(Appended form No. 3)

Abstract of Doctoral Thesis

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Title: 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）

Abstract of thesis:

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

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(Appended form No. 3)

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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学位論文審査の結果の要旨

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題　　目	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）

審査結果の要旨：

凍結保存精液は家畜の人工授精や生殖補助技術に不可欠であるが、凍結融解過程において精子の運動性や受精能が低下することが大きな課題となっている。現在、融解後精子の選別には遠心分離法が広く用いられているが、遠心操作は精子に物理的ストレスや酸化ストレスを与え、精子機能をさらに低下させる可能性が指摘されている。本研究は、遠心分離を必要としない新規精子選別デバイスMIGLIS（Migration-Gravity Sedimentation Device）を用い、ウシおよびブタの凍結融解精子に対する有効性を検証するとともに、その最適な利用条件を明らかにすることを目的として実施された。

第1章では、ウシ凍結融解精子を用い、MIGLIS法と従来の洗浄法およびPercoll密度勾配遠心法との比較を行った。まず、MIGLIS法における精子希釈条件を最適化した後、精子機能、活性酸素種（ROS）産生および体外受精後の胚発生能について評価した。その結果、MIGLIS法では回収率は低いものの、機能性の高い精子が選択され、精子および初期胚におけるROS産生が有意に低下したことから、酸化ストレスを軽減する低侵襲な精子選別法であることが示された。

第2章では、通常の精液検査では差が認められないにもかかわらず受精成績が異なる高受胎群および低受胎群のウシ凍結融解精子を用いて、MIGLIS法の有効性を検討した。その結果、低受胎群ではDNA断片化率が高く、これが受精能低下の主要因であることを明らかにした。また、MIGLIS法によりDNA断片化率が低減し、低受胎群における胚発生能が改善されたことから、本法は従来の精液検査では検出できない精子機能異常の改善にも有効であることが示された。

第3章では、耐凍性が低いブタ凍結融解精子を対象として、MIGLIS法の適用条件を検討し

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た。カフェイン添加濃度および処理時間を最適化した結果、2.5 mM カフェイン添加および10 分間処理が最適条件であり、この条件下では精子機能が維持されるとともに、体外受精後の胚発生率が有意に向上した。

本研究は、ウシおよびブタを対象として、遠心分離を用いない新規精子選別法 MIGLIS の有効性を初めて体系的に検証するとともに、種差を考慮した最適条件を明らかにした点で高く評価される。さらに、本法が精子の酸化ストレスおよび DNA 損傷を軽減し、生殖補助技術における受精成績や胚発生能の向上に寄与する可能性を示したことは、家畜繁殖分野のみならず獣医繁殖学の発展に貢献する重要な成果である。

以上により本論文は博士（獣医学）の学位論文として十分な価値を有するものと判定した。