POST-THAW SPERM ISOLATION EFFICIENCY USING A CENTRIFUGE-FREE DEVICE (MIGLIS) IN ANIMAL MODELS

動物モデルにおける融解後精子選別に対する遠心分離不要型デバイス（MIGLIS）の有効性

Joint Graduate School of Veterinary Medicine

Yamaguchi University

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We hereby recommended that the thesis prepared under supervision by Nguyen Thi Suong, entitled "Post-thaw sperm isolation efficiency using a centrifuge-free device (MIGLIS) in animal models" should be accepted as fulfilling in part for the degree of Doctor of Philosophy.

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ABSTRACT

Sperm function is known to decline substantially following cryopreservation and thawing, despite efforts to optimize freezing protocols. Accordingly, post-thaw sperm selection approaches are being refined to enhance the elimination of damaged sperm subpopulations and undesirable by-products, thereby ensuring that only functionally competent sperm are utilized in fertilization programs.

In this study, we evaluated for the first time a centrifugation-free sperm selection approach using the MIGLIS device, which operates based on migration-gravity sedimentation. Given the well-recognized interspecies differences in sperm structure and physiology, which influence susceptibility to cryo-induced damage, the efficiency of sperm selection may vary between species and thus requires species-specific evaluation. Therefore, frozen-thawed bovine and porcine sperm, representing models with distinct cryotolerance profiles, were used to systematically assess the effectiveness of this approach.

Chapter 1 investigated the efficacy of bovine sperm selection using the MIGLIS device and compared its performance with conventional centrifugation-based techniques, including simple washing and Percoll density gradient centrifugation. An initial experiment was conducted to determine the optimal sperm dilution ratio for efficient application of the MIGLIS system. Thereafter, selection efficiency was evaluated with respect to overall sperm functional quality, post-processing reactive oxygen species (ROS) generation and subsequent in vitro blastocyst development. Although the MIGLIS system yielded a lower sperm recovery rate, it selectively enriched a subpopulation with higher functional competence. Notably, intracellular ROS levels were significantly reduced in both MIGLIS-selected spermatozoa and the resulting early-stage blastocysts. No significant differences were observed in blastocyst formation rates among the selection methods. Overall, these results demonstrate that MIGLIS improves sperm functional integrity by limiting oxidative stress, supporting its use as a less invasive alternative to centrifugation-based sperm selection strategies.

In **Chapter 2**, we further clarified the functional role of the MIGLIS device in bovine sperm selection when samples exhibit comparable conventional quality parameters but result in markedly different reproductive outcomes, as observed between the high-fertility (HF) and low-fertility (LF) sperm groups. Although standard laboratory-based semen evaluation showed no differences between groups in motility, viability, or acrosome integrity, clear

disparities emerged at the functional level, with the HF group exhibiting higher cleavage and blastocyst formation rates. This functional advantage was accompanied by elevated mitochondrial activity during early embryogenesis, while ROS levels remained unchanged. Notably, sperm DNA fragmentation undetected by routine assessments, was identified as the key factor underlying reduced fertilizing capacity. DNA fragmentation was also significantly correlated with the plasma membrane integrity index, suggesting a close association between chromatin stability and membrane functionality. Importantly, MIGLIS selection effectively reduced DNA fragmentation in both groups and partially rescued developmental outcomes in the LF group, highlighting its potential to target hidden sperm defects and improve the efficiency of cryopreserved semen in assisted reproduction.

Chapter 3 extended the investigation to porcine sperm model, with the objective of evaluating and optimizing MIGLIS-based selection for frozen-thawed porcine semen. Given the higher susceptibility of porcine spermatozoa to cryo-induced stress and their shorter post-thaw functional lifespan compared to bovine sperm, protocol refinement was essential to ensure effective selection while minimizing additional damage. To address this, caffeine supplementation was introduced to support sperm function during separation, and incubation time within the MIGLIS system was systematically optimized. The combination of 2.5 mM caffeine and a 10 min incubation period provided the best balance between sperm recovery and functional quality. Under these optimized conditions, MIGLIS-selected spermatozoa achieved significantly improved embryo development rates, indicating that moderate caffeine supplementation enhances sperm resilience during MIGLIS-based selection and improves subsequent developmental competence.

Overall conclusion: Findings from both bovine and porcine models suggest that MIGLIS has the potential to serve as an effective platform for post-thaw sperm selection. With species-specific optimization, it can enhance functional sperm quality and support embryonic development, particularly in sperm populations exhibiting lower overall fertilization efficiency.

Limitations and future perspectives: Despite promising results, this study relied mainly on in vitro assessments, and sperm recovery rates were relatively low. Future research should focus on validating outcomes through in vivo fertility trials.

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LIST OF ABBREVIATIONS

a.u.	Arbitrary units
BO	Brackett-Oliphant
BSA	Bovine serum albumin
CASA	Computer-assisted sperm analysis system
COCs	Cumulus-oocyte complexes
DCF	Dichlorofluorescein
DCFH-DA	Dichlorodihydrofluorescein Diacetate
DFI	DNA fragmentation index
DGC	Density gradient centrifugation
DNA	Deoxyribonucleic acid
FITC-PNA	Fluorescein isothiocyanate-labelled peanut agglutinin
HF	High fertility
HOST	Hypo-osmotic swelling test
IVF	In vitro fertilization
IVM	In vitro maturation
IVP	Vitro embryo production
L/D	Live/Dead
LF	Low fertility
MS	Migration-sedimentation
PBM	Porcine blastocyst medium
PBS	Phosphate Buffered Saline
PFM	Porcine fertilization medium
PI	Propidium iodide
PVA	Polyvinyl alcohol
PZM	Porcine zygote medium
ROS	Reactive oxygen species
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling assay

GENERAL INTRODUCTION

Sperm cryopreservation has become an indispensable tool in both commercial livestock production and reproductive research, as it enables rapid global dissemination of elite genetics while reducing biosecurity risks. It facilitates timely artificial insemination, large-scale genetic improvement, and standardized assisted reproductive technologies. Although cryopreservation is designed to slow metabolic activity, extend sperm lifespan, and maintain functional competence, the freezing-thawing process inevitably subjects spermatozoa to osmotic, thermal, and biochemical stress, which can impair their performance [1]. Freezing promotes ice crystal formation and extracellular hyperosmotic conditions, resulting in cellular dehydration and shrinkage [2]. Upon thawing, a transient hypoosmotic environment leading to irreversible plasma membrane disruption. Phase transitions in membrane lipids increase permeability and disturb ion homeostasis, particularly Ca^{2+} and bicarbonate influx, thereby compromising metabolic regulation [3-6]. Structural alterations, including changes in disulfide cross-linking, may impair DNA integrity, while damage to the perinuclear theca, F-actin localization, and mitochondrial function further undermines sperm stability [3, 5, 7]. Moreover, excessive generation of ROS during and after cryopreservation exacerbates lipid peroxidation and DNA fragmentation [8]. Collectively, these cryo-induced injuries impair post-thaw sperm quality, capacity for capacitation, and fertilization potential.

As a result, post-thaw semen represents a heterogeneous population in which functionally competent sperm coexist with cells exhibiting sublethal or irreversible damage. Although increasing sperm numbers per dose is commonly used to compensate for these losses, this strategy may inadvertently elevate the proportion of damaged cells, which can negatively affect viable sperm through the release of harmful factors and lead to unnecessary sperm wastage [9]. Therefore, the development of effective sperm selection strategies is critical to improve the functional quality of post-thaw samples.

Conventional techniques, including swim-up, washing, and density gradient centrifugation, primarily rely on sperm motility and centrifugation-based separation. More recent approaches such as thermotaxis-based systems and magnetic-activated cell sorting have attempted to incorporate functional or apoptotic markers into selection processes [10-12]. In parallel, advanced technologies, including flow cytometry, microfluidic platforms, electrophoresis, hyaluronic acid-binding assay, and Raman spectroscopy and artificial intelligence have shown promise for high-precision sperm selection and sex-sorting

applications [13-16]. However, centrifugation-based systems depend on high gravity forces that may exacerbate membrane destabilization in cryo-compromised sperm, and even highly motile spermatozoa may still exhibit elevated levels of DNA fragmentation [17, 18]. Although advanced platforms offer improved accuracy, they are often technically complex, costly, and less suitable for routine application in livestock production systems.

In this context, the MIGLIS® device has recently been introduced as a novel, label-free sperm selection platform based on migration-gravity sedimentation principles. By selecting sperm according to their sustained migration capacity under low mechanical stress, MIGLIS® avoids centrifugation-associated damage and has shown promising improvements in sperm quality and embryo development in human studies [19].

However, its application in livestock species remains largely unexplored, particularly in bovine and porcine, which exhibit marked differences in cryo-sensitivity. Bovine sperm are generally more resilient to cryo-induced injury, whereas porcine sperm are more susceptible to oxidative stress, membrane disruption, and reduced post-thaw longevity. These differences are primarily attributed to species-specific variations in membrane lipid composition and chromatin organization [20]. As a result, post-thaw sperm quality differs markedly between species. While both show substantial declines in motility and viability (~50% in bovine and up to 60% in porcine), porcine sperm exhibit greater membrane, acrosomal, and mitochondrial damage. In contrast, bovine sperm tend to show higher DNA fragmentation (~9%), whereas porcine sperm display minimal fragmentation (~1%) but increased chromatin decondensation (~10%) [3, 21, 22]. Therefore, these interspecies differences underscore the need for species-specific optimization of sperm selection strategies, particularly when applying MIGLIS®, to ensure its effectiveness across different livestock species.

Overall objective

The overall objective of this study was to evaluate the efficiency and biological relevance of post-thaw sperm isolation using migration-gravity sedimentation principles through application of the MIGLIS® device in bovine and porcine sperm models.

In the bovine model, **Chapter 1** aimed to evaluate the efficiency of sperm selection using the MIGLIS® device and to compare its performance with conventional centrifugation-based methods. **Chapter 2** aimed to elucidate the hidden factors underlying differences in reproductive performance between sperm populations with comparable conventional semen

parameters, with particular emphasis on evaluating whether MIGLIS®-based selection can effectively mitigate these latent defects by enriching a functionally superior sperm subpopulation.

In the porcine model (**Chapter 3**), we aimed to optimize MIGLIS®-based sperm selection for frozen-thawed semen by defining optimal caffeine supplementation and incubation time to maximize recovery while preserving functional integrity.

CHAPTER 1

Selection of spermatozoa with high motility and quality from bovine frozen-thawed semen using the centrifuge-free device

ABSTRACT

This study aimed to assess the potential of the centrifuge-free commercial device (MIGLIS®) in selecting functional frozen-thawed bovine sperm by migration-gravity sedimentation, its effect on embryo development, and compare the potential with that of centrifugation-based techniques, including washing and Percoll® density gradient centrifugation (DGC). In experiment 1, different dilutions (1.5×, 2×, and 3×) of frozen-thawed spermatozoa were assessed to identify the adequate one for the MIGLIS® method. In experiment 2, the recovery rates, quality, and reactive oxygen species (ROS) concentrations of the spermatozoa selected using MIGLIS®, washing, and Percoll® DGC were compared. In experiment 3, the result in vitro fertilized embryos from spermatozoa selected using the three methods were evaluated for blastocyst formation rates and intracellular ROS concentrations at the 2-4 cell stage. Using the MIGLIS® device, significantly more spermatozoa were recovered at 2× dilution compared with the other dilution ratio, while motility was not affected by the dilution ratio. Compared with centrifugation-based methods, MIGLIS® resulted in lower sperm recovery rates; however, spermatozoa selected by MIGLIS® exhibited significantly higher motility, viability, and acrosome integrity. ROS concentration of spermatozoa in the MIGLIS® method was significantly lower than that in the washing method. Although blastocyst formation rates did not differ among the three methods, embryos derived from MIGLIS®-selected spermatozoa showed significantly lower intracellular ROS levels at the early cleavage stage compared with those produced using Percoll® DGC. In conclusion, the MIGLIS® method has the potential to select functional, high-quality frozen-thawed bovine spermatozoa.

Keywords: *bovine; IVF; migration-sedimentation; ROS; spermatozoa.*

1. Introduction

The number of embryos produced each year is significantly increasing due to the current demand for in vitro embryo production (IVP) in cattle and assisted reproductive technology implementation. However, the results vary considerably owing to the impact of many unknown or known factors. Sperm quality is a crucial factor that strongly influences the outcomes in IVP. Sperm from different bulls, or belonging to different batches from the same bull, also affect embryonic development [23]. Although the use of frozen semen in IVP offers some advantages, the recovery of sperm quality after thawing is reduced appreciably. This has driven scientists to develop several advanced techniques for selecting high-quality sperm for IVP. The desired sperm selection methods require the removal of dead or nonfunctional sperm, debris, leukocytes, bacteria, cryoprotective agents, and other undesired substances but without detrimental effects on sperm function [24].

Currently, centrifugation-based methods such as washing and density gradient centrifugation (DGC) for sperm preparation are widely used in both humans and mammals. These conventional methods remove extraneous material but also distinguish various cell types and motile spermatozoa [25]. Recently, it has been reported that spermatozoa selected by single-layer centrifugation had a higher production of superoxide but significantly reduced hydrogen peroxide production [26]. However, a drawback of conventional methods is that using centrifugation induces ROS production [27]. In general, ROS play crucial roles in motility, fertilization capacity acquisition, acrosome reactions, and interactions with oocytes [28]. ROS are produced by white blood cells in semen and sperm cells during freezing and thawing [29]. Since all metabolizing cells produce ROS, it would assume that ROS production may be linked to the metabolic activity of the spermatozoa. However, excessive physiological ROS levels cause oxidative stress, limiting the antioxidant capacity of cells and causing DNA damage, resulting in reduced sperm function [30, 31]. Furthermore, the cell membrane of sperm, which is composed of polyunsaturated fatty acids and has few scavenging enzymes that can neutralize ROS, is particularly susceptible to ROS [32]. In addition, DGC methods using discontinuous silica-based gradient media such as Percoll® have been associated with increased sperm DNA damage, which cannot be fully attributed to ROS generation during centrifugation alone. The presence of metal components in this medium may promote reactive oxygen species formation near DNA, thereby adversely affecting fertilization and embryonic development [33, 34].

Recently, an alternative method for semen preparation without centrifugation was proposed to reduce sperm damage [35, 36]. Migration-sedimentation (MS) of spermatozoa settles motile sperm to the bottom of the tube without centrifugation, owing to sperm motility and gravity [36]. As a commercial device, MIGLIS® (Menicon Life Science Co., Ltd., Aichi, Japan) is available for sperm preparation based on the MS principle and consists of three parts: an outer lid, an inner lid, and a tube. When the medium is layered on top of the sperm sample dispensed outside the central tube, the motile sperm swims in the medium, crosses the top of the tube, and drops to the bottom of the tube by gravity, where they aggregate. In contrast, dead or less motile sperm remains outside the central tube. Human spermatozoa selected using MIGLIS® were less damaged than those selected by centrifugation-based methods [19, 35]. However, the efficiency of MS for selecting cryopreserved bovine semen has not yet been reported.

Therefore, we first established the optimal conditions for using MIGLIS® in frozen-thawed bovine semen. Second, we compared the selection efficiency and intracellular ROS concentrations of spermatozoa selected using MIGLIS® and centrifugation-based methods (washing and Percoll® DGC). Moreover, we evaluated the development and intracellular ROS concentrations of embryos fertilized with spermatozoa selected using MIGLIS® and centrifugation-based methods.

2. Materials and methods

2.1. Experimental design

Experiment 1: Sperm dilution for selection by MIGLIS® method

To evaluate the adequate dilution of spermatozoa for selection using MIGLIS® device, frozen-thawed spermatozoa were diluted with Brackett-Oliphant (BO) medium [37] supplemented with 0.35% of bovine serum albumin (BSA, Sigma Aldrich Japan, Tokyo, Japan) (BO+BSA solution). They were then divided into four groups: undiluted, 1.5× dilution, 2× dilution, and 3× dilution. Spermatozoa from each dilution group were selected using the MIGLIS® device, and the concentration and motility of the selected spermatozoa were evaluated as described below. The sperm recovery rate was calculated by dividing the number of sperm recovered after selection by the number of sperm in the original frozen-thawed semen. Frozen-thawed spermatozoa from one semen straw were divided into four groups, and each experiment was performed five times.

Experiment 2: Quality of selected spermatozoa

To evaluate the quality of selected spermatozoa using the three different methods, the recovery rate, motility, viability, plasma membrane integrity, acrosomal integrity, and intracellular ROS concentrations of spermatozoa after selection by the MIGLIS®, Percoll® DGC, or washing methods were assessed as described below. In the MIGLIS® group, spermatozoa were diluted 2-fold (based on the results of Experiment 1) and selected using the MIGLIS® device. Frozen-thawed spermatozoa from one semen straw were divided into groups, and sperm quality and intracellular ROS concentrations were assessed nine and three times, respectively.

Experiment 3: Development and intracellular ROS concentrations of embryos fertilized with selected spermatozoa

In-vitro fertilization was performed using spermatozoa selected by the MIGLIS®, Percoll® DGC, or washing methods as described below. The blastocyst formation rates of embryos after culturing for 8 days and intracellular ROS concentrations of embryos at the 2–4 cell stage in each group were evaluated. Frozen-thawed spermatozoa from one semen straw were divided into groups, and each IVF experiment was performed three times. The washing method was used as a batch control because the washing method was usually used for sperm preparation for IVF in our IVF procedure.

2.2. Sperm preparation

Commercial cryopreserved semen straws from the same batch of a Japanese Black bull provided by the Yamaguchi Prefectural Agriculture Technical Center, Department of Livestock Technology (Yamaguchi, Japan) were used for all experiments. One cryopreserved semen straw (0.5 mL) was immediately thawed in warm water at 37 °C for 30 s for each experiment. Frozen-thawed spermatozoa were selected using the three methods described below.

2.2.1. Sperm preparation by MIGLIS® method

The MIGLIS® device consists of a small central tube inside a cylindrical container (Fig. 1.1). Frozen-thawed semen was injected outside the central tube as described by Tatsumi *et al*. [35]. An inner lid was placed, and the BO+BSA solution was gently poured from the bottom of the central tube to cover the semen with a total volume of 3.7 mL. Subsequently, the outer lid was covered to prevent evaporation of liquid, and the device was left in the dark

at 37 °C for 1 h to allow the swimming sperm to fall to the bottom of the central tube by gravity. Finally, sperm suspensions (400 µL per sample) collected from the bottom of the central tube were used for the experiments.

2.2.2. Sperm preparation by washing method

Frozen-thawed semen was transferred to a sterile tube containing 3 mL BO+BSA solution and centrifuged at 540 × *g* for 5 min. The supernatant was discarded, and the resulting pellet was resuspended in 3 mL of BO+BSA solution and centrifuged again under identical conditions. After removing the supernatant, 400 µL of BO+BSA solution was added, and the resultant sperm suspensions (400 µL per sample) were used for the experiments.

2.2.3. Sperm preparation by Percoll® density gradient centrifugation (DGC) method

The Percoll® solution consisted of 90% (v/v) Percoll (Cytiva, Tokyo, Japan), 1.97 mM calcium chloride solution, 0.39 mM magnesium chloride solution, 0.37% (v/v) sodium DL-lactate, 25 mM sodium bicarbonate, 0.01% (v/v) phenol red, and 10% (v/v) sperm TL solution. The sperm TL solution consisted of 0.8 M sodium chloride, 0.03 M potassium chloride, 3 mM sodium dihydrogen phosphate, and 0.1 M HEPES. Frozen-thawed semen was layered onto 3 mL 90% Percoll® solution in a sterile tube and centrifuged at 740 × *g* for 10 min. The supernatant was discarded, and the resulting pellet was suspended in 3 mL of the BO+BSA solution and centrifuged at 540 × *g* for 5 min. After removing the supernatant, 400 µL of BO+BSA solution was added, and the resultant sperm suspensions (400 µL per sample) were used for the experiments.

2.3. Sperm quality assessments

Motility analyses were performed using a computer-assisted sperm analysis system with a Sperm Class Analyzer® (SCA® v.4.2) device (MICROPTIC, Barcelona, Spain). Motility was analyzed based on the examination of 25 consecutive digitized images obtained from three to five fields using a 10× phase-contrast objective, and at least 500 spermatozoa per sample were analyzed. An aliquot (5 µL) of sperm suspension was loaded into a pre-warmed Makler® chamber and placed on a warm plate at 37 °C. The analysis time was 1 s per field, and images were taken with a time-lapse of 1 s. Therefore, the image capture speed was one every 40 msec. After acquiring the representative fields, total (curvilinear velocity >10 µm/s) and progressive motility (curvilinear velocity >100 µm/s) were recorded.

Sperm viability was assessed using a live/dead stain combination (SYBR-14/propidium iodide [PI], LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA) according to the manufacturer's protocol with minor modifications [38]. Briefly, an aliquot of semen (5 µL) was mixed with 50 µL of a solution containing 3 µL of PI (diluted 1:10 in distilled water) and 3 µL of SYBR-14 (diluted 1:500 in DMSO) and incubated according to the manufacturer's instructions. One hundred spermatozoa were assessed in each of two duplicate aliquots of each sample and were evaluated using a fluorescence microscope (200 ×, Nikon Eclipse 80i; Nikon Corp.) with a 488-nm filter. The live sperm nuclei were stained with the SYBR-14. They fluoresced bright green, whereas the dead sperm nuclei exhibited red (PI) fluorescence.

The plasma membrane integrity of the sperm was assessed using the hypo-osmotic swelling test [39]. Briefly, an aliquot of semen (20 µL) was mixed with 80 µL of hypo-osmotic solution (150 mOsm/kg) containing 13.5 mg/mL D-fructose and 7.35 mg/mL trisodium citrate dehydrate in distilled water. The samples were incubated at 37 °C for 10 min. Then 10 µL of each sample was placed on a slide and overlaid with a coverslip. One hundred spermatozoa in three fields from one aliquot of each sample were assessed using a phase-contrast microscope (400×, Nikon TE300; Nikon Corp.). The plasma membrane integrity of the sperm was expressed as the percentage of sperm with curled tails (intact plasma membrane) out of the total number of spermatozoa.

The acrosomal integrity of the sperm was measured using fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA). The samples were spread on slides, air-dried at room temperature, and fixed with absolute ethanol for 10 min at room temperature. After drying, the slides were spread with 30 µL of FITC-PNA (100 µg/mL) in PBS and incubated in a dark, moist chamber for 30 min at 37 °C. The slides were then rinsed with PBS, air-dried, and overlaid with coverslip. The acrosomal status (intact or reacted) was determined from the FITC-PNA staining pattern observed using fluorescence microscopy (400×, Nikon Eclipse 80i; Nikon Corp.) with a 488-nm filter [40]. In all, 100 spermatozoa were counted over at least three different fields. The acrosomal integrity was expressed as the mean percentage of sperm with intact acrosomes.

2.4. Quantification of sperm intracellular ROS level

Intracellular ROS concentrations were measured using flow cytometry-based 2',7'-Dichlorodihydrofluorescein Diacetate (DCFH-DA) staining (Dojin Chemical, Kumamoto,

Japan), according to the methods described by Kiani-Esfahani *et al*. [41]. The DCFH-DA is a non-fluorescent probe that can pass through the cell membrane. In cells, DCFH-DA is deacetylated by intracellular esterases and converted to DCFH. It reacts with intracellular ROS to produce 2,7-dichlorofluorescein (DCF). The fluorescence intensity of DCF is proportional to the intracellular ROS concentrations. Approximately 200 μL of selected sperm adjusted to a final concentration of 3.0×10^6 sperm/mL was incubated with 1 mmol/L DCFH-DA at 37 °C for 30 min, and 3 μL PI was added for double staining. The samples were then analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Tokyo, Japan) in which a blue laser provided excitation at 488 nm. DCF was detected using a 525/40 BP filter, and PI was detected using a 690/50 BP filter. Data collection and preparation for analysis were performed using the CytExpert 2.3 software (Beckman Coulter). The gating strategies and analytical processes were performed, according to the methods described by Sharma *et al.* [42]. Only sperm of the expected size were selected using forward scatter versus side scatter (Fig. 1.2A). Sperm aggregation was eliminated in the P1 population (Fig. 1.2B). The P2 population (10,000 spermatozoa) was counted. The PI-negative events were selected from the P2 population (Fig. 1.2C). The FITC-fluorescence intensity of DCF in the P3 population (Fig. 1.2D) was used to determine the sperm intracellular ROS concentrations.

2.5. In vitro embryo production

Ovaries were obtained from the slaughterhouse and transported to the laboratory in physiological saline supplemented with 100 IU/mL penicillin (Meiji Seika Pharma Co., Ltd. Tokyo, Japan) and 0.1 mg/mL streptomycin (Meiji Seika Pharma Co., Ltd.) at 20–25 ℃. Cumulus-oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass were collected from follicles that were 2–8 mm in diameter. The COCs were then transferred into a maturation medium (BO-IVM; IVF Bioscience, Falmouth, UK) in four-well dishes (Nunc, Roskilde, Denmark) and incubated for 22 h at 38.5 ℃ in a humidified incubator containing 5% CO_2 in the air.

Frozen-thawed spermatozoa selected by the three methods described above were diluted to a final concentration of 1.0×10^6 sperm/mL with IVF medium (BO-IVF; IVF Bioscience, Falmouth, Great Britain). After in vitro maturation, the COCs were randomly divided into three groups and co-incubated with spermatozoa selected by each method for 20 h at 38.5 °C in a humidified incubator containing 5% CO_2 in air. Each IVF experiment was performed four times.

After IVF, cumulus cells surrounding the presumptive zygotes were removed by mechanical pipetting. The zygotes were transferred to a culture medium (BO-IVC, IVF Bioscience, Falmouth, Great Britain) covered with mineral oil in a four-well dish and incubated for 8 days at 38.5 °C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. The blastocyst formation was evaluated 8 days after IVF.

2.6. Quantification of intracellular ROS in early embryos

Embryos at the 2-4 cell stage were stained with DCFH-DA to assess intracellular ROS concentrations according to the methods described by Javvaji *et al.* [43]. Briefly, 2–4 cell stage embryos (seven embryos per group) were collected 42 h after the start of IVF. Embryos were washed with PBS and incubated in PBS supplemented with 10 µM DCFH-DA for 30 min at 37 °C in the dark. After incubation, embryos were gently washed with PBS, transferred onto slides with 20 µL of PBS, and observed under a confocal fluorescence microscope using an FITC filter (excitation: 485 nm; emission: 535 nm). The fluorescence intensity of DCF was analyzed using image analysis software (ImageJ), according to the methods described by Jensen *et al.* [44]. After opening the image, the fluorescence intensity was measured by selecting the required area and a homogeneous background for each area to compensate for the fluorescence intensity.

2.7. Statistical analysis

The results of sperm recovery, motility, sperm quality, IVF, and ROS concentration were evaluated by a one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). The values expressed in percentage for sperm recovery rate, sperm quality, and blastocyst formation rate were subjected to arcsine transformation before statistical analysis. Data was presented as a mean value ± standard error (SE). The differences with a probability value of $P \leq 0.05$ were considered statistically significant.

3. Results

3.1. Experiment 1: Sperm dilution for selection by MIGLIS® method

As shown in Fig. 1.3, the recovery rate of spermatozoa was significantly higher ($p < 0.05$) in the two-fold dilution group than in the other groups. However, there were no significant differences in progressive motility or total motility irrespective of the sperm dilution ratio.

3.2. Experiment 2: Quality of selected spermatozoa

As shown in Table 1.1, sperm recovery rates were significantly lower ($p < 0.05$) with the MIGLIS® and Percoll® DGC methods than with the washing method. The rates of motility, viability, and acrosomal integrity of the sperms selected using the MIGLIS® method were significantly higher ($p < 0.05$) than those of the sperms selected using the other methods. Moreover, the DCF fluorescence intensity of sperm in the MIGLIS® method was significantly lower than that in the washing method ($p < 0.05$).

3.3. Experiment 3: Development and intracellular ROS concentrations of embryos fertilized with selected spermatozoa

As shown in Fig. 1.4, the blastocyst formation rates were similar among the embryos developed from the sperms selected using the three methods. However, the DCF fluorescence intensity of early-stage embryos produced using the MIGLIS® method was significantly lower ($p < 0.05$) than that of embryos produced using the Percoll® DGC method (40.3 a.u. vs. 65.3 a.u.).

4. Discussion

The selection of high-quality spermatozoa during sperm preparation is an important factor in IVF embryo production. The present study showed that, despite a lower recovery rate, the MIGLIS® method was superior to centrifugation-based methods in terms of the quality and ROS concentrations of selected spermatozoa.

Accurate determination of the semen dilution ratio is necessary because highly viscous cryoprotectants such as glycerol and Tris-egg yolk may affect sperm motility and gravity after thawing. Therefore, in the first experiment, we investigated the optimal sperm dilution concentration for the MIGLIS® method. We found that the two-fold diluted sperm exhibited a higher recovery rate after selection using the MIGLIS® method than the undiluted sperm and the sperm diluted to other ratios. This supports the fact that as cryoprotectants inhibit the progressive movement of sperm, viscosity reduction by appropriate dilution increases the sperm recovery rate [45]. However, we observed that progressive and total motility values did not differ, irrespective of the sperm dilution ratio. These results could be explained by the fact that only vigorous spermatozoa collected from the inner area were able to swim over the barrier subject to the inner area. Inactive spermatozoa that could not swim up or over the barrier were presumed to have been excluded.

Washing is a simple, quick, and cost-effective process; however, it requires centrifugal force and does not completely separate debris and undesirable components from sperm. Immature and damaged sperm, such as those with abnormalities in the head or plasma membranes, produce more ROS than mature sperms [46]. The Percoll® DGC method may partially resolve the limitations of the washing method and exclude undesired cells. Although optimized formulations and protocols, such as single-layer centrifugation, may not cause excess ROS production, the centrifugation-based method is a double-edged sword when it is a source of excess ROS production. When oxidative stress exceeds physiological thresholds, it impacts sperm function and the functioning of proteins, lipids, and nucleic acids detrimentally, leading to sperm death [31]. The MIGLIS® method, a sperm selection method based on motility, creates a sediment owing to gravity at the bottom of the tube where centrifugation is unnecessary. Thus, using the MIGLIS® method helps to avoid these deleterious effects, which have been attributed to centrifugation [35, 36]. In the present study, we observed that the ROS concentrations of spermatozoa selected using the MIGLIS® method were significantly lower than those of spermatozoa selected using the washing method, albeit with lower recovery rates as well. Moreover, the motility, viability, and acrosome integrity of spermatozoa selected by the MIGLIS® method were higher to those of the spermatozoa selected by the centrifugation-based methods including washing and Percoll® DGC. These results indicate that MIGLIS® method without centrifugation would not only decrease the ROS concentrations of the selected spermatozoa but would also recover spermatozoa with high motility and quality.

Oxidative stress in sperm affects fertilization by damaging sperm integrity, epigenetic reprogramming, and paternal genome transfer, subsequently affecting embryo quality [47]. In the present study, we observed that the concentrations of intracellular ROS in early-stage embryos generated from spermatozoa selected using the MIGLIS® method were lower than those in the embryos selected using the Percoll® DGC method; however, the blastocyst formation rates were similar when using the two methods. These results indicate that there is no relationship between oxidative stress in early-stage embryos and blastocyst outcomes. A previous study demonstrated no relationship between sperm oxidative stress and in vitro fertilization or blastocyst outcomes in humans [48]. The effect of pre-fertilization oxidative stress in sperm on embryonic development has been suggested to be ROS substance-dependent [33]. Moreover, this may be related to the self-repair mechanism by inhibiting wild-type p53-induced phosphatase 1 activity in the oocyte/zygote, during which the

oocyte/zygote repairs itself in response to sperm damage and restores embryo quality [49, 50]. Conversely, this ability may not be effective if oxidative DNA damage exceeds the repair threshold and adversely affects embryonic development [51]. Since our results did not show any effect of ROS concentrations in pre-fertilization sperm or early-stage embryos on blastocyst formation, the self-repair mechanism in the oocyte/zygote should be investigated in subsequent studies.

In conclusion, the spermatozoa selected using the MIGLIS® method maintained higher quality and lower ROS concentrations than those selected using centrifugation-based methods. Moreover, early-stage embryos derived from spermatozoa selected using MIGLIS® method had decreased concentrations of intracellular ROS, the ROS concentrations in pre-fertilized sperm and early-stage embryos had no effect on blastocyst formation rates. Taken together, these results suggest that the MIGLIS® method has potential for the selection of spermatozoa with higher quality but needs further elucidation in terms of the relationship between intracellular ROS concentrations and embryo development.

FIGURES

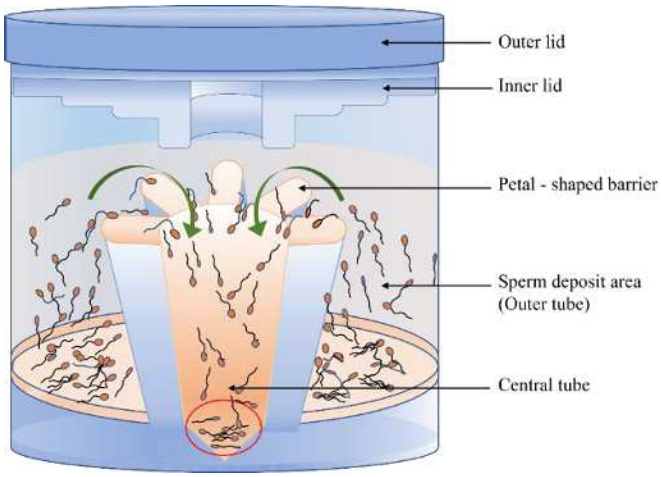


Figure 1.1. MIGLIS® device structure and selection workflow. MIGLIS® device consists of a cylindrical construction divided into a central and an outer tube, separated by a petal-shaped central barrier; the top is covered by an inner lid, which opens along the central tube, and the outer lid. The semen sample is deposited into the outer tube and then covered with the inner lid; medium is then added to the central tube until a total volume of 3.7 mL is achieved. During incubation, only motile sperm swims up through the petal-shaped barrier and falls to the bottom of the central tube owing to gravity and sedimentation. Motile sperm suspension is collected at the bottom (red circle).

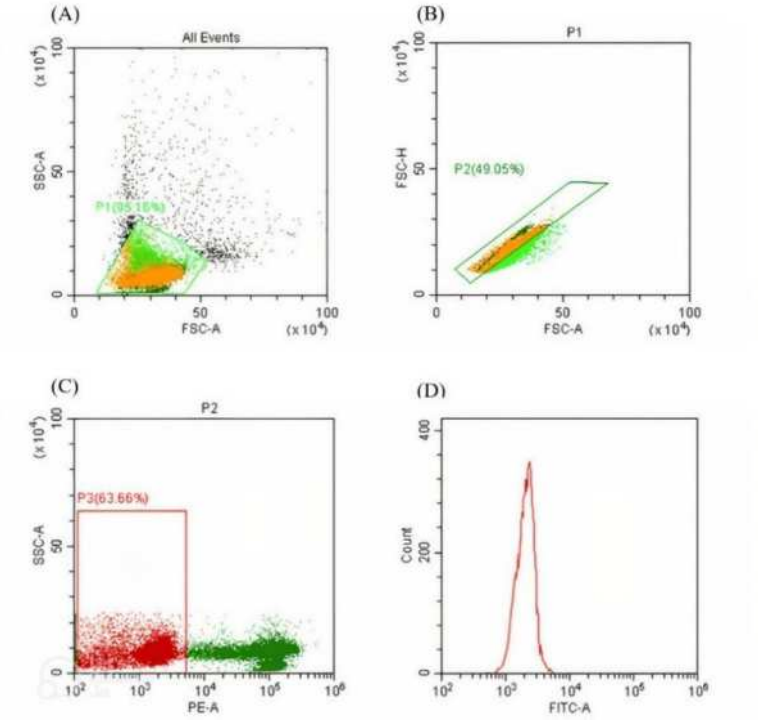


Figure 1.2. Representative examples of flow cytometry analyses assessing the 2',7'-dichlorofluorescein (DCF) fluorescence intensity of frozen-thawed bull spermatozoa. (A)

Selecting only sperm with the expected size (P1). (B) De-aggregating sperms isolated from the P1 population (P2). (C) Gating PI-negative events in the P2 population (P3). (D) DCF fluorescence intensity of the sperms in the P3 population.

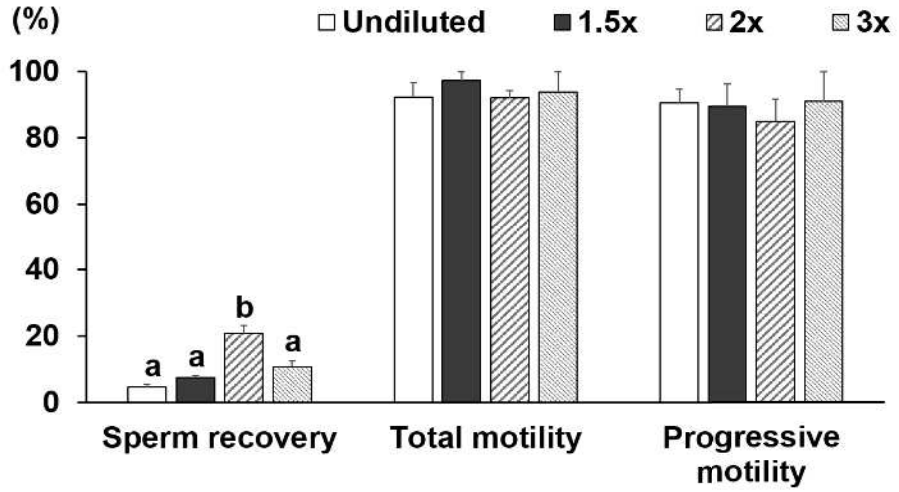


Figure 1.3. Recovery rate and motility of frozen-thawed sperm diluted at various concentrations before selection using the MIGLIS® device. Frozen-thawed spermatozoa were diluted with BO+BSA solution and divided into four groups: undiluted, 1.5 × dilution, 2 × dilution, and 3 × dilution. Data is presented as a mean value ± standard error in five replicate experiments. [a-b] Bars with different letters differ significantly ($p < 0.05$).

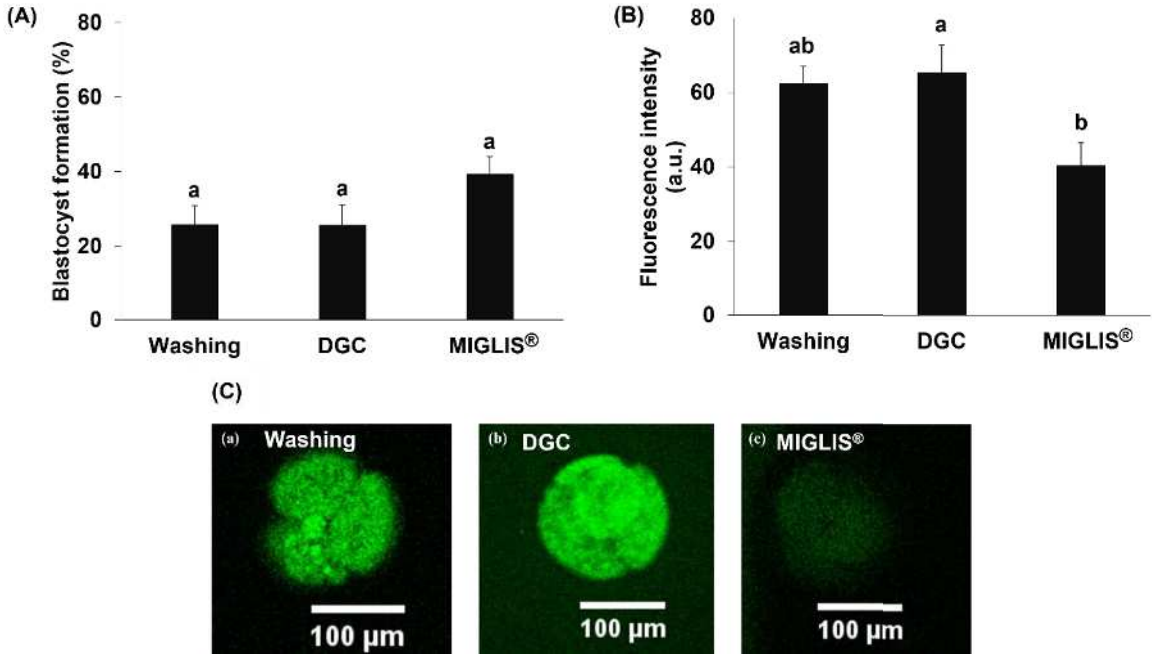


Figure 1.4. Development and intracellular ROS concentrations of embryos fertilised with spermatozoa selected using washing, Percoll® density gradient centrifugation (DGC), or MIGLIS® methods. The intracellular ROS concentrations were estimated based on 2',7-Dichlorodihydrofluorescein Diacetate (DCFH-DA) staining. (A) Blastocyst formation of embryos fertilised with spermatozoa selected using washing, Percoll® DGC, and MIGLIS® methods. The trials were performed three times. (B) 2',7'-dichlorofluorescein (DCF) fluorescence intensity of the 2–4 cell stage embryos fertilised with spermatozoa selected using washing, Percoll® DGC, and MIGLIS® methods. (C) Representative examples of DCF fluorescence intensity of embryos fertilised using the sperm selected using washing (a), Percoll® DGC (b), and MIGLIS® methods (c). ^{ab} Bars with different letters differ significantly ($p < 0.05$).

TABLE

Table 1.1. Recovery rates, quality, and intracellular ROS concentrations of sperm after selection using the washing, Percoll® density gradient centrifugation (DGC), and MIGLIS® methods*.

Group**	Sperm recovery (%) ***	Motility (%)		Viability (%)	Plasma membrane integrity (%)	Acrosome integrity (%)	DCFH - DA intensity (a.u.) ****
		Total	Progressive				
Washing	87.3 ± 6.7[a]	67.5 ± 5.4[a]	56.9 ± 5.0[a]	60.8 ± 3.7[a]	65.4 ± 0.9[a]	96.9 ± 0.6[a]	1938.7 ± 130.6[a]
Percoll® DGC	33.9 ± 5.5[b]	82.6 ± 2.1[b]	73.5 ± 1.9[b]	78.8 ± 1.2[b]	71.3 ± 3.7[ab]	97.1 ± 0.2[a]	1633.5 ± 111.5[ab]
MIGLIS®	19.8 ± 1.5[b]	97.1 ± 0.7[c]	93.3 ± 0.8[c]	92.4 ± 1.5[c]	76.9 ± 3.6[b]	99.2 ± 0.3[b]	1447.4 ± 35.0[b]

*Sperm quality and intracellular ROS concentrations were assessed nine and three times, respectively. The data are shown as mean ± SEM.

**Frozen-thawed sperm were selected using the washing, Percoll® DGC, or MIGLIS® methods.

***The sperm recovery rate was calculated by dividing the number of sperm recovered after selection by the number of sperm in the original frozen-thawed semen.

****The intracellular ROS concentrations were estimated based on 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining.

[a-c] Values with different superscript letters in the same column are significantly different ($p < 0.05$). DCF: 2',7'-dichlorofluorescein, a.u.: arbitrary units.

CHAPTER 2

MIGLIS® sorting enhances embryo development by reducing DNA fragmentation in sperm of similar conventional quality

ABSTRACT

Frozen semen quality is closely associated with reproductive performance, yet substantial variation in fertility persists even among samples that meet standard artificial insemination criteria. This study aimed to identify functional determinants that distinguish high-fertility (HF) from low-fertility (LF) sperm beyond conventional evaluation and to assess whether MIGLIS®-based selection can reduce hidden sources of dysfunction by enriching a functionally competent subpopulation. Although HF and LF samples showed similar motility, viability, and acrosome integrity, they differed markedly in DNA integrity, with LF sperm exhibiting significantly higher DNA fragmentation index (DFI). This elevated DFI, which is not captured by routine semen assessment, was strongly associated with reduced embryonic competence, as evidenced by the high incidence of arrest at the 1–2 cell stage. Early embryos derived from HF sperm displayed greater mitochondrial activity during early development, while ROS levels and later-stage mitochondrial function and apoptosis remained comparable between groups. Importantly, MIGLIS® selection significantly reduced DFI and improved embryo development, particularly in low-fertility samples. These findings highlight DNA fragmentation and membrane functionality as complementary indicators of fertility beyond conventional assessments and support MIGLIS® as a practical strategy to enrich functional sperm and reduce wastage of lower-quality semen.

Key words: bovine, sperm, DNA fragmentation, MIGLIS®

1. Introduction

Cryopreservation causes significant damage to sperm, reducing viability and motility by up to 50% after the freeze-thaw process; however, it remains indispensable in artificial insemination programs [52, 53]. This highlights the importance of selecting sperm with high cryotolerance to improve post-thaw quality [54, 55]. Despite decades of use, conventional semen evaluation, particularly sperm motility, remains a poor predictor of fertility, as samples with comparable parameters can differ markedly in conception rates by 20–25% [56]. Consistently, motility accounts for only a minor fraction of fertility variation (9.5%), whereas functional parameters such as viability, acrosome integrity, reactive oxygen species (ROS), and DNA fragmentation index explain substantially more (30.4%) [57]. These findings highlight important limitations in current semen evaluation strategies.

Sperm DNA fragmentation reflects paternal genome integrity and influencing embryo development [58]. DNA fragmentation encompasses various forms of damage, including abasic sites, base modifications, single- and double-strand breaks, and DNA-protein crosslinks, arising from both endogenous and external factors [59, 60]. While low levels of fragmentation are a normal feature of spermatogenesis, defective apoptosis or abnormalities in chromatin-packaging proteins can lead to the persistence of sperm with highly compromised DNA. Notably, a certain degree of DNA damage may not impair motility, suggesting that conventional semen assessments and selection are insufficient to identify and eliminate spermatozoa with impaired genomic integrity.

This limitation may be exacerbated by semen processing and cryopreservation, which increase DNA damage [60-63]. Therefore, post-thaw sperm selection strategies capable of enriching functionally competent spermatozoa with preserved genomic integrity are critically needed. The MIGLIS® device is a novel, label-free platform based on migration-sedimentation principles that isolate highly motile sperm without mechanical bias. Previous studies have reported improved motility and embryo development following MIGLIS® selection [64], but its potential to enrich sperm with intact DNA remains unclear.

Accordingly, we hypothesize that differences in DNA fragmentation contribute to fertility variation among semen samples with similar conventional semen parameters, and that MIGLIS® selection improves embryonic development by preferentially enriching spermatozoa with preserved genomic integrity.

2. Materials and Methods

2.1. Semen samples

Frozen semen samples were obtained from the Yamaguchi Prefectural Agriculture & Forestry General Technology Center (Yamaguchi, Japan) and classified into low-fertility (LF; ~36.0%) and high-fertility (HF; ~59.5%) groups based on field conception rates.

2.2. Experimental design

2.2.1. *Conventional quality of sperm and its correlation with DNA fragmentation*

Frozen thawed sperm from multiple batches of each LF and HF groups were incubated in phosphate-buffered saline (PBS) supplemented with 1% BSA at 37°C during the evaluation period. Sperm motility was measured immediately after thawing (0 h), and after 1 h and 3 h of incubation. Viability, plasma and acrosome membrane integrity were assessed immediately post-thaw. Each assessment was performed with seven to eight replicates. DNA fragmentation was performed using six to nine replicates in each batch.

2.2.2. Embryo developmental potential following in vitro fertilization

To assess the potential for in vitro blastocyst development, in vitro matured oocytes were fertilized with sperm from LF and HF groups. Early embryo development was recorded by measuring cleavage rates and embryonic cell count at 42 h post-fertilization. Concurrently, some early embryos were randomly collected to assess intracellular ROS levels and mitochondrial activity. On day 8, blastocyst formation rates, mitochondrial activity, and apoptotic rate were evaluated. Four replicates were performed.

2.2.3. Effects of MIGLIS®-based sperm sorting on DNA fragmentation and embryo development

Sperm samples from each group were sorted using MIGLIS® device. A portion of the sorted sperm was evaluated for DNA fragmentation, while the remaining sorted sperm was subjected to in vitro fertilization. Cleavage rates (42 h post-fertilization) and blastocyst formation (day 8 post-fertilization) were evaluated. Four replicates were performed.

2.3. Sperm handling and routine quality assessment

Frozen sperm straws (0.5 ml) were thawed in warm water at 37°C for 30 seconds. Routine sperm quality assessments were performed using a computer-assisted sperm analysis system (CASA) with the Sperm Class Analyzer® (SCA® v.4.2) device (Microptic, Barcelona, Spain).

Sperm handling and routine assessment procedures were conducted in the laboratory mimic the procedures of a sperm collection center as described by Shimazaki *et al.* [65] and other functional parameters were performed as in Nguyen *et al.* [64] with some minor modifications.

Sperm motility

Motility evaluation was performed by transferring 5 µL of sperm suspension from each group onto a warm chamber (MUR-500; Matsunami Glass Ind. Ltd., Osaka, Japan) and quickly observing under a phase-contrast microscope (Nikon TE300; Nikon Corp.). Analysis was conducted on 25 consecutive digitized images captured from three to five different fields using 10x phase-contrast objective. At least 500 sperms per sample were analyzed at an image capture speed of 40 milliseconds. Following the acquisition of representative fields, sperm were categorized and recorded based on overall motility (curvature velocity exceeding 10 µm/s) and progressive motility (curvature velocity exceeding 100 µm/s).

Sperm viability

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (SYBR-14/propidium iodide PI, Molecular Probes, Inc., Eugene, OR, USA). Briefly, 50 µL of semen was mixed with 3 µL of PI (ratio 1:10 in distilled water) and 3 µL of SYBR-14 (ratio 1:500 in DMSO), followed by incubation for 10 min for each labeling step. At least 200 sperms were evaluated under a fluorescence microscope (20× magnification, Nikon Eclipse 80i, Nikon) using a 488 nm filter. Live sperm exhibited bright green fluorescence from SYBR-14 staining, while dead sperm displayed red fluorescence from PI staining.

Sperm plasma membrane integrity

Plasma membrane integrity was assessed using the Hypo-Osmotic Swelling Test (HOST). Hypo-osmotic medium was prepared by dissolving 7.35 mg/mL of citric acid dihydrate (Wako, Gunma) and 13.5 mg/mL of fructose (Sigma-Aldrich Japan, Tokyo) in milli-Q water. A 10 µL aliquot of sperm suspension was gently mixed with 90 µL of the pre-warmed hypo-osmotic medium and incubated at 37°C for 10 minutes. After incubation, 10 µL of the mixture was placed on a microscope slide and covered with a cover slip. Approximately 200 sperms per sample were evaluated and classified as HOST-positive (showing coiled tails) or HOST-negative (non-coiled tails).

Acrosome integrity

Acrosome integrity was assessed using fluorescein isothiocyanate-peanut agglutinin (FITC-PNA; Sigma-Aldrich Japan, Tokyo, Japan). A 10 µL aliquot of sperm was placed on a glass slide, smeared evenly, and air-dried. The smear was then fixed in 95% ethanol for 10 minutes. After drying, 30 µL of a FITC-PNA/DPBS(-) solution (1:100 dilution) was applied to the smear and incubated in a humidified chamber at 37°C for 10 minutes. Subsequently, 8 µL of propidium iodide (PI) solution was added and incubated for another 10 minutes. Following several washes with cold DPBS(-), slides were examined under a fluorescence microscope. Sperm showing uniform green fluorescence over the acrosomal region were classified as acrosome-intact (positive), while those lacking fluorescence were considered acrosome-reacted or damaged (negative). Approximately 200 sperm cells per sample were evaluated.

2.4. Sperm DNA fragmentation index

Frozen-thawed semen samples of each batch were diluted into final concentration 10×10^6 cells/ml with TNE buffer containing 0.15M NaCl (Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan), 0.01M Tris HCl (Nippon Gene Corporation, Tokyo, Japan), 1mM EDTA (Dojin Chemical Laboratory, Kumamoto, Japan). Next, 0.08M HCl (Fujifilm Wako Pure Chemicals Corporation, Osaka, Japan), 0.15M NaCl and 0.1% TritonX-100 (Sigma-Aldrich, USA) were added to make a volume 200 µl of the diluted sample, and cell membrane permeabilization treatment for exactly 30 seconds. Finally, 1.2 ml of acridine orange staining solution contain 200 mM sodium bicarbonate (Wako Pure Chemical Industries, Osaka, Japan), 0.1 M citrate buffer (citric acid monohydrate (Wako Pure Chemical Industries, Osaka, Japan), trisodium citrate dihydrate (Wako Pure Chemical Industries, Osaka, Japan), 1 mM EDTA, 150 mM NaCl and 6 µg acridine orange (Kanto Chemical, Tokyo) were added and allowed to stand at room temperature for 15 minutes.

The samples were analyzed by flow cytometer system (CytoFLEX - BeckmannCoulter, Tokyo). The acridine orange staining solution was passed through the flow cytometer for approximately 15 minutes to saturate the tubes with acridine orange molecules before measurements were initiated. Acridine orange was excited with a blue laser 488 nm with adjusted green and red fluorescence gain and detected the following filters 525/40BP and 690/50BP. Data was collected on 10,000 gated events for each sample.

2.5. In vitro embryo production

In vitro embryo production was performed as previously described [64]. Briefly, ovaries were collected from a slaughterhouse and stored in physiological saline supplemented with

100 IU/mL penicillin (Meiji Seika Pharma Co., Ltd., Tokyo, Japan) and 0.1 mg/mL streptomycin (Meiji Seika Pharma Co., Ltd.) at 20–25°C.

Follicular fluid containing cumulus-oocyte complexes (COCs) was aspirated using a 5 mL syringe with an 18 G needle (Terumo Corporation, Tokyo), transferred into a tube containing 1 mL of prewarmed BO-WASH solution (IVF Bioscience, UK), and kept at 38.5°C on a warm plate. The sediment was then transferred to a petri dish containing PBS solution with 100 IU/mL penicillin and 100 μg/mL streptomycin for COCs collection. Only COCs with more than four layers of cumulus cells and uniform cytoplasmic morphology were selected under a stereomicroscope. Selected COCs were washed three times each in BO-WASH and BO-IVM (IVF Bioscience, UK) solutions, then cultured in prewarmed BO-IVM medium. Incubation was performed for 22 hours at 38.5°C in a 5% CO_2 atmosphere.

Matured oocytes were fertilized with sperm from HF and LF at a final concentration of 1.0×10^6 sperm/mL in BO-IVF medium (IVF Bioscience, UK) for 20 hours at 38.5°C. After 20 hours of co-incubation, presumptive zygotes were denuded by gentle pipetting and transferred to BO-IVC medium (IVF Bioscience, UK), covered with mineral oil, and incubated for 8 days at 38.5°C in a humidified atmosphere containing 5% CO_2, 5% O_2, and 90% N_2.

2.6. Reactive oxygen species and mitochondrial activity in early embryos

Early embryos (42 h post-IVF) at the 2–8 cell stage were double-stained to assess intracellular ROS and mitochondrial activity using ROS Assay Kit -Photo-oxidation Resistant DCFH-DA- (Dojin Chemical Co.) and MitoTracker™ Red CMXRos (Invitrogen, Eugene, OR, USA), respectively. Briefly, early embryos were washed in PBS containing 0.3% (w/v) polyvinyl alcohol (PVA; Sigma-Aldrich Japan, Tokyo) and incubated with DCFH-DA at 38.5°C with 5% CO_2 in the dark for 30 minutes. After incubation, embryos were gently washed in PBS-PVA and transferred to MitoTracker Red CMXRos diluted to 400 nM in PBS. They were then incubated for 45 minutes, protected from light by aluminum foil. Following staining, embryos were washed with PBS-PVA and mounted on slides with 10 μL of PBS. Fluorescent images were acquired using a Leica Mica WideFocal Live-cell confocal laser microscope controlled by LAS X software (Leica Microsystems). Intracellular ROS was measured at excitation/emission wavelengths of 488/500–550 nm, while mitochondrial activity was measured at 579/599 nm. Fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Embryo area

was selected as the region of interest (ROI), and the mean fluorescence intensity within the ROI was recorded for each embryo. Fluorescence intensity with DCFH-DA- and MitoTracker was expressed in arbitrary units (a.u.).

2.7. Mitochondria activity and apoptotic rate in blastocyst

Mitochondrial activity and apoptotic rate were assessed by triple staining with MitoTracker Red CMXRos kit (Invitrogen, Eugene, OR, USA) and terminal deoxynucleotidyl transferase dUTP nick end labeling assay (Tunel assay, Roche Diagnostics, Tokyo, Japan) combined bisbenzimide (Hoechst 33342; Sigma-Aldrich), respectively. Briefly, blastocysts were incubated in MitoTracker Red CMXRos (Invitrogen, Eugene, OR, USA) diluted to 400 nM in PBS and protected from light with aluminum foil in a two-gas incubator (38.5°C, 5% CO_2, 5% O_2) for 45 minutes. Following incubation, embryos were washed four times with PBS containing 0.3% PVA (PBS-PVA) and fixed overnight at 4°C in PBS containing 3.7% (w/v) paraformaldehyde, shielded from light. The next day, embryos were washed four times with PBS-PVA, permeabilized with PBS containing 0.1% Triton X-100 for 40 minutes, then washed again and incubated in blocking solution (PBS/Triton with 1% BSA, 1mM glycine) overnight at 4°C in the dark. On the following day, embryos were stained for apoptosis using a Tunel mixture for 1 hour at 38.5°C in the dark. After four PBS-PVA washes, embryos were treated with 1.9 mM bisbenzimide in 90% (v/v) glycerol for 30 minutes at room temperature. After final washes (four with blocking solution and three with PBS-PVA), embryos were mounted on slides using Anti-fade mounting medium (SlowFade, Molecular Probes, USA), covered with a coverslip, and stored at 4°C in the dark until imaging. Fluorescent images of blastocysts were acquired using a Leica Mica WideFocal Live-cell confocal microscope. The apoptotic rate (Tunel-positive cells/total cells) were manually counted from the images. Mitochondrial activity was quantified using ImageJ software (NIH, Bethesda, MD, USA) by selecting each blastocyst as ROI and measuring the integrated fluorescence intensity (IntDen). Mitochondrial activity was measured at excitation/emission wavelengths of 579/599 nm.

2.8. Sorting sperm by MIGLIS® device

The method was performed as described previously [64]. Briefly, thawed spermatozoa were injected into the deposition area of MIGLIS® device. BO solution containing 1% BSA was gently poured from the bottom of the central tube to cover the semen by a total volume of 3.7 mL and incubated at 37°C for 1 h. Sorted sperm sub-population was obtained from the

bottom of the central tube (400ml) by micropipette and then subjected to evaluation of DFI and in vitro embryo production.

2.9. Statistical analysis

All percentage data were subjected to arcsine transformation prior to statistical analysis to normalize variances. Differences between groups were evaluated using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) test for post hoc comparisons, using STATVIEW software (Abacus Concepts, Inc., Berkeley, CA, USA). Correlations between DNA fragmentation index and sperm quality parameters were assessed using Spearman's rank correlation test. Data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Conventional quality of sperm and its correlation with DNA fragmentation

The quality of thawed sperm was evaluated using conventional semen analysis techniques, regardless of semen batch (Fig. 2.1). Among the assessed parameters, sperm in HF group exhibited a significantly higher HOST value compared to LF group (65.5 ± 2.6% vs. 40.2 ± 4.2%, $p < 0.001$). No significant differences were observed between the groups in other semen quality indices.

DFI value regardless of batch in HF group was significantly lower than that in LF group (3.5 ± 0.4% vs. 12.2 ± 1.0%, respectively, $p < 0.0001$, Fig. 2.1). To assess whether this difference was influenced by different batches, comparisons were performed between batches within the same group, no statistical differences were found ($p > 0.05$).

Correlations between DFI and other sperm quality parameters are presented in Table 2.1. In which DFI showed a moderate negative correlation with HOST (r = -0.68, $p < 0.01$) and viability (r = -0.62, $p < 0.05$). No significant correlations were found between DFI and total motility, progressive motility, or acrosomal integrity ($p > 0.05$).

3.2. *Embryo development potential following in vitro fertilization*

At 42 hours post-fertilization (Fig. 2.2), cleavage rate was significantly higher in HF group compared to LF group (72.3 ± 10.0% vs. 42.5 ± 5.5%, $p < 0.05$). Early embryonic cell stage analysis showed that embryos derived from HF sperm more frequently reached the 4–8 cell stage than that of LF group (50.7 ± 9.9% vs. 14.3 ± 3.3%, respectively, $p < 0.05$), whereas

those from LF sperm predominantly remained at the 1–2 cell stage (85.7 ± 3.3% vs. 47.9 ± 10.2%, respectively, $p < 0.05$).

Mitochondrial activity in early embryos produced by in vitro fertilization with HF sperm was significantly higher than in those fertilized with LF sperm (83.8 ± 5.1 vs. 57.9 ± 4.1 a.u., $p < 0.01$). However, no significant difference in intracellular ROS levels was observed between the two groups (53.9 ± 3.8 vs. 72.3 ± 12.1 a.u., $p > 0.05$).

Blastocyst formation rates were also significantly higher in HF group compared to LF group (43.3 ± 4.8 vs. 11.5 ± 1.9 %; $p < 0.01$). No statistically significant differences were found between groups in mitochondrial activity or apoptotic index at the blastocyst stage ($p > 0.05$).

3.3. Effects of MIGLIS®-based sperm sorting on DNA fragmentation and embryo development

Sperm fractionation using the MIGLIS® device (Fig. 2.3) resulted in a significantly lower DFI level compared to the unsorted control in both the HF (0.8 ± 0.3 vs. 3.7 ± 0.4 a.u., $p < 0.001$) and LF (2.2 ± 0.2 vs. 13.8 ± 1.6 a.u., $p < 0.001$). Especially, sperm from the LF group showed a significant improvement in DNA integrity after selection using the MIGLIS® device, becoming comparable to that of the HF group ($p > 0.05$).

The cleavage rate and blastocyst formation rate (Fig. 2.3) were both significantly higher after MIGLIS® selection than unsorted sperm only in LF group (56.0 ± 4.3 vs. 29.6 ± 9.4 %, $p < 0.05$ and 27.5 ± 5.0 vs. 6.0 ± 3.8 %, $p < 0.05$). In contrast, no significant differences were observed in the HF group for either cleavage rate (76.9 ± 6.2 vs. 62.0 ± 14.1 %, $p > 0.05$) or blastocyst formation rate (50.6 ± 4.3 vs. 44.7 ± 5.2 %, $p > 0.05$) between MIGLIS®-selected and unselected sperm.

4. Discussion

This study identifies key determinants of fertility that are not captured by routine semen assessments. Although sperm motility has traditionally been emphasized as an important indicator of fertility, our findings showed no significant differences in motility between HF and LF sperms. All sperm samples in this study exceeded the widely accepted threshold of 60% total motility considered adequate for successful conception [66, 67]. Differences in reproductive performance may be attributed to the durability and functional longevity of sperm within female reproductive tract, which were assessed by evaluating thermotolerance over a 3-hour incubation period [68]. However, our thermotolerance testing showed no

significant difference between groups. These findings suggest that neither initial motility nor motility stability is a reliable standalone predictor of fertility within scope of this study. To date, literature remains divided on the predictive value of motility indices, with some studies supporting their relevance [69-71] or no association [72-74].

Notably, sperm DNA fragmentation strongly correlates with fertility outcomes. Samples with reduced fertility exhibited significantly higher DFI, whereas those with better fertility consistently remained below the critical 7–8% threshold [13, 75]. Sperm DNA damage has been associated with alterations in protein components associated with spermatogenesis, chromatin remodeling, fertilization, and pronuclear fusion [58]. Given the multifactorial nature of sperm quality, integrating conventional parameters with indicators such as DFI provides a more accurate prediction of fertility [76]. The observed negative correlation between DFI and HOST further suggests a link between membrane functionality and DNA integrity, although its predictive value remains context dependent. In humans, specific HOST swelling patterns (types B, C, D/E) are associated with reduced DNA fragmentation [77-80]. In particular, type D patterns have been linked to higher expression of phospholipase C zeta, a key factor in oocyte activation [81]. Because our study lacked detailed swelling patterns analysis and involved a relatively small cohort, a robust predictive model could not be established. Future studies with larger cohorts and more detailed HOST data are needed to better elucidate the relationship between plasma membrane functionality, DNA integrity, and fertility outcomes. This supports the importance of functional parameters beyond conventional semen evaluation.

Sperm DNA fragmentation may not significantly affect motility at certain levels of damage, as flagellar function primarily governs movement [17, 59, 82-84]. Instead, the impact of high DFI becomes evident after fertilization. Embryos derived from sperm with elevated DFI showed reduced cleavage and blastocyst formation compared to those from lower DFI groups. Early cleavage (1–2 cell stage) was unaffected, but developmental arrest occurred prior to embryonic genome activation (4–8 cell stage), consistent with previous reports [85]. Mechanistically, sperm DNA damage disrupts paternal DNA demethylation and impairs genomic reprogramming in early embryo stage [47, 86]. The severity of the effect depends on the extent of DNA lesions, which can delay pronuclear replication, trigger G2/M cell cycle arrest, and induce γH2AX signaling, leading to delayed cleavage or complete developmental arrest. Some embryos may temporarily overcome these defects but ultimately arrest and degenerate without classical apoptosis [87]. Furthermore, different types of DNA

damage exert stage-specific effects: single-strand breaks generally do not affect fertilization but can disrupt early cleavage (2–6 cell stage), whereas double-strand breaks are more likely to impair later stages of preimplantation development [83].

Sperm DNA damage is primarily driven by oxidative stress, and embryos can inherit such lesions. However, ROS levels in early embryos remained similar between groups, suggesting that maternal repair and antioxidant mechanisms efficiently neutralize sperm-derived oxidative damage, likely via base excision repair pathways, mitochondria activity [47, 50, 88-90]. In this context, mitochondria play a central role in maintaining redox balance and supporting repair-associated energy demand. Consistently, embryos derived from HF sperm exhibited higher mitochondrial activity, potentially reflecting reduced oxidative burden and lower requirement for DNA repair during early development, supporting higher cleavage and blastocyst formation. In addition, no differences in apoptosis or mitochondrial activity were detected at the blastocyst stage, suggesting a potential self-selection process during early development, whereby embryos with severe damage fail to progress. Importantly, DFI is not solely driven by oxidative stress; defects in chromatin remodeling, such as incomplete histone-to-protamine transition, abnormal condensation, nucleosome retention, or defective heparin binding, may also underline sperm DNA damage [58, 91, 92]. These findings underscore the importance of assessing sperm chromatin integrity alongside routine assessments.

Despite growing evidence supporting biomarkers as superior predictors of male fertility [71, 93], their routine use in breeding centers is limited. An alternative strategy involves improving post-thaw sperm selection systems, with an emphasis on practicality and non-invasive techniques. Our study highlights the potential of MIGLIS® device, which utilizes a migration-sedimentation mechanism and avoids centrifugation-induced stress. MIGLIS®-based sperm selection significantly reduced DFI in both high- and low-fertility groups. Notably, the low-quality semen group benefited more from MIGLIS® based selection, showing higher embryonic developmental outcomes, including cleavage and blastocyst formation rates. This differential response can be explained by the ability of MIGLIS® to enrich sperm populations with improved DNA integrity, with the magnitude of benefit depending on the initial semen quality. In the low-DFI group, MIGLIS® induced only a modest further reduction in DNA fragmentation, and both pre- and post-selection levels likely remained within the repair capacity of the oocyte, resulting in limited impact on embryonic development. In contrast, in the high-DFI group, MIGLIS® substantially reduced

DNA fragmentation relative to unprocessed samples, increasing the proportion of spermatozoa within a range that can be effectively managed by embryonic DNA repair mechanisms. However, because baseline damage was higher, a fraction of spermatozoa likely still exceeded the oocyte's compensatory capacity, resulting in a more pronounced improvement in embryonic development.

In conclusion, incorporating DNA fragmentation index may improve sperm fertility assessment. MIGLIS® sperm sorting offers a non-invasive approach to select sperm with lower DNA fragmentation and improve embryo development. Further in vivo studies are needed to confirm effects on conception rates.

FIGURES

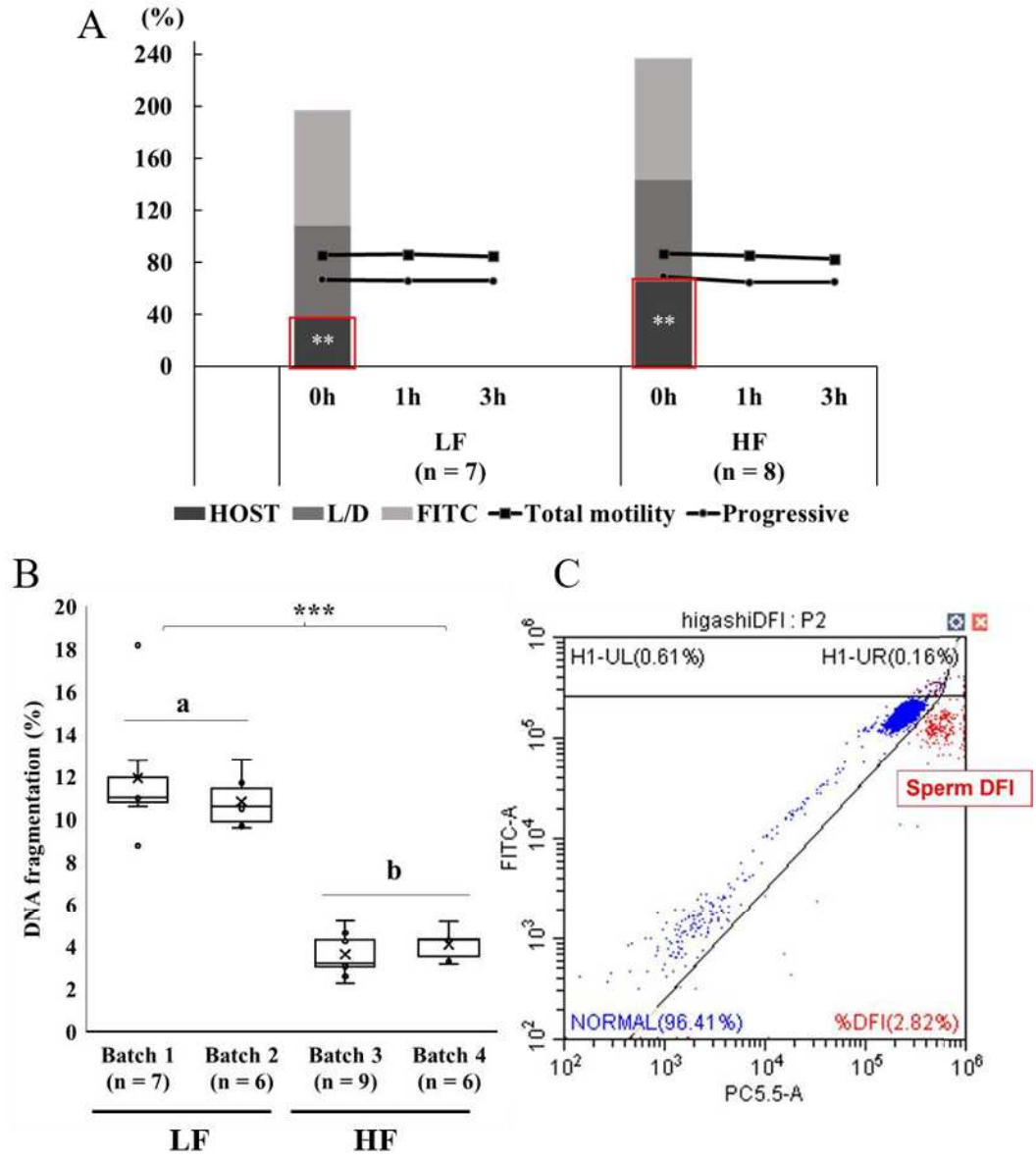


Figure 2.1. Sperm quality assessment. (A) Conventional sperm quality parameters. (B) DNA fragmentation index (DFI) levels across batches. (C) Representative flow cytometry gating strategy used to distinguish sperm with fragmented and intact DNA. Values are presented as mean ± SEM. Six to eight replicates were performed. Different letters denote statistically significant differences among batches within each group. $^{**}p < 0.001$; $^{***}p < 0.0001$.

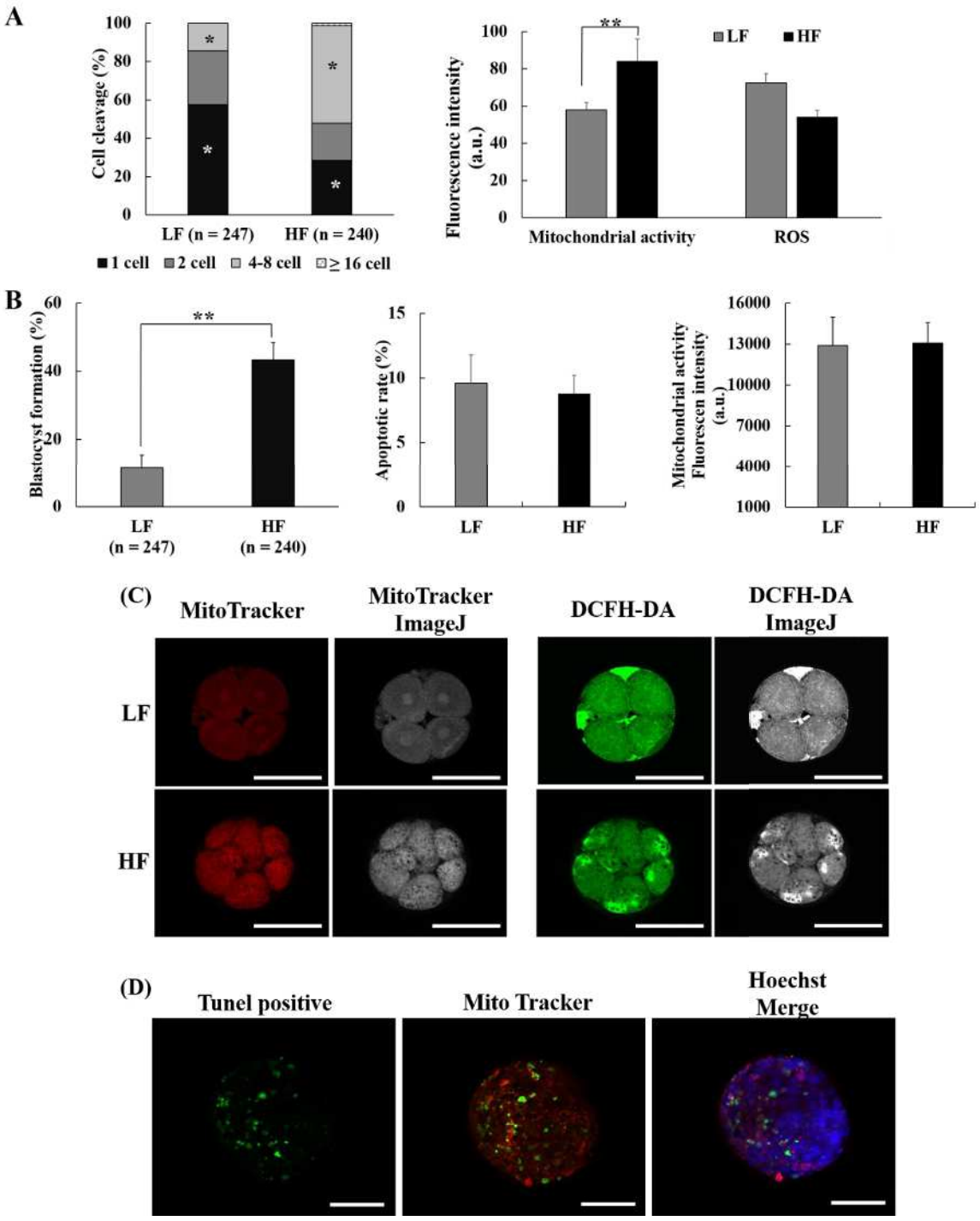


Figure 2.2. Embryo production following in vitro fertilization using frozen-thawed low-fertility (LF) and high-fertility (HF) sperm. (A) Number of blastomeres in early embryos at 42 h post-fertilization, along with quantification of fluorescence intensity reflecting mitochondrial activity and ROS levels. (B) Blastocyst formation rate, apoptotic rate, and fluorescence intensity of mitochondrial activity. (C) Representative fluorescence images of early blastocysts with double staining; Red fluorescence indicates MitoTracker staining (mitochondrial activity), green fluorescence indicates DCFH-DA staining (ROS), and

grayscale images correspond to MitoTracker and DCFH-DA channels exported as TIFF files for ImageJ analysis. (D) Representative fluorescence images of blastocysts triple-stained with TUNEL (green, apoptotic cells), MitoTracker (red, mitochondrial activity), and Hoechst (blue, nuclei). Values are presented as mean ± SEM. Four to five replicates were performed. *$p < 0.05$, **$p < 0.01$. Scale bar = 100 μm. a.u., arbitrary units.

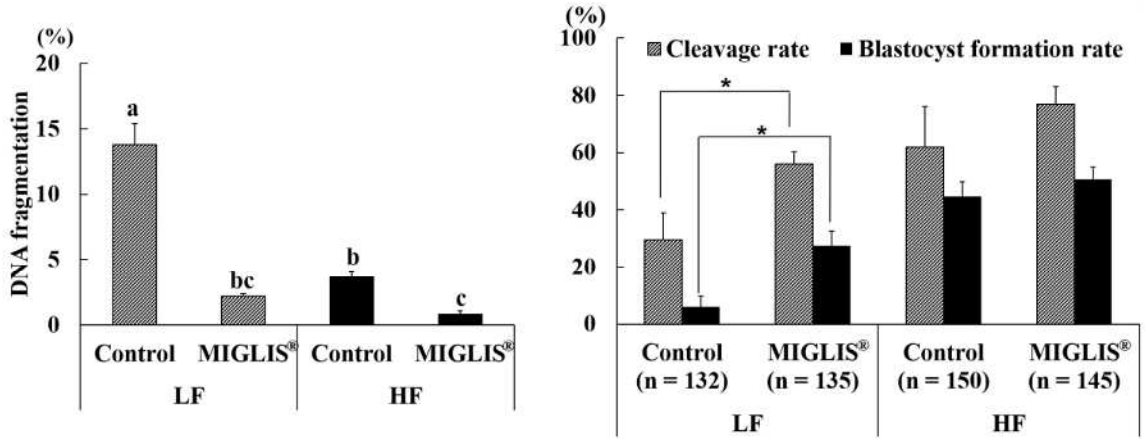


Figure 2.3. (A) DNA fragmentation levels in sperm sorted using the MIGLIS® device compared with the unsorted (control) group. Different letters indicate statistically significant differences among groups. (B) In vitro embryo production following sperm sorting using the MIGLIS® device compared with the unsorted group; *$p < 0.05$. Values are presented as mean ± SEM. Four replicates were performed.

TABLE

Table 2.1. Spearman's correlation coefficients (*r*) among sperm quality parameters

	DFI	Total motility	Progressive	Live/Dead	HOST	FITC
DFI	1					
Motility	0.025079	1				
Progressive	0.006955	0.571526*	1			
Live/Dead	-0.62304*	0.011657	0.122537	1		
HOST	-0.68045**	-0.06707	0.242163	0.647108**	1	
FITC	-0.08139	-0.26787	0.24667	-0.22925	0.224318	1

The values represent the correlation coefficient (r) between each pair of parameters. Asterisks indicate statistically significant correlations: *$p < 0.05$, **$p < 0.01$. Seven to eight replicates were performed. HOST: Hypo-osmotic swelling test; FITC: fluorescein isothiocyanate.

CHAPTER 3

Quality and fertilizing ability of frozen-thawed porcine sperm separated using a migration sedimentation method

ABSTRACT

This study evaluated the potential of the MIGLIS® device for functional selection of frozen-thawed porcine spermatozoa. Porcine sperm are particularly susceptible to cryo-induced oxidative stress and exhibit rapid functional decline after thawing, which may compromise the efficiency of post-thaw sperm sorting procedures. To address this limitation, MIGLIS®-based selection was optimized by combining caffeine supplementation as an antioxidant during separation, along with the determination of an appropriate incubation time. Frozen-thawed sperm were loaded into the MIGLIS® device and incubated with different concentrations of caffeine (0, 1, 2.5, 5, and 10 mM). The optimal incubation time was subsequently evaluated using 2.5 mM caffeine for 5, 10, 15, or 20 min. Sperm recovered from the inner regions of the device were assessed for quality parameters and used for in vitro fertilization of in vitro-matured oocytes followed by 7 days of embryo culture. Sperm collected from the inner region after incubation with 2.5 mM caffeine showed improved quality parameters compared with those incubated without caffeine, with progressive motility significantly increased after 10 min of incubation. Furthermore, oocytes fertilized with sperm recovered from the inner collecting region of the MIGLIS® device following incubation with 2.5 mM caffeine for 10 min exhibited significantly higher blastocyst formation rates than those fertilized with sperm from the outer region containing unselected spermatozoa, irrespective of the porcine. These findings indicate that MIGLIS®-based selection, optimized with 2.5 mM caffeine and a 10 min incubation period, can effectively enrich functionally competent porcine spermatozoa and improve subsequent embryonic development.

Keywords: porcine, sperm, MIGLIS®, caffeine, in vitro fertilization.

1. Introduction

Thus far, sperm sorting methods such as swim-up, density gradient centrifugation (DGC), microfluidic, have been used to select high-quality sperm for use in assisted reproductive technologies [94]. Recently, a commercial device (MIGLIS®, Menicon Life Sciences, Nagoya, Japan) was reported to collect motile sperm without centrifugation based on a sperm migration sedimentation method (MS) [19, 35]. The device is composed of a small conical cup (inner space) inserted into an outer deposit area (outer space), where only motile spermatozoa can migrate from the outer chamber and subsequently settle in the inner space. Sperm sorting using the MIGLIS® device is a new sperm preparation method for selecting sperm with high motility and fertilization abilities [35].

In our previous study on bovine sperm, we demonstrated that the MS method not only separated sperm with high motility and quality but also decreased ROS concentrations of the selected sperm compared with centrifugation-based methods [64]. Excessive ROS production is known to induce oxidative stress, overwhelm cellular antioxidant defenses, and cause DNA damage, ultimately impairing sperm function [30, 31].

In porcine, in vitro fertilization has a high incidence of abnormal fertilization (polyspermy), which compromises embryo developmental competence [95]. Therefore, the selection of high-quality sperm prior to IVF is especially critical in this species. However, sperm characteristics and lifespan are species specific, and sperm selection methods often require species-specific optimization. Although the effectiveness of MIGLIS®-based sperm selection has been demonstrated in human [19] and bovine sperm [64], its efficiency and optimal conditions for selecting cryopreserved porcine sperm remain unclear.

Porcine spermatozoa exhibit heightened sensitivity to oxidative stress compared with other species, owing to the unique composition of their plasma membrane, which is rich in polyunsaturated fatty acids and low in cholesterol, rendering them particularly vulnerable to oxidative damage during in vitro handling and selection [96]. Consequently, strategies that both minimize oxidative stress and enhance sperm motility are especially relevant for porcine sperm selection, particularly for migration-based methods such as MIGLIS®. Caffeine is known to stimulate sperm motility by inhibiting phosphodiesterase, thereby increasing intracellular cAMP levels and improving sperm motility and longevity [97]. By prolonging the half-life of cAMP and activating protein kinases, caffeine further promotes motility, cyclic hyperactivation, capacitation, and the acrosome reaction [98, 99]. Previous

studies have demonstrated that the effects of caffeine on sperm motility are species dependent. In livestock, supplementation with 2.5 mM caffeine increased the motility of bovine sperm selected by swim-up [100], whereas in horses, the addition of 5 mM caffeine before swim-up increased sperm recovery and enriched highly motile sperm [101]. Moreover, sperm incubation time is known to influence sperm functional properties, and migration-sedimentation requires sufficient time for spermatozoa to migrate through the medium and subsequently sediment into the collection zone [102].

Therefore, determining the optimal caffeine concentration and incubation time is essential for the effective selection of porcine sperm using the MIGLIS®-based selection method. In this study, we examined the optimal conditions, including caffeine concentration and incubation time, for selecting frozen-thawed porcine sperm using the MIGLIS® device. Furthermore, we evaluated fertilization outcomes and subsequent embryo development following in vitro fertilization using sperm recovered from the selecting space (inner) and the non-selecting space (outer) of the device using semen across different boars.

2. Material and methods

All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. The procedures used in this study were approved by the Animal Research Committee of Tokushima University. Furthermore, the guidelines for animal experiments at Tokushima University were followed to protect animal welfare.

2.1. Sperm separation using the MIGLIS® device

Semen from three large white boars (boars A-C) were cryopreserved according to a previously described method [103]. Cryopreserved semen straws (0.25 mL) were thawed in a water bath at 38°C for 30 s before examination. After thawing, the sperm washed once by centrifugation, because preliminary examination showed that the recovery rate was extremely low when separating sperm by the MIGLIS® device without washing. Briefly, the frozen-thawed semen was washed by centrifugation at 550 × g for 2 min in an incubation solution consisting of TCM-199 with Earle's salts (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 4 mM calcium lactate (Nacalai Tesque, Kyoto, Japan), 10 mM glucose (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 25 mM HEPES (Dojindo, Tokyo, Japan), 12% fetal bovine serum (HyClone, Logan, UT, USA), and 0.1% gentamicin (pH ranged from 7.6–7.8). The supernatant was removed and the sediment was injected into the outer region of the central tube in the MIGLIS® device consisting of a small

central tube inside a cylindrical container [104]. An inner lid was inserted, and the incubation solution was gently poured from the bottom of the central tube to cover the semen with a total volume of 3.7 mL. Subsequently, the outer lid was covered to prevent evaporation of the liquid, and the device was placed in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2 at 39°C to allow the swimming sperm to fall to the bottom of the central tube by gravity. Finally, sperm suspensions (400 μL per sample) collected from the bottom of the inner and outer tubes were used for the experiments.

2.2. Assessment of sperm quality

Motility analyses were performed using a computer-assisted sperm analysis system (Sperm Class Analyser®: SCA® v.4.2; MICROPTIC, Barcelona, Spain). The analysis of motility was based on the examination of 25 consecutive, digitized images obtained from 3 to 5 fields using a 10× phase contrast objective, and at least 500 sperm per sample were analyzed using an image capture speed of 40 ms. Analyses of the viability, plasma membrane integrity, and acrosome integrity were conducted using a live/dead stain (SYBR-14/propidium iodide [PI], LIVE/DEAD Sperm Viability Kit; Molecular Probes, Inc., Eugene, OR, USA), the hypo-osmotic swelling test [39], and fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA; Vector Laboratories, Inc., Burlingame, CA, USA), respectively, according to the methods described by Taniguchi *et al*. [105]. The quality of frozen-thawed sperm was assessed after separating the semen (Figure 3.1).

2.3. Assessment of penetration ability of sperm and oocyte development

Oocyte collection, in vitro maturation, fertilization, and embryo culture were performed as previously described [47]. Briefly, ovaries were obtained from prepubertal crossbred gilts (Landrace × Large White × Duroc) at a local slaughterhouse. Cumulus-oocyte complexes (COCs) with a uniform ooplasm and compact cumulus cell mass were collected by ovary slicing and cultured in a maturation medium at 39°C in a humidified incubator containing 5% CO_2. The maturation medium consisted of tissue culture medium 199 with Earle's salts (TCM 199; Invitrogen Co., Carlsbad, CA, USA), supplemented with 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 50 μM sodium pyruvate, 2 mg/mL D-sorbitol (Wako Pure Chemical Industries Ltd., Osaka, Japan), 50 μM β-mercaptoethanol (Wako Pure Chemical Industries Ltd.), 10 IU/mL equine chorionic gonadotropin (Kyoritsu Seiyaku, Tokyo, Japan), 10 IU/mL human chorionic gonadotropin (Kyoritsu Seiyaku), and 50 μg/mL gentamicin.

After maturation for 20–22 h, COCs were cultured for an additional 24 h in a maturation medium without hormones under the same conditions.

For IVF, sperm collected from the bottom of the inner and outer tubes of the MIGLIS® device were transferred into 5 mL of porcine fertilization medium (PFM; Research Institute for Functional Peptides Co., Yamagata, Japan) and washed by centrifugation at 500 × g for 5 min. The sperm pellet was resuspended in PFM to achieve a concentration of 2.0×10^6 cells/mL. Sperm (250 µL) was added to 250 µL PFM containing oocytes (about 45 oocytes per well) in four-well dishes. The final sperm concentration was adjusted to 1×10^6 cells/mL. The oocytes were co-incubated for 5 h at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2.

The zygotes were mounted on glass slides 10 h after insemination and fixed with acetic acid: ethanol (1:3, v/v) for 48–72 h to assess oocyte fertilization. The fixed zygotes were stained with acetic orcein (1% orcein in 45% acetic acid) and examined under a phase-contrast microscope. Oocytes containing both female and male pronuclei were considered fertilized and categorized as normal or polyspermy based on the number of swollen sperm heads and/or pronuclei in the cytoplasm [106].

After IVF, the zygotes were washed with porcine zygote medium (PZM-5; Research Institute for Functional Peptides Co.) and cultured continuously at 39°C in a humidified incubator containing 5% CO_2, 5% O_2, and 90% N_2. Embryos were cultured for three days and subsequently incubated in porcine blastocyst medium (PBM; Research Institute for Functional Peptides Co.) for 4 days. Cleavage and blastocyst formation of embryos were evaluated at 3 days and 7 days after IVF, respectively. To evaluate the total cell number in the blastocysts, all embryos at the blastocyst and expanded blastocyst stages were fixed and stained with Hoechst 33342 at the end of the culture procedure.

2.4. Experimental designs

Experiment 1: To determine the optimal concentration of caffeine in the incubation solution for separation, frozen-thawed sperm were incubated with different concentrations of caffeine (0, 1, 2.5, 5, and 10 mM) for 10 min in the MIGLIS® device, as described above. After incubation, sperm suspensions collected from the bottom of the inner tube were used to assess sperm recovery and quality. Four replicates were performed.

Experiment 2: A caffeine concentration of 2.5 mM was most suitable for improving sperm quality after separation in Experiment 1. To determine the appropriate incubation time for frozen-thawed sperm in the MIGLIS® device, sperm were incubated with 2.5 mM caffeine for 5, 10, 15, or 20 min. After incubation, sperm suspensions collected from the bottom of the inner tube were used to assess sperm recovery and quality. Four replicates were performed.

Experiment 3: Incubation for 10 min was optimal for maintaining sperm quality after separation in Experiment 2. To evaluate the fertilization and embryo development of oocytes fertilized with frozen-thawed sperm separated into two regions (outer and inner), sperm from three boars (A-C) were incubated with 2.5 mM caffeine for 10 min, fertilized with in vitro-matured oocytes, and then cultured in vitro for 7 d. Four replicates were performed.

2.5. Statistical analysis

Sperm recovery, motility, and quality were evaluated using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test using STATVIEW (Abacus Concepts, Inc., Berkeley, CA, USA). The percentages of sperm recovery and sperm quality were subjected to arcsine transformation before statistical analysis. The rates of fertilization, embryo development, and cell numbers between the two regions (outer and inner) of each boar were evaluated using Student's *t*-test. Data is presented as a mean value ± standard error. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Effects of caffeine concentration on sperm separation by MIGLIS®

The recovery rates of sperm collected from the bottom of the inner tube were higher ($p < 0.05$) after incubation with 5 mM caffeine than after incubation without caffeine (Table 3.1). Sperm quality parameters, including total and progressive motility, sperm viability, plasma membrane integrity, and acrosome integrity of sperm collected after incubation with 2.5 and 5 mM caffeine were higher ($p < 0.05$) than sperm incubated without caffeine and with 10 mM caffeine. Moreover, sperm viability was higher ($p < 0.05$) after incubation with caffeine (2.5 mM) than after incubation with 5 mM caffeine.

3.2. Effects of incubation time on sperm separation by MIGLIS®

The recovery rate of sperm collected from the bottom of the inner tube was higher ($p < 0.05$) after incubation for 10 min than after incubation for 5 or 15 min (Table 3.2). Moreover, sperm

collected after incubation for 10 min had higher progressive motility than other incubation times ($p < 0.05$). All sperm quality parameters after incubation for 20 min were lower than those of sperm incubated for 5 or 10 min ($p < 0.05$).

3.3. Fertilization and embryonic development of porcine oocytes fertilized with separated sperm

When sperm were incubated with 2.5 mM caffeine for 10 min and used to fertilize in vitro-matured oocytes, the percentages of total and monospermic fertilization of sperm collected from the bottom of the inner space (selecting space) in boar B were higher ($p < 0.05$) than those of sperm from the bottom of the outer space (non-selecting space) (Table 3.3). Moreover, the blastocyst formation rates from sperm collected from the inner space were higher ($p < 0.05$) than those of sperm collected from the outer space, regardless of the individual boar.

4. Discussion

In this study, MIGLIS®-based selection of frozen-thawed porcine spermatozoa was optimized by combining the procedure with supplementation of 2.5 mM caffeine and an incubation period of 10 min. Prior to MIGLIS® processing, porcine semen was subjected to a single centrifugation step to remove extender components and standardize sperm concentration. This preparatory centrifugation was applied uniformly to all experimental groups and therefore did not constitute the selective step. Functional sperm enrichment occurred exclusively during the MIGLIS® phase, which relies on migration-gravity sedimentation and active sperm migration under low mechanical stress rather than centrifugal stratification.

When the MIGLIS® device is applied to human sperm collection, motility and mitochondrial integrity increase compared with the swim-up and Percoll® DGC methods; however, the sperm recovery rate is reduced [19, 35]. In our previous study, we demonstrated that the MS method using the MIGLIS® device increased the motility, viability, and acrosome integrity rates of frozen-thawed bovine sperm compared to washing and Percoll® DGC methods and decreased the recovery rates [104]. Consistent with these findings, the present study showed that sperm recovery in the inner tube was reduced to less than one-fifth (~20%), however, the number of recovered sperm remained sufficient for IVF applications.

Porcine spermatozoa exhibit high metabolic demands to sustain motility and therefore rely heavily on efficient energy metabolism. During sperm sorting procedures, motility decline may occur due to ATP depletion and mitochondrial metabolic disturbance, while oxidative imbalance under in vitro conditions can further impair sperm motility and viability [107, 108]. Caffeine, a methylxanthine compound, inhibits phosphodiesterase activity and increases intracellular cAMP levels, which in turn modulates Ca^{2+} channel activity and promotes Ca^{2+} influx. This signaling pathway enhances flagellar beating and supports sperm motility. Accordingly, caffeine supplementation in the separation medium during MIGLIS®-based sorting was intended to maintain sperm metabolic activity and stabilize motility, thereby facilitating the recovery of functionally competent spermatozoa.

In the present study, supplementation with 2.5 mM caffeine provided the most favorable balance between sperm recovery and functional quality during MIGLIS®-based selection. Although higher caffeine concentrations (>5 mM) increased sperm recovery rates, exposure to 10 mM negatively affected sperm quality, particularly motility, indicating a concentration-dependent response. The optimal performance observed at 2.5 mM caffeine suggests that moderate stimulation is sufficient to support sustained motility without inducing detrimental overstimulation. Previous studies have shown that caffeine can enhance sperm motility in a dose-dependent manner through Ca^{2+}-mediated regulation of flagellar activity [109, 110]. Moderate increases in intracellular Ca^{2+} promote coordinated and progressive flagellar movement, facilitating the sustained motility required for effective migration-sedimentation within the MIGLIS® device. In contrast, excessive Ca^{2+} influx at higher caffeine concentrations may disrupt flagellar coordination, leading to dysregulated or arrested motility characterized by persistent flagellar curvature [111]. Under MIGLIS® separation conditions, which rely on continuous and energy-efficient motility, such dysregulation likely reduces the ability of spermatozoa to successfully migrate into the inner collection region.

As incubation time increases, ROS accumulate and may exceed the buffering capacity of endogenous antioxidant systems, leading to redox imbalance and functional deterioration [112]. Therefore, it is important to elucidate the effect of the incubation time on sperm sorting using the MIGLIS® device to ensure enough recovered sperm, while maintaining adequate sperm quality. In the present study, we observed that incubation for 10 min increased the recovery rate of sperm in the inner tube and progressive motility but substantially decreased the sperm quality after separation with 20 min of incubation. These results suggest that sperm quality after separation using the MIGLIS device is incubation time dependent.

The barrier designed between the deposit (outer) and collecting area (inner) of the MIGLIS® device separates the motile and non-motile sperm, facilitating the collection of only the motile sperm-rich fraction. In the present study, we confirmed that the use of sperm collected from the inner tube increased blastocyst formation rates compared with sperm collected from the outer tube, regardless of the individual boar. Moreover, the percentages of total and monospermic fertilization improved in one of the boars; the other two boars had higher values for spermatozoa collected from the inner tube. In humans, the MS method using the MIGLIS® device has been recommended for selecting sperm with higher motility and greater fertilization ability for IVF [35]. Our results also indicate that the MS method may select sperm with higher fertilization ability for IVF.

In conclusion, the present study indicates that the recovery rate and quality of sperm collected after separation using the MS method with the MIGLIS® device depend on the caffeine concentration and incubation time. Furthermore, IVF using sperm separated by the MIGLIS® device has the potential to improve fertilization and embryonic development. Therefore, the MIGLIS® device may be useful for separating high-quality sperm and improving IVF outcomes.

FIGURES

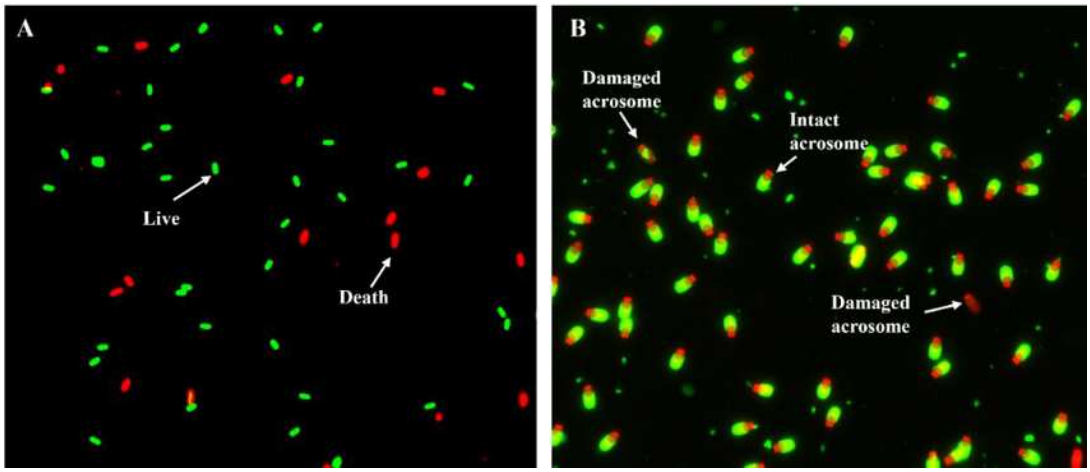


Figure 3.1. Representative fluorescent staining images of sperm viability and acrosome integrity. (A) Viability assessed using SYBR-14/propidium iodide (PI) staining; live spermatozoa show green fluorescence, whereas dead spermatozoa show red fluorescence. (B) Acrosome integrity evaluated using FITC-PNA/PI staining; spermatozoa acrosomes with green fluorescent were considered intact, whereas spermatozoa showing red fluorescence was considered damaged.

TABLES

Table 3.1. Effects of caffeine concentration on sperm separation using the MIGLIS® device.*

Caffeine concentration (mM)	Sperm recovery (%) **	Motility (%)		Viability (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
		Total	Progressive			
0	11.7 ± 1.7^{a}	15.4 ± 0.8^{a}	4.3 ± 0.7^{a}	8.0 ± 0.6^{ab}	6.6 ± 0.7^{a}	66.1 ± 1.2^{a}
1	16.5 ± 2.8^{ab}	29.8 ± 3.3^{b}	20.4 ± 2.8^{b}	9.9 ± 1.0^{bd}	9.7 ± 0.7^{bd}	73.1 ± 1.6^{bc}
2.5	14.7 ± 1.7^{ab}	30.9 ± 3.7^{b}	22.3 ± 3.5^{b}	18.1 ± 0.8^{c}	14.1 ± 1.0^{c}	77.7 ± 2.3^{b}
5	20.8 ± 2.9^{b}	29.5 ± 2.8^{b}	18.8 ± 2.4^{b}	11.4 ± 1.5^{d}	12.2 ± 1.0^{bc}	77.8 ± 3.1^{b}
10	19.5 ± 2.3^{b}	13.5 ± 1.4^{a}	4.8 ± 0.9^{a}	6.4 ± 0.7^{a}	9.2 ± 1.0^{ad}	68.0 ± 2.2^{ac}

Note: $^{a-d}$ Values with different superscript letters in the same column are significantly different ($p < 0.05$).

* Recovery rate and quality of frozen-thawed sperm migrated from the outer space to the inner space of the MIGLIS® device. Four replications were carried out. Percentages are expressed as mean ± SEM.

** Sperm recovery rate was calculated by dividing the number of sperm recovered from inner space by the number of sperm in the original frozen-thawed semen.

Table 3.2. Effects of incubation time on sperm separation using the MIGLIS® device. *

Incubation time (min)	Sperm recovery (%) **	Motility (%)		Viability (%)	Plasma membrane integrity (%)	Acrosome integrity (%)
		Total	Progressive			
5	8.7 ± 2.2[a]	25.3 ± 2.5[a]	16.2 ± 2.2[a]	7.2 ± 0.5[a]	8.1 ± 0.8[a]	74.9 ± 3.7[a]
10	19.1 ± 2.7[b]	31.0 ± 2.1[a]	21.8 ± 1.7[b]	10.5 ± 0.5[a]	10.8 ± 0.8[a]	77.1 ± 3.7[a]
15	12.6 ± 1.4[ac]	15.1 ± 1.6[b]	5.8 ± 1.0[c]	7.4 ± 0.6[a]	4.8 ± 0.5[b]	79.1 ± 2.0[a]
20	16.9 ± 2.3[bc]	16.6 ±1.0[b]	8.0 ± 0.8[c]	4.4 ± 0.5[b]	4.8 ± 0.5[b]	64.1 ± 1.2[b]

Note: [a-c] Values with different superscript letters in the same column are significantly different ($p < 0.05$).

* Recovery rate and quality of frozen-thawed sperm migrated from the outer space to the inner space of the MIGLIS® device. Four replications were carried out. Percentages are expressed as mean ± SEM.

** Sperm recovery rate was calculated by dividing the number of sperm recovered from inner space by the number of sperm in the original frozen-thawed semen.

Table 3.3. Fertilization and embryo development of porcine oocytes fertilized with sperm separated by the MIGLIS® device. *

Boar	Selected space	No. of examined oocytes	No. (%) of oocytes		No. of examined oocytes	No. (%) of oocytes		Total cell number of blastocysts
			Fertilized	Monospermy **		Cleaved	Developed to blastocysts	
A	Inner	49	37 (75.7 ± 2.2)	24 (64.9 ± 5.1)	418	323 (76.3 ± 6.1)	55 (13.1 ± 1.3)[a]	42.5 ± 2.2
	Outer	51	28 (54.5 ± 11.0)	12 (46.4 ± 8.8)	423	285 (66.6 ± 5.0)	25 (5.9 ± 0.5)[b]	45.8 ± 4.1
B	Inner	53	45 (85.1 ± 4.2)[a]	26 (57.8 ± 2.1)[a]	375	257 (68.7 ± 2.4)	55 (14.7 ± 1.1)[a]	39.7 ± 1.9
	Outer	50	30 (58.3 ± 5.6)[b]	8 (28.2 ± 5.5)[b]	373	228 (60.5 ± 3.0)	15 (3.8 ± 0.9)[b]	37.8 ± 4.0
C	Inner	39	31 (79.7 ± 9.0)	18 (59.1 ± 3.6)[a]	353	212 (62.1 ± 3.2)	29 (8.4 ± 0.4)[a]	47.2 ± 3.4
	Outer	40	21 (51.9 ± 12.2)	9 (43.3 ± 4.1)[b]	348	176 (52.6 ± 4.9)	11 (3.8 ± 0.9)[b]	44.3 ± 5.5

Note: [a-b] Values with different superscript letters on the same porcine are significantly different ($p < 0.05$).

* Four replications were carried out. Percentages are expressed as mean ± SEM.

** The proportions of monospermic fertilization were calculated by dividing the number of monospermic fertilized oocytes by the total number of fertilized oocytes.

GENERAL DISCUSSION

In the present study, migration-gravity sedimentation-based selection using the MIGLIS® device consistently enriched sperm populations with superior functional parameters in both bovine and porcine models. In Chapter 1, we evaluated the efficiency of MIGLIS® in selecting frozen-thawed bovine sperm and demonstrated that, compared with centrifugation-based approaches, MIGLIS® yielded a higher-quality sperm population. Importantly, the reduced oxidative burden observed in MIGLIS®-selected sperm likely reflects both intrinsic functional discrimination during migration and decreased mechanical stress during processing. Therefore, the observed improvements can be attributed to a combined effect of selective enrichment and reduced procedure-induced damage.

Notably, conventional semen parameters such as total and progressive motility only partially reflect biological heterogeneity [17, 18, 113]. Although motility remains a key functional attribute, it does not fully represent genomic integrity or metabolic resilience. Indeed, even highly motile spermatozoa may exhibit elevated levels of DNA fragmentation. In chapter 2, bovine sperm samples showed comparable routine quality parameters but markedly different reproductive performance in vitro and in vivo, and divergence in fertility was primarily associated with DNA fragmentation and closely linked to plasma membrane stability. These findings are consistent with previous recommendations to integrate DFI assessment with conventional semen evaluation [113]. Importantly, MIGLIS® significantly reduced the proportion of spermatozoa with fragmented DNA regardless of initial semen quality, supporting the concept that MIGLIS® based selection captures aspects of functional stability beyond motility alone. When applied to in vitro embryo production, MIGLIS®-selected sperm improved developmental outcomes; however, the magnitude of improvement depended on baseline semen quality.

Mechanistically, the efficiency of MIGLIS® appears to be associated with its migration-dependent selection principle. Sustained active migration requires coordinated maintenance of membrane integrity, mitochondrial function, and redox balance. Spermatozoa with cryo-induced sublethal damage may retain short-term motility yet lack the metabolic sustainability necessary for prolonged migration. Under the low-mechanical-stress conditions of MIGLIS®, separation is not governed by centrifugal force or density gradients but by the capacity of spermatozoa to maintain functional stability throughout the migration phase. Within this framework, migration may function as a biological endurance filter rather

than a purely kinematic sorter. While this model is supported by the present functional observations, detailed molecular characterization of mitochondrial efficiency, oxidative dynamics, and chromatin remodeling will be required to further substantiate the proposed mechanism.

A major strength of this study is the consistency of functional enrichment across two physiologically distinct species. Although bovine and porcine semen required different preparatory adjustments, the core selection event occurred exclusively during the migration phase. In porcine semen (Chapter 3), preliminary centrifugation was necessary for extender removal and concentration standardization, along with antioxidant supplementation and shorter incubation to prevent excessive oxidative stress and ATP depletion. Importantly, these steps were uniformly applied to all porcine groups and did not constitute the selective mechanism. Bovine spermatozoa, exhibiting greater cryotolerance, underwent MIGLIS® processing without additional protective adjustments. The comparable functional improvements observed across species suggest that the efficiency of MIGLIS® is primarily attributable to migration-based discrimination rather than species-specific preprocessing.

Despite these promising findings, several limitations should be acknowledged. Fertility outcomes were inferred primarily from laboratory-based quality indicators and in vitro embryo development rather than large-scale field fertility trials. Therefore, validation under commercial breeding conditions is necessary to determine translational applicability.

Overall, the present findings support the potential utility of MIGLIS® device-based sperm selection as a low-mechanical-stress strategy for enriching functionally competent spermatozoa following cryopreservation, particularly in samples exhibiting elevated baseline heterogeneity.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

General conclusions

This study demonstrates that MIGLIS® device-based sperm selection has the potential to effectively enrich functionally competent spermatozoa after cryopreservation in both bovine and porcine models.

Scientific significance

This work provides the first systematic evaluation of MIGLIS® device-based sperm selection system in livestock. The results contribute to improved understanding of post-thaw sperm heterogeneity and support the development of species-specific, low-mechanical-stress selection systems aimed at enhancing fertilization efficiency and embryo development in livestock reproduction.

Limitations and future perspectives

Although the present study provides invaluable insights, several limitations should be considered. Reproductive performance was evaluated predominantly through laboratory-based quality assessments and in vitro embryo development, rather than through large-scale field fertility trials. Consequently, confirmation under commercial breeding conditions will be essential to establish the practical and translational relevance of MIGLIS®-based selection.

Furthermore, the proposed mechanistic links were derived mainly from indirect functional indicators. Future investigations should incorporate comprehensive oxidative stress profiling, in-depth analyses of mitochondrial bioenergetics, molecular assessment of chromatin stability, and well-designed in vivo fertility trials to more precisely define the underlying biological mechanisms and long-term reproductive benefits of this selection approach.

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