Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model

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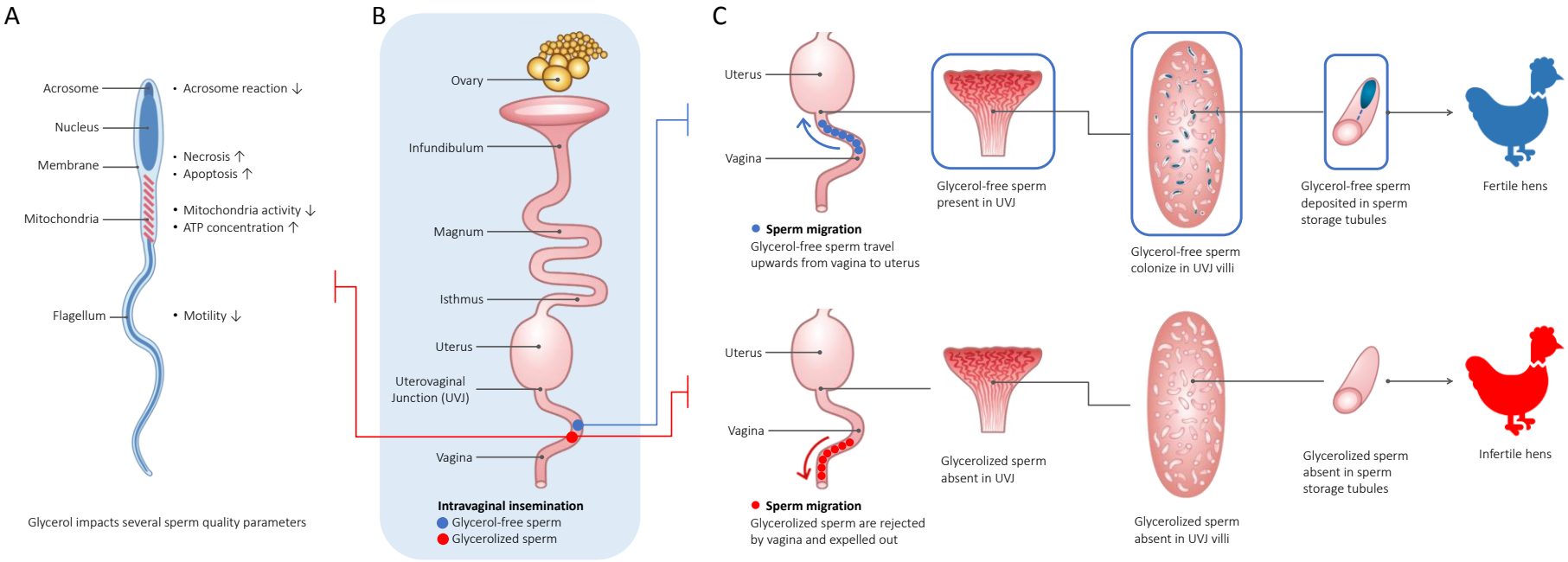
Highlights

1. Glycerol provides excellent cryoprotection and shows cytotoxicity to sperm cells.
2. In hens, 2% or 6% glycerol added into semen induce partial or total infertility.
3. Glycerol affects sperm migration and storage in the oviduct.
4. Glycerol causes more negative modifications of sperm biology at 41°C than at 4°C.
5. Removing glycerol prior to insemination is the most valuable solution so far to avoid fertility reduction.

Graphical Abstract



Proposed model of glycerol impacts on sperm fertilizing capacity in chickens. (A) indicates glycerol impacts on sperm biology: increasing cell deaths and ATP contents, and decreasing acrosomal reaction, motility and mitochondria activity. (B) exhibits the intravaginal insemination of glycerol-free or glycerolized sperm in hen's oviduct. (C) illustrates glycerol-free sperm deposited in sperm storage tubules of uterovaginal junction, leading hens to produce fertile eggs. While glycerolized sperm are rejected and expelled by vagina, leading sperm absence in sperm storage tubules and infertile hens.

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ABSTRACT

Glycerol is a cryoprotectant used worldwide for sperm cryopreservation in animals but it is associated with decreasing fertility. The mechanism underlying glycerol effects remains unclear, thus here we aimed to better understand using the chicken model. First, we checked the effect of increasing glycerol concentration at insemination on hen fertility, showing that 2 and 6% glycerol induced partial and total infertility, respectively. Subsequently, we examined sperm storage tubules (SST) colonizing ability during *in vivo* insemination and *in vitro* incubation of Hoechst stained sperm containing 0, 2 or 6% glycerol. Furthermore, we conducted perivitelline membrane lysis tests and investigated sperm motility, mitochondrial function, ATP concentration, membrane integrity and apoptosis, after 60 min of incubation with different glycerol concentrations (0, 1, 2, 6 and 11%), at two temperatures to mimic pre-freezing (4°C) and post-insemination (41°C) conditions. Whereas 2% glycerol significantly reduced 50% of SST containing sperm, 6% glycerol totally inhibited SST colonization *in vivo*. On the other hand, *in vitro* incubation of sperm with SST revealed no effect of 2% glycerol, and 6% glycerol showed only a 17% reduction of sperm filled SST. Moreover, glycerol reduced sperm-egg penetration rates as well as affected sperm motility, bioenergetic metabolism and cell death at 4°C when its concentration exceeded 6% and caused greater damages at 41°C, especially decreasing sperm motility. These data altogether reveal important effects of glycerol on sperm biology, sperm migration, SST colonization, and oocyte penetration, suggesting at least a part of fertility reduction by glycerol and open ways for improving sperm cryopreservation.

Key words: Cryoprotectant; Fertility; Glycerol; Sperm storage tubules (SST); Toxicity

1. Introduction

Sperm cryopreservation is a practical strategy to struggle the biodiversity crisis of wild animals (Bolton et al., 2022), to exchange superior genotypes in domestic animals (Bebbere and Succu, 2022), and to preserve fertility in humans (Tao et al., 2020). The cryopreservation involves successive steps: semen collection, cryoprotectant addition, cellular dehydration, freezing, storage in liquid nitrogen, and thawing prior to sperm use (Ugur et al., 2019). Cryoprotectants are chemical compounds that protect cells from cryodamages. Among them, glycerol, an intracellular cryoprotectant, is the most commonly used one for sperm cells in most species (Villaverde et al., 2013; Papa et al., 2015). However, it is associated with undesirable cytotoxic effects on sperm (Holt, 2000; Morrier et al., 2002), affecting sperm biological characteristics and fertility (Fahy, 2010; Best, 2015) in various species including humans (McLaughlin et al., 1992), mice (Katkov et al., 1998), donkeys, horses (Vidament et al., 2009), pigs (Gutiérrez-Pérez et al., 2009), sheep (Abdelhakeam et al., 1991) and marsupials (Taggart et al., 1996).

To avoid its cytotoxic effects, many studies have proposed glycerol alternatives during cryopreservation process (Yildiz et al., 2007; Silva et al., 2012), but still lacking encouraging fertilization success (Abouelezz et al., 2015, 2017; Gloria et al., 2019) or fail to fit safety requirements and clear identification for cryobanking (Tselutin et al., 1999). Another strategy is related to glycerol removal before insemination (Seigneurin and Blesbois, 1995; Purdy et al., 2009). While these methods can partly alleviate its negative effects, little is known about the mechanisms involved in glycerol toxicity, directly on sperm biology and/or through modifications of the female genital tract.

In chickens, glycerol present in semen may dramatically induce total infertility with only half of the concentration used for cryopreservation (Polge, 1951; Neville et al., 1971).

During the production and use of chicken cryopreserved semen, sperm face different temperatures: 4°C during the glycerol addition, equilibration, decreasing to -196°C during freezing and storage, 4°C again after thawing, and finally 41°C after insemination into the female reproductive tract (Lin et al., 2022). While this cryopreservation method is quite efficient, the combination of glycerol presence at 4°C and 41°C may have direct effects on sperm biology in addition to their journey inside the oviduct, glycerol may impair sperm migration and storage into the sperm storage tubules (SST), a specific well developed organ involved sperm selection, survival, and maintenance of fertilizing capacity, similar to the sperm reservoir of mammalian fallopian tubes (Camara Pirez et al., 2020; Mahé et al., 2021). The deficit of sperm storage in SST and its release to reach the oocyte in the oviduct may impair final fertility (Machado et al., 2019; Kölle, 2022). Furthermore, glycerol toxic effects may also reduce sperm ability to penetrate the coverage of the oocyte, known as the inner perivitelline membrane (IPVM) in birds, analogous to the mammalian zona pellucida (ZP) (Ichikawa et al., 2017), leading to a fertilization failure.

Consequently, using the avian model, here we aimed to decipher the effects of glycerol on sperm fertilizing ability, including direct effects on sperm biology as well as the interaction of sperm with female genital tract. We firstly determined the critical concentrations of glycerol in semen associated with chicken fertility reduction by insemination tests. Subsequently, we explored the sperm capacity to reach and be stored in the oviduct with both *in vivo* and *in vitro* SST colonization experiments. Then, we investigated sperm ability to penetrate perivitelline membrane (PVM) *in vitro*. Finally, we evaluated the direct effects of glycerol on sperm biology during pre-freezing (4°C) and post-insemination (41°C) temperature conditions, on sperm motility, mitochondrial activity, ATP generation, membrane integrity and apoptosis.

2. Materials and methods

2.1. 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 number: N° APAFIS#4026-2016021015509521 and APAFIS#34415-202112141205965). A total of 20 adult T44 roosters (Gallus gallus domesticus, SASSO, France) were randomly allocated to 2 groups for fertility tests and sperm biology parameters study (motility, mitochondrial activity, ATP concentration, viability, sperm-SST storage and PVM penetration). Two herds of 60 adult Lohmann hens were housed by groups of 4, one for fertility tests and the other for SST and PVM experiments. All animals were under a lighting regimen of 14h light:10h dark, controlled temperature at 20°C, feeding with a standard diet and water *ad libitum* at the INRAE Poultry Experimental unit (https://doi.org/10.15454/1.5572326250887292E12). Animals were between 35 and 85-week-old during this study.

2.2. Semen collection and processing

Rooster semen was collected in a tube containing 200 µL of Lake PC diluent (Lake and Ravie, 1981) by abdominal massage (Burrows and Quinn, 1937). Semen samples were then pooled and diluted to 2 000 × 10^6 cells/mL with Lake PC diluent at room temperature for the following treatments. The number of all experimental replicates are given in the figure legends.

2.3. Fertility tests

After collection, pooling and adjustment of glycerol concentration to 0, 1, 2, 6 and 11% (v/v), semen was immediately used for intravaginal insemination (4 cm depth), with a dose of 100×10^6 sperm/hen for 2 consecutive days (12 hens/experimental group). Eggs were collected from day 2-7 after first insemination and stored at 15°C/85% humidity before incubation at 37.7°C/55% humidity. Fertile and infertile eggs were determined by candling at the 7^{th} day of incubation (Long and Kulkarni, 2004). Repeated manipulation with exchanged group of hens used to confirm the previously observed results (Supplementary Data 1).

2.4. Sperm- SST test

2.4.1. Sperm staining

Sperm was labeled with the previous methodologies (McDaniel et al., 1997; King et al., 2002). After collection, pooling and dilution, 40 µg of Hoechst-33342 (bisbenzimide, Sigma-Aldrich) was added to 1 mL of diluted semen and gently mixed on an orbital shaker at 4°C for 4 h. The fertilizing ability of stained sperm is given in Supplementary Data 2, showing that Hoechst labeling did not affect fertility tendency.

2.4.2. *In vivo* insemination

Hoechst stained sperm were divided to 3 groups and added glycerol to a final concentration of 0 (control), 2 and 6% of glycerol. A total of 3 hens per group were inseminated twice at 24 h intervals with a dose of 200×10^6 sperm/female (King et al., 2002) then slaughtered 24 h after the second insemination and oviducts were isolated. The villi (*n* = 6 per animal) containing SST were separated randomly from uterovaginal mucosa (Cordeiro et al., 2021). Villi pieces were fixed in 4% paraformaldehyde solution at 37°C for 30 min then mounted in Fluoromount-G™ medium (ThermoFisher) on microscope slides. Images were acquired using a microscope slide scanner (Axio Scan.Z1, Zeiss) with an

objective lens 20X. Fluorescence imaging was performed with X-Cite Illumination System and emission Band Pass of EM BP 445/50 (DAPI) and EM BP 690/50 (Alexa Fluor 633) used respectively for Hoechst labeled sperm and SST autofluorescence. The presences of sperm-filled or sperm-empty SST were identified manually with QuPath image analysis software and the percentage of SST filled with stained sperm was calculated.

2.4.3. *In vitro* incubation

For this experiment, sperm staining and the uterovaginal villi collection were performed as previously described. Hoechst labeled sperm were washed in Lake PC diluent to remove extra staining solution and centrifuged at 600×*g* for 20 min at 4°C. Sperm pellets were resuspended and adjusted with Lake PC diluent to 2 000 × 10^6 cells/mL then mixed with glycerol to final concentration of 0 (control), 2 and 6% glycerol. For each treatment, 6 pieces of villi were placed individually in 1 mL of Advanced DMEM/F-12 medium (ThermoFisher, Gibco#12634-010) containing 0, 2 and 6% of glycerol in a 4-well culture dish. Each well was inseminated with 200 × 10^6 sperm and incubated at 39°C for 30 min in 5% CO_2 and 95% humidity. After incubation, villi pieces were washed in Dulbecco's phosphate-buffered saline (DPBS) to remove sperm adhering outside the SST, then fixed and analyzed as previously described.

2.5. Sperm-PVM perforation test

2.5.1. Isolation of perivitelline membrane (PVM)

PVM was isolated according to the method described previously (Steele et al., 1994; Bongalhardo et al., 2009). Yolk membrane was separated from freshly unfertilized chicken eggs and washed several times in DPBS to remove adherent yolk. Segments excluding

germinal disc area of PVM were cut in the size of 75 mm × 75 mm and placed in Advanced DMEM/F-12 medium before use.

2.5.2. Sperm glycerolized treatment

After collection and pooling, semen was diluted with glycerol-Lake PC diluent to 200 × 10^6 sperm cells/mL and final concentration of 0 (control), 1, 2, 6 and 11% glycerol then incubated at 4 or 41°C for 10 min.

2.5.3. *In vitro* incubation of PVM and glycerolized sperm

A piece of PVM placed in 1 mL of Advanced DMEM/F-12 medium was incubated with 10 × 10^6 sperm of each condition (0, 1, 2, 6 and 11%) in a 24-well culture dish at 41°C for 20 min. After incubation, PVM segments were washed in DPBS and fixed in 4% paraformaldehyde solution at 37°C for 1 min (Ichikawa et al., 2017). The PVM sections were then mounted on microscope slides, stained with Schiff's reagent (Sigma-Aldrich) and air-dried (Akhlaghi et al., 2014). The images of holes that formed in the PVM were captured by the microscope slide scanner with brightfield optics of an objective lens 20X. Three regions were randomly selected from each PVM and the number of holes was counted manually with QuPath software to calculate the number of holes/mm^2.

2.6. Evaluation of sperm quality parameters

After semen collection and dilution, glycerol was added to reach final concentration of 0 (control), 1, 2, 6 and 11% and then incubated at 4 or 41°C for 60 min.

2.6.1. Sperm motility

Sperm motility were examined with a computer-assisted sperm analysis system (CASA, IVOS, IMV Technologies) as previously described (Vitorino Carvalho et al., 2021). The evaluation was performed with a concentration of 30 × 10^6 sperm/mL. Based on several

motility parameters, i.e. average path velocity (VAP), straight line velocity (VSL) and straightness (STR = VSL/VAP), motility results were indicated as percentage of motile sperm and progressive sperm, which were defined as the percentage of sperm showing a VAP > 5 µm/sec and VAP > 50 µm/sec with STR > 75%, respectively.

2.6.2. Mitochondrial activity

Sperm mitochondrial parameter was evaluated with a fluorescent probe JC-1 (Sigma-Aldrich, CAS NO.: 3520-43-2), a green-fluorescent monomer at low membrane potential and forms red-fluorescent aggregates at higher potential (Gliozzi et al., 2017). Semen was diluted to 1×10^7 sperm/mL in PBS to final volume of 200 µL and then incubated with 1 µg/mL of JC-1 dye at 37°C for 30 min. A total of 5 000 events of each sample were analyzed by Guava® easyCyte (IMV Technologies). The results were expressed as the percentage of sperm with mitochondrial membrane depolarization.

2.6.3. ATP concentration

A luciferase reaction assay (CellTiter-Glo® Luminescent Assay Kit, Promega#G7570) was used to measure sperm ATP concentration here (Nguyen et al., 2015). After incubation with glycerol, semen samples were immediately centrifuged at 4°C, 800×*g* for 10 min, supernatant was removed and the pellets were frozen at -20°C. Before ATP assay, sperm pellets were thawed and resuspended in 100 µL PBS in the 96-well white polystyrene microplate. After 30 min of equilibration at room temperature, 100 µL luciferin/luciferase reagent was added to each well and plates were placed on an orbital shaker for 2 min to induce cell lysis then waited for 10 min to stabilize the luminescent signal. The luminescence was recorded with the luminometer plate reader (CLARIOstar, BMG LABTECH) and transformed to ATP concentration based on a standard curve.

2.6.4. Membrane breakage

This parameter was explored by SYBR 14/Propidium iodide (PI) double fluorescent staining technique (Molecular Probes, Invitrogen #L7011) combined with a flow cytometer Guava® easyCyte (Gliozzi et al., 2017). PI positive sperm (red fluorescence) were recognized as membrane damaged sperm and SYBR 14 ones (green fluorescence) were considered as intact sperm. The results were expressed as percentage of sperm showing membrane breakage.

2.6.5. Apoptosis

Annexin V-binding technique (Hoogendijk et al., 2009) was used to determine sperm apoptotic cells according to the manufacturer's instructions (Novus Biologicals™#NBP2-29373). Briefly, 1.5 µL semen was washed in 1 mL of PBS twice. Sperm pellets were obtained by centrifuge at 400×*g* for 5 min and resuspended in 55 µL of Annexin V-FITC and PI mix staining buffer. After 20 min of incubation in the dark, 200 µL of assay buffer were added and samples were analyzed by Guava® easyCyte within 1 h.

2.7. Statistical Analysis

Statistical analyses were performed with GraphPad Prism version 6.07. The impact of glycerol concentration on fertility was analyzed by Chi-square test and Fisher's exact test. Percentage of SST filled with sperm were analyzed by Kruskal-Wallis and Dunn's multiple comparisons tests to evaluate the effect of glycerol concentration. Significant differences were considered when p-value was under 5%. Sperm-PVM penetration data were normally distributed and analyzed by two-way ANOVA test to examine the effects of glycerol concentration and exposure temperature. All sperm quality parameters results were analyzed by two-way ANOVA test to examine the effects of glycerol concentration and exposure time. Since exposure time had no effect on all sperm quality parameters at 4°C

(Table 1), data from different timepoints were pooled in a mixed model to highlight the effects of glycerol concentration. Differences in the means were analyzed by Tukey's HSD Post Hoc test.

3. Results

3.1. Impact on animal fertility

Whereas 1% glycerol had no effect on the number of fertilized eggs (Figure 1), the presence of 2% glycerol significantly reduced the fertility by about 50% while 6% and 11% of glycerol resulted in total infertility. Same effect was reproduced in the second replicate test (Supplementary Data 1).

3.2. Impact on sperm presence in SST

The concentrations of 0, 2 and 6% glycerol were chosen in SST experiments based on the fertility results. Indeed, the presence of 2 and 6% glycerol in semen led to a partial and total loss of fertility, respectively, compared to 0%. We firstly confirmed that this effect was the same after Hoechst staining protocol (Supplementary Data 2). Subsequently, the capacity of sperm to reach and be stored in SST was investigated by inseminating Hoechst stained sperm then SST dissection (Figure 2A). The percentage of sperm filled SST significantly decreased as the glycerol concentration increased (Figure 2B): 2% and 6% glycerolized sperm dramatically reduced the percentage of SST containing sperm respectively by 50% and by nearly 100%.

To focus our work on the SST capacity to host sperm (avoiding possible motility effects preventing sperm to reach SST), same approach was applied with *in vitro* incubation of uterovaginal villi and glycerolized sperm (Figure 3A). No effect was observed with 2%

glycerol (Figure 3B), while 6% glycerol significantly decreased 17% the proportion of SST colonized by stained sperm.

3.3. Effect on sperm-PVM penetration

In order to evaluate the effect on fertilizing capacity, sperm was incubated 10 min with different glycerol concentration at 4 and 41 °C and the number of holes generated in PVM by sperm hydrolysis (Figure 4A) was analyzed. Whereas no interaction between glycerol concentration and the temperature was detected, each variable independently affected the number of holes in PVM (Figure 4B), showing less holes with increasing glycerol concentrations. While a higher reduction of fertilizing ability was observed with the incubation at 41°C than at 4°C (Supplementary Data 3), no significant difference was found by pair-wise comparisons.

3.4. Effect on sperm biology with an incubation at 4°C

In order to collect new data relating to sperm biology affected at the specific stages during the pre-freezing procedure, several aspects of sperm quality parameters were evaluated including sperm motility, mitochondrial function, ATP concentration, membrane integrity and apoptosis in presence of various concentrations of glycerol within 1 hour of incubation at 4°C. At this temperature, no effect of exposure time and its interaction with glycerol concentration was observed on all evaluated parameters (Table 1). Consequently, we further combined all timepoints to highlight only the effects of glycerol concentration (Supplementary Data 4). When compared to 0% glycerol, no effect of 1 and 2% glycerol was observed on all sperm biological characteristics (Figure 5A-F), except that 2% glycerol significantly increased ATP concentration (Figure 5D). Furthermore, 6% and 11% glycerol

decreased sperm motility and increased the values of all other sperm parameters when compared to control condition. Moreover, 11% glycerol caused more pronounced negative influence than 6%, except on mitochondrial function (Figure 5).

3.5. Effect on sperm biology with an incubation at 41°C

We then explored the same sperm biology functions after glycerol exposure at 41°C, to mimic the impact of temperature after sperm insemination into female reproductive tract, within 1 hour (the physiological time necessary for sperm to reach the fertilization site in the oviduct). Based on our preliminary results, sperm motility traits were observed within 30 min of incubation time due to their fast alterations when exposed to glycerol at 41°C. The tendencies of percentage of motile and progressive sperm were very similar and were both significantly affected by glycerol concentration, exposure time and their interaction (Table 1). Indeed, whereas no impact of 1, 2 and 6% of glycerol was revealed at 0 min, the addition of 11% glycerol immediately dropped sperm motility (Figure 6). A similar negative effect was also induced by 6% glycerol after 10 min of exposure (Figure 6). In addition to the reduction of sperm motility classically induced by the incubation of chicken sperm at 41°C (decreasing pattern observed with control condition over time), at 20 and 30 min, all glycerol concentrations reduced drastically sperm motility in a dose dependent manner, leading to sperm nearly immobile with 11% of glycerol at 20 min.

Significant effects of glycerol concentration, exposure time and their interaction were observed on mitochondrial function (Table 1). Whereas at 0 min, no difference was observed in all glycerol concentrations, a significant increase of mitochondria depolarization was observed at 30 min in absence of glycerol when compared to 0 min. Interestingly, mitochondria depolarization was higher in 60 min than all other timepoints and it

significantly decreased with the increase of glycerol concertation (Figure 7A). Glycerol concentration, exposure time and their interaction also significantly affected energy metabolism as revealed by sperm ATP concentration evaluation (Table 1). Indeed, whereas no significant influence was observed for 1 and 2% glycerol conditions at all timepoints (Figure 7B), an important increase of ATP concentration was revealed with 11% of glycerol at 30 min as well as 6% of glycerol at 60 min, when compared to control, and to 1 and 2% glycerol conditions. At 60min, the ATP concentration observed with 11% was higher than the one obtained with 6% of glycerol.

In order to clarify the effect of glycerol on sperm cell survival, two parameters were evaluated: the presence of sperm membrane breakage and sperm apoptosis. Both parameters presented very closed patterns with a significant increase of their level due to glycerol concentration and exposure time, with no significant interaction (Table 1). At 0 min, no impact was observed on the membrane breakage rate whereas an increase in apoptotic cell percentage was found immediately after the addition of 11% glycerol. At 30 and 60 min, the rates of membrane breakage and apoptotic sperm cells significantly increased only for 6 and 11% when compared to the other glycerol concentrations as well as to these experimental conditions at 0 min (Figure 8).

4. Discussion

Whereas glycerol is one of the most widely used cryoprotectant to preserve sperm at very low temperature in animals, its presence is also associated to undesired negative effects on fertility (Macías García et al., 2012). In chickens, this negative influence could lead to total infertility depending on the glycerol concentration present in inseminated semen (Polge, 1951; Seigneurin and Blesbois, 1995). However, the cellular and molecular

mechanisms underlying the balance between maintaining sperm integrity and inducing toxic effects when glycerol acts as a cryoprotectant remain unclear.

Here, using insemination program with chicken semen combined with different glycerol concentrations, we demonstrated that the presence of 2% glycerol harmfully reduced animal fertility to half (91 to 43%) and that 6% glycerol induced total infertility (Figure 1 and Supplementary Data 1). These results are consistent with previous studies performed with different insemination programs and animal genotypes (Polge, 1951; Neville et al., 1971), suggesting that glycerol toxic effects appear regardless of animal genetic background, age or insemination protocols in this species. Whereas 7% glycerol has no effect on pregnancy rates in cattle (Papa et al., 2015), the addition of only 2.2% glycerol in donkey semen induces infertility (Vidament et al., 2009). Consequently, although a cytotoxic glycerol effect could be presumed for all animal species, some important variability of sensitivity can be observed. However, very little information is available concerning about the stage and target of this toxicity, i.e. direct impacts on sperm biology or/and effects on the events occurring inside the female tract.

Once semen is inseminated into hen vagina, a population of sperm first reaches the SST to be stored before continuing their journey to fertilize the oocyte in the oviduct. Our data revealed that semen containing 2% glycerol showed a distinguishable decreased ability to colonize the SST and that 6% glycerol led to sperm absence in SST (Figure 2). This diminution or absence of sperm in SST, already mentioned previously (Marquez and Ogasawara, 1977), is certainly one of the major causes of fertility reduction induced by the glycerol presence (Supplementary Data 2). However, this experiment did not discriminate that sperm absence in SST was due to the failure of sperm migration (from vagina to SST) or of entering and binding into SST. To answer this question, the *in vitro* experiment was

designed to exclude the defect of sperm migration from vagina to UVJ to see if glycerolized sperm can be found in SST if they are deposited close from SST. Different from *in vivo* experiment, sperm were present in SST after *in vitro* incubation of sperm with UVJ villi. Intriguingly found that no impact of 2% glycerol on the rate of sperm filling in SST (Figure 3) and just a partial reduction of 17% with 6% glycerol, a relatively slight loss when compared to *in vivo* result (100% reduction), suggesting that small amount of glycerol impairs sperm travel from vagina to SST *in vivo*, and that higher quantity of glycerol also inhibits sperm depositing into SST. These negative effects may be due to motility defect and/or sperm rejected by vagina selection (Brillard, 1993; Bakst et al., 1994) as well as another barrier at the SST level acting as a gatekeeper (Brady et al., 2022) to exclude glycerolized sperm.

After 30 min *in vitro* incubation of sperm with UVJ villi, we found that 6% glycerol did not impair sperm motility (Supplementary Data 5), showing a completely different result from another sperm motility observation. In that experiment, sperm were incubated with 6% glycerol without UVJ villi, leading sperm to lose almost their entire motility within 30 min (Figure 6). This finding may suggest that UVJ tissues provide a positive effect on motility maintenance, which has been proposed in a previous study (Spreen et al., 1990).

In addition to affect sperm transport and storage, glycerol may also directly influence sperm fertilizing ability including its potentiality to release proteolytic enzymes to hydrolyze the perivitelline membrane (PVM), a crucial step to penetrate and fertilized the oocyte (Lemoine et al., 2011; Priyadarshana et al., 2020). The higher the glycerol concentration was, the lesser the sperm hydrolysis activity was (Figure 4), proving that glycerol impairs chicken sperm fertilizing capacity and, this effect was more pronounced at 41°C than at 4°C (Supplementary Data 3), suggesting a synergic effect between temperature and glycerol concentration. To our best knowledge, these data are the first revealing glycerol effects

directly on sperm-egg penetration in birds. However, a different observation was reported in rams, indicating that glycerol accelerates egg penetration and polyspermy (Slavik, 1987), suggesting a species specific effect remaining to be confirmed.

The sperm processing for freezing, thawing and insemination involves mainly two temperatures: low temperature (4°C) used during sperm preparation and physiological body temperature (41°C) after insemination into the female tract. These temperatures were considered here to decipher the direct impacts of glycerol on sperm biology to better understand which stages of the process the sperm are affected. At 4°C, despite the presence of less than 2% of glycerol had no effect on sperm biology within 60 min, more than 6% of glycerol significant decreased sperm motility and viability, and increased mitochondrial activity and ATP concentration (Figure 5), revealing that the modification for current freezing protocol might be useful to reduce undesired glycerol effects since the most used glycerol concentration for chicken sperm cryopreservation is more than 8% (Mocé et al., 2010; Zong et al., 2022). Decreasing the amount of glycerol to less than 2% may be a possible solution by replacing its requirement with other cryoprotectants. However, cytotoxic effects of other cryoprotectants, such as DMA and DMSO, cannot be ignored (Best, 2015; Mosca et al., 2019). An option would be to mix several cryoprotectants to have a sufficient cryoprotection while maintaining each individual under its own toxicity threshold. Designing a satisfactory and harmless sperm cryopreserved method remains an endeavor and a challenge even after 70 years of development. Another finding in our study was the absence of effect of exposure time on sperm biology at 4°C within 1 h (Table 1 and Figure 5). This information could be very useful to add more flexibility to insemination programs, by keeping semen at 4°C maximum 1 hour in farm environment. However, *in vivo* data on animal fertility are still needed to verify this potential procedure modifications.

At 41°C (physiological body temperature), the situation is dramatically different. Indeed, sperm motility was rapidly decreased by glycerol in a concentration dependent way, leading to a strong motility reduction more than 90% within 20 min in the presence of 11% glycerol (Figure 6). Consequently, this severe impairment of sperm motility could disturb normal sperm progression to reach SST or the fertilizing site in the oviduct since this whole journey takes only 1 hour *in vivo* (Bakst et al., 1994). Short-term exposure of glycerol at room temperature in 20 min also led to a significant reduction of sperm motility in humans (McLaughlin et al., 1992) and mice (Katkov et al., 1998), suggesting that it's not a chicken specific problem. At this temperature, higher concentration of glycerol (6 and 11%) also increased sperm membrane breakage, a typical feature of necrosis, and apoptosis as evidenced by Annexin V test (Figure 8). Whereas similar results were observed for membrane breakage of stallion sperm under 5% glycerol condition, no effect was observed on apoptotic rate (Macías García et al., 2012), suggesting a different sensitivity of glycerol effects on sperm cell death patterns depending on the species. Alternatively, modifying a glycerol freezing diluent for chicken sperm by adding apoptotic regulators such as agonist/antagonist of death receptor signaling or caspase inhibitors (Fischer and Schulze-Osthoff, 2005) might be a possible way to limit glycerol cell death induction. Furthermore, this increase of sperm death may also be one of the causes of the reduction of sperm presence in SST. Since SST are known to select and maintain higher quality sperm, sperm presenting death signals may be rejected (Brillard, 1993; Blesbois and Brillard, 2007).

Lastly, glycerol changes chicken sperm energy metabolism with a dramatic increase of ATP concentration and a lower mitochondrial depolarization (Figure 7), corresponding to previous studies in stallions (Macías García et al., 2012) and boars (Malcervelli et al., 2020). During ATP synthesis via mitochondrial respiration, the displacement of protons generates a

voltage gradient to polarize the membrane with positive charge outside, defined as mitochondrial membrane potential (Wang et al., 2022). Thus mitochondria depolarization, refers to sperm with low mitochondrial membrane potential, generally associates with ATP deficiency and lower sperm motility (Alamo et al., 2020). Here sperm incubated at 41°C for 30 min showed no difference of mitochondria depolarization in each glycerol condition (Figure 7A), theoretically generating a comparable ATP content in each of them (Pandey et al., 2021). However, glycerol concentrations of 6 and 11% led to a 25 and 60-fold higher ATP concentrations than the other conditions (Figure 7B), raising the hypothesis that excessive ATP is synthesized via glycolysis or pentose phosphate pathways (Setiawan et al., 2020). However, further studies will be needed to determine direct evidence of the activity of these pathways.

Dynein, a specific ATPase uses energy from ATP hydrolysis and distributes it along the axonemal microtubules, the central cytoskeletal structures of sperm flagellum, to generate sperm movement (Alamo et al., 2020). Theoretically, sperm with higher ATP contents show higher motility. However, here we observed that at 30 min ATP accumulation in sperm under 6 and 11% glycerol conditions (Figure 7B) contrastingly decreased their motility performance (Figure 6). This finding raises another hypothesis that glycerol may disrupt dynein enzymatic activity or flagellar axonemal structure, leading to the accumulation of unused ATP.

5. Conclusion

To conclude, we used the chicken model to demonstrate that glycerol affects severely sperm capacity to interact with female reproductive tract, disturbing sperm migration and storage and reducing sperm ability to penetrate the ovum and achieve fertilization in the oviduct. We also proved that these effects occur mainly at the physiological body

temperature, suggesting a good sperm capacities preservation by the glycerol during cryopreservation/thawing process. Consequently, although understanding the entire mechanisms of glycerol toxicity remains a challenge, it is clear that removing glycerol prior to insemination is a valuable solution to avoid fertility reduction in chickens and should be also considered in mammalian species.

CRediT authorship contribution statement

Hsiu-Lien Herbie Lin: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing - original draft. **Pascal Mermillod:** Conceptualization, Writing - original draft, Visualization, Supervision. **Isabelle Grasseau:** Methodology, Investigation, Data curation. **Jean-Pierre Brillard:** Methodology, Writing - review & editing. **Nadine Gérard:** Methodology, Writing - review & editing. **Karine Reynaud:** Methodology, Writing - review & editing. **Lih-Ren Chen:** Writing - review & editing. **Elisabeth Blesbois:** Conceptualization, Supervision. **Anaïs Vitorino Carvalho:** Conceptualization, Formal analysis, Writing - original draft, Visualization, Supervision.

Declaration of interest

The authors declare that there are no conflicts of interest.

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Figures

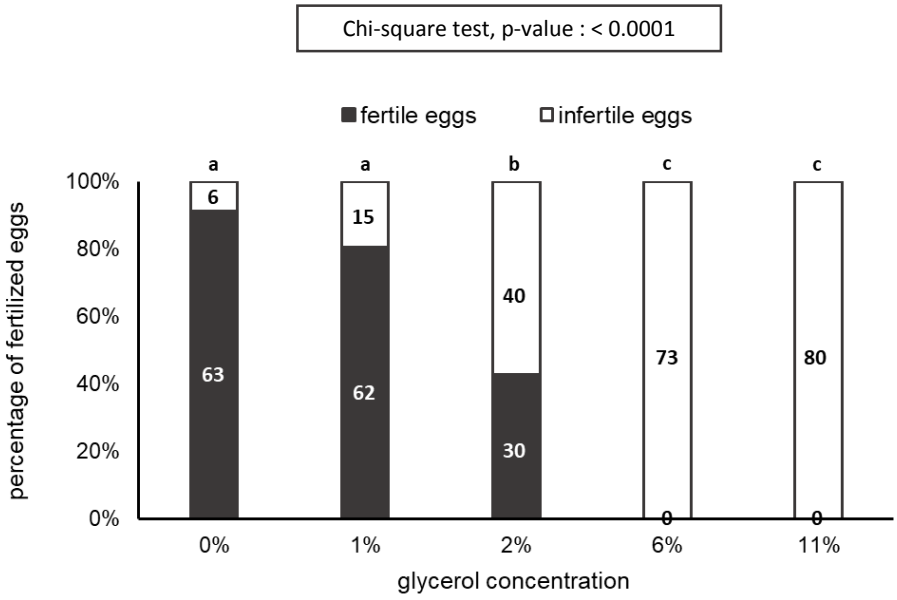
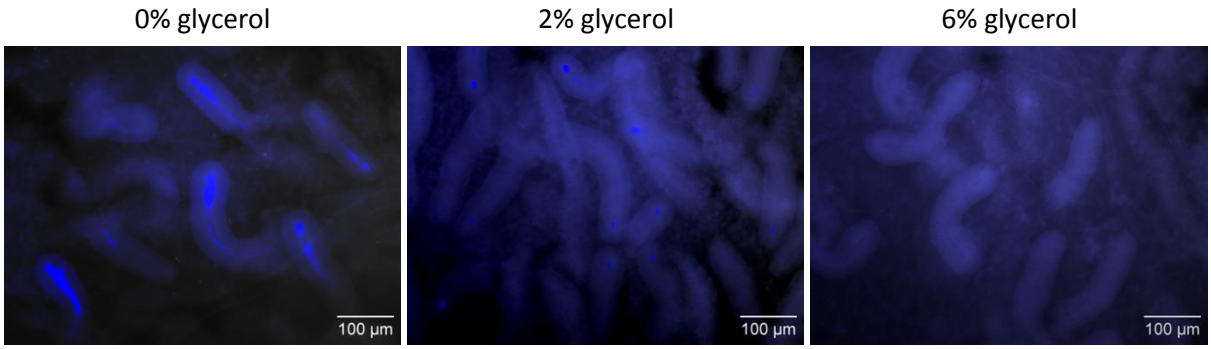


Figure 1. Effect of glycerol presence in semen samples on sperm fertility capacity. Semen was collected and pooled from 10 roosters. Number of hens=12 for each experimental condition. Eggs were collected between 2nd and 7th day after first insemination in a dose of 100 × 10^{6} sperm per hen for 2 consecutive days. Black and white bars represent the percentage of fertile and infertile eggs. The numbers of considered eggs are indicated on the bars. Different letters indicate significant differences (p-value < 0.05).

A



B

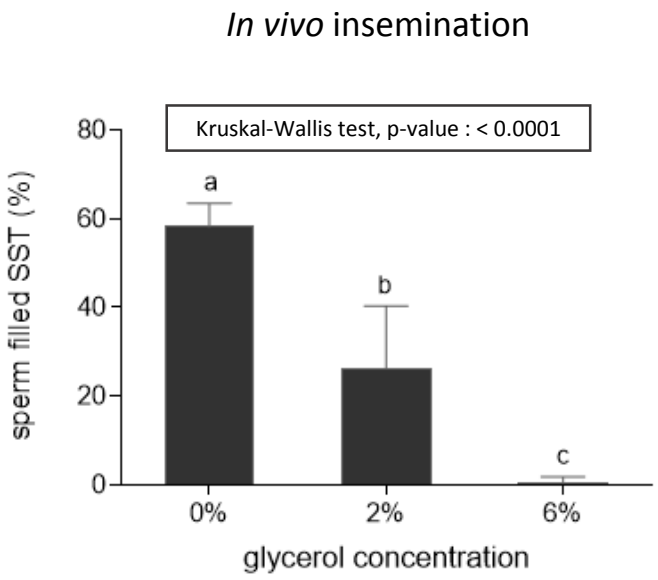
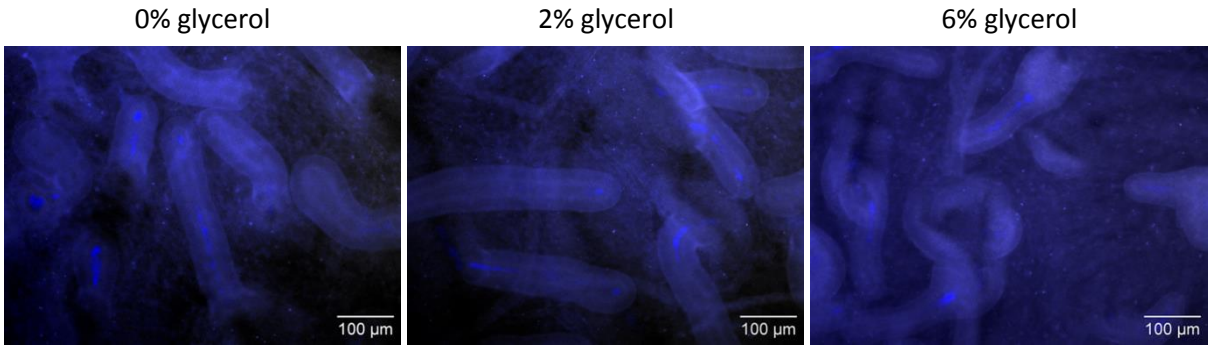


Figure 2. The presence of sperm in sperm storage tubules (SST) after *in vivo* insemination. A and B represent sperm identification (A) and the percentage of SST containing sperm (B) after *in vivo* insemination with 0, 2 and 6% glycerolized semen. Sperm stained with Hoechst 33342 at 4°C for 4 h then mixed with glycerol just before insemination. SST were detected by autofluorescence. Data collected from 6 villi of each treatment. The bars and lines correspond to the mean (3 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

A



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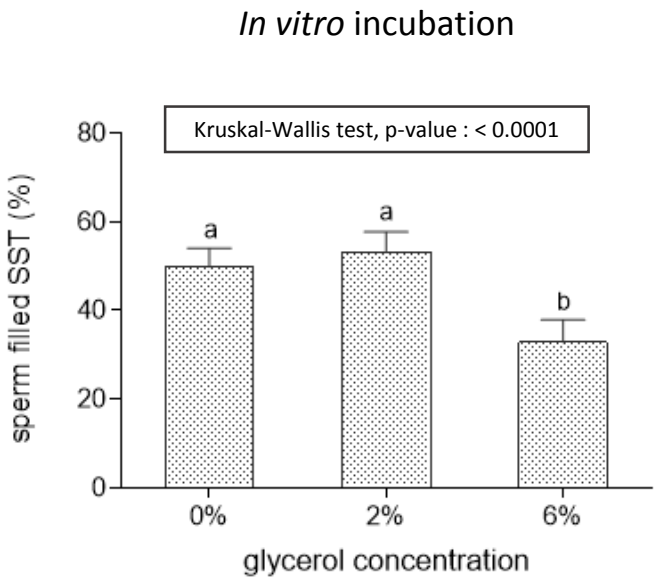
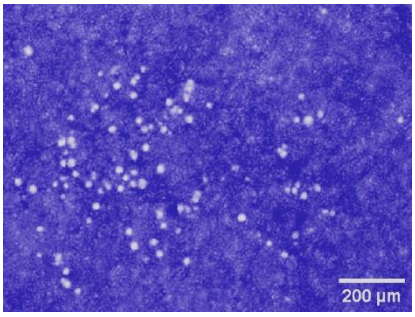


Figure 3. The presence of sperm in sperm storage tubules (SST) after *in vitro* incubation. A and B represent sperm identification (A) and the percentage of SST containing sperm (B) after *in vitro* incubation of sperm and uterovaginal villi in 0, 2 and 6% glycerol medium. Sperm stained with Hoechst 33342 at 4°C for 4 h and washed in Lake PC diluent then mixed with glycerol just before incubation. SST were detected by autofluorescence. Data collected from 3 villi of each treatment. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

A



B

two-way ANOVA test (p-value)			
	GLY	temperature	GLY × temperature
number of holes in PVM	**0.0044**	**0.0003**	0.6720

Abbreviation: GLY=glycerol concentration.

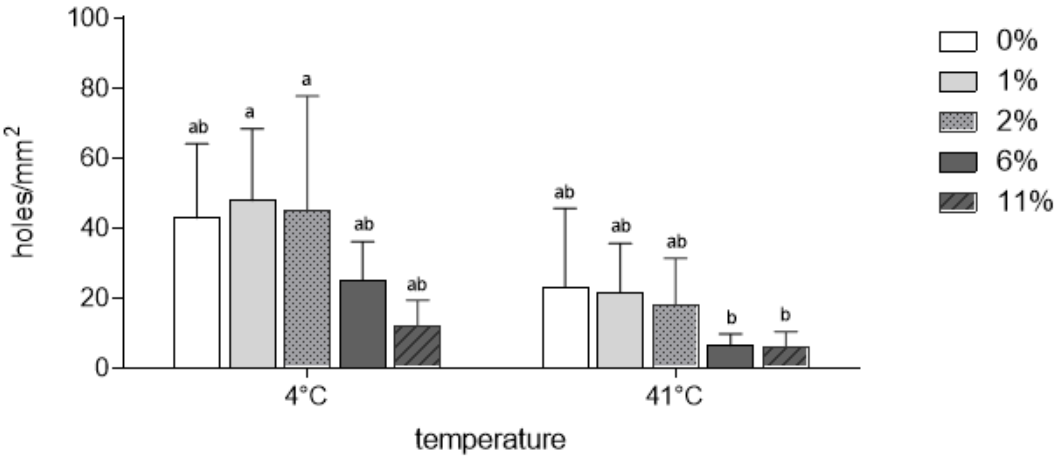


Figure 4. Sperm penetration in perivitelline membrane (PVM) after pre-incubation with different glycerol concentrations. A and B represent the image (A) and the number of holes (B) after PVM incubated with glycerolized sperm. Semen samples were collected and pooled from 10 roosters and subjected to 0, 1, 2, 6 and 11% glycerol at 4 or 41°C for 10 min before incubation with PVM (41°C for 20 min). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

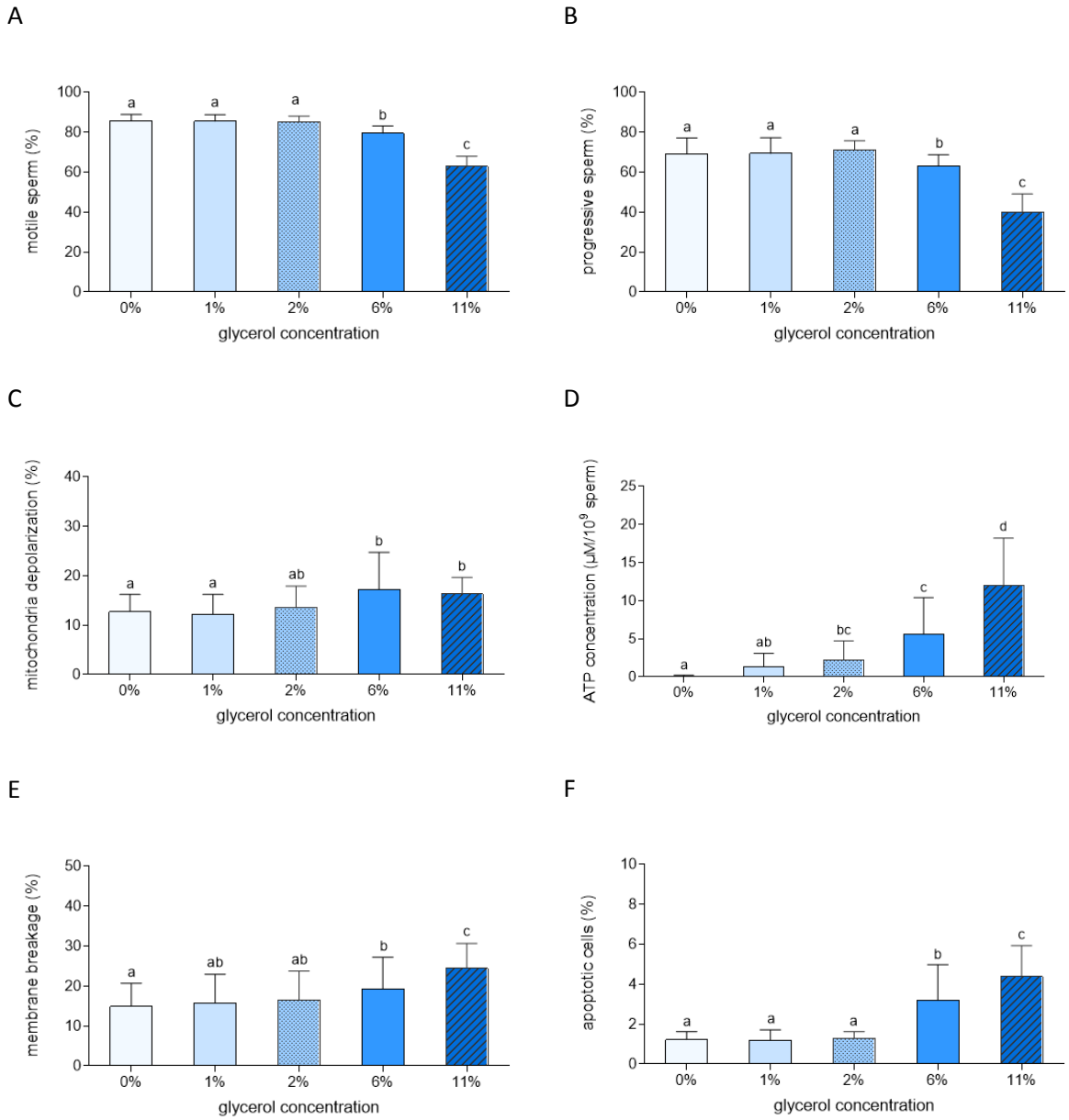
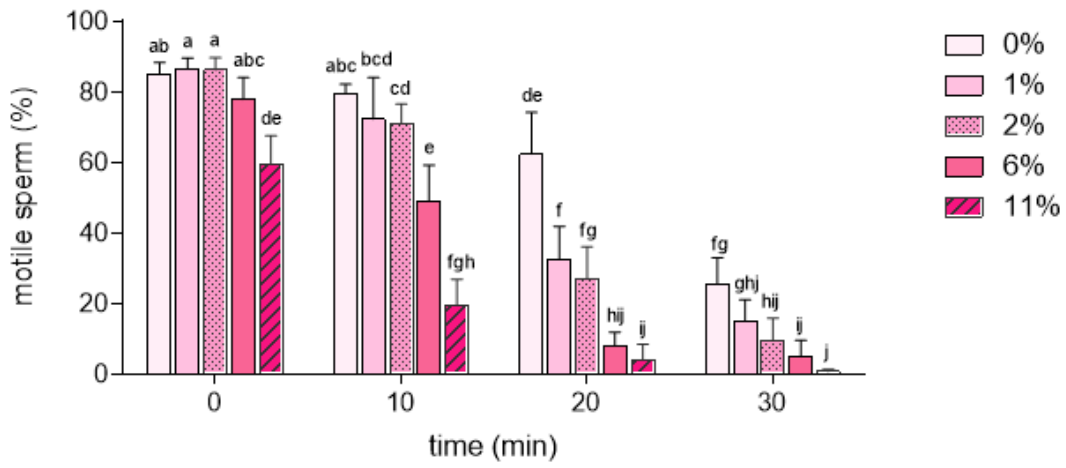


Figure 5. Sperm quality parameters of glycerolized sperm at 4°C. A-F represent evaluations of different sperm quality parameters. Semen samples were collected and pooled from 10 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

A



B

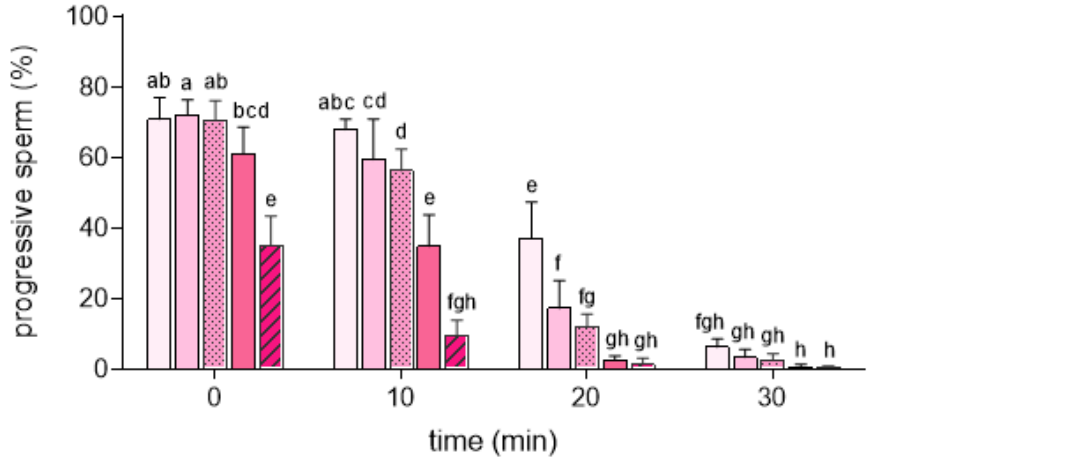
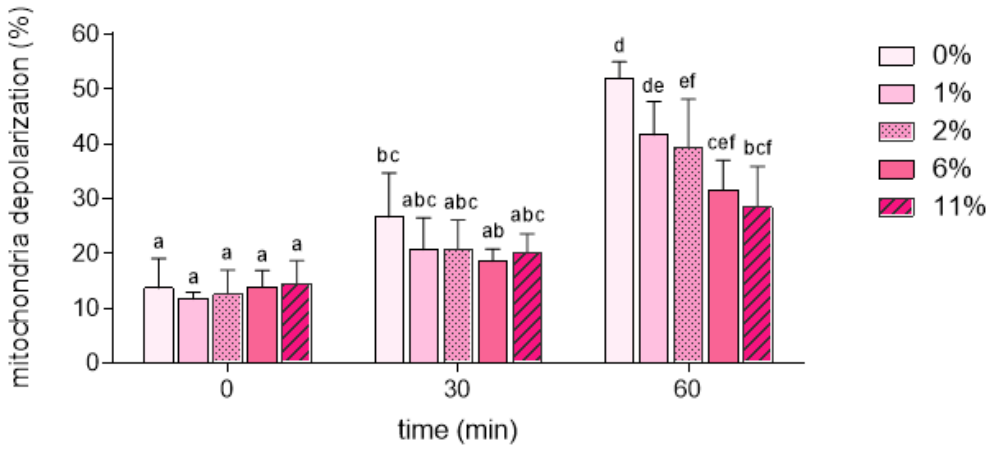


Figure 6. Sperm motility of glycerolized sperm at 41°C. A and B represent the percentages of motile (A) and progressive sperm (B). Semen samples were collected and pooled from 10 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

A



B

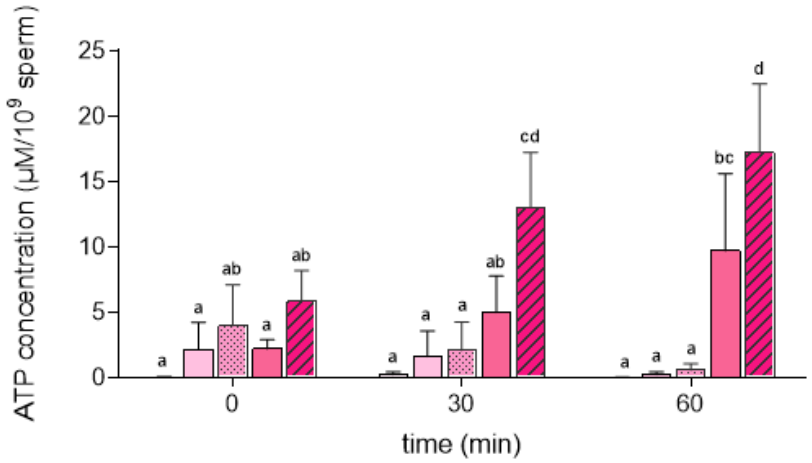
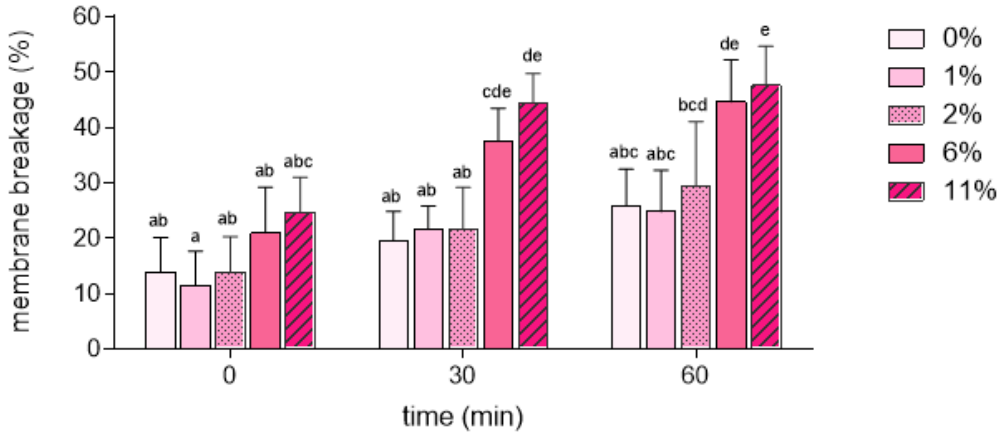


Figure 7. Sperm mitochondria function and ATP concentration of glycerolized sperm at 41°C. A and B represent the percentages of mitochondria depolarization (A) and ATP concentration (B). Semen samples were collected and pooled from 10 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

A



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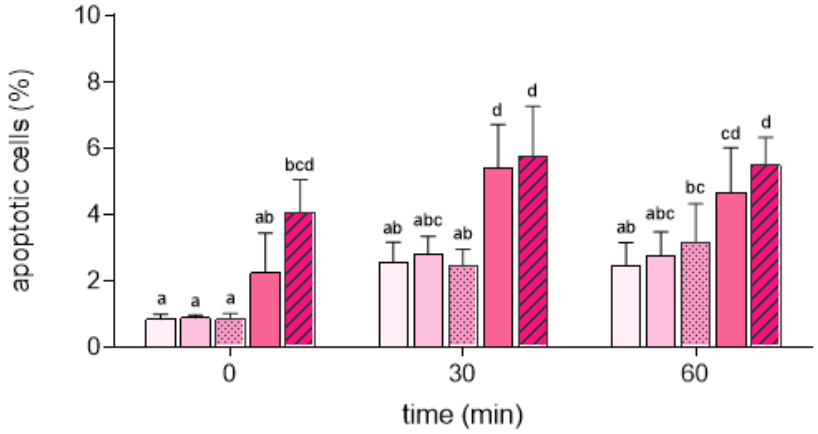

Figure 8. Sperm membrane breakage and apoptosis of glycerolized sperm at 41°C. A and B represent the percentages of membrane breakage (A) and apoptosis (B). Semen samples were collected and pooled from 10 roosters. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

TABLE

Table 1. Effect of glycerol concentration (GLY) and exposure time (T) on *in vitro* sperm quality parameters.

	two-way ANOVA test (p-value)					
	4°C			41°C		
parameter	GLY	T	GLY × T	GLY	T	GLY × T
motile sperm	**< 0.0001**	0.9103	0.8219	**< 0.0001**	**< 0.0001**	**< 0.0001**
progressive sperm	**< 0.0001**	0.9633	0.9839	**< 0.0001**	**< 0.0001**	**< 0.0001**
mitochondria activity	**0.0192**	0.0676	0.9894	**< 0.0001**	**< 0.0001**	**0.0004**
ATP concentration	**< 0.0001**	0.9520	0.9767	**< 0.0001**	**0.0049**	**< 0.0001**
membrane breakage	**0.0041**	0.4032	0.9609	**< 0.0001**	**< 0.0001**	0.3196
apoptosis	**< 0.0001**	0.0851	0.9361	**< 0.0001**	**< 0.0001**	0.5577

P-values lesser than 0.05 were considered as significant (in bold). Data of motile and progressive sperm were observed at 0, 10, 20 and 30 min and the other parameters were observed at 0, 30 and 60 min.

Author declaration

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

We further confirm that any aspect of the work covered in this manuscript that has involved experimental animals has been conducted with the ethical approval of all relevant bodies and that such approvals are acknowledged within the manuscript.

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