, and Thawing

The collection of semen samples and the processes of freezing and thawing were the same as previously described (Lin et al., 2023a). Eighteen roosters were initially selected based on sperm tests, which included assessing motility (> 80%) and membrane integrity (> 80%). Due to the limitations of handling capacity for sperm cryopreservation, the roosters were randomly divided into 3 groups. Semen ejaculate was collected in the morning twice a week using the abdominal massage method (Burrows and Quinn, 1937). It was then pooled and diluted 1:1 with Lake PC diluent (Lake and Ravie, 1981) at room temperature. The mixture was gently mixed and placed at 4°C for 10 min. The resulting semen suspension was diluted to a final concentration of 11% glycerol. It was then equilibrated at 4°C for 10 min before being loaded into 0.5 mL plastic freezing straws (IMV Technologies) and sealed. The straws were then frozen using a controlled cooling rate of −7°C/min, starting from 4°C and reaching −35°C. After that, the cooling rate was increased to −60°C/min, bringing the temperature down to −140°C (Nguyen et al., 2015). Finally, the straws were plunged into liquid nitrogen (−196°C) and stored until thawing. Frozen semen straws were thawed in a water bath at 4°C for 3 min, gently pooled, mixed, and then evenly distributed to each treatment. The number of biological replicates for each experiment is provided in the legend of each figure.

Glycerol Removal Protocols

In this study, we compared 3 different methods for removing glycerol: SP centrifugation at either 4°C or 20°C (4°C-SP or 20°C-SP), and SD before centrifugation. Control in these experiments involved frozen-thawed semen without any treatment, which was stored at 4°C during the glycerol removal process of treatment straws.

SP Centrifugation In the present study, the working solution used for SP centrifugation was actually the glycerol removal solution from our previous study (Lin et al., 2020,2023a). A mixture of 36% Percoll and 80 mM sucrose was prepared in phosphate buffered saline (PBS), and 2 mL of the SP mixture was transferred into a 15 mL centrifuge tube. Then, 500 μL of frozen-thawed semen was gently layered on top of the SP and centrifuged for 15 min at 800 × g at either 4°C or 20°C. After centrifugation, the supernatant was discarded, and the sperm pellets were resuspended in 100 μL of Lake 7.1 diluent (Lake et al., 1981) for subsequent experiments.

SD Centrifugation SD centrifugation is the current method used to remove glycerol from cryopreserved chicken semen (Seigneurin and Blesbois, 1995). Briefly, the frozen-thawed semen was gradually diluted with Lake C diluent (Lake and Stewart, 1978) to a final dilution of 1:4. This was achieved by adding the diluent 6 times, with a 2-min interval between each addition at 4°C with gentle agitation. The diluted semen was then immediately centrifuged for 15 min at 500 × g at 4°C. After removing the supernatant, the sperm pellets were resuspended in 100 μL of Lake 7.1.

Fertility Test

A total of 36 hens were randomly divided into 3 groups. Each group contained 12 hens that were inseminated with thawed semen treated with 1 of the 3 different glycerol removal protocols (4°C-SP, 20°C-SP, and SD). After treatment, the sperm were immediately used for intravaginal insemination at a depth of 3 to 4 cm. Each hen received a dose of 200 × 10^6 sperm (King et al., 2002; Thélie et al., 2019a) for 5 consecutive inseminations, with 2 inseminations per week. Eggs were collected from the 2nd day after the first insemination until the 13th day after the last insemination. Fertility was categorized into early fertility (referring to eggs between the 2nd day after the first insemination and the 6th day after the final one) and late fertility (referring to eggs between the 7th and 13th day after the final insemination). Collected eggs were stored at 15°C with 85% humidity for 7 d before being incubated at 37.7°C with 55% humidity. Fertile and infertile eggs were determined by candling on the 7th day of incubation (Long and Kulkarni, 2004).

Glycerol Concentration Determination

Glycerol assay kit (#MAK117, Millipore Sigma, Burlington, MA) was used to measure the concentration of glycerol, following the instructions provided by the manufacturer. Briefly, glycerol standard solution was first diluted to obtain concentrations of 0, 0.3, 0.6, and 1 mM. Then, 10 μL of semen or standard samples was transferred into wells of a 96-well microplate and added directly 100 μL of the reaction solution to each well. The microplate was examined using spectrophotometry (Tecan Infinite M200 Plate Reader, Tecan Life Science, Männedorf, Zürich, CH) to measure the absorbance at a wavelength of 570 nm after 20 min of incubation at room temperature in the dark. Finally, the absorbance value was used to calculate the concentration of glycerol-based on a standard curve.

Evaluation of Sperm In Vitro Quality Parameters

In this study, we analyzed the in vitro quality parameters of sperm, including motility, membrane integrity, apoptotic cells, and mitochondrial activity. The methodologies used in the previous study (Lin et al., 2023a) were employed and briefly described as follows.

Sperm Motility Sperm motility parameters were tested using a computer-assisted sperm analysis system (**CASA**, IVOS Motility Analyzer, IMV Technologies, L'Aigle, Orne, FR). An aliquot of 2 μL of semen (30 × 10^6 cells/mL) was placed on a Makler Counting Chamber to capture images from 4 fields for evaluating the average path velocity (**VAP**), straight line velocity (**VSL**), and straightness (**STR** = VSL/VAP). Motility results were presented as the percentage of motile sperm and progressive sperm. Motile sperm were defined as sperm showing a VAP greater than 5 μm/s, while progressive sperm were defined as sperm with a VAP greater than 50 μm/s and a STR greater than 75%.

Membrane Integrity A dual staining technique using SYBR 14 and Propidium iodide (**PI**) fluorescent dyes (LIVE/DEAD Sperm Viability Kit #MAK117) in combination with a flow cytometer (Guava easyCyte 5HT, IMV Technologies, L'Aigle,Orne, FR) was employed to assess sperm membrane integrity. Briefly, semen was diluted to a concentration of 1 × 10^7 sperm/mL in PBS, with a final volume of 200 μL. It was then incubated with 1 μL of SYBR 14 (2 μM) and 1 μL of PI (240 μM) for 10 min at 37°C. After staining, the fluorescent signals were excited using a single blue laser (488 nm) and detected through green (525/30 nm), yellow (583/26 nm), and red (680/30 nm) channels. A total of 5,000 events were analyzed for each sample. PI-positive sperm (red) were considered as sperm with damaged membranes. Thus, sperm showing SYBR 14-positive (green) with PI-negative were classified to have intact membranes, and the results were then indicated as a percentage of sperm with intact membranes.

Apoptosis Apoptotic cells were detected using the Annexin V-binding technique, following the manufacturer's instructions (Annexin V Apoptosis Kit #NBP2-29373, Novus Biologicals, Centennial, CO). Briefly, 1.5 μL of semen was washed twice in 1 mL of cold PBS and centrifuged at 400 × g for 5 min to obtain sperm pellets. Recovered pellets were resuspended in 55 μL of staining buffer containing Annexin V-FITC and PI. After 20 min of incubation in the dark, the sperm suspension was supplemented with 200 μL of assay buffer and then analyzed by Guava easyCyte within 1 h. A total of 5,000 sperm cells from each sample were tested, and the results were presented as a percentage of apoptotic sperm cells.

Mitochondria Activity A fluorescent probe, JC-1 (CAS NO.: 3520-43-2, Millipore Sigma, Burlington, MA), was used to evaluate mitochondria membrane potential (**MMP**). Semen was diluted to 1 × 10^7 sperm/mL in PBS to a final volume of 200 μL and then incubated with 2 μL of JC-1 dye (100 μg/mL) for 30 min at 37°C. A total of 5,000 sperm from each sample were analyzed using the Guava easyCyte. The results were expressed as the ratio of red to green fluorescence of JC-1.

SST Filling Test

The protocols for sample preparation were modified from a previous study (Lin et al., 2023b) and were

adapted for the use of frozen-thawed semen in this study. The modifications included adjusting the staining dose and time, which are described as follows.

Sperm Staining Procedure After thawing and glycerol removal as previously described, 10 μL of Hoechst-33342 (bisbenzimide 1 mg/mL, Millipore Sigma, Burlington, MA) were added to each sperm suspension (100 μL) and gently mixed on an orbital shaker at 4°C for 90 min before use.

Artificial Insemination A total of 12 hens, 3 for each glycerol removal treatment (including the control group), received intravaginal insemination with Hoechst-labeled thawed sperm twice at 24-h intervals, with a dose of 200×10^6 sperm cells per female.

Isolation of Uterovaginal Junction Villi and Observation of SST Hens were slaughtered 24 h after the second insemination, and reproductive tracts were isolated (Supplementary Data 1A). The villi (n = 5 per animal) containing SST distributed in the uterovaginal junction (**UVJ**) (Supplementary Data 1B and 1C) were randomly dissected from the underlying mucosa. Pieces of villi were fixed in a 4% paraformaldehyde solution at 37° C for 30 min and then mounted on microscope slides using Fluoromount-G medium (ThermoFisher). Images were acquired using a microscope slide scanner (Axio Scan.Z1, Zeiss) with a 20 × objective lens. Fluorescence imaging was performed using the X-Cite Illumination System. The emission Band Pass filters used were EM BP 445/50 (DAPI) for Hoechst-33342 labeled sperm and EM BP 690/50 (Alexa Fluor 633) for SST autofluorescence. The presence of sperm-filled SST and sperm-empty SST in each UVJ villus was identified manually using QuPath image analysis software and the percentage of SST filled with stained sperm was determined.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 9.0.2. A chi-square test was initially employed to examine the association between the glycerol removal methods (4°C-SP, 20°C-SP, and SD) and fertility (the number of fertile and infertile eggs). Subsequently, a Fisher's exact test was applied to determine the significance of the differences between each individual experimental condition. A Kruskal-Wallis test was conducted to investigate the effects of the glycerol removal protocol on various parameters, including sperm motility, membrane integrity, apoptosis, mitochondria activity, and sperm SST filling experiments. When the Kruskal-Wallis test showed statistical significance, a Dunn's multiple comparisons test was used to analyze the differences in means among the groups. However, the results regarding glycerol concentration, measured in mM, showed a very large range of data between the control and the treatments. Under this condition, smaller values can be overwhelmed by larger values. To make the visualization clearer, it is necessary to take the logarithmic transformation of each variable (Metcalf and Casey, 2016; Pyle et al., 2016). After transforming the glycerol concentrations into logarithmic values, an ANOVA test was conducted to investigate the effects of the glycerol removal protocol. This was followed by a Tukey's multiple range comparisons post-test to examine the differences between the groups.

RESULTS

Different Fertility Achieved Among Glycerol Removal Methods

A significant impact of the glycerol removal method was observed on both early and late fertility (Figure 1). Insemination of 4°C-SP centrifuged sperm resulted in 67.4% early fertility (Figure 1A), which was significantly higher than that of 20°C-SP (47.6%) and SD treated sperm (34.8%). The rate of late fertility (Figure 1B) was 27.5% for semen treated with 4°C-SP glycerol removal, which was significantly higher than the rates for semen treated with 20°C-SP (8.8%) and SD (10.1%).

SP Centrifugation Removed More Glycerol Than SD Method

The concentration of glycerol in thawed semen significantly decreased after all treatments aimed at removing glycerol (Figure 2). Despite both SP and SD centrifugation methods efficiently removing glycerol, SP centrifugation, both at 4°C and 20°C, showed a more pronounced effect in eliminating glycerol (removal rate = 96.1 and 96.8%, respectively) compared to the SD treatment, which decreased glycerol by 93.3% (Supplementary Data 2).

Glycerol Removal Enriched Sperm Progressive Motility but Not Other Parameters

Glycerol removal treatment had no impact on the percentage of motile sperm (Figure 3A). However, compared to the control group, SD centrifugation significantly increased the proportion of sperm with progressive movement, but no significant difference was observed between the treatments themselves (Figure 3B). A significant effect of glycerol removal treatment was observed on the percentage of sperm membrane integrity and apoptotic cells (Figures 3C and 3D). This effect was supported by the fact that SP centrifugation at 4°C decreased membrane integrity (Figure 3C), while SD centrifugation induced apoptosis (Figure 3D) compared to the control. However, no significant difference was found in these 2 parameters between the treatments for glycerol removal. No impact of glycerol removal treatment was observed on mitochondria activity (Figure 3E).

Glycerol Removal Increased Sperm Storage in Hen's Oviduct

While no sperm was observed in the SST after insemination of control semen, the thawed sperm

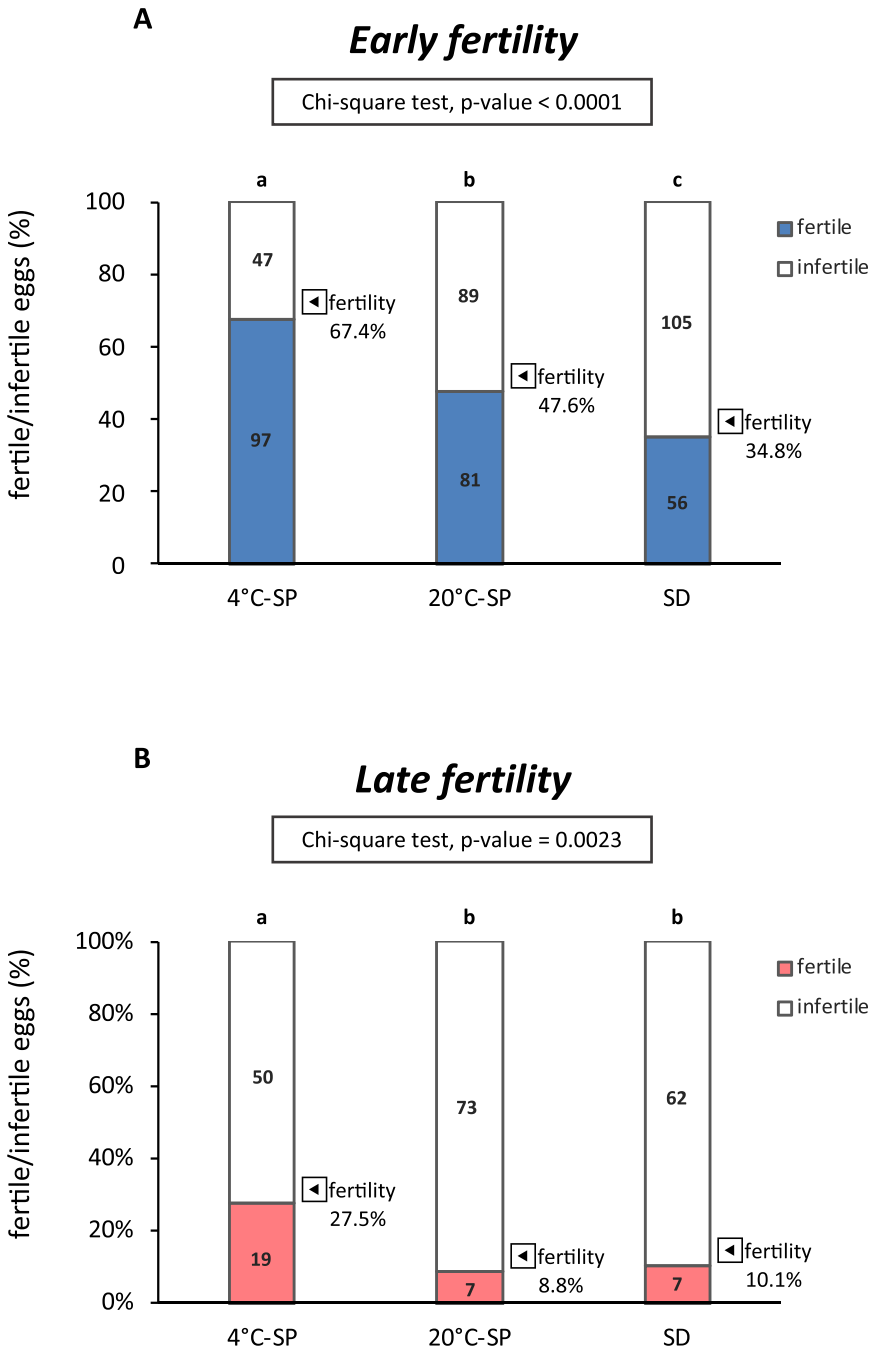

Figure 1. Fertility of frozen-thawed semen after glycerol removal treatment. (A) represents early fertility, which involves collecting eggs from the 2nd day after the first artificial insemination (AI) to the 6th day after the final one. (B) represents late fertility, the eggs collected between the 7th and 13th day after the last AI. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4°C or 20°C). SD: Protocol of serial dilution followed by centrifugation. The number of hens is 12 for each experimental condition. Deep color bars represent the percentage of fertility. The number of eggs being considered is indicated on the bars. The different letters show a significant difference between glycerol removal methods with P-value < 0.05.

reached the SST after the glycerol removal treatments (Figure 4). Semen treated with 4°C-SP centrifugation resulted in 50.1% of sperm-filled SST, which was significantly higher than the SD protocol (23.0%). However, no significant difference was found between the 4°C-SP and 20°C-SP treatments, or between the 20°C-SP and SD treatments (Figure 4A). Different UVJ villi exhibited varying capacities for sperm filling, particularly for the 20°C-SP treated sperm, which ranged from 0% to 65%.

DISCUSSION

Glycerol, a potent cryoprotectant used for freezing sperm, has been found to be linked to fertilization dysfunction in hens. As a result, it is necessary to remove glycerol before insemination (Long, 2006). Historically, glycerol was removed by SD centrifugation (Lake and Stewart, 1978; Seigneurin and Blesbois, 1995). Recently, we developed a new approach called SP centrifugation, which is based on a composition of colloidal gel, Percoll, and sucrose. This method

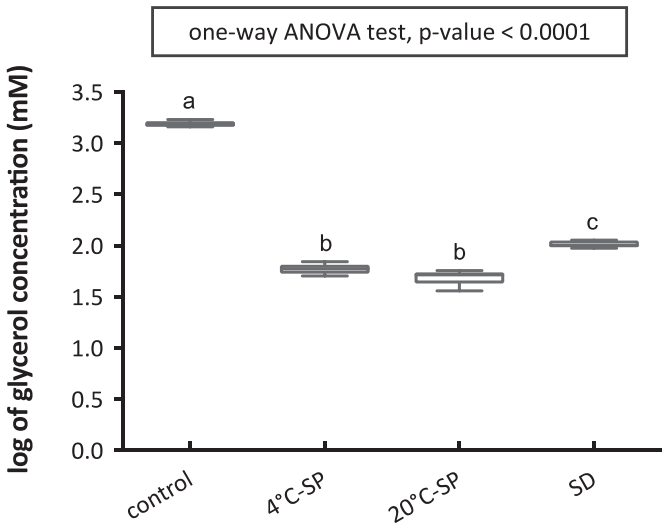

Figure 2. Logarithmic glycerol concentration after glycerol removal treatment. Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4°C or 20°C). SD: Protocol of serial dilution followed by centrifugation. The lines (—) in the diagram boxes represent the median values of observations. The boxes extend from the 25th to the 75th percentile of the observations. The upper and lower bars indicate the maximum and minimum values of observations (5 biological replicates). The different letters show a significant difference between glycerol removal methods with P-value < 0.05.

can be performed at both 4°C and 20°C. Our previous results revealed a better fertility with SP compared to the SD method (Lin et al., 2023a). However, no information was provided on how these different glycerol removal methods achieved varying levels of fertilizing capacity. Therefore, this study was conducted to understand how these protocols affect fertility and to identify the factors contributing to these differences.

Here, we divided the fertility observations into 2 stages: early (between 2nd and 6th days after AI) and late (between 7th and 13th days after AI) fertility. This division allows us to emphasize the effect of glycerol removal methods on fertility duration, including sperm interaction with the female genital tract − that is, storage in the SST, and resulting sperm fertilizing capacities. The 4°C-SP centrifuged sperm exhibited a higher fertility capacity than the 20°C-SP, both in early and late stages. This could be explained by the fact that keeping semen at a low temperature (4°C−5°C) reduces sperm metabolism and retains sperm fertilizing capacity (Clarke et al., 1982; Giesen and Sexton, 1983; Sarkar, 2020). However, sperm treated with 20°C-SP exhibited higher early fertility but similar late fertility compared to the SD treated sperm. Thawed semen, after undergoing SP centrifugation, contained only approximately half the amount of glycerol remaining compared to the samples treated with SD. However, all concentrations are far lower than the reported threshold of 2% glycerol, which has been shown to affect chicken fertility (Polge, 1951; Neville et al., 1971). This difference in glycerol concentrations after SP or SD treatments did not affect sperm motility, membrane integrity, apoptosis, or mitochondrial activity. However, sperm treated with 4°C-SP exhibited a higher SST filling capacity when compared to semen treated with SD centrifugation. These observations suggest that the difference in fertility duration is not caused by variation in sperm in vitro quality, as measured in this study. Instead, it is likely influenced by the interaction between sperm and the female reproductive tract, including storage in SST. The identification of the molecules involved in sperm-SST binding and their fate during freezing, thawing, and glycerol removal will be necessary to decipher the differences in terms of SST filling capacity of sperm after different thawing treatments.

The sperm filling capacity of 20°C-SP treated sperm showed a much larger variation in different villi (from 0 to 65%) when compared to 4°C-SP sperm (from 36 to 62%). Indeed, maintaining rooster semen at low temperatures can reduce their metabolism and preserve their fertilizing capacity (Clarke et al., 1982; Sarkar, 2020). In addition, glycerol may exhibit stronger cytotoxicity to sperm cells at higher temperatures (Macías García et al., 2012; Lin et al., 2023b), which could cause more significant effects on the biological characteristics of sperm. Conducting the experiment at the molecular level may provide a solution for gaining a better understanding of the temperature effects of glycerol because we did not observe any differences in the in vitro quality parameters of sperm after glycerol removal treatments. At least at this moment, this information suggests that the optimal way to use SP centrifugation during field practice is to perform the protocol at 4°C. This is because the only requirement is a temperature-controlled centrifuge, which is simpler than the SD protocol. Nevertheless, if the context does not allow for the 4°C condition, the 20°C-SP treatment still achieves higher fertility than the conventional method of SD centrifugation.

A decrease in sperm membrane integrity was observed in all semen samples after the removal of glycerol, compared to the control. This suggests that centrifugation may cause membrane breakage (Marzano et al., 2020), which is a characteristic of necrotic cell death (Choi et al., 2019). Indeed, centrifugal force may impact sperm membrane integrity (Varisli et al., 2009), however, our objective is to recover a sufficient amount of sperm for insemination while preserving their fertilization ability. It is impossible to ignore the need for a significantly larger quantity of retrieved sperm compared to what is used for in vitro fertilization (Morrell et al., 2016; Hungerford et al., 2023). Therefore, the centrifugation step should aim to achieve a balance between ensuring optimal sperm recovery and minimizing damage to the sperm membranes. In addition to sperm membrane integrity, a significantly higher number of apoptotic sperm was observed in SD treated sperm compared to the control group, but this was not found in SP treated sperm. In this study, we utilized the Annexin-V binding method to detect sperm apoptosis by assessing the externalization of phosphatidylserine on the surface of sperm (Hichri et al., 2018). This externalization is an early sign of cell damage (Muratori et al., 2004) and also indicates the occurrence of sperm capacitation (Juan-Manuel et al., 2017). This

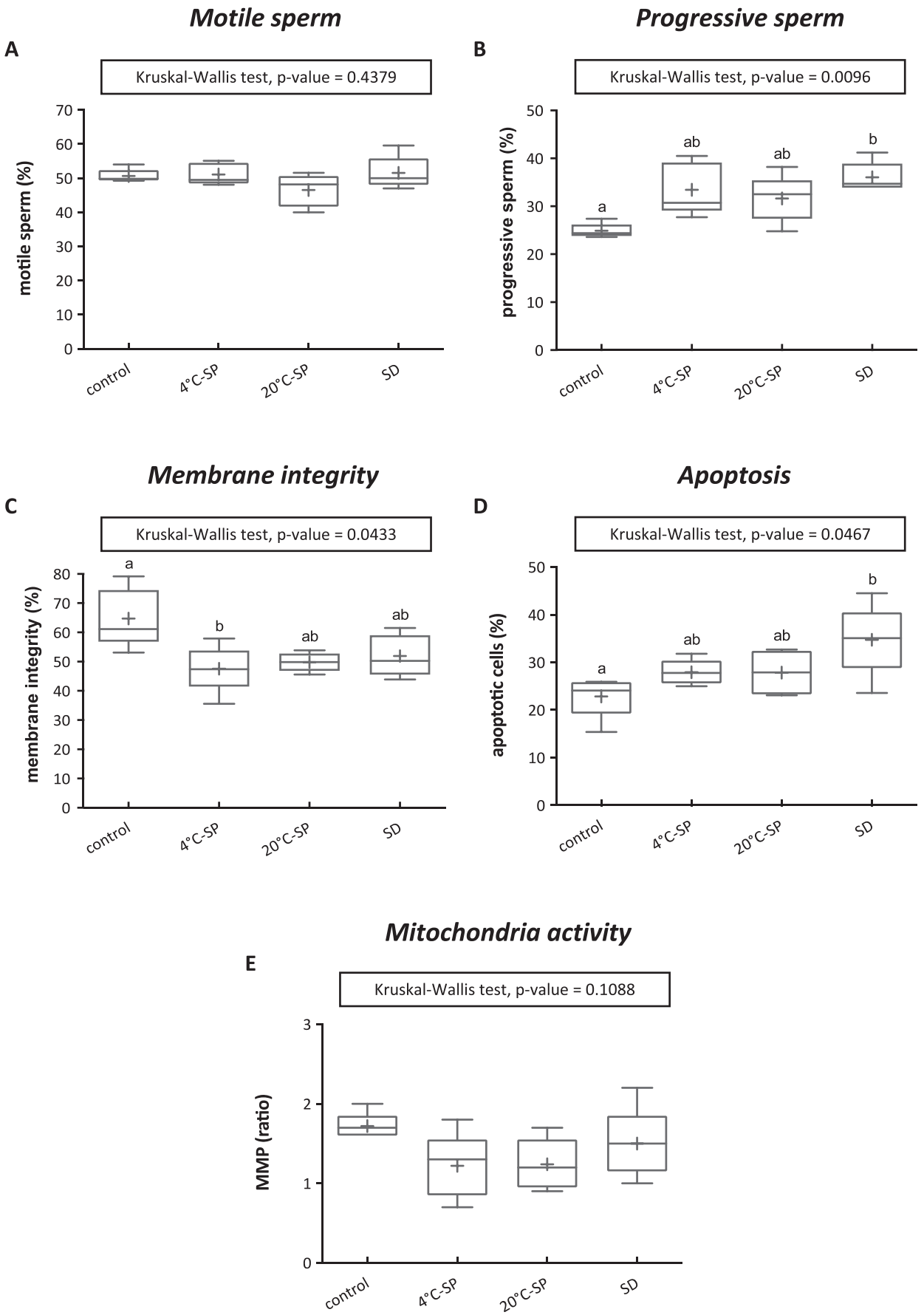

Figure 3. Sperm in vitro quality parameters after glycerol removal treatment. (A–E) represent the percentage of motile sperm (A), progressive sperm (B), membrane integrity (C), apoptosis (D) and mitochondria membrane potential (MMP) (E). Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4°C or 20°C). SD: Protocol of serial dilution followed by centrifugation. The lines (—) and crosses (+) in the diagram boxes represent the median and mean values of observations. The boxes extend from the 25th to the 75th percentile of the observations. The upper and lower bars indicate the maximum and minimum values of observations (5 biological replicates). The different letters show a significant difference between glycerol removal methods with P-value < 0.05.

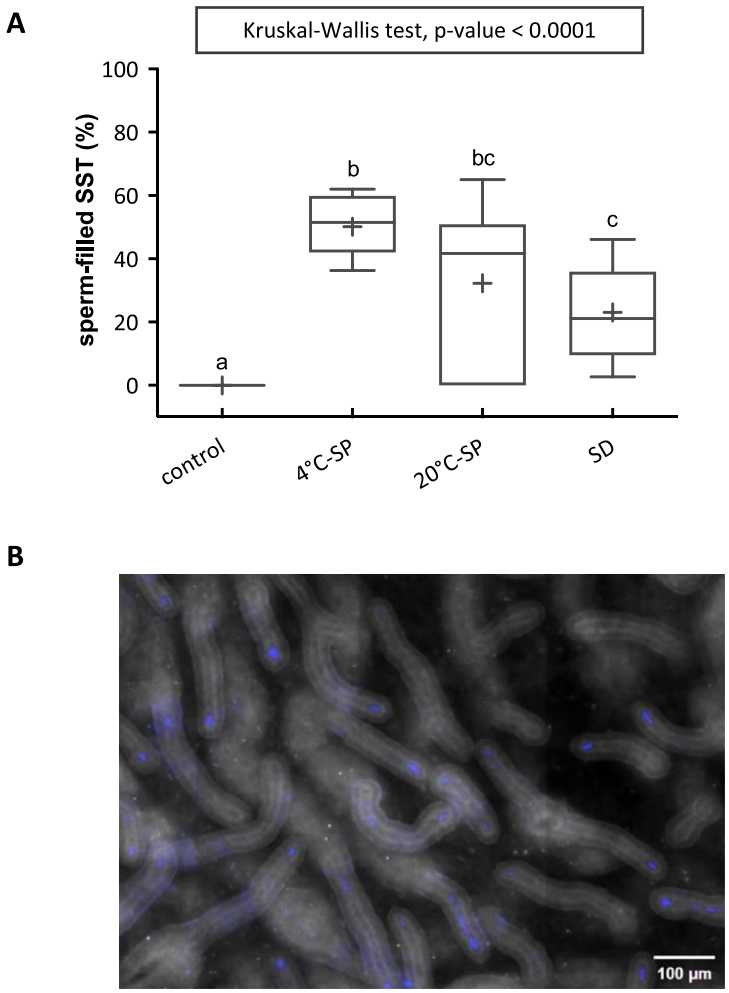

Figure 4. The presence of sperm in sperm storage tubules (SST) after insemination of frozen-thawed semen. (A) and (B) represent the percentage of SST filled with sperm (A) and sperm deposition patterns (B). Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4°C or 20°C). SD: Protocol of serial dilution followed by centrifugation. Data collected from 5 villi of each treatment. The lines (—) and crosses (+) in the diagram boxes represent the median and mean values of observations. The boxes extend from the 25th to the 75th percentile of the observations. The upper and lower bars indicate the maximum and minimum values of observations (3 biological replicates). The different letters show a significant difference between glycerol removal methods with P-value < 0.05.

may subsequently lead to the disruption of sperm storage and the fertilization process (Thélie et al., 2019b) in the oviduct, resulting in decreased fertility. All of these observations might inspire advancements in the design of the glycerol removal protocol.

In conclusion, we have demonstrated that the SP method leads to an increased fertilizing potential of glycerol-cryopreserved sperm compared to the standard SD protocol, due to improved fertility rates and longer sperm storage in SST, with the simpler methodology. This could be attributed to the more efficient removal of glycerol and the greater capacity of SP centrifugation to preserve sperm storage in the hen's reproductive tract. However, it is not linked to sperm functional parameters. To further investigate the influences of glycerol removal methods, additional molecular and biochemical analysis, such as proteomic approaches and oxidative damage (Soler et al., 2016; Rui et al., 2017; Vitorino Carvalho et al., 2021), would be helpful to identify molecular pathways impacted by the different post-thawing treatments, and for future improvements.

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DISCLOSURES

The authors declare that there are no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103448.

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