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Chicken sperm cryopreservation: improving the use of glycerol as a cryoprotectant

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Je me souviens encore de la première fois où j'ai pris le bus de l'INRAE du centre de Tours à Nouzilly. Le ciel était couvert, le vent soufflait fort et la température était amèrement froide. En tant que fille originaire d'un pays tropical, cet environnement m'a profondément attristée. J'avais grandi entourée de soleil chaud et de brises douces, donc ce contraste brutal m'a déçu. Cela a brisé l'image romantique que j'avais de commencer un nouveau voyage en France. J'ai appelé ma famille pour partager mes sentiments de dépression, et ils m'ont offert des paroles d'encouragement, me rassurant que tout irait bien. Ce qui m'a le plus touché, c'est quand ils ont dit : "Si tu veux abandonner et rentrer chez toi immédiatement, tu peux le faire ! Nous soutiendrons toujours toute décision que tu prendras." Je tiens donc d'abord à exprimer ma gratitude d'avoir une famille qui me soutient et qui fait preuve de gentillesse et de générosité de manière constante. Ils me rassurent en me disant que je n'ai pas à craindre l'échec, car ils seront toujours là pour me rattraper si je trébuche.

Peu de temps après, j'ai commencé mon parcours académique à l'INRAE. Heureusement, j'ai eu le privilège de travailler aux côtés d'Isabelle Grasseau et de Luiz Cordeiro au laboratoire. Ils ont été constamment solidaires et compréhensifs, non seulement en m'aidant dans mes expériences, mais aussi en offrant des conseils pour ma vie personnelle. Leur aide a été cruciale pour m'aider à m'adapter à la vie en France. Pendant ce temps, j'étais basée au deuxième étage de BOA, ce qui m'a permis d'interagir avec des personnes de l'équipe DOVE de l'INRAE. J'ai particulièrement apprécié les conversations stimulantes que j'ai eues avec eux pendant l'heure du déjeuner. J'ai acquis de précieuses connaissances sur des concepts philosophiques français, tels que "Choisir, c'est renoncer" de Joël Gautron et "Qui garde une âme d'enfant ne vieillit jamais" de Nelly Bernardet. De plus, j'ai eu de nombreuses discussions enrichissantes sur la culture asiatique avec Jacky Ezagal, abordant des sujets tels que les dim sum, les mangas et les sushis. Ces amitiés significatives m'ont apporté un soutien pendant les défis de mon premier hiver rigoureux en France. Progressivement, la saison a laissé place au printemps délicieux puis à l'été radieux. Je me sentais pleinement prête à embrasser les expériences académiques et culturelles qui m'attendaient dans ce magnifique pays.

Jour après jour, semaine après semaine, mois après mois, j'ai consacré quatre ans à mener des expériences, à rédiger quatre articles, un manuscrit de thèse et à présenter ma thèse. Je suis

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Poursuivre un doctorat à l'étranger est une aspiration de longue date pour moi, depuis que j'ai participé à une conférence internationale en tant qu'étudiant en master. Je suis reconnaissant à Ming-Che Wu et Lih-Ren Chen, mes superviseurs au TLRI, de m'avoir offert de nombreuses opportunités d'apprentissage qui m'ont aidé à grandir et à évoluer. Je tiens également à remercier Jeng-Fang Huang, le directeur du TLRI, ainsi que mes collègues, pour leur soutien constant et leur assistance tout au long de mes études en France.

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I am very grateful

I can still recall the first time I boarded the INRAE bus from Tours center to Nouzilly. The sky was overcast, the wind was strong, and the temperature was bitterly cold. As a girl from a tropical country, this environment brought me deep sadness. I had grown up surrounded by warm sunshine and gentle breezes, so the stark contrast was disappointing. It shattered the romanticized image I had of starting a new journey in France. I called my family to share my feelings of depression, and they offered words of encouragement, assuring me that everything would be okay. What moved me the most was when they said, "If you want to give up and return home immediately, you can! We will always support any decision you make." So, I first want to express my gratitude for having a supportive family that consistently shows kindness and generosity. They reassure me that I need not fear failure, as they will always be there to catch me when I stumble.

Shortly after, I began my academic journey at INRAE. Fortunately, I had the privilege of working alongside Isabelle Grasseau and Luiz Cordeiro in the laboratory. They were consistently supportive and understanding, not only assisting me with my experiments but also offering guidance in my personal life. Their assistance was crucial in helping me adapt to life in France. During that time, I was based on the second floor of BOA, which allowed me to interact with people from the INRAE DOVE team. I particularly enjoyed engaging in thought-provoking conversations with them during lunchtime. I gained valuable insights into French philosophical concepts, such as "Choisir c'est renoncer" by Joël Gautron and "Qui garde une âme d'enfant ne vieillit jamais" by Nelly Bernardet. Additionally, I had many engaging discussions about Asian culture with Jacky Ezagal, covering topics such as dim sum, manga, and sushi. These meaningful friendships provided support during the challenges of my first harsh winter in France. Gradually, the season transitioned into the delightful spring and then the radiant summer. I felt fully prepared to embrace the academic and cultural experiences that awaited me in this beautiful country.

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Pursuing a PhD abroad has been a long-standing aspiration of mine ever since I participated in an international conference as a master's student. I am grateful to Ming-Che Wu and Lih-Ren Chen, my supervisors at TLRI, for providing me with numerous learning opportunities that have helped me to grow and develop. I would also like to extend my thanks to Jeng-Fang Huang, the director of TLRI, as well as my colleagues, for their constant support and assistance throughout my studies in France.

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in France will not signify the end of our stories. I am looking forward to seeing all of you again, wherever our paths may cross in the world, and to engaging in a delightful new conversation with you, my dear friends.

Finally, this brief paragraph is dedicated to Mr. Jedispas, who has been a constant source of love and joy in my life. I apologize for not including his full name, as there is a superstition in my country that including a loved one's name in the acknowledgement of a thesis can kill the relationship. ANYWAY, I just want to let him know that I appreciate him for bringing light into my life, always making me laugh, and showing me what a life filled with love is like.

Hsiu-Lien Herbie LIN

Cryoconservation des spermatozoïdes de coq : améliorer l'utilisation du glycérol comme agent cryoprotecteur

Résumé en français : (1700 caractères max.)

Le glycérol (GLY) est un cryoprotecteur couramment utilisé pour congeler les spermatozoïdes (SPZ) chez la plupart des espèces animales. Néanmoins, il présente une toxicité notable pour les SPZ, ce qui entraîne une réduction de la fertilité. En effet, chez la poule, la dose efficace pour la cryoconservation est de 11%, alors que la présence de seulement 6% de GLY lors de l'insémination induit une infertilité complète. Pour mieux comprendre les effets toxiques du GLY sur les SPZ, le premier objectif de ma thèse était d'étudier les effets du GLY sur les caractéristiques fonctionnelles des SPZ de coq et ses impacts sur leur stockage dans les voies femelles. Les paramètres de SPZ ont été évalués in vitro pendant 60 min en présence de différentes concentrations de GLY (0, 1, 2, 6 et 11%), à 4 et 41°C. Ensuite, nous avons évalué la capacité des SPZ à coloniser les glandes de stockage (GSS) après une insémination in vivo et lors d'une co-incubation in vitro en présence de 0, 2 ou 6% de GLY. Les seules conditions qui réduisaient les capacités des SPZ à 4°C étaient 6 et 11% de GLY. De plus, des modifications plus graves ont été observées sur les caractéristiques fonctionnelles des SPZ à 41°C, notamment sur leur mobilité. In vivo, la présence de seulement 2% de GLY a réduit de moitié la présence des SPZ dans les GSS et une absence totale a été observée à partir de 6%. In vitro, la diminution des SPZ dans les GSS ne s'observait qu'à partir de 6% de GLY. Pour limiter les effets du GLY lors de l'insémination, le deuxième objectif de ma thèse était de développer un nouveau protocole pour éliminer le GLY après la décongélation. Pour ce faire, j'ai développé une solution à base de sucrose et de Percoll (SP) qui à l'aide d'une centrifugation permet d'éliminer le GLY. Cette nouvelle solution de SP élimine plus efficacement le GLY, améliore le stockage des SPZ dans les GSS et augmente de la fertilité par rapport à la méthode conventionnelle de dilutions sériées suivies d'une centrifugation.

Mots clés : cryoconservation, fertilité, toxicité du glycérol, spermatozoïdes, glandes de stockage des spermatozoïdes (GSS)

Chicken sperm cryopreservation: improving the use of glycerol as a cryoprotectant

Summary : (1700 caractères max.)

Glycerol (GLY) is a commonly used cryoprotectant for sperm in many species. However, it has a toxic effect on sperm cells, causing a decrease in fertility. In chickens, the optimal glycerol dose used for sperm freezing is 11%, but complete infertility is associated with only 6% GLY present in inseminated semen. To better understand the toxicity of glycerol on chicken sperm, the first objective of my thesis was to investigate the adverse effects of GLY on sperm functional characteristics and its impacts on sperm storage in the female genital tract. Sperm in vitro quality parameters were evaluated during a 60-min incubation with various concentrations of GLY (0, 1, 2, 6 and 11%) at two temperatures of 4 and 41°C. Then, the ability of sperm to colonize sperm storage tubules (SST) was evaluated after in vivo insemination and during in vitro co-incubation in the presence of 0, 2 or 6% GLY. The only concentrations that affected sperm characteristics at 4°C were 6 and 11% GLY. Moreover, greater changes were observed in the functional characteristics of sperm at 41°C, particularly in terms of their motility. In vivo, the presence of only 2% GLY reduced the arrival of sperm in SST by half, and a complete absence of sperm was observed with 6% GLY. On the other hand, co-incubation of sperm with 2% GLY in vitro did not decrease the number of SST filled with sperm. To mitigate the adverse effects of GLY during insemination, the second objective of my thesis was to develop a new protocol for the elimination of GLY during thawing. For this purpose, I designed a solution using sucrose and Percoll (SP) combined with centrifugation to effectively remove GLY from thawed semen. Compared to the conventional method of serial dilution centrifugation, this new SP method can efficiently eliminate glycerol, resulting in increased sperm storage in SST. Additionally, it improves fertility rate and persistence, reduces processing time and equipment requirements, and allows for flexible operations.

Keywords : cryopreservation, fertility, glycerol toxicity, sperm, sperm storage tubules (SST)

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ABSTRACT

Germplasm cryopreservation is an important tool for conserving genetic heritage. Among all the approaches, sperm cryopreservation is a highly valuable biotechnology for males due to its ability to handle large sample sizes, respect animal welfare, and achieve a higher success rate in producing offspring compared to other methods. However, this technique requires the use of chemical cryoprotectants to protect sperm from cryodamage. Glycerol (GLY) is a commonly used cryoprotectant for sperm in many species. However, it has a toxic effect on sperm, causing a decrease in fertility. The most significant case occurs in chickens, where the optimal freezing dose is 11%, but complete infertility is associated with only 6% GLY present in inseminated semen. During the cryopreservation protocol, GLY impacts can occur during two distinct stages: the pre-freezing manipulation at 4°C and the oviductal environment at 41°C after insemination. These two different temperatures may be partially responsible for the toxic effects of GLY. To gain a better understanding of the cytotoxic effects of GLY on sperm, it is necessary to consider these temperature variations.

Therefore, the first objective of my thesis was aimed to investigate the adverse effects of GLY on sperm functional characteristics and its impacts on sperm storage in the female genital tracts. Sperm motility, membrane integrity, apoptosis, mitochondrial activity, ATP concentration, and perivitelline membrane penetration capacity were therefore evaluated during a 60-min incubation with various concentrations of GLY (0, 1, 2, 6 and 11%) at two temperatures of 4 and 41°C. Then, the ability of sperm to colonize sperm storage tubules (SST) was evaluated after *in vivo* insemination and during *in vitro* co-incubation in the presence of 0, 2 or 6% GLY. The only concentrations that affected sperm characteristics at 4°C were 6 and 11% GLY. Moreover, greater changes were observed in the functional characteristics of sperm at 41°C, particularly in terms of their motility. *In vivo*, the presence of only 2% GLY reduced the sperm arrival in SST by half, and a total absence of sperm was observed with 6% GLY. On the other hand, 2% GLY during sperm-SST co-incubation *in vitro* did not decrease the number of SST filled with sperm. The reduction was only observed with 6% GLY. These data reveal a greater impact of GLY at 41°C compared to 4°C. GLY significantly affects the functional capacities of sperm, particularly their motility and interaction with the female reproductive tract, including the storage of sperm in SST.

To mitigate the adverse effects of GLY during insemination, the second objective of my thesis was to develop a new protocol for the elimination of GLY during thawing. For this purpose, I designed a solution using sucrose and Percoll (SP) combined with centrifugation to effectively eliminate GLY from thawed semen. I further investigated the effects of various sucrose/Percoll ratios on sperm quality and compared them to the conventional serial dilution (SD) centrifugation method. The results showed that this new technique can effectively be applied

to cryopreserved chicken semen for removing GLY, increasing sperm storage in SST, improving fertility rate and persistence, reducing processing time and equipment requirements, and allowing for flexible operations.

To conclude, I have demonstrated that GLY induces sperm cell death, reduces acrosomal reaction, and alters energy production pathways, thereby impairing motility. These negative changes may decrease sperm storage in SST, leading to reduced fertility. Therefore, I have developed a streamlined and effective process for removing GLY to enhance the success of using cryopreserved chicken semen. This new approach improves the elimination of GLY and increases the capacity of sperm storage in the oviducts compared to the conventional SD method. As a result, it provides higher and longer-lasting fertility in hens.

RÉSUMÉ

La cryoconservation des cellules germinales est une approche indispensable pour la préservation du patrimoine génétique. Pour les mâles, la cryoconservation des spermatozoïdes (SPZ) est la plus utilisée car il est possible de collecter des échantillons de grande taille, de le faire dans le respect du bien-être animal, et d'obtenir un taux de réussite élevé par rapport aux autres méthodes. Cependant, cette technique nécessite l'utilisation d'agents chimiques pour protéger les SPZ contre les dommages causés par les températures négatives utilisées pour le stockage des cellules. Le glycérol (GLY) est un cryoprotecteur couramment utilisé chez la plupart des espèces animales. Néanmoins, il présente une certaine toxicité pour les SPZ, entraînant une diminution de la fertilité. En effet, chez la poule, la dose efficace pour la cryoconservation est de 11%, alors que la présence de seulement 6% de GLY lors de l'insémination induit une infertilité complète. Au cours du protocole de cryoconservation, les impacts du GLY peuvent se produire lors de deux étapes importantes : le traitement pré-congélation des SPZ et après l'insémination. Ces deux étapes s'effectuent à des températures différentes, qui peuvent être en partie liées aux effets toxiques du GLY : respectivement, 4 et 41°C (température interne de la poule). Pour mieux comprendre les effets toxiques du GLY sur les SPZ, il est donc nécessaire de prendre en compte ces variations de température.

Ainsi, le premier objectif de ma thèse était d'étudier les effets du GLY sur les caractéristiques fonctionnelles des SPZ de coq et ses impacts sur leur stockage dans les voies femelles. Leur mobilité, leur intégrité, leur activité mitochondriale, leur contenu en ATP et leur capacité à pénétrer la membrane périvitelline ont été évalués pendant 60 min en présence de différentes concentrations de GLY (0, 1, 2, 6 et 11%), à 4 et 41°C. Ensuite, nous avons évalué la capacité des SPZ à coloniser les glandes de stockage (GSS) après une insémination *in vivo* et lors d'une co-incubation *in vitro* en présence de 0, 2 ou 6% de GLY. Les seules conditions qui réduisaient les capacités des SPZ à 4°C étaient 6 et 11% de GLY. De plus, des modifications plus graves ont été observées sur les caractéristiques fonctionnelles des SPZ à 41°C, notamment sur leur mobilité. *In vivo*, la présence de seulement 2% de GLY a réduit de moitié la présence des SPZ dans les GSS et une absence totale a été observée à partir de 6%. *In vitro*, la diminution des SPZ dans les GSS ne s'observait qu'à partir de 6% de GLY. Ces données révèlent un impact plus important du GLY à 41°C qu'à 4°C, avec une altération des capacités des SPZ, notamment sur leur mobilité et leur interaction avec le tractus femelle.

Pour limiter les effets du GLY lors de l'insémination, le deuxième objectif de ma thèse était de développer un nouveau protocole pour éliminer le GLY lors de la décongélation. Pour ce faire, j'ai développé une solution à base de sucrose et de Percoll (SP) qui à l'aide d'une centrifugation permet d'éliminer le GLY. Pour cette étude, j'ai étudié l'efficacité de différentes concentrations de sucrose/Percoll par rapport à la qualité de la semence obtenue par la méthode

conventionnelle de dilutions sériées suivies d'une centrifugation (SD). Notre nouvelle solution élimine efficacement le GLY, améliore le stockage des SPZ dans les GSS et augmente de la fertilité.

En conclusion, après avoir démontré les effets négatifs du GLY sur l'intégrité et la mobilité des SPZ ainsi que sur leurs interactions avec les voies femelles, j'ai développé un protocole simple et efficace pour éliminer le GLY des semences cryoconservées. L'utilisation de ce nouveau protocole sur le terrain ouvre de nouvelles perspectives concernant l'exploitation en routine des semences de coq cryoconservées.

ABBREVIATIONS

AI = Artificial Insemination

AMP = Adenosine Monophosphate

AMPK = AMP Activated Protein Kinase

AO/LD = Acridine Orange and LIVE/DEAD Fixable Blue assay

AQP = Aquaporins

AR = Acrosomal Reaction

ART = Assisted Reproductive Technology

ATP = Adenosine Triphosphate

CASA = Computer-Assisted Sperm Analysis

ClTI = Chicken Liver Trypsin Inhibitor

DMA = Dimethyl Acetamide

DMSO = Dimethyl Sulfoxide

CPA = Cryoprotectant Agent

DAMP = Damage-Associated Molecular Pattern

DISC = Death-Inducing Signaling Complex

ET = Embryo Transfer

ETC = Electron Transport Chain

FITC = Fluorescein Isothiocyanate

FSH = Follicle Stimulating Hormone

GD = Germinal Disc

GLUT = Glucose Transporter

GLY = Glycerol

GnRH = Gonadotropin Releasing Hormone

ICSI = Intracytoplasmic Sperm Injection

iPSC = induced Pluripotent Stem Cell

IPVM = Inner Perivitelline Membrane

IVF = In Vitro Fertilization

LH = Luteinizing Hormone

mPR = membrane Progestin Receptor

NGS = Next-Generation Sequencing

OPVM = Outer Perivitelline Membrane

OXPHOS = Oxidative Phosphorylation

PGC = Primordial Germ Cell

PGF2α = Prostaglandin F2 Alpha

PI = Propidium Iodide

PKA = Protein Kinase A

PNA = Peanut Agglutinin

PS = Phosphatidylserine

PUFA = Polyunsaturated Fatty Acid

PVM = Perivitelline Membrane

ROS = Reactive Oxygen Species

SB = Sperm-associated Body

SCNT = Somatic Cell Nuclear Transfer

SD = Serial Dilution

SDC = Serial Dilution Centrifugation

sncRNA = small non-coding RNA

SP = Sucrose-Percoll Solution

SSC = Spermatogonial Stem Cell

SST = Sperm Storage Tubule

TGFβ = Transforming Growth Factor Beta

TNFα = Tumor Necrosis Factor Alpha

UVJ = Uterovaginal Junction

ZP = Zona Pellucida

INTRODUCTION

1. Conservation of genetic biodiversity

Biodiversity refers to the variety of living organisms present on the planet Earth, which is currently experiencing a significant loss of species and individuals, named the sixth mass extinction, caused by human activities (Díaz and Malhi, 2022). In this notable decline of biodiversity, approximately 13% of avian and 27% of mammalian species have disappeared (IUCN, 2023), indicating extinction rates that are 1,000-10,000 times higher than natural events (Bolton et al., 2022). *In situ* and *ex situ* conservation programs may offer hope for preventing species extinction.

1.1. Genetic conservation in live animals

In situ conservation is defined as the protection of genetic resources of living species in their natural habitats, such as national parks, sanctuaries, and biosphere reserves. Another strategy, *ex situ-in vivo* conservation, involves raising live animals outside of their natural habitats, such as zoos, captive breeding programs, and aquariums. However, using an *ex situ-in vivo* strategy to preserve genetic diversity can sometimes be ineffective due to reproductive failure caused by the disruption of natural social structures and breeding behaviors. This is in addition to the problems associated with inbreeding, such as reduced fertility (Lees and Wilcken, 2009). Conservation of live animals is always at risk of unexpected infectious diseases, as well as the high costs of feed and housing. Besides, the transportation of animals involves challenges related to sociopolitical issues of animal welfare, high shipping costs, and the transmission of diseases (Johnston et al., 1993; Pukazhenthi and Wildt, 2004). Therefore, *ex situ* cryopreservation of germplasm, which is a long-term survival strategy (Bolton et al., 2022), is considered an efficient approach for genetic conservation (Nakamura, 2016).

1.2. *Ex situ* cryopreservation of germplasm

Germplasm is defined as the reproductive cells used to generate progeny, including gametes, gonads, embryos, and primordial germ cells (PGCs). The *ex situ-in vitro* conservation technique, cryobanking, involves the long-term storage of viable reproductive cells or tissues in liquid nitrogen at -196°C. These cryopreserved materials can be used to recreate offspring through assisted reproductive technology (ART). ART includes artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), embryo transfer (ET), and PGCs transplantation (Howard et al., 2016; Hikabe et al., 2016; Briski and Salamone, 2022). Despite not being applicable to every species, cryopreservation of somatic cells and induced pluripotent stem cells (iPSCs) is another approach for achieving the same purpose (Gomez et al., 2008; Herrick, 2019). These techniques are widely applied in various species. However, it is necessary

to optimize the protocol specific to each species in order to obtain successful results (Herrick, 2019; Mastromonaco and Songsasen, 2020).

1.2.1. Cryopreservation of oocytes and embryos

Slow freezing, one of the cryopreservation methods, involves a controlled and gradual decrease in temperature procedure, which is associated with the risk of ice crystallization that can cause damage to cell membranes (Marques et al., 2019). Mammalian oocytes and embryos are large cell masses that contain a significant amount of water. However, their membrane has poor and variable permeability to cryoprotectant agents (CPA) (Arav, 2014; García-Martínez et al., 2021), which results in increased ice crystal formation during freezing. This, in turn, leads to the disruption of cellular structures **(Figure 1.1)**. Vitrification, which involves working in a high osmolarity solution prepared with a high concentration of CPA and rapidly immersing samples directly into liquid nitrogen, has been found to be more successful for freezing oocytes and embryos compared to conventional slow freezing methods (Morrell and Mayer, 2017). Due to the high viscosity of the vitrification medium and the rapid cooling speed, a glass-like state is achieved quickly, preventing ice crystallization **(Figure 1.1)** and minimizing structural damage (Arav, 2014; Rienzi et al., 2017). However, intracellular ice can still form during vitrification if the concentration of CPA is not sufficiently high. This presents a dilemma, as high volumes of CPA can be toxic to cells. Thus, a more recent strategy named ultrarapid vitrification has been introduced to minimize cryoinjury by increasing the cooling and warming rates. This allows for vitrification with lower CPA concentrations, resulting in reduced toxicity to oocytes and embryos. Several specific materials, such as the open pulled straw (Vajta et al., 1999), cryoloop (Lane et al., 1999), piece of nylon mesh (Matsumoto et al., 2001), and the gel-loading tip (Tominaga and Hamada, 2001), have been developed to achieve higher cooling and warming rates.

Whereas embryo production, cryopreservation, and transfer methods are well-established in some species, such as ruminants and rodents (Dochi, 2019; Ferré et al., 2020; Lamas et al., 2020), they still require improvement in other species due to significant differences in embryo structure and molecular contents (Boes et al., 2023). In general, unfertilized oocytes are more sensitive to cryoinjury than embryos (Kasai, 2002). Additionally, *in vitro* produced embryos are more sensitive to cryopreservation than *in vivo* derived embryos (Leibo et al., 1996).

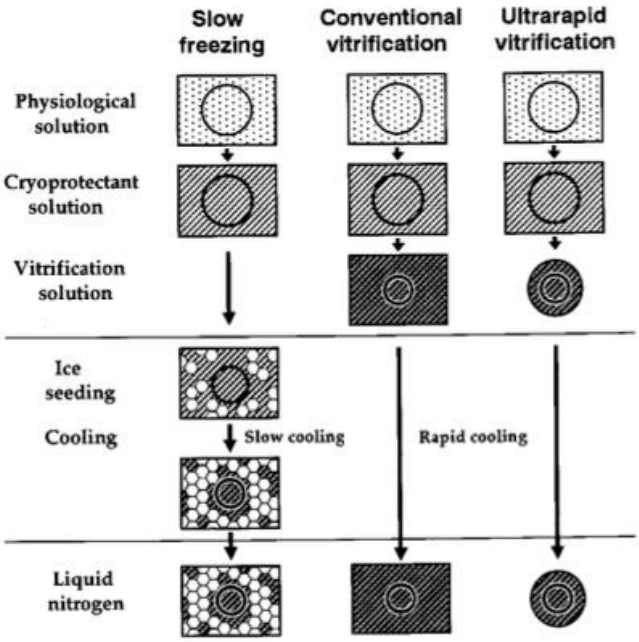

Figure 1.1. Schematic representation of an oocyte/embryo (circle) during slow freezing, conventional vitrification, and ultrarapid vitrification (Kasai, 2002). White hexagons represent ice crystals. The concentration of cryoprotectant is indicated by the intensity of shading.

1.2.2. Cryopreservation of somatic cells and stem cells

Donor somatic cells can be cryopreserved using CPA (Dai et al., 2006), without CPA (Hoshino and Saeki, 2010), or even freeze-dried (Wakayama et al., 2022) before conducting the somatic cell nuclear transfer (SCNT) technique. SCNT is an advanced biotechnology used to produce genetic replicas of an individual, also known as clones. The first animal obtained by SCNT was the famous sheep Dolly (Campbell et al., 1996), produced by transferring the nucleus from a donor somatic cell into an enucleated oocyte cytoplasm. After being artificially activated, the oocytes reprogram the exogenous donor nucleus through dedifferentiation and develop into early embryos *in vitro*. These embryos can then be transferred into the uterus of a suitable recipient and successfully implant. SCNT has potential for various purposes, such as rescuing endangered species, reviving extinct species, producing transgenic animals, and cloning farm animals with desirable phenotypes (Oback, 2008; Iqbal et al., 2021). However, the survival rates of live offspring are generally very low (Chavatte-Palmer et al., 2013).

Isolation of spermatogonial stem cells (SSC) from cryopreserved testicular tissue and transplanting them into surrogate males is a potential method for generating viable sperm for fertilization (Takashima and Shinohara, 2018; Ciccarelli et al., 2020). A more recent technique,

called induced pluripotent stem cells (iPSCs), involves generating stem cells directly from somatic cells through genetic reprogramming and transfection with pluripotency-related genes. These iPSCs can be differentiated *in vitro* into sperm or oocytes for subsequent fertilization (Hayashi et al., 2012; Bolton et al., 2022). With this approach, it is important to choose viable tissues that can be reprogrammed for iPSC generation after cryopreservation. Fibroblasts are widely used for this purpose (Li et al., 2009; Liu et al., 2014).

1.2.3. Advantages and disadvantages of sperm cryopreservation

Compared to other germ cells, sperm are relatively easier to collect in large amounts (Comizzoli and Wildt, 2013; Santiago-Moreno and Blesbois, 2022) since sperm are continuously produced by males from sexual maturity until the end of their lives, allowing for individual mass collection (Bolton et al., 2022). In addition to ejaculates, sperm can also be collected from mature individuals within 48 hours after animal death or castration. This technique can be achieved by flushing sperm from the deferent ducts in birds (Villaverde-Morcillo et al., 2016) or from the epididymis in mammals (Martínez-Fresneda et al., 2019; Galarza et al., 2021).

Due to the unique characteristics of sperm in each species, protocols for sperm cryopreservation should be customized to suit the specific species (Leibo and Songsasen, 2002; Comizzoli, 2015). However, the general stages of the method remain the same in all species. This process involves several steps: semen collection, addition of CPA, cellular dehydration, freezing, storage in liquid nitrogen, and thawing before use (Ugur et al., 2019). However, the choice of CPA and their dosage may vary among different species, depending on the specific sperm requirements and tolerance levels (Schiewe et al., 1991; Stoops et al., 2007; Zee et al., 2008; Robeck et al., 2011). Additionally, the cooling rate and container material are other factors that need to be tested (Comizzoli and Wildt, 2013). A more comprehensive explanation of chicken sperm cryopreservation can be found in our review article (Lin et al., 2021) in the following chapter (see page 45-66).

In mammals, the male is heterogametic, producing X and Y-bearing sperm. The cryopreservation of mammalian sperm allows for the preservation of the entire nuclear genome and the restoration of the same genetic material after thawing. However, the male avian is homogametic (ZZ) and produces only Z sperm. Consequently, sperm cryopreservation alone does not enable the preservation of the entire genome in birds. To achieve this goal, it needs to be combined with other germplasm cryopreservation techniques, such as preserving the PGCs.

1.2.4. The specific case of avian species

In birds, sperm cryopreservation is indeed the most convenient, least expensive, and least invasive method for germplasm conservation. However, it does not allow for the conservation

of the W chromosome and mitochondrial DNA. The cryopreservation of oocytes and embryos is currently not possible in avian species due to their megalecithal nature, which means they contain large quantities of lipids (Santiago-Moreno and Blesbois, 2022). Thus, cryopreservation and transplantation of PGCs are valuable alternative methods for preserving the female genome **(see Figure 1.2)**. PGCs, which are precursor cells for gametes that generate sperm and ova in adults, can be isolated from the blood and gonads of avian embryos during the early stages of development (Nakamura, 2016; Lázár et al., 2021). In general, PGCs can be multiplied *in vitro* before freezing and/or after thawing to increase their numbers prior to transfer into host embryos. This process is performed to create chimeric animals and generate functional gametes with the genotype of the donor (Whyte et al., 2015). It is necessary to remove as many endogenous PGCs as possible from the hosts prior to transplanting donor PGCs in order to increase the homogeneity of donor-derived gametes in the recipients. In addition to their interest in conservation, PGCs can undergo genome editing before being transferred into recipient animals. This is currently the only available method for producing genome-edited birds (Serralbo et al., 2020; Ichikawa et al., 2022).

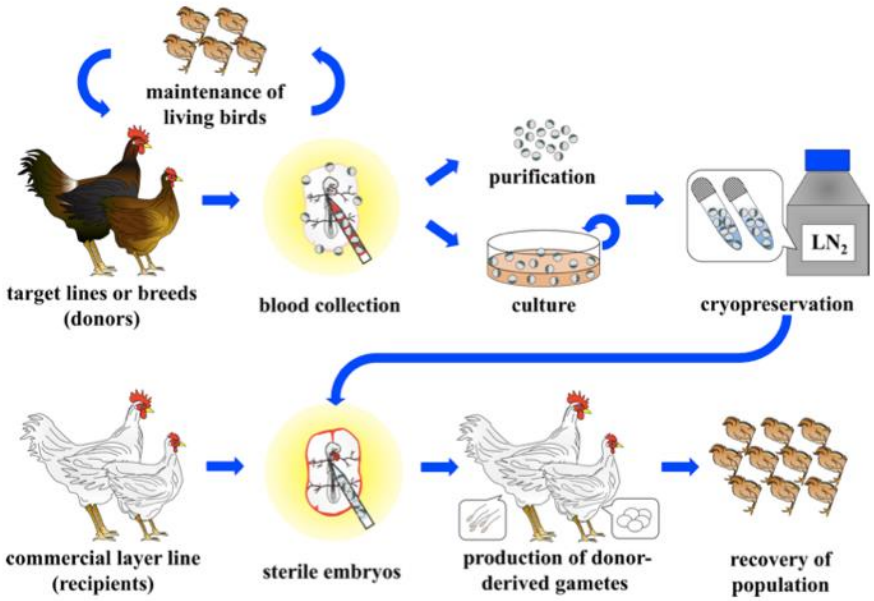

Figure 1.2. Outline of a poultry PGCs-bank program (Nakamura, 2016). The procedures for PGCs conservation consist of five steps: 1) collecting PGCs from target lines or breeds, 2) purifying and culturing PGCs, 3) storing PGCs long-term in liquid nitrogen, 4) transplanting PGCs into sterilized recipient embryos, and 5) recovering populations through mating of male and female recipients.

2. Avian sperm journey: from production to fertilization

2.1. In the male reproductive tract

Avian sperm spend a shorter period of time within the male genital tract compared to the average of 45.8 days observed for mammalian sperm (Jones and Lin, 1993). In chickens, spermatogenesis takes 13 days (Deviche et al., 2011). Afterward, the sperm transit through the epididymis and the deferent ducts for 1-3 days (de Reviers, 1975) before being stored for a maximum of 7 days (Santiago-Moreno and Blesbois, 2020).

2.1.1. Sperm production

The male reproductive system in birds consists of a pair of testes, epididymis, deferent ducts **(Figure 2.1A and B)**, and accessory reproductive organs (Vizcarra et al., 2022; Valerie, 2023). These include the paracloacal vascular bodies, dorsal proctodeal gland, and lymphatic folds (Fujihara, 1992), instead of the accessory sex glands found in mammals. Unlike mammalian testes, which need to be maintained at a temperature 2-8°C below the core body temperature to support normal spermatogenesis and testicular functions (Abd El-Emam et al., 2023), avian testes are located within the abdominal cavity (Deviche et al., 2011) at an internal body temperature of 40-41°C (Padhi et al., 2016). In such warm conditions, their functions are not suppressed, and they efficiently produce sperm while exhibiting several distinct characteristics compared to mammals (Słowińska et al., 2020) **(Table 2.1)**.

The testes consist of interstitial tissue and seminiferous tubules, which are respectively the sites of androgen production and spermatogenesis (Estermann et al., 2021). The epididymis consists of a series of ducts that eventually empty into the deferent duct. The ducts within the epididymis include the rete testis, efferent ducts, connecting ducts, and the epididymal duct. The epididymal ducts and the deferent duct are collectively known as the excurrent ducts of the testis **(Figure 2.1C)**. The seminiferous tubules connect with the rete testis at specific locations along the interface between the testis and epididymis (Vizcarra et al., 2022).

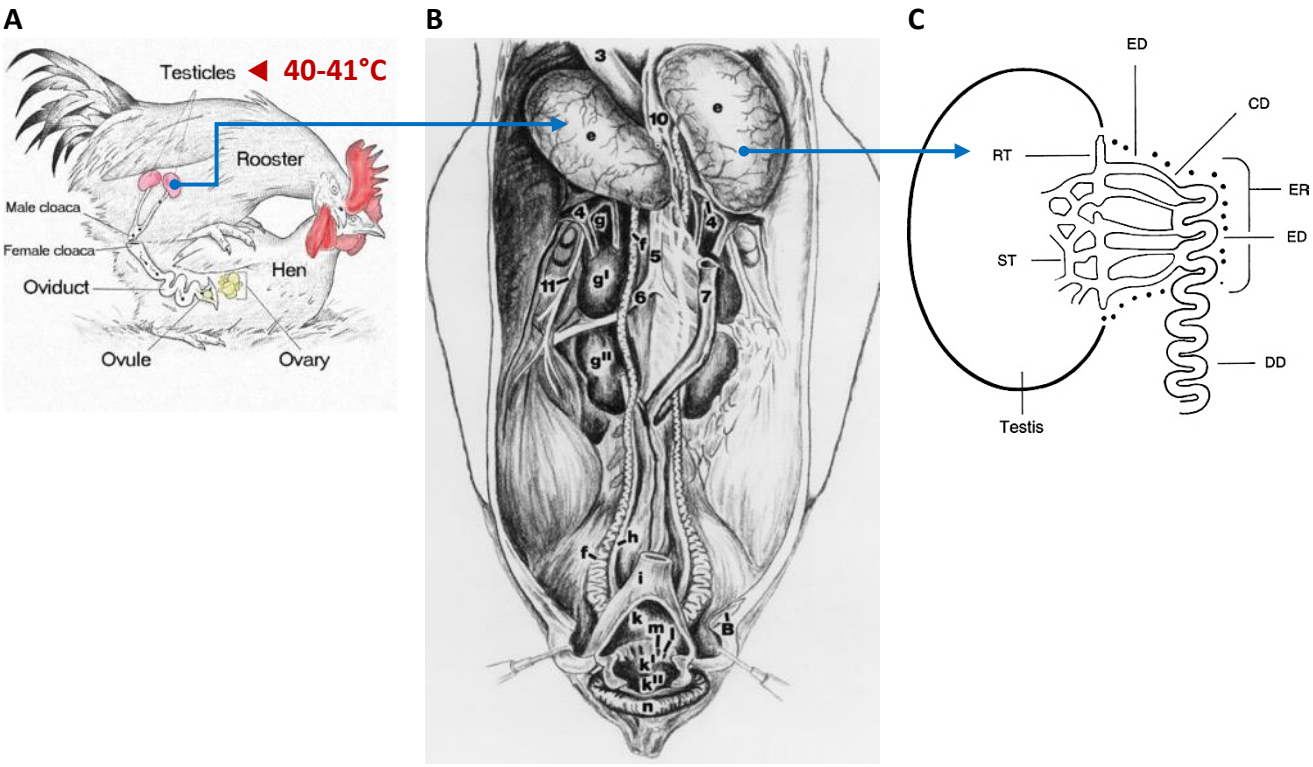

Figure 2.1. (A) A simple illustration of the reproductive anatomy in male and female chickens, as well as their mating behavior, known as the "cloacal kiss", (B) Topography of the dorsal body wall of the rooster and (C) Schematic of the excurrent ducts of the testis (Vizcarra et al., 2022 and Valerie 2023). Abbreviations of (A): e-testis, f- deferent duct, h-ureter, l-opening of left deferent duct, m-opening of left ureter, (n) anus. Abbreviations of (C): ST-seminiferous tubules, RT-rete testis, ED-efferent duct, CD-connecting duct, ER-epididymal region, ED-epididymal duct, DD-deferent duct.

Table 2.1. Comparisons of sperm production in the Japanese quail, laboratory rat, and domestic ram (Modified from Jones and Lin, 1993)

Parameter	Quail	Rat	Ram
Body mass (g)	148	559	35 000
Testes/body mass (%)	2.26	0.67	0.91
Spermatogenesis cycle length (day)	2.69	12.5	10.4
Daily sperm production (10^6/g testis)	92.5	23.7	19
Sperm transit through the extragonadal ducts (day)	1	8.4	16.4
No. of extragonadal sperm (10^6/animal)	308	735	>165 000
No. of ejaculates:			
produced by testes/day	26	1	2
available in extragonadal ducts*	1-?	5	14-26

* The number of ejaculates that can be collected in a day (and that contain sufficient sperm for fertilization) minus the number of ejaculates produced by the testes per day.

Spermatogonia reside in the seminiferous tubules and undergo several distinct stages, known as spermatogenesis, to transform into active sperm **(Figure 2.2)**. In the first stage, spermatogonia cells initially proliferate and differentiate into primary spermatocytes through mitosis. This is followed by two consecutive divisions via meiosis, resulting in the formation of secondary spermatocytes and then haploid spermatids. The final stage is spermiogenesis, which primarily focuses on the morphological maturation of spherical spermatids into elongated ones. This process includes the condensation and elongation of the acrosome and nucleus, as well as the development of the flagellum (Deviche et al., 2011; Haseeb et al., 2019). Apoptosis (Zakariah et al., 2022) and autophagy (Haseeb et al., 2019) are crucial processes for normal spermiogenesis, resulting in a dramatic loss of 97% in cytoplasm, while nuclear volume changes account for 96% of this reduction (Sprando and Russell, 1988). Mature spermatids are equipped with a head that contains an acrosome, a cylindrical nucleus, mitochondria, and a tail (flagellum). Then, they are released from Sertoli cells into the seminiferous tubule lumen, a process known as spermiation, and subsequently transit into the epididymis (Vizcarra et al., 2022).

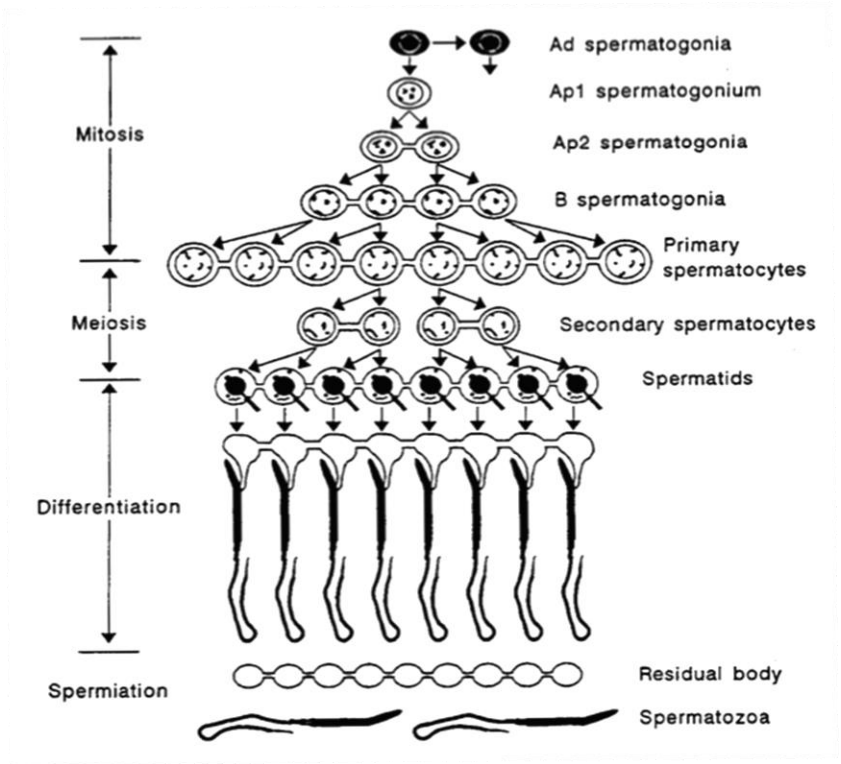

Figure 2.2. Spermatogenesis in the Japanese quail (Jones and Lin, 1993). The process involves three major phases: (1) spermatocytogenesis, which is the stage of spermatogonia renewal and proliferation through two or more mitotic divisions of one of the products of the renewal division; (2) meiosis, which is the stage of spermatocyte division through two reduction divisions; and (3) spermiogenesis, which is the stage of spermatid differentiation to produce sperm.

2.1.2. Sperm maturation

Chicken seminal plasma is derived from the proximal efferent ducts, epididymis, and deferent ducts (Santiago-Moreno et al., 2019) and is known to enhance sperm fertilizing capacity. However, the precise molecular mechanisms behind this regulation have yet to be fully elucidated (Santiago-Moreno and Blesbois, 2020). Furthermore, since avian sperm cells are capable of fertilizing the oocyte, unlike what is observed in mammals, the role of post-testicular maturation in avian sperm is still controversial (Nixon et al., 2014; Asano and Priyadarshana, 2022). Despite the fact that testicular sperm can bind to the perivitelline membrane and undergo acrosomal reaction, they lack normal motility (Ashizawa and Wishart, 1987; Nixon et al., 2014). This suggests that avian sperm fully acquire their motility capacities in the epididymis. Some other functions are boosted during the journey of sperm within the male reproductive tract. This maturation process includes acrosin activation, acrosomal proteolytic activity (Thélie et al., 2019b), chromatin compaction (Bernal et al., 2022), DNA protection (Santiago-Moreno et al., 2019), antioxidant capacity (Li et al., 2020), survival capacity during *in vitro* storage (Ahammad et al., 2011), and extension of sperm lifespan in the hen oviducts (Borziak et al., 2016). These findings demonstrate the importance of post-testicular maturation for avian sperm, as it does in mammals. However, the capacitation process, which occurs in mammals, has never been found in avian sperm (Howarth, 1971). Besides, avian sperm undergo an acrosomal reaction immediately after contacting the perivitelline membrane *in vitro* (Lemoine et al., 2008). This indicates that capacitation in the female genital tract is not essential for fertilization in birds.

2.2. Travel from male reproductive tract to the oviduct

2.2.1. Semen transmission through a cloacal kiss

During natural mating, the rooster's phallus protrusion first forms a tumescent lymphatic tissue and then immediately rotates into the cloaca before ejaculation (Vizcarra et al., 2022; Valerie, 2023). In chickens, the phallus is non-intromittent **(Figure 2.1A)**. Semen is transferred to the female through a process known as the "cloacal kiss" (Briskie and Montgomerie, 2001).

2.2.2. Artificial insemination

In chickens, the most commonly used assisted reproductive technology is artificial insemination, which involves the collection of semen and the insemination process.

Chicken semen collection is most often performed by dorso-abdominal massage to induce ejaculation (Burrows and Quinn, 1937). This method is much less invasive than electrical stimulation and does not require anesthesia. While collecting semen for further treatments, it is important to exclude cloacal products such as feces, urates, or transparent fluid in order to ensure efficient *in vitro* storage (Santiago-Moreno and Blesbois, 2020).

When conducting cloacal insemination, sperm are deposited into the chamber of the cloacal opening. This procedure mimics the cloacal kiss of natural mating and is considered the easiest and least stressful method for animals (Barna et al., 2020). However, this technique is less efficient and requires more sperm doses than intravaginal insemination. During intravaginal insemination, a gentle pressure is applied to the hen's abdomen to evert the vaginal orifice through the cloaca. Semen is then transferred into the vaginal region at a depth of 2-3 cm (Quinn and Burrows, 1936). With this method, hen laying performance is not disturbed, and it allows for the selection of functional sperm during vaginal transit to achieve good fertility (Bakst et al., 1994). Other AI techniques have been developed, as reviewed by Lin et al. (2021), including intrauterine, intramagnal, and intraperitoneal inseminations. However, all of these methods are invasive, bypass vaginal sperm selection, and have deleterious effects on laying activity.

Performing inseminations in the afternoon/evening usually results in higher fertility rates compared to those done in the morning. This was first hypothesized by Parker and Arscott (1971), who suggested that the presence of a hard-shelled egg in the uterus at the time of insemination could be the reason for this difference. However, the event of oviposition was also proposed as the primary cause, supported by a decrease in fertility when insemination was conducted within a few hours before or after oviposition, regardless of the presence or absence of the hard-shelled egg in the reproductive tract (Johnston and Parker, 1970; Giesen et al., 1980). While the physiological basis has not yet been established, the hormonal rhythm associated with oviposition may explain these observations (Hughes and Parker, 1972).

2.3. Sperm sojourn in sperm storage tubules (SST)

In the female reproductive tract, the journey of sperm involves vaginal selection and then storage within the sperm storage tubules (SST) in the uterovaginal junction (UVJ). After a quiescent period in SST, sperm are then released into the oviductal lumen again and travel upward to the infundibulum for fertilization to complete their adventure. Once chicken sperm enter the female genital tract, they can survive for up to 3 weeks (Blesbois and Brillard, 2007), which is significantly longer than the 3-5 days typically observed in most mammals (Kölle, 2022). Such differences in sperm survival characteristics highlight the importance of interactions with maternal tissues for sperm performance. Recent studies have demonstrated that communication between sperm and SST cells may involve extracellular vesicles produced by SST epithelium (Cordeiro et al., 2021a; b). This suggests a potential mechanism for regulating sperm activities while they reside in the hen's oviduct.

2.3.1. Vaginal selection

In chickens, more than 80% of sperm are expelled within 30 minutes after semen is deposited into the vagina, and less than 1% of inseminated sperm ultimately reach the SST at the UVJ

(Brillard, 1993). These findings suggest that the vagina is the primary site for sperm selection in avian species. Once they arrive in the hen oviducts, sperm start heading towards the UVJ with the help of their intrinsic motility, smooth muscle peristaltic contractions, and the beating of ciliated cells on the mucosal surface of the vagina (Bakst, 2011).

2.3.2. Sperm arrival in SST of UVJ

In chickens, sperm reach the SST within 1 hour of insemination (Bakst et al., 1994), while the filling of the SST with sperm continues to increase up to 24 hours after insemination (Sasanami et al., 2013). Successive inseminations result in late-inseminated sperm being filled to a different SST than early ones (King et al., 2002). The exact mechanisms of how sperm enter, reside, and are released from the SST are still not clear. It has been generally accepted that only motile sperm are allowed to enter the UVJ (Brillard, 1993; Bakst et al., 1994). Scientists have proposed that alterations in the glycoprotein-glycolipid on the surface of sperm cells impair their binding to UVJ tissue (Froman and Thursam, 1994; Steele and Wishart, 1996; El-Sherry et al., 2022). This suggests the presence of a molecular passport on the sperm membrane that is necessary for SST binding and subsequent fertilization. A recent hypothesis suggests that the immune system in the avian oviduct also plays a role in vaginal selection (Das et al., 2008). This is supported by evidence of increased immune activity following sperm migration through the vaginal region (Higaki et al., 1995).

2.3.3. Long-term stay in SST

Sperm storage capacity of SST varies among species and is genetically determined. The duration of fertilization is up to 12 days in Japanese quails, 21 days in chickens, and over 70 days in turkeys (Blesbois and Brillard, 2007).

Sperm charged into SST

It is generally accepted that the SST in the UVJ is the primary sperm storage site (Bakst, 1987; McKone, 2023), which is located in the anterior 2-3 cm section of the vagina **(Figure 2.3)**. The fertilizing capacity of hens is related to the number and size (length) of SST (Bakst et al., 2010). The variations in luminal pH levels in SST and serotonin release in UVJ (Holm et al., 1996; Bakst and Akuffo, 2008) are believed to enhance sperm motility, frequency of vaginal cilia beating, and smooth muscle activity. These changes work in coordination with the fluid counterflow produced by ciliated cells at the opening of SST together to guide sperm into SST (Blesbois and Brillard, 2007). Sperm are then distributed either in widely spaced or packed, parallel bundles with their heads towards the distal blind end of the SST **(Figure 2.4)**.

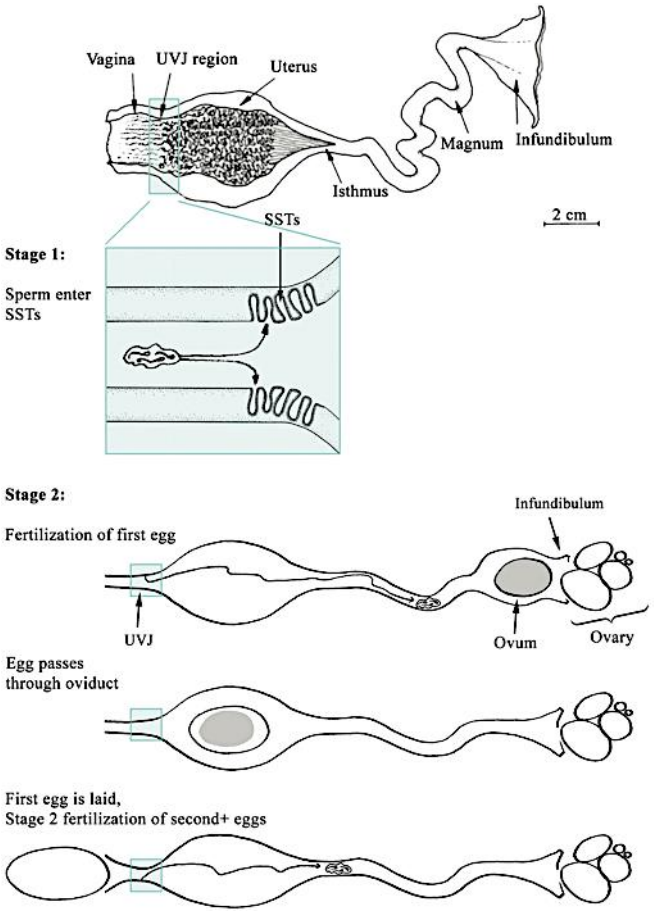

Figure 2.3. Avian fertilization (McKone, 2023). During the process of fertilization, sperm travel through the avian oviduct (top) from the site of ejaculation in the vagina to the infundibulum in two distinct stages.

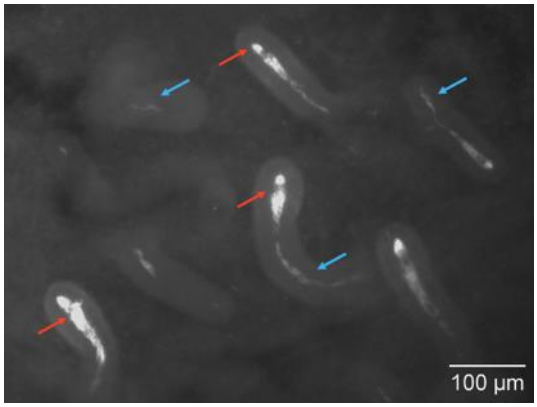

Figure 2.4. Rooster sperm storing patterns inside SST (H.-L. H. Lin). Blue arrows indicate that sperm are deposited separately and widely spaced. Red arrows indicate that sperm are packed in parallel bundles with their heads towards the distal blind end of the SST.

Prolongation of sperm residence in SST

The SST provide a delicate microenvironment not only for sperm survival but also for maintaining their fertilizing capacity. Despite the fact that regulatory mechanisms are still not elucidated, several hypotheses have been proposed:

Inactive sperm motility: The luminal pH value of the SST has been proposed to regulate sperm motility (Holm et al., 1996), as an acidic condition can decrease sperm mitochondrial activity (Mishra et al., 2018). A more recent finding indicates that lactic acid inactivates sperm motility in SST through the inhibition of ATPase activity of dynein, which is the motor protein responsible for flagella movement (Matsuzaki et al., 2015). This system seems reasonable not only to conserve energy consumption but also to protect sperm from ROS attacks by reducing sperm respiration inside the SST (Matsuzaki and Sasanami, 2022).

Metabolic regulation: SST contains significant amounts of lipidic components, including cholesterol esters and complex lipids. These components are considered accessible energy sources that help maintain sperm functions during storage (Renden et al., 1981; Blesbois and Brillard, 2007). Besides, glycogen is abundant in the cytoplasm of chicken SST, which is considered a potential energy source during prolonged sperm storage (Bakst, 1987; Bréque et al., 2003). In addition, sperm are assumed to utilize endogenous fatty acids as well (Froman, 2003).

Anti-oxidation: Chicken sperm contain a high level of polyunsaturated fatty acids (PUFAs), which increases the risk of membrane damage caused by lipid peroxidation (Surai et al., 2001). In addition to antioxidants such as vitamin C and vitamin E, the enzymes GSH, GSH-Px, and SOD are also observed to be activated in the SST. The antioxidant enzymatic activities in the SST are found to be highly active, suggesting that the anti-oxidation system protects the hosted sperm against peroxidation (Bréque et al., 2003).

Sperm immunosuppression: The immune response in the UVJ may affect sperm viability and fertility (Das et al., 2005). Therefore, one hypothesis is that immune privilege exists for resident sperm in the SST to facilitate their storage and survival (Das et al., 2008). This immune privilege is estrogen-dependent (Das et al., 2006b) and also involves TGFβ-mediated immunosuppression (Das et al., 2006a).

Housekeeping: Aquaporins (AQP) are a group of small membrane proteins that function as water channels, regulating water movement in cellular tissues (Verkman and Mitra, 2000). They are found in the apical part of SST (Zaniboni and Bakst, 2004) probably to participate in expelling sperm metabolites accumulating in SST lumen to maintain homeostasis of SST microenvironment.

Sperm discharged from SST for fertilization

A successful fertilization depends on sperm encountering the ovum precisely at the right location and at the right time. Avian sperm take advantage of the female's ability to store sperm, allowing them to wait inside the female reproductive tract for several successive ovulations. Once sperm are released from SST, they ascend to the anterior infundibulum to fertilize the upcoming ovum. Alternatively, they can enter the SST in that region for a second storage (Van Krey et al., 1967).

Despite the lack of solid evidence on how sperm are released from the lumens of SST and transported to the fertilization site, several theories regarding sperm discharge and delivery have been suggested.

Hormonal regulation: Resident sperm flow out of the SST close to the time of ovulation/oviposition (Bobr et al., 1964), suggesting that this process may be regulated by the ovulatory cycle and stimulated by progesterone **(Figure 2.5)** (Ito et al., 2011; Sasanami et al., 2013). There is at least a 5-hour gap between the elevation of progesterone levels and the next ovulation, which is a good strategy to prevent released sperm from being disturbed by the descending egg (Sasanami et al., 2013). In addition, prostaglandin F2α (PGF2α), produced in the male cloacal gland and composing the seminal plasma, induces vaginal contractions and regulates the orifice of SST (Sasanami et al., 2015), thereby contributing to sperm transportation for fertilization (Khillare et al., 2018).

Fluid flow: Another theory proposes that when sperm are depleted of ATP and lose their oscillatory movement during their residency in the SST (Froman, 2003), stored sperm flow out of the SST lumens with a fluid current (El-Sherry et al., 2022). As previously described, AQP are present in SST tissues, which may facilitate the movement of water surrounding the orifice of SST. This movement could potentially expel not only metabolic waste but also resident sperm.

No need for sperm intrinsic motility: Dead sperm, when inseminated in the uterus, can reach the infundibulum with similar sperm numbers as insemination with fresh semen (Allen and Grigg, 1957). This evidence may fully explain that sperm motility is not necessary for sperm transport in the avian oviduct after passing through the vagina.

Controversial neural factors: The mechanical pressure of a passing egg was once proposed to induce the random release of sperm from SST based on the evidence that no contractile tissue was found around SST (Van Krey et al., 1967). However, another study presented that SST were surrounded by smooth muscle and neural tissue elements, including numerous neurons and small ganglia. In addition, F-actin, a linear polymer microfilament of the neuronal cytoskeleton, was found abundantly localized in the apical region of the epithelial cells of SST and in the smooth muscle in UVJ (Freedman et al., 2001). This suggests that innervation may play a role in the release of sperm from SST.

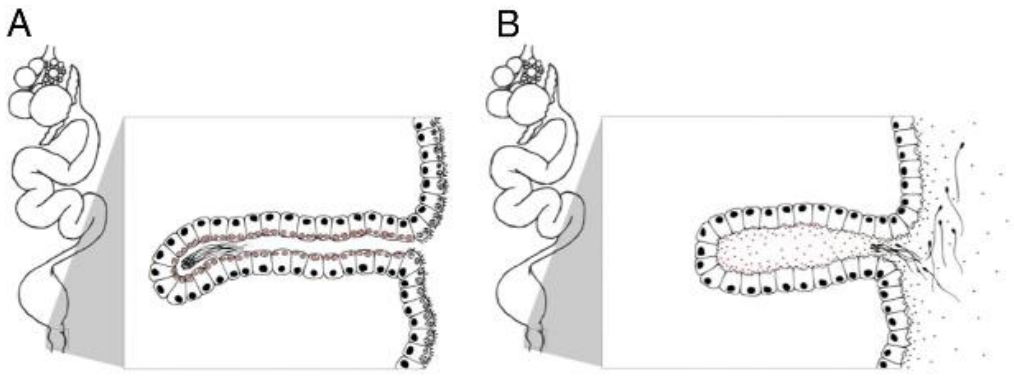

Figure 2.5. Schematic model of the proposed mechanism for sperm release from the SST in the quail oviduct (Ito et al., 2011). Before the onset of the progesterone surge (A), the sperm in the SST are inactive, and the lumen of the SST is not expanded. When ovulation is imminent (B), the blood concentration of progesterone increases, stimulating the release of the resident sperm from the SST. This release is accompanied by contraction-like morphological changes in the SST, which are likely initiated through the membrane progestin receptor (mPR) that mediates signal transduction. This process might be supported by the lubricant effect of cuticle materials secreted from the ciliated cells of the UVJ (black dots) as well as the unknown materials supplied from the SST epithelial cells (red dots), the events coincidently triggered under the progesterone control.

2.4. Meet the ovum and complete fertilization

After sperm undergo the first selection from the vagina to the UVJ (only 1% remaining), they encounter a second, more strict selection while moving from the UVJ to the infundibulum. In this stage, less than 0.0001% of inseminated sperm can be found (Bakst et al., 1994), and only a few hundred are able to interact with the perivitelline membrane of a laid egg (Wishart, 1987). Such rigorous selection may choose the most competitive sperm for fertilization and could potentially lead to the low embryonic mortality observed in birds (Blesbois and Brillard, 2007).

2.4.1. Ovulation

Although both the left and right ovaries and oviducts are formed during embryogenesis, the paired structures are only present 5-10 days after hatching in most avian species (Idahor, 2022). Soon after, the right ovary and oviduct regress, leaving only the left ones functional. Unlike mammalian species, the avian infundibulum is located further away from the ovary, which makes it more difficult to capture large and fragile ova that are filled with fluid yolk (Shimada and Tanabe, 1982). Thus, the electrical engulfing activity of the infundibulum, which is active

around the time of ovulation, is a crucial process to ensure that the ova can be effectively picked up.

Preovulatory follicles are present in a hierarchy based on their size **(Figure 2.6)**. The largest follicle (F1) is waiting for the next ovulation, while the second largest follicle (F2) will ovulate the following day, and so on (van der Klein et al., 2020). Similar to mammals, the regulation of ovulation in chickens occurs through the hypothalamus-pituitary-ovarian axis, which controls follicular development and ovum formation **(Figure 2.7)**. In brief, GnRH is released in the hypothalamus after photoperiodic stimulation, and it subsequently triggers the synthesis and secretion of FSH and LH in the anterior pituitary. Then, these hormones travel to the ovary, where FSH stimulates follicular growth and LH stimulates steroidogenesis from the follicles (Bahr and Johnson, 1984; Sah and Mishra, 2018). A follicle consists of an oocyte and steroidogenic tissues, including the theca layer and granulosa layer. The theca layer is responsible for the production of estradiol in smaller follicles, while the granulosa layer is responsible for the production of progesterone in F1 and F2 follicles. Plasma estradiol increases 4-6 hours before ovulation, playing a crucial role in the formation of egg yolk by stimulating the liver to produce yolk precursors. LH and progesterone surges occur 4-7 hours before ovulation in laying chickens (Liu et al., 2001). Progesterone continues to drive LH release, leading to the rupture and release of the mature F1 yellow follicle. It also associates with avidin production, contraction of the myometrium, and eggshell formation (Mishra et al., 2019). In chickens, the interval between two ovipositions is 24-27 hours, while the interval between oviposition and ovulation of the next ovum is about 30 minutes (Scanes, 2022).

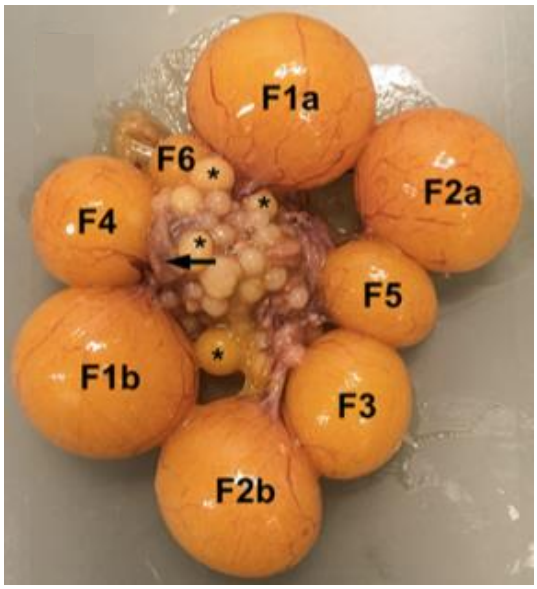

Figure 2.6. Hierarchy of chicken ovarian follicles (van der Klein et al., 2020). The image shows the ovary of a broiler breeder. Arrows indicate postovulatory follicles, asterisks (*) indicate examples of pre-recruitment follicles, and preovulatory follicles are indicated as F1-F6, with "a" and "b" indicating follicles that are similar in size (double hierarchy).

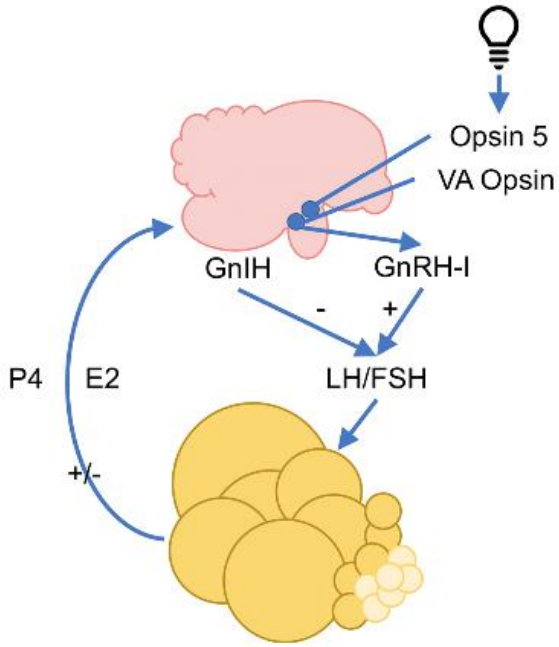

Figure 2.7. Schematic overview of the hypothalamus-pituitary-gonadal axis (van der Klein et al., 2020). Abbreviations: GnIH = gonadotropin inhibitory hormone; GnRH-I = gonadotropin releasing hormone I; LH = luteinizing hormone; FSH = follicle stimulating hormone; P4 = progesterone; E2 = estradiol-17β. Blue circles represent the paraventricular organ, the pink area represents the brain, and the yellow circles represent the ovary. GnIH and GnRH are released from the hypothalamus and regulate the release of LH and FSH from the pituitary into the circulatory system.

2.4.2. Fertilization

At ovulation, the ovum is surrounded by an inner perivitelline membrane (IPVM) that forms between the granulosa cells and the oolemma during folliculogenesis in the ovary (Brégeon et al., 2022). IPVM is composed of glycoproteins and is orthologous to the mammalian zona pellucida (ZP) (Sasanami et al., 2011). Soon after ovulation, the ovum is captured and guided to the fertilization site in the infundibulum (Bellairs and Osmond, 2014) **(Figure 2.8)**. At the same time, a highly competitive population of sperm is transported to the infundibulum and waits for the ovum there in order to achieve their genetic mission. Once in contact with the IPVM, avian sperm undergo the acrosomal reaction (Okamura and Nishiyama, 1978), releasing acrosomal enzymes to hydrolyze and penetrate the IPVM to fertilize the ovum (Howarth, 1990). There is only a very short window for successful fertilization, approximately 15 minutes **(see Table 2.2)**. Immediately, the outer perivitelline membrane (OPVM) is deposited onto the IPVM by secretory cells in the infundibulum to act as an antimicrobial protection for the embryo and yolk content (Brégeon et al., 2022). In chickens, the average number of sperm penetrations in a fertilized egg is 82, with a range of 4 to 868 (Hemmings and Birkhead, 2015). Therefore,

physiological polyspermy (the penetration of the ovum by multiple sperm) is considered an essential aspect of the fertilization process in birds. However, an excessive number of sperm interacting with the ovum, known as pathological polyspermy, increases embryo mortality (Bakst and Howarth, 1977). This necessitates the use of OPVM as a physical barrier to prevent its occurrence (Brégeon et al., 2022).

Table 2.2. Passage of the egg through the oviduct (Bellairs and Osmond, 2014).

Oviduct	Time spent	Event
Ovary	0 h	Ovulation
Infundibulum	15 min	Fertilization/thin albumen
Magnum	3 h	Albumen added
Isthmus	15 min	Shell membranes added
Shell gland	20 h	Shell added
Vagina	Negligible	Egg laid

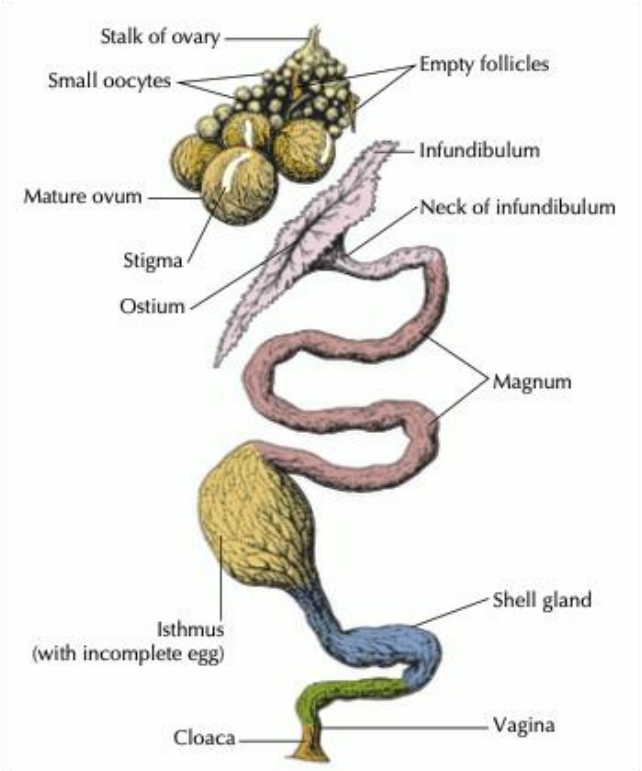

Figure 2.8. Left ovary and oviduct of the domestic fowl (Bellairs and Osmond, 2014).

The germinal disc (GD) is a specific area that can be found on the IPVM. It represents approximately 2% of the total IPVM surface (8 mm^2) (Steele et al., 1994), containing the female genetic material and organelles (Perry et al., 1978). Under *in vivo* insemination conditions, the holes caused by sperm acrosomal hydrolysis are most commonly observed in the GD area (Okamura and Nishiyama, 1978). However, during *in vitro* incubation of sperm and IPVM segments, no differences were observed between different regions in the number of holes or in the profile of IPVM proteins (Steele et al., 1994). These findings suggest that the factors involved in sperm-ovum attraction are not associated with IPVM. Sperm-associated body (SB) is a hydrophobic protein found in both layers of the PVM and oviductal infundibulum. SB contains Ca^{2+} and releases it to activate sperm, which is a necessary process for acrosomal reaction and hydrolytic holes (Rabbani et al., 2007). However, Ca^{2+} does not induce hyperactivation in chicken sperm as it does in mammalian species (Lemoine et al., 2008). This could be a plausible case of divergent evolution, as the acrosomal reaction is triggered by the simultaneous presence of Ca^{2+} and IPVM. There is no need for motility hyperactivation in avian sperm to approach the ovum, as is the case in mammals for crossing the cumulus cells and ZP.

2.4.3. Egg formation

After the formation of the OPVM, the thick albumen surrounds the yolk in the tubular region of the infundibulum. The fertilized ovum then enters the magnum, which is a region with a rich distribution of mucus-secreting epithelium. The watery albumen is added to the ovum before it moves to the isthmus for the deposition of the inner shell membrane. The uterine fluid is derived from the blood, with components selected by epithelial cells and enriched by their own secretions. The uterus, also known as the shell gland, passes this fluid into the albumen to expand the egg to its full size. It also utilizes its mineral ions to generate the outer shell membrane. Then, the longer stay of 20 hours in the oviduct is necessary to complete the formation of the calcareous eggshell. During this period in the uterus, the egg rotates slowly to ensure an even distribution of calcium ions and the proper orientation of the embryo. For more comprehensive reviews on hen's egg formation, see Bellairs and Osmond (2014).

2.4.4. Fertility test

A fertility test is the most reliable way to evaluate the sperm's fertilizing capacity. However, it involves a series of intensive and time-consuming tasks compared to *in vitro* evaluations of sperm quality conducted in the laboratory. After insemination (for a detailed introduction, see section 2.2.2), the fertilized eggs are collected and stored at a temperature of approximately 15-20°C and a humidity level of 75-80% for no longer than 7 days (Fasenko, 2007). Eggs are then placed into an incubator at a temperature of 37-38°C and a humidity level of 50-55% (French, 1997; Noiva et al., 2014). In general, fertility can be tested after 7 days of incubation (Long and Kulkarni, 2004; Thananurak et al., 2019) by using candling. This test involves placing a light bulb on the surface of the egg and observing the internal features from the other side.

A fertile egg can be determined by the presence of an embryo shadow and blood vessels. In contrast, an infertile egg exhibits clear contents.

3. Predictions of sperm fertilizing capacity through *in vitro* evaluations

The chicken sperm are thin and long cells (Lake et al., 1968) **(Figure 3.1)**. The anterior compartment is composed of an acrosome vesicle and an elongated haploid nucleus, which together form the sperm head (Korn et al., 2000). The sperm of non-passerine birds contain a structure known as the perforatorium or acrosomal spine. This structure, composed of fibers and containing actin, is located between the acrosome and the anterior part of the nucleus (Etches, 1996). Passerine birds and most mammals, except rodents, do not possess a similar structure in the subacrosomal region (Baccetti et al., 1980). The length of the perforatorium varies among bird species, with the turkey and rooster having a shorter length of 1.0 µm compared to the guinea fowl's 1.9 µm. The perforatorium is believed to have a skeletal function, providing internal conical shape of the acrosome (Baccetti et al., 1980).

The sperm neck consists in the proximal and distal centrioles, which are composed of microtubules surrounded by a thickened centriole wall. The midpiece includes the mitochondria and an encircled axoneme, followed by an extended fibrous sheath called the principal piece **(Figure 3.2)**. The last part, known as the endpiece, is surrounded by the plasma membrane but does not have a fibrous sheath. The midpiece, principal piece, and endpiece constitute the flagellum (Aire, 2014). For more details on chicken sperm morphology, refer to Jamieson (2011).

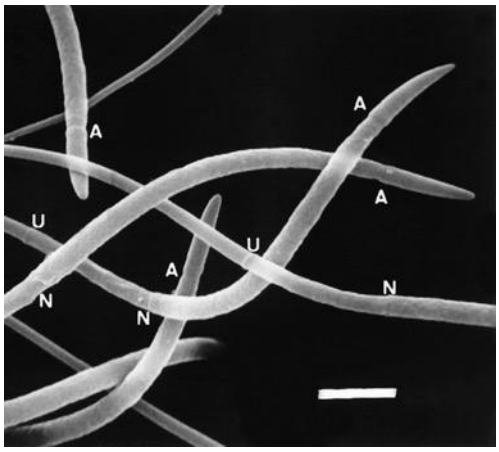

Figure 3.1. Scanning electron micrograph of chicken sperm cells (Vizcarra et al., 2015). The structure at the anterior end of the sperm cell (A) marks the posterior boundary of the acrosome. The nucleus extends posteriorly from point (A) to the neck region (N), which marks the anterior end of the midpiece. The midpiece, which contains the anterior portion of the axoneme and the highly modified mitochondria, extends back to the raised annulus (U). The tail of the sperm extends from the annulus to the end of the cell. At the nucleus, the diameter of chicken sperm is about 0.5 µm, and the overall length of the cell is approximately 90 µm. Bar = 2 µm.

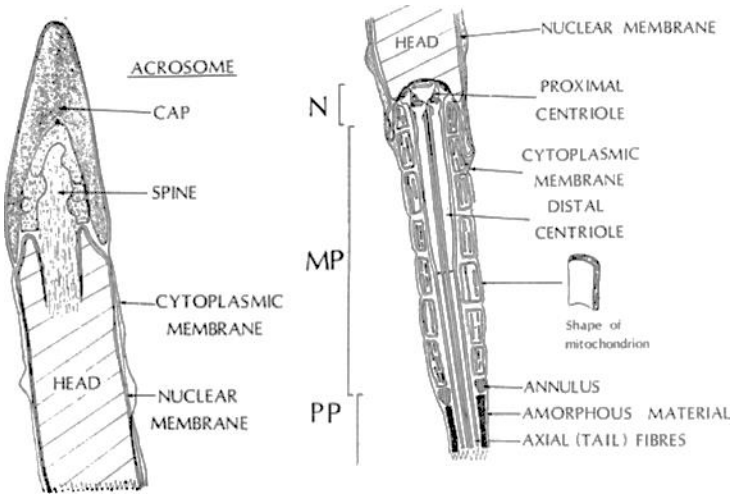

Figure 3.2. Diagrammatic representation of chicken sperm (Lake et al., 1968). Left: A longitudinal section through the acrosome and anterior part of sperm head. The granular material between the acrosome cap and acrosome spine is shown. Right: A longitudinal section through the base of the head, the neck (N), the middle-piece (MP) and the anterior part of the principal-piece (PP) of sperm.

Each sperm feature serves a specific function that is necessary for the fertilization process. In brief, sperm need an intact acrosome and nucleus to penetrate the ovum, achieve fertilization, and support normal embryonic development. Healthy mitochondria and flagella are essential for generating the correct movement pattern when approaching the sperm storage sites and the ovum. Thus, evaluations of these functional structures are regarded as *in vitro* sperm quality parameters, which have the potential to predict *in vivo* sperm fertilizing capacity **(Table 3.1)**. Here, several *in vitro* sperm parameters, including their physiology, will be described, and their analysis methodology will be discussed.

Table 3.1. Correlations (r) between *in vitro* sperm quality parameters and fertility (H.-L. H. Lin).

parameter	Correlation	Reference
Mass motility	**Moderate**, $p > 0.05$	Blesbois et al. 2008
CASA motility	**Moderate**, $p < 0.05$	Blesbois et al. 2008
Membrane integrity	**Strong**, $p < 0.01$	Blesbois et al. 2008
Apoptosis	**Moderate**, $p > 0.05$	Anzar et al. 2002
Sperm-egg penetration	**Strong**, $p < 0.01$	Barbato et al. 1998
DNA integrity	**Strong**, $p < 0.01$	Evenson 2016
ATP production	**Weak**, $p < 0.01$	Chan and Wang 1987
Mitochondrial activity	**No direct evidence***	Gallon et al. 2006

Weak = $r < 0.4$; Moderate = $0.4 < r < 0.6$; Strong = $r > 0.6$; *correlated to sperm morphology, motility and acrosomal reaction.

3.1. Sperm motility

3.1.1. Chicken sperm motility is temperature-dependent

Low temperatures can decrease metabolic activity, greatly inhibiting chicken sperm motility at 0-5°C (Ashizawa and Nishiyama, 1978; Clarke et al., 1982). However, chicken sperm exhibits a specific heat inhibition from 30°C to 40°C, which is regulated by the intracellular Ca^{2+} level through the protein kinase A pathway (Ashizawa et al., 1994, 2009). This phenomenon has been observed in certain avian species, such as rooster, turkey, and drake sperm, but not in Japanese quail (Wishart and Wilson, 1999). These findings highlight the need to modify the measurement conditions of sperm motility among different species. Interestingly, this information also raises the hypothesis that the mechanisms of sperm transport and storage may differ between species, such as chickens and Japanese quails. For the preservation of sperm, the activity of chicken sperm may be hindered when stored in SST due to increased temperature. However, quail sperm may be regulated by different mechanisms.

3.1.2. Mass motility

Sperm flagella generate movement through helical and dextral waves, resulting in a clockwise spinning motion when a group of sperm move together (Vernon and Woolley, 1999). The mass motility is a parameter that evaluates the overall movement performance of a group of sperm. The motility score is defined by the movement (yes or no, fast or slow) and the motion pattern (forward and whirlwind). It can be classified on different scales in various laboratories, making it quite subjective. Mass motility can be observed using a light microscope, making it an economical, fast, and simple method. While a strong correlation has been found between mass motility and fertility in sheep (David et al., 2015), a moderate correlation ($r = 0.47$, $p > 0.05$) has been observed between mass motility and fertility **(Table 3.1)** in chickens (Blesbois et al., 2008).

3.1.3. Individual motility

Computer-assisted sperm analysis (CASA) is another technique used to evaluate sperm motility. It analyzes individual cell movements instead of global ones. It was first introduced in the 1970s (Dott and Foster, 1979) with the development of a successive image capturing system and an automatic sperm tracking algorithm platform for quantitative analysis of individual sperm movement characteristics. Despite the fact that a CASA system can provide accurate and reliable examinations, implementing a standard protocol is essential to prevent bias. For example, the accurate classification of sperm motility relies on correctly configuring cell sizes and sperm trajectory parameters in the analysis software (Amann and Waberski, 2014). In addition, the preparation of semen samples also needs to be considered. Factors such as sperm concentration, diluent compositions, pH value, temperature, and slide module can all lead to significantly different results (Broekhuijse et al., 2011; Kathiravan et al., 2011; Yang et al., 2019).

CASA is an efficient method for obtaining numerous results of sperm motion kinetics within seconds of operation. It has been found to have a moderate correlation with chicken fertilizing capacity (Blesbois et al., 2008) **(Table 3.1)**, supported by $r = 0.5$ and $r = 0.6$ ($p < 0.05$) respectively for the percentage of motile and progressive sperm.

3.2. Sperm viability

3.2.1. Membrane integrity is an indicator of sperm viability

The plasma membrane serves as a direct barrier against changes in the extracellular environment and enables cells to maintain homeostatic regulation. The loss of plasma membrane integrity would definitely cause disruptions in normal cell functions and ultimately result in cell death. Necrosis, apoptosis, and autophagy are three classical types of cell death, each displaying distinguishable features through specific signaling pathways (Fink and Cookson, 2005; Chen et al., 2018). Despite autophagy being generally regarded as a degradation pathway, it can also induce cell death (Ryter et al., 2014). Necrosis is a passive and accidental process of cell death, typically caused by environmental injuries. It leads to cell swelling and membrane breakage (Choi et al., 2019). In contrast, apoptosis is characterized as an active, programmed, and autonomous cellular process. It involves the regulated expression of specific genes and induces particular morphological features, such as cytoplasmic and nuclear shrinkage, DNA fragmentation, and the formation of apoptotic bodies. This process is characterized by blebbing but an intact plasma membrane (D'Arcy, 2019).

3.2.2. Losing membrane integrity: necrosis

Necrosis is activated when cells are exposed to external injuries, such as extreme temperatures, pressure, chemicals, or osmotic shock (Murao et al., 2021). It is usually a rapid process that causes cell swelling and bursting due to the loss of osmotic pressure. Cell rupture leads to the release of biomolecules, known as damage-associated molecular patterns (DAMPs), also referred to as danger signals or alarms. DAMPs are subsequently recognized by immune cells, which stimulate an inflammatory response. This response ultimately triggers the regeneration process or cell lysis (Vénéreau et al., 2015).

3.2.3. Apoptosis

Apoptosis involves two primary signaling mechanisms, namely the extrinsic and intrinsic pathways. The extrinsic event, known as the death receptor-mediated apoptosis pathway, is activated by the binding of the corresponding Fas ligand, TRAIL, or TNFα (tumor necrosis factor α) receptors (Lavrik et al., 2005; Chen et al., 2018). The adaptor protein FADD and the procaspase-8 protein together form a complex known as the death-inducing signaling complex (DISC). In DISC, procaspase-8 is transformed into caspase-8, which either directly activates caspase-3 to induce apoptosis or first hydrolyzes Bid to truncated Bid (tBid). Then, it interacts

with Bax/Bak in mitochondria to induce apoptosis **(Figure 3.3)**. The other event, known as the intrinsic mitochondrial-dependent pathway, begins when an injury occurs within the cell. The Bcl-2 protein family controls the intrinsic pathway by regulating the permeability of the mitochondrial outer membrane, which enables the release of cytochrome c and Apaf-1 into the cytoplasm. This leads to the activation of caspase-9, which initiates a series of proteolytic events.

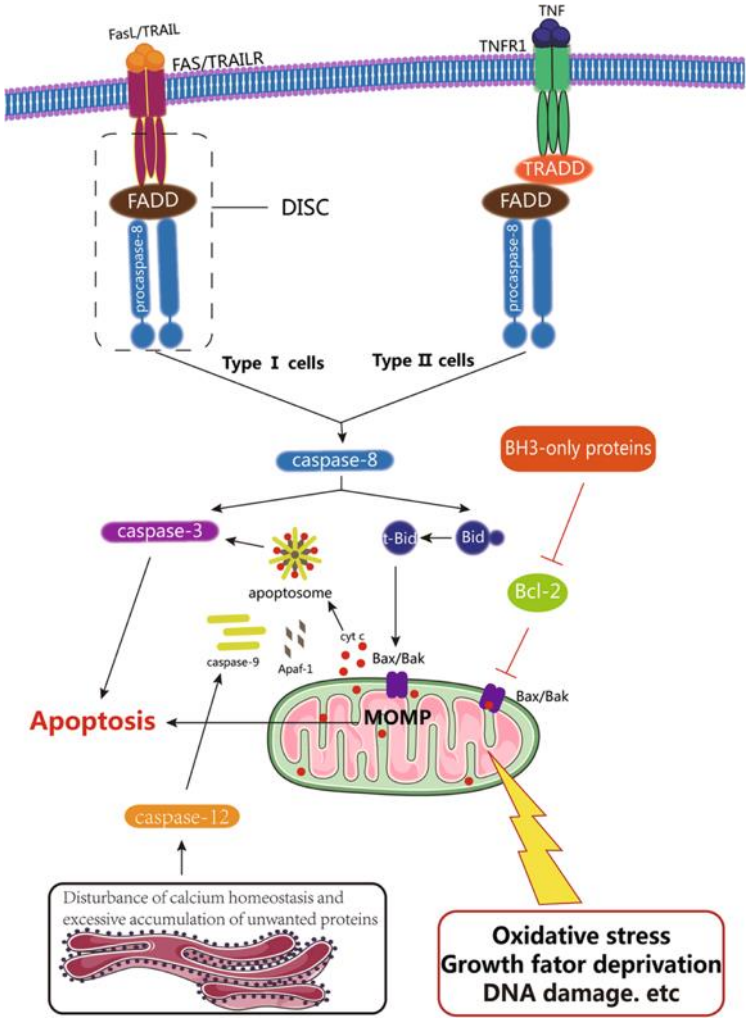

Figure 3.3. Mechanisms of apoptosis (Chen et al., 2018).

3.2.4. Cell death tests

Membrane integrity

The definition of viable sperm is often associated with the integrity of the plasma membrane, rather than its ability to move. This is because the membrane is crucial for sperm to interact with other cells and the external environment (Hossain et al., 2011). The correlation between sperm viability and fertility has been recognized in both avian and mammalian species (Gillan

et al., 2005; Blesbois et al., 2008) **(Table 3.1)**. Therefore, the routine evaluation of sperm quality in a spermatology lab often includes the analysis of sperm motility kinetics and membrane integrity. A dual fluorescence staining of SYBR-14 and Propidium iodide (PI) is commonly used to assess the integrity of the plasma membrane. SYBR-14 (green fluorescence) can penetrate cells and bind to DNA, regardless of whether the membrane is intact or damaged. In contrast, PI (red fluorescence) can only penetrate cells with a damaged membrane and then bind to DNA. As an alternative to using a fluorescence microscope, eosin and nigrosin stains (Sharma and Agarwal, 2021) can be used for the differential staining of intact and damaged cells. Eosin can only penetrate damaged membranes (pink), thus the intact membrane remains unstained (white) against a blue background (nigrosin). However, fluorescent staining allows for greater contrast and interpretation between viable and dead cells, resulting in a more accurate and effective outcome compared to eosin-nigrosin staining (Chalah and Brillard, 1998; Watts, 2019; Glazar and McCue, 2021).

For the observation of fluorescent signals, both fluorescence microscopy and flow cytometry can be used to detect the quantity of fluorescent cellular components. Flow cytometry cannot distinguish the exact location of the fluorescence inside the cells. The data is presented at a whole-cell level. In contrast, fluorescence microscopy can show how fluorescence is distributed inside the cells. However, flow cytometry is capable of handling a greater number of cells than fluorescence microscopy, up to several thousand cells, which allows for a more representative observation (Ryding, 2018).

Apoptosis

Several studies have shown that necrosis and apoptosis coexist in semen fractions (Lachaud et al., 2004; Feitosa et al., 2008; Fraczek et al., 2015), and both have effects on fertility **(Table 3.1)**. At the onset of apoptosis, cells lose their phospholipid membrane asymmetry and expose phosphatidylserine (PS) on the cell surface, which is a unique characteristic of apoptotic cells (Naito et al., 1997; Lee et al., 2013). Annexin V is a 36-kDa calcium-binding protein that specifically binds to anionic phospholipids, including PS. Commercially available fluorescently tagged Annexin V can be used to detect early apoptotic cells (Crowley et al., 2016; Abbady et al., 2017). The advantages of this technique include the ability to detect the initiation of apoptosis and its high sensitivity, which allows for the detection of a single apoptotic cell (Elmore, 2007). Annexin V is typically conjugated with fluorescein isothiocyanate (Annexin V-FITC), which emits green fluorescence. Dual staining with propidium iodide (PI), a red-fluorescent label for necrotic cells, can be employed to differentiate necrotic cells from early apoptotic cells within the same semen fraction. In addition, this dual staining technique allows for the detection of both apoptotic and necrotic cells simultaneously, resulting in a more efficient methodology and potentially improving the prediction of sperm fertilizing capacity.

3.3. Acrosome integrity

3.3.1. Acrosomal reaction (AR) in avian sperm

A successful fertilization depends on a series of signaling pathways during the interaction between sperm and ovum. First, sperm cells need to bind to and penetrate the outer coverage of the ovum. This structure is known as the inner perivitelline membrane (IPVM) in birds, and it is analogous to the zona pellucida (ZP) in mammals. However, sperm interaction with the ovum in birds does not exhibit such a stringent species-specific barrier as in mammals and marine invertebrates (Wassarman et al., 1999; Stewart et al., 2004). In chickens, the IPVM has identified six glycoproteins: ZP1, ZP2, ZP3, ZP4, ZPD, and ZPAX (Smith et al., 2005). Among these, ZP1 and ZP3 are the major glycoproteins (Ichikawa et al., 2017). Once the acrosomal area of the sperm contacts the ovum, the IPVM immediately triggers the acrosomal reaction (AR). This reaction involves the fusion of the outer acrosomal membrane with the plasma membrane, as well as the rupture of the apical region to release the contents of the acrosome. This release is necessary for the degradation of the extracellular matrix, allowing for sperm penetration **(Figure 3.4)**. Sperm acrosin, an acrosomal proteolytic enzyme, is released during AR and is responsible for the hydrolysis of IPVM. It binds to N-linked glycans on ZP1, inducing local lysis of the IPVM (Horrocks et al., 2000; Bausek et al., 2004; Sasanami et al., 2007, 2011). In addition to ZP1 and ZP3, a recent study showed that ZP4 may be critical for sperm binding, specifically at the germinal disk region (Benson et al., 2022).

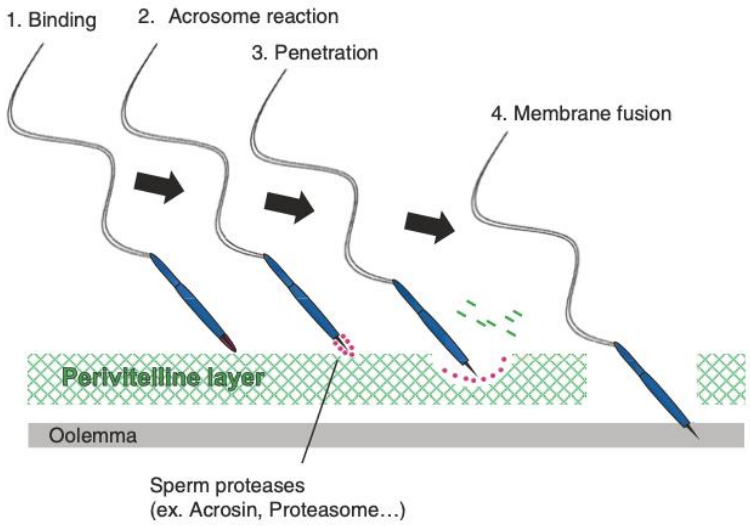

Figure 3.4. Sperm binding, proteolytic degradation, dissolution of the ovum perivitelline layer matrix, and penetration (Nishio and Matsuda, 2017). A series of interactions between sperm and the IPVM of the ovum presumably occur in the infundibulum of the oviduct during a short period of time (around 15 minutes in chickens) after ovulation.

In mammals, successful fertilization requires the prior capacitation of sperm in the female genital tract. This process includes the hyperactivation of flagella beating, which facilitates the penetration of the zona pellucida (ZP) and the oocyte. This can be reproduced *in vitro* through species-specific sperm treatments such as heparin, caffeine, and ionophores. It has been accepted that avian sperm can achieve fertilization without the need for the capacitation process in the female reproductive tract. However, recent studies have shown that chicken sperm, when incubated under mammalian capacitating conditions, can increase their spontaneous AR (Lemoine et al., 2008; Priyadarshana et al., 2018). This suggests that avian and mammalian sperm may have a similar mechanism for inducing AR. Despite the mechanisms of how avian sperm initiate AR remaining poorly understood, recent studies have shown a novel glucose signaling pathway **(Figure 3.5)** responsible for inducing AR through AMPK activation (Ushiyama et al., 2019). Another pathway, mediated by sperm membrane rafts **(Figure 3.6)**, regulates AR via PKA (Priyadarshana et al., 2018).

Keeping acrosome integrity until the time for AR is necessary for sperm cells to ensure their fertilizing ability. The CITI1/SPINK2 protein, identified in chicken seminal plasma, is responsible to keep acrosin inactive (Thélie et al., 2019b). Considering that avian sperm generally stay inside SST for a longer period of time, leading to a hypothesis of a specific controlling pathway to keep sperm acrosomal functionality during SST storage (Priyadarshana et al., 2020).

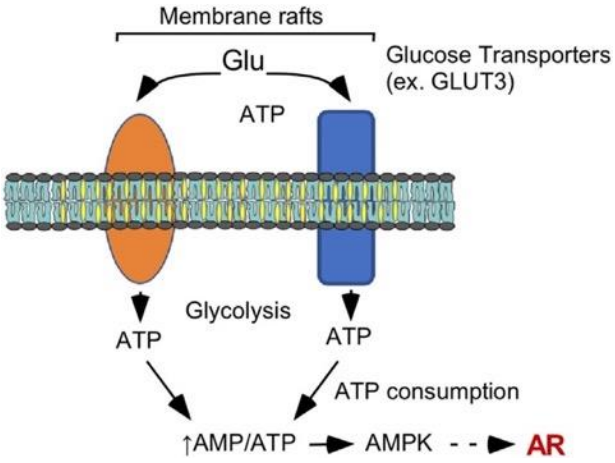

Figure 3.5. A schematic model for the mechanism of glucose-dependent AR (Ushiyama et al., 2019). The plasma membrane of sperm contains multiple membrane rafts that mediate glucose uptake, which is dependent on specific transmembrane transporters, such as GLUT3. An increase in glucose uptake results in the elevation of adenosine monophosphate (AMP) relative to adenosine triphosphate (ATP), possibly through glycolysis and ATP-consuming pathways. This leads to AMPK activation and further enhances acrosomal responsiveness to IPVL stimulation.

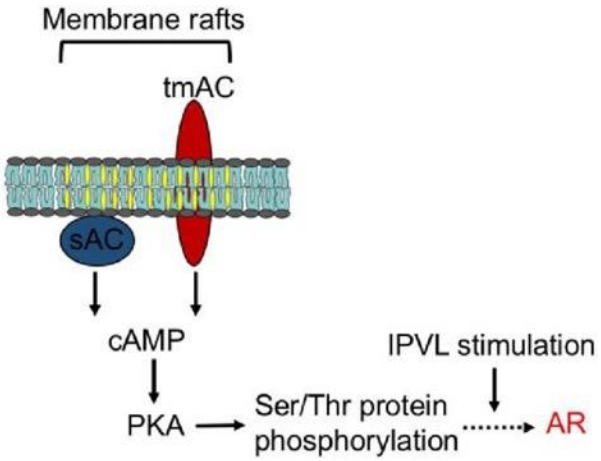

Figure 3.6. Schematic model of membrane raft regulation of AR in chicken sperm (Priyadarshana et al., 2018). The plasma membranes of sperm consist of multiple membrane rafts that act as scaffold/assembly platforms for the functional regulation of sAC and tmAC. This interaction supports the production of cAMP, thereby enhancing the phosphorylation of PKA substrate proteins, leading to the induction of AR in response to IPVL stimulation.

3.3.2. Sperm acrosome tests

Sperm-egg penetration test: counting the number of hydrolytic holes in the PVM

In birds, the ovum acquires yolk in the same compartment with PVM synthesis, leading to the difficulty of *in vitro* observation of sperm-egg interaction. Therefore, several alternative experimental models have been developed. For example, the huge size of avian oocytes offers the possibility to purify ZP proteins for sperm-egg interaction studies (Bausek et al., 2004; Sasanami et al., 2007; Ichikawa et al., 2017). The observation of holes in IPVM is another model to qualify sperm acrosomal status. The number of holes observed after *in vitro* incubation of sperm with IPVM sheets reflects the number of sperm that bind to IPVM and successfully perform normal acrosomal functions to create holes for sperm penetration, which is necessary for fertilization to occur. This measurement is considered predictive of *in vivo* fertility (Donoghue, 1999) **(Table 3.1)**. In addition to collecting IPVM from follicular oocytes (Howarth, 1990; Lemoine et al., 2008; Sasanami et al., 2011), the isolation of PVM from laid, nonfertilized eggs has been introduced to test sperm fertilizing ability as well (Steele et al., 1994; Robertson et al., 1997). This method is certainly more convenient and respectful of animal welfare.

The vitrification of PVM is possible to prolong its storage for a more flexible use (Bongalhardo et al., 2009). A novel sperm-binding assay, designed by coating chicken PVM extract onto microplates and directly incubating it with sperm, is able to quantify the number of sperm binding (Barbato et al., 1998). In addition to their use in avian species, these novel techniques may also be applicable to mammals. This is because PVM has already been used to detect the fertilizing potential of mammalian sperm (Amann et al., 1999; Losano et al., 2015; Campos et al., 2017).

Test combined with AR stimulation and FITC-PNA staining

Peanut agglutinin (PNA), which exclusively binds to the glycoconjugates of the outer acrosomal membrane or matrix, is widely used to assess the acrosomal status of sperm (Cheng et al., 1996; Lemoine et al., 2011). The PNA, which is often labeled with fluorescein isothiocyanate (FITC), is used to detect acrosomal loss in sperm cells due to its intense green fluorescence. The examination of hydrolysis holes in the PVM serves as a valuable method for evaluating the sperm's capacity to undergo AR and predicting fertility. However, manipulating thin PVM and calculating hole quantities pose technical complexities and require a significant amount of time. Therefore, an alternative approach that can be considered is the induction of AR through the utilization of IPVM (Koyanagi et al., 1988; Lemoine et al., 2011), followed by staining with FITC-PNA. The impact of the treatment that induces AR will indicate the percentage of sperm undergoing AR within the population.

3.4. Nucleus

3.4.1. DNA integrity in avian sperm

One of the primary functions of sperm is to transfer male genetic material, the integrity of which is critical for successful embryonic development (Kumaresan et al., 2020). The sperm nucleus appears as an extremely condensed chromatin structure, which serves to protect the genome during its long journey before fusing with the haploid genome of the oocyte (Ward, 2010; Blesbois, 2012). However, such protection is still not sufficient to avoid DNA damage during the harsh freezing-thawing processes, which can result in a significant level of DNA fragmentation (Gliozzi et al., 2011; Santiago-Moreno and Blesbois, 2020). In addition to the risk of DNA damage occurring during freezing and thawing, it can also occur in germ cells during spermatogenesis, particularly during the crossing-over recombination that takes place at the meiotic prophase. Thus, it may be important to confirm the DNA integrity in individual sperm cells to ensure their fertilizing capacity.

3.4.2. DNA integrity tests

DNA integrity analyses were introduced in the 1980s (Küçük, 2018), including the single cell gel electrophoresis (COMET), sperm chromatin structure assay (SCSA), acridine orange test (AOT), and terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay. Recently, an innovative protocol called double staining of acridine orange (AO) and LIVE/DEAD Fixable Blue (LD) has been developed to distinguish sperm populations with intact membranes and no DNA fragmentation (Da Costa et al., 2021). In addition to its simplicity, sperm populations still retain the potential for insemination or cryopreservation after AO/LD staining.

3.5. Sperm bioenergetic network

3.5.1. Adenosine triphosphate (ATP) production in avian sperm

Adenosine triphosphate (ATP) is the primary molecule responsible for storing and releasing energy in cells (Pinna et al., 2022). When ATP is hydrolyzed to adenosine diphosphate (ADP), energy is released to meet the cell's energy needs. The reversible switches between ATP and ADP are achieved through the addition or removal of phosphate groups. Sperm movement requires ATP hydrolysis as the bioenergetic source (Cosson, 2012), mainly generated via two pathways: glycolysis and mitochondrial oxidative phosphorylation (OXPHOS). The primary mechanism of sperm ATP generation may vary among species and remains controversial regarding the main energy factory, glycolysis or OXPHOS (Hereng et al., 2011; Asano and Tajima, 2017). In sperm, the main challenge is the distant energy export along the entire flagellum, which leads to the hypothesis that sperm possess various metabolic strategies for ATP generation (Du Plessis et al., 2015). In birds, mitochondrial ATP synthesis has been suggested as a significant energy source for sperm motility (Froman and Feltmann, 1998). However, recent studies propose that both glycolysis and OXPHOS are both responsible for flagellar motility (Setiawan et al., 2020). Furthermore, OXPHOS is considered the primary pathway for providing the energy required for sperm movement to penetrate the IPVM (Setiawan et al., 2021).

Glycolysis

Although the production of ATP molecules is less efficient, the glycolytic pathway is faster, does not require oxygen, and produces ATP that is localized in the principal piece **(Figure 3.7a)**. This localization is crucial as it provides ATP where it is most needed for generating sperm movement (Amaral, 2022). In chickens, the membrane transporter GLUT1 has been confirmed to play a significant role in sperm motility mediated by glucose (Setiawan et al., 2020). Glycolysis converts glucose into pyruvate through a series of enzyme-catalyzed reactions, resulting in a net production of two ATP molecules from each glucose molecule consumed **(Figure 3.7b)**. According to the cellular oxygen levels, pyruvate subsequently enters two possible pathways. Under aerobic conditions, pyruvate is oxidized to acetyl-CoA, which enters the Krebs cycle **(Figure 3.7c)**. In the Krebs cycle, acetyl-CoA is further oxidized to CO_2, and NAD+ is regenerated. In anaerobic conditions, pyruvate is fermented to lactate, which is associated with the conversion of NADH to NAD+. This conversion allows for the reintegration of the glycolytic pathway and enables additional ATP production (Sengupta et al., 2020).

Oxidative phosphorylation (OXPHOS)

Mitochondrial OXPHOS, which occurs only when oxygen is available, is the most efficient pathway for generating ATP (Castellini et al., 2021). OXPHOS is a series of oxidation-reduction reactions that occur in the inner mitochondrial membrane **(Figure 3.7c)**, which is referred to as

the electron transport chain (ETC). It consists of five protein complexes and two electron-transfer molecules, ubiquinone (Q) and cytochrome C (C). The complexes I, II, III, and IV correspond to the successive transport of electrons from NADH, succinate, and then O_2, pumping 4 protons into the intermembrane space. Subsequently, the complex V (ATP synthase) drives protons back into the mitochondrial matrix while facilitating ATP synthesis (Amaral, 2022).

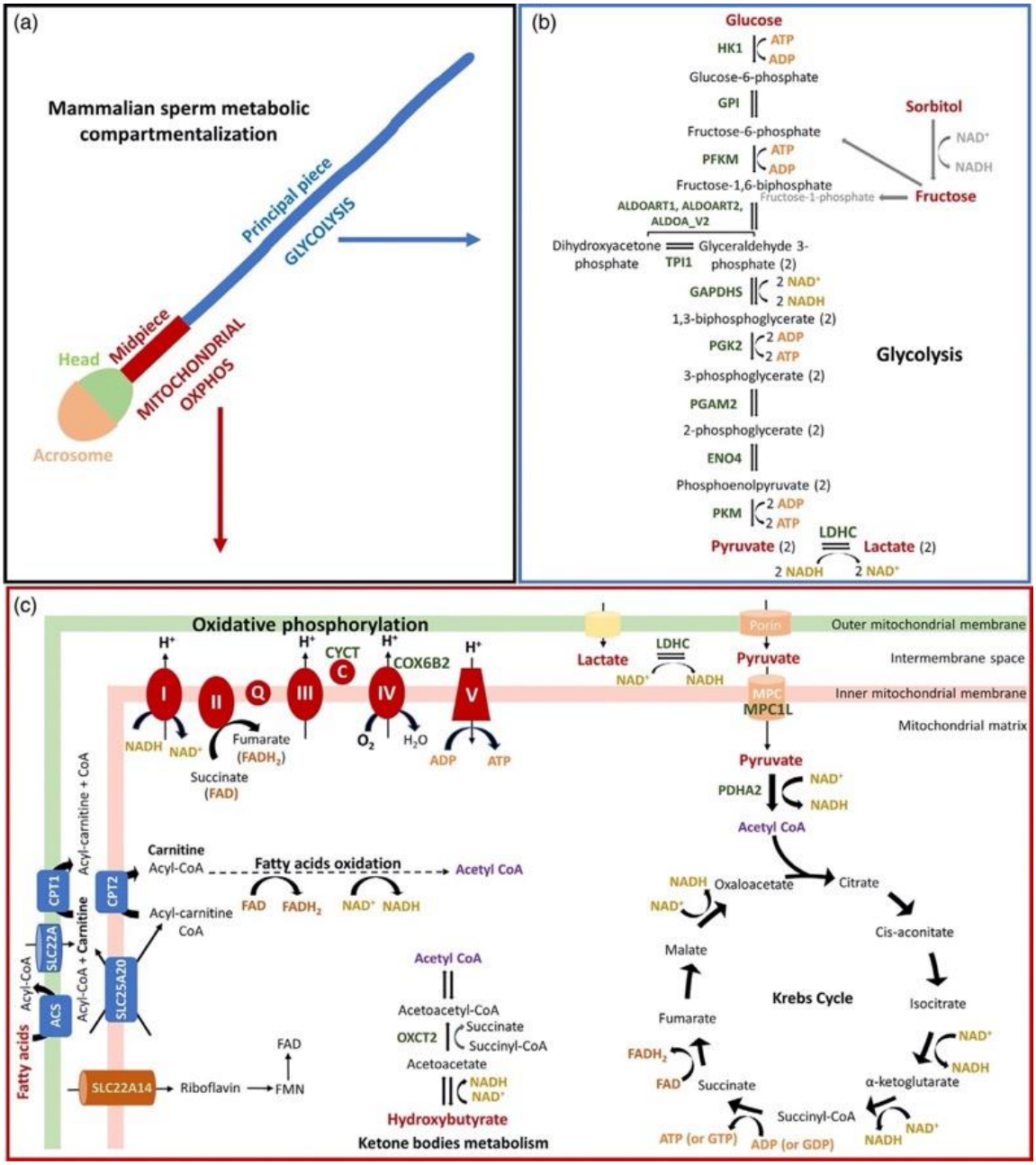

Figure 3.7. Overview of the energy metabolic pathways responsible for ATP generation in mammalian sperm. (Amaral, 2022). (a) represents the mammalian sperm cell, with the main subcellular parts and bioenergetics compartmentalization indicated: oxidative phosphorylation (OXPHOS) in the midpiece and glycolysis in the principal piece. (b) and (c) indicate the detailed metabolic pathways of glycolysis and oxidative phosphorylation.

3.5.2. ATP synthesis tests

ATP is certainly essential to support sperm movement and fertilization. Therefore, its correlations with other sperm quality parameters and fertility can be expected (Comhaire et al., 1983). However, the results vary across studies (Jeyendran et al., 2022; Nikitkina et al., 2022). Such differences may result from the dynamic nature of ATP content, as sperm continue to produce and consume ATP after ejaculation. In addition to directly monitoring ATP content, measuring mitochondrial activity (Paoli et al., 2011; Amaral et al., 2013; Gallo et al., 2021) can be an alternative method for assessing mitochondrial OXPHOS energy production. The fluorescent carbocyanine probe, JC-1, is commonly used to detect mitochondrial membrane potential (Gravance et al., 2000). Sperm mitochondria are labeled with JC-1, which is a green-fluorescent monomer that distinguishes them at low membrane potential. At higher potential, JC-1 forms red-fluorescent aggregates (Gliozzi et al., 2017).

3.6. Searching for additional sperm biomarkers

Sperm quality evaluation is primarily divided into two categories: structural evaluation and functional evaluation. Generally, multiple tests are required on a single semen sample to more accurately predict fertility. However, the truth is that it is still difficult to link *in vitro* quality tests to *in vivo* fertilizing capacity, even when combining several *in vitro* evaluations (Wang and Swerdloff, 2014; Castro et al., 2018; Morrell et al., 2018). Occasionally, animals with normal sperm quality parameters may experience infertility, and the reason for this is unknown. This highlights the need for additional biomarkers to improve fertility predictions.

3.6.1. Transcriptomics profile

Transcriptomic technology (next-generation sequencing or NGS) is a high-throughput method for identifying and quantifying RNA transcripts, understanding global cell functions, and studying gene regulation, which serves as a parameter for male fertility (Urena et al., 2022; Indriastuti et al., 2022). In addition to NGS, single-cell sequencing can provide extensive information to discriminate between different cell types related to male infertility, based on their RNA contents (Hermann et al., 2018; Di Persio et al., 2021). Sperm chromatin is tightly packed during spermiogenesis, leading to the hypothesis of very limited transcriptional events in sperm (Ren et al., 2017). However, it is generally accepted that mature sperm carry thousands of small non-coding RNAs (sncRNAs), which accumulate during spermatogenesis and post-testicular modifications (Alves et al., 2020; Sellem et al., 2022). Although their roles remain to be fully interpreted, a growing number of sperm-borne microRNAs have been found to be associated with early embryo development (Liu et al., 2012; Wang et al., 2017; Alves et al., 2019). This suggests their potential as relevant biomarkers for predicting fertility.

3.6.2. Proteomics profile

Since it is widely acknowledged that translation is highly limited in mature sperm (Gur and Breitbart, 2006; Urena et al., 2022), semen proteomics approaches using high-resolution mass spectrometry for identification and quantification may enable the identification of biomarkers for individual male fertilizing capacity (Peddinti et al., 2008; Kwon et al., 2015; Selvam et al., 2019). Recently, differences in the nature and/or abundance of protein compositions have been identified between fertile and subfertile males, either in sperm or seminal plasma (Labas et al., 2015; Soler et al., 2016). The differentially expressed proteins are involved in molecular pathways such as sperm movement, mitochondrial activities, sperm maturation, storage, and fertilization (Vitorino Carvalho et al., 2021). They may represent new tools to distinguish the reproductive potential of animals.

4. Chicken semen cryopreservation

Review paper: Chicken semen cryopreservation: importance of cryoprotectants

4.1. Abstract

Conservation of animal genetic resources is a crucial strategy for maintaining biodiversity and facilitating the rapid spread of genetic progress in animal husbandry. Keeping genotypes of living animals is feasible, but it always carries the risk of disease transmission and involves expensive maintenance fees. As an alternative strategy, *in vitro* germplasm cryopreservation overcomes these disadvantages and offers additional advantages, such as long-term storage, long-distance and low-cost delivery, and efficient management of cryobanking space due to the compact size of sample storage. Different from mammals, it is currently not possible to preserve oocytes or embryos in avian species using existing techniques. However, the same goal can be achieved through the cryopreservation of sperm, primordial germ cells (PGCs) and gonadal tissues. In these methods, sperm cryopreservation is the most practical option due to its efficient protocols for sample collection, simple procedures for generating offspring, respect for animal welfare through non-invasive manipulations, and high success rate compared to the others.

Beginning with semen collection, chicken sperm undergo a series of *in vitro* processes for cryopreservation in order to keep able to fulfill their biological mission of fertilization, provided they can overcome the challenges along this lengthy journey. These include pre-freezing, freezing, thawing, and insemination. In addition, it is necessary to screen the quality of sperm in individuals before collection to ensure their fertilizing capacity after thawing. Several factors, such as proper management and operational proficiency, also influence the quality of fresh semen. After entering the pre-freezing stage, several decisions need to be made. These include determining the dilution method, selecting cryoprotectant agents (CPA), choosing the storage device type, determining the cooling rate, and deciding on the thawing method.

Glycerol (GLY) is the first discovered CPA that has successfully cryopreserved chicken sperm, resulting in the production of chick offspring. However, GLY is associated with fertilization failure when inseminated, which has been a persistent issue since the 1950s, particularly in chickens. The standard protocol, which includes 11% GLY, provides the best protection for rooster sperm against cryodamage. However, only 2% of GLY present in semen at insemination is enough to induce a reduction in fertility, while 6% of GLY leads to complete infertility. So far, we have partially discovered that GLY can impact the biological characteristics of sperm, including motility, respiration metabolism, and acrosomal reaction. It may also alter sperm deposition and protein secretion in the female reproductive tract. Despite having been discussed for the past 70 years, there is still very limited information available to explain why it is so harmful to fertilizing capacity of sperm cells.

It is a dilemma whether to appreciate its excellent cryoprotection or to describe its toxic properties. Therefore, it remains the most commonly used CPA for chicken sperm cryopreservation. Meanwhile, a specific technique known as serial dilution centrifugation (SDC) has been developed for GLY removal prior to insemination, which has achieved acceptable fertility rates in chickens. SDC certainly gives GLY the opportunity to participate in sperm cryobanking. However, this technique requires specific equipment, complex working procedures, and is time-consuming. Therefore, there is a need to develop an easy, fast, and flexible method to improve the efficiency of utilizing frozen-thawed chicken semen. At the same time, gaining a better understanding of the cellular and molecular mechanisms that contribute to GLY toxicity and fertilization failure could be beneficial in developing a new and effective strategy to manage GLY toxicity.

This introduction section is summarized as a review article titled "Chicken semen cryopreservation: importance of cryoprotectants", that has been published by World's Poultry Science Journal in November 2021.

4.2. Résumé

La conservation des ressources génétiques animales est une stratégie cruciale pour maintenir la biodiversité. Elle peut aussi faciliter et accélérer la diffusions des progrès génétiques obtenus par la sélection. Conserver les génotypes par l'élevage d'animaux vivants est réalisable mais entraîne des frais d'entretiens élevés et n'évite pas les risques de transmission de maladies. Une stratégie alternative, la cryoconservation *in vitro* des génotypes d'intérêt, passe outre ces inconvénients et présente également d'autres avantages tel que le stockage à long terme, la possibilité de transport sur de longues distances et une réduction des couts. Contrairement aux mammifères, la conservation des ovocytes ou des embryons n'est pas possible dans les espèces aviaires avec les techniques existantes. Nous pouvons atteindre le même objectif avec la cryoconservation des spermatozoïdes (SPZ), des cellules germinales primordiales (CGP) et des tissus gonadiques. Parmi ces méthodes, la cryoconservation des SPZ est la plus efficace en raison de protocoles simples pour le prélèvement d'échantillons, et pour générer des descendants (insémination), dans le respect du bien-être animal grâce à des manipulations non invasives et présentant un taux de réussite élevé par rapport aux manipulations des CGP ou des tissus gonadiques.

En commençant par la collecte de la semence, les SPZ de coq subissent une série d'aventures *in vitro* afin de remplir leur mission biologique, la fécondation. La réussite dépend de la façon dont ils peuvent surmonter les défis rencontrés pendant ce long voyage. Les SPZ doivent faire face et vaincre plusieurs obstacles au cours des processus de cryoconservation par la pré-congélation, la congélation, la décongélation et l'insémination. Il est nécessaire de contrôler la qualité des SPZ chez les individus d'intérêt pour s'assurer de ses capacités de fécondation après décongélation. Plusieurs points, tels que la gestion des animaux en routine et la compétence opérationnelle, déterminent la qualité de la semence fraîche. Après être entré dans l'étape de pré-congélation, d'autres décisions d'être prises, y compris la méthode de dilution, le choix des argents cryoprotecteurs (ACP), le type de contenant de stockage, la vitesse de refroidissement et la méthode de décongélation et d'insémination.

Le glycérol (GLY) est le premier ACP avec lequel on a pu congeler les SPZ de coq et donner naissance à des poussins. Cependant, le GLY est associé à un échec de fécondation s'il est présent dans la dose inséminée. C'est un problème historique depuis les années 1950, en particulier chez les poulets. Le protocole standard, 11% de GLY, offre la meilleure protection des SPZ de coq contre les dommages liés à la congélation. Malheureusement, la présence de seulement 2% de GLY présent dans la semence lors de l'insémination est suffisante pour induire une réduction de la fertilité alors que 6% de GLY rend les inséminations totalement infertiles. Jusqu'à présent, nous savons partiellement que le GLY peut avoir un impact sur les caractéristiques biologiques des SPZ en matière de mobilité, de métabolisme respiratoire et de réaction acrosomique. Il peut aussi altérer le dépôt des SPZ et la sécrétion de protéines par le

tractus génital femelle. Même si la toxicité du GLY a été étudiée au cours de ces 70 années, encore très peu d'informations ont été présentées pour expliquer pourquoi il est si nocif lors de l'insémination.

Il y a donc un dilemme entre l'excellente cryoprotection du GLY et sa toxicité, il reste cependant l'ACP le plus utilisé pour la cryoconservation des SPZ. Une technique spécifique, connue sous le nom de dilution sériée et centrifugation (DSC), a donc été développée pour l'élimination du GLY avant l'insémination, atteignant des taux de fertilité acceptables. Cette technique implique des équipements spécifiques, des procédures de travail complexes et longues, ce qui nous a conduit développer une méthode plus facile, rapide et flexible pour améliorer l'efficacité d'utilisation de la semence congelée de coq. Dans le même temps, une meilleure compréhension des mécanismes cellulaires et moléculaires sous-jacents à la toxicité du GLY et à l'échec de la fécondation pourrait être utile pour obtenir une nouvelle stratégie pour contrôler la toxicité du GLY.

Cette introduction a fait l'objet d'un article de revue intitulé "La cryoconservation de la semence de coq : l'importance des agents cryoprotecteurs", publié par le World's Poultry Science Journal en novembre 2021.

4.3. Article

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Chicken semen cryopreservation: importance of cryoprotectants

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SUMMARY

Sperm cryopreservation is an important strategy to conserve animal genetic diversity and transmit superior genetic background, facilitated by a non-invasive sampling and collection of large amounts of sperm. In chickens, various freezing protocols and cryoprotectants (CPAs) have been developed to freeze sperm, but each lab still debates and competes to disclose the most suitable freezing conditions. Many key points, such as semen donor selection, collection tips, diluent compositions, CPA selection, pre-freezing manipulation, semen packaging type, freezing and thawing rates, are all impactful to the efficiency of chicken sperm cryopreservation. Whereas glycerol is believed as the most efficient and the less toxic CPA, it also presents an unwanted contraceptive effect after insemination. Thus, one of the major developed strategies to conquer this issue is removing glycerol before insemination, but current protocols remain not efficient to completely abolish glycerol contraceptive effect. Novel methodologies will bring us a better understanding of the cellular and molecular mechanisms involved in sperm cryobiology to decipher the challenges of chicken sperm cryopreservation.

KEYWORDS

Chicken; semen; freezing; cryoprotectant; glycerol; contraception

Introduction

Conservation of animal genetic resources is an indispensable strategy to protect biodiversity, to restore endangered breeds or species, to assure animal population from unpredictable epidemics or breeding accidents in animals (Boettcher *et al.* 2013; Mara *et al.* 2013; Joost *et al.* 2015), including fish (Cabrita *et al.* 2010; Torres *et al.* 2016), birds (Blesbois 2011; Rakha *et al.* 2016; Svoradová *et al.* 2018) and mammals (Moore and Hasler 2017; Morrell and Mayer 2017). Conservation of genetic resources for the restoration of genetic diversity can be achieved by different methods of storage of cells able to show a reproductive potential, depending on the species. They mainly include the cryopreservation of sperm, embryos (in mammals), gonadal tissues, and, in specific species, primordial germ cells (chicken)(FAO guideline 2021 *in press)* or stem cells (mouse) (Silva *et al.* 2015). Unlike mammalian species, it is not presently possible to preserve avian oocytes or embryos due to the macrolecithal characteristics of the egg

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(Blesbois 2018), including the presence of a very big oocyte (3–4 cm of diameter in the hen) with a very high amount of lipids (yolk) extremely difficult to cryopreserve (Long 2006, 2013; Nakamura 2016). Three germplasm types showing complementary approaches are presently available for cryobanking in poultry: semen, primordial germ cells (PGCs) and gonadal tissue (FAO guideline 2021 *in press)*. Whereas all of these methods show advantages and limits, sperm cryopreservation based on the collection of large amounts of cells in the respect of animal welfare remains the most suitable approach for long-term storage, long-distance genetic exchange and genetic resources management (Çiftci and Aygün 2018; Thélie *et al.* 2019; Cardoso *et al.* 2020). Chicken sperm cryopreservation consists of a long *in vitro* journey for sperm cells including several stages: collection, preparation, freezing and thawing before restoring sperm fertilising capacity and oocyte fertilisation through artificial insemination (AI) process (Figure 1). Whereas current procedures result from more than 60 years of progress, started with the first experiments of semen cryopreservation in 1940s (Shaffner *et al.* 1941) resulting to the first progeny 10 years later using glycerol as CPA (Polge 1951), no standardised protocol has been developed for all chicken breeds/lines. In addition to the relative high cost of sperm freezing and storage compared to maintain the rooster population, and the noticeable decrease in fertility compared to fresh semen, sperm cryopreservation remains in a confidential usage to the progress and commercial application in poultry industry (Donoghue and Wishart 2000). Currently, glycerol is definitely the most broadly used CPA for freezing chicken sperm. It can provide excellent protection for sperm to resist the damages from frozen/thawed processes (Blesbois and Brillard 2007; Blesbois *et al.* 2007; Thélie *et al.* 2019). However, it needs a complex removal procedure before AI. Conversely, CPAs such as dimethyl acetamide (DMA), dimethyl sulphoxide (DMSO) and dimethyl formamide (DMF) are not necessary to be removed prior to AI but give less protection for chicken sperm to oppose the cryodamages.

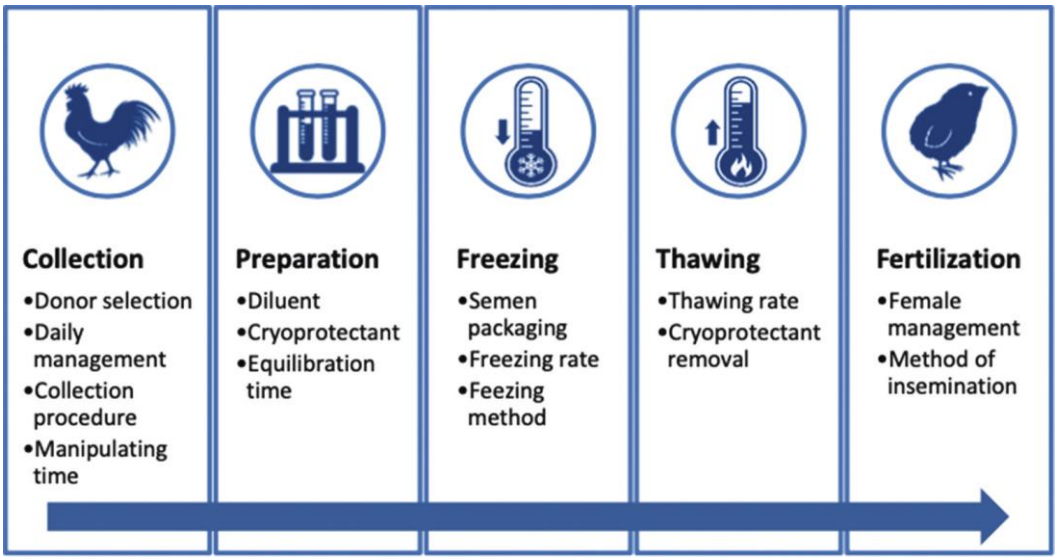


Figure 1. The different stages and influencing factors of chicken sperm cryopreservation.

In this review, we first expose the different main factors impacting chicken sperm cryopreservation, with a special focus on CPAs as one of the major sources of success influence. Subsequently, the issues relative to the historical and widely used CPA, the glycerol, are discussed. Finally, we will present new inspirations for implementing frozen/thawed chicken semen as a tool for practical reproductive strategy in animal husbandry.

Critical points of chicken semen cryopreservation

Initial semen quality

Male fertility

One of the major factors influencing male fertility is the genetic background. Thus, different chicken breeds show different capacities for sperm cryopreservation (Blesbois *et al.* 2007). Another very important point is the male age. Indeed, at the puberty, the young males do not show rapidly an optimal sperm production and quality and then show variations with the annual cycle of reproduction under photoperiodic control (De Reviers *et al.* 1981). This quality increases after some weeks but usually decreases in the second part of the sexual season. As an example, in commercial broiler breeder males reared under daily photoperiod 14 h light:10 h dark and proper management, the optimal age to collect semen for cryopreservation is usually between 30 and 40 weeks (unpublished observations). The length and efficiency of the sexual seasons vary also a lot with external factors including climate, photoperiod, food and water, consequently influencing sperm production and quality (Tabatabaei *et al.* 2010; Shanmugam *et al.* 2014; Rakha *et al.* 2017).

Chicken semen collection

Chicken semen is generally collected by a non-invasive method, the dorso-abdominal massage (Burrows and Quinn 1937). This method usually involves two persons, one for abdominal massage to make a gentle eversion of the cloacum and to access the opening of the deferent duct and the ejaculation reflex (Mohan *et al.* 2018), and the other for the semen flow into a container. There is a risk of contamination of semen with other cloaca products (i.e. faeces, urates, transparent fluid, reviewed by Etches 1996), which is deleterious to sperm conservation, that could be avoided by the well-training and experienced skills of the collectors (Mohan *et al.* 2018). Another point to reduce the possibility of contamination is temporarily restriction of feed and water (12 hr) before semen collection (Yadav *et al.* 2019), that is why it is recommended to collect in the morning. Furthermore, routinely collecting semen from donors (2–3 times a week) increases semen quality (De Reviers 1973).

Fresh semen quality

It is necessary to scan fresh semen quality from males to select the candidates for semen collection. *In vitro* quality tests, especially sperm motility (e.g. mass motility and percentage of motile spermatozoa), sperm viability and sometimes membrane fluidity, have been reported to explain 85% of the variation of fertility of cryopreserved sperm in chickens (Blesbois *et al.* 2008).

Key points of the cryopreservation process

After making sure the best animal conditions, the success of chicken sperm cryopreservation depends on several biophysical and biochemical factors (Figure 1).

Manipulating time

Duration of operating chicken semen from collection to freezing highly impacts sperm cryopreservation. Indeed, neat semen stored *in vitro* at an ambient temperature more than 15 min gradually lose the fertility potential (Blesbois and Brillard 2007), due to a combination of factors including very high metabolic, functional and also proteolytic activities (Lemoine *et al.* 2009; Nguyen *et al.* 2014). Thus, the first recommendation is to manage the shorter possible the time between semen collection and freezing (Vasicek *et al.* 2015).

Diluents, CPAs and equilibration time

Semen diluents can supply continuous physicochemical stability to sperm during *in vitro* storage (Blesbois and Brillard 2007). Iso-osmotic (330–450 milliosmoles) and closely natural pH (6.8–7.4) are adjusted by mixing balanced salt/buffer solutions (Blesbois 2012). They usually contain energetic substrates and products expected to make easier the freeze-thaw process (see after). Dilution rates are also important; the rates 1:3 to 1:5 are usually suggested (Sexton 1981; Seigneurin and Blesbois 1995; Santiago-Moreno *et al.* 2011). Dilution rates might lead to different sperm concentrations during freezing. However, few studies discussed this issue in chicken sperm and showed no significant differences in the fertility (Moghbeli *et al.* 2016b; Pérez-Marín *et al.* 2019).

There are two classes of CPAs: the intracellular and the extracellular ones. Intracellular CPAs such as glycerol, dimethyl sulphoxide (DMSO), dimethyl acetamide (DMA), methyl-acetamide (MA), ethylene glycol (EG), dimethyl formamide (DMF) and also some monosaccharides, are amphipathic and low molecular weights components that can diffuse through cell membrane to minimise cell damages by regulating the formation of intracellular ice crystals, and have been used extensively for chicken sperm cryopreservation (Donoghue and Wishart 2000). Extracellular CPAs, e.g. polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), sucrose, trehalose (disaccharides) and raffinose (trisaccharide), generally form a shield surrounding cells, which can protect cells by reducing extracellular ice crystals and making easier the dehydration of the freezing process (Massip *et al.* 2004; Motta *et al.* 2014; Thananurak *et al.* 2019).

Sperm metabolism must be decreased for *in vitro* storage, which is practically achieved by lowering semen temperature (Lake and Ravie 1982; Giesen and Sexton 1983). Notably, semen temperature must be decreased gradually to prevent harmful cold shock effects (Clarke *et al.* 1982; Wishart 1984). A 2–4°C equilibration is usually applied to semen to add the CPAs with the lowest possible interaction with sperm metabolism before freezing and to ensure an equilibration of CPAs and sperm (Blanco *et al.* 2000; Mphaphathi *et al.* 2016). The best equilibration time depends on the CPAs and on the experimental conditions, varying from 1 min with DMA to 1–2 hours with DMSO (Sexton 1981; Santiago-Moreno *et al.* 2011; Zaniboni *et al.* 2014).

Semen packaging types

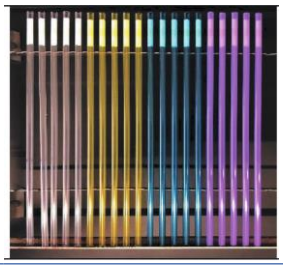
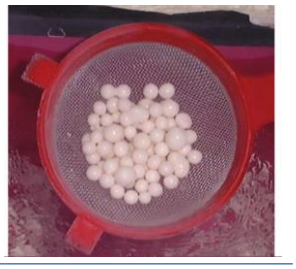
There are three most popular packaging: glass ampoules, plastic straws and pellets (Table 1). Glass ampoule was the first type of container introduced for chicken semen storage (Lake and Stewart 1978). Then, plastic straws and pellets (semen drops) were developed (Sexton 1980; Tselutin *et al.* 1995). Plastic straws facilitate individual identification and storage and also are less harmful than glass ampoule if the burst happens (Pickett and Berndtson 1974; Pickett *et al.* 1978; Mortimer 2004). Pellets are a rapid freezing method, increasing freezing homogeneity compared to plastic straws or glass ampules, and require less equipment (Brotherton 1990). Among these alternatives, considering handling and storing capacity, biosecurity and precise identification of individual animal, plastic straws might be the priority to use for the cryobanking (Tselutin *et al.* 1999; Woelders *et al.* Hiemstra 2006; Blesbois 2007b, 2007a).

Whereas no impact of packaging was observed by Abouelezz *et al.* (2015b), Tselutin *et al.* (1999) reported higher fertility with chicken semen frozen with 6% DMA in pellets than with 6% DMA in straws. The fertility obtained from frozen/thawed semen with 6% DMA in pellets was equivalent to 11% glycerol in straws, which suggested both were optimal combinations of CPAs/packaging. However, the differences between studies might be owing to other side effects (lab conditions, chicken breeds management) confirming that a lot remains to investigate to precisely define the optimal CPA/packaging combination.

Freezing rates

The first really efficient chicken sperm cryopreservation method using glycerol and slow freezing rates, was reported by Lake and Stewart (1978). This method was further improved and one of its adaptation (Seigneurin and Blesbois 1995) was validated for retaining sperm fertilising capacity up to 18 years (Thélie *et al.* 2019). Freezing rates can be conducted by a programmable freezer or by layering semen straws in different distances of the vapour above the surface of liquid nitrogen. Certainly, the former

Table 1. Comparisons of different semen packaging types.

	Glass ampoule	Plastic straw	Pellets
Packaging types			
Advantages	• First introduced type • Precise identification	• Less harmful when the burst happens • High biosecurity • Precise identification • Efficient storage system	• Simple equipment • Efficient heat homogeneity
Disadvantages	• High risk of shattering • Inefficient heat homogeneity	• Heat homogeneity less efficient than pellets	• Low biosecurity • Indistinguishable identification

method is more precise to control the freezing rates. However, it is expensive and not always available, especially in the field working. Different freezing rates affect post-thaw sperm motility, viability and fertilising ability (Madeddu *et al.* 2016; Thélie *et al.* 2019). Furthermore, optimal freezing rates depends on the CPAs/packaging combination (Sexton 1979). For example, the use of glycerol/straws is conducted with quite low semen freezing rate in the first part of the curve (i.e. 7°C/min up to −35°C), while the use of DMA/pellets may allow a direct plunging in liquid nitrogen. In this last case, the freezing rate depends on the size of the pellet but approximately 600°C/min (Woelders *et al.* 2006). The semen freezing rates suggested for DMF/straws and EG/straws are, respectively, −15 and −1°C/min (Thélie *et al.* 2019).

Thawing rates

Adjusting thawing rates moderate the water crystals growing and minimise damage to sperm. Chicken semen thawed at 5°C or 60°C, respectively, with semen frozen with glycerol or DMA could lead to desirable fertility (Tselutin *et al.* 1999). Chicken sperm better respond to slow thawing, which depends on freezing rate (Bellagamba *et al.* 1993). Nevertheless, keeping semen at low temperature (5°C) after thawing could be benefit to chicken sperm quality since the infra-physiological temperature might reduce the sperm metabolism (Lake and Ravie 1982; Giesen and Sexton 1983) and the reaction of lipid peroxidation (Fujihara and Howarth JR 1978), and the physiological temperature would be reached only at the time of insemination.

Critical points related to post thawing conditions

CPAs removal before AI – glycerol case

The glycerol shows an original effect possibly specific to chicken species, leading an inevitable contraception. One important strategy against glycerol contraceptive effect is to remove it before AI (Table 2). One suggested solution is the dialysis of frozen/thawed fowl semen using Cellophane bags (Polge 1951) or CryoCell® container (Buss 1993). However, this method is time-consuming and causes cell damages due to the imbalanced rates between glycerol and water transport.

Another approach is stepwise dilutions followed by centrifugation (Clark and Shaffener 1960; Lake and Stewart 1978). Although this method is time-consuming (but much less that dialysis) and complex, it has become the most common operation to reduce glycerol concentrations from chicken frozen/thawed semen (Seigneurin and Blesbois 1995; Tselutin *et al.* 1999; Abouelezz *et al.* 2017; Thélie *et al.* 2019) (Figure 2).

Table 2. Comparisons of glycerol removal methods.

Removal methods	Species	Time	Fertility	References
Slow dialysis	Chicken	2 hr	54%	Polge (1951)
CryoCell® dialysis	Chicken	1.5–2 hr	56%	Buss (1993)
One-step dilutions + centrifugation	Chicken	7 min	40%	Clark and Shaffener (1960)
Stepwise dilutions + centrifugation	Chicken	33–45 min	63%	Lake and Stewart (1978)
Stepwise dilutions + centrifugation (simplified)	Chicken	27 min	76%	Seigneurin and Blesbois (1995)
Accudenz centrifugation	Chicken	25 min	37%	Long and Kulkarni (2004)
Accudenz centrifugation	Turkey	25 min	49%	Long and Kulkarni (2004)
Percoll® centrifugation	Turkey	30 min	19%	Long and Kulkarni (2004)

The third method for glycerol removal implied colloidal centrifugation, based on Accudenz or Percoll® solution to separate sperm from glycerolised semen (Long and Kulkarni 2004). Purdy *et al.* (2009) compared the glycerol removal effects of stepwise dilutions and Accudenz centrifugation and showed higher motility obtained with Accudenz procedure but a higher number of recovered sperm with stepwise dilution centrifugation, suggesting efficiency specificities of each procedure. Improvement of concerning colloidal centrifugation is still considered and recently, Lin *et al.* (2020) reported a new protocol for the selection of fresh motile chicken sperm based on Percoll® single-layer centrifugation (SLC). Further investigation will be necessary to confirm if this procedure would be suitable for chicken sperm cryopreserved with glycerol.

Methods of AI for frozen/thawed chicken semen

Intravaginal insemination (IV) (the place of natural mating) is the most common AI technique, developed by Quinn and Burrows (1936). Including welfare-concerned issue, it involves applying gently pressure to the hen's abdomen and everting the vaginal orifice through the cloacum, then sperm deposit into female vagina (3–4 cm deep), allowing normal selection of the best sperm for fertilisation and not disturb the lay. However, to improve the fertility of frozen/thawed chicken semen, other AI methods have been developed, including intrauterine (IU), intramagnal (IM), and intraperitoneal (IP) inseminations (Figure 3). With IU procedure, semen is directly deposed into female uterus by a glass cannula (Allen and Bobr 1955). IM insemination surgically deposits semen into the magnum part of the female oviduct (Bacon *et al.* 1986). With the IP technique, the surgical sperm deposition is performed into peritoneal cavity near the infundibulum (Brown *et al.* 1963). IU, IM and IP show deleterious effects on the laying rate (Allen and Bobr 1955; Brown *et al.* Hobbs 1963; Long and Kulkarni 2004), and IV insemination is also the cheaper technique.

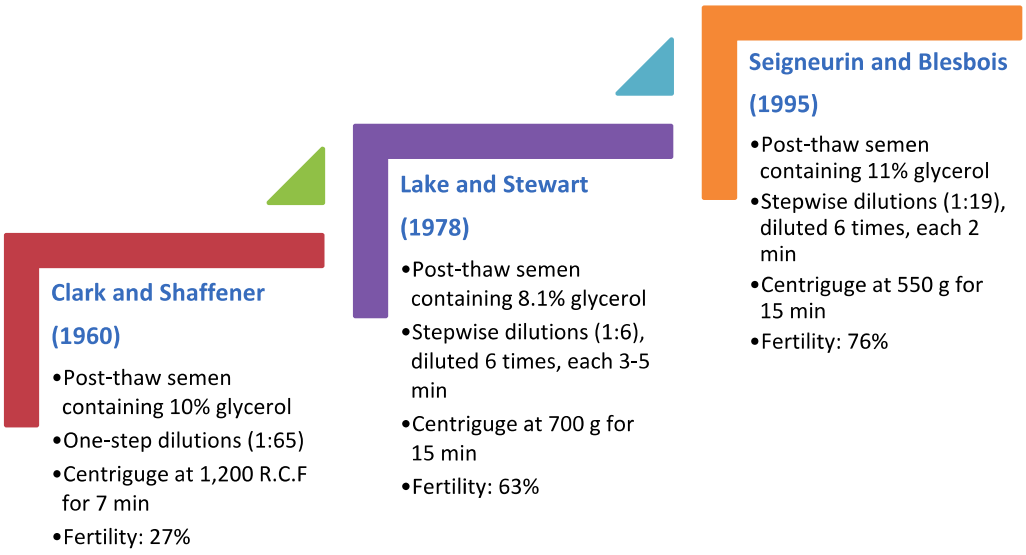


Figure 2. Progress of stepwise dilutions for chicken post-thaw semen.

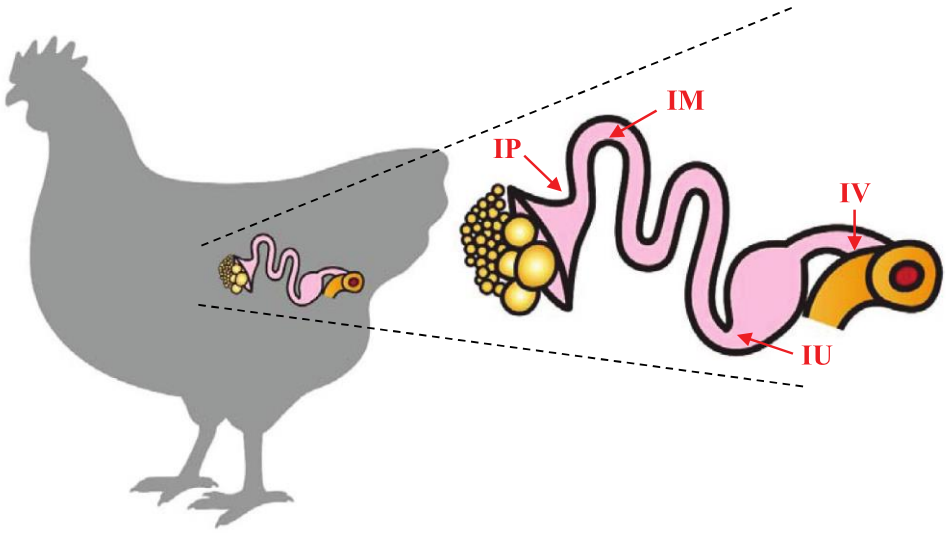


Figure 3. Methods of AI in chicken. Intravaginal (IV), intrauterine (IU), intramagnal (IM) and intraperitoneal (IP) insemination deposit sperm respectively in the vagina, uterus, magnum and peritoneal cavity near the infundibulum.

The complex choice of cryopreservation medium

As previously described, intracellular CPAs are extensively used for chicken sperm cryopreservation. Their length of entry in sperm is highly variable (one min for glycerol (Lemma 2011) to nearly 3 times longer for DMSO in human sperm (Gilmore *et al.* 1997) and depends also on sperm specificity. Each intracellular CPA can cause specific cytotoxicity (membrane breach, oxidative stress, osmotic shock and chilling injury) (Best 2015), leading no consensus to identify the most suitable CPA for chicken sperm cryopreservation (Table 3).

Whereas Miranda *et al.* (2018) concluded that EG was the most suitable CPA to conserve the motility of frozen/thawed sperm when compared to DMF, DMA and MA; Thélie *et al.* (2019) showed higher fertilising ability of semen frozen/thawed with glycerol when compared to DMF, DMA and EG that showed the lowest results. But Abouelezz *et al.* (2017) showed contrasted effects of glycerol and DMA. The tremendous differences between these results certainly results from complex interactions involving CPAs concentration, extenders, freeze-thaw procedures, animal characteristics and differences in semen quality tests.

In addition to intracellular CPAs, the use of extracellular CPAs, alone or in combination with intracellular CPAs has been explored (Table 3). PVP is classically employed in complement to glycerol (Lake and Stewart 1978; Seigneurin and Blesbois 1995). Egg yolk or its purified components (e.g. egg low-density lipoprotein, LDL) have been used to

Table 3. The effects of various intracellular and extracellular cryoprotectants on chicken frozen/thawed semen.

Cryoprotectants	Diluent	Packaging	Semen quality test	The more recent references
Intracellular only				
6% DMA	Kobidil	Straw	Motility	Miranda *et al.* (2018)
6% DMF	BHSV	Straw	Fertility	Thélie *et al.* (2019)
7.5% DMF	Kobidil	Straw	Motility	Miranda *et al.* (2018)
8% EG	Kobidil	Straw	Motility	Miranda *et al.* (2018)
10% EG	BHSV	Straw	Fertility	Thélie *et al.* (2019)
9% MA	Kobidil	Straw	Motility	Miranda *et al.* (2018)
Intracellular + extracellular				
5% DMA + 0.3% PVP	Lake	Pellet	Fertility	Abouelezz *et al.* (2017)
6% DMA + PVP	FEB	Pellet	Fertility	Tselutin *et al.* (1999)
6% DMA + PVP	FEB	Straw	Fertility	Thélie *et al.* (2019)
6% DMA + 0.1 M trehalose + 0.3% PVP	Lake	Straw	Motility	Mosca *et al.* (2016)
6% DMF + 1 mM sucrose	BHSV	Straw	Motility Membrane Acrosome integrity Mitochondria Fertility	Thananurak *et al.* (2019)
2% Glycerol + 4% LDL	Beltsville	Straw	Motility Membrane Mitochondria Fertility	Shahverdi *et al.* (2015)
5% Glycerol + 10% egg-yolk	Ringer lactate	Straw	Motility Viability	Junaedi *et al.* (2016)
8% Glycerol + 0.3% PVP	Lake	Straw	Fertility	Abouelezz *et al.* (2017)
11% Glycerol + 0.3% PVP	Lake	Straw	Fertility	Thélie *et al.* (2019)
Extracellular only				
15% egg yolk	RFE	Straw	Motility Viability Membrane Acrosome integrity Fertility	Rakha *et al.* (2018)
6% PVP	RFE	Straw	Motility Viability Membrane Acrosome integrity Fertility	Rakha *et al.* (2017)

DMA: dimethyl acetamide; DMF: dimethyl formamide; EG: ethylene glycol; MA: methyl-acetamide; LDL: low-density lipoprotein; PVP: polyvinylpyrrolidone; RFE: red fowl extender; Kobidil is a commercial diluent; BHSV contains myo-inositol.

cryopreserve successfully domestic chicken semen (Lake 1968; Shahverdi *et al.* 2015; Junaedi *et al.* 2016), or the Red jungle fowl ancestor (Rakha *et al.* 2017, 2018). Moreover, the addition of sucrose to DMF or of trehalose to DMA (Shahverdi *et al.* 2015; Junaedi *et al.* 2016; Mosca *et al.* 2016; Thananurak *et al.* 2019) has been reported to increase post-thaw chicken sperm quality and/or fertility capacities.

Other potential beneficial additives

Avian sperm membrane contains abundant polyunsaturated fatty acids (PUFAs) (Blesbois *et al.* 1997; Blesbois and Hermier 2003; Cerolini *et al.* 2006; Partyka *et al.* 2012; Fattah *et al.* 2017), which can easily be peroxidised (lipid peroxidation – LPO) in the presence of reactive oxygen species (ROS), leading to membrane, acrosome, mitochondria and DNA disruption (O'Connell *et al.* 2002; Bollwein, Fuchs, and Koess 2008). ROS are active during the frozen/thawed process, increasing the sperm cryoinjuries (Isachenko *et al.* 2003). Antioxidants addition to freezing extenders is a possible solution improving sperm quality after thawing (Salmani *et al.* 2013; Zanganeh *et al.* 2013) (Table 4). Many antioxidants have been tested including vitamins, polyphenols, carotenoids and amino acids (Blesbois *et al.* 1993; Mangiagalli *et al.* 2007; Asmarawati 2010; Tabatabaei *et al.* 2011; Al-Daraji 2014; Moghbeli *et al.* 2016a; Partyka *et al.* 2017; Thananurak *et al.* 2020) with variable results.

Sperm membrane fluidity is a biophysical factor linked to the cholesterol/phospholipid ratio of sperm membrane in addition to membrane PUFAs and others. Freezing is a rigidifying process (Blesbois *et al.* 2005; Chuaychu-Noo *et al.* 2017) and modifying the cholesterol/phospholipid ratio of sperm membrane through the addition of cyclodextrins – cyclic oligosaccharide able to remove or add cholesterol into sperm membranes – may affect positively or negatively semen cryopreservation (Purdy and Graham 2004; Mocé *et al.* 2010). Partyka *et al.* (2016; Partyka *et al.* 2018) used cyclodextrins to increase

Table 4. The effects of various beneficial additives on chicken frozen/thawed semen.

Additive	Diluent	Cryopreotectant	Semen quality test	References
Enhance anti-oxidative capacity				
Vitamin E	Beltsville	11% glycerol	Motility Viability Membrane	Moghbeli *et al.* (2016a)
L-carnitine	EK	6% DMA	Mitochondria Apoptosis LPO DNA fragmentation	Partyka *et al.* (2017)
Hypotaurine	EK	6% DMA	Mitochondria Apoptosis LPO DNA fragmentation	Partyka *et al.* (2017)
Serine	BHSV	6% DMF	Acrosome integrity Mitochondria Fertility	Thananurak *et al.* (2020)
Adjust membrane fluidity				
2-hydroxypropyl-β-cyclodextrin	EK	6% DMA	Motility Mitochondria	Partyka *et al.* (2018)

the frozen-thawed chicken sperm quality (Table 4). This is opposite to many observations made on mammalian species (Purdy and Graham 2004) and illustrates well the complexity of the parameters involved in membrane fluidity.

Specific role of glycerol on chicken sperm cryopreservation

Historically, 10–20% of glycerol was used to freeze chicken sperm (Polge *et al.* 1949), but the presence of at least 2% glycerol in inseminated semen was associated with contraceptive effect (Polge 1951), leading to the necessary of removing glycerol before AI. Nowadays, 11% glycerol associated to its removal before AI is certainly the most used freezing conditions for chicken sperm cryopreservation (Blesbois and Brillard 2007; Blesbois *et al.* 2007; Abouelezz *et al.* 2015a; Thélie *et al.* 2019). However, despite the development of efficient approach to obtain progeny from chicken sperm frozen with glycerol, the mechanisms underlying the glycerol contraceptive effect are still unclear, but several theories have been proposed, including direct effects on sperm and on female reproductive tract (Figure 4).

Direct impacts of glycerol on sperm biology

Glycerol (4% and 8%) increased the respiration rate of sperm cells for at least 60 min but reduced sperm motility for only 15 min when chicken sperm were incubated at physiological temperature (41°C) (Sexton 1973), which was not observed with DMSO or EG (Sexton 1974). Glycerol also impacts sperm penetration into the oocyte by reducing the presence of trypsin-like enzyme (TLE) (Johnson *et al.* 1976) involved in sperm penetration of the vitelline membrane of the ovum during fertilisation (Howarth and Digby 1973). However, this reduction of TLE did not correspond to the severe reduction of fertility that may involve other actors of the acrosome reaction pathway (Johnson *et al.* 1976). Indeed, 11% of glycerol in fresh chicken sperm diluents dramatically decreased the ability of sperm to undergo acrosome reaction (Mocé *et al.* 2010). A modification of sperm acrosome reaction capacity induced by glycerol may be one of the causes of the reduction sperm penetration into ovum perivitelline layer. In all experiments, the hatchability/fertility rate was not affected, indicating that glycerol seemingly impaired partial functions for sperm fertilisation but did not disturb embryonic viability (Neville *et al.* 1971; Sexton 1973). These observations do not exclude potential more discreet effects on the development through potential epigenetic changes as we will see in the paragraph IV.

Glycerol impacts on sperm progression in the female reproductive tract

In general, fowl sperm appear in the uterus within 30 min or in the infundibulum within 1 hr after IV insemination (Bakst *et al.* 1994). However, no sperm was found at the top of oviduct when hens were inseminated by IV with semen containing 15–20% glycerol (Polge 1951), which presumed that glycerol may also impact the sperm evolution into the female reproductive tract, as potentially partly resulting from decrease of sperm motility. However, fertility was partly restored when 15% glycerolised fresh semen was inseminated IU (Allen and Bobr 1955; Allen 1958), indicating the problem was not only due to sperm biology but

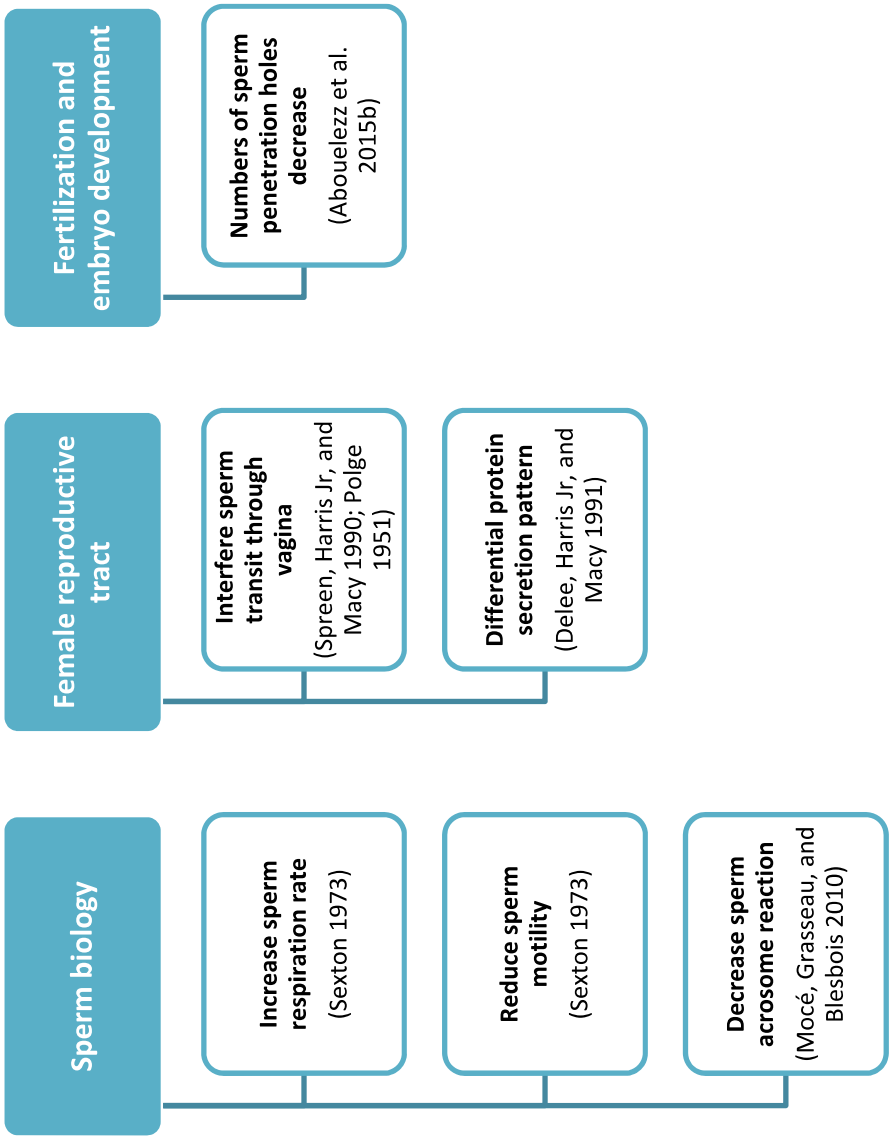


Figure 4. Glycerol deleterious effects in chicken reproduction.

also relative to female physiology especially in the lower oviduct part. These authors suggested that the uterovaginal junction might be a barrier to limit glycerolised sperm reaching the upper parts of the oviduct.

Chicken sperm incubated with glycerol and cells derived from female reproductive tract resulted to higher sperm motility and viability, suggesting these cells were beneficial for mitigating cytotoxicity from glycerol (Latorre *et al.* 1988). Spreen *et al.* (1990) indicated the motility of glycerolised sperm significantly decreased in vaginal culture, which was not observed in other reproductive organ-slice cultures, suggesting a specific deleterious effect of glycerol during the sperm transit through vagina. Differential protein secretion pattern was observed in vaginal-slice culture systems in presence or absence of glycerol, but further investigations will be necessary to decipher the proteins associated to the consequence of glycerol on vagina physiology (Delee *et al.* 1991).

Glycerol introduced 5 min before or after fresh semen IV insemination reduced the fertility more in 5 ~ 7 days than in the first 4 days, which suggested that glycerol disturbs sperm transport and/or storage, this is possibly due to an inflammatory response of hen's vagina which would block general sperm transport for a short time (Westfall and Howarth JR 1977). Interestingly, the fertility was not affected when glycerol was inseminated 10 min before semen, which indicated that the antifertility effect of glycerol only would last less than 10 min in the vagina.

Is glycerol the only CPA necessary to be removed before insemination?

Removing glycerol from post-thaw chicken sperm before AI is a necessity procedure. As developed for glycerol, other CPAs may largely influence the sperm transit into female reproductive tract (Harris JR 1968; Mitchell and Buckland 1976), and this point would be questioned for the future. For instance, IU resulted in superior fertility than IV when the DMSO was used (Mitchell and Buckland 1976). IP also led to higher fertility from chicken semen cryopreserved with DMSO than with IV (Harris JR 1968). However, the fertility obtained with these techniques remains lower than with glycerol stepwise dilutions protocol (Brown *et al.* 1963; Seigneurin and Blesbois 1995; Tselutin *et al.* 1999; Thélie *et al.* 2019). Unquestionably, for specific application, such as cryobank programmes when only few offsprings are needed to restore a genetic breed, these techniques may be useful. But bypassing the IV procedure requires well-trained manipulator but also induces much stress to hens which can easily lead to the dramatic drop in egg production and possibility presents other problem such as infections (Allen and Bobr 1955).

New developments in chicken semen cryopreservation

Improvement in the use of CPAs and their removal after thawing is necessary for the future development of chicken sperm cryopreservation, as well as increasing knowledge in the biochemical mechanisms involved.

We pointed the importance of sperm membrane lipids in the success of freezing, in membrane fluidity demand and biochemical actions (Blesbois *et al.* 1997; Blesbois *et al.* 1997; Douard *et al.* 2000; Blesbois *et al.* 2005), which could possibly be partly re-established by the use of cyclodextrin transporters (Chuaychu-Noo *et al.* 2017; Partyka *et al.* 2018) or other different transporters including lipoprotein complexes.

More discreet mechanisms may be impacted by the cryopreservation procedure. Recent development in omics methods leads to understand better semen molecular actors involved in sperm fertilising ability, such as proteomic studies of chicken semen particularly will be very useful to decipher and identify biomarkers of sperm freezing capacities and fertility (Labas *et al.* 2015). Cheng *et al.* (2015) identified the change in protein abundance in chicken sperm before and after frozen/thawed processes and revealed that most impacted proteins were involved in energy metabolism, hydrolase activity, signal transduction and sperm motility. Other changes have been observed on sperm transcriptome especially on the genes related to proteins involved in stressful conditions such as CIRBP, RHOA, HSP70 and HSP90 (Qi *et al.* 2020). Interestingly, the addition of recombinant HSP90 protein into the extender improved sperm motility and viability, confirming the interest of molecular investigation for the improvement of sperm cryopreservation. Moreover, epigenetic modifications in mature sperm play an important role for the embryo development (Cassuto *et al.* 2016), that may be impacted by sperm cryopreservation as revealed for DNA methylation and histone modifications (Flores *et al.* 2011; Zeng *et al.* 2014; Hezavehei *et al.* 2018). Sperm cryopreservation associated with an elevation of ROS, led to significant reductions of DNA methylation, H3K9 acetylation and H3K4 methylation compared to the fresh semen (Salehi *et al.* 2020). This information could be one clue for the constitution of freezing solutions, including the CPAs, extenders and even antioxidants, which are able to avoid sperm intracellular ROS alterations in epigenetic patterns.

Conclusion

Sperm cryopreservation is an important strategy to protect animal biodiversity, to transmit superior genetic background and to prevent the animal population devastation from epidemics. It is not yet adapted to commercial chicken industry mainly due to the noticeable reduction of fertilising capacity of chicken sperm after freeze/thaw processes. Glycerol is deemed as the most effective and universal cryoprotectant for chicken sperm, but it can also lead contraception to hens if not removed properly before artificial insemination. In contrast, other frequent cryoprotectants, such as DMA, DMSO, DMF, do not require to be removed before insemination, but are less efficient to protect chicken sperm from cryodamages. Therefore, looking for a novel and powerful sperm cryopreservation procedures based on efficient cryoprotectants adapted to most chicken breeds and able to remove any contraceptive effects is mandatory to make chicken sperm cryopreservation more acceptable in practical industry.

In the past half century, scientists made a lot of efforts to verify the best cryopreservation protocol for chicken sperm. Presently, freezing and storing chicken sperm over a long period of time is no longer a fairy tale. Understanding the cellular and molecular regulations involved in the cryopreservation process can be very useful to optimise the use of frozen/thawed semen. Modern omics technology are very powerful techniques that can dig out abundant and insightful information about the mechanisms related to cryobiology, which may help us to find out the solutions to overcome the obstacles.

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5. Glycerol toxicity

5.1. Glycerol is a good intracellular cryoprotectant agent (CPA)

Cryoprotectant agents (CPA) are chemical compounds that provide cell cryoprotection during freezing-thawing processes (Bhattacharya, 2018). Glycerol is one CPA that replaces water and prevents ice crystal formation, thereby reducing internal cryodamage in cells. It can also lower the freezing point of the diluent and improve the sudden increase in sodium chloride concentration during dehydration. In addition, it increases the viscosity during cooling (Brisson et al., 2001; Morris et al., 2006), leading to a kinetic retardation of both ice crystal growth and dehydration speed (Han and Bischof, 2004). These properties make glycerol a highly suitable CPA. However, it works by interfering with the hydrogen bonds of water molecules (Towey and Dougan, 2012), causing non-specific toxicity to the cells (Fahy et al., 2004; Best, 2015). Despite its potential toxicity to sperm cells, glycerol remains the preferred CPA used in the freezing diluent for most species (Villaverde et al., 2013; Papa et al., 2015; Ma et al., 2022; Bang et al., 2022) due to its high cryoprotective efficiency (Holt, 2000).

5.2. Glycerol causes a decline in bird fertility

Glycerol is the first discovered CPA, which made the concept of sperm freezing a reality (Polge et al., 1949). The first offspring was born in 1950 from chicken semen that had been frozen with glycerol (Polge, 1951). Meanwhile, studies have demonstrated that the presence of as little as 2% glycerol in inseminated semen can decrease fertility in chickens (Polge, 1951; Long and Kulkarni, 2004). Furthermore, a higher concentration of 6% glycerol can lead to complete infertility, necessitating the implementation of an additional procedure to eliminate glycerol prior to the insemination of thawed semen. However, the mechanisms underlying the negative effects of glycerol on sperm's fertilizing capacity are still not understood.

5.3. Evidence of glycerol toxicity in mammalian sperm

Changes in sperm structures and functions after exposure to glycerol are illustrated in different species as follows. These findings confirm that glycerol toxicity also affects mammalian sperm **(refer to Figure 5.1)**. It is a seemingly universal issue in sperm biology, with a species-specific pattern of known damage.

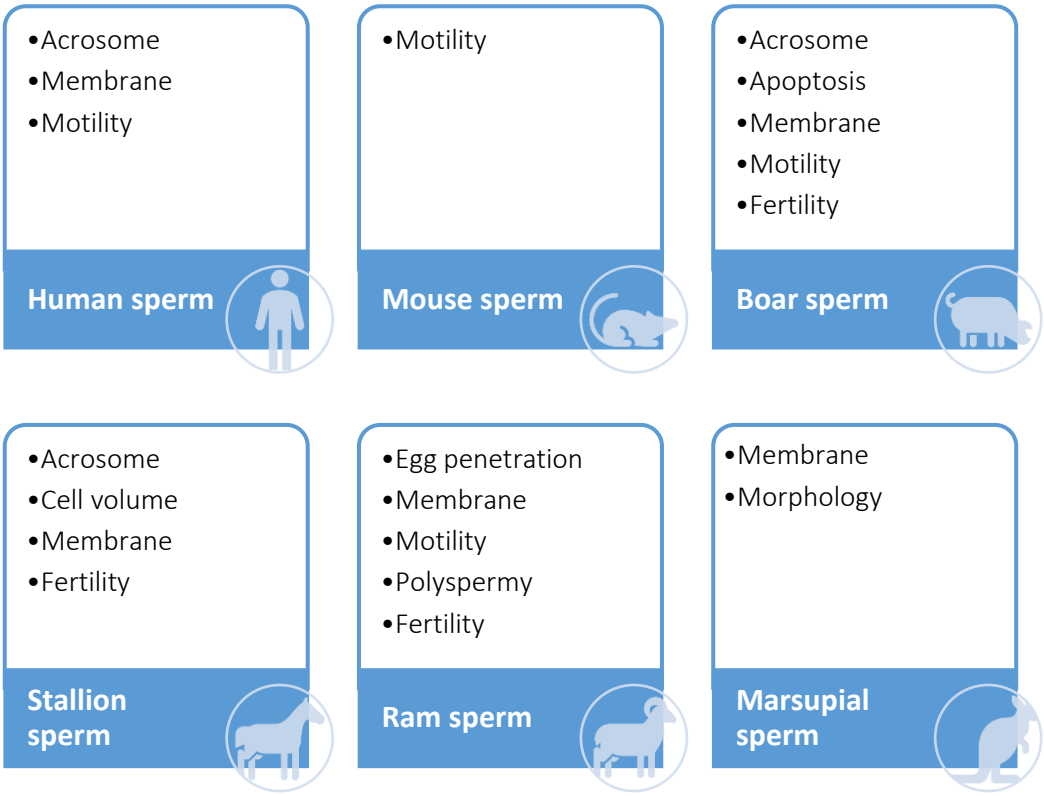

Figure 5.1. Glycerol toxic impacts on sperm biology in mammals (H.-L. H. Lin).

5.3.1. Glycerol affects sperm biology

In humans

During the entire cryopreservation process, human sperm lose 73% of their progressive motility, with 42% of this loss occurring during the glycerol addition. This step has a greater impact on the loss of motility than the freezing-thawing process (McLaughlin et al., 1992). Except for motility, glycerol also affects sperm membrane and acrosome integrity (Barthelemy et al., 1990). Notably, in these studies, sperm were exposed to 7.5% glycerol at room temperature.

In mice

Motility reduction is observed in mouse sperm with increasing glycerol concentrations (from 2% to 6%) at either 0 or 22°C (Katkov et al., 1998). In that study, glycerol was added in several steps to eliminate the effects of osmotic shrinkage or swelling. However, the reduction in sperm motility could not be avoided, indicating that glycerol can have both chemical toxicity and osmotic damage effects.

In boars

A low concentration of 2-3% glycerol is recommended for boar sperm cryopreservation. This is because there is evidence that higher concentrations of glycerol can decrease motility and viability, as well as induce apoptosis (Zeng et al., 2014). Glycerol induces apoptotic death in sperm through both extrinsic and intrinsic signaling pathways. In addition to triggering death signals, glycerol also initiates alterations in acrosomal morphology (Wilmut and Polge, 1974; Buhr et al., 2001).

In stallions

Glycerol is a commonly used CPA for freezing stallion sperm, with concentrations typically ranging from 2.5% to 6% (Vidament et al., 2001). However, a more recent study suggests that the concentration of glycerol in the freezing diluent should not exceed 2.5% to prevent disturbances in sperm biology caused by glycerol toxicity (Macías García et al., 2012). A low concentration of 0.5% glycerol causes an increase in cell volume after a 10-minute incubation at 37°C. Sperm death occurs when the glycerol level exceeds 2.5% during initial exposure, resulting in necrosis rather than apoptosis. Furthermore, glycerol induces rapid depolymerization of F-actin at concentrations exceeding 1.5% (Macías García et al., 2012), suggesting that glycerol may initiate early capacitation (Breitbart et al., 2005).

In rams

Despite the presence of 7% glycerol in ram semen, it does not affect sperm motility and viability at 5°C within 24 hours (Morrier et al., 2002). However, a decrease in fertility was observed after 6 hours of storage with 3% glycerol at 5°C (Abdelhakeam et al., 1991). Sperm exposed to 10% glycerol at room temperature for 10 minutes show higher rates of egg penetration and polyspermy, while penetration activity decreases with longer exposure time of 120 minutes. In general, ram sperm gradually penetrate the zona-free eggs during a 16-hour culture. However, sperm treated with glycerol showed a higher rate of penetration, particularly during the first 4-5 hours of culture. This suggests that glycerol accelerates and synchronizes the induction of the acrosomal reaction (Slavík, 1987). These findings suggest that capacitation events may be induced earlier in glycerolized sperm, similar to what was previously described in stallion sperm (Macías García et al., 2012). This may explain why the deposition of glycerolized sperm directly into the uterus can achieve a higher conception rate than cervical insemination (Armstrong and Evans, 1984).

In marsupials

High tolerance of glycerol is an unusual case in marsupial sperm (Holt, 2000). For example, possum sperm frozen with 17.5% glycerol achieves the best motility after thawing (Rodger et al., 1991), whereas koala and kangaroo sperm are extreme cases, frozen with higher glycerol concentrations of up to 20-28% (Johnston et al., 1993; Rodger et al., 2009). Despite showing good motility, sperm exhibit poor membrane quality and morphology (head bending), as well

as unclear fertility (Rodger et al., 1991; Taggart et al., 1996). These findings explain that a high concentration of glycerol is necessary for cryopreservation in order to achieve reasonable sperm motility after thawing. However, it also induces certain toxic effects on sperm cells (Rodger et al., 1991; McClean et al., 2006). Such a situation leads to the development of alternative CPA, such as DMA and DMSO, to reduce the need for glycerol in the freezing diluent (Rodger et al., 2009).

5.3.2. Glycerol toxicity is temperature-dependent

Boar sperm showed decreasing fertility when exposed to 5% glycerol at 20°C for 6 hours, while no effect was observed at 5°C. This suggests that the toxic impact of glycerol on sperm fertilizing capacity is temperature-dependent (Wilmut and Polge, 1974). Interestingly, sperm storage with 10% glycerol for 6 hours at 5°C led to higher fertility than storage without glycerol, proving that glycerol can protect boar sperm at low temperatures. In cattle, the addition of glycerol after semen cooling achieved higher values of motility and viability. This explains that glycerol has a temperature-dependent effect on bull sperm (Ma et al., 2022). Different from boar sperm, the storage of stallion or donkey sperm with glycerol at 5°C for only 1-2 hours induces a significant reduction in fertility (Demick et al., 1976; Vidament et al., 2009). This highlights that sperm in different species exhibit varying tolerances to glycerol exposure time, as well as different tolerances to glycerol exposure concentration (Holt, 2000; Simonik et al., 2022).

5.4. Inspiration from the glycerol toxicity in mammalian sperm for chicken sperm

In the previous chapter titled "Chicken semen cryopreservation," we discussed a specific section "Specific role of glycerol in chicken sperm cryopreservation" in our review article. This section presents two relevant topics, which are outlined below:
1) Direct impacts of glycerol on sperm biology
2) The impact of glycerol on sperm progression in the female reproductive tract

Although the information presented is very limited, it is currently all we have to partially understand why glycerol decreases the fertilizing ability of chickens. As previously mentioned, glycerol is a widely used CPA in most species. However, when we compare the differences in the use of cryopreserved semen between avian and mammalian species, several interesting questions arise. For example, why is chicken sperm more sensitive to glycerol toxicity compared to mammalian sperm? Why has the glycerol removal protocol never been introduced for thawed semen in mammals? Is glycerol toxicity a unique occurrence in avian sperm or a widespread issue in all species? Do differences in female reproductive tract structure or fertilization mechanisms alter or rescue the fate of glycerolized sperm in mammals compared to birds? Searching for answers to these questions may provide useful information to better understand the interaction between glycerol and chicken sperm.

5.5. Restore fertility of glycerolized sperm

5.5.1. Sperm deposition in the upper genital tract

Intrauterine insemination of glycerol containing sperm directly into the upper part of the hen's oviduct can prevent fertilization failure, which would otherwise occur when using intravaginal insemination (Allen and Bobr, 1955). In addition to chicken sperm, the same observation was found in mammalian species as well. In cattle, a glycerol concentration of up to 7% has no effect on the pregnancy rate (Papa et al., 2015). This may be due to the fact that the semen is inseminated using a catheter that passes through the cervix into the uterus. In sheep, satisfactory pregnancy rates are achieved with both vaginal and cervical inseminations using fresh semen. Nevertheless, poor fertility is observed when inseminating with sperm cryopreserved in glycerol (Tervit et al., 1984). However, the pregnancy rate can be enhanced when applying intrauterine insemination (Armstrong and Evans, 1984; Abdelhakeam et al., 1991; Salamon and Maxwell, 1995). These examples demonstrate that the toxicity of glycerol can be overcome when sperm is deposited in the upper female reproductive tract.

5.5.2. Glycerol removal

The most significant case of glycerol toxicity occurs in chickens to cause their fertilizing dysfunction (Polge, 1951; Neville et al., 1971). This has led to the development of specific techniques for isolating sperm from glycerol during thawing as a routine process (Seigneurin and Blesbois, 1995; Long and Kulkarni, 2004; Purdy et al., 2009). Unlike in birds, frozen mammalian sperm is directly inseminated after thawing without an additional glycerol removal procedure. Indeed, the removal of glycerol using the dialysis method was tested for glycerolized ram sperm, but it did not improve its fertilizing ability (Abdelhakeam et al., 1991).

THESIS OBJECTIVES

In birds, sperm cryopreservation is the most practical strategy for preserving genotypes. It is used for genetic breeding purposes and to prevent the extinction of breeds. Glycerol is the first discovered cryoprotectant, which brought sperm cryopreservation from imagination to reality. It also allowed for the birth of the first offspring from frozen sperm in 1950. Regardless of other cryoprotectants that have been successively tested, glycerol still remains the effective agent to preserve sperm cells. However, glycerol is toxic to sperm and can even cause infertility when its presence in inseminated semen exceeds 6% in chickens. Despite the fact that its toxicity has been noticed since its first use, the mechanisms involved are still unclear. Therefore, in order to enhance understanding, the first part of this thesis was conducted.

1. The objective is to investigate the impact of glycerol on the fertilizing ability of chicken sperm by assessing the fertility of hens after insemination with glycerolized sperm.
2. To assess the impact of glycerol on sperm colonization in the hen's oviduct by observing the patterns of sperm storage after their exposure to glycerol.
3. To investigate the effects of glycerol on sperm biological characteristics by evaluating sperm quality parameters *in vitro* after exposing them to glycerol at two different temperatures. These temperatures were chosen to simulate freezing, thawing conditions (4°C) and post-insemination conditions (41°C).

Glycerol removal before insemination is currently the only method to prevent the negative effects of glycerol in chickens. A conventional method, serial dilution centrifugation, was introduced into the thawing process 25 years ago. Despite achieving an acceptable fertility rate through this technique, it requires specialized equipment, intricate workflows, and is time-consuming. In order to simplify the practical use of cryopreserved chicken semen, the second part of this thesis was conducted.

1. The objective is to develop a new protocol utilizing centrifugation on a colloid gel, specifically Percoll, to simplify and improve the efficiency of glycerol removal.
2. To demonstrate the efficiency of this new approach by evaluating the fertility of the hens, observing the *in vitro* quality characteristics, and examining sperm colonization in the hens' oviduct after glycerol removal treatment.

Part 1: Exploring the impacts of glycerol toxicity on the fertilizing capacity of chicken sperm

1. Abstract

Glycerol, one of the permeable cryoprotectant agents (CPA), functions to replace water, preventing ice crystal formation and reducing interior cryodamage during freezing and thawing processes. Seventy years ago, glycerol was discovered as a CPA for sperm cryopreservation and successfully resulted in the first production of chicken offspring using frozen semen. Even though other CPA have been consecutively introduced, glycerol still remains the favored one added to the sperm freezing diluent of most species. However, it is unfortunate that glycerol is also associated with certain toxic effects on sperm cells, which can lead to a decrease in fertility when glycerol is present in the inseminated dose. The most apparent example occurs in chickens, where the presence of only 6% glycerol in semen can cause all females to lose their entire fertilizing ability. It is worth noting that 11% glycerol is the most commonly used concentration for freezing rooster sperm. Unlike the case of inseminating thawed sperm together with glycerol in mammals, chicken sperm must be separated from glycerol before insemination. This is done through a technique known as serial dilution centrifugation, which is necessary to prevent glycerol toxicity from affecting their fertility. Despite this glycerol removal method being able to maintain the chicken sperm fertilizing ability, the mechanisms involved in the toxic effects of glycerol on sperm and the female reproductive tracts remain poorly understood. Thus, none of the clues provided allowed to design strategies to control glycerol toxicity when sperm cells are exposed to it. Based on these reasons, one of the objectives of my thesis was to investigate the effects of glycerol on the fertilizing capacity of chicken sperm. This was done by examining their fertility, their storage capacity within the hens' oviduct, and the changes in their *in vitro* quality parameters following exposure to glycerol.

In this study, we first inseminated hens with sperm containing 0 (control), 1, 2, 6 and 11% glycerol to confirm the dose-dependent reduction of fertility. Subsequently, we conducted both *in vivo* and *in vitro* experiments on sperm storage tubules (SST) and perivitelline membrane (PVM) penetration to understand the effects of glycerol on female fertilization characteristics. Finally, to determine the stage at which sperm damage occurs during glycerol exposure, we exposed glycerolized sperm to temperatures of 4 and 41°C. These temperatures were chosen to mimic the freezing and thawing processes, as well as the conditions in the oviduct after insemination. We then observed various *in vitro* sperm quality parameters, such

as sperm motility, mitochondrial function, ATP concentrations, membrane integrity, and apoptosis.

Here, we observed that the presence of 2% glycerol at insemination caused a 50% reduction in fertility, while 6% and 11% glycerol resulted in complete infertility. These results corresponded to the observations of *in vivo* SST experiments, which showed that insemination of control sperm (0% glycerol) resulted in 58% of the SST being filled with sperm. However, the addition of 2% and 6% glycerol significantly reduced the proportions to 27% and almost 0% respectively. Interestingly, the results of *in vitro* SST experiments differed from the effects observed after insemination. The presence of 2% glycerol during coincubation of sperm with SST tissues did not decrease the percentage of SST filled with sperm. However, when 6% glycerol was used, the percentage decreased from 50% (control) to 33%. As another indicator of fertilization potential, the ability of PVM penetration was primarily impaired by glycerol at 41°C, rather than at 4°C. In addition, we found that glycerol concentrations of 6% and 11% were the only ones that slightly impaired sperm quality parameters at 4°C. However, the presence of glycerol at the same levels at 41°C severely damaged the sperm, particularly impacting sperm motility traits.

Altogether, our data revealed important links between glycerol toxicity and chicken fertilization dysfunction. The evidence here shows that glycerol may affect sperm characteristics, particularly sperm motility. Such defects may lead to sperm failing to colonize the SST, either due to vaginal selection or to SST barrier. This could explain, at least in part, the reduction in fertility.

As previously mentioned, glycerol is a CPA broadly used in most species. Despite the limited number of conducted studies, glycerol toxicity is recognized as a common issue in both avian and mammalian sperm. In addition, SST are analogous to the sperm reservoir in mammalian oviducts. Thus, the data presented here may provide valuable insights for further studying the mechanisms of glycerol toxicity and designing innovative cryopreservation strategies, not only in chickens but also in other species.

This section of the thesis is presented as a research article titled "Is glycerol a good cryoprotectant for sperm cells? New exploration of its toxicity using avian model" has been published by the journal Animal Reproduction Science in September 2023.

2. Résumé

Le glycérol est un agent cryoprotecteur (ACP) perméable, il a pour fonction de remplacer l'eau, d'empêcher la formation de cristaux de glace et ainsi de réduire les dommages internes dans les cellules au cours de la congélation. Il a été utilisé il y a 70 ans comme cryoprotecteur pour la cryoconservation des spermatozoïdes (SPZ) et a permis la production de poussins par insémination de sperme dégelé. Même si d'autres ACP ont été testés ultérieurement, le glycérol reste toujours le plus utilisé dans le dilueur de congélation des SPZ de la plupart des espèces. Malheureusement, le glycérol produit aussi certains effets toxiques sur les SPZ, provoquant une diminution de la fertilité. Le cas le plus apparent se produit chez les poulets, où la présence de seulement 6% de glycérol dans la semence peut induire une perte totale de la fertilité, alors que 11% de glycérol est la concentration la plus utilisée pour congeler les SPZ de coq. Contrairement au cas de l'insémination de SPZ décongelé avec du glycérol chez les mammifères, les SPZ de coq doivent être séparés du glycérol avant l'insémination avec une dilution sériée suivie de centrifugation pour éviter une diminution de fertilité. Bien que cette méthode d'élimination du glycérol permette de préserver le pouvoir fécondant des SPZ, les mécanismes impliqués dans les effets toxiques du glycérol sur les SPZ et sur l'appareil reproducteur femelle restent mal compris. Ceci rend difficile l'élaboration de stratégies préventives pour diminuer la toxicité lorsque les SPZ sont exposés au glycérol. Dès-lors, l'un des objectifs de ma thèse était de décrypter l'impact du glycérol sur le pouvoir fécondant des SPZ de coq en observant leur fertilité, leur capacité de stockage à l'intérieur des oviductes et les altérations de leurs paramètres fonctionnels après exposition au glycérol.

Dans cette recherche, nous avons d'abord inséminé les poules avec les SPZ contenant 0 (témoin), 1, 2, 6 et 11% de glycérol pour confirmer la diminution dose-dépendante de la fertilité. Par la suite, nous avons suivi la capacité de colonisation des glandes de stockage des spermatozoïdes (GSS) *in vivo* et *in vitro*, ainsi que la capacité de pénétration de la membrane périvitelline (MPV) pour appréhender les impacts du glycérol sur les caractéristiques de fécondation. Enfin, pour déterminer à quel stade de l'exposition au glycérol les SPZ sont endommagés, nous avons suivi les SPZ glycérolés à 4 et 41°C, imitant les températures du processus de congélation et de décongélation, ainsi que dans l'oviducte après insémination, puis observé les paramètres de qualité de SPZ *in vitro*: la mobilité, la fonction mitochondriale, la concentration d'ATP, la perturbation de la membrane et l'apoptose.

Nous avons observé que la présence de 2% de glycérol lors de l'insémination provoquait une réduction de 50% de la fertilité alors que 6 et 11% de glycérol entraînaient une infertilité totale. Ces résultats correspondaient aux observations d'expériences de colonisation de GSS *in vivo*, montrant que 58% de GSS contiennent des SPZ lors de l'insémination de semence témoin (0% de glycérol), alors que 2 et 6% de glycérol réduisaient considérablement les proportions à 27% et près de 0%, respectivement. Fait intéressant, les expériences de colonisation de GSS *in vitro*

ont eu un résultat différent de l'effet après insémination. La présence de 2% de glycérol pendant l'incubation des SPZ et le tissu de GSS n'a pas diminué le pourcentage de GSS remplies de SPZ, alors que 6% de glycérol l'ont seulement réduit de 50% (contrôle) à 33%. Un indicateur de capacité de fécondation, la pénétration du MPV était principalement altérée par le glycérol à 41°C plutôt qu'à 4°C. De plus, nous avons constaté que les concentrations de glycérol de 6% et 11% étaient les seules à avoir légèrement altéré les paramètres de qualité de SPZ à 4°C. Cependant, la présence de glycérol aux mêmes niveaux à 41°C a gravement endommagé les SPZ, ce qui a eu un impact sur les caractéristiques de motilité des SPZ.

Dans l'ensemble, nos données montrent les liens importants entre les effets toxiques du glycérol et le dysfonctionnement de la fécondation des poules. En effet, le glycérol peut avoir un impact sur les caractéristiques des SPZ, en particulier sur leur mobilité. De tels défauts peuvent empêcher les SPZ de coloniser les GSS soit à cause de la sélection dans le vagin, soit à cause d'une barrière au niveau des GSS, expliquant au moins une partie de la réduction de la fertilité.

Comme indiqué précédemment, le glycérol est un ACP beaucoup utilisé dans la plupart des espèces. Malgré très peu d'études, la toxicité du glycérol est considérée comme un problème général à la fois pour les SPZ aviaires et de mammifères. De plus, les GSS sont analogues par leur fonction au réservoir de SPZ dans les oviductes chez les mammifères. Ainsi, nos données peuvent être précieuses pour étudier plus avant les mécanismes de la toxicité du glycérol ou pour concevoir des stratégies innovantes de cryoconservation, non seulement chez les poulets mais aussi chez d'autres espèces.

Cette partie de la thèse est présentée dans un article de recherche intitulé "Le glycérol est-il un bon cryoprotecteur pour les spermatozoïdes ? Des nouvelles explorations de sa toxicité sur un modèle aviaire", publié par le journal Animal Reproduction Science en septembre 2023.

3. Article 1

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

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ABSTRACT

Glycerol is a cryoprotectant used widely for the cryopreservation of animal sperm, but it is linked to a decrease in fertility. The mechanism underlying the negative effects of glycerol remains unclear. Therefore, in this study, we aimed to gain a better understanding by using the chicken model. First, we investigated the impact of increasing the concentration of glycerol during insemination on hen fertility. Our findings revealed that 2% glycerol resulted in partial infertility, while 6% glycerol led to complete infertility. Subsequently, we examined the ability of sperm to colonize sperm storage tubules (SST) during *in vivo* insemination and *in vitro* incubation. The sperm used in the experiment were stained with Hoechst and contained 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 simulate pre-freezing (4 °C) and post-insemination (41 °C) conditions. Whereas 2% glycerol significantly reduced 50% of sperm containing SST, 6% glycerol completely 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 in sperm-filled SST. Moreover, glycerol reduced sperm-egg penetration rates and also affected sperm motility, bioenergetic metabolism, and cell death at 4 °C. These effects were observed when the concentration of glycerol exceeded 6%. Furthermore, at 41 °C, glycerol caused even greater damage, particularly in terms of reducing sperm motility. These data altogether reveal important effects of glycerol on sperm biology, sperm migration, SST colonization, and oocyte penetration. This suggests that glycerol plays a role in reducing fertility and presents opportunities for improving sperm cryopreservation.

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1. Introduction

Sperm cryopreservation is a practical strategy to address the biodiversity crisis in wild animals (Bolton et al., 2022), to introduce superior genotypes in domestic animals (Bebbere and Succu, 2022), and to preserve fertility in humans (Tao et al., 2020). The cryopreservation process involves several steps: semen collection, addition of cryoprotectant, cellular dehydration, freezing, storage in liquid nitrogen, and thawing before using the sperm (Ugur et al., 2019). Cryoprotectants are chemical compounds that protect cells from cryodamage. Among them, glycerol, an intracellular cryoprotectant, is the most commonly used 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), which can affect 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 the cytotoxic effects, several studies have suggested alternatives to glycerol during the cryopreservation process (Yildiz et al., 2007; Silva et al., 2012). However, these alternatives still lack promising results in terms of fertilization success (Abouelezz et al., 2015, 2017; Gloria et al., 2019) or fail to meet the safety requirements and clear identification needed for cryobanking (Tselutin et al., 1999). Another strategy is related to the removal of glycerol before insemination (Seigneurin and Blesbois, 1995; Purdy et al., 2009). While these methods can partially alleviate the negative effects, little is known about the mechanisms involved in glycerol toxicity, either directly on sperm biology or through modifications of the female genital tract.

In chickens, the presence of glycerol in semen may significantly reduce fertility, even at half the concentration used for cryopreservation (Polge, 1951; Neville et al., 1971). During the production and use of cryopreserved chicken semen, sperm are exposed to different temperatures: 4 °C during glycerol addition and equilibration, decreasing to − 196 °C during freezing and storage, returning to 4 °C after thawing, and finally reaching 41 °C after insemination into the female reproductive tract (Lin et al., 2022). While this cryopreservation method is quite efficient, the presence of glycerol at both 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). The SST is a well-developed organ that plays a role in sperm selection, survival, and maintenance of fertilizing capacity. It is similar to the sperm reservoir found in 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 overall fertility (Machado et al., 2019; Kölle, 2022). Furthermore, the toxic effects of glycerol may also impair the ability of sperm to penetrate the inner perivitelline membrane (IPVM) in birds, which is analogous to the mammalian zona pellucida (ZP) (Ichikawa et al., 2017). This can result in a failure of fertilization.

Consequently, using the avian model, we aimed to decipher the effects of glycerol on sperm fertilizing ability. This included investigating the direct effects of glycerol on sperm biology, as well as studying the interaction between sperm and the female reproductive tract. We first determined the critical concentrations of glycerol in semen associated with a reduction in chicken fertility through insemination tests. Subsequently, we explored the capacity of sperm to reach and be stored in the oviduct through both *in vivo* and *in vitro* SST colonization experiments. Then, we investigated the ability of sperm to penetrate the 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. We assessed its impact on sperm motility, mitochondrial activity, ATP concentration, 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 numbers: N° APAFIS#4026–2016021015509521 and APAFIS#34415–202112141205965). A total of 20 adult T44 roosters (Gallus gallus domesticus, SASSO, France) were randomly allocated into two groups for fertility tests and the study of sperm biology characteristics, including motility, mitochondrial activity, ATP concentration, viability, sperm-SST storage, and PVM penetration. Two groups of 60 adult Lohmann hens were housed separately, with each cage consisting of 4 hens. One group was used for fertility tests, while the other group was used for SST and PVM 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 provided with a standard diet and unlimited access to water at the INRAE Poultry Experimental Unit (https://doi.org/10.15454/1.5572326250887292E12). Animals were between 35 and 85 weeks old during this study.

2.2. Semen collection and processing

Rooster semen was collected by abdominal massage (Burrows and Quinn, 1937) and placed in a tube containing 200 µL of Lake PC diluent (Lake and Ravie, 1981). Semen samples were then pooled and diluted to 2000 × 10^6 cells/mL with Lake PC diluent at room temperature for the subsequent treatments. The number of all experimental replicates is given in the figure legends.

2.3. Fertility test

After collecting, pooling, and adjusting the glycerol concentration to 0%, 1%, 2%, 6% and 11% (v/v), the semen was immediately

used for intravaginal insemination at a depth of 4 cm. Each experimental group received a dose of 100×10^6 sperm per hen for two consecutive days (Cordeiro et al., 2021a), with a total of 12 hens per group. Eggs were collected from day 2 to day 7 after the first insemination and stored at a temperature of 15 °C with a humidity level of 85% before being incubated at a temperature of 37.7 °C with a humidity level of 55%. Fertile and infertile eggs were determined by candling on the 7th day of incubation (Long and Kulkarni, 2004). A repeated insemination was performed with a different group of hens (Supplementary Data 1) to confirm whether the impact of glycerol on fertility was consistent with the previously observed results (Fig. 1).

2.4. Sperm- SST test

2.4.1. Sperm staining

Sperm were labeled according to the procedures described by McDaniel et al. (1997) and 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.

2.4.2. In vivo insemination

Hoechst-stained sperm were divided into three groups and glycerol was added to achieve final concentrations of 0 (control), 2% and 6%. A total of 3 hens per group were inseminated twice at a 24-hour interval with a dose of 200×10^6 sperm per female (King et al., 2002). The hens were then slaughtered 24 h after the second insemination, and their oviducts were isolated. The villi ($n = 6$ per animal) containing SST were randomly separated from the uterovaginal mucosa (Cordeiro et al., 2021b). Villi pieces 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 20X 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-labeled sperm and EM BP 690/50 (Alexa Fluor 633) for SST autofluorescence. The presence of sperm-filled or sperm-empty SST was identified manually using 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 collection of uterovaginal villi were performed as previously described. Hoechst-labeled sperm were washed in Lake PC diluent to remove excess staining solution and then centrifuged at 600g for 20 min at 4 °C. Sperm pellets were resuspended and adjusted with Lake PC diluent to a concentration of 2000×10^6 cells/mL. They were then mixed with glycerol to final concentrations of 0 (control), 2% and 6%. For each treatment, six pieces of villi were individually placed in 1 mL of Advanced DMEM/F-12 medium (ThermoFisher, Gibco#12634–010) containing 0, 2% and 6% glycerol in a 4-well culture dish. Each well was supplemented with 200×10^6 sperm and incubated at 39 °C for 30 min in an environment of 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, they were 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; Barbato et al., 1998). The yolk membrane was separated from freshly unfertilized chicken eggs and washed several times in DPBS to remove any adherent yolk. Segments, excluding the germinal disc area of PVM, were cut to a 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, the semen was diluted with glycerol-Lake PC diluent to a concentration of 200×10^6 sperm cells/mL. Different concentrations of glycerol (0% as control, 1%, 2%, 6%, and 11%) were added, and the mixture was then incubated at either 4 °C or 41 °C for 10 min.

2.5.3. In vitro incubation of PVM and glycerolized sperm

A piece of PVM was placed in 1 mL of Advanced DMEM/F-12 medium and incubated with 10×10^6 sperm from 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 a 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 microscope slide scanner captured images of the holes that formed in the PVM using brightfield optics and a 20X objective lens. Three regions were randomly selected from the anterior, middle, and posterior parts of 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 characteristics

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

2.6.1. Sperm motility

Sperm motility was examined using a computer-assisted sperm analysis system (CASA, IVOS, IMV Technologies), as previously described (Vitorino Carvalho et al., 2021). After exposure to glycerol, semen samples were diluted with Beltsville Poultry Semen Extender (BPSE) (Sellier et al., 2006) to achieve a final concentration of 30×10^6 sperm cells/mL. Then, a 2 µL aliquot of semen was placed on a Makler Counting Chamber to capture images from four fields for evaluating the average path velocity (VAP), straight line velocity (VSL), and straightness (STR = VSL/VAP). Motility results were indicated as the percentage of motile sperm and progressive sperm. Motile sperm were defined as the percentage of sperm showing a VAP greater than 5 µm/sec, while progressive sperm were defined as the percentage of sperm with a VAP greater than 50 µm/sec and STR greater than 75%.

2.6.2. Mitochondrial activity

Sperm mitochondrial characteristic was evaluated using a fluorescent probe called JC-1 (Sigma-Aldrich, CAS NO.: 3520–43–2). JC-1 is a green-fluorescent monomer at low membrane potential that turns into 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 5000 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 (Nguyen et al., 2015). After incubation with glycerol, semen samples were immediately centrifuged at 4 °C, 800g for 10 min. The supernatant was then removed, and the pellets were frozen at − 20 °C. Before conducting the ATP assay, the sperm pellets were thawed and then resuspended in 100 µL of PBS in a 96-well white polystyrene microplate. After 30 min of equilibration at room temperature, 100 µL of luciferin/luciferase reagent was added to each well. The plates were then placed on an orbital shaker for 2 min to induce cell lysis, followed by a 10-min wait to stabilize the luminescent signal. The luminescence was recorded using the luminometer plate reader (CLARIOstar, BMG LABTECH) and then converted to ATP concentration using a standard curve.

2.6.4. Sperm plasma membrane integrity

This characteristic was explored using the SYBR 14/Propidium iodide (PI) double fluorescent staining technique (Molecular Probes, Invitrogen #L7011) in combination with a flow cytometer, Guava® easyCyte (Gliozzi et al., 2017). PI-positive sperm (red fluorescence) were recognized as sperm with damaged membranes. Thus, sperm showing SYBR 14-positive (green fluorescence) with PI-negative were considered to have intact membranes. The results were expressed as a percentage of sperm showing intact plasma membrane.

2.6.5. Apoptosis

Annexin V-binding technique was used to determine sperm apoptotic cells according to the manufacturer's instructions (Novus Biologicals™ #NBP2–29373). Briefly, 1.5 µL of semen was washed twice in 1 mL of PBS. Sperm pellets were obtained by centrifugation at 400g 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 was added, and the samples were analyzed by Guava® easyCyte within 1 h.

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.07. The impact of glycerol concentration on fertility was analyzed using the Chi-square test and Fisher's exact test. The percentage of SST filled with sperm was analyzed using Kruskal-Wallis and Dunn's multiple comparisons tests to evaluate the effect of glycerol concentration. Significant differences were considered when

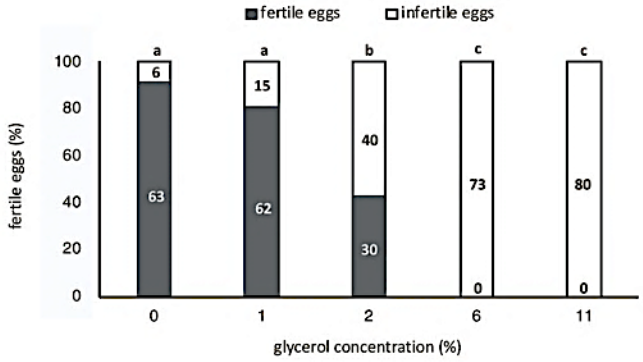


Fig. 1. Fertility (%) of chicken semen samples added with different concentrations of glycerol. Black and white bars represent the fertile and infertile eggs, and the number of eggs is reported in the bars. The different letters show a significant difference between glycerol concentrations with p-value < 0.05.

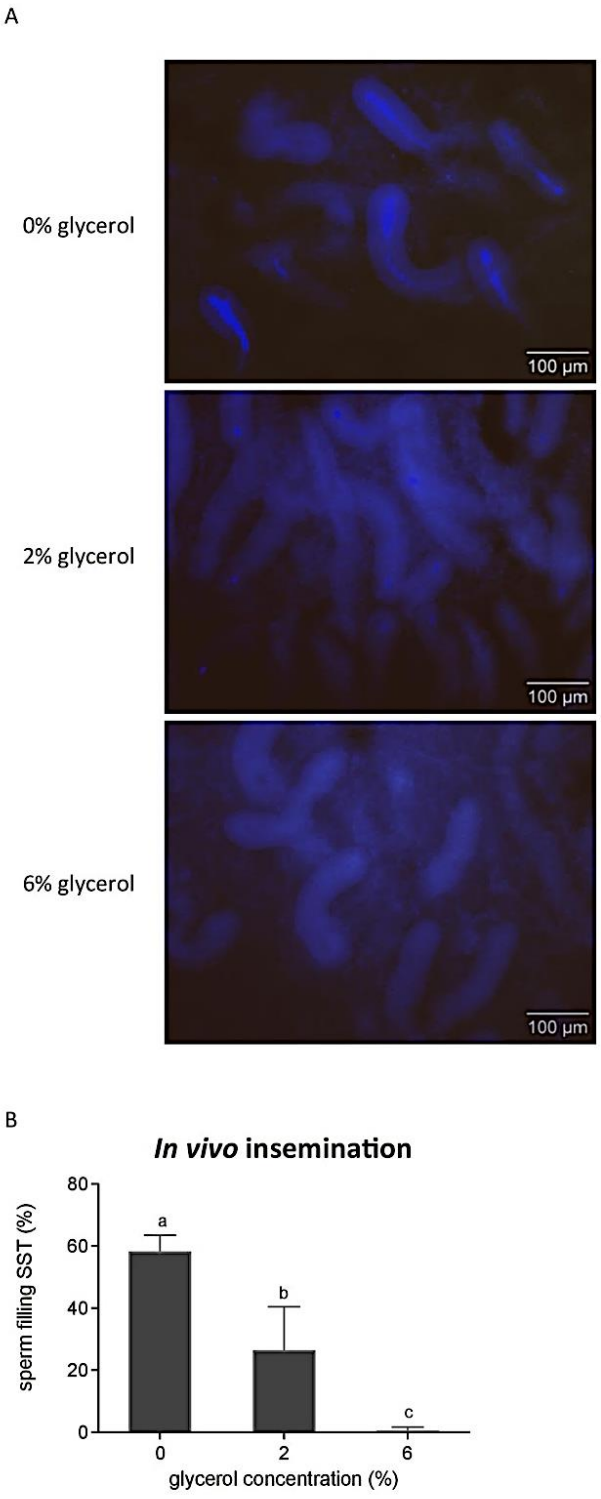


Fig. 2. The presence of sperm in sperm storage tubules (SST) after *in vivo* insemination. A and B represent sperm identification (A) and the percentage of SST containing sperm (B). The bars and lines correspond to the mean (3 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations with p-value < 0.05.

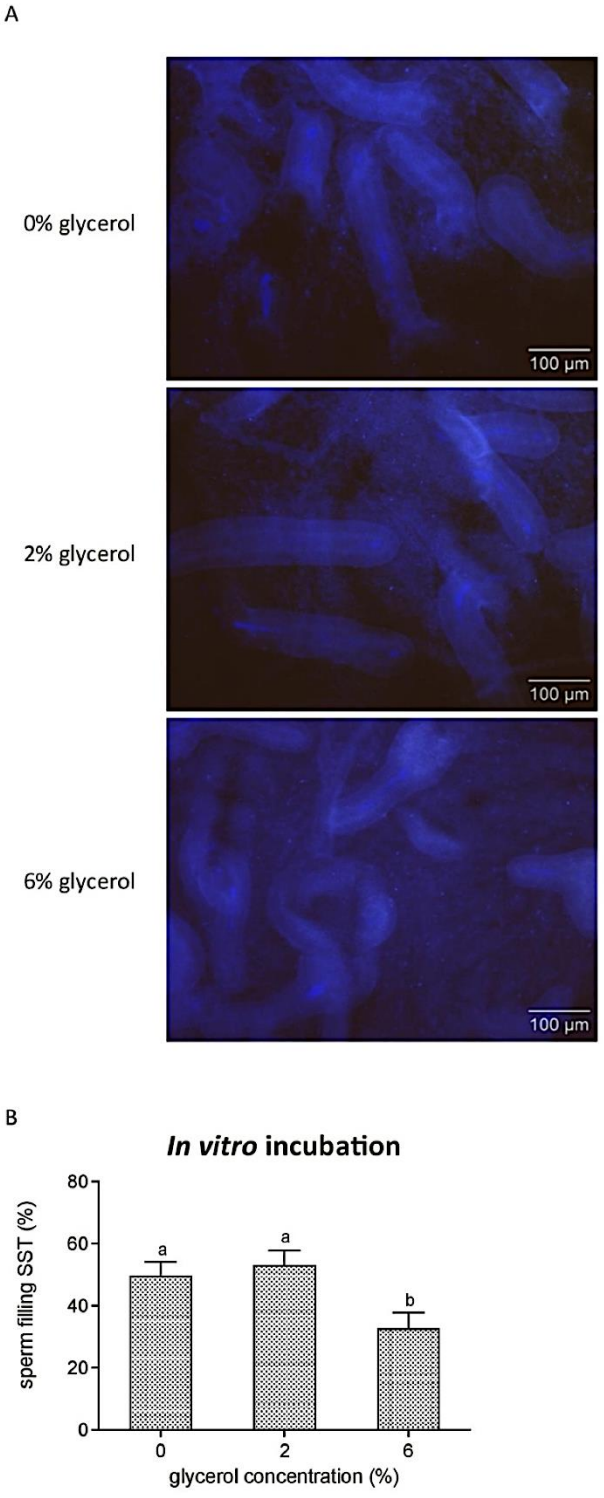


Fig. 3. The presence of sperm in sperm storage tubules (SST) after *in vitro* incubation. A and B represent sperm identification (A) and the percentage of SST containing sperm (B). Data collected from 3 villi of each treatment. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations with p-value < 0.05.

the p-value was below 5%. Sperm-PVM penetration data were normally distributed and analyzed using a two-way ANOVA test to examine the effects of glycerol concentration and exposure temperature. All sperm quality characteristics results were analyzed using a two-way ANOVA test to investigate the impact of glycerol concentration and exposure time. Because exposure time had no effect on all sperm quality characteristics at 4 °C (Table 1), data from different time points were pooled in a mixed model to highlight the effects of glycerol concentration. Differences in the means were analyzed using Tukey's HSD Post Hoc test.

3. Results

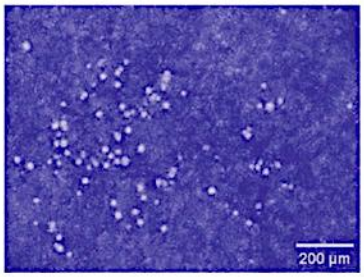
3.1. Impact on animal fertility

The effect of increasing concentrations of glycerol on sperm fertility is reported in Fig. 1. Whereas 1% glycerol had no effect on the percentage of fertilized eggs, the presence of 2% glycerol significantly reduced fertility by about 50%, while 6% and 11% glycerol resulted in complete infertility. The same effect was confirmed in the second trial replication (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. In addition, we confirmed that Hoechst labeling did not affect the fertility decrease caused by glycerol effect (Supplementary Data 2). Subsequently, the capacity of sperm to reach and be stored in SST was investigated by inseminating Hoechst-stained sperm, followed by SST

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.

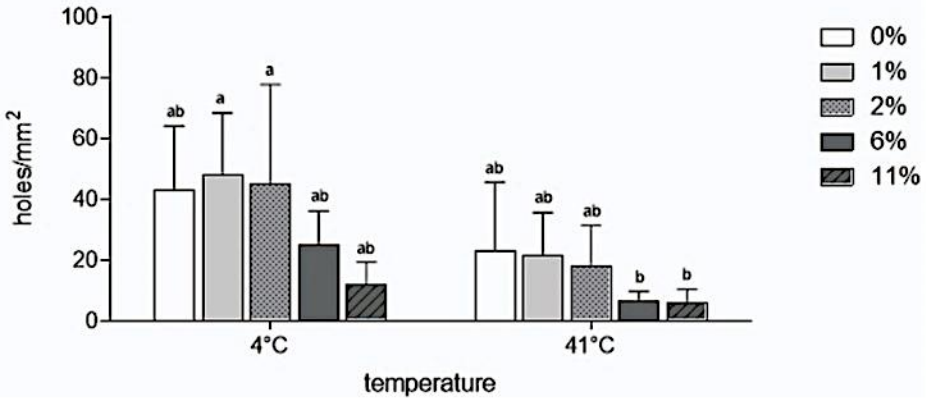


Fig. 4. Sperm penetration in perivitelline membrane (PVM) after pre-incubation with different glycerol concentrations. A and B represent the image (A) and the number of holes (B). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations and temperatures with p-value < 0.05.

dissection (Fig. 2A). The percentage of sperm-filled SST significantly decreased as the concentration of glycerol increased (Fig. 2B). Specifically, 2% and 6% glycerolized sperm resulted in a significant reduction in the percentage of SST containing sperm, with a decrease of 50% and nearly 100% respectively.

To focus our work on the capacity of the SST to host sperm and avoid any potential motility effects that could prevent sperm from reaching the SST, we used the same approach for *in vitro* incubation of uterovaginal villi and glycerolized sperm (Fig. 3A). No effect was observed with 2% glycerol (Fig. 3B), while 6% glycerol significantly decreased the proportion of SST colonized by stained sperm by 17%.

3.3. Effect on sperm-PVM penetration

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

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

In order to collect new data on the effects of the pre-freezing procedure on sperm biology at specific stages, several aspects of sperm quality characteristics were evaluated. These included sperm motility, mitochondrial function, ATP concentration, membrane integrity, and apoptosis. The evaluations were conducted within 1 h of incubation at 4 °C, in the presence of various concentrations of glycerol.

At this temperature, no effect of exposure time or its interaction with glycerol concentration was observed on any of the evaluated characteristics (Table 1). Consequently, we further combined all time points to highlight only the effects of glycerol concentration (see Supplementary Data 4). When compared to 0% glycerol, no effect of 1% and 2% glycerol was observed on all sperm biological characteristics (Fig. 5A-F), except that 2% glycerol significantly increased ATP concentration (Fig. 5D). Furthermore, the presence of 6% and 11% glycerol decreased sperm motility and membrane integrity, while increasing the values of all other sperm characteristics when compared to the control condition. Moreover, 11% glycerol caused a more pronounced negative influence than 6%, except on mitochondrial function (Fig. 5).

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

We then investigated the same functions of sperm biology after exposing them to glycerol at 41 °C. This temperature was chosen to simulate the effects of the female reproductive tract on sperm after insemination, within a timeframe of 1 h. This duration is considered the physiological time required for sperm to reach the fertilization site in the oviduct. Based on our preliminary results, we observed changes in sperm motility traits within 30 min of incubation time when exposed to glycerol at 41 °C. These changes occurred rapidly. The percentages of motile and progressive sperm showed similar tendencies and were both significantly affected by glycerol concentration, exposure time, and their interaction (Table 1). Indeed, while there was no impact of 1%, 2% and 6% glycerol at 0 min, the addition of 11% glycerol immediately decreased sperm motility (Fig. 6). A similar negative effect was also observed after 10 min of exposure to 6% glycerol (Fig. 6). In addition to the reduction of sperm motility classically induced by incubating chicken sperm at 41 °C (as observed with the control condition over time), all glycerol concentrations at 20 min and 30 min significantly decreased sperm motility in a dose-dependent manner. This resulted in nearly immobile sperm with 11% 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 in mitochondrial depolarization was observed at 30 min in the absence of glycerol when compared to 0 min. Interestingly, mitochondrial depolarization

Table 1
Effect of glycerol concentration (GLY) and exposure time (T) on *in vitro* sperm quality characteristics, as reported by P-values.

	4 °C			41 °C		
characteristic	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 integrity	**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

Sperm variables are represented by the rows, while the sources of variation are represented by the columns. Data were analyzed using a two-way ANOVA test at 4 °C and 20 °C, respectively. 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 characteristics were observed at 0, 30 and 60 min

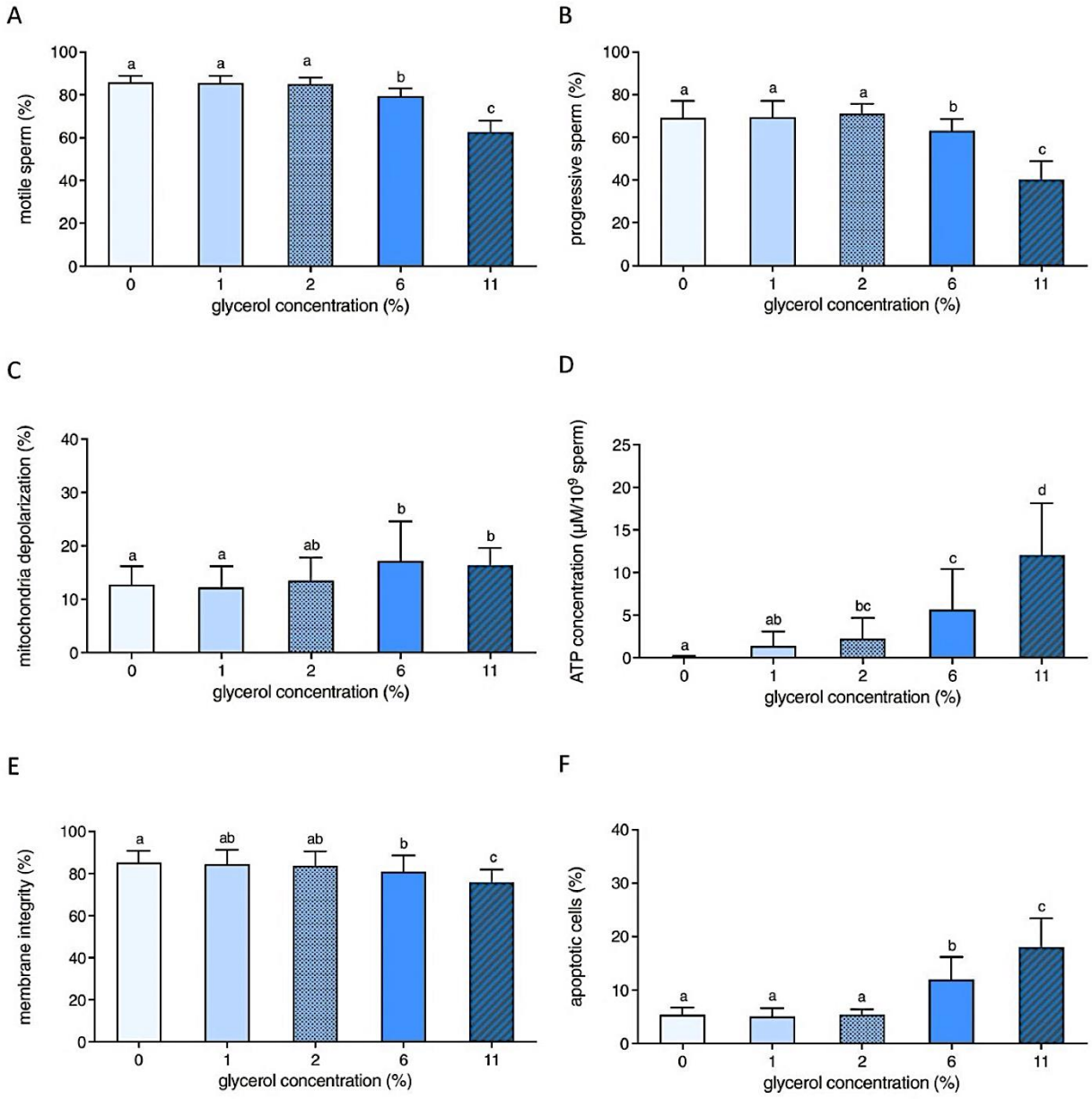


Fig. 5. Sperm quality characteristics of glycerolized sperm at 4 °C. A-F represent evaluations of different sperm quality characteristics. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations with p-value < 0.05.

was higher at 60 min compared to all other time points, and it significantly decreased with an increase in glycerol concentration (Fig. 7A). Glycerol concentration, exposure time, and their interaction also significantly affected energy metabolism, as revealed by the evaluation of sperm ATP concentration (Table 1). Indeed, no significant influence was observed for the 1% and 2% glycerol conditions at all time points (Fig. 7B). However, there was a significant increase in ATP concentration at 30 min with 11% glycerol and at 60 min with 6% glycerol, compared to the control and the 1% and 2% glycerol conditions. At 60 min, the ATP concentration observed with 11% glycerol was higher than the one obtained with 6% glycerol.

In order to clarify the effect of glycerol on sperm cell survival, two characteristics were evaluated: the sperm plasma membrane integrity and the presence of apoptosis. Both characteristics presented a significant effect due to glycerol concentration and exposure time, with no significant interaction (Table 1). At 0 min, no impact was observed on the rate of sperm plasma membrane integrity. However, an increase in apoptotic cell percentage was found immediately after the addition of 11% glycerol. At 30 and 60 min, the rates of sperm membrane integrity and apoptotic sperm cells significantly decreased and increased by only 6% and 11% respectively, when compared to the other glycerol concentrations, as well as to the experimental conditions at 0 min (Fig. 8).

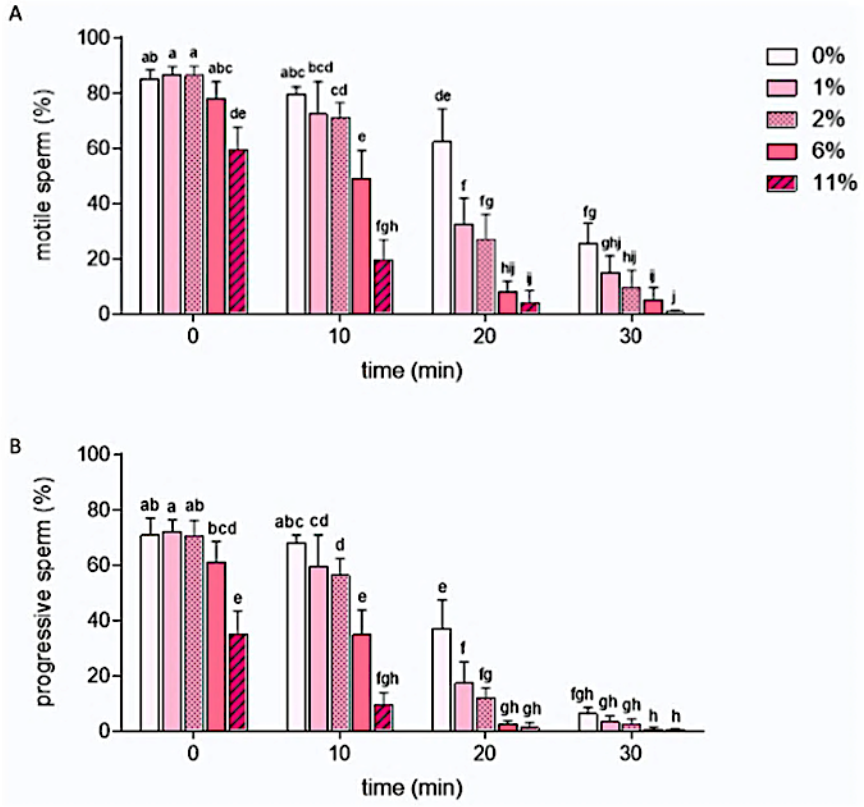


Fig. 6. Sperm motility of glycerolized sperm at 41 °C. A and B represent the percentages of motile (A) and progressive sperm (B). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations and times with p-value < 0.05.

4. Discussion

Whereas glycerol is one of the most widely used cryoprotectants to preserve sperm at very low temperatures in animals, its presence also contributes to undesired negative effects on fertility (Macías García et al., 2012). In chickens, this negative influence could lead to complete infertility, depending on the concentration of glycerol present in the inseminated semen (Long, 2006; Blesbois and Brillard, 2007). 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 an insemination program with chicken semen combined with different glycerol concentrations, we demonstrated that the presence of 2% glycerol significantly reduced animal fertility by half (from 91% to 43%), and that 6% glycerol resulted in total infertility. These results are consistent with previous studies performed using different insemination programs and animal genotypes (Long and Kulkarni, 2004; Zong et al., 2022). This suggests that the toxic effects of glycerol occur regardless of the animal's 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 effect of glycerol could be presumed for all animal species, some significant variability in sensitivity can be observed. However, very little information is available regarding the stage and target of this toxicity, specifically the direct impacts on sperm biology and/or the effects on the events occurring inside the female reproductive tract.

Once semen is inseminated into the hen's 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 noticeable decrease in its ability to colonize the SST, while 6% glycerol resulted in the absence of sperm in the SST. This decrease or absence of sperm in SST, as previously mentioned (Bakst et al., 2010; Hemmings et al., 2015), is certainly one of the major causes of reduced fertility induced by the presence of glycerol. However, this experiment did not determine whether the absence of sperm in the SST was due to a failure of sperm migration from the vagina to the SST or a failure of sperm entering and binding to the SST. To address this question, an *in vitro* experiment was designed to eliminate the issue of sperm migration from the vagina to the UVJ. The aim was to determine whether glycerolized sperm could be detected in the SST when they were deposited in close proximity to the SST. Different from the *in vivo* experiment, sperm were present in the SST after *in vitro* incubation of sperm with UVJ villi. Intriguingly, it was found that there was no impact of 2% glycerol on the rate of sperm filling in the SST. However, there was a partial reduction of 17% with 6% glycerol. This reduction is relatively slight when compared to the *in vivo* result, which showed a 100% reduction. These findings suggest that a small

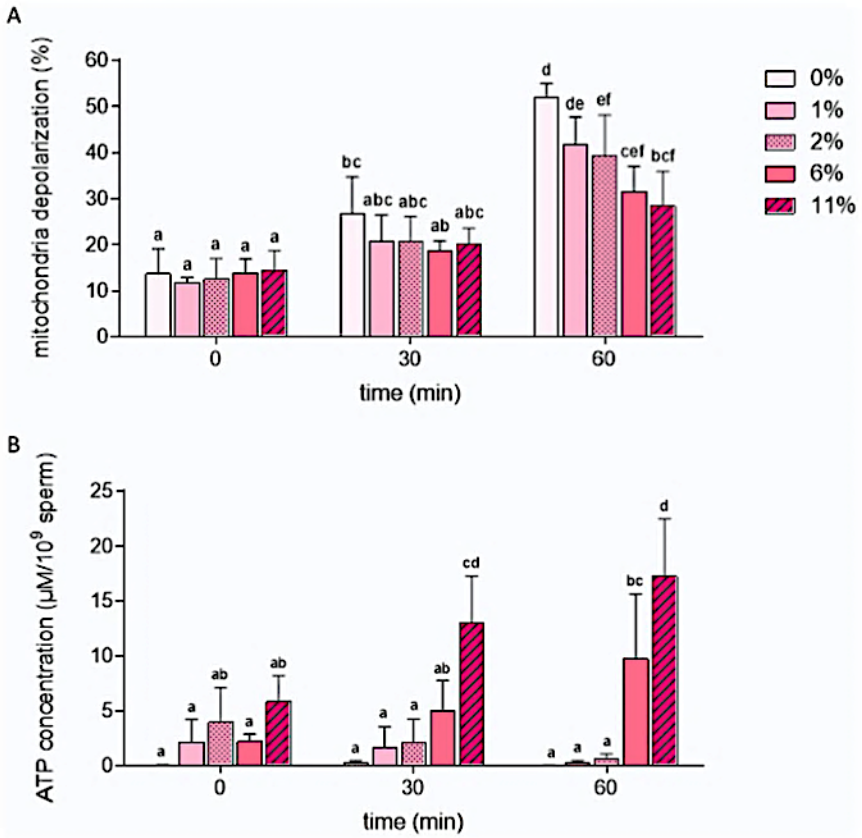


Fig. 7. Sperm mitochondria function and ATP concentration of glycerolized sperm at 41 °C. A and B represent the percentages of mitochondria depolarization (A) and ATP concentration (B). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations and times with p-value < 0.05.

amount of glycerol impairs sperm travel from the vagina to the SST *in vivo*, and that a higher quantity of glycerol also inhibits sperm deposition into the SST. These negative effects may be due to a motility defect and/or sperm being rejected by vaginal selection (Brillard, 1993; Bakst et al., 1994). There may also be another barrier at the SST level acting as a gatekeeper (Brady et al., 2022) to exclude glycerolized sperm.

After a 30-min *in vitro* incubation of sperm with UVJ villi, we discovered that the presence of 6% glycerol did not affect sperm motility (Supplementary Data 5). This finding contrasts with a previous observation of sperm motility. In that experiment, sperm were incubated with 6% glycerol without UVJ villi, which resulted in the sperm losing almost all of their motility within 30 min. This finding may suggest that UVJ tissues have a positive effect on maintaining motility, as proposed in a previous study (Spreen et al., 1990).

In addition to affecting sperm transport and storage, glycerol may also directly influence sperm fertilizing ability, including its potential to release proteolytic enzymes that hydrolyze the perivitelline membrane (PVM). This step is crucial for penetrating and fertilizing the oocyte (Lemoine et al., 2011; Priyadarshana et al., 2020). The higher the concentration of glycerol, the lower the activity of sperm hydrolysis was. This demonstrates that glycerol negatively affects the fertilizing capacity of chicken sperm. Furthermore, this effect was more significant at 41 °C compared to 4 °C, indicating a synergistic relationship between temperature and glycerol concentration. To the best of our knowledge, this data is the first to reveal the direct effects of glycerol on sperm-egg penetration in birds. However, a different observation was reported in rams, indicating that glycerol accelerates egg penetration and polyspermy (Slavik, 1987). This suggests a species-specific effect that still needs to be confirmed.

The process of freezing, thawing, and inseminating sperm involves primarily two temperatures: a low temperature of 4 °C during sperm preparation and the physiological body temperature of 41 °C after insemination into the female reproductive tract. These temperatures were considered in order to decipher the direct impacts of glycerol on sperm biology and better understand which stages of the process are affected. At 4 °C, the presence of less than 2% glycerol had no effect on sperm biology within 60 min. However, when the glycerol concentration exceeded 6%, there was a significant decrease in sperm motility and viability, as well as an increase in mitochondrial activity and ATP concentration. These findings suggest that modifying the current freezing protocol could be beneficial in reducing the undesired effects of glycerol. It is worth noting that the most commonly used glycerol concentration for chicken sperm cryopreservation is over 8% (Mocé et al., 2010; Zong et al., 2022). Decreasing the amount of glycerol to less than 2% may be a potential

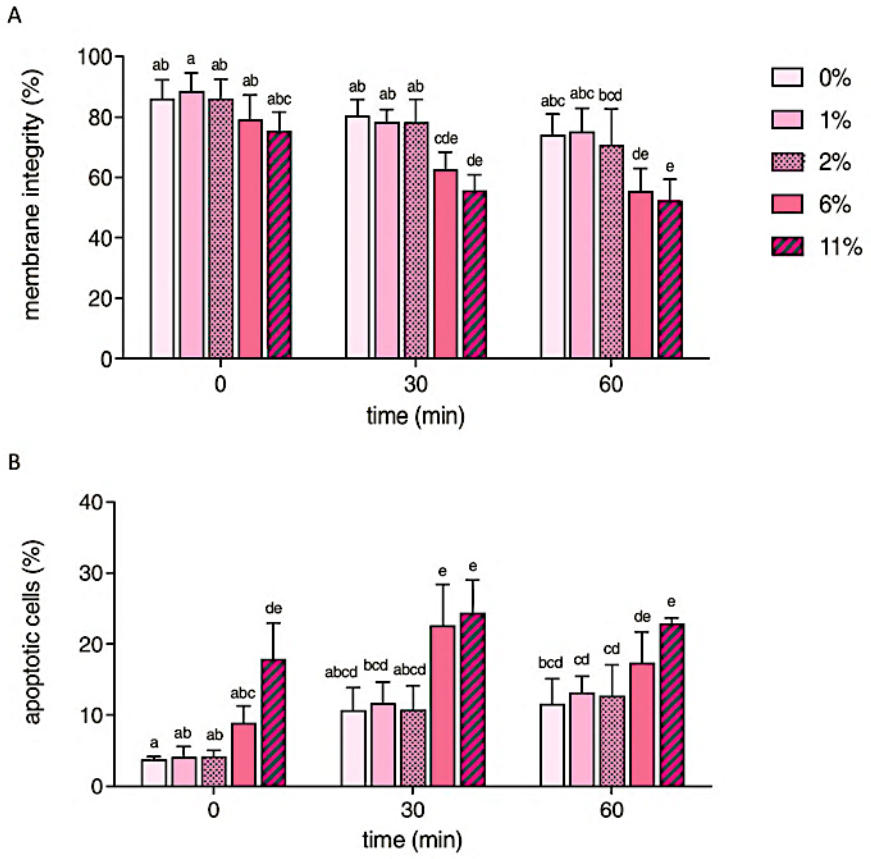


Fig. 8. Sperm membrane integrity and apoptosis of glycerolized sperm at 41 °C. A and B represent the percentages of membrane integrity (A) and apoptosis (B). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The different letters show a significant difference between glycerol concentrations and times with p-value < 0.05.

solution by substituting its need with alternative cryoprotectants. However, the cytotoxic effects of other cryoprotectants, such as DMA and DMSO, cannot be ignored (Best, 2015; Mosca et al., 2019). An option would be to combine multiple cryoprotectants to achieve sufficient cryoprotection while ensuring that each individual remains below its own toxicity threshold. Designing a safe and effective method for cryopreserving sperm remains an ongoing endeavor and challenge, even after 70 years of development. Another finding in our study was the absence of any impact of exposure time on sperm biology at 4 °C within 1 h. This information could be very useful for improving flexibility in insemination programs by keeping semen at a temperature of 4 °C for 1 h in a farm environment. However, *in vivo* data on animal fertility is still needed to verify these potential procedure modifications.

At 41 °C (the physiological body temperature), the situation is dramatically different. Indeed, sperm motility was rapidly decreased by glycerol in a concentration-dependent manner, resulting in a significant reduction in motility of over 90% within 20 min in the presence of 11% glycerol. Consequently, this severe impairment of sperm motility could disrupt the normal progression of sperm to reach the SST or the fertilization site in the oviduct, as this entire journey typically takes only 1 h *in vivo* (Bakst et al., 1994). Short-term exposure to glycerol at room temperature for 20 min also resulted in a significant decrease in sperm motility in both humans (McLaughlin et al., 1992) and mice (Katkov et al., 1998). This suggests that the issue is not specific to chickens. At this temperature, a higher concentration of glycerol (6% and 11%) also resulted in the loss of plasma membrane integrity and the induction of apoptosis in sperm cells, which are typical features of cell death (Chen et al., 2018). Whereas similar results were observed for the membrane integrity of stallion sperm under 5% glycerol condition, no effect was observed on the apoptotic rate (Macías García et al., 2012). This suggests that the sensitivity of glycerol effects on sperm cell death patterns may vary depending on the species. Alternatively, a possible approach to limit glycerol-induced cell death in chicken sperm could be to modify the glycerol freezing diluent by adding apoptotic regulators, such as agonists/antagonists of death receptor signaling or caspase inhibitors (Fischer and Schulze-Osthoff, 2005). Furthermore, this increase in sperm mortality may also be one of the causes of the decrease in sperm count in SST. Since the SST are known to choose and preserve higher quality sperm, any sperm displaying signs of death may be rejected (Brillard, 1993; Blesbois and Brillard, 2007).

Lastly, glycerol alters the energy metabolism of chicken sperm, resulting in a significant increase in ATP concentration and a decrease in mitochondrial depolarization. This finding is consistent with previous studies conducted on stallions (Macías García et al., 2012) and boars (Malcervelli et al., 2020). During ATP synthesis through mitochondrial respiration, the displacement of protons

generates a voltage gradient that results in the polarization of the membrane, with a positive charge on the outside. This is known as the mitochondrial membrane potential (Wang et al., 2022). Thus, mitochondrial depolarization refers to sperm with a low mitochondrial membrane potential, which is generally associated with ATP deficiency and lower sperm motility (Alamo et al., 2020). Here, sperm incubated at 41 °C for 30 min showed no difference in mitochondrial depolarization under each glycerol condition, indicating a comparable ATP content in each condition (Pandey et al., 2021). However, glycerol concentrations of 6% and 11% led to a 25-fold and 60-fold higher ATP concentration, respectively, compared to the other conditions. This raises the hypothesis that excessive ATP is synthesized through glycolysis or the 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, utilizes energy from ATP hydrolysis to distribute it along the axonemal microtubules, which are the central cytoskeletal structures of the sperm flagellum, in order to generate sperm movement (Alamo et al., 2020). Theoretically, sperm with higher ATP content show higher motility. However, we observed that ATP accumulation in sperm at 30 min under 6% and 11% glycerol conditions resulted in a contrasting decrease in their motility performance. This finding raises another hypothesis that glycerol may disrupt the enzymatic activity of dynein or the structure of the flagellar axoneme, resulting in the accumulation of unused ATP.

5. Conclusion

To conclude, we used the chicken model to demonstrate that glycerol significantly affects sperm capacity to interact with the female reproductive tract. This disruption leads to impaired sperm migration and storage, as well as a reduced ability to penetrate the ovum and achieve fertilization in the oviduct. We also demonstrated that these effects primarily occur at the physiological body temperature, indicating that glycerol effectively preserves sperm functionality during the cryopreservation/thawing process. Consequently, although understanding the complete mechanisms of glycerol toxicity remains a challenge, it is clear that removing glycerol prior to insemination is a valuable solution to prevent fertility reduction. This approach has been proven to be useful in chickens.

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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 Competing Interest

There is no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anireprosci.2023.107330.

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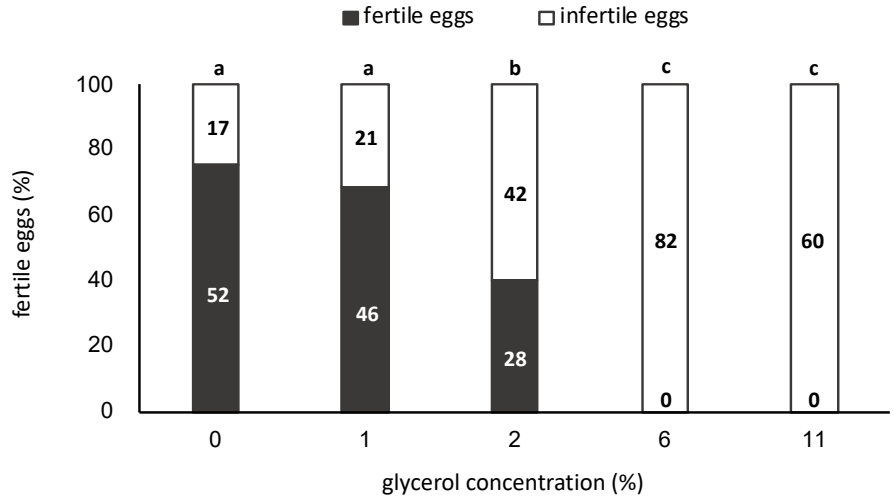
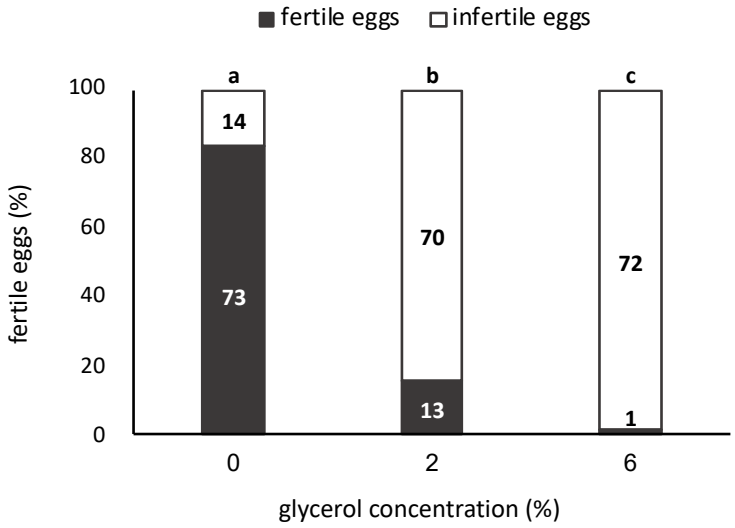
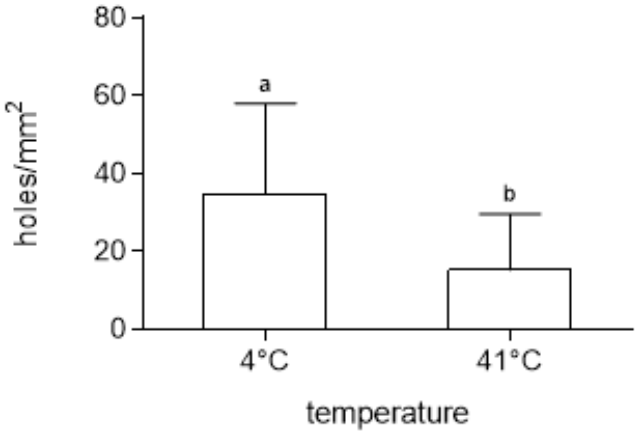
[Supplementary Data of Article 1]



Supplementary Data 1. Fertility (%) of chicken semen samples added with different concentrations of glycerol. Repeated experiment of Figure 1. Black and white bars represent the fertile and infertile eggs, and the number of eggs is reported in the bars. The different letters show a significant difference between glycerol concentrations with p-value < 0.05.



Supplementary Data 2. Fertility of Hoechst 33342-stained glycerolized chicken sperm. Semen collected and pooled from 10 roosters. Number of hens=8 for each treatment (2 experimental replicates). Eggs were collected between 2nd and 7th day after insemination in a dose of 200 × 10^{6} sperm per hen for 2 consecutive days. Black and white bars represent the fertile and infertile eggs, and the number of eggs is reported in the bars. The Different letters show a significant difference between glycerol concentrations with p-value < 0.05.

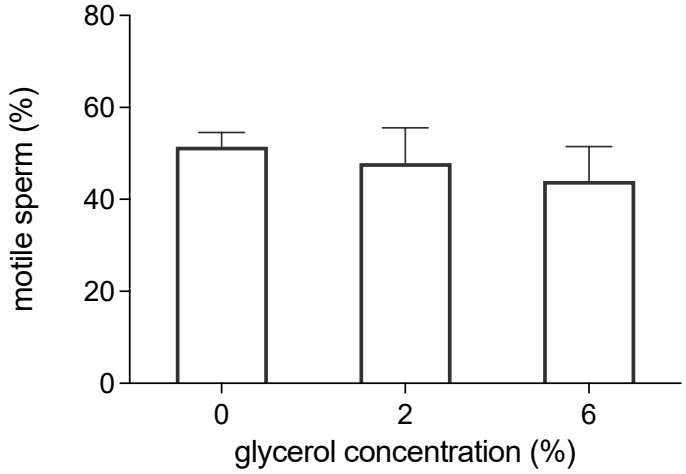


Supplementary Data 3. The number of holes after PVM incubated with glycerolized sperm. The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean. The Different letters show a significant difference between temperatures with p-value < 0.05.

Supplementary Data 4. Effect of glycerol concentration on *in vitro* sperm quality characteristics at 4°C, as reported by P-values

	one-way ANOVA test (p-value)
motile sperm	**< 0.0001**
progressive sperm	**< 0.0001**
mitochondria activity	**0.0017**
ATP	**< 0.0001**
membrane integrity	**< 0.0001**
apoptosis	**< 0.0001**

A



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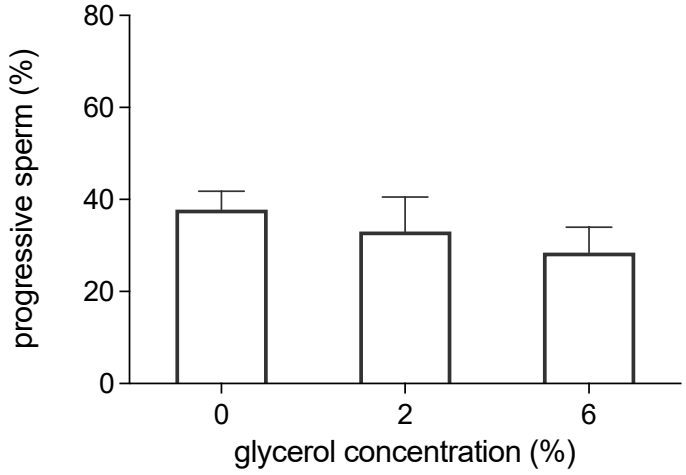

Supplementary Data 5. Glycerolized sperm motility after *in vitro* incubation with sperm storage tubules (SST) for 30 min. A and B represent the percentages of motile (A) and progressive sperm (B). The bars and lines correspond to the mean (5 experimental replicates) and the standard deviation of the mean.

Part 2: Development of a colloidal method to prevent glycerol toxicity from impairing the fertilizing capacity of cryopreserved chicken sperm

1. Abstract

Conservation of animal genetic resources is essential not only for maintaining biodiversity, but also as a crucial opportunity to restore animal populations after sanitary culling. In the field of animal husbandry, it is also an efficient strategy to manage animal breeding programs and reduce workload on the farm. Among all techniques, sperm cryopreservation is considered the most practical one in birds due to its efficient sample collection, simplicity in offspring production, respect for animal welfare, and higher success rate compared to the use of primordial germ cells or gonadal tissue. Glycerol is a commonly used cryoprotectant for sperm cryopreservation. Despite its highly protective properties in helping sperm cells during the freezing and thawing processes, glycerol possesses unwanted cytotoxicity that reduces the fertilizing ability after insemination. This has been reported in several avian and mammalian species. Chicken is the most illustrative example, as a protocol using 11% glycerol is commonly used to freeze rooster sperm. However, hens completely lose their fertility when inseminated with semen containing only 6% glycerol. Thus, a protocol for glycerol removal, namely serial dilution (SD) and centrifugation, was introduced in the thawing process to maintain the fertilizing capacity of thawed sperm. With the development of SD centrifugation, the use of cryopreserved sperm for offspring production has become a viable strategy in the field of chicken breeding. This technique has shown an average fertility rate of 30-50%. However, this method requires specific equipment, involves complex operations, and is time-consuming. It is also difficult to establish a routine in chicken breeding. In addition to these historical descriptions, the previous results section of my thesis once again demonstrates that glycerol toxicity is an inevitable consequence when it is present during insemination. This suggests that glycerol removal is necessary before sperm are deposited in the hens' vagina. Thus, the other purpose of my thesis was to develop a new technique for removing glycerol in order to simplify the use of frozen-thawed semen and potentially enhance its reproductive efficiency. This would allow for better utilization of cryopreserved sperm in chickens.

For this study, we first designed a new protocol for glycerol removal, called sucrose-Percoll (SP) centrifugation. The SP solution consisted of sucrose and Percoll. Sucrose was primarily added here to maintain the osmolarity of the solution for thawed sperm, in addition to its beneficial effects on cell membrane structure. Percoll is a well-established colloid that has been used for selecting thawed sperm in mammalian *in vitro* fertilization. More recently, it has also been used

for selecting viable sperm in fresh semen for insemination in chickens. We prepared four compositions of SP solution (SP 40, 50, 60 and 70%) and conducted centrifugation at two temperatures (4 and 20°C) to evaluate their effects on sperm *in vitro* quality parameters and *in vivo* fertility tests. Finally, we determined that the optimal condition was SP 40% at either 4 or 20°C. The novelty of this study was the addition of sucrose in Percoll to increase the osmolarity of the working solution. This is important to avoid thawed sperm from suffering osmotic shock, and it is also a key point in adapting this new SP solution for chicken thawed semen. To the best of our knowledge, this is the first study to successfully apply colloidal centrifugation to remove glycerol from thawed chicken semen and achieve successful egg fertilization after insemination.

Thereafter, we performed the new SP centrifugal method at both 4 and 20°C (4°C-SP and 20°C-SP) and compared it with SD centrifugation to demonstrate how they restored the fertilizing capacity of glycerol cryopreserved sperm. We found that SP centrifugation removed more glycerol from thawed semen than the SD method. We also observed that sperm centrifuged at 4°C-SP showed a higher percentage of sperm filled SST compared to sperm centrifuged at 20°C-SP, which was higher than sperm treated with SD. This tendency was also consistent with fertility rates. In addition, centrifugation at 4°C-SP led to frozen-thawed sperm achieving a longer fertilizing duration compared to the other two treatments. Here, we have demonstrated that our new SP centrifugation approach has superior potential compared to the SD method for removing glycerol from cryopreserved chicken semen. This is supported by direct evidence of *in vivo* sperm storage and its impact on fertility outcomes, as well as its simplicity and time efficiency.

Collectively, this new SP glycerol removal protocol is valuable for a chicken sperm cryobanking program. This is supported by direct evidence of successful restoration of sperm fertilizing capacity through the reimplantation of sperm storage in hens' oviducts, resulting in increased fertility. We highly recommend using SP centrifugation under 4°C conditions.

This part of the thesis includes two research articles (Article 2 and Article 3). The first article, titled "A simple and fast alternative method to remove glycerol from chicken semen after cryopreservation", has been published in the journal Cryobiology in August 2023. Another article titled "Exploring how glycerol removal approaches successfully preserve fertilizing capacity of cryopreserved chicken sperm" will be submitted to the journal Poultry Science.

2. Résumé

La conservation des ressources génétiques animales est essentielle non seulement pour maintenir la biodiversité, mais aussi pour restaurer les populations animales après un abattage sanitaire. Elle est aussi une stratégie efficace pour gérer les programmes d'élevage et réduire la charge de travail à la ferme. Parmi toutes les techniques, la cryoconservation des spermatozoïdes (SPZ) est la plus pratique chez les oiseaux en raison d'une collecte d'échantillons efficace, d'une utilisation simple pour la production de poussins, du respect du bien-être animal et d'un taux de réussite plus élevé que les cellules germinales primordiales ou le tissu gonadique. Le glycérol est un agent cryoprotecteur souvent utilisé pour la cryoconservation des SPZ. Bien que le glycérol soit hautement protecteur pour aider les SPZ à surmonter les processus dangereux de congélation et de décongélation, il possède une cytotoxicité indésirable pouvant diminuer la capacité de fécondation, comme signalé dans plusieurs espèces aviaires et mammifères. Le poulet est le cas le plus significatif puisque 11% de glycérol est le protocole le plus utilisé pour congeler les SPZ de coq, alors que les poules sont infertiles lorsqu'elles sont inséminées avec de la semence contenant 6% de glycérol. Ainsi, un protocole d'élimination du glycérol, à savoir la méthode de dilution sériée et centrifugation (DSC), a été introduit dans le processus de décongélation pour le maintien de la capacité de fécondation des SPZ décongelés. En effet, avec le développement de la DSC, l'utilisation de la semence cryoconservée pour la production de progéniture devient une stratégie réalisable chez les poulets avec 30 à 50% de fertilité en moyenne. Cependant, cette méthode nécessite un équipement spécifique, des opérations complexes ainsi que du temps, difficile à adapter en routine dans un élevage de poulet. De plus, la partie précédente des résultats de ma thèse montre encore une fois que la toxicité du glycérol est un événement inévitable s'il est présent lors de l'insémination, suggérant que l'élimination du glycérol est obligatoire avant l'insémination des poules. Ainsi l'autre objectif de ma thèse visait à développer une nouvelle technique d'élimination du glycérol pour simplifier l'utilisation de la semence décongelée et probablement améliorer l'efficacité de l'insémination, afin de faire une meilleure utilisation des SPZ cryoconservés chez les poulets.

Pour cette étude, nous avons d'abord conçu une nouvelle méthode d'élimination du glycérol, à savoir la centrifugation sur sucrose-Percoll (CSP). Le sucrose a été ajouté ici principalement pour maintenir l'osmolarité de la solution pour les SPZ décongelé en plus de son avantage sur la structure de la membrane. Le Percoll est un gel colloïdal classique utilisé pour la sélection de SPZ décongelés lors de la fécondation *in vitro* chez les mammifères et plus récemment pour la sélection de SPZ de la semence fraîche pour l'insémination chez les poulets. Nous avons donc préparé quatre compositions de solution SP (SP 40, 50, 60 et 70%) et opéré la centrifugation à deux températures (4 et 20°C) puis évalué les effets en observant les paramètres de qualité des SPZ *in vitro* et par des tests de fertilité *in vivo*. Finalement, nous avons déterminé que la

meilleure condition était SP 40% à 4 ou à 20°C. La nouveauté de cette étude était l'ajout de sucrose au Percoll pour augmenter l'osmolarité de la solution de travail et limiter le choc osmotique lors du retrait du glycérol. À notre connaissance, il s'agit de la première étude à appliquer avec succès la centrifugation colloïdale pour éliminer le glycérol des SPZ décongelés de coq et produire des œufs fertiles après insémination.

Par la suite, nous avons testé cette nouvelle méthode de CSP à 4 et 20°C (4°C-CSP et 20°C-CSP) et comparée avec la DSC, pour évaluer la capacité fécondante des SPZ cryoconservés au glycérol. Nous avons constaté que la CSP éliminait plus de glycérol de la semence décongelée que la DSC. Nous avons également observé que les SPZ traités avec la condition 4°C-CSP colonisaient un pourcentage plus élevé de SST que la condition 20°C-CSP,les deux conditions étant supérieures aux SPZ traités par la DSC. Cette tendance était également cohérente avec la fertilité. De plus, les SPZ traités avec 4°C-CSP montré une durée de fécondation plus longue que les deux autres traitements. Ici, nous avons prouvé que notre nouvelle méthode est une efficace pour l'élimination du glycérol, améliorant le stockage des SPZ et la fertilité.

En conclusion, ce nouveau protocole d'élimination du glycérol, la CSP, sera précieux pour les programmes de cryobanque de SPZ chez les poulets, grâce à la restauration efficace de la capacité et la durée de fécondation des SPZ en améliorant le stockage des SPZ dans les oviductes et en augmentant la fertilité. Nous recommandons particulièrement d'utiliser la CSP à 4°C.

Cette partie de la thèse est présentée dans deux articles de recherche (Article 2 et Article 3). "Une méthode alternative simple et rapide pour éliminer le glycérol de la semence de coq après la cryoconservation" est le titre d'un article publié par le journal Cryobiology en août 2023. Une autre étude intitulée "La restauration de la capacité fécondante des spermatozoïdes de coq cryoconservés au glycérol" est soumise au journal Poultry Science.

3. Article 2

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A simple and fast alternative method to remove glycerol from chicken semen after cryopreservation

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ABSTRACT

A concentration of 11% of glycerol is the standard one for sperm cryopreservation in chickens, however, the presence of just 2% glycerol already causes severe fertility reduction, suggesting the necessity of removing glycerol before artificial insemination (AI). The major approach developed for this purpose is serial dilution followed by centrifugation (SDC), which demands special equipment (such as a refrigerate room) to maintain post-thaw semen at 4 °C, besides being time consuming. Therefore, we attempted to develop a simple method to remove glycerol from chicken frozen-thawed semen based on a colloidal gel, Percoll, which is ordinarily used to select motile and viable sperm in mammals as well as in fresh chicken semen. In this study, we used a Percoll based glycerol removal solution (GRS) containing sucrose to avoid frozen-thawed sperm suffering from osmotic stress. Subsequently, several conditions including GRS compositions (GRS A, B, C and D) and centrifugation temperatures (4 and 20 °C) were compared by their influence on sperm *in vitro* parameters. Afterwards, GRS A and D were selected for fertility evaluation, compared to conventional SDC method. Our results showed that the fertility with GRS A at both 4 and 20 °C were higher than GRS D ($p < 0.05$) and similar or even superior to the fertility obtained with SDC method. Altogether, our novel GRS protocol is a valuable method for chicken sperm cryobanking policy, supported by its notable results of fertility as well as saving 44% of time, with a simple equipment at flexible operation temperatures of 4 or 20 °C.

1. Introduction

Preservation of animal genetic resources is a crucial strategy not only to conserve the biodiversity of species and breeds, but also to restore animal populations after sanitary culling [4,45,51]. Sperm cryopreservation allows the collection of large quantities of cells, respect animal welfare, and offers the possibility of long-distance resource exchanges as well as long-term storage [9,10].

Glycerol is a preferable cryoprotectant to freeze chicken sperm because it provides to sperm cells an excellent protection against cryodamages, ensures long-term storage for cryobanking, retains fertilizing capacity and achieves normal embryonic development [1,3,5,41,47]. However, the presence of glycerol in frozen-thawed semen harshly affects fertility [24,25,36], and the mechanisms underlying this negative effect remain unclear. Therefore, the only solution nowadays is to remove this cryoprotectant before artificial insemination (AI) to avoid fertilization failure. Currently, the most common method used to reduce glycerol concentration from chicken frozen-thawed semen is the serial dilutions followed by centrifugation procedure (SDC) [22,39]. However, SDC implies special equipment to maintain semen temperature at 4 °C (i.e. refrigerated room) and is time consuming (requiring approximately 30 min to treat 10 straws of thawed semen) [24].

Density gradient centrifugation based on colloidal solutions, such as Accudenz or Percoll, has been used to select highly motile sperm cells [31]. In chickens, Accudenz was once reported to successfully remove glycerol from frozen-thawed semen and select motile sperm cells in 6 lines of roosters acquired from USDA [37], however, no information about fertility post-treatment was reported in the study. Meanwhile, Percoll, a nondialyzable polyvinylpyrrolidone (PVP) coated silica particles in a salt solution, was used to select motile sperm for *in vitro* fertilization after sperm cryopreservation in mammals [14,34] and was recently introduced as single-layer centrifugation method to select motile sperm from fresh semen in chickens [23]. Taking the advantage of our previous experiments [23], we aimed to develop a novel, easy and

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rapid protocol based on Percoll to remove glycerol from chicken frozen-thawed semen and select motile sperm.

Osmotic shock may cause avian sperm damages leading to loss of motility [28]. The addition of sucrose could be useful to maintain solution osmolarity for frozen-thawed chicken sperm in addition to its beneficial effect on cell membrane structure [46]. Besides, sucrose is also known as a selection agent for motile sperm in boars [17]. Hence, in order to avoid any osmolarity issue, we intended to add sucrose to a Percoll based solution, to remove glycerol from cryopreserved chicken semen.

Another factor that may influence the efficiency of a glycerol removal procedure is the temperature. Indeed, keeping semen at low temperature (4–5 °C) reduces sperm metabolism and retains sperm motility and fertilizing capacity [11,15,38], however, a procedure temperature of 20 °C is certainly more adapted to farm conditions, thus we decided to compare both temperatures.

In this work, we first defined the best condition of the glycerol removal solution (GRS) (including 4 compositions and 2 temperatures of centrifugation) to efficiently remove glycerol from frozen-thawed semen and meanwhile retaining sperm performances. Finally, we evaluated the impact of GRS treatment on fertility, compared to the one obtained with conventional serial dilution method.

2. Materials and methods

2.1. Animal management

A total of 18 adult T44 roosters (31–45-week-old, *Gallus domesticus*, Sasso, France) were used as semen donors and 96 adult Lohmann hens (26–50-week-old) were used for fertility tests. All roosters were housed in individual cages and the hens were housed in cages by groups of 4 at the INRAE Poultry Experimental unit UE-PEAT INRAE (2018, https://doi.org/10.15454/1.5572326250887292E12). All animals were under a lighting regimen of 14 h light:10 h dark, controlled temperature at 20 °C, feeding with a standard diet and water *ad libitum*. All experiments were carried out 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 #4026–2016,021,0 15,509,521 and APAFIS #34415–202,112,141,205,965).

2.2. Semen collection, cryopreservation, and thawing

Eighteen roosters were selected for this study based on sperm evaluations including motility (>80%), viability (>80%) and fertility (>85%). Considering the handling capacity of sperm cryopreservation operation, animals were randomly divided in 3 groups for semen collection. Semen was collected twice a week by abdominal massage method [8] and cares were taken to avoid any possible contamination from cloacal products. Individual semen of each group (6 animals) was collected, pooled in 2.5 mL, diluted 1:1 with Lake PC diluent [20] at room temperature then gently mixed and placed at 4 °C for 10 min. The resulting suspension was then diluted 1:1 with Lake PC diluent containing 22% (vol/vol) glycerol to reach final concentration of 11% glycerol. After dilution, sperm concentration was between 1200 and 1500 × 10^6 cells/mL and equilibrated at 4 °C with gentle agitation for 10 min [33]. After equilibration, semen was filled into 0.5 mL plastic freezing straws (IMV Technologies) and sealed. The straws were transferred into a programmable freezer (MiniDigitcool, IMV Technologies) and frozen with the controlled freezing rate of −7 °C/min from 4 to −35 °C and then of −60 °C/min from −35 to −140 °C [33]. The straws were then plunged into liquid nitrogen (−196 °C) and stored at least 2 weeks [47] until thawing. For each experiment, the straws were retrieved equally from 3 rooster groups and thawed in a water bath at 4 °C for 3 min. After thawing, semen was gently mixed and distributed equally to each treatment. The number of all experimental replicates are given in the Figure legends.

2.3. Glycerol removal protocol

2.3.1. Testing sucrose level in percoll solution

Sucrose addition to Percoll solution was necessary to avoid frozen-thawed sperm suffering from osmotic stress [28] and different sucrose concentrations were compared here to establish its workable range. Briefly, sucrose was firstly added to Percoll 50% solution to obtain sucrose-Percoll solution in different sucrose concentration (0, 100, 200 and 400 mM; and 0, 50, 100 and 150 mM, respectively for 2 sets of experiment) and transferred 2 mL to a 15 mL conical plastic centrifuge tube. Then, 500 μL of frozen-thawed semen was gently layered on the top of the sucrose-Percoll solution and centrifuged 15 min at 800 g at 20 °C. After centrifugation, the supernatants were discarded and sperm pellets were resuspended in 100 μL of Lake 7.1 diluent [21] to evaluate sperm motility, which has been reported as an indicator of their fertilizing capacity in chickens [5]. Control in this study was frozen-thawed semen without Percoll treatment and stored at 20 °C during the centrifugation process.

2.3.2. Comparison of glycerol removal conditions

Percoll based solution was developed and named as glycerol removal solution (GRS). Glycerol removal protocols, including 4 compositions of GRS and 2 centrifugation temperatures, were compared in this study. Based on the results of previous experiment, 4 compositions of GRS were designed and named as GRS A, B, C and D respectively containing sucrose 80, 100, 120 and 140 mM and Percoll at 36, 45, 54 and 63% (osmolarity were 408, 440, 472 and 506 mOsmol) (Supplementary Table 1). A volume of 500 μL of frozen-thawed semen was softly placed on each 2 mL of GRS in a 15 mL conical plastic tube and centrifuged 15 min at 800 g at 4 or 20 °C. Control in this experiment was frozen-thawed semen without GRS treatment and stored at 4 or 20 °C during centrifugation. The supernatants and sperm pellets were manipulated as previously described and glycerol concentration, sperm cells retrieval, sperm motility, membrane integrity and mitochondria activity (mitochondria membrane potential, MMP) and fertilizing ability were evaluated.

2.4. Sperm in vitro evaluation

2.4.1. Glycerol concentration

Glycerol Assay Kit (Sigma-Aldrich) was performed here to determine glycerol concentration according to the manufacturer's instructions. Briefly, 10 μL semen were loaded in wells of the 96-well microplate then 100 μL of reaction solution were added directly to each well and examined by spectrophotometry (Tecan Infinite M200 Plate Reader, Tecan Life Science) to record the absorbance at a wavelength of 570 nm after incubation for 20 min at room temperature in darkness. Finally, the absorbance was transformed to glycerol concentration based on a standard curve.

2.4.2. Sperm cells retrieval

After GRS centrifugation, sperm pellet volume was determined by weighing and sperm concentration was measured by a photometer (Accucell photometer, IMV Technologies) at a wavelength of 540 nm. Sperm numbers after GRS centrifugation = pellet volume (mL) × pellet concentration (10^6 sperm/mL), and sperm numbers before GRS centrifugation = 0.5 (mL) × sperm concentration (10^6 cells/mL) of post-thaw semen. The calculation of sperm cells recovery was as follows:

$$\text{Recovery } (\%) = \frac{\text{sperm numbers after GRS centrifugation}}{\text{sperm numbers before GRS centrifugation}}$$

2.4.3. Sperm motility

Sperm motility parameters were evaluated with a computer-assisted

sperm analysis (CASA, IVOS Motility Analyzer, Hamilton Thorne, IMV Technologies). Sperm concentration was adjusted to 30×10^6 cells/mL and a 2 μL aliquot was placed on a Makler Counting Chamber to capture four field images of each sample and analyze average path velocity (VAP), straight line velocity (VSL) and straightness (STR = VSL/VAP). Motility results were presented as percentage of motile sperm and progressive sperm, which were defined as the percentage of sperm showing a VAP >5 μm/s and VAP >50 μm/s with STR >75%, respectively.

2.4.4. Membrane integrity

SYBR 14 and Propidium iodide (PI) fluorescent dyes (Molecular Probes, LIVE/DEAD® Sperm Viability Kit, #L7011) combined with a flow cytometer (Guava® easyCyte, IMV Technologies) were used to detect sperm membrane integrity. Semen was diluted to 1×10^7 sperm/mL in PBS to final volume of 200 μL and then incubated with 1 μL of SYBR 14 (2 μM) and 1 μL of PI (240 μM) for 10 min at 37 °C. A total of 5000 events of each sample were analyzed with Guava® easyCyte and the results were expressed as percentage of sperm with intact membrane [23].

2.4.5. Mitochondria activity

Mitochondria membrane potential (MMP) was assessed with a fluorescent probe JC-1 (Sigma-Aldrich, CAS NO.: 3520-43-2) which is a green-fluorescent monomer at low membrane potential and forms red-fluorescent aggregates at higher potential. Semen was diluted to 1×10^7 sperm/mL in PBS to 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 5000 events of each sample were analyzed by Guava® easyCyte and the results were expressed as the ratio of red to green fluorescence of JC-1.

2.5. Fertility tests

Since serial dilution centrifugation (SDC) is the ordinary method used to remove glycerol from chicken frozen-thawed semen, it was used as the control treatment to compare with GRS centrifugation in this study. Briefly, frozen-thawed semen was gradually diluted to a final dilution of 1:4 (adding diluent 6 times totally with an interval of 2 min) with Lake C diluent [22] at 4 °C with gentle agitation. Serial diluted semen was immediately centrifuged 15 min at 500 g at 4 °C. Supernatants were discarded and sperm pellets resuspended in 100 μL of Lake 7.1. Intravaginal AI (4 cm depth) was performed with frozen-thawed semen treated after GRS centrifugation or SDC in a dose of 200×10^6 sperm per hen, totally 12 hens per experimental group. Three AI trials were conducted individually to compare GRS A and D at 4 °C, at 20 °C, and GRS An at both 4 and 20 °C (Fig. 1). Eggs were collected from day 2 after the first AI to day 5 after the last AI and stored at 15 °C for 7 days before the onset of incubation at 37.7 °C. Fertile and infertile eggs were

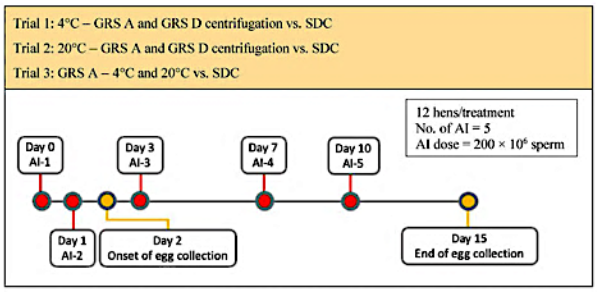

Fig. 1. Artificial insemination (AI) trials of frozen-thawed semen treated with GRS centrifugation and SDC. GRS: glycerol removal solution. SDC: serial dilution centrifugation. Each AI trial contained 5 successive inseminations. Sperm number of each AI was 200×10^6 sperm per hen. Eggs were collected from day 2 of the first AI to day 5 of the final AI in each trial.

determined by candling at the 7th day of incubation [25,46]. AI trials were performed with different AI frequency and different population of 18 males and 36 females (Supplementary Fig. 1) to validate the tendency and results provided as Supplementary Fig. 2.

2.6. Statistical analysis

All statistical analyses of sperm *in vitro* evaluations were performed with RStudio software version April 1, 1717. For the experiment of testing sucrose level in Percoll solution, the results of sperm motility presented normal distribution and applied one-way ANOVA test to evaluate the effect of sucrose concentration in sucrose-Percoll solution. For the experiment of comparison of glycerol removal conditions, data of glycerol removal, sperm cells retrieval, sperm motility, membrane integrity, and mitochondria activity were normally distributed and analyzed using two-way ANOVA to examine the effects of GRS composition and centrifugation temperature. Differences in the means were analyzed by Tukey's HSD Post Hoc test with a significance of 5%. Statistical analyses of fertility tests were performed with GraphPad Prism version 6.07. The impact of glycerol removal method on fertility was analyzed with a Chi2 test with a statistical significance of 5%.

3. Results

3.1. Impact of sucrose presence in percoll solution on sperm motility

Sucrose concentration in Percoll solution impacted sperm motility at both 0–400 mM and 0–150 mM ranges (Fig. 2 and Supplementary Table 2), compared to non-centrifuged semen (control). Percoll centrifugation in the absence of sucrose had no impact on the percentage of motile sperm (Fig. 2A) but reduced the percentage of progressively motile sperm of 10% compared to control (Fig. 2B). Sucrose addition in the range of 100–400 mM resulted in higher motile and progressive sperm percentage than 0 mM (Fig. 2A–B), whereas a significant decrease of these sperm motility parameters was observed at 400 mM of sucrose compared to 100 and 200 mM. The positive effect of sucrose addition on sperm motility was observed as well in the narrower range of sucrose concentration (Fig. 2C–D), showing no significant difference between 50 and 150 mM.

3.2. GRS centrifugation eliminates glycerol efficiently

The impact of different GRS compositions (GRS: A, B, C and D) and temperatures (T: 4 and 20 °C) on glycerol removal efficiency were investigated. While no effect of T or interaction of GRS and T were found (Supplementary Table 3), a significant decrease in glycerol concentration with all GRS compositions (Fig. 3) was observed when compared to control condition. In fact, a concentration inferior to 120 mM was observed after GRS centrifugation, i.e. a reduction of 93.6% of glycerol in frozen-thawed semen samples.

3.3. GRS centrifugation retrieves sperm successfully

GRS composition impacted on sperm concentration (Supplementary Table 3), mainly due to the significant decrease induced by GRS D when compared to GRS A and B at 20 °C (Fig. 4A). No significant difference was observed at 4 °C. Among all conditions, GRS A at 20 °C obtained the highest sperm concentration with no difference with GRS A at 4 °C ($p < 0.05$) and GRS B at 20 °C ($p < 0.05$) (Fig. 4A). The interaction of GRS and T was significant for sperm recovery (Supplementary Table 3), supported by the lower sperm recovery observed with GRS D at 20 °C when compared with all other conditions (Fig. 4B).

3.4. GRS centrifugation positively selects for sperm motility

Significant impacts of GRS, T and their interaction were observed on

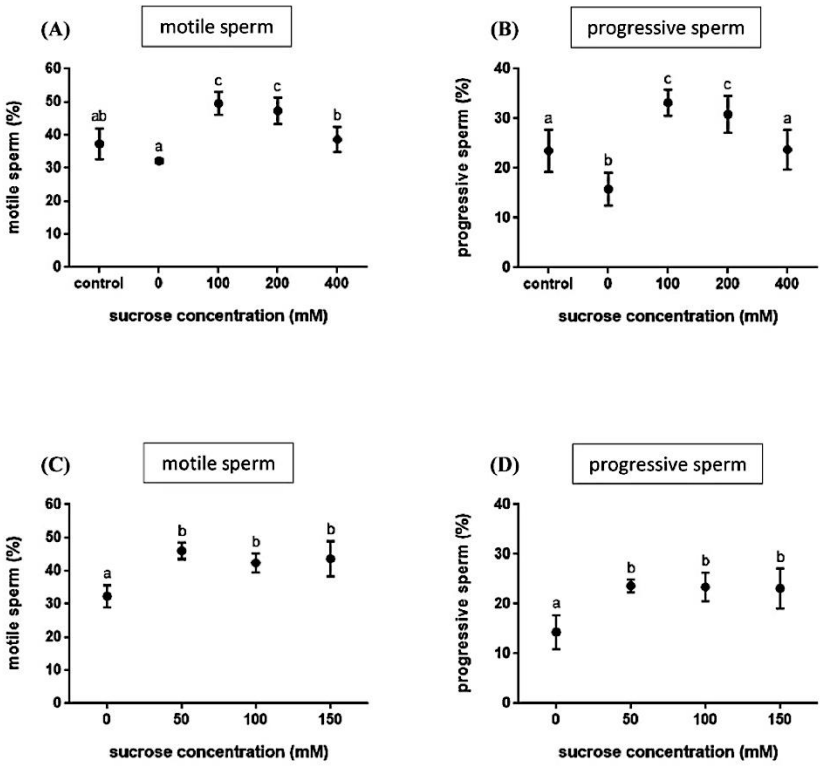

Fig. 2. Effect of sucrose concentration in Percoll solution on sperm motility. (A) (B) and (C) (D) represent sperm motility parameters of post-thaw semen treated with Percoll solution contains sucrose of 0~400 mM and 0–150 mM, respectively. Control: frozen-thawed semen without any treatment and stored at 20 °C. The dots and lines correspond to the mean (n = 6) and the standard deviations to the mean. Different letters indicate significant differences between two conditions (p-value <0.05).

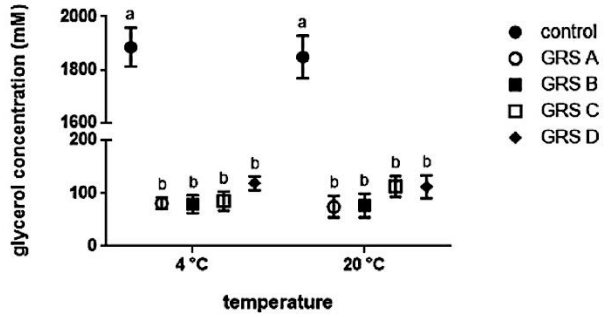

Fig. 3. Effect of GRS composition and temperature (T) on glycerol removal efficiency. GRS: glycerol removal solution. Control: frozen-thawed semen without any treatment and stored at 4 or 20 °C. The dots and lines correspond to the mean (n = 5) and the standard deviations to the mean. Different letters indicate significant differences between two conditions (p-value <0.05).

sperm motility parameters (percentages of motile and progressive sperm cells, Supplementary Table 3). In control conditions (without GRS centrifugation), storing post-thaw semen at 20 °C rather than at 4 °C induced a significant reduction in sperm motility (Fig. 5). While no impact of GRS treatment on sperm motility was observed at 4 °C, the same treatment at 20 °C increased the sperm motility parameters when compared to control, thus compensating the loss of motility induced by storing at 20 °C and reaching similar levels of sperm motility as those stored at 4 °C.

3.5. *GRS centrifugation influences sperm membrane integrity and mitochondria activity*

While GRS composition impacted sperm membrane integrity and mitochondria activity, T only influenced membrane integrity (Supplementary Table 3). GRS C and GRS D centrifugation at 4 °C showed a higher percentage of sperm cells with intact membrane than control with near 50% of disrupted membrane, while this increase was not observed at 20 °C. Besides, a significant reduction was found with GRS A at 20 °C (Fig. 6A). A significant reduction of mitochondria membrane potential (MMP) was observed at 4 °C with GRS B and at 20 °C with GRS B, C and D when compared with control (Fig. 6B).

3.6. *GRS centrifugation benefits fertility rate*

Based on the results of all *in vitro* parameters (summarized as Table 1), we chose to test the fertility of post-thaw semen treated with GRS A and GRS D since they achieved respectively superior results of sperm retrieval and/or positive selection for sperm quality parameters. Compared with control SDC treatment, GRS A centrifugation resulted in a superior fertility at 4 °C (Fig. 1-Trial 1 and Fig. 7A) and comparable fertility at 20 °C (Fig. 1-Trial 2 and Fig. 7B). Furthermore, the fertility of semen treated with GRS A was higher than the ones obtained with GRS D at both temperatures (Fig. 7A and B and Supplementary Figs. 2A and 2B). We therefore clarified the effect of temperature of GRS A treatment on fertility and observed higher fertility rate at 4 °C than at 20 °C, both higher than the ones obtained with control (Fig. 1-Trial 3 and Fig. 7C). Nevertheless it showed a different tendency of temperature effect in another AI experiment (Supplementary Figs. 1 and 2).

4. Discussion

Inspired by density characteristics for selecting motile sperm from

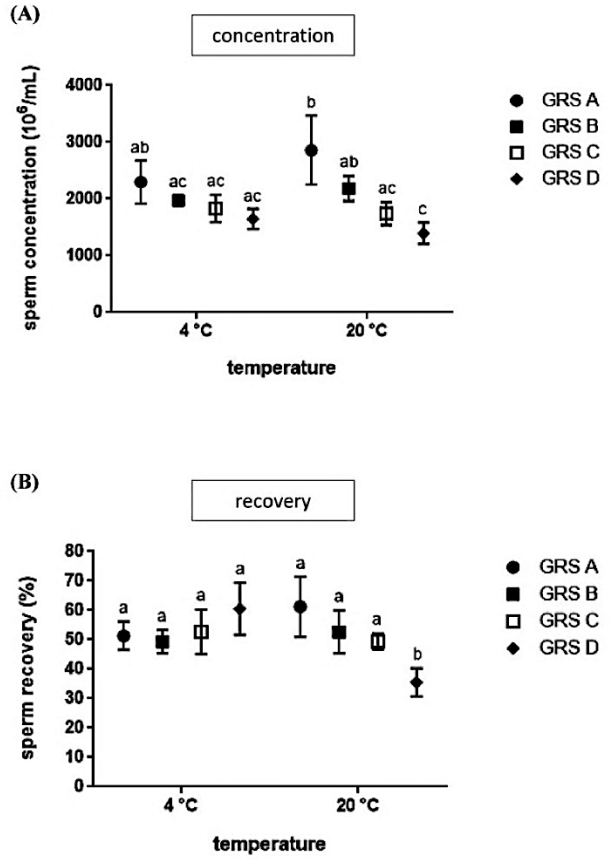

Fig. 4. Effect of GRS composition and temperature (T) on sperm retrieval. GRS: glycerol removal solution. Sperm recovery (%) = percentage of sperm numbers harvested from 500 μL of frozen-thawed semen. (A) and (B) represent sperm concentration and recovery after GRS treatment. Control: frozen-thawed semen without any treatment and stored at 4 or 20 °C. The dots and lines correspond to the mean (n = 5) and the standard deviations to the mean. Different letters indicate significant differences between two conditions (p-value <0.05).

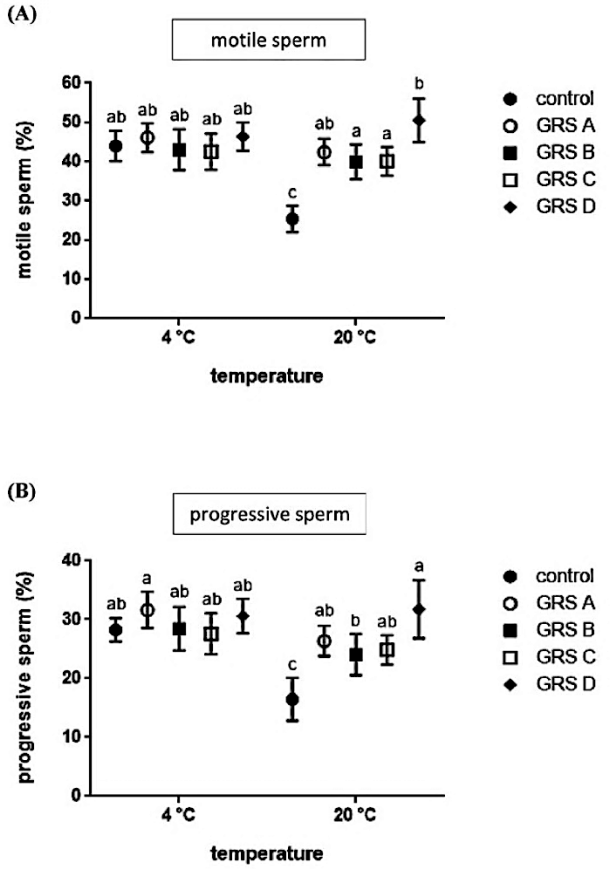

Fig. 5. Effect of GRS composition and temperature (T) on sperm motility. GRS: glycerol removal solution. (A) and (B) represent the percentage of motile and progressive sperm after GRS treatment. Control: frozen-thawed semen without any treatment and stored at 4 or 20 °C. The dots and lines correspond to the mean (n = 5) and the standard deviations to the mean. Different letters indicate significant differences between two conditions (p-value <0.05).

cryopreserved semen in mammals [14,34] and fresh semen in chickens [23], we aimed to develop a new strategy based on Percoll colloid centrifugation, to remove glycerol and select sperm with high motility from chicken frozen-thawed semen. However, our preliminary experiments revealed that the single use of Percoll caused a decrease of sperm motility, suggesting that Percoll alone did not work for chicken frozen-thawed semen. We hypothesized that the loss of sperm motility may be due to acute change of osmolarity [28] between freezing solution (1800 mOsmol) and Percoll solution (335 mOsmol). Thus, we decided to use sucrose to increase the osmolarity of Percoll solution. Sucrose also presents other beneficial effects such as its capacity to stabilize sperm membrane structure in chickens [46] and to select motile sperm in boars [17]. Surprisingly, only slight addition of 50 mM of sucrose, increasing the osmolarity of 58 mOsmol, was already enough to improve sperm motility. This beneficial effect was observed within a range of sucrose concentration between 50 mM and 200 mM, which was the reference for the preparation of glycerol removal solution (GRS) with Percoll and sucrose in the present study.

Here we showed for the first time that centrifugation of thawed semen on GRS can eliminate glycerol efficiently. Previous work reported that chicken semen containing 2% of glycerol impaired the fertilizing ability in hens [32,36]. In our study, glycerol concentration dropped to less than 120 mM (equivalent of <1% of glycerol) in post-thaw semen after GRS treatment. In other words, our novel GRS centrifugation method achieved glycerol concentrations compatible with AI conditions and combined with other sperm quality parameters could potentially predict fertilizing ability of glycerol cryopreserved semen after thawing.

In addition to efficiently removing glycerol, GRS treatment successfully retrieved sperm cells with a satisfying concentration for AI manipulation. A different protocol based on another colloid centrifugation, Accudenz (AD) has been used to remove glycerol from frozen-thawed chicken semen [37] and recovered semen in concentration of 239×10^6 sperm cells/mL. Our new GRS centrifugation method could retrieve 5–10 times higher sperm concentration than AD centrifugation with an AI volume of less than 150 μL per hen (dose = 200×10^6 sperm cells), which is a well-adapted volume for intravaginal insemination without losing extra sperm from cloaca [6].

AI tests in this study revealed that GRS A obtained higher fertility than GRS D, supporting GRS A as the optimal GRS composition to treat frozen-thawed chicken semen. Such results were unexpected since our *in vitro* observations showed that GRS D treatment led to no difference of sperm motility and mitochondria activity but better performance of sperm membrane integrity than GRS A. However, sperm membrane integrity evaluation is not enough to predict fertility potential of frozen-thawed semen and should be associated with other molecular analysis

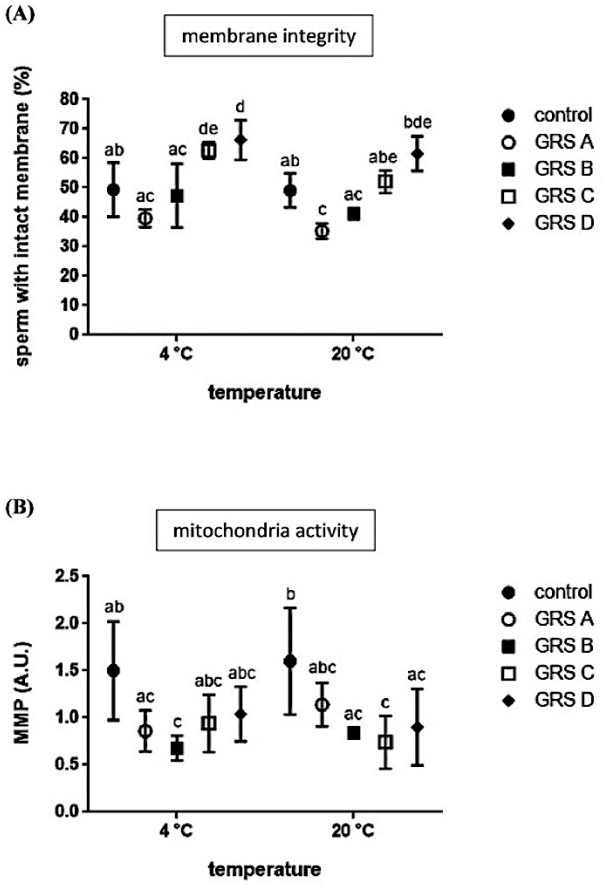

Fig. 6. Effect of GRS composition and temperature (T) on sperm membrane and mitochondria status. GRS: glycerol removal solution. MMP: mitochondria membrane potential. (A) and (B) represent the membrane integrity (%) and MMP (A.U.) after GRS treatment. Control: frozen-thawed semen without any treatment and stored at 4 or 20 °C. The dots and lines correspond to the mean (n = 5) and the standard deviations to the mean. Different letters indicate significant differences between two conditions (p-value <0.05).

such as proteomic approaches [42,43,49]. Additionally, GRS D treated semen showed a lower sperm concentration, requiring more volume than GRS A to reach same AI sperm dose, equivalent to inject more glycerol into vagina, that might cause enough damage to sperm transport and storage in the female tract to consequently impair fertility [12,50]. Moreover, polyvinylpyrrolidone (PVP), one of the components of Percoll, reported to damage sperm structures and induce defective embryo development [19,44]. As GRS D contains more Percoll colloid, thus more PVP, it could be more toxic to chicken sperm cells.

Despite negative impact on sperm membrane integrity at 20 °C, GRS A centrifugation took 15 min to complete glycerol removal process (Table 2), allowed more than 50% of sperm recovery with suitable concentration, achieved a positive selection for sperm motility and obtained higher fertility than GRS D. Altogether this suggests GRS A as the optimal treatment for processing post-thaw chicken semen. We further investigated the influence of temperature of GRS A treatment on fertility since low temperature (4–5 °C) is beneficial to sperm metabolism and fertility [11,15,38], and 20 °C is indeed more practical than 4 °C under farm conditions. The results revealed that higher fertility was obtained after treatment at 4 °C than at 20 °C. However, no effect of temperature was detected in another repeated manipulation (Supplementary Fig. 1-Trial 3). Further investigation through AI trials will be required to validate this results. Although it is complex and time consuming, SDC is still the most common and the only successful procedure for processing glycerol cryopreserved chicken semen to obtain fertile eggs by intravaginal insemination [39,47]. Therefore, we used SDC treatment semen as control condition in this study. Taking all the fertility tests together, GRS A centrifugation whether at 4 or 20 °C led to an equivalent or even superior fertility than conventional SDC method.

Apart from glycerol, other cryoprotectants could be used for chicken sperm cryopreservation and may have negative effects in hen's reproductive tract and fertility [16,29]. However, no link between CPA removal for other cryoprotectant and fertility rates was ever demonstrated. It would be interesting to apply this GRS centrifugation for removing other cryoprotectants from post-thaw semen before insemination and observe how it affects fertility.

Glycerol is the most broadly used cryoprotectant for animal sperm cryopreservation [48], however it can influence sperm structures and metabolism both due to osmotic and non-osmotic effects at physiological body temperature [7,26]. Removing glycerol from post-thaw semen before AI is a necessary procedure in chickens [37,47] but has never been recommended in mammalian species. Thus, it might be worthy to test this glycerol removal method on frozen-thawed semen of other species to examine if it improves fertility. Despite glycerol possibly causes fertilization failure in birds, it remains the favor cryoprotectant used in the freezing diluent in most avian species regarding to its broadly cryoprotective efficiency [18,51]. Thus, our new protocol shall be useful to be included in the AI procedure of conservation breeding programs in rare/local avian breeds to improve the use of cryopreserved semen. However, *in vitro* and *in vivo* trials should be performed to investigate potential adaptation.

Percoll discontinuous gradient centrifugation has been used for cryopreserved sperm selection in boars, bulls and humans [13,27,35], resulting in increased efficiency of *in vitro* fertilization (IVF) [2,34,40]. Nevertheless, our GRS method did not show such positive effects on sperm structures (i.e. membrane or mitochondria), possibly resulted from the usage of single Percoll layer in our study instead of Percoll gradients. However, single layer centrifugation is simpler, cost efficient and yields more sperm recovery than gradient centrifugation [23,30,

Table 1
Summary of *in vitro* sperm evaluation after GRS centrifugation.

		4 °C				20 °C			
GRS[a]		A	B	C	D	A	B	C	D
glycerol removal	concentration	+	+	+	+	+	+	+	+
sperm retrieval	concentration	+	=	=	=	+	+	=	=
	recovery	=	=	=	=	=	=	=	-
sperm motility	motile	++	++	++	++	++	+	+	++
	progressive	++	++	++	++	++	+	++	++
sperm quality parameters	membrane	=	=	+	+	-	=	=	+
	MMP	=	-	=	=	=	=	-	=
score		6	4	6	6	5	4	3	5

[a] Glycerol removal solution; +, = and – respectively mean 1, 0 and −1 point.

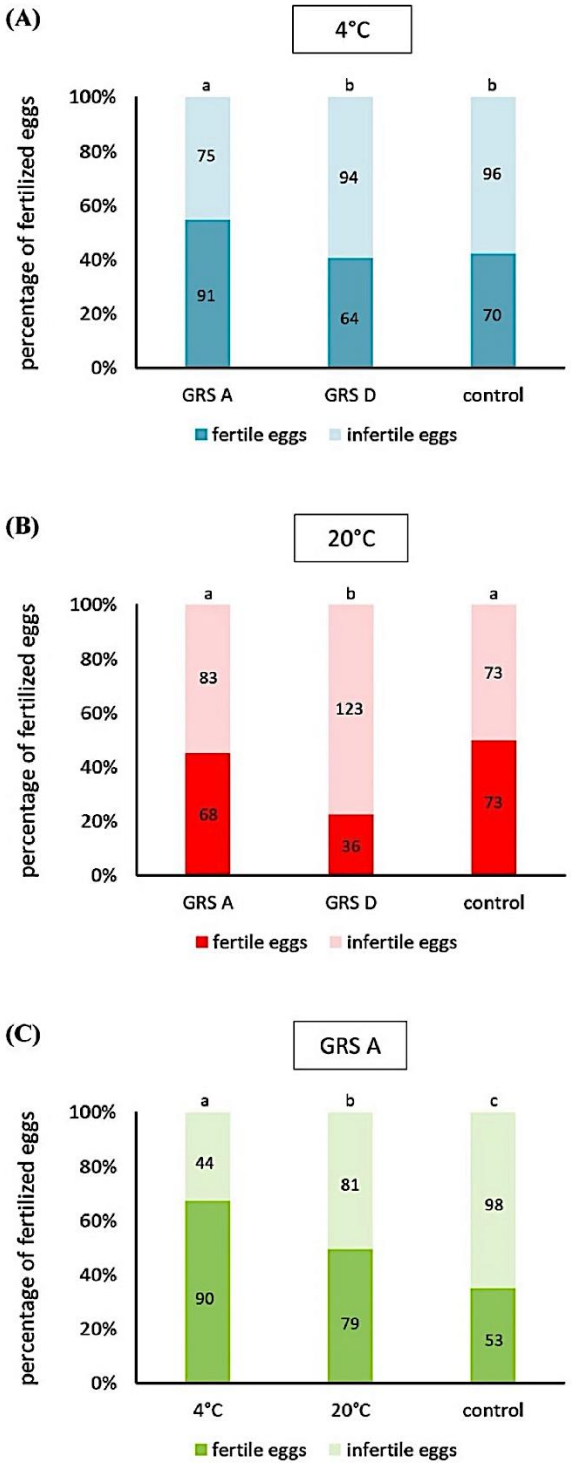

Fig. 7. Effect of glycerol removal methods on fertility capacity. Figure A, B and C correspond respectively GRS at 4 °C (Trial 1), 20 °C (Trial 2) and GRS An at 4 °C and 20 °C (Trial 3). GRS: glycerol removal solution. Control: serial dilution centrifugation (SDC). Eggs were collected from day 2 of the first AI to days 5 of the final AI. Deep color bars represent the percentage of fertilized eggs (%). The numbers of eggs are indicated on the bars.

Table 2
Comparisons of glycerol removal methods.

Method	Solution	Dilution		Centrifugation		
		T (°C)	time (min)	force (g)	T (°C)	time (min)
GRSC[a]	Sucrose-Percoll	–	0	800	4 or 20	15
ADC[b]	Accudenz	–	0	300	5	25
SDC[c]	Lake C	4	12	500	4	15

[a] Glycerol removal solution centrifugation.
[b] Accudenz discontinuous gradient centrifugation (12% over 30%).
[c] Serial dilution centrifugation.

52], which are essential key factors to be considered for practical AI in farms.

5. Conclusions

Altogether, we developed a new protocol to remove glycerol from frozen-thawed chicken semen, leading to a superior fertility than the conventional method, saving almost half of operating time, doubling semen straws handling capacity and simplifying equipment requirements. Several factors such as semen layer volume, centrifuge speed and time that could influence the result of Percoll centrifugation, could be studied to further improve the efficiency of this new technique.

Conflict of interest and authorship conformation form

- The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript.
- All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.
- This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2023.104567.

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[Supplementary Data of Article 2]

Supplementary Table 1. Composition of GRS A, B, C and D

GRS[A]	Sucrose-Percoll[B] (%)	PBS (%)	Percoll (%)	Sucrose (mM)	osmolarity (mOsmol)
A	40	60	36	80	408
B	50	50	45	100	440
C	60	40	54	120	472
D	70	30	63	140	506

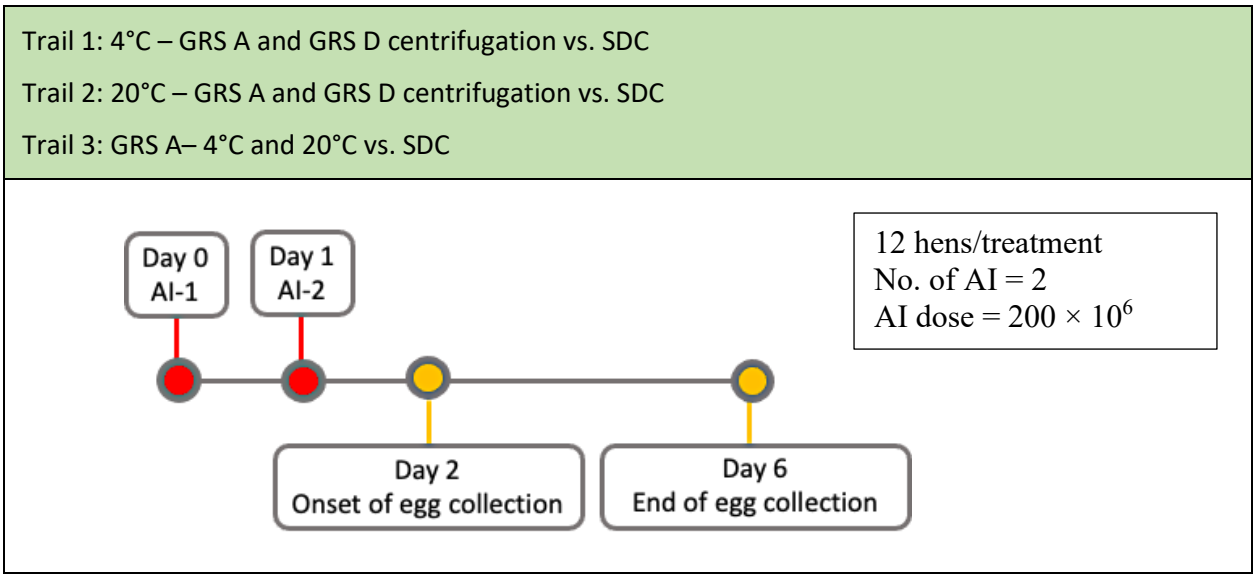
[A]glycerol removal solution; [B]Sucrose-Percoll contains 200 mM sucrose, 90% Percoll in PBS

Supplementary Table 2. Effect of sucrose concentration in Percoll solution on sperm motility

	one-way ANOVA (p-value)			
parameter	Sucrose concentration (0-400 mM)		Sucrose concentration (0-150 mM)	
motile sperm (%)	< 0.001	< 0.001	< 0.001	< 0.001
progressive sperm (%)	< 0.001	< 0.001	< 0.001	0.002

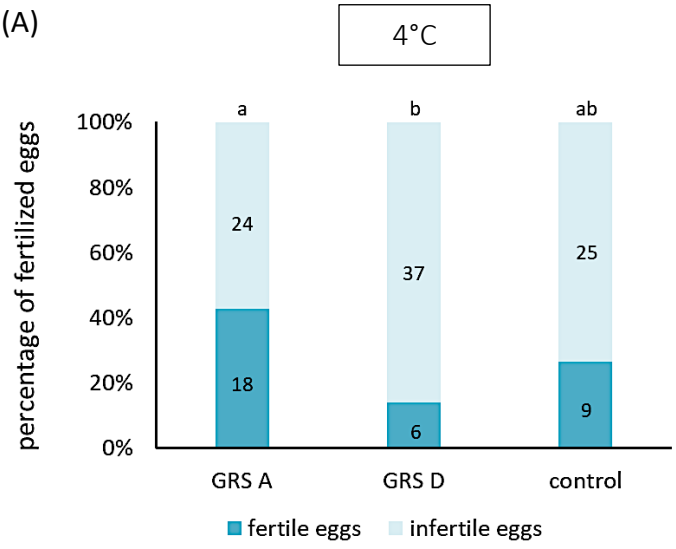
Supplementary Table 3. Effect of glycerol removal solution (GRS) composition and temperature (T) on sperm quality parameters

	two-way ANOVA (p-value)		
parameter	GRS	T	GRS × T
glycerol concentration	< 0.001	0.614	0.125
sperm concentration (10^6 sperm/mL)	< 0.001	0.324	0.063
sperm recovery (%)	0.069	0.086	< 0.001
motile sperm (%)	< 0.001	< 0.001	< 0.001
progressive sperm (%)	< 0.001	< 0.001	0.002
membrane integrity (%)	< 0.001	0.004	0.434
MMP (A.U.)	< 0.001	0.726	0.493

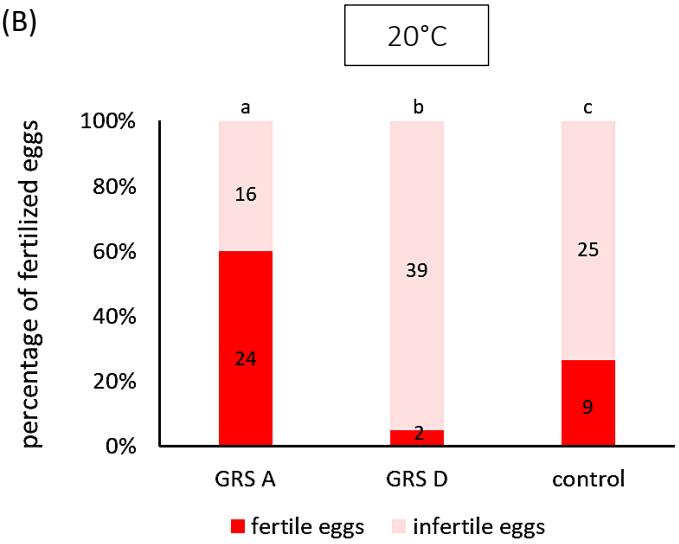


Supplementary Figure 1. Artificial insemination (AI) trials of frozen-thawed semen treated with GRS centrifugation and SDC. GRS: glycerol removal solution. SDC: serial dilution centrifugation. Each AI trial contained 2 successive inseminations. Sperm number of each AI was 200×10^6 sperm per hen. Eggs were collected from day 2 to day 6.

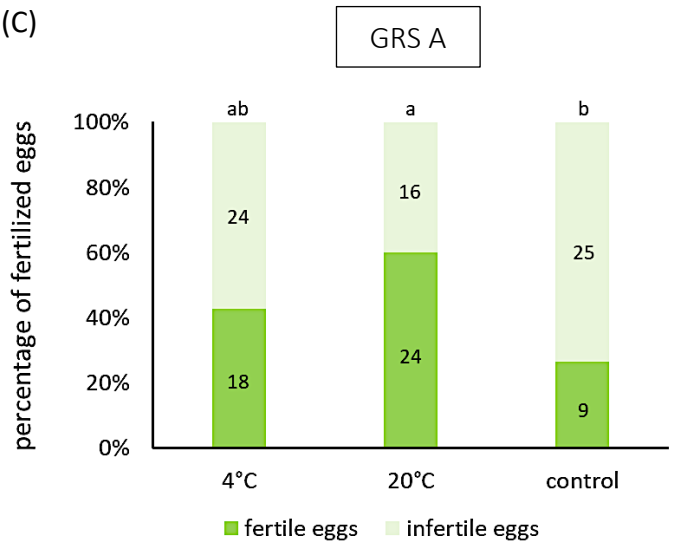
(A)


4°C
percentage of fertilized eggs
100%
80%
60%
40%
20%
0%
a
b
ab
24
37
25
18
6
9
GRS A
GRS D
control
fertile eggs
infertile eggs

(B)


20°C
percentage of fertilized eggs
100%
80%
60%
40%
20%
0%
a
b
c
16
39
25
24
2
9
GRS A
GRS D
control
fertile eggs
infertile eggs

(C)


GRS A
percentage of fertilized eggs
100%
80%
60%
40%
20%
0%
ab
a
b
24
16
25
18
24
9
4°C
20°C
control
fertile eggs
infertile eggs

Supplementary Figure 2. Effect of glycerol removal methods on fertility capacity. Figure A, B and C correspond respectively GRS at 4°C (Trial 1), 20°C (Trial 2) and GRS A at 4°C and 20°C (Trial 3). GRS: glycerol removal solution. Control: serial dilution centrifugation (SDC). Eggs were collected from day 2 of the first AI to days 5 of the final AI. Deep color bars represent the percentage of fertilized eggs (%). The numbers of eggs are indicated on the bars.

4. Article 3

Exploring how glycerol removal approaches successfully preserve fertilizing capacity of cryopreserved chicken sperm

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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. Serial dilution (SD) method was developed to eliminate glycerol and mitigate its adverse effects. However, it is complex, time-consuming, and requires specific equipment, limiting its application in the poultry industry. We have recently developed a new method for removing glycerol using a colloid gel called sucrose-Percoll (SP), that can be performed at either 4°C (4°C-SP) or 20°C (20°C-SP). It is simpler and faster than the conventional SD method, and it also enhances fertility in hens. However, the effectiveness of these protocols in terms of fertility remains uncertain and requires a better understanding for future improvement of glycerol removal. Here, we examined the effects of SP and SD protocols on the fertility persistence. 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 six days after insemination, and also higher than sperm processed using SD. No difference was observed between 20°C-SP and SD between 7 and 13 days. However, a 2.7-time higher fertility was showed with 4°C-SP. In addition, SP method demonstrated a two-fold greater ability to remove glycerol than 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 confirm that SP centrifugation is more effective than SD method. This is supported by observations of its stronger ability to remove glycerol from semen samples and its higher capacity to store sperm in the female reproductive tract, which explains the variation in fertility rates. No significant differences were observed in the sperm quality characteristics,

suggesting that further experiments are necessary to thoroughly investigate the molecular impacts of glycerol removal treatments.

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

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., 2022). 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 inclusion 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). 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 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., 2022), which explains its limited utilization in the poultry industry.

Recently, we described a new protocol called sucrose-Percoll (SP) centrifugation to remove glycerol from cryopreserved chicken semen (Lin et al., 2023a). This technique is based on the passage of glycerolized sperm through a sucrose-Percoll solution using 15 minutes of

centrifugation. 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 at 4°C or 20°C is higher than the SD method, confirming its potential use in the poultry industry. Whereas SP and SD methods are known to limit the glycerol concentration in treated semen samples (Zong et al., 2022; Lin et al., 2023a), the cellular mechanisms underlying the difference in efficiency remain unknown.

To better understand the varying impacts of glycerol removal approaches (i.e. SP centrifugation 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 one week after insemination (late fertility). Subsequently, we evaluated sperm quality parameters in vitro after glycerol removal treatments to clarify the sperm selection efficiency of each method. 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 to 45 weeks old, Gallus gallus domesticus, Sasso, France), housed in individual cages, were used as semen donors. A herd of 45 adult Lohmann hens (35 to 85 weeks old), housed in groups of four in cages, were used for fertility tests and SST experiments. All animals were subjected to a lighting regimen of 14 hours of light and 10 hours 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 three groups. Semen was collected 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 minutes. The resulting semen suspension was diluted to a final concentration of 11% glycerol. It was then equilibrated at 4°C for 10 minutes 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 minutes, 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 three different methods for removing glycerol: sucrose-Percoll (SP) centrifugation at either 4°C or 20°C (4°C-SP or 20°C-SP), and serial dilution (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 (GRS) from our previous study (Lin et al., 2023a). A mixture of 36% Percoll and 80 mM sucrose was prepared in 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 minutes 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 six times, with a 2-minute interval between each addition at 4°C with gentle agitation. The diluted semen was then immediately centrifuged for 15 minutes 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 three groups and inseminated with thawed semen treated with one of the three 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 3-4 cm. Each hen received a dose of 200 × 106 sperm 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 days 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 (Sigma-Aldrich, #MAK117) was used to measure the concentration of glycerol, following the instructions provided by the manufacturer. Briefly, 10 µL of semen was transferred into wells of a 96-well microplate. Then, 100 µL of the reaction solution was added directly to each well. The microplate was examined using spectrophotometry (Tecan Infinite M200 Plate Reader, Tecan Life Science) to measure the absorbance at a wavelength of 570 nm after 20 minutes 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, Hamilton Thorne, IMV Technologies). An aliquot of 2 µL of semen (30 × 106 cells/mL) was placed on a Makler Counting Chamber to capture images from four 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/sec, while progressive sperm were defined as sperm with a VAP greater than 50 µm/sec and a STR greater than 75%.

Membrane Integrity. A dual staining technique using SYBR 14 and Propidium iodide (PI) fluorescent dyes (Molecular Probes, LIVE/DEAD® Sperm Viability Kit, #L7011) in combination with a flow cytometer (Guava® easyCyte 5HT, IMV Technologies) was employed to assess sperm membrane integrity. Briefly, semen was diluted to a concentration of 1 × 107 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 minutes 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, Novus Biologicals™, #NBP2-29373). Briefly, 1.5 µL of semen was washed twice in 1 mL of cold PBS and centrifuged at 400×g for 5 minutes to obtain sperm pellets. Recovered pellets were resuspended in 55 µL of staining buffer containing Annexin V-FITC and PI. After 20 minutes of incubation in the dark, the sperm suspension was supplemented with 200 µL of assay buffer and then analyzed by Guava® easyCyte within 1 hour. 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 (Sigma-Aldrich, CAS NO.: 3520-43-2), was used to evaluate mitochondria membrane potential (MMP). Semen was diluted to 1 × 107 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 minutes 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.

Sperm Storage Tubules (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, Sigma-Aldrich, B2261) were added to each sperm suspension (100 µL) and gently mixed on an orbital shaker at 4°C for 90 minutes before use.

Artificial Insemination. A total of 12 hens, three for each glycerol removal treatment (including the control group), received intravaginal insemination with Hoechst-labeled thawed sperm twice at 24-hour intervals, with a dose of 200 × 106 sperm cells per female.

Isolation of UVJ Villi and Observation of SST. Hens were slaughtered 24 hours 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 minutes 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 (Pyle et al., 2016; Metcalf and Casey, 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 (Figure 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 two parameters between the treatments for glycerol removal. No impact of glycerol removal treatment was observed on mitochondria membrane potential (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 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, the Percoll, and sucrose. This method can be performed at both 4°C and 20°C. Our previous results revealed a better

fertility with SP compared to 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 two 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 – i.e. 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 and SD sperm, both in early and late stages. 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 a low temperature of 4-5°C 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 in 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 (PS) 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 may subsequently lead to the disruption of sperm storage and the fertilization process (Thélie et al., 2019b) in the oviduct, resulting in decreased fertility.

In conclusion, we have demonstrated that our novel method for glycerol removal, SP centrifugation, offers a new opportunity to enhance the utilization of cryopreserved semen in the poultry industry. This is supported by the fact that the SP process results in a higher fertilizing capacity of glycerol frozen-thawed sperm compared to the usual SD protocol, leading to better results in terms of fertility rates and the longevity of sperm storage in SST. This may be attributed to the stronger ability of SP centrifugation to remove glycerol and its greater capacity to restore sperm storage in the hen's reproductive tract. To further investigate the mechanisms of glycerol removal methods, additional molecular analysis, such as proteomic approaches (Soler et al., 2016; Vitorino Carvalho et al., 2021), may be helpful in identifying the molecules involved in sperm-SST binding and determining their fate during different post-thawing treatments.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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[Figures]

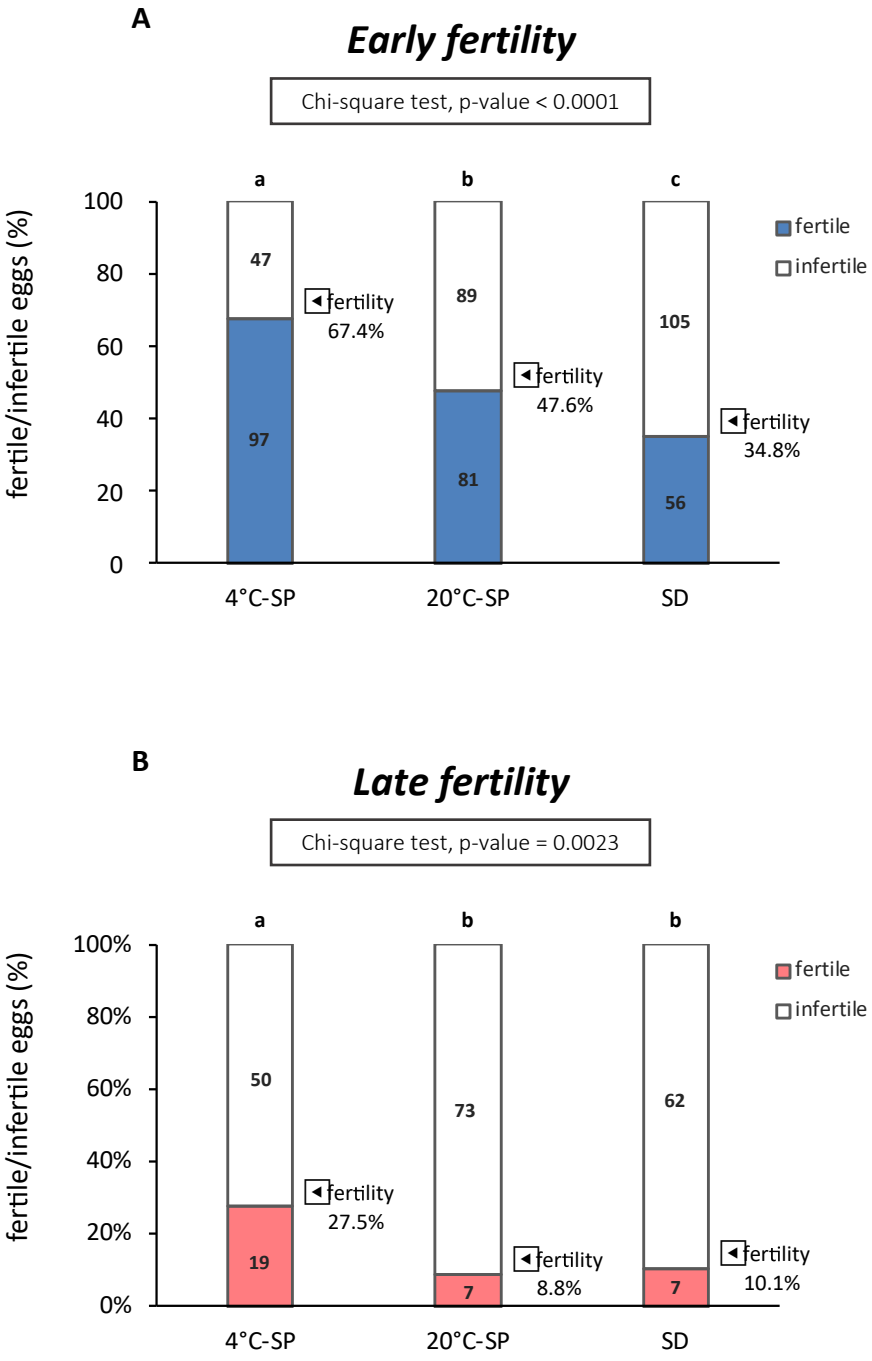


Figure 1. Fertility of frozen-thawed semen after glycerol removal treatment. A represents early fertility, which involves collecting eggs from the 2nd day after the first artificial insemination (AI) to the 6th day after the final one. B represents late fertility, the eggs collected between the 7th and 13th day after the last AI. SP: Protocol for centrifugation with sucrose-

Percoll solution (at 4 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.

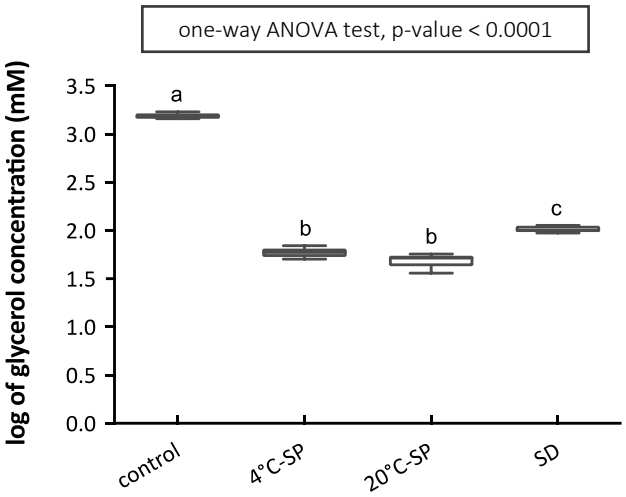


Figure 2. Logarithmic glycerol concentration after glycerol removal treatment. Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4 or 20°C). SD: Protocol of serial dilution followed by centrifugation. The lines (—) 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.

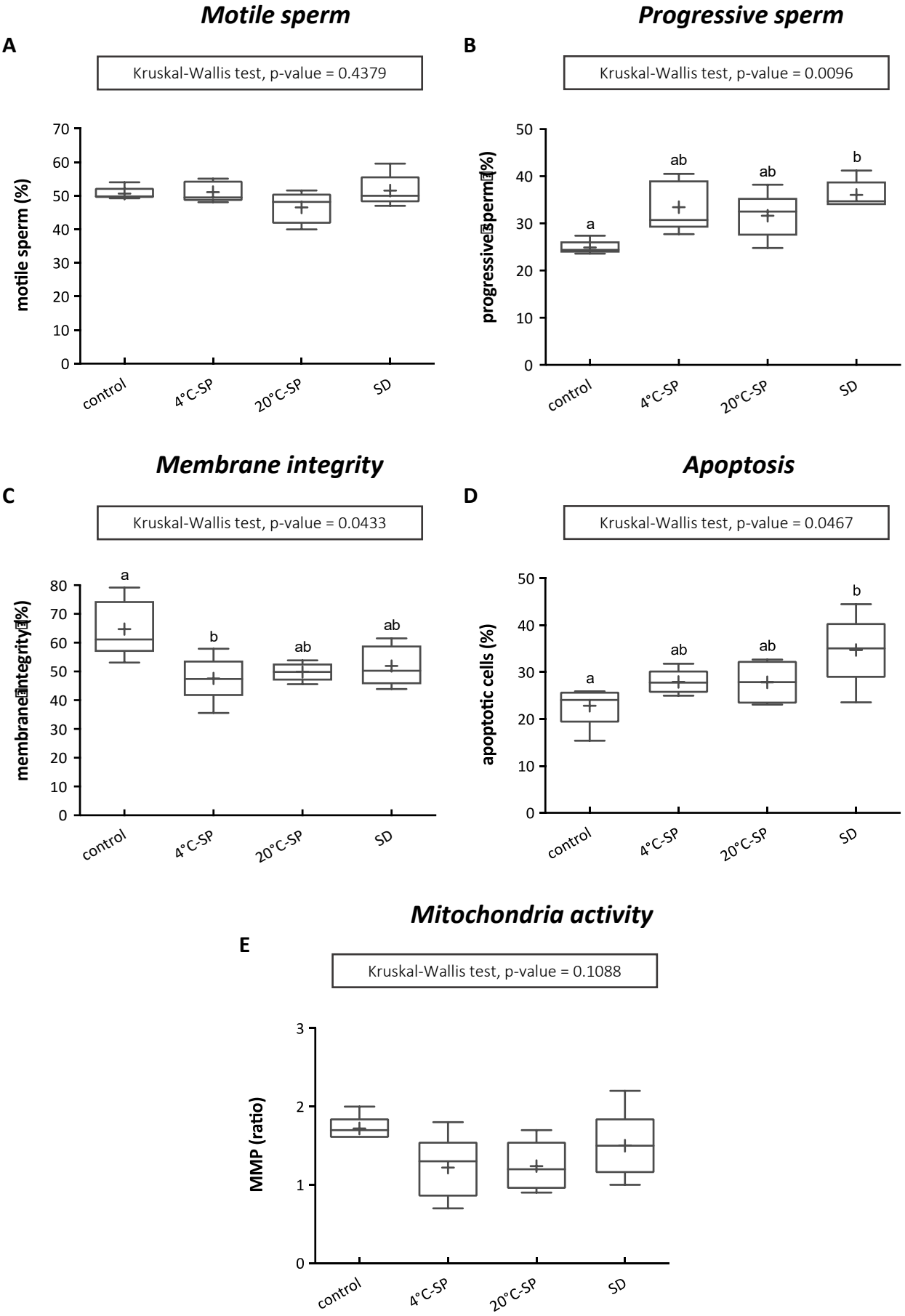

Motile sperm
A
Kruskal-Wallis test, p-value = 0.4379
motile sperm (%)
0
10
20
30
40
50
60
70
control
4°C-SP
20°C-SP
SD
Progressive sperm
B
Kruskal-Wallis test, p-value = 0.0096
progressive sperm (%)
0
10
20
30
40
50
a
ab
ab
b
control
4°C-SP
20°C-SP
SD
Membrane integrity
C
Kruskal-Wallis test, p-value = 0.0433
membrane integrity (%)
0
10
20
30
40
50
60
70
80
a
b
ab
ab
control
4°C-SP
20°C-SP
SD
Apoptosis
D
Kruskal-Wallis test, p-value = 0.0467
apoptotic cells (%)
0
10
20
30
40
50
a
ab
ab
b
control
4°C-SP
20°C-SP
SD
Mitochondria activity
E
Kruskal-Wallis test, p-value = 0.1088
MMP (ratio)
0
1
2
3
control
4°C-SP
20°C-SP
SD

Figure 3. Sperm *in vitro* quality parameters after glycerol removal treatment. A, B, C, D and E represent the percentage of motile sperm (A), progressive sperm (B), membrane integrity (C), apoptosis (D) and mitochondria membrane potential (MMP). Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4 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.

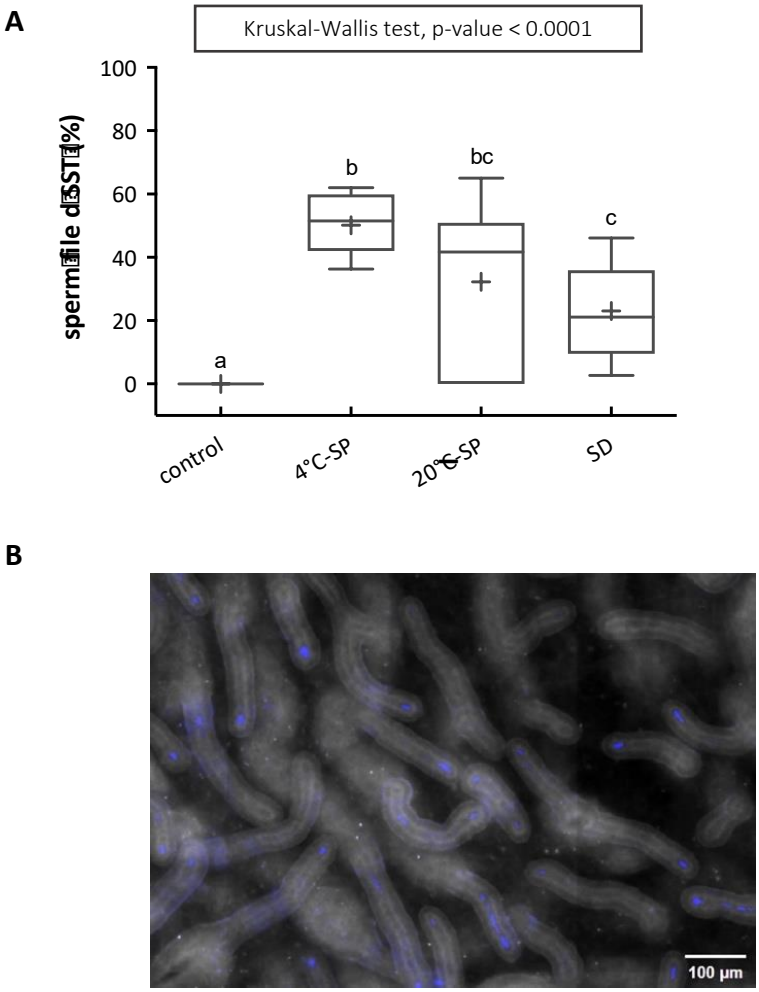
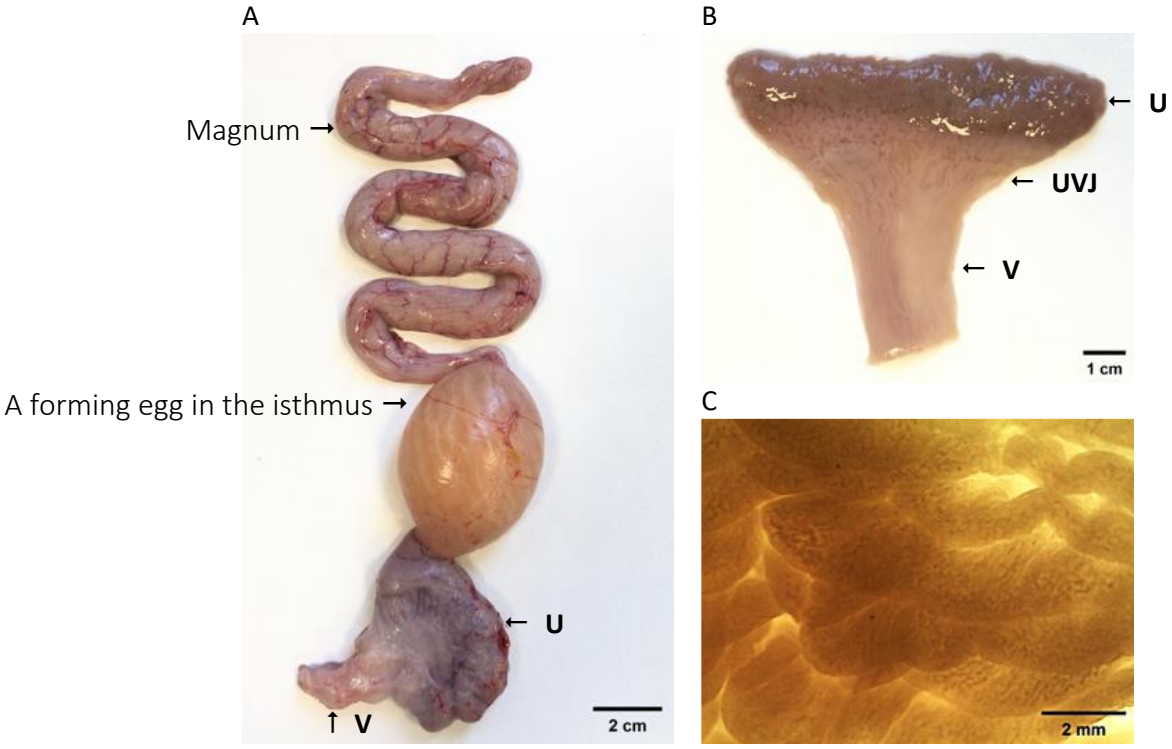
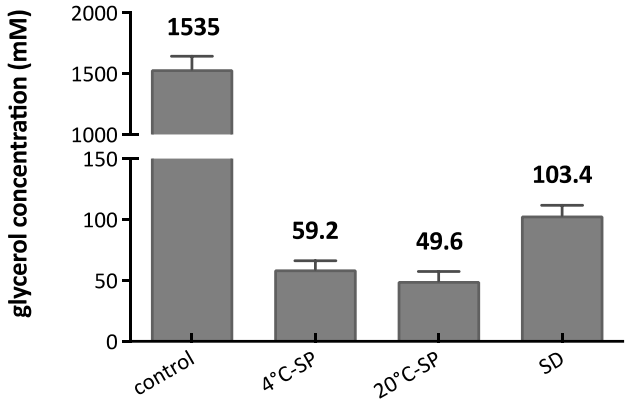


Figure 4. The presence of sperm in sperm storage tubules (SST) after insemination of frozen-thawed semen. A and B represent the percentage of SST filled with sperm (A) and sperm deposition patterns (B). Control: Frozen-thawed semen without any treatment and stored at 4°C. SP: Protocol for centrifugation with sucrose-Percoll solution (at 4 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.

[Supplementary Data of Article 3]



Supplementary Data 1. Dissection of a hen's reproductive tract. A, B, and C represent the hen's oviduct (A), uterovaginal junction (B), and UVJ mucosa villi containing numerous sperm storage tubules (C). Abbreviation: U = uterus, UVJ = uterovaginal junction, V = vagina.



Supplementary Data 2. 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 or 20°C). SD: Protocol of serial dilution followed by centrifugation. The mean glycerol concentration is shown above the bar of each treatment (5 biological replicates). The bars and lines correspond to the mean and the standard deviation to the mean.

GENERAL DISCUSSION AND PERSPECTIVES

1. How to mitigate glycerol toxicity when using it as a cryoprotectant

1.1. Glycerol toxicity is not a unique case in chicken sperm

In fact, glycerol toxicity also occurs in mammalian sperm. This is supported by evidence of structural or functional damage in the sperm of boars, donkeys, and stallions (Wilmut and Polge, 1974; Demick et al., 1976; Vidament et al., 2009; Macías García et al., 2012). This damage includes impairments in sperm motility, membrane integrity, acrosome function, and fertilization ability. In addition, sperm exposed to glycerol at room temperature (20°C) suffered more serious damage than at a cooling temperature (5°C) (Wilmut and Polge, 1974; Ma et al., 2022). These studies were conducted at 5 and 20°C, primarily focusing on the standard process of dilution and glycerol addition commonly employed for sperm cryopreservation.

Glycerol is a widely used sperm cryoprotectant in many species (Papa et al., 2015; Ma et al., 2022; Bang et al., 2022). However, it's toxicity is not extensively discussed in the field of mammalian sperm cryobiology, likely because it is more pronounced in poultry, where a high concentration of glycerol is required to achieve optimal cryoprotection (Long and Kulkarni, 2004). In addition, we have acknowledged one possible reason that may be attributed to the different insemination techniques used in avian and mammalian species. In cattle, glycerol (up to 7% in semen) had no effect on pregnancy rates (Papa et al., 2015) because the sperm is inseminated into the uterus using a catheter that passes through the cervix (Diskin, 2018). In sheep, successful fertilization occurred when glycerolized sperm were deposited in the uterus, while fresh semen can be successfully inseminated into the vagina (Armstrong and Evans, 1984; Maxwell et al., 1984). This suggests that stored semen cannot pass through the cervix, as confirmed by microendoscopy (Druart et al., 2009). In donkeys, the presence of only 2.2% glycerol in semen induced infertility because the sperm were delivered before reaching the cervix (Vidament et al., 2009). These cases may highlight the importance of the insemination site for glycerolized sperm in relation to fertility.

1.2. Use glycerol for chicken sperm cryopreservation

1.2.1. Glycerol concentration

Here, we present that exposing chicken sperm to 6% glycerol at 4°C resulted in a slight decrease in sperm quality characteristics. However, when the same treatment was applied at 41°C, it caused more severe damage to the sperm cells. The most commonly used concentration of glycerol to freeze chicken sperm is 11% (Tselutin et al., 1999; Long and Kulkarni, 2004). This

concentration provides better cryoprotection and results in higher fertility compared to sperm cryopreserved in 8% glycerol (Abouelezz et al., 2015; Thélie et al., 2019a).

1.2.2. Intrauterine insemination technique is needed

High concentrations of glycerol, which are known to cause significant damage to chicken sperm quality parameters, can still result in successful fertility if inseminated beyond the vagina. For example, when 15% glycerolized sperm is inseminated directly into the hen's uterus, fertility rates of up to 73% can be achieved (Allen and Bobr, 1955). This suggests that sperm preserved in glycerol can have most of their fertility once passing through the vagina, regardless of the presence of glycerol. In addition, uterine tissue may have positive effects on sperm biology parameters, similar to its effect on sperm motility (Spreen et al., 1990). Further studies are needed to compare the effects of glycerol on sperm quality parameters in the presence of vaginal or uterine tissues.

1.3. Perspectives

1.3.1. Alternative freezing protocol

As previously indicated, a concentration of 6% glycerol was found to be harmful to sperm quality parameters in chickens, while a concentration of 11% is required to achieve optimum glycerol cryoprotection. A strategy to reduce the concentration of glycerol used would be to mix it with other CPA. However, several CPA, such as DMA and DMSO, have been found to exhibit both cryoprotective action and some toxicity, which can have negative effects on living cells (Mitchell and Buckland, 1976; Best, 2015; Mosca et al., 2019). Mixing them may allow us to keep each individual one below the toxicity threshold, and an optimal mixture with low individual concentrations remains to be designed.

Unlike intracellular CPA, extracellular CPA are large and hydrophilic molecules that act as osmoprotectants and help with cell dehydration (Aisen et al., 2002). They can be added to the freezing diluent to reduce the need for permeable CPA concentrations. DMA combined with trehalose, and DMF mixed with sucrose, have been tested to freeze chicken sperm and have shown positive effects on sperm fertilizing capacity (Thananurak et al., 2019; Mosca et al., 2019).

Mammalian oocytes and embryos contain a large amount of water, and their membranes have limited permeability to CPA (Arav, 2014). This led to the development of a non-equilibrium cryopreservation technique that involves a short exposure to a high concentration of a mixture of permeable and non-permeable CPA. This technique promotes cell dehydration and increases the viscosity of the solution. Together with an extremely high cooling rate (>2,000°C per minute), these methods, called vitrification, prevent the formation of ice crystals, resulting in the solution being frozen in an amorphous, glass-like structure (Rall and Fahy, 1985; Massip,

2001). Vitrification techniques use a combination of highly permeable CPA at low individual concentrations, such as glycerol, DMSO, and ethylene glycol, along with non-permeable ones like sucrose and trehalose. The cells are briefly exposed to this mixture in a small volume before being rapidly immersed in liquid nitrogen. The success of vitrification depends on the viscosity of the solution, the speed of cooling, and the volume of the sample. Glycerol, when combined with other CPA has been introduced and has shown potential for sperm cryopreservation in humans (Le et al., 2019; Tao et al., 2020). Thus, the use of vitrification may be interesting to test on rooster sperm to observe its effectiveness with low doses of glycerol and other CPA. However, to date, the only species in which successful sperm vitrification has been reported is humans (Isachenko et al., 2004). Considerable chromatin damage was induced in dog sperm by vitrification, much more than by conventional freezing (Sánchez et al., 2011). Even with human sperm, there are conflicting reports about whether vitrification offers any advantages over conventional slow freezing (Agha-Rahimi et al., 2014). The size of the sperm head appears to be a crucial factor in determining cryosensitivity during vitrification. Smaller sperm heads are better able to withstand the severe osmotic effects compared to the larger, paddle-shaped sperm heads found in most livestock species (Morrell and Mayer, 2017). Therefore, further developments are needed before this technique can provide a viable alternative to slow freezing of sperm.

1.3.2. Regulation of glycerol toxicity based on sperm biology

In addition to motility, glycerol also affects sperm energy generation, including its impact on mitochondrial function and ATP concentration, as observed in our study. We believe that sperm motility dysfunction may be associated with alterations in the energetic network. For example, we observed an enormous increase in ATP concentration, suggesting that the elevated ATP levels may have resulted from an alternative ATP synthesis pathway, such as glycolysis (Du Plessis et al., 2015; Davila et al., 2016), or a disruption in the ATP/ADP balance (Tourmente et al., 2015; Yu et al., 2019). Thus, designing a new formula for energy compositions in glycerol freezing diluent might be useful for restoring the balance of energy synthesis (Darr et al., 2016; Sengupta et al., 2020). We observed that apoptosis induction also occurred in glycerolized sperm, raising a hypothesis of adding apoptotic regulators (Fischer and Schulze-Osthoff, 2005; Woo et al., 2020) to compose of freezing diluent may modulate glycerol impact on cell death. Dysregulation of mitochondrial ATP production and apoptosis may indicate the cellular response to environmental stress, which can result in impaired oxidative phosphorylation and the formation of reactive oxygen species (ROS). The use of suitable antioxidants may help reduce the detrimental impact of ROS on various cell components, thereby preventing DNA degradation and cell death.

2. Glycerol impairs chicken sperm transit from the vagina to the uterus

Pioneer studies have already shown that the thresholds for partial and total fertility loss in chicken semen are 2% and 6% glycerol, respectively (Polge, 1951; Neville et al., 1971). This study confirms the same trend. This demonstrates that glycerol toxicity has a universal effect on chicken fertilization, regardless of genetic background or age.

2.1. An increasing temperature in hen's oviduct induces more glycerol toxicity to sperm

In our study, we compared the toxicity of glycerol at 4 and 41°C to simulate the conditions experienced during freezing, thawing, or post-insemination stages. A significant temperature-dependent effect was observed, indicating that glycerol at 41°C had a greater detrimental impact on sperm quality parameters compared to 4°C. This suggests that the majority of the toxic effects of glycerol are exerted after it is deposited into the hen's vagina, rather than during the freezing and thawing processes.

2.2. Glycerol impairs sperm motility and prevents them from reaching the storage site

Our study revealed a significant decline in motility parameters due to the addition of glycerol. Sperm lost more than 90% of their motility within 20 minutes after being exposed to 6% glycerol at 41°C. Meanwhile, we noticed that there were almost no sperm present in the SST after inseminating semen containing 6% glycerol. Despite very limited knowledge about the mechanisms of vaginal selection in chickens, such as the selection of what sperm parameters. It is generally accepted that only motile sperm are capable of passing through the vagina and depositing into the SST (Brillard, 1993; Bakst et al., 1994). Therefore, we hypothesized that the decrease in sperm motility caused by glycerol toxicity prevents sperm from reaching and colonizing the SST due to their rejection during vaginal selection. This ultimately led to infertility in hens (Bakst et al., 2010).

Another observation in our study may also support this theory. We conducted *in vitro* incubation of uterovaginal villi and sperm in a medium containing 6% glycerol. While inseminating sperm containing 6% glycerol resulted in the complete absence of sperm in the SST. However, after 30 minutes of *in vitro* incubation at 41°C, the SST showed a sperm filling rate of 33%, which is only a slight decrease of 17% compared to the control sperm of 50%. Interestingly, at the end of incubation, the sperm unexpectedly retained approximately 50% of their motility. This suggests that the difference in sperm storage between *in vivo* insemination and *in vitro* incubation may be attributed to two possibilities: either the sperm escape from vaginal selection, or their remaining motility is crucial for them to reach the SST.

2.3. Glycerol toxicity lasts for a short time in the hen's oviduct

In addition to sperm, the effect of glycerol on the hen's vagina has been investigated (Westfall and Howarth, 1977). In that study, 100 µL of 15% glycerol was loaded at intervals of 0, 5, and 10 minutes before or after the deposition of 100 µL of semen. All treatments showed a decrease in fertility, except for glycerol when given 10 minutes before sperm. This suggests that glycerol did not permanently modify maternal tissues, but its toxicity directly affected the sperm. The toxic effect of glycerol in the vagina lasted no longer than 10 minutes, likely due to glycerol being cleared by the vaginal epithelium. In our experiment, we mixed glycerol with sperm just before insemination, similar to the method used in the study where glycerol was loaded 0 minutes before sperm deposition. While insemination of sperm containing 6% glycerol produced completely infertile eggs in our study, their study found that inseminating sperm together with 7.5% glycerol still achieved approximately 35% fertility. It is likely that the difference might be due to the fact that glycerol and sperm were deposited in two different aliquots, which may have disrupted the complete mixing of the solutions. In other words, the glycerol and sperm were not in direct contact.

Despite the fact that glycerol toxicity is not long-lasting, individual hens exhibited varying levels of tolerance to glycerol toxicity, as evidenced in our study by the differences in sperm storage and fertility after insemination with sperm containing 2% glycerol **(Figure A)**. This may be related to the vaginal mucosa's ability to eliminate glycerol. However, the mechanism for overcoming glycerol toxicity in the vagina is still unknown.

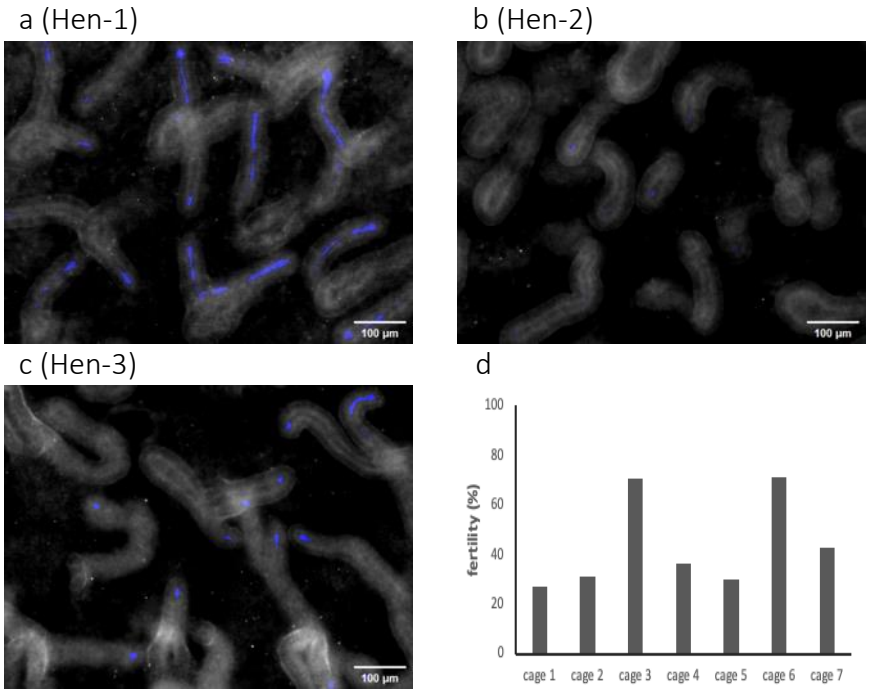


Figure A. Variation of sperm storage patterns inside SST in individual hens (a, b, and c) and fertility (d) in different cages of 4 animals, after inseminating semen containing 2% of glycerol (H.-L. H. Lin).

2.4. When glycerolized sperm escape from the vaginal selection

Indeed, intrauterine insemination could restore fertility to 73% using 15% glycerolized sperm in chickens (Allen and Bobr, 1955). However, this operation caused stress in hens and severely suppressed egg production. As a result, it has never been adapted for practical use, unlike in mammalian species where it is possible to inseminate through the cervix using a catheter. Another piece of direct evidence was obtained from our study of *in vitro* uterovaginal villi and sperm incubation. We deposited sperm directly outside of villi containing SST in culture. In this model, sperm entered the SST without traveling through the vagina. After 30 minutes of incubation, we observed that 6% glycerol sperm was distributed in 33% of the SST. However, when performing *in vivo* insemination of sperm under the same glycerol condition, no sperm was observed in the SST. This suggests that the vagina may be responsible for rejecting glycerolized sperm from passing through and entering the uterovaginal area. As previously described, the sperm were unable to move forward in the vagina due to a loss of motility. Thus, searching for a method to assist glycerolized sperm in overcoming vaginal selection might help them reach the SST.

2.5. Glycerol impacts fertilization even after a successful sperm migration

Acrosomal reaction (AR) is a mandatory process for successful fertilization, which can be assessed by measuring sperm hydrolysis activity in the perivitelline membrane (PVM) in avian species (Lemoine et al., 2011). Here, we have demonstrated that glycerol significantly reduces AR primarily at 41°C, which is the physiological temperature in the oviduct, rather than at 4°C. Our findings were in accordance with a previous study, which showed that glycerol reduces the hydrolysis holes in PVM (Abouelezz et al., 2015). In our study, we conducted an *in vitro* sperm-PVM experiment, which provided more direct evidence compared to another study that involved inseminating glycerolized sperm and then collecting eggs to assess sperm-egg penetration in the PVM. This earlier study did not differentiate between the effects of sperm migration defect and AR dysfunction.

2.6. Perspectives

2.6.1. Assist in the passage of glycerolized sperm through vaginal selection

Despite our conclusion that exposing sperm to glycerol at 41°C is very harmful, we cannot avoid subjecting cryopreserved sperm to this temperature as it is intended to be inseminated inside the female genital tract. We hypothesized that the main cause for the unsuccessful migration of glycerolized sperm in oviducts was likely the loss of sperm motility, resulting in their expulsion from the vagina. Thus, a method that can facilitate the transportation of glycerolized sperm from the vagina to the SST could be a potential solution to overcome glycerol toxicity.

An unexpected increase in sperm motility was observed in our study when sperm were incubated with uterovaginal villi in a medium containing 6% glycerol. Sperm motility was surprisingly maintained at 50% after 30 minutes of incubation at 41°C, unlike when sperm were incubated alone in the same concentration of glycerol, which resulted in a complete loss of motility. This suggests that uterovaginal villi may protect sperm from the negative effects of glycerol, allowing for the preservation of sperm motility. This finding is consistent with a previous study conducted by Spreen et al. (1990). Therefore, it would be interesting to identify the molecules responsible for this protective effect of the uterovaginal villi. Understanding their cellular mechanisms could be used to design a novel molecular strategy to maintain the motility of glycerol cryopreserved sperm during their deposition in the vagina. This would allow them to safely reach the uterovaginal area without the need to remove glycerol.

It is unclear if sperm motility is essential for their migration in the oviducts. After inseminating dead sperm into the uterus, they can be found in the infundibulum (Allen and Grigg, 1957). This suggests that sperm motility is not essential for sperm transport in the hen's oviduct after passing through the vagina. The authors also observed that the injection of sperm-free fluid into the vagina caused a spasmodic movement in the cloaca, resulting in the expulsion of most of the fluid within a few minutes. In normal conditions, when sperm enter the vagina, they begin to move towards the uterovaginal junction (UVJ) with the coordination of smooth muscle peristaltic contractions and the beating of ciliated cells in the vagina (Bakst, 2011). Thus, we can presume that an important crosstalk between sperm and vaginal tissues is missing when inseminating the sperm-free fluid. This raises the question of whether glycerolized sperm lose their ability to communicate with the vagina when they are inseminated, thus excluding them as a case of sperm-free fluid. If we can better understand the mechanisms involved in sperm transport within the oviduct, it would be more feasible to develop innovative solutions for facilitating the passage of glycerolized sperm from the vagina to the uterus.

Glycerol is known to induce peristaltic contractions in the bowel (Tomaru et al., 1993) and is largely used as a laxative treatment, inducing the expulsion of feces and absorption of water. It may have a similar effect in the female genital tract and cause the expulsion of semen after insemination. It would be interesting to inseminate dead sperm alone or with glycerol in the vagina and in the uterus to separate the respective effects of reduced sperm motility and glycerol-induced peristaltic movements.

2.6.2. Selection of hens with oviducts that are more tolerant to glycerol

As previously described, we hypothesized that hens would exhibit varying levels of resistance to glycerol toxicity **(Figure A)**, which would result in differences in their fertilization capacity. Indeed, changes in protein secretion patterns have been found when the chicken vagina was exposed to glycerol (Delee et al., 1991). Thus, we can presume that the vaginal protein secretion may vary among individual hens after glycerol exposure, resulting in different

responses to the transportation of damaged sperm. However, further studies are necessary to identify these proteins, particularly to distinguish them from the hens with high and low tolerance to the effects of glycerol. In addition, we can also imagine that the absorption of glycerol by vaginal epithelial cells may probably differ in individuals as well (Hjelm et al., 2010), leading to varying abilities to reduce the local toxic effects of glycerol.

3. Glycerol removal technique

3.1. Development of sucrose-Percoll (SP) centrifugation

Despite Percoll centrifugation having been introduced for sperm wash and selection for decades (Gorus and Pipeleers, 1981; Long and Kulkarni, 2004), the novelty of our study was the use of this technique, with the addition of sucrose, to prevent osmotic stress during the removal of glycerol from thawed chicken sperm. Due to this simple modification, we successfully applied this technique to cryopreserved chicken sperm and achieved a fertility rate of up to 66%. To the best of our knowledge, this study is the first to demonstrate the application of colloidal centrifugation on cryopreserved chicken sperm for insemination, resulting in satisfactory fertility in avian species.

3.2. Use SP centrifugation for mammalian sperm

Although glycerol impacts sperm fertilization in mammals, glycerol removal protocol has never been introduced to remove glycerol from cryopreserved mammalian sperm. In fact, the dialysis method was tested to remove glycerol from ram semen (Abdelhakeam et al., 1991). This study concluded that glycerol removal with dialysis did not improve ewe fertilizing ability, probably because dialysis is a long process, showing the necessity to find a faster solution for glycerolized sperm cleaning. However, intrauterine insemination of mammalian sperm is not highly technical and sufficient to obtain good fertility in ewe without glycerol removal from inseminated sperm to ignore the glycerol toxic problem. However, this technique involves complex manipulations such as laparoscopic or transcervical techniques (Gibbons et al., 2019), leading to difficult use under field conditions. Since ram sperm can be kept up to 24 hours at 18°C before successful vaginal insemination, the sheep industry did not invest in cryopreservation research. However, the development of a simple glycerol removal technique, as we did in our study, may be helpful to achieve satisfying fertility with intravaginal insemination of frozen semen, which would be more flexible than liquid conservation and improve the possibilities of genetic exchanges in sheep.

3.3. Use SP centrifugation for research

One of our studies aimed to observe how SP centrifugation restores the fertilizing capacity of glycerol cryopreserved sperm using an *in vitro* model. Unfortunately, we did not succeed in this experiment due to several reasons. The sperm required a lengthy Hoechst 33342 staining

procedure after thawing in order to become visible within the SST. Since thawed sperm are more fragile than fresh ones, not to mention the additional washing and centrifugation protocol required after prolonged staining to remove excess fluorescence. All of these operations may cause thawed sperm to lose their normal fertilizing functions. According to this unsuccessful experience, staining sperm with Hoechst 33342 before freezing may be a solution to make this experiment feasible. By the way, when thawed semen is subjected to a glycerol removal protocol for experiments, any excess fluorescence can be eliminated simultaneously.

3.4. Perspectives

Despite our SP centrifugation being able to achieve satisfactory fertility, certain specific conditions, such as the volume of semen treated, centrifugal forces, and duration, can certainly be adjusted to enhance its efficiency. For the other method of removing glycerol, serial dilution centrifugation, it is possible to enhance its efficiency by increasing the dilution factors, which will result in a decrease in the final glycerol concentration. However, it can be anticipated that more time will be required to remove the supernatant after centrifugation.

4. Improving knowledge limitations

4.1. Proteomics analysis

Sperm quality evaluations are routinely performed to predict fertility, particularly through motility and viability analysis. However, the link between sperm parameters and fertilizing capacity is not clearly established. In sheep, mass motility is well correlated with fertility, but this is not the case in chickens. Thus, additional biomarkers are required for accurate prediction of sperm fertility. Due to recent advancements in high-throughput proteomics methods and technology, it would be beneficial to explore proteomic markers that can indicate sperm fertility.

An obvious example was found in Article 3 of this thesis. Despite no difference in sperm quality parameters being found, sperm treated with SP centrifugation achieved twice the fertility compared to sperm treated with serial dilution method. In addition, we also observed that glycerol affected sperm colonization in the hen's sperm storage structures. Thus, exploring how glycerol changes sperm or oviduct proteomics profiles may allow us to identify molecular information and markers of successful insemination.

4.2. Acquire more knowledge on sperm storage tubules (SST)

We observed that the SST at two ends of one uterovaginal villus exhibited different capacities for receiving glycerolized sperm. Despite numerous pioneering studies that have provided extensive information on the structures and functions of SST (Brillard, 1993; Bakst et al., 1994; King et al., 2002; Sasanami et al., 2015; Matsuzaki et al., 2015), this issue has never been

addressed before. We hypothesized that glycerolized sperm may accumulate more at the vaginal end of the SST than at the uterine end. We are curious to determine whether this difference is caused by a defect in sperm transport or by the varying molecular compositions of the vaginal and uterine ends of the SST.

4.3. Interaction between glycerol concentration and insemination volume

Although many previous studies have revealed that the presence of glycerol at insemination can cause fertilization failure in chickens, the effect depends on the concentration of glycerol in semen (Polge, 1951; Neville et al., 1971; Long, 2006). Considering the variation in sperm concentration, it results in different volumes of inseminating semen (with a fixed number of inseminated sperm), which also affects the quantity of glycerol deposited in the vagina. In cases of low sperm concentration, hens are inseminated with a higher amount of glycerol into their vagina. We can imagine that glycerol toxicity may persist longer in the vagina and induce more adverse effects.

4.4. Using glycerol for sperm liquid storage

Preserving sperm cells in the extremely low temperature of liquid nitrogen is certainly an ideal method for long-term storage in cryobanking (Long, 2006; Blesbois, 2011). In chickens, sperm after 18 years of cryopreservation are still capable to reproduce offspring (Thélie et al., 2019a). Despite this method being able to retain well sperm fertilizing capacity, it shows a certain level of inconvenience to use in practice. Perhaps, liquid storage can be an alternative option in practices requiring a short conservation (Donoghue and Wishart, 2000). Despite chicken sperm showing no marked changes in membrane or DNA integrity after prolonged storage at 5 °C (Blank et al., 2021), damages to sperm motility, mitochondrial function, and fertility were observed within 24 hours (Kheawkanha et al., 2023). These observations suggest the need to improve the strategy for liquid storage of chicken sperm. In boars, it has been reported that sperm preserved at 5°C for 6 hours in diluent containing 10% glycerol obtained higher fertility than 0% of glycerol (Wilmut and Polge, 1974). This suggested that glycerol may exert an anti-cooling effect on sperm cells and inspired the idea of using glycerol for chicken sperm liquid preservation. According to our study, the addition of 1-2% glycerol has no negative effect on the fertilizing capacity of chicken sperm. We can also imagine that slight dehydration of sperm cells can be expected under this low concentration of glycerol, which may have an effect on metabolic regulation of sperm cells.

GENERAL CONCLUSION

After conducting experiments on the insemination of glycerolized sperm to assess the effects of glycerol on fertility and distribution in sperm storage tubules, as well as to compare the alterations of sperm quality parameters *in vitro*, we have established the following findings **(Figure 3)**.

1. The presence of 2% and 6% glycerol in semen respectively resulted in a 50% and 100% loss of fertility after insemination.
2. Glycerolized sperm were unable to reach the uterovaginal junction and colonize the sperm storage tubules, which partially explains the decrease in fertility.
3. Glycerol significantly affects sperm biological characteristics, including motility, cell death induction, acrosomal function, and bioenergetic metabolism. In particular, it caused a significant decrease in sperm motility within 20 minutes at 41°C. Glycerol mainly impaired sperm biology at 41°C, which is the temperature of the oviductal environment, rather than at 4°C, during pre- and post-freezing procedures.

After conducting experiments to design a solution for eliminating glycerol and performing inseminations using thawed chicken sperm after glycerol removal protocols, we have established the following findings **(Figure B)**.

1. A new formula for a glycerol removal solution, composed of sucrose and Percoll, has been designed to remove glycerol from cryopreserved chicken sperm.
2. Treating thawed chicken semen with this innovative sucrose-Percoll solution enabled effective removal of glycerol and restoration of sperm colonization in sperm storage tubules, leading to satisfactory fertility. Furthermore, it achieved a two-fold increase in fertility using simpler equipment and half the processing time compared to the conventional serial dilution method.

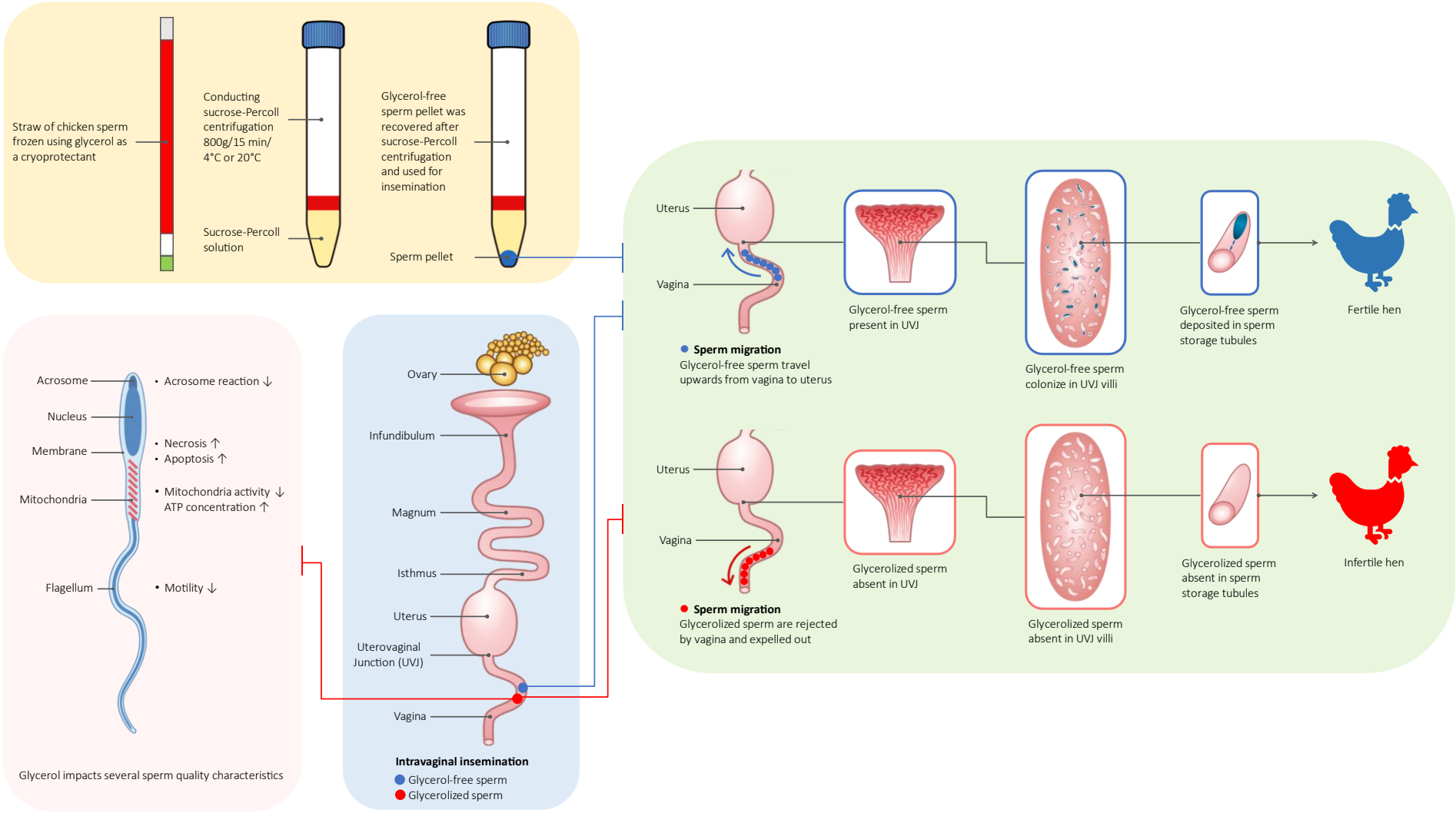


Figure B. Summary diagram illustrating the detrimental impact of glycerol on sperm parameters *in vitro*, as well as the consequences of inseminating control (blue) or glycerolized sperm (red) on sperm migration and final fertilizing capacity. However, our novel sucrose-Percoll centrifugation technique can avoid the disruptive effects of glycerol toxicity (Modified from Lin et al., 2023).

LIST OF PUBLICATIONS AND COMMUNICATIONS

1. **First insights on seminal extracellular vesicles in chickens of contrasted fertility**

   Reproduction 161(5):489-498 (May 2021)

2. **25ème Journée Thésards de l'Unité PRC**

   Mini-poster and oral presentation (16-17 June 2021)

3. **Les 30 ans de la Fête de la science**

   Video and poster (2-3 October 2021)

4. **Les 14èmes Journées de la Recherche Avicole et Palmipèdes à Foie Gras**

   Full paper and poster (9-10 March 2022)

5. **26ème Journée Thésards de l'Unité PRC**

   Mini-poster and oral presentation (15-16 June 2022)

6. **19th International Congress on Animal Reproduction**

   Abstract and poster (26-30 June 2022)

7. **26th World's Poultry Congress**

   Abstract and oral presentation (7-11 August 2022)

8. **Avian Research Symposium 2023**

   Abstract and oral presentation (7-9 March 2023)

REPRODUCTION
RESEARCH

First insights on seminal extracellular vesicles in chickens of contrasted fertility

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Abstract

Male subfertility causes are very varied and sometimes related to post-gonadic maturation disruption, involving seminal plasma constituents. Among them, extracellular vesicles are involved in key exchanges with sperm in mammals. However, in birds, the existence of seminal extracellular vesicles is still debated. The aim of the present work was first to clarify the putative presence of extracellular vesicles in the seminal plasma of chickens, secondly to characterize their size and protein markers in animals showing different fertility, and finally to make preliminary evaluations of their interactions with sperm. We successfully isolated extracellular vesicles from seminal plasma of males showing the highest differences in semen quality and fertility by using ultracentrifugation protocol (pool of 3 ejaculates/rooster, *n*=3/condition). Size characterization performed by electron microscopy revealed a high proportion of small extracellular vesicles (probably exosomes) in chicken seminal plasma. Smaller extracellular vesicles appeared more abundant in fertile than in subfertile roosters, with a mean diameter of 65.12 and 77.18 nm, respectively. Different protein markers of extracellular vesicles were found by western blotting (*n*= 6/condition). Among them, HSP90A was significantly more abundant in fertile than in subfertile males. In co-incubation experiments (*n*= 3/condition), extracellular vesicles enriched seminal fractions of fertile males showed a higher capacity to be incorporated into fertile than into subfertile sperm. Sperm viability and motility were impacted by the presence of extracellular vesicles from fertile males. In conclusion, we successfully demonstrated the presence of extracellular vesicles in chicken seminal plasma, with differential size, protein markers and putative incorporation capacity according to male fertility status.

Reproduction (2021) **161** 489–498

Introduction

Seminal plasma, the fluid part of semen, is a highly complex fluid originating from secretions of the male tract. It interacts actively with sperm, and plays a key role in sperm maturation and fertilizing ability (Maxwell *et al.* 2007, Rodríguez-Martínez *et al.* 2011, Druart & de Graaf 2018). The composition and functions of seminal plasma differ greatly between species. For example, due to the lack of specific secretion glands such as prostate or seminal vesicles in the male tract, the seminal plasma of birds is very different from the mammalian seminal plasma, and consequently, the post-gonadic maturation process also (reviewed by Blesbois & Brillard 2007, Blesbois 2012, Santiago-Moreno & Blesbois 2020). The various investigations performed in mammals revealed that extracellular vesicles (EVs) present in seminal plasma are involved in the post-gonadic sperm maturation (Ronquist & Brody 1985, Sahlén *et al.* 2002, Saez *et al.* 2003, Sullivan *et al.* 2005, Frenette *et al.* 2010).

EVs are membrane vesicles involved in the transfer of proteins, lipids and nucleic acids between two cells, as signaling vehicles to maintain the normal cell homeostasis or respond to pathological events (Van Niel *et al.* 2018). There are two main categories of EVs defined from their size and biogenesis process: the exosomes (30–150 nm mean diameter) produced from endosomal system and the microvesicles (50–500 nm mean diameter) derived from the plasma membrane (Raposo & Stoorvogel 2013, Van Niel *et al.* 2018). However, mainly due to the overlapping range of size and the similar morphology, the definition of the proportion of sub-species of EVs remains a true challenge from the current available protocols of isolation (Van Niel *et al.* 2018). EVs formation involves specific proteins to regulate their biogenesis (e.g. programmed cell death 6-interacting protein – PDCD6IP), to maintain protein folding and membrane fusion (chaperones including Heat Shock Protein 90 kDa alpha – HSP90A, Valosin-containing protein-VCP), to regulate EVs-fusion with the target cells

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(annexins like ANXA5) (Buschow *et al.* 2010, Théry *et al.* 2018, Van Niel *et al.* 2018, Munuce *et al.* 2019).

Seminal EVs are secreted by different part of the mammalian male reproductive tract. They are generally named after the production localization, such as epididymosomes if secreted by the epididymis (Frenette *et al.* 2002, Saez *et al.* 2003, Frenette *et al.* 2010, Chen *et al.* 2016), or prostasomes if produced by the prostate (Renneberg *et al.* 1997, Sahlén *et al.* 2002, Sullivan *et al.* 2005, Burden *et al.* 2006, Ronquist 2012). Seminal EVs have multiple impacts on sperm biology including sperm maturation (Sullivan *et al.* 2005) especially by modifying sperm motility (Fabiani *et al.* 1994, Arienti *et al.* 2004), plasma membrane integrity (Du *et al.* 2016), and capacity to acrosome reaction (Murdica *et al.* 2019). They are also involved in the embryonic development (Conine *et al.* 2018) and may be sufficient to transfer a paternal phenotype to offspring (Chan *et al.* 2020).

Due to their importance in sperm biology, numerous authors suggest that the EVs may be markers of in/subfertility in mammals (Gabrielsen & Lipshultz 2019, Vickram *et al.* 2020). Despite no difference of shape or size, Murdica *et al.* (2019) revealed a difference of action of EVs on sperm biology (motility and acrosome reaction) depending on the patient fertility status. Furthermore, recent proteomic investigations revealed a list of more than 90 proteins differentially abundant between EVs isolated from seminal plasma of normozoospermic and asthenozoospermic patients (Lin *et al.* 2019). Collectively, these data suggest that EVs contents modulate sperm fertilizing ability. Despite the presence of EVs protein markers in rooster seminal plasma (Labas *et al.* 2015, Borziak *et al.* 2016, Li *et al.* 2020), the presence of EVs could not be described (Alvarez-Rodriguez *et al.* 2020). Consequently, the EVs existence in the seminal plasma of roosters is still debating and their potential link to the animal fertility status remains unexplored.

In this context, the first aim of the present study was to isolate rooster seminal plasma EVs. Then, we evaluated size and protein markers according to the fertility ability of the animals. Finally, we also investigated the interaction of enriched fractions of seminal EVs with sperm activity.

Material and methods

Animals

All experiments were carried out in accordance with the legislation governing the ethical treatment of birds and were approved by the French Ministry of Higher Education, Research and Innovation, and the Val-de-Loire Animal Ethics Committee (authorization no. DAP: APAFIS#4026-2016021015509521). On the 40 adult T44 roosters (*Gallus* gallus domesticus, Sasso, France), 39 were semen donors. 80 ISA Brown adult hens were used for fertility tests. All roosters were breeding into individual cages and the hens reared in cages of four animals, housed at the INRAE experimental unit UE-PEAT INRAE Poultry Experimental Facility (2018, https://doi.org/10.15454/1.5572326250887292E12). All the animals were 35–40 weeks old. They were maintained under a lighting regimen 14 h light:10 h darkness cycle, controlled temperature at 21°C, feeding with a standard diet and water *ad libitum*.

Semen collection

Semen from each rooster was collected individually twice a week (2–3 days between two collections) in 200 µL of a specific collection extender according to experiment (see subsequently), by dorso-abdominal massage as previously described (Burrows & Quinn 1937). Care was taken to avoid any contamination with transparent fluid and other cloacal products.

On the 40 tested animals, only one was infertile (absence of ejaculate) and was not used in this investigation.

In vitro semen evaluation and fertility test

Semen of the 39 males was collected in Beltsville poultry semen extender (BPSE) (Sexton 1977). Sperm volume was defined by weighing (mg) and sperm concentration was determined by light absorption using a photometer (Accucell photometer, IMV Technologies, L'Aigle, France) at a wavelength of 540 nm (Brillard & McDaniel 1985). Sperm viability (%) was determined with SYBR-14/Propidium iodide fluorescent dyes (Molecular Probes™ LIVE/DEAD™ SYBR-14/propidium iodide fluorescent dyes, L7011, Invitrogen, France), and revealed by flow cytometry (EasyCyte Guava, IMV Technologies, France) (Thélie *et al.* 2019). The proportion of live and dead sperm was assessed on a total of 5000 sperm per sample. Mass motility (movement of sperm group) was defined on a motility scale previously described (Blesbois *et al.* 2008): zero as a total lack of movement and nine as the presence of representing whirlwinds covering 30–60% of the observed area. Objective measurements of percentage of motile sperm (%) were evaluated by the computer-assisted sperm analysis (CASA) system with an HTM-IVOS (Hamilton-Thorn Motility Analyzer, IVOS, IMV Technologies), as previously described (Nguyen *et al.* 2014). The percentage of motile sperm was retained as the most robust parameter for further analyses.

A dose of 100×10^6 sperm/female was used for intravaginal inseminations of 8 hens per male (females laying rate higher than 95%). The eggs were collected daily and put into incubation. The fertility rate (number fertile eggs/number incubated eggs × 100) was estimated after 8 days of incubation by candling the eggs laid between days 2 and 8 after a single insemination. The highly fertile group (F) was defined by fertility > 90% and the subfertile group (SF) by fertility <45%. The experimental candidates selected for the EVs enriched fractions were selected according to their fertility level (F > 90%, SF < 45%) and also percentage of motile sperm (F > 50%, SF < 30%) and mass motility (F ≥ 6.5, SF < 3) differentials.

Seminal extracellular vesicles isolation

For this specific preparation, semen was collected individually (with a minimum interval of 3 days between collects)

in PBS (Sigma) added with protease inhibitor EDTA-free (04693159001, Roche Diagnostics). The first seminal plasma fraction (clarification) was obtained individually from total semen centrifugation at 959 ***g*** (Centrifuge 5417R, Eppendorf, France) for 10 min at 4°C. The supernatant, clarified seminal plasma, was collected and stored in ice. Sperm pellet was gently resuspended with 500 µL PBS with protease inhibitor and centrifuged as performed before. Supernatant were collected and added to clarified seminal plasma. Pellet was resuspended a second time in 500 µL PBS with protease inhibitor and placed on agitation 500 rpm (Thermomixer compact, Eppendorf, France), 10 min at 37°C. A third centrifugation was performed as described before and the supernatant was pooled with the previous supernatant fractions. The total volume of seminal plasma was centrifuged at 15,294 ***g*** for 15 min at 4°C. The supernatant was collected containing seminal plasma without cellular debris and stored at −20°C for further EVs isolation.

EVs isolation was carried out on resulting purified seminal plasma (pool of three collections per animal per replicate). EVs isolation was performed by two successive ultracentrifugations at 100,000 ***g*** for 90 min at 4°C (Beckman model L8-M with SW-55-Ti rotor, adjusted k-factor: 163, using the formula $k = (2.533 \times 10^{11}) \times \ln(rmax/rmin)/rpm^2$, were r(min) = 60.8mm and r(max) = 108.5mm at 30,000 rpm), with a washing step with PBS with protease inhibitor (Théry *et al.* 2006, Doyle & Wang 2019). The pellet obtained (EVs-enriched) from the second ultracentrifugation was suspended in 50 µL of PBS with protease inhibitor for transmission electron microscopy observations and western blotting experiments or in 50 µL Lake 7.1 (Lake & Ravie 1981) with protease inhibitor for EVs co-incubation tests with sperm.

Western blotting analysis

Western blotting experiments were performed for EVs protein markers (Buschow *et al.* 2010, Théry *et al.* 2018, Van Niel *et al.* 2018, Munuce *et al.* 2019) on individual samples of EVs suspension (6 F and 6 SF roosters). In each sample was added 100 µL lysis buffer (150 mM NaCl ,10 mM Tris HCl, 1 mM EGTA, 1 mM EDTA, 200 mM sodium fluoride, 4 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 % Triton X-100 and 0,5 % NP40) with protease inhibitor EDTA-free, sonicated and centrifuged at 12,000 ***g*** during 10 min at 4°C. Supernatant was collected and protein concentration was determined by the DC™ Protein Assay kit (5000112, BioRad, USA) according to the manufacturer's instructions. Samples were diluted in protein loading buffer (928-40004, LI-COR Biosciences, USA) added 10% (v/v) 2-Mercaptoethanol (M-7522, Sigma-Aldrich) and heated at 95°C for 5 min. From diluted samples, 20 µg of proteins were loaded on 10% SDS-PAGE, before being transferred to nitrocellulose membranes (10-600001, Amersham Protran 0.2 µm NC, GE Healthcare Life Science), in triplicate. Full-length protein loaded on gel were measured from images obtained from membranes stained with Revert™ 700 Total Protein Stain (926-11011, LI-COR Biosciences, USA) according to the manufacturer's instructions, using Odyssey CLx Imaging System (LI-COR Biosciences, USA) and quantified using Image Studio™ Lite Software (LI-COR Biosciences, USA). Membranes were washed with TBST (TBS, N14581, Interchim Life Sciences, France; 0.1 % (v/v) Tween-20, P9516, Sigma, Germany), incubated 1 h in the blocking solution (5 % (w/v) non-fat dry milk in TBST) at room temperature, and then incubated with the primary antibody overnight at 4°C in the blocking solution under agitation. The primary antibodies used were against: ANXA5 (36 kDa, 1:1000, rabbit, CSB-PA06384A0RB, Cusabio), and HSP90A (90 kDa, 1:1000, mouse, ab59459, Abcam), VCP (90 kDa, 1:1000, mouse, ab11433, Abcam) and PDCD6IP (96 kDa, 1:500, rabbit, HPA011905, Sigma). After washing, membranes were incubated at room temperature for 2 h with IRDye 800CW goat anti-rabbit (926-32211, LI-COR Biosciences, USA) or IRDye 800CW goat anti-mouse (926-32210, LI-COR Biosciences, USA) diluted 1:2000 in the Odyssey blocking buffer (927-50000, LI-COR Biosciences, USA) 1:1 (v/v) in TBS. The image of protein band signals was captured using Odyssey CLx Imaging System and quantified using Image Studio™ Lite Software and relative concentration was calculated using full-length protein quantification on membrane stained with Revert™ 700 to band signal normalization.

Characterization by transmission electron microscopy

From EVs-enriched suspension, 10 µL aliquot was thawed in ice during 1 h with the same volume of Trump's fixative solution (4% formaldehyde, 1% glutaraldehyde in PBS). The 3 µL aliquot was placed on a Formvar carbon-coated grid for 5 min. Samples were washed with distilled water (three times, 10 s), then for negative contrast stained with 2% uranyl acetate (three times, 10 s) and air dried at room temperature. The electron-micrographs were obtained using TEM HITACHI HT 7700 Elexience at 80 kV (with a charge-coupled device camera AMT) and JEM 1011 (JEOL, Japan) equipped with a Gatan digital camera driven by Digital Micrograph software (Gatan, Pleasanton, USA) at 100 kV. From each sample (3 F and 3 SF), all extracellular vesicles were measured from the 10 field photos using ImageJ software version 1.51n (http://imagej.nih.gov/ij, NIH).

Co-incubation of sperm with extracellular vesicles

Individual semen from 3 F and 3 SF roosters was collected in tube containing 200 µL Lake 7.1 added with protease inhibitor EDTA-free. Seminal plasma was removed by centrifugation at 600 ***g*** (2-16PK, Sigma), 10 min at 20°C and sperm concentration was determined as previously described. Diluted at 100×10^6/mL, sperm were incubated with 10 or 50 µg of EVs-enriched suspension pooled from 3 F animals, or without EVs, at 37°C, in water bath. Co-incubations EVs-sperm were proceeded for motility and viability measurements, as previously described, after short periods of co-incubation (5, 10 and 15 min) since the sperm quality decreases very rapidly for longer time at this temperature (Lemoine *et al.* 2008). Experiments were performed in triplicate.

Extracellular vesicles incorporation by rooster sperm

To evaluate the EVs incorporation by sperm, lipophilic green fluorescence dye PKH67 (MINI67, Sigma) was used to stain the EVs as previously described (Saadeldin *et al.* 2014). EVs-

enriched fraction of 3 F or 3 SF roosters (10 µL EVs-enriched fraction/animal; 30 µL final solution) was stained with PKH67 according to the manufacturer's instructions. Same volume of PBS was stained in parallel and used as negative control. Stained EVs were stored at −20°C, protected from light until co-incubation with sperm.

Semen from the 3 F and 3 SF roosters were prepared as described above in Lake 7.1. The F and SF sperm were co-incubated with stained EVs (10 µg of total protein) originating from F or from SF animals, in 1 mL Lake 7.1, protected from light, at 37°C in water bath during 1 h. One microliter of each incubation was applied in glass pre-treated with poly-L-lysine (VWF International, Germany) and coverslips were applied with Fluoroshield mounting medium with DAPI (Sigma). Individual sperm cells were analyzed using a confocal laser-scanning microscope (LSM700, Zeiss) equipped of excitation wavelengths 488 to 555 nm. All experiments were performed in triplicate.

Statistical analysis

All statistical analyses were performed with R software version 3.6.3 (R Core Team 2017) using the following packages: nlme (version 3.1-147) (Pinheiro & Bates 2013), lsmeans (version 2.30-0) (Lenth 2016) and multcomp (version 1.4–13) (Piepho 2004). Statistical testing was performed on datasets at a statistical significance of 5%.

In vitro semen quality

The Student's *t*-test was used for comparison of each parameter of *in vitro* semen evaluation among different groups. $P < 0.05$ was considered statistically significant.

TEM analysis

Size was determined for 3724 EVs from 3 F roosters and 1660 obtained from 3 SF animals. EVs was classified in three categories: < 70 nm, between 70 and 100 nm, > 70 nm and their distribution in F and SF groups was analyzed by *chi*^{2} test.

Protein abundance

Relative abundances of proteins present in EVs and obtained by western blotting on 6 F and 6 SF roosters were analyzed by Wilcoxon–Mann–Whitney test.

PKH67 relative intensity

Three animals of each fertility groups were used to investigate PKH67 relative intensity in sperm. First, the impact of the fertility group of EVs and the fertility group of sperm was investigated with a linear model (ANOVA test).

Post-hoc analyses with Tukey's multiple comparisons adjustment were performed depending to the significant terms. Then, the presence and origin of EVs was focused in only F sperm with a linear model (ANOVA test) Post-hoc analyses were performed with Tukey's multiple comparisons adjustment.

Viability and motility in co-incubation experiments

Based on the data collected from 3 F and 3 SF roosters, the impact of EVs, time and their interaction on sperm viability and motility was estimated following a mixed linear model for modeling heteroscedasticity and correlated errors. Each fertility group and each EVs quantity was analyzed separately. Post-hoc analyses with Tukey's multiple comparisons adjustment were performed depending to the significant terms.

Results

Fertility test

The individual fertility rates of the 39 males producing ejaculates ranked from 20 to 96%. Seven representative roosters were used in this work to constitute F samples with an average fertility rate of 95.07 ± 0.90%, means % motile sperm 57.4 ± 4.88%, and mass motility 6.79 ± 0.22. The 6 representative selected SF animals had an average fertility rate of 33.69 ± 2.81% and a mean % motile sperm 23.88% ± 7.05, and mass motility 3.61 ± 0.80. All these parameters were significantly different between groups ($P < 0.002$). Among all the animals analyzed, 7 F and 6 SF, there was no significant differences in the mean sperm concentration.

Extracellular vesicles characterization by TEM

Among the animals analyzed, three animals from each group (F and SF) were selected regarding the most representative differences in fertility and semen tests: fertility status ($P < 0.0001$; F: 97.44 ± 0.51%; SF: 30.45 ± 3.38%), means % motile sperm ($P < 0.001$; F: 66.83 ± 2.37%; SF: 14.38 ± 5.60%), mass motility ($P < 0.001$; F: 6.94 ± 0.24; SF: 2.33 ± 0.19) and concentration ($P < 0.01$; F: 3.01×10^9 sperm/mL ± 0.18; SF: 1.88×10^9 sperm/mL ± 0.13). From these animals, EVs-enriched samples were observed by transmission electron-microscopy. EVs presented the characteristic morphology of nanometric extracellular rounded and chapped vesicles in F and SF animals. Size characterization revealed EVs of 25–444 nm in F roosters and of 17–348 nm in SF animals (Fig. 1A and B) with a mean diameter of 65.12 nm for F roosters and 77.18 nm for SF animals (Fig. 1B). EVs size differential analysis showed distinct distribution between groups (Fig. 1C). Seminal plasma EVs of diameter less or equal to 70 nm were more abundant in F than in SF roosters (Fig. 1C). By contrast, EVs sized 70–100 nm or above 100 nm were more abundant in SF than in F seminal plasma (Fig. 1C).

Extracellular vesicle protein markers

The measurement of relative signal intensity indicated EVs with significant intense signal of HSP90A ($P < 0.001$) in F animals in comparison with SF roosters (Fig. 2A and B). There were no significant intensity differences between F and SF for the EVs markers ANXA5, PDCD6IP and VCP.

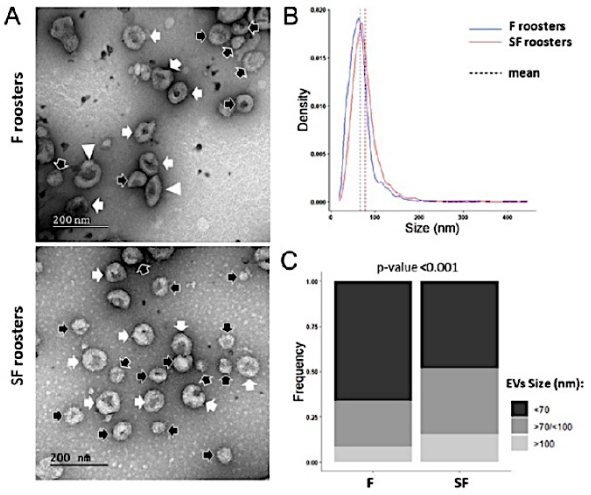

Figure 1 Characterization by transmission electron-microscopy (TEM) of extracellular vesicles (EVs) isolated from fertile (F) and subfertile (SF) seminal plasma (three animals per condition). (A) EVs sizes 20–70 nm (black arrow), 71 < 100 nm (white arrow), and >100 nm (white arrow-head) were observed in seminal plasma from F and SF roosters. (B) Representative graphic of EVs size distribution in experimental groups (F in blue and SF in red). Dotted lines indicate the mean size of characterized EVs in each experimental group (F and SF). (C) Frequency analysis of the EVs size (<70 nm, 70 < 100 nm, and >100 nm) analyzed by chi^2 test.

Extracellular vesicles incorporation by sperm

In vitro experiments were performed by EVs-sperm co-incubation assays on 3 F and 3 SF animals regarding the most representative differences in fertility and semen tests, as described above. EVs incorporation into sperm cells were observed predominantly in the head of the sperm and a faint signal in the intermediate piece. On the head of the sperm, intense droplets signals could be observed. No signal was observed on the sperm tail (Fig. 3A).

Co-incubation experiments revealed significant impacts of the sperm origin, of the EVs-enriched fraction origin and a significant interaction between these two variables on the PKH67 staining (Fig. 3B, 3 roosters per fertility status, in triplicate). Indeed, the highest PKH67 staining was observed in F sperm co-incubated with EVs isolated from F animals ($P < 0.001$). Furthermore, PKH67 staining was lower in sperm of SF compared to sperm of F rooters, when cultured with EVs from F chickens. No significant impact of the EVs origin on PKH67 staining was revealed in SF sperm, presenting lower staining than F sperm.

In order to better understand the EVs incorporation in F sperm, we performed a new statistical analysis of the experimental data obtained from the previous experiment, focusing on conditions containing F sperm and including a new condition of F sperm in absence of EVs (Fig. 3C). A significant impact of EVs presence was observed, supported by a higher PKH67 staining in experimental conditions with EVs when compared to F sperm alone ($P < 0.001$), confirming that the PKH67 staining was linked to the presence of EVs. As revealed by previous statistical model, the PKH67 staining of F sperm was higher with EVs from F animals than in all other conditions ($P < 0.001$).

Sperm viability and motility in presence of extracellular vesicles

The presence of 10 µg of EVs-enriched fraction had no impact on the viability of F or SF sperm. The dose of 50 µg significantly increased the viability of F sperm after 15 min of co-incubation ($P < 0.001$) (Fig. 4).

On F sperm, there were significant impacts of the experiment duration and of the interaction between the experiment duration and the EVs presence, with higher F sperm viability at 15 min in presence of EVs when compared to without EVs ($P < 0.001$). Concerning the

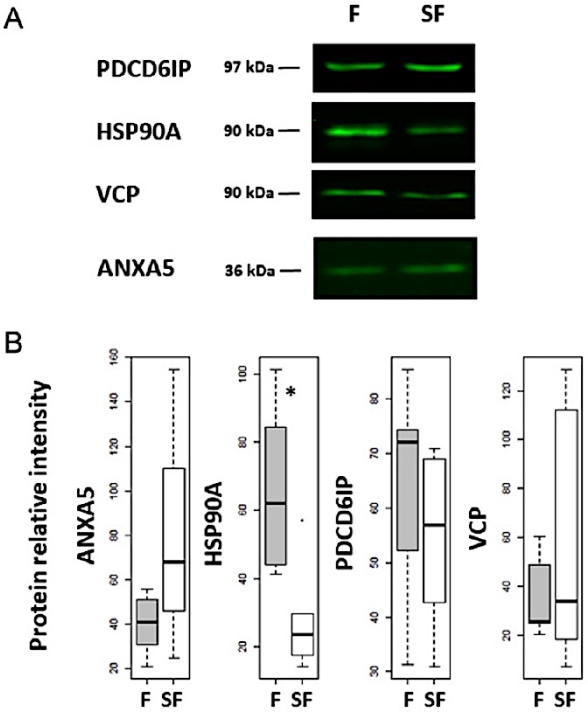

Figure 2 Western blotting results of ANXA5, HSP90A, PDCD6IP and VCP protein abundances in extracellular vesicles (EVs) isolated of seminal plasma from fertile (F) and subfertile (SF) roosters (six animals per condition, in triplicate). (A) Signal images obtained by Western blotting using specific antibodies. (B) Boxplots of the relative signal intensity of protein EVs markers immunodetected from F (gray bar) and SF (white bar) animals. Protein relative abundance intensity (mean of intensities of Western blotting quantifications performed from six animals per group) as arbitrary units normalized on the total quantity of proteins loaded. Internal black line in the bars indicates the median of the intensities of the experimental group and (*) indicates the statistical difference between the groups ($P < 0.001$).

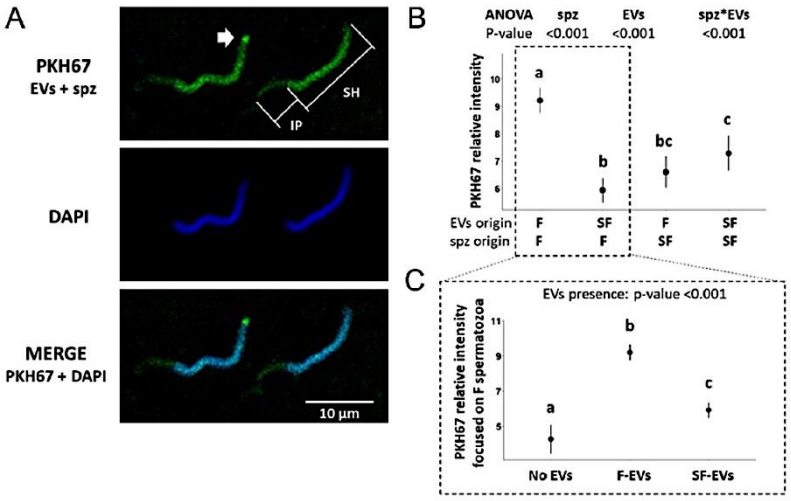

Figure 3 Incorporation in rooster sperm of extracellular vesicles (EVs) marked with PKH67 lipophilic stain (three roosters per fertility status, in triplicate). (A) Sperm head (SH) and intermediate piece (IP) stained after 1 h co-incubation with 10 µg EVs isolated from fertile (F) animals and PKH67-stained (PKH67, green) on F sperm. Sperm nucleus were stained with DAPI (blue). Merging image were resultant from PKH67 and DAPI signal images (Merge). Arrow indicates dropped concentration of green signal. (B) Representative graphic of EVs PKH67-stained from F and subfertile (SF) animals incorporated by F and SF sperm, after 1 h co-incubation. (C) Detail of incorporation intensities in F sperm incubated with (isolated from F or SF animals) or without EVs (No EVs). PKH67 emission intensities quantification was normalized by DAPI signals. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$). Scale bare (10 µm).

SF sperm, only a significant impact of the experiment duration was observed with the 50 µg dose ($P < 0.008$), as revealed by a general increase of the SF sperm viability.

A difference of proportion of motile sperm was observed between F and SF sperm with a higher motility observed in F sperm (Fig. 5). For a given time, the supplementation with 10 µg of EVs-enriched fraction isolated from F animals did not significantly affect the proportion of motile sperm, but the dose of 50 µg induced a significant reduction of the percentage of F motile sperm after 15 min of incubation ($P < 0.001$). This effect was not observed with SF sperm. The duration of the experiment significantly affected the proportion of motile sperm with a decrease for F sperm ($P < 0.001$), and more complicated time-EVs interactions effect in SF sperm.

The 10 µg dose showed a significant effect of interaction between experiment duration and EVs presence in F sperm supported by higher motility in F sperm with EVs at 5 min ($P < 0.001$) when compared to the samples without EVs at the same time, whereas the higher motility was observed in samples without EVs at 15 min.

Discussion

During the last two decades, seminal EVs have been largely studied in mammals (Sullivan *et al.* 2005), at the difference of other animal classes such as birds. In the present study, we were able to demonstrate, for the first time, the presence of EVs in the rooster seminal fluid. We could also report a difference of seminal EVs size

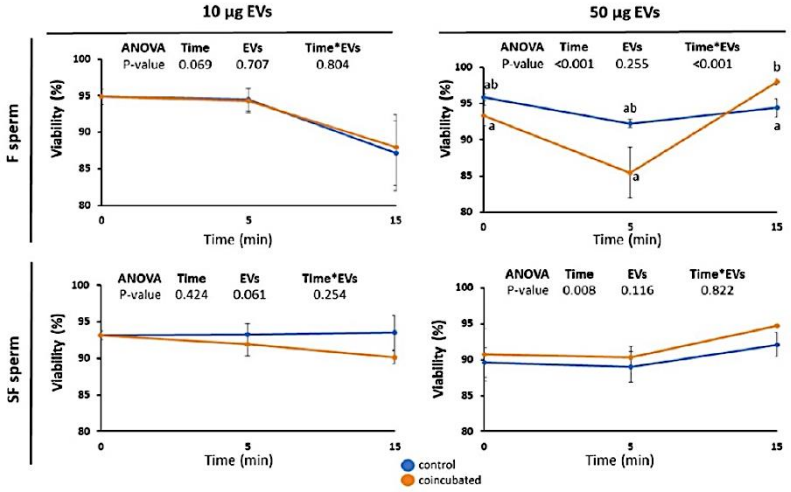

Figure 4 Sperm viability (three animals per conditions, in triplicate) evaluated by flow cytometry after incubation at 37°C in the presence of 10 or 50 µg of extracellular vesicles (EVs, red lines) isolated from seminal plasma of fertile animals during 0, 5 and 15 min. The control without EVs is represented in blue lines. Data are presented as the average of the percentages (%) and standard deviation. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$).

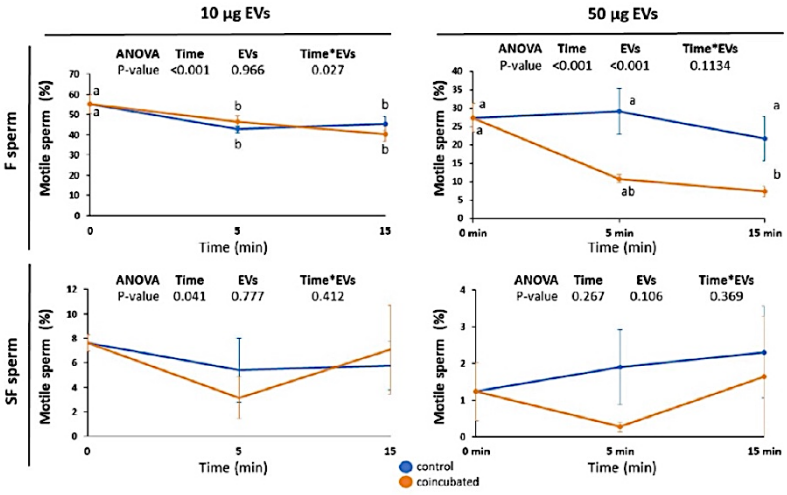

Figure 5 Sperm motility analysis from fertile (F) and subfertile (SF) roosters (three animals per conditions, in triplicate) co-incubated with 10 µg or 50 µg of extracellular vesicles (EVs, red lines) isolated from seminal plasma of fertile animals during 0, 5 and 15 min. Percentage (%) of motile sperm evaluated by CASA. The control without EVs is represented in blue lines. Data are presented as the average of the percentages (%) and S.D. Significant difference in pairwise *post hoc* analysis between conditions were expressed by different letters ($P < 0.05$).

between the most contrasted F and SF roosters. These EVs expressed classical EVs protein markers and among the tested ones, HSP90A content was significantly higher in F roosters when compared to SF animals. Our incorporation experiments also revealed a difference of EVs fusion to sperm depending on fertility status. This incorporation could be involved in the observed modulation of sperm motility in presence of EVs.

Despite the presence of EVs protein markers in seminal plasma (Labas *et al.* 2015, Borziak *et al.* 2016, Li *et al.* 2020), Alvarez-Rodrigues *et al.* (2020) presented EVs as potentially very rare in this fluid in chickens. It seems clear that the observation of EVs is not easy to reveal in chickens due to specific features of the species (high sperm metabolic rate, rapid degradation after semen collection, high sensitivity of the membranes to physical disruption) (Lemoine *et al.* 2008, Blesbois 2012, Nguyen *et al.* 2014). Thus, in order to succeed in the observation of seminal EVs in roosters, we built a protocol of extraction including immediate use of antiproteases, successive centrifugations and successive accelerations adapted to rooster semen, in order to preserve the EVs structures.

With this approach, we successfully isolated and observed round shaped EVs in rooster seminal plasma of F and SF animals. These EVs are predominantly smaller than 100 nm of diameter, suggesting a large proportion of small EVs (such as exosomes) in the chicken seminal EVs population (Van Niel *et al.* 2018) as observed in mammalian species such as boars (Alvarez-Rodriguez *et al.* 2019, Barranco *et al.* 2019) and humans (Aalberts *et al.* 2012). However, recent evidence suggested that size definition of EVs was not enough to define the different EVs subpopulations (such as exosomes or microvesicles) (Van Niel *et al.* 2018). The EVs classification also relies on the cellular compartment involved in their biogenesis, that is, inner cell part for exosomes and cellular plasma membrane for microvesicles (Van Niel *et al.* 2018). Consequently, further analyses will be necessary to precisely evaluate the different EVs subpopulations, especially by identifying their biogenesis cellular location.

Furthermore, our data revealed a difference of chicken seminal EVs size distribution according to the individual fertility status, with smaller EVs in F animals. No EVs size difference has been observed between seminal plasma EVs from patients with normospermia when compared to individuals with asthenozoospermia (Murdica *et al.* 2019). Our data suggest a modification of the EVs production or subtypes according to the sperm fertility ability.

In order to precise seminal EVs, we investigated the presence of classical EVs markers, including ANXA5, HSP90A, PDCD6IP and VCP (Bobrie *et al.* 2012, Théry *et al.* 2018, Almiñana & Bauersachs 2019, Riou *et al.* 2020). All of them were present in the EVs-enriched fractions. One of these investigated proteins, HSP90A is reported as expressed in the sperm cells of various species (Yue *et al.* 1999) including chickens (Soler *et al.* 2016). HSP90A expression is crucial for the male fertile since its depletion in *Drosophila* is associated to male sterility, relative to a motility reduction (Yue *et al.* 1999). Consequently, we can hypothesize that chicken EVs are involved in the presence or maintenance of HSP90A in the sperm. Moreover, HSP90A was present in higher amount in the EVs of F males when compared to SF animals. Since HSP90A content was previously reported to be higher in the sperm cells of subfertile males (Soler *et al.* 2016), by opposite to seminal EVs, this suggest complex mechanisms of exchanges and integration of EVs information by the sperm.

Even if EVs isolated from F and SF males were efficient to incorporate F sperm, this efficiency was higher concerning EVs-enriched fraction extracted from F roosters. This EVs incorporation was predominantly observed in rooster sperm head as already observed

in mammals (Arienti *et al.* 1997, 2004, Du *et al.* 2016, Zhou *et al.* 2019). This suggests a difference of the EV cell-targeting signal according to the male fertility status. Furthermore, no differential EVs incorporation has been revealed in SF sperm. Consequently, since the EVs isolated from the F sperm could merge with F sperm, the little incorporation of EVs into SF sperm suggests a perturbation of the fusion mechanisms. On one hand, due to the putative role of EVs in exchanges between the sperm and their extracellular environment, this may suggest a reduction of these exchanges in case of SF sperm and thus a perturbation of the sperm maturation process. On the other hand, we can hypothesize that F animals contain a predominant subtype of EVs, with different protein content and greater capacity for incorporation, as suggested for the impact of female physiological status on endometrial EVs incorporation into spermatozoa (Murdica *et al.* 2020), helping them to keep high fertilization capacity.

These hypotheses are consistent with the absence of EVs impact on sperm motility and viability of SF animals, by opposite to F sperm. Thus, the EVs presence could not reestablish a normal percentage of motile sperm in SF animals. However, the absence of EVs impact on SF sperm motility and viability do not conclude of the total absence of EVs impact on SF biology. Other effects need to be further explored such as the impact on acrosome reaction (Murdica *et al.* 2019, 2020).

Interestingly, in addition to the dose-dependent response of F sperm to EVs (observed only with 50 µg EVs-enriched fraction), the sperm viability of F males increased after 15 min of incubation while motility decreased. At 37°C, incubation of bird sperm is still a delicate operation since the rooster sperm are extremely active at this temperature and may lose very fast their fertilizing capacity. In this condition, it seems that the dose of 50 µg EVs isolated from F individuals may be helpful to maintain the sperm viability. The role of EVs in the regulation of sperm function before fertilization has been reviewed by Barkalina *et al.* (2015) and Machtinger *et al.* (2016). Particularly, modification of the sperm motility in the presence of EVs was already reported in humans (Murdica *et al.* 2019), porcines (Alcântara-Neto *et al.* 2020) and boars (Du *et al.* 2016). In chickens, we can hypothesize that the observed decrease of motility could contribute to the maintenance of viability by lowering a too intense metabolic activity of rooster sperm, allowing longer storage or increased survival capacity. However, since, we used successive and differential (ultra)centrifugation approach to reveal seminal EVs, our study of EVs-sperm co-incubation must be confirmed by further different approaches in the future. Indeed, EVs may be isolated by different methods. All of them show specific advantages and limits that may affect their content and functionalities (Doyle & Wang 2019, Jeon *et al.* 2020). Thus, complementary approaches based on other EVs separation technics will be necessary to confirm our functional hypothesis.

In conclusion, we successfully demonstrated the presence of EVs in rooster seminal plasma, with a differential size distribution, protein compositions and efficient incorporation to sperm. These results share new information helpful for further comprehension of the mechanisms involved in sperm fertilizing capacities and for the exploration of new phenotypic tools of reproduction capacity of males.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

L C and E B designed the experiments; L C, I G, H H L L and R U performed the experiments; L C and A V C analyzed the results; L C, A V C and E B wrote the manuscript. All authors approved the final manuscript.

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New advances in chicken sperm cryopreservation-the issue of glycerol

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INTRODUCTION

Currently, glycerol is the most used cryoprotectant for chicken sperm cryopreservation[1]. 11% glycerol is the standard concentration for freezing chicken sperm but needs to be removed before insemination to avoid the contraceptive effect[2]. The mechanisms underlying this contraceptive effect remains unclear. Here we explore how glycerol impacts on sperm motility at 41°C to mimic the temperature in the female tract. Furthermore, we also try to develop a new strategy based on Percoll™ single layer centrifugation (SLC)[3,4] to remove glycerol prior to insemination in order to facilitate the application of cryopreserved chicken sperm.

METHODES

1. Chicken semen was diluted with glycerol-free PC diluent to final concentration of 0, 1, 2, 6 and 11% glycerol respectively. Diluted semen was incubated at 41°C and assessed sperm motility with the computer-assisted sperm analysis (CASA, IVOS) at 0, 10, 20 and 30 min.
2. Glycerol removal solution (GRS) was prepared from Percoll™ added with 0.2 M sucrose in different concentration (40, 50, 60 and 70%). 0.5 ml of post thaw semen was layered on top of 2 ml of GRS and centrifuged at 800 g for 15 min at 4 and 20 °C. Sperm pellets were resuspended to assess glycerol concentration with Glycerol Assay Kit (Sigma-Aldrich) and sperm motility with CASA.
3. Data were presented as mean ± SD and analyzed by two-way ANOVA. Significant difference was identified by different letters ($p<0.05$).

RESULTAT-1 Impacts of glycerol on sperm motility

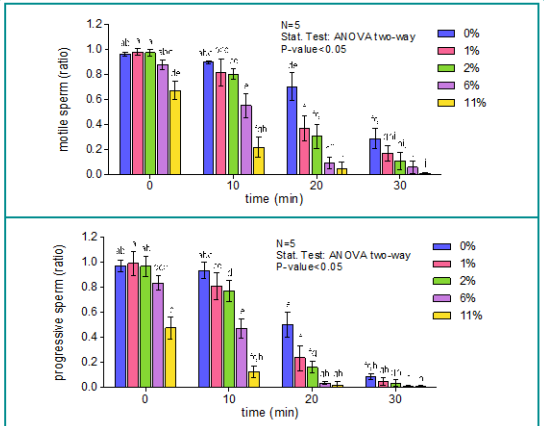

Figure 1. The effects of glycerol concentration on the ratio of motile and progressive sperm after 0, 10, 20 and 30 min incubation at 41°C. Ratio = sperm (%) after treatment : sperm (%) before treatment

1. The ratio of motile and progressive sperm reduced with time, and more reduction appeared when glycerol concentration increased.
2. The ratio of motile sperm of 0, 1 and 2% glycerol sperm was significantly higher than 6 and 11% glycerol sperm at 10 and 20 min, and similar result was obtained for progressive sperm at 30 min.

RESULTAT-2 Development of new glycerol removal method

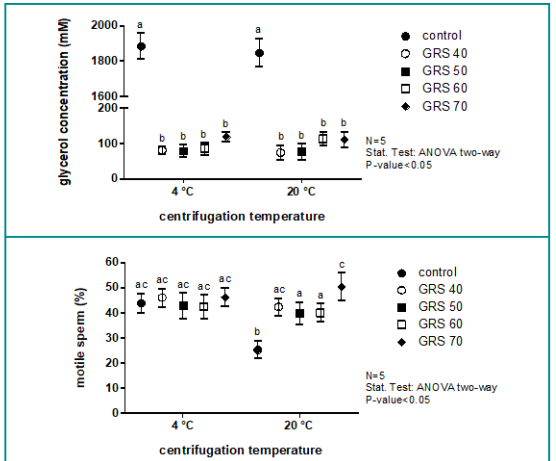

Figure 2. Glycerol concentration and sperm motility after GRS SLC at 4 and 20 °C.

1. GRS SLC efficiently removed glycerol from chicken post-thaw semen.
2. GRS SLC at 20 °C provided a direct selection for motile and progressive sperm (data no. shown).

CONCLUSIONS

1. Contraceptive effects of glycerol may be partly due to a reduction of sperm motility and thus, a putative impairment of sperm transportation in the female tract. More sperm physiology parameters (acrosome and mitochondria integrity, apoptosis, ...) are currently investigated to better understand glycerol impact on chicken sperm.
2. Our new glycerol removal method succeed in efficiently removing glycerol saving 1/3 of time when compared to conventional method (stepwise dilutions followed by centrifugation)[2] and functionally selecting sperm with higher motility which is the parameter strongly correlated with fertility[1]. Further insemination test are needed to ensure the practical application of this method.

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ÉLIMINATION DU GLYCÉROL DE LA SEMENCE DÉCONGELÉE DE COQ PAR CENTRIFUGATION DANS UN GEL COLLOÏDAL SYNTHÉTIQUE

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RÉSUMÉ

La cryoconservation des spermatozoïdes est une méthode cruciale dans la conservation de la biodiversité des espèces et des races, mais aussi pour assurer la restauration des lignées lors de catastrophes sanitaires. Actuellement, le glycérol est l'agent cryoprotecteur le plus utilisé pour la cryoconservation des spermatozoïdes de coq, étant le plus efficace dans le maintien des fonctions du spermatozoïde, mais il est associé à un taux de réussite faible voir une totale stérilité lors de l'insémination. Par conséquent, nous cherchons à développer une méthode simple d'élimination du glycérol par centrifugation dans un gel colloïdal synthétique, le Percoll®. Après collecte, la semence de coqs a été congelée en glycérol 11% en paillettes avec un congélateur programmable. Lors de la décongélation, la semence a été déposée sur une solution de sucrose (200 mM) en présence de Percoll® (Solution Sucrose-Percoll®, SSP) dans du PBS puis centrifugée 15 min. Afin de définir les meilleures conditions, différentes concentrations de SSP (40, 50, 60 ou 70%) ainsi que deux températures (4 ou 20°C) ont été testées. La concentration de glycérol et la mobilité individuelle des spermatozoïdes ont été évaluées après centrifugation respectivement par le kit Glycerol Assay et par Computer-Assisted Sperm Analysis system (CASA). Nos données montrent que toutes les concentrations de SSP réduisent la présence de glycérol à moins de 120 mM (soit une élimination de 94% du glycérol initial). La centrifugation à 20°C permet une bonne sélection des spermatozoïdes mobiles et progressifs, en particulier pour SSP 40 et 70%. Notre nouvelle méthode permet donc d'éliminer efficacement le glycérol des semences de coq cryopréservées tout en sélectionnant les spermatozoïdes présentant une bonne mobilité. Comparée à la méthode conventionnelle de dilutions successives, elle permet de gagner 44% de temps de traitement des échantillons lors de l'élimination du glycérol.

ABSTRACT

Sperm cryopreservation is a crucial method not only to conserve the biodiversity of species and breeds, but also to restore animal populations during emergent epidemics. Currently, glycerol is the most widely used cryoprotectant to freeze chicken sperm due to its effectiveness for maintaining sperm functions, but it is associated with a reduction of fertility or a total infertility during insemination. Therefore, we tried to develop a simple method to remove glycerol from frozen-thawed chicken semen with a centrifugation in a synthetic colloidal gel, the Percoll®. After collecting, chicken semen was frozen in a diluent of 11% glycerol in straws with a programmable freezer. Thawed semen was layered on the solution containing 200 mM sucrose and Percoll® (Solution Sucrose-Percoll®, SSP) in PBS and centrifuged 15 min after. Different concentrations of SSP (40, 50, 60 or 70%) combined with two different temperatures (4 or 20°C) were tested to determine the best conditions of SSP centrifugation. Glycerol concentration and sperm individual motility were evaluated respectively by Glycerol Assay kit and Computer-Assisted Sperm Analysis system (CASA). Our results showed that all concentrations of SSP efficiently reduce glycerol concentration to less than 120 mM (equal to remove 94% of glycerol from thawed semen). Centrifugation at 20°C allowed an efficient selection for motile and progressive sperm especially with SSP 40 and 70%. It indicated that our novel method can efficiently remove glycerol from post-thaw chicken semen with a positive selection of motile sperm. Compared to the conventional method of serial dilutions, it can save 44% of time of glycerol removal process.

INTRODUCTION

La conservation des ressources génétiques est une stratégie cruciale pour protéger la biodiversité, restaurer des espèces ou races menacées et contrer les épidémies ou les accidents de reproduction (Morrell and Mayer, 2017; Svoradová *et al*., 2018). Trois types cellulaires sont actuellement disponibles pour la cryobanque chez les oiseaux : les spermatozoïdes, les cellules germinales primordiales et le tissu gonadique. Parmi toutes ces méthodes, la cryoconservation des spermatozoïdes reste encore l'approche la plus répandue car elle se base sur la collecte de grandes quantités de cellules, le respect du bien-être animal, et la possibilité d'un stockage long ainsi que d'échanges de ressources à longue distance (Çiftci and Aygün 2018; Thélie *et al*., 2019; Cardoso *et al*., 2020). Actuellement, le glycérol est le cryoprotecteur le plus utilisé pour congeler les spermatozoïdes de coq (Abouelezz *et al*., 2015 ; Lin *et al*., 2021). Il fournit une excellente protection des spermatozoïdes leur permettant de résister aux impacts négatifs du processus congélation/décongélation (Blesbois and Brillard, 2007; Blesbois *et al*., 2007). Cependant, il est associé à une baisse de la fertilité pouvant être totale et il doit donc être éliminé avant l'insémination (Seigneurin and Blesbois, 1995; Thélie *et al*., 2019). Néanmoins jusqu'à présent, le processus d'élimination du glycérol est compliqué et se base sur des équipements particuliers (dont une chambre froide). Par conséquent, nous cherchons à développer une nouvelle méthode simple d'élimination du glycérol de la semence de coq décongelée, se basant sur le Percoll®, un gel classiquement utilisé pour sélectionner les spermatozoïdes mobiles chez les mammifères mais également sur de la semence fraiche de coq (*Lin et al*., 2020).

1. MATERIELS ET METHODES

1.1. Animaux

18 coqs adultes (entre 32 et 42 semaines d'âge) de lignée T44 (*Gallus gallus domesticus*, Sasso, France) ont été utilisés comme donneurs de semence. Tous les coqs ont été hébergés en cages individuelles à l'unité expérimentale INRAe UE-PEAT (2018, https://doi.org/10.15454/1.5572326250887292E12), nourris avec un régime standard, de l'eau à volonté et élevés à 20°C et sous un cycle lumineux de 14h de lumière et 10h d'obscurité par jour. Ce protocole expérimental a été approuvé par le Ministère de l'Éducation Supérieure, de la Recherche et de l'Innovation (MESRI) et le comité d'éthique Val-de-Loire (N° APAFIS #4026-2016021015509521).

1.2. Collecte, congélation et décongélation de la semence

La semence de coq a été collecté régulièrement deux fois par semaine par la méthode de massage abdominal (Burrows et Quinn, 1937). Trois groupes de 6 animaux ont été formés de façon aléatoire pour regrouper les semences dans un volume de Lake PC similaire à celui de collecte puis mélangés doucement et refroidis à 4°C pendant 10 min. Un volume égal de Lake PC contenant 22% de glycérol a été ajouté pour atteindre une concentration finale de 11% de glycérol, puis l'échantillon a été équilibré à 4°C avec une agitation douce pendant 10 min. Par la suite, la semence a été déposée dans des paillettes de 0,5 mL (IMV Technologies, France). Toutes les paillettes ont été scellées, transférées dans un congélateur programmable (MiniDigitcool, IMV Technologies, France) et congelées à vitesse contrôlée de 4 à -35°C à -7 °C/min puis de -35 à -140°C à -20 °C/min. Finalement, les paillettes ont été plongées dans de l'azote liquide pour une conservation d'au moins 14 jours et décongelées au bain-marie à 4°C pendant 3 min pour la suite des expériences.

1.3. Préparation de Solution Sucrose-Percoll® et élimination du glycérol

La solution de Sucrose-Percoll® (SSP) a été préparée en ajoutant du Percoll® (Sigma-Aldrich, USA) isotonique et du sucrose 200 mM pour composer 40, 50, 60 ou 70% de SSP dans du PBS, puis 2 mL de SSP ont été transférés dans un tube conique de 15 mL. La semence décongelée (5 répliques) a été déposée sur 2 mL de SSP et centrifugée 15 min à 800×g à 4 ou 20°C. Le culot de spermatozoïdes a été collecté et remis en suspension avec le dilueur Lake 7.1. Les témoins étaient la semence décongelée sans élimination du glycérol.

1.4. Évaluation de la concentration de glycérol et de la mobilité individuelle des spermatozoïdes

La concentration de glycérol a été mesurée selon les instructions du kit Glycerol Assay (Sigma-Aldrich, USA) sur 10 µL d'échantillon. La mobilité individuelle des spermatozoïdes a été mesurée au Computer-Assisted Sperm Analysis system (CASA, IVOS, IMV Technologies, France) sur 2 µL d'échantillon à 30×10^6 cellules/mL. Quatre champs d'observation ont été capturés pour chaque échantillon. La trajectoire de chaque spermatozoïde a été enregistrée afin de calculer sa vitesse moyenne (velocity average path, VAP). Les résultats de mobilité ont été exprimés en pourcentage de spermatozoïdes mobiles et progressifs qui ont présenté respectivement une VAP > 5 mm/s et VAP > 50 mm/s.

1.5. Analyses statistiques

Toutes les analyses statistiques sont réalisées avec le logiciel R (version 1.4.1717) par ANOVA à deux facteurs. Les résultats sont exprimés en moyennes et écart-type. Une différence significative est révélée par une p-valeur inférieure à 0,05%.

2. RESULTATS ET DISCUSSION

2.1. Concentration de glycérol après centrifugation

Nos données révèlent une réduction significative de la concentration de glycérol après traitement de la semence par une solution SSP (quelque soit la concentration de Percoll)(Tableau 1). En effet, alors que la concentration moyenne de glycérol de la semence décongelée est de 1866,3 mM, après centrifugation dans SSP, elle est réduite à moins de 120 mM dans tous les traitements (Tableau 2). Ces résultats révèlent que notre nouvelle méthode d'élimination du glycérol peut éliminer au moins 93,6% du glycérol de la semence décongelée. Des expériences antérieures indiquent que 2% de glycérol (soit environ 273.4 mM) est le seuil maximal pour obtenir la capacité maximale de fécondation (Polge, 1951; Neville *et al*., 1971). Ainsi, notre méthode permet d'obtenir une concentration de glycérol compatible avec de bonnes conditions d'insémination.

2.2. Mobilité des spermatozoïdes après centrifugation

La mobilité des spermatozoïdes décongelés est un indicateur de leur capacité fécondante chez les poulets (Blesbois *et al*., 2008). C'est pourquoi nous avons choisi d'étudier ce critère comme prédicteur potentiel de la fertilité. La concentration de SSP, la température mais également l'interaction de ces deux facteurs ont un impact significatif sur la mobilité des spermatozoïdes (Tableau 1). La mobilité des spermatozoïdes dans l'échantillon témoin diminue de façon importante à 20°C mais pas à 4°C dans les 15 min suivant la décongélation (Tableau 2). L'élimination du glycérol par notre protocole à 4°C ne montre aucune différence significative de la mobilité des spermatozoïdes. Cependant, le même protocole à 20°C améliore la mobilité des spermatozoïdes, en particulier dans le traitement SSP 70% qui maintient le pourcentage de spermatozoïdes mobiles et progressifs à respectivement de 25,1% et 15,2% plus élevés que le témoin. Cette observation est en accord avec celle précédemment rapportée par Lin *et al*., (2020) qui signalent que la centrifugation de semence sur une solution de Percoll® est bénéfique pour sélectionner les spermatozoïdes mobiles d'une semence fraîche de coq. En outre, un protocole basé sur un autre gel colloïdal, le gel Accudenz (AD), est déjà utilisé pour éliminer le glycérol de la semence décongelée de coq par centrifugation dans la solution AD discontinue (12% sur 30%) à 5°C (Purdy *et al*., 2009). Cependant, nos données suggèrent une meilleure mobilité des spermatozoïdes. La comparaison des deux protocoles dans les mêmes conditions permettrait d'identifier la méthode plus efficace.

De plus, les taux de mobilité observés à 20°C après centrifugation en SSP sont équivalents à ceux obtenus à 4°C, suggérant que ce protocole peut facilement être développé à température ambiante.

2.3. Durée du protocole d'élimination du glycérol

Actuellement, la centrifugation avec dilutions en série (CDS) est la méthode la plus courante pour réduire la concentration de glycérol de la semence décongelée de coq (Blesbois and Brillard, 2007). Toutefois, cette méthode nécessite de travailler pendant de long temps (Tableau 3). De plus, cette méthode ainsi que celle précédemment présentée basée sur le gel AD nécessite de travailler à 4°C, et donc la présence d'une chambre froide, ce qui est difficile à mettre en pratique dans les élevages. Au contraire, notre nouvelle méthode d'élimination du glycérol n'a pas besoin de la chambre froide et la centrifugation ne prend que 15 min, ce qui permet gagner respectivement 40% et 44,4% de temps par rapport à la méthode d'AD et de CDS.

CONCLUSIONS

Nos données montrent l'efficacité d'une nouvelle méthode d'élimination du glycérol de la semence décongelée de coq basée sur le gel Percoll®. Cette méthode permet d'éliminer efficacement le glycérol de la semence décongelée tout en sélectionnant les spermatozoïdes présentant une meilleure mobilité. Par rapport à la méthode CDS conventionnelle ou celle basée sur le gel AD, elle est plus économique, à la fois en temps et en équipement car elle peut être employée à température ambiante et éviter donc la nécessité d'une chambre froide. Actuellement, des expériences sont en cours pour déterminer l'impact de cette méthode sur la réussite de l'insémination artificielle.

REMERCIEMENTS

Les auteurs tiennent à remercier Philippe Didier, Joël Delaveau et Jérémy Bernard (INRAe UE-PEAT) pour leur investissement dans la gestion des animaux. Ce projet est financé par le projet [ANR-11- INBS-0003].

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Tableau 1. Effets de la température et la concentration de Solution Sucrose-Percoll® sur la concentration de glycérol, le pourcentage des spermatozoïdes mobiles et progressifs

	Statistique (p-valeur)		
	Température	**Concentration de SSP**	**Température* Concentration de SSP**
Concentration de glycérol	0.633	<0,001	0.460
Spermatozoïdes mobiles	<0,001	<0,001	<0,001
Spermatozoïdes progressifs	<0,001	<0,001	0.002

SSP : Solution Sucrose-Percoll®. Une p-valeur inférieure à 0,05 est considérée comme significative.

Tableau 2. Effets sur la concentration de glycérol, le pourcentage des spermatozoïdes mobiles et progressifs de la semence décongelée de coq après centrifugation dans Solution Sucrose-Percoll®

	4°C					**20°C**				
	TEM	**SSP 40%**	**SSP 50%**	**SSP 60%**	**SSP 70%**	**TEM**	**SSP 40%**	**SSP 50%**	**SSP 60%**	**SSP 70%**
Concentration de glycérol (mM)	1885,1 ± 73,1^{a}	80.8 ± 10,5^{b}	79.1 ± 16,7^{b}	85.0 ± 17,9^{b}	118.8 ± 12,9^{b}	1847.5 ± 80,5^{a}	74.4 ± 20,3^{b}	76.8 ± 22,7^{b}	112.5 ± 19,5^{b}	111.9 ± 21,5^{b}
Spermatozoïdes mobiles (%)	44.0 ± 3,9^{ab}	46.1 ± 3,7^{ab}	43.1 ± 5,2^{ab}	42.5 ± 4,6^{ab}	46.4 ± 3,6^{ab}	25.4 ± 3,3^{c}	42.5 ± 3,4^{ab}	39.9 ± 4,4^{a}	40.1 ± 3,7^{a}	50.5 ± 5,5^{b}
Spermatozoïdes progressifs (%)	28.3 ± 2,0^{ab}	31.6 ± 3,1^{a}	28.5 ± 3,7^{ab}	27.6 ± 3,5^{ab}	30.6 ± 2,9^{ab}	16.5 ± 3,7^{c}	26.4 ± 2,6^{ab}	24.1 ± 3,5^{b}	24.9 ± 2,5^{ab}	31.7 ± 4,9^{a}

TEM : échantillon témoin ; SSP : Solution Sucrose-Percoll® ; Les valeurs sont exprimées en moyenne ± écart-type (n = 5) ; ^{abc} Des exposants différents indiquent des différences significatives (p-valeur<0,05).

Tableau 3. Comparaisons des méthodes d'élimination du glycérol entre la centrifugation par Sucrose-Percoll® solution, Accudenz et avec dilutions en série

	Solution	Dilution		Centrifugation		
		T (°C)	Time (min)	Force (g)	T (°C)	Time (min)
SPS	Sucrose-Percoll®	-	0	800	4 or 20	15
AD	Accudenz discontinu	-	0	300	5	25
CDS	Lake C	4	12	500	4	15

SSP : Solution Sucrose-Percoll® ; AD : Accudenz ; CDS : centrifugation avec dilutions en série.

Élimination du glycérol de la semence décongelée de coq par centrifugation dans un gel colloïdal synthétique

Lin Hsiu-Lien Herbie[1,2], Grasseau Isabelle[1], Mermillod Pascal[1], Blesbois Elisabeth[1], Vitorino Carvalho Anaïs[1]

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INTRODUCTION

La conservation des ressources génétiques est cruciale pour protéger la biodiversité, restaurer des espèces ou races menacées et contrer les épidémies[1,2]. La cryoconservation des spermatozoïdes (spz) est l'approche la plus répandue car elle offre la possibilité d'un stockage et transport longs[3,4,5] des échantillons. Actuellement, le glycérol est le cryoprotecteur le plus utilisé pour conserver au froid la semence de coq [6,7]. Cependant, **il est associé à une baisse de la fertilité pouvant être totale et il doit donc être éliminé avant l'insémination**[8]. Les méthodes actuelles de retrait du glycérol (dilutions en série (CDS)[8] ou gel Accudenz (AD)[9]) sont longues et/ou nécessitent du matériel particulier rarement disponible en élevage. **Notre objectif est donc de développer une nouvelle méthode simple de retrait du glycérol**, en utilisant le Percoll®, un gel classiquement employé pour sélectionner les spz mobiles[10], combiné à du sucrose afin de limiter un potentiel choc osmotique. Pour identifier les meilleures conditions d'utilisation de cette **Solution de Sucrose-Percoll® (SSP)**, nous avons **testé différentes concentrations et température de centrifugation sur l'efficacité de retrait du glycérol, la mobilité et les capacités fécondantes des spz** (obtenues après insémination artificielle – IA).

MATÉRIELS ET MÉTHODES

Collecte de la semence par la méthode de massage abdominal[11]

- 4°C pendant 10 min
- Ajout de 11% de glycérol
- 4°C sous agitation pendant 10 min

- Paillette de 0,5 mL
- Congélation: de 4 à -35°C à -7 °C/min puis de -35 à -140°C à -20 °C/min
- Maintien en azote liquide (>14 jours)

Ingrédient de SSP

SSP	A	B	C	D
40	40	60	80	408
50	50	50	100	440
60	60	40	120	472
70	70	30	140	506

A: SSP basique = 90% Percoll® + 10% 10X PBS + 200mM sucrose
B: 1XPBS (%)
C: sucrose (mM)
D: Osmolalité (mOsmol/kg)

-Décongélation au bain-marie à 4°C pendant 3 min
-Dépôt sur 2 mL de SSP et centrifugation 15 min à 800×g à **4 ou 20°C**.

Resuspension en Lake 7.1[8]. Témoins (TEM): Semence décongelée sans élimination du glycérol (évaluation *in vitro)* ou avec la technique CDS[4,8] (IA)

Cinq IA à une dose de 200×10^6 spz pendant 2 semaines. Collecte des œufs sur 13 jours après la première IA.

- Analyse du retrait du glycérol : Glycerol Assay kit (Sigma)
- Mobilité: Computer-Assisted Sperm Analysis system (IVOS, Hamilton).

- Impact du traitement SSP et de la température sur concentration du glycérol et mobilité: ANOVA à deux facteurs (R).
- Impact sur la fertilité : Chi2 (GraphPad Prism)

RÉSULTATS ET DISCUSSION

I. Concentration de glycérol

1. On observe une baisse significative de la concentration en glycérol après traitement par SSP.
2. Notre méthode permet d'éliminer le glycérol pour atteindre des concentrations compatibles avec le maintien de la fertilité lors d'inséminations (273 mM ou moins[12,13]).

	Statistique (p-valeur)		
	Température	Traitement par SSP	Température* Traitement par SSP
Concentration de glycérol	0.633	<0,001	0.460

	4°C					20°C				
	TEM	SSP 40%	SSP 50%	SSP 60%	SSP 70%	TEM	SSP 40%	SSP 50%	SSP 60%	SSP 70%
Glycérol (mM)	1885,1 ± 73,1^a	80.8 ± 10,5^b	79.1 ± 16,7^b	85.0 ± 17,9^b	118.8 ± 12,9^b	1847.5 ± 80,5^a	74.4 ± 20,3^b	76.8 ± 22,7^b	112.5 ± 19,5^b	111.9 ± 21,5^b

II. Mobilité des spermatozoïdes

1. Le traitement par SSP, la température et leur interaction ont un effet significatif sur la mobilité des spermatozoïdes.
2. La mobilité des spermatozoïdes témoins diminue de façon importante à 20°C mais pas à 4°C dans les 15 min suivant la décongélation.
3. L'élimination du glycérol par notre protocole à 4°C ne montre pas d'effet mobilité des spermatozoïdes, alors que le même protocole à 20°C augmente la proportion de spermatozoïdes mobiles, en particulier avec la SSP 70%.

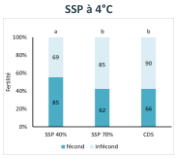
	Statistique (p-valeur)		
	Température	Traitement par SSP	Température* Traitement par SSP
Spermatozoïdes mobiles	<0,001	<0,001	<0,001
Spermatozoïdes progressifs	<0,001	<0,001	0.002

	4°C					20°C				
	TEM	SSP 40%	SSP 50%	SSP 60%	SSP 70%	TEM	SSP 40%	SSP 50%	SSP 60%	SSP 70%
Spermatozoïdes mobiles (%)	44.0 ± 3,9^{ab}	46.1 ± 3,7^{ab}	43.1 ± 5,2^{ab}	42.5 ± 4,6^{ab}	46.4 ± 3,6^{ab}	25.4 ± 3,3^c	42.5 ± 3,4^{ab}	39.9 ± 4,4^{ab}	40.1 ± 3,7^a	50.5 ± 5,5^b
Spermatozoïdes progressifs (%)	28.3 ± 2,0^{ab}	31.6 ± 3,1^a	28.5 ± 3,7^{ab}	27.6 ± 3,5^{ab}	30.6 ± 2,9^{ab}	16.5 ± 3,7^c	26.4 ± 2,6^{ab}	24.1 ± 3,5^b	24.9 ± 2,5^{ab}	31.7 ± 4,9^a

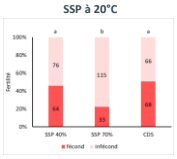
III. Fertilité

SSP 40 et 70% semblent être les deux conditions les plus favorables. Les expériences d'insémination ont montré une meilleur fertilité à 4°C avec SSP 40% comparé à SSP 70% et CDS. A 20°C, SSP 40% a obtenu le même niveau de fertilité que CDS et supérieur à SSP 70%.

SSP à 4°C



SSP à 20°C



Les chiffres sur les barres représentent le nombre d'œufs

IV. Durée du protocole d'élimination du glycérol

Notre nouvelle méthode serait plus facilement applicable en élevage car ne nécessite pas de chambre froide, ni de centrifugeuse réfrigérée et est plus rapide que les autres méthodes actuellement disponibles.

	Solution	Dilution		Centrifugation		
		T (°C)	Temps (min)	Force (g)	T (°C)	Temps (min)
SSP	Sucrose-Percoll®	-	0	800	4 or 20	15
AD	Accudenz discontinu	-	0	300	5	25
CDS	Lake C	4	12	500	4	15

CONCLUSIONS

1. Nos données montrent l'efficacité de la SSP sur l'élimination du glycérol de la semence décongelée de coq avec sélection des spermatozoïdes mobiles.
2. L'utilisation de SSP 40% à 4 ou 20°C permet d'obtenir des résultats de fertilité au moins similaires voir meilleurs que la méthode de décongélation conventionnelle.
3. Notre méthode est plus économique en temps (gain de 40%) et en équipement.

Centre Val de Loire

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5. 26ème Journée Thésards de l'Unité PRC

26ème Journée Thésards de l'unité PRC INRAE Val de Loire – 15 et 16 Juin 2022

Exploring how glycerol impacts fertility capacity of chicken sperm

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INTRODUCTION

Glycerol is an excellent cryoprotectant for vertebrate sperm against cryodamages[1]. In chicken, 11% glycerol is the standard concentration for freezing sperm, but it needs to be eliminated before inseminating hens to avoid infertility[2]. This phenomenon has been discussed since 1950s[3], but the mechanisms involved in negative glycerol effects are still unknown. Here we hypothesized that glycerol disturb sperm biology before (during freezing and thawing processes) and after (in the female tract) insemination. Thus, we firstly confirmed the effect of increasing glycerol concentrations on fertility. Then we investigated how sperm biology was impacted by glycerol presence at 4 and 41°C within 60 min (time necessary for cryopreservation, thawing, insemination and progression in the female tract).

METHODS

1. Semen of 10 adult T44 roosters was collected, pooled, and diluted with Lake PC diluent combined to 0, 1, 2, 6 and 11% glycerol at 4°C.
2. For fertility test (n=2), 80 Isabrown hens were inseminated immediately after dilution with 100×10^6 pooled glycerolized sperm/female.
3. For *in vitro* tests (n=5), sperm membrane integrity, mitochondria activity, apoptosis, ROS balance and ATP content were assessed at 0, 30 and 60 min at 4 and 41°C with fluorescent probes and ATP-luciferase reaction kit by flow cytometry or luminometer.
4. Data of fertility and *in vitro* sperm biology were analyzed by Chi2 test and two-way ANOVA with Rstudio and GraphPad softwares, respectively. Significant difference was identified by different letters ($p<0.05$).

RESULT-1 Impacts of glycerol on fertility

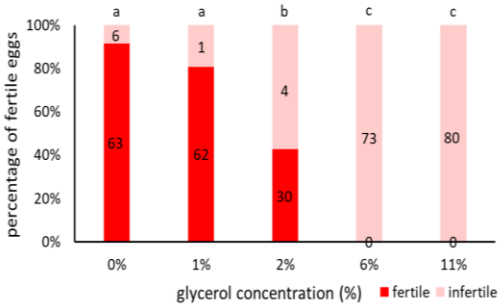

Figure 1. Effects of glycerol on hen fertility. Red bars represent the percentage of fertile eggs (%). The numbers on the bars represent the eggs numbers.

2% glycerol in semen caused more than 50% loss of fertility and 6% led to a complete infertility.

CONCLUSIONS and PERSPECTIVES

1. Glycerol causes distinguishable impacts on sperm motility and biology at 41°C, which might probably alter normal functions of sperm progression, selection and storage in the female tract, resulting in decreased fertility.
2. ROS balance and ATP content are two major factors regulating sperm motility. Our future *in vitro* studies will narrow down to verify their connections.
3. Further *in vivo* and *ex vivo* experiments will be applied to decipher the deleterious effects of glycerol during sperm progression or storage in the female tract.

RESULT-2 Impacts of glycerol on sperm biology

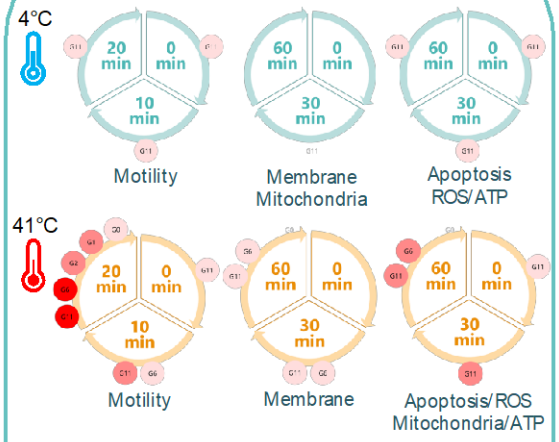

Figure 2. Effects of glycerol concentration on sperm biology. G0, 1, 2, 6, 11 represent glycerol 0, 1, 2, 6, 11%. ● ● ● represent mild, medium and severe impacts.

1. At 4°C, only 11% glycerol caused mild impacts on sperm biology.
2. At 41°C, glycerol severely impaired sperm motility in 10-20 min.
3. Glycerol induced sperm metabolism modifications mainly occurred when concentration higher than 6%.

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POSTERS

WEDNESDAY 29TH JUNE

W155
RECOMBINANT PORCINE JUNO BEADS SUPPORT SPECIES-SPECIFIC SPERM BINDING

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BACKGROUND-AIM
Juno protein is localized on the egg's membrane and it is essential in mammals fertilization due its binding to the sperm receptor IZUMO1. Knock out for some of these two proteins show absence of fertilization. So, we propose the use of beads coated with recombinant JUNO protein to bind those spermatozoa with the greatest capacity to fertilise.
METHODS
We used two plasmid vectors pcDNA3.1 to cloning the predicted sequence encoding JUNO gene with a 6-histidine tag added to the C-terminus, from Sus scrofa and Homo sapiens. Human Embryonic Kidney (HEK) 293T cells were transiently transfected with the plasmid vectors to produce the recombinant porcine and human JUNO protein (rpJUNO and rhJUNO). Secreted proteins were purified and conjugated to commercial magnetic beads (His Mag Sepharose Excel GE Healthcare) to generate the 3D models pJUNO-beads and hJUNO-beads. 3D models were co-incubated for 1, 2, 3 and 4 hours with fresh ejaculated boar semen from fertile males to assess the number of spermatozoa bound per bead and the number of bound spermatozoa reacted acrosome. Beads without protein (Ctrl-beads) were used as internal control for the assay. Beads were fixed in glutaraldehyde and stained with Hoechst and PNA-FITC. We studied a total of 605 beads and statistical analysis was performed by one-way ANOVA and when P-value was <0.05, differences among groups were analysed by a Tukey's.
RESULTS
Expression of proteins and its conjugation to commercial beads was confirmed by SDS-PAGE and Western-Blot. The greatest significant differences in the number of spermatozoa bound were observed at 4 hours (P= 0) with 12,58±1,38 for pJUNO-beads, 7,96±0,98 for hJUNO-beads and 5,69±0,61 for Ctrl-beads. The number of reacted spermatozoa at 4 hours was significative higher (P= 0) in pJUNO-beads with 5,68±0,67 while in human and control models had 3,43±0,39 and 2,64±0,23 respectively.
CONCLUSIONS
These preliminary data show that JUNO proteins were successfully expressed and secreted by HEK cells and conjugated to commercial beads. The generated pJUNO-beads model binds more reacted sperms than control and human models showing that porcine sperm specifically recognize porcine JUNO protein hence, indicating that the Juno-sperm binding is species-specific.
Supported: Fundación Séneca 20887/PI/18; Ministerio de Ciencia e Innovación PID2020-114109GB-I00

W156
IMPACT OF GLYCEROL ON SPERM FERTILIZING CAPACITY IN CHICKEN

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BACKGROUND-AIM
Glycerol is the most common cryoprotectant of vertebrate semen because it provides an excellent protection of sperm against cryodamages. In chicken, 11% glycerol is the standard concentration for freezing sperm, but it needs to be removed before inseminating hens, otherwise the fertility is dramatically decreased. This phenomenon has been discussed since 1950s, but the mechanisms involved remain unclear. Here we hypothesize that glycerol preserves sperm capacities at low temperature whereas interfere with sperm biology at physiological temperature (i.e. 41°C). Thus, we firstly investigated the effect of increasing glycerol concentrations on fertility. Secondly, we explored how sperm biology, especially sperm motility and membrane integrity, could be impaired by glycerol presence at 41°C within 60 min (physiological time of sperm evolving from vagina to infundibulum).
METHODS
Semen of 10 adult T44 roosters was collected, pooled, and diluted with glycerol-Lake PC diluent to final concentration of 0, 1, 2, 6 and 11% glycerol at 4°C. For fertility test (n=2), 80 Isabrown hens were inseminated immediately after dilution with 100×106 pooled glycerolized sperm/female. For in vitro tests (n=5), sperm mass and individual motilities were assessed at 0, 10, 20 and 30 min at 41°C by microscope and computer-assisted sperm analysis (CASA), respectively, and membrane integrity was evaluated at 0, 30 and 60 min by fluorescence flow cytometry. Data were analysed by ANOVA tests.
RESULTS
Results revealed that 2% glycerol led to 50% decline of fertility, and infertility was observed with 6 and 11% glycerol. No impact of glycerol on sperm mass motility was revealed but 1% glycerol significantly reduced sperm individual motility after 10 min, and more severe reduction was observed with higher concentrations. Moreover, whereas no impact was observed with 1 and 2% glycerol, 6 and 11% glycerol induced sperm membrane integrity failure after 30 min of incubation at 41°C.
CONCLUSIONS
Collectively, the presence of glycerol in semen samples at very low concentration impacted fertility and sperm motility, whereas higher concentrations are also associated with membrane defects, supporting the need to remove glycerol before insemination to stop the disturbances of sperm transportation and integrity in female tract.

Impact of glycerol on sperm fertilizing capacity in chicken

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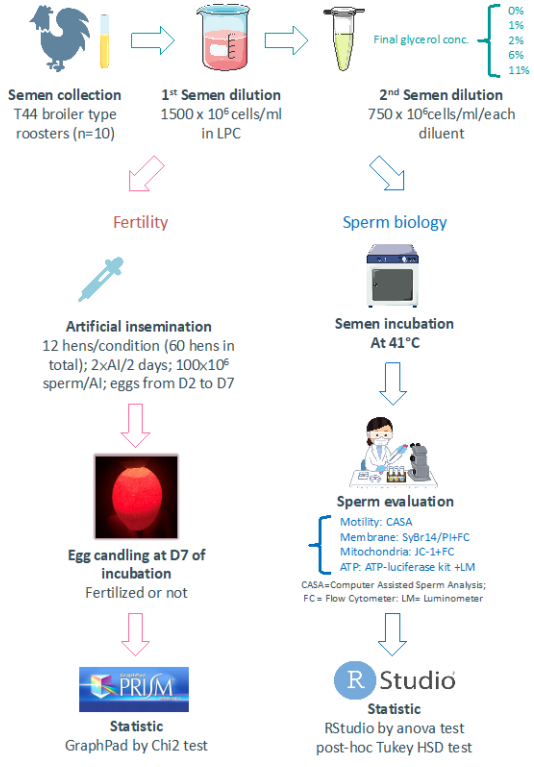
INTRODUCTION

Glycerol is the most common cryoprotectant used for vertebrates semen because it provides an excellent protection of sperm against cryodamages(1). In chicken, 11% glycerol is the standard concentration for freezing sperm, but it needs to be removed before inseminating hens, otherwise the fertility is dramatically decreased(2). This phenomenon has been discussed since 1950s(3), but the mechanisms involved remain unclear.

We hypothesize that the major impacts of glycerol on sperm biology occurs at physiological temperature of 41°C. To test this hypothesis, we firstly confirmed the effect of increasing glycerol concentrations in semen sample on fertility. Secondly, we explored how sperm biology could be impaired by glycerol presence at 41°C within 60 min (physiological time of sperm transit from vagina to fertilization site).

MATERIALS AND METHODS



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RESULT 1 Fertility

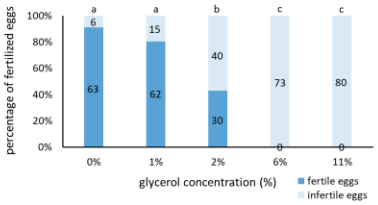

Figure 1. Effects of glycerol at different concentrations on hen fertility. Deep blue bars represent the percentage of fertilized eggs (%). The numbers of eggs are indicated on the bars.

2% glycerol in semen caused more than 50% loss of fertility, and 6% or higher glycerol led to a total infertility.

RESULT 2 Sperm biology

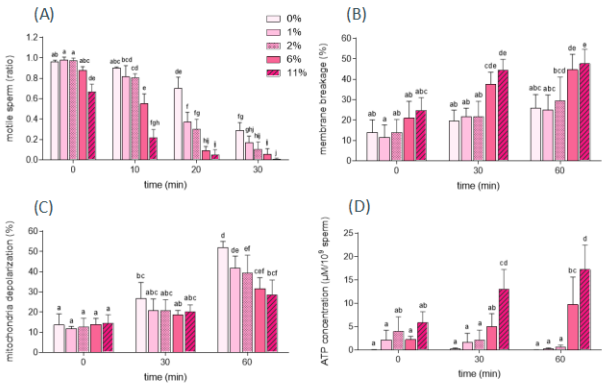

Figure 2. Effects of glycerol concentration on sperm (A) motility (B) membrane (C) mitochondria and (D) ATP content. The results correspond to the mean (n = 5) and the standard deviation of the mean. Different letters indicate significant differences (p-value < 0.05).

1. At 41°C, glycerol severely impaired sperm motility in 20 min.
2. Glycerol also induced modifications of sperm membrane, mitochondria and ATP which mainly occurred when concentration was higher than 6%, and increased with time.

CONCLUSIONS AND PERSPECTIVES

1. The decrease of fertility observed when the semen is in presence of glycerol at 41°C seems to be associated to effects on sperm motility, membrane integrity and energy production.
2. Our results suggest that glycerol might probably alter normal functions of sperm progression, selection and storage in the female tract.
3. ATP content is a major regulating factor for sperm motility. Further studies will be aimed at understanding the mechanisms of increasing ATP accumulation under glycerol treatment.
4. Further *in vivo* and *ex vivo* experiments of sperm storage tubule (SST) tissue will be applied to decipher the deleterious effects of glycerol during sperm progression or storage in the female tract.

26th World's Poultry Congress, abstracts selected in 2022

ID: 1939

APPLICATION OF COLLOIDAL CENTRIFUGATION TO REMOVE GLYCEROL FROM CHICKEN FROZEN-THAWED SEMEN

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**Presenting author*

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Glycerol is the most used cryoprotectant to freeze chicken sperm due to an excellent protection of sperm cells against cryodamage. However, the presence of glycerol in post-thawed semen causes severe fertility reduction, leading to the necessity of removing glycerol before insemination. The main approach developed to remove glycerol is serial dilution (SD) protocol, which implies special equipment to maintain the thawed semen at 4°C and is time consuming. Therefore, we developed a simple method to remove glycerol from chicken frozen-thawed semen based on centrifugation in a colloidal gel, Percoll®, which is ordinarily used to select motile sperm in mammals as well as in fresh chicken semen. Eighteen adult T44 roosters were randomly divided between three groups and used as semen donors. Semen was collected from each group and frozen in Lake PC diluent containing 11% glycerol in straws in a programmable freezer. Frozen semen was thawed at 4°C for 3 min, layered on PBS solution containing 200 mM sucrose and isotonic Percoll® (Sucrose-Percoll® Solution, SPS) and centrifuged at 20°C, 800×g for 15 min. Different concentrations of SPS in PBS (40, 50, 60 or 70%) were tested to identify the best conditions by performing in vitro tests including glycerol concentration assay and sperm individual motility evaluation, as indicators of in vivo fertility. Artificial inseminations were performed on 36 hens with frozen-thawed semen treated by SPS and by SD protocols to compare their efficiencies. Data of in vitro and fertility tests were analysed by two-way ANOVA and Chi2 tests. Our results indicated that all concentrations of SPS efficiently decreased glycerol concentration to less than 120 mM while the concentration was 1866.3 mM before treatment. A beneficial selection of motile and progressive sperm was achieved by all concentrations of SPS, especially SPS 70% showed the best improvement of 25,1% and 15,2%. SPS 40 and 70% were two conditions selected for insemination test and reached 45.8% and 21.6% of fertility respectively, compared to 49.1% after SD treatment. Collectively, our new colloidal method can efficiently remove glycerol from chicken frozen-thawed semen with a positive selection of sperm motility. SPS 40% obtained better fertility than SPS 70% but was not significantly different from SD. Compared to the conventional SD protocol, this method can save 44% of the time while achieving similar fertility.

Session 6: 4:15pm March 9th

Impacts of glycerol on sperm fertilizing ability in chickens

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Abstract:

Glycerol is the most efficient cryoprotectant to freeze chicken sperm, but it is associated with fertilization failures, requiring its removal before insemination. The cellular mechanisms underlying these negative effects on fertility remain unknown. Here, we firstly investigated the impacts of glycerol (0,1, 2, 6 and 11%) on sperm biology within 60 min of incubation at two temperatures to mimic freezing procedures (4°C) and physiological temperature (41°C). After collection, semen of 10 roosters was pooled, incubated with different doses of glycerol and sperm motility, membrane integrity, apoptosis, mitochondria activity and ATP production were assessed. Secondly, we explored how glycerol modulates sperm storage in oviduct. Nine hens were inseminated with Hoechst stained sperm (200×106 sperm/female), slaughtered after 24 hours, and the presence of sperm was explored in sperm storage tubules (SST) at uterovaginal junction. 11% glycerol is the only concentration interfering with sperm biology at 4°C whereas 6 and 11% glycerol significantly impair sperm characteristics at 41°C, especially in terms of motility. Furthermore, % of SST containing sperm decreased with increasing glycerol concentrations. Collectively, our data revealed important impacts of glycerol on sperm motility, potentially responsible of the reduction of sperm storage in SST observed in vivo after insemination, explaining reduced fertility.

Keywords: chicken, sperm, glycerol l

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