Evaluation of bovine semen quality based on sperm organelle functions and motility subpopulation structures

（精子の細胞小器官機能および運動様式を指標にした

牛精液品質評価法の検討）

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Abbreviation

AI: Artificial insemination

ALH: Amplitude of lateral head displacement

ANOVA: Analysis of variance

BCF: Beat cross frequency

BO: Brackett and Oliphant medium

BSA: Bovine serum albumin

CASA: Computer-assisted sperm analysis

DPBS: Dulbecco's phosphate-buffered saline without calcium and magnesium

EYT: Egg yolk-tris

EYTG: Egg yolk-tris containing glycerol

FITC-PNA: Fluorescein peanut agglutinin FITC conjugate

hr: hour

JC-1: 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethyl- benzimidazolyl-carbocyanine iodide

LIN: Linearity

LN_2: Liquid nitrogen

min: minute

MTDR: MitoTracker Deep Red

PE-PNA: Phycoerythrin-conjugated peanut agglutinin

PI: Propidium Iodide

ROS: Reactive oxygen species

SD: Standard deviation

sec: second

SEM: Standard error of mean

SOF: Synthetic oviductal fluid

STR: Straightness

VAP: Average path velocity of sperm

VCL: Curvilinear velocity

VSL: Straight line velocity

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Preface

Artificial insemination (AI) with frozen-thawed semen has become a fundamental technique for the reproduction of dairy and beef cattle. All processing steps of semen cryopreservation induce several damages to sperm and reduce their fertility[87]. Therefore, quality control of semen is required and many kinds of laboratory assays for the evaluation of semen quality have been developed. Evaluation of sperm motility has long been one of the most common factor for predicting male fertility[3]. Then, cellular structures, such as plasma membrane, acrosome, and morphology and functions, such as mitochondrial membrane potentials have also been used as indicators of sperm fertility[21, 48, 78, 80]. However, results from any single laboratory assay could not estimate accurately the fertilizing potential of semen samples[29]. In addition, most of the conventional examinations were subjective and the results could vary among observers[42]. Combined multiple examinations are necessary, therefore, to estimate the characteristics of sperm more accurately.

Fluorescent staining for evaluating by fluorescent microscopy or flow cytometry has also become in widespread use as a tool to evaluate sperm characteristics that include plasma membrane integrity, acrosomal integrity, mitochondrial membrane potential, DNA integrity, and oxidative stress[53, 56, 84]. Recently, the technique of multicolor staining for simultaneous evaluation of multiple sperm characteristics has been used[14, 64] because evaluation of multiple sperm characteristics simultaneously can be a powerful tool for the prediction of semen fertility. However, it is difficult to evaluate multiple sperm characteristics simultaneously by using fluorescent probes because some probes have similar emission and overlap each other. In the last several decades, computer-assisted sperm motility analysis (CASA) was introduced as the tool for objective evaluation method of sperm characteristics[83]. Cluster analysis of CASA data was reported as a suitable method to classify the sperm to different motility subpopulations, helping to take advantage of the information contained in CASA datasets in many species[52].

However, the application of sperm motility subpopulation analysis for bull semen was limited and there were a few reports[24, 61-63]. Therefore, the relationship between sperm motility subpopulation structures and fertility was unclear.

In order to evaluate the relationship between sperm characteristics and fertility accurately, a comparison of the results derived from the laboratory assay of sperm characteristics and semen fertility is necessary. However, the conception rate after AI in cattle fluctuates among practitioners[27], season[13], and reproductive management[66]. Therefore, it is necessary to use some models, which can provide the obvious difference in fertility. Semen before and after sex-sorting may be a candidate of the model for decreased fertility. Sex-sorted semen shows lower fertility than non-sorted semen because sorting process has detrimental effects on sperm to reduce their fertilization capacity[70]. A set of two successive ejaculates at the same day may be another candidate. Because these semen shows the different sperm motility and fertility[82].

To achieve the above-mentioned purpose, 4 studies were carried out in the present study. In Chapter I, the author developed the quadruple staining procedure for bovine sperm by 4 fluorescent dyes as an objective simultaneous evaluation procedure for plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm. In Chapter II, the effect of freezing and chilling preservation on plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm were evaluated by using the quadruple fluorescent staining. Then, the author also examined the usefulness of cluster analysis to evaluate the structures of sperm motility subpopulation in the first and second ejaculates as the model of different motility and fertility sperm in Chapter III. In Chapter IV, the quality of sex-sorted and non-sorted semen were compared by using evaluation methods developed in Chapters I, II, and III for predicting the fertility of frozen-thawed semen.

Chapter I

Simultaneous evaluation of plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential in bovine sperm by flow cytometry

Introduction

AI using frozen-thawed bull semen is a generally used technique for the reproduction of dairy and beef cattle. It was reported that the improvement of frozen-thawed semen quality, such as motility, malformation, and concentration of sperm in semen, was positively correlated with the pregnancy rate[10]. Semen collected from bulls is diluted, cooled, and frozen for long-term storage until insemination into the female genital tract. All processing steps of semen cryopreservation may induce damage to the plasma membrane and cellular structure of sperm[32, 73, 87]. Therefore, the evaluation of sperm characteristics by laboratory assays is very important to achieve a high pregnancy rate by AI using frozen-thawed semen. There are several reports dealing with criteria for the evaluation of various sperm characteristics: motility, viability, morphological abnormality, and organelle functions[21, 48, 78, 80]. However, most of the evaluation methods in these reports are subjective because they are achieved by microscopic observation and the obtained results may fluctuate depending on the practitioner. Therefore, objective and quantitative methods should be chosen for the evaluation of sperm characteristics. Moreover, it is thought that the results from any single laboratory assay will not effectively estimate the fertilizing potential of a semen sample[29]; therefore, combined multiple assays are necessary to estimate the characteristics of sperm more accurately. Recently, flow cytometry has been used as an objective tool for evaluating multiple characteristics of a large number of sperm[84]. Nagy et al.[64] demonstrated that triple staining by SYBR-14, propidium iodide (PI), and phycoerythrin-conjugated peanut agglutinin (PE-PNA) was effective for evaluation of the

viability and acrosomal integrity of bovine sperm simultaneously. In addition, Thomas et al.[80] proved that mitochondrial membrane potential of sperm could be assessed by flow cytometry using 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethyl- benzimidazolyl-carbocyanine iodide (JC-1) as a probe. If these two methods can be combined, I can evaluate 3 items, viability, acrosomal integrity and mitochondrial membrane potential, of sperm simultaneously and it is possible to obtain more detailed information about each sperm. However, the combination of these reagents for flow cytometry cannot be achieved because such staining uses the same excitation (488 nm) and the broad-emission spectral properties of JC-1 (green, 510-520 nm, and red-orange, 590 nm) overlap with SYBR-14 (517 nm). It is also difficult to distinguish JC-1 from PI (617 nm) and PE-PNA (580 nm) by flow cytometry. Celeghini et al.[14] reported simultaneous evaluation of viability, acrosome integrity, and mitochondrial membrane potential using fluorescent microscopy. However, their method cannot be applied to flow cytometry because they used PI and JC-1. Hallap et al.[31] reported that sperm having high mitochondrial membrane potential, which were judged by double staining with SYBR-14 and MitoTracker Deep Red (MTDR), showed high motility. An excitation laser of MTDR (640 nm) is different from those of SYBR-14, PI, and PE-PNA (488 nm). Moreover, MTDR is known as a highly specific probe for mitochondria[53]. Therefore, MTDR is a candidate for evaluating mitochondrial membrane potential simultaneously with triple staining mentioned above.

In the present study, I aimed to develop an objective evaluation procedure for plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm simultaneously using flow cytometry after staining with SYBR-14, PI, PE-PNA, and MTDR.

Materials and Methods

Semen

Frozen semen, which was diluted with egg yolk-tris (EYT) containing 6% (v/v) glycerol extender and packed in 0.5-ml straw, derived from the same ejaculates of 5 Holstein bulls donated from Genetics Hokkaido Association (Sapporo, Japan), were used for this study. The semen was thawed at 37˚C for 45 sec in water and expelled into a 1.5-ml tube. The thawed semen was used for different staining, as follows. Dead sperm used in experiment I-1 were prepared by thawing at 37˚C in water and refreezing in liquid nitrogen twice.

Double staining for evaluation of mitochondrial membrane potential

Staining solution was prepared as described in a previous study[31]. In brief, 100 µl of MTDR (final concentration 10 nM; M22426, Life Technologies, Carlsbad, CA, USA), 1 µl of SYBR-14 (final concentration 100 µM; L-7011 LIVE/DEAD Sperm Viability Kit, Molecular Probes, Eugene, OR, USA) and 800 µl of Dulbecco's phosphate-buffered saline without calcium and magnesium (DPBS) were mixed (staining solution). Then, 100 µl of semen was mixed with staining solution and warmed at 37˚C for 10 min in the dark.

Triple staining for evaluation of viability and acrosomal integrity of sperm

Staining solution was prepared as described in a previous study[64]. Briefly, 1 µl of SYBR-14, 2.5 µl of PE-PNA (final concentration 2.5 µg/ml; GTX01509, GeneTex, Irvine, CA, USA), 5 µl of PI (final concentration 12 µM; L-7011, LIVE/DEAD Sperm Viability Kit, Molecular Probes), and 900 µl of DPBS were mixed (staining solution). Then, 100 µl of semen was mixed with staining solution and warmed at 37˚C for 10 min in the dark.

Quadruple staining for simultaneous evaluation of viability, acrosomal integrity, and mitochondrial membrane potential of sperm

The same volume and types of fluorescent dye as in the triple staining along with 100 µl of MTDR solution were added to 800 µl of DPBS (staining solution). Then, 100 µl of semen was mixed with the staining solution and warmed at 37˚C for 10 min in the dark.

Analysis by flow cytometry

After staining, 10 µl of 10% (v/v) formaldehyde (final concentration 0.1%) was added to all samples (1,000 µl) to immobilize the living sperm in the staining solution, as described in a previous study[34]. Subsequently, 100 µl of stained sample was mixed with 400 µl of DPBS and subjected to flow cytometry. Sperm suspensions were run through a flow cytometer (FACS VerseTM, BD Biosciences, San Jose, CA, USA). SYBR-14, PI, and PE-PNA were excited using a 488-nm excitation laser and detected in an FITC filter (527/32 nm), PE-filter (586/42 nm), and Per-CP-Cy5.5 filter (700/54 nm), respectively. MTDR was excited at 640 nm and detected in an APC filter (660/10 nm). Flow cytometric gating of sperm was performed as reported by Hallap et al.[31] and Nagy et al.[64]. The gating of quadruple staining was performed as described in Fig. I-1. Briefly, particles stained with SYBR-14 or PI were judged as sperm (Fig. I-1 A). Sperm were divided into 2 groups (live and dead) by PI emission (Fig. I-1 B) and then each group was gated by PE-PNA and MTDR (Figs. I-1 C and D). Fluorescent data of all events were collected until 10,000 gated events were counted. Triplicate measurements per sample were conducted and the average was used as a value of the sample.

Analysis by fluorescent microscopy

After staining and immobilization, an 8-µl sample was loaded on a slide, coverslipped, and evaluated immediately under a fluorescent microscope (ECLIPSE Ci, Nikon, Tokyo, Japan)

equipped with a B-2A filter (excitation 450-490 nm and emission >520 nm) and a G2-A filter (excitation 510-560 nm and emission >590 nm) at × 400 magnification. Microscopic examination was mainly conducted by using a B-2A filter. A G2-A filter was used for the evaluation of plasma membrane integrity when PI emission was not clear. Two hundred sperm per slide were examined and classified based on the fluorescence emitted from each probe (Table I-1). Three slides per sample were examined and the average was used as the value of the sample.

Evaluation of sperm characteristics

Sperm characteristics estimated by flow cytometry are described in Table I-1. Briefly, when sperm were stained with PI, they were evaluated as dead because the damage to the plasma membrane allowed the PI to penetrate inside them. When acrosome was stained with PE-PNA, it was evaluated as damaged. When the midpiece of sperm was stained with MTDR, it was evaluated that the sperm had high mitochondrial membrane potential.

Sperm characteristics evaluated by fluorescent microscopy are expressed in Fig. I-2. Sperm stained with PI were evaluated as dead in the same way as by flow cytometry. When acrosomal region of sperm was stained with PE-PNA, it was evaluated as damaged acrosome. Some sperm were stained with PE-PNA intermediately (Fig. I-2 C), they were also judged as sperm with a damaged acrosome.

Experimental design

Experiment I-1: Frozen-thawed semen was mixed with 0, 25, 50, 75, and 100% dead sperm. These samples were subjected to triple staining. After staining, half of the sample was evaluated by flow cytometry and the other half by fluorescent microscopy; the obtained results were then compared. Semen derived from 5 bulls was used for this experiment.

Experiment I-2: Frozen-thawed semen derived from a bull was subjected to double, triple, and quadruple staining. Then, the results of the sperm characteristics estimated by quadruple staining were compared with those of double or triple staining samples. The experiment was repeated 4 times on independent samples.

Statistical analysis

Statistical analysis was performed using JMP 9.0.2 (SAS, NC, USA). The correlation between each characteristic of sperm estimated by flow cytometry and by fluorescent microscopy was analyzed by linear regression analysis. The percentages of sperm characteristics examined using different equipment and different staining procedures were compared by Student's *t*-test. Differences with $P<0.05$ were recognized as significant.

Results

Experiment I-1

The viability and acrosomal integrity of sperm evaluated by flow cytometry and fluorescent microscopy were significantly correlated ($r>0.9$, $P<0.01$), except for the live sperm with a damaged acrosome ($P=0.866$), as shown in Fig. I-3. The percentages of each characteristic evaluated by flow cytometry and fluorescent microscopy are shown in Table I-2. There were no significant differences in all characteristics (live sperm with an intact acrosome, live sperm with a damaged acrosome, dead sperm with an intact acrosome, and dead sperm with a damaged acrosome) evaluated by the two types of equipment ($P>0.05$). The percentages of live sperm with a damaged acrosome were low among the samples with different mixed ratios of dead sperm.

Experiment I-2

The viability, acrosomal status, and mitochondrial membrane potential of sperm evaluated by flow cytometry after quadruple staining and by the other staining procedures are shown in Table I-3. There were no significant differences in all characteristics evaluated by quadruple staining and the other procedures ($P>0.05$). By quadruple staining, more than 95% of the live sperm having an intact acrosome showed high mitochondrial membrane potential. In addition, more than 95% of dead sperm having an intact acrosome showed low mitochondrial membrane potential.

Discussion

In the present study, the results of sperm characteristics evaluated by flow cytometry and fluorescent microscopy were similar, except for live sperm with a damaged acrosome. High correlation may be due to the criteria of sperm evaluation. The count of fluorescent intensity obtained by flow cytometry indicated two obvious peaks, those evaluated as negative and positive. However, PE-PNA positive peak had a broad base toward the low intensity (10^3-10^4) as shown in Fig. I-1 E, and this small peak might be a subpopulation of sperm those observed as intermediately stained and judged as positive under fluorescent microscopy. Therefore, evaluation of sperm using two equipment could evaluate sperm characteristics by same criteria and provide us similar results. This exception may have been caused by quite a small population (<1%) of live sperm with a damaged acrosome in semen. The number of sperm evaluated by flow cytometry was about 50 times greater than by fluorescent microscopy, which examined about 200 sperm[14, 74]. In spite of evaluating a large number of sperm, quite a small population of this type of sperm may indicate that sperm die immediately after damage to the acrosome.

In the present study, the viability, acrosomal integrity, and mitochondrial membrane potential of sperm could be evaluated accurately by quadruple staining without interference of fluorescent dye. The present results mean that most of the sperm with damage to the plasma membrane had impaired mitochondrial membrane potential, even though about two-third of them had the intact acrosome. Mitochondria produce ATP, which is required for housekeeping of the plasma membrane of sperm[73]. In the present study, most of the live sperm high mitochondrial membrane potential, while the dead sperm showed a low one. Low mitochondrial membrane potential indicates a decrease or lack of ATP production. A decrease of ATP production may become a cause of sperm death without acrosomal damage. Mitochondrial activity is crucial and correlates with the fertilization ability of sperm[2]. In further study, the relationship between fertility and sperm characteristics as evaluated by flow cytometry using quadruple staining should be carried out.

A staining method to estimate the viability of sperm, acrosomal integrity, and mitochondrial functions simultaneously by using four fluorescent dyes under a fluorescent microscope (Hoechst 33342, PI, FITC-PSA, and JC-1) has also been reported[14]. However, in this previous study[14], only hundreds of sperm could be evaluated subjectively. On the other hand, the method developed in the present study enables the objective estimation of more than 10,000 sperm by flow cytometry in a short time. This means that the characteristics of sperm can be evaluated more accurately and quickly than ever by our procedure.

In conclusion, the quadruple staining using SYBR-14, PI, PE-PNA, and MTDR for flow cytometry can evaluate the plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bovine sperm simultaneously. The procedure can be applied to the quality control of bovine frozen-thawed semen.

Table I-1. Staining patterns and evaluation of sperm characteristics by each staining method

Staining procedure	Staining pattern			Sperm characteristics		
	PI	PE-PNA	MitoTracker Deep Red	Viability	Acrosome	Mitochondrial membrane potential
Double	n.e.	n.e.	+	n.e.	n.e.	high
	n.e.	n.e.	-	n.e.	n.e.	low
Triple	-	-	n.e.	Live	Intact	n.e.
		+	n.e.		Damaged	n.e.
	+	-	n.e.	Dead	Intact	n.e.
		+	n.e.		Damaged	n.e.
Quadruple	-	-	+	live	Intact	high
			-			low
		+	+		Damaged	high
			-			low
	+	-	+	dead	Intact	high
			-			low
		+	+		Damaged	high
			-			low

+; fluorescence-positive.
-; fluorescence-negative.
n.e.; not evaluated.

Table I-2. Characteristics of bovine sperm, the mixture of thawed semen and dead sperm, evaluated by flow cytometry and fluorescent microscopy after triple staining

Equipment	Sperm characteristics		% of sperm classified for each characteristic				
	Viability	Acrosome	1:0*	3:1*	1:1*	1:3*	0:1*
Flow cytometry	Live	intact	63.7±5.6	47.7±4.6	31.5±3.8	15.3±2.4	0.0±0.0
		damaged	0.3±0.2	0.2±0.1	0.2±0.1	0.0±0.0	0.0±0.0
	dead	Intact	22.0±4.7	17.6±3.8	12.9±2.0	7.6±1.0	1.8±1.0
		damaged	14.0±3.2	34.5±2.7	55.3±2.5	77.0±1.9	98.0±1.1
Fluorescent microscopy	live	intact	60.0±5.4	44.8±3.8	29.0±5.1	10.4±5.4	0.0±0.0
		damaged	0.1±0.2	0.0±0.1	0.1±0.1	0.0±0.1	0.0±0.0
	Dead	intact	24.4±6.2	17.5±3.8	12.3±2.9	7.5±1.8	1.3±0.7
		damaged	15.5±6.5	35.7±7.8	58.6±6.3	79.6±1.6	98.7±0.7

Values are the mean ± standard deviation (SD, 5 bulls/group).
*; Mixed ratio of frozen-thawed semen and dead sperm.

Table I-3. Sperm characteristics evaluated by flow cytometry using different staining procedures

Sperm characteristics			% of sperm characteristics evaluated by each staining		
Viability	Acrosome	Mitochondrial membrane potential	Quadruple	Triple	Double
Live	intact	High	64.7±1.5	-	-
		Low	2.0±0.5	-	-
		Total	66.8±2.3	67.0±1.4	-
	damaged	High	0.1±0.1	-	-
		Low	0.0±0.0	-	-
		Total	0.1±0.1	0.1±0.1	-
Dead	intact	High	0.9±0.4	-	-
		Low	21.2±1.1	-	-
		Total	22.0±1.8	21.7±1.6	-
	damaged	High	0.4±0.1	-	-
		Low	10.7±1.3	-	-
			11.1±1.4	11.2±0.4	-
Total of high mitochondrial activity			66.1±1.5	-	67.9±1.5
Total of low mitochondrial activity			33.9±1.5	-	32.1±1.5

Values are the mean ± SD (4 replicates).

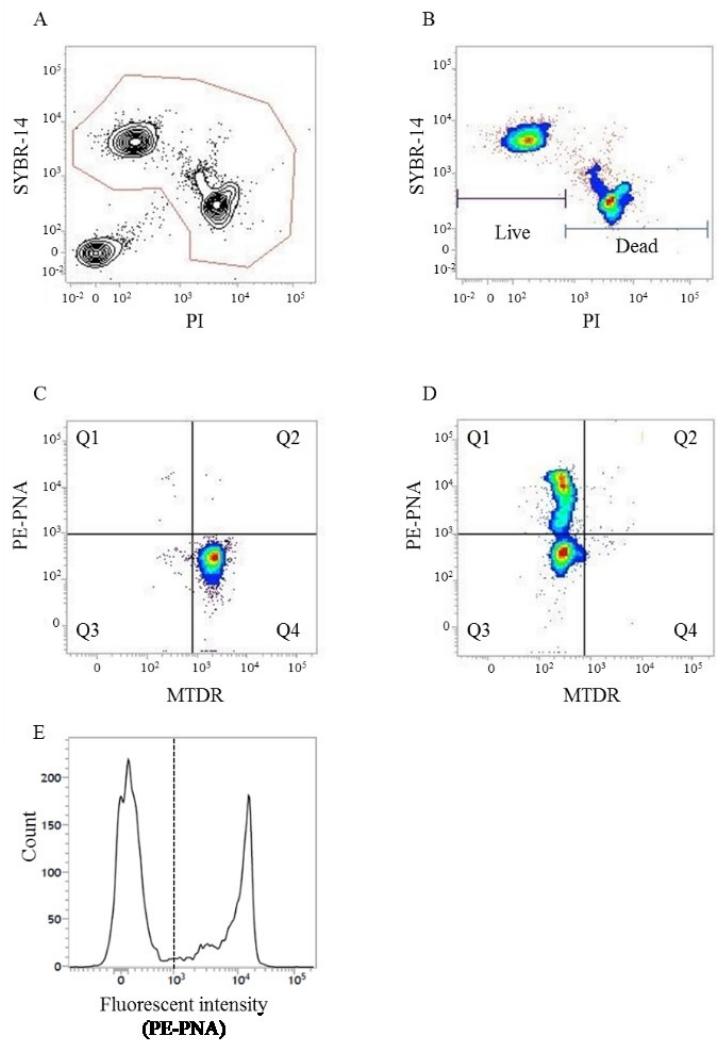

Fig. I-1. Gating procedure and judgement for quadruple staining analysis by flow cytometry
Items stained with SYBR-14 and propidium iodide (PI) were distinguished as sperm and gated from all events (area in red line; A). Gated sperm were divided into live and dead clusters (B) followed by classification into 4 groups (Q1-4) by acrosome integrity and mitochondrial membrane potential in live (C) and dead (D) sperm. (Q1) damaged acrosome with low mitochondrial membrane potential, (Q2) damaged acrosome with high mitochondrial membrane potential, (Q3) intact acrosome with low mitochondrial membrane potential, and (Q4) intact acrosome with high mitochondrial membrane potential. PE-PNA: phycoerythrin-conjugated peanut agglutinin, MTDR: MitoTracker Deep Red. The judgement of each sperm characteristic by flow cytometry depended on fluorescent intensity. Sperm with low fluorescent intensity ($<10^3$) was judged as PE-PNA negative (intact acrosome) and sperm with high fluorescent intensity ($\geq 10^3$) was judged as PE-PNA positive (damaged acrosome) (E).

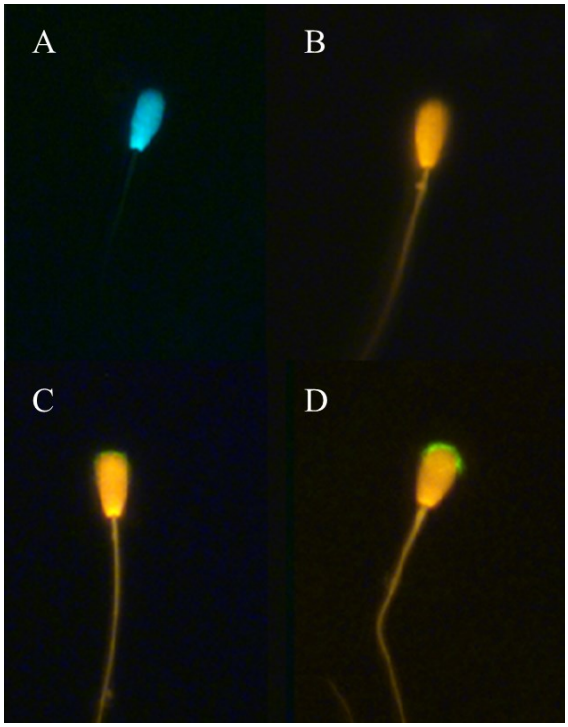

Fig. I-2. The photographs of sperm triple staining taken by a florescent microscopy
The head of sperm stained with SYBR-14 and acrosomal region not stained with PE-PNA (A) were judged as a live sperm with an intact acrosome. The head of sperm stained with PI but acrosomal region not stained with PE-PNA (B) was judged as dead sperm with an intact acrosome. The heads of sperm stained with PI and acrosomal region stained intermediately (C) and completely with PE-PNA (D) were both judged as a dead sperm with a damaged acrosome.

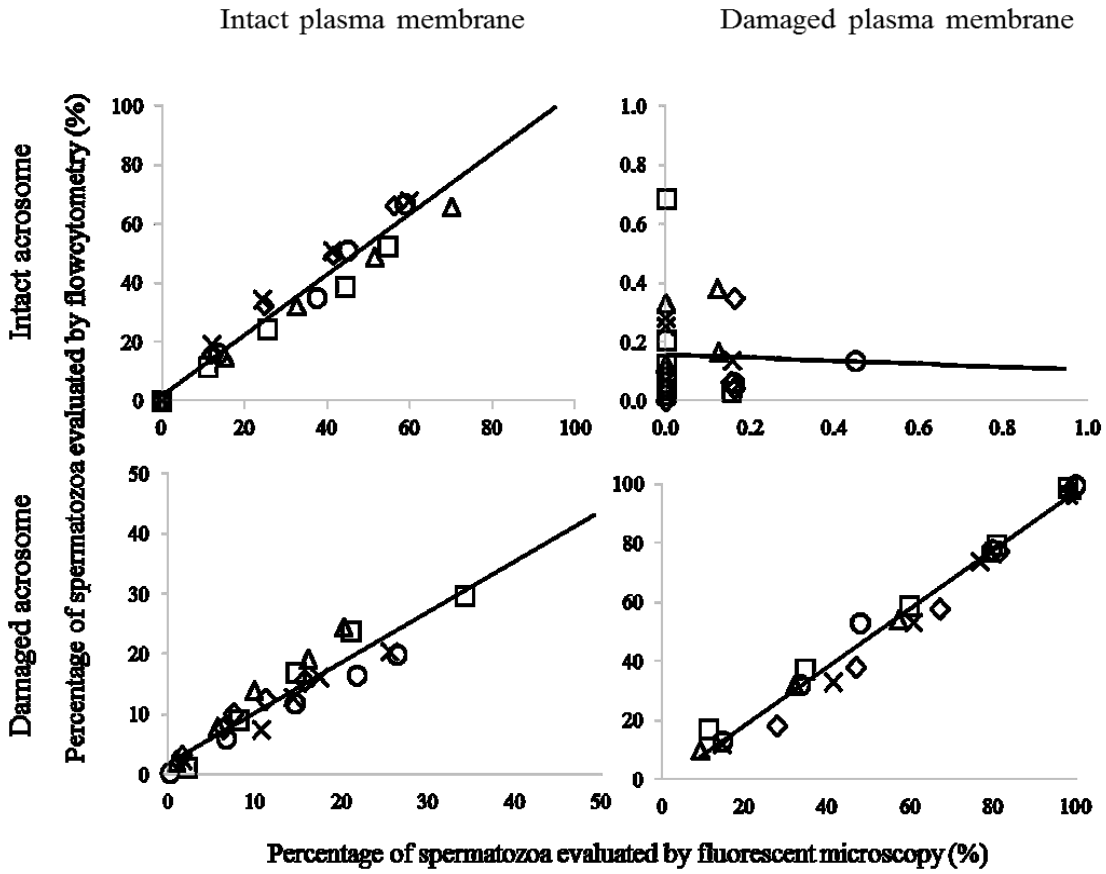

Fig. I-3. Scatter plots and regression lines of percentages of sperm evaluated by flow cytometry and fluorescent microscopy

Semen from 5 bulls was used and data from the same bull are indicated by the same symbol.

Summary

The present study aimed to develop an objective evaluation procedure to estimate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bull sperm simultaneously by flow cytometry. Firstly, I used frozen-thawed semen mixed with 0, 25, 50, 75, and 100% dead sperm. Semen was stained using three staining solutions: SYBR-14, propidium iodide (PI), and phycoerythrin-conjugated peanut agglutinin (PE-PNA), for the evaluation of plasma membrane integrity and acrosomal integrity. Then, the characteristics evaluated by flow cytometry and by fluorescent microscopy were compared. In terms of the results, the characteristics of sperm (plasma membrane integrity and acrosomal integrity) evaluated by flow cytometry and by fluorescent microscopy were similar. Secondly, I attempted to evaluate plasma membrane integrity, acrosomal integrity, and also mitochondrial membrane potential of sperm by flow cytometry using conventional staining with three dyes (SYBR-14, PI, and PE-PNA) combined with MitoTracker Deep Red (MTDR) staining (quadruple staining). Then, the sperm characteristics evaluated by flow cytometry using quadruple staining were compared with those of staining using SYBR-14, PI, and PE-PNA and staining using SYBR-14 and MTDR. From the obtained results, there were no significant differences in all characteristics (viability, acrosomal integrity, and mitochondrial membrane potential) evaluated by quadruple staining and the other procedures. In conclusion, quadruple staining using SYBR-14, PI, PE-PNA, and MTDR for flow cytometry can evaluate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bovine sperm simultaneously.

Chapter II

Effects of semen preservation procedure in egg yolk-tris based extender on bull sperm characteristics

Introduction

AI has become a fundamental technique for the reproduction of dairy and beef cattle. Semen freezing has been generally used for the preservation of sperm, and liquid preservation at approximately 5˚C is also used in some countries[25, 85]. In a typical semen freezing procedure, semen is pre-diluted by buffered extender, cooled to low temperature (approximately 5˚C), and second dilution by buffered extender containing cryoprotectant agents[85]. Then, semen is preserved in liquid condition at low temperature or in liquid nitrogen (LN_2) after freezing. Semen dilution by a buffered extender is necessary for liquid preservation[25], same as freezing procedure[85]. All steps of semen preservation can cause damages to the plasma membrane and other cell structures of sperm due to the effect of changing osmolality, temperature, and generating reactive oxygen species (ROS)[87]. These damages reduce semen fertility[73, 90]. Therefore, development of a novel preservation method is needed for improving semen quality and fertility in field.

In addition, the use of sex-sorted semen has become spread all over the world[5, 69]. However, sex-sorted semen has indicated lower fertility than unsorted semen because sorting process and extended preservation period before sorting have been attributed to the low fertility[69]. Sex-sorting process needs longer time, because the number of sperm sorted per hr is only 20 million cells[69]. Typically, semen is sorted in a small portion, and a part of semen is exposed to buffered solution over night before sorting. This situation may be similar to liquid preserved semen. Therefore, it is necessary to estimate the damage to sperm in detail

for the determination of critical points of preservation process and to improve the preservation procedures.

Many researchers reported the correlation between a procedure of bull semen preservation and bull semen quality or fertility[72, 81]. Recently, some reports showed the methods to evaluate sperm characteristics simultaneously by using fluorescent staining[14]. In addition, a previous study suggested that combination of the evaluating oxidative stress, acrosomal integrity, DNA compaction, mitochondrial activity, sperm viability, movement of velocity, and morphologic abnormalities give us a reasonable prediction of bovine semen fertility[71]. However, there are few reports that estimated the damages of sperm during semen preservation procedure by using evaluation method of multiple characteristics simultaneously. To fine-tune semen preservation procedure to improve fertility of semen, we should know which steps or treatments compromise the function of sperm. The purpose of this study is to assess the effect of freezing and liquid preservation procedures on plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm preserved in a conventional extender, EYT[25] by using multiple fluorescent staining.

Materials and Methods

Semen collection, processing and sampling for experiment

Three Japanese black bulls used in the present study (4-6 years old) were kept for the commercial production of frozen semen in the AI center (Genetics Hokkaido). Fertility of the bulls was proved by AI in the field. In the present study, semen was collected by artificial vagina, and only the first ejaculate was used. Immediately after collection, the glass tube containing ejaculate was removed from artificial vagina. Then the volume of semen was evaluated by visually checking the scale on the tube, while concentrations of sperm were

assessed using a photometer (SDM 5 12300/0007 DE, Minitube, Tiefenbach, Germany). Five ejaculates were obtained from each bull (15 ejaculates in total), and semen collection were performed twice a week. Semen volume and concentrations of sperm of samples were within the ranges from 3.5 to 7 ml and from 800 to 2,030 × 10^6 cells/ml, respectively.

Each semen sample was divided into two parts to use for the following experiments. For assessing the relationship between sperm damage and the steps of semen preservation procedure, we sampled the processing semen at various timing as described below. Firstly, 2 ml of an ejaculate were mixed with a same volume of EYT (200 mM Tris, 63.76 mM citric acid, 55.5 mM fructose, 20% (v/v) egg yolk; pH: 6.6)[25] warmed in hot water at 37˚C for pre-dilution. Diluted sample was cooled by conventional method. Namely, the glass tube including diluted sample was immersed in approximately 200 ml of 32˚C water in a 500-ml plastic beaker. Then, the beaker was set in a cold room and leaved for approximately 90 min until sample temperature decreased to 4˚C (conventional cooling). Then to adjust sperm concentration to 80 × 10^6 cells/ml, EYT was added to cooled sample. After that, same volume of EYT containing 12% (v/v) glycerol (12% EYTG) was added (final concentration of glycerol, 6%) and drawn into 0.5-ml straws and frozen by a program freezer (DIGITcool, IMV Technologies, L'Aigle, France). The final concentration of sperm in the straws were 40 × 10^6 cells/ml. Straws containing frozen samples were preserved in LN_2 (frozen sample) for at least 1 week until the evaluation of semen quality. Some samples drawn into straws but not frozen were kept at 4˚C until the evaluation (conventional cooling sample). Five samples of conventionally cooled were kept at 4˚C for 5 days and used for the evaluation of sperm characteristics in liquid preservation, and each tube were used per day for the evaluation of the effect of long-term exposure to glycerol and low temperature on sperm characteristics (days 1-5). Secondly, remaining semen samples were also mixed with same volume of EYT. Diluted samples (0.5 ml each) were transferred to 1.5-ml tubes and some of them were kept at 20˚C until the evaluation (pre-dilution sample). To evaluate the cooling effects on sperm more clearly, other

pre-diluted samples were immersed directly in a cold water at 4˚C and kept for approximately 90 min (rapid cooling sample). Then, samples of rapid cooling and same volume of 12% EYTG were mixed and brought to our laboratory at 4˚C. Pre-dilution, conventional cooling, and rapidly cooling samples were brought to our laboratory within 3.5 hr after collection, and examined sperm characteristics immediately after arriving at the laboratory.

Straws containing frozen and conventionally cooled samples were immersed in water at 37˚C for 1 min, and samples were expelled into 1.5-ml tubes. All semen samples in 1.5-ml tubes were also warmed by immersing them into 37˚C water for 1 min. In addition, pre-diluted samples were mixed with the same volume of 12% EYTG. Therefore, sperm in all samples were suspended in 6% EYTG to evaluate in same condition for sperm. To adjust sperm concentration at 10×10^6 cells/ml, all samples were diluted again by 6% EYTG warmed at 37˚C, and 200 µl of diluted samples were transferred into new 1.5-ml tubes for multiple fluorescent staining.

Quadruple staining for simultaneous evaluation of plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm

Evaluation of sperm characteristics were performed as previously reported[14] with a slight modification. Briefly, 2 µl of Hoechst 33342 (final concentration 0.8 µg/ml, Molecular Probe, Eugene, OR, USA) stock solution was mixed with sample and incubated for 10 min. After incubation, 1 µl of JC-1 (final concentration 1 µg/ml, Mitochondrial Membrane Potential Detection kit, Cell Technology Inc., Fremont, CA, USA) solution was mixed with sample and incubated for 3 min. Then, 0.5 µl of PI (final concentration 10 µg/ml, Sigma-Aldrich, St. Louis, MO, USA) solution and 0.5 µl of fluorescein peanut agglutinin FITC conjugate (FITC-PNA; final concentration 25 µg/ml, Vector Laboratories, Burlingame, CA, USA) were mixed with sample and incubated for 8 min. All incubation was conducted at 37˚C in the dark. After staining with fluorescent probes, 2 µl of 10% (v/v) formaldehyde was added to samples

for stabilization of sperm. Then 5 μl of sperm suspension was mounted on a slide glass and covered with a cover glass. More than 200 sperm on a slide glass were evaluated under fluorescent microscope (Eclipse Ci) with triple band filter (DAPI/FITC/TRITC, Nikon), and sperm were categorized into 8 groups as shown in Fig. II-1. Briefly, plasma membrane integrity was assessed as intact when the head of sperm was stained by Hoechst 33342 (final concentration 0.8 μg/ml, Molecular Probe, Eugene, OR, USA) and as damaged when the head was stained by PI (red). Acrosomal integrity was assessed as damaged when acrosome was stained by FITC-PNA (green). If acrosome was not stained, the acrosome was judged as intact. Mitochondrial membrane potential was determined by the color of the midpiece of sperm stained by JC-1. When the midpiece was stained by orange, it was assessed as high mitochondrial membrane potential. Sperm with intact plasma membrane, intact acrosome and high mitochondrial membrane potential (type A) were defined as normal sperm.

Statistical analysis

The data of 3 bulls were pooled to compare the effect of preservation processes on sperm characteristics. Differences in the mean values of the characteristics evaluated by fluorescent staining were analyzed by Tukey-Kramer's HSD test. Differences were considered significant at $P<0.05$. All analyses were performed using JMP pro 12 (SAS, NC, USA).

Results

Sperm characteristics at each step of freezing procedure were shown in Table II-1. Pre-diluted sample showed the highest proportion of normal (type A) sperm, however, the percentage decreased to approximately 50% as the advancement of the semen freezing process.

Proportions of type F sperm were higher in conventional cooling and frozen-thawed samples than in pre-diluted samples ($P<0.05$). Rapid cooling sample showed the lower proportion of type A sperm, and higher proportion of type H than pre-diluted and conventional cooling samples ($P<0.05$), and these proportions were similar to those of frozen-thawed sample. Proportions of type B, similar to type A but with low mitochondrial membrane potential, was higher in conventional and rapid cooling samples than in others ($P<0.05$). Sperm characteristics during liquid preservation were shown in Table II-2. After conventional cooling, the proportion of type A sperm decreased markedly ($P<0.05$). The proportion of type A sperm decreased gradually during liquid preservation, and became approximately 40% at day 5. The proportion of type B sperm was highest at day 3 ($P<0.05$). Proportions of types F and H sperm significantly increased immediately after conventional cooling, however, the increase became gradual until day 5. The proportions of types C and D sperm were rarely observed in freezing and liquid preservation processes.

Discussion

Cooling and addition of glycerol to semen extender are important procedure for semen cryopreservation, because low temperature decreases energy consumptions of sperm[49] and glycerol is one of the most popular cryoprotectants and gives a better result in cryopreservation of sperm than other different cryoprotectants[26]. It is also well known that these processes decrease the plasma membrane integrity of sperm. The results in the present study confirmed that most of sperm were damaged to plasma membrane by preservation procedure as previously reported[26, 85, 90].

Also, the present study indicated that another pathway of damages to sperm during preservation. After conventional and rapid cooling, the proportion of sperm with intact

plasma membrane, intact acrosome, and low mitochondrial membrane potential increased. It might indicate that cooling process disrupts mitochondrial activity and sperm compromised mitochondrial activity lose plasma membrane integrity in a minute. Mitochondria produces ATP which is necessary to maintain plasma membrane integrity of sperm[73]. Therefore, semen cooling process injures mitochondria, leading to the impairment of plasma membrane integrity of sperm. The result of higher proportion of sperm with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential at day 3 of liquid preservation also support this hypothesis.

The present results suggest that rapid cooling would give serious damages to sperm because rapid cooling sample showed lower level of normal sperm similarly to frozen-thawed sample. Yoon et al.[90] described that frozen-thawed process was the most critical step to sperm because plasma membrane defects were caused by ice crystal formation in semen extender[85]. In the present study, sperm with intact plasma membrane and damaged acrosome were very few, and sperm with damaged acrosome dramatically increased after frozen-thawed. These results might suggest that acrosome damage is occurred after plasma membrane injury following mitochondrial dysfunction or simultaneously with plasma membrane injury of sperm, because of extensive injury by cryoinjury, such as high osmolality and ice nucleation in semen[7]. Although cryoinjury could not occur during rapid cooling to 4˚C, the proportion of sperm with damaged plasma membrane, damaged acrosome, and low mitochondrial membrane potential was high similar to that of frozen-thawed sperm and significantly higher than that of conventional cooling sperm. It is reported that high cooling rate of semen causes cold shock, which induce damage to sperm viability, motility and functions[28]. It is also reported that cold shock induces the production of ROS[15]. ROS produced by cold shock has been known as an inducer of cryocapacitation of sperm, which means the change of the cholesterol component in plasma membrane and the damage of acrosome[75]. There are no reports to indicate the relationship between ROS production and cooling rate. In further study, I should investigate

the relationship between them and clarify the optimal cooling rate for producing sperm having higher normality after frozen-thawed and liquid preservation.

In conclusion, during cooling process to 4˚C, sperm may be firstly injured to mitochondrial membrane, and low mitochondrial function may cause the impairment of plasma membrane and subsequent cell death other than direct critical damages to plasma membrane. Also, rapid cooling process causes dramatic injuries in sperm similar to frozen-thawed process, although the mechanism is unclear. In further study, the mechanism of cold-shock on membranes of sperm should be investigated, and a novel preservation procedure protecting mitochondrial function should be developed.

Table II-1. Characteristics of sperm during freezing processes evaluated by fluorescent staining

Characteristics of sperm			Category	% of sperm at each step of sperm freezing			
Plasma membrane	Acrosome	Mitochondrial membrane potential		Pre-dilution	Conventional cooling	Frozen-thawed	Rapid cooling
Intact	Intact	High	A	76.0±6.6[a]	59.2± 6.4[b]	51.0± 5.1[c]	50.4±7.1[c]
		Low	B	0.8±1.3[a]	2.7±2.4[b]	0.4±0.5[a]	2.8±1.5[b]
	Damaged	High	C	0.0±0.1	0.1±0.4	0.0±0.1	0
		Low	D	0.0±0.1	0	0	0
Damaged	Intact	High	E	3.4±2.5[a]	1.9±1.4[ab]	0.9±0.9[b]	0.5±0.5[b]
		Low	F	10.0±4.1[a]	20.0±5.5[b]	23.0±4.0[b]	21.6±4.6[b]
	Damaged	High	G	1.7±1.7[a]	0.7±0.5[ab]	0.2±0.3[b]	0.8±0.7[ab]
		Low	H	8.0±2.1[a]	15.4±3.1[b]	24.5±1.8[c]	23.9±5.0[c]

Values are the mean ± SD (3 bulls/group, 5 replicates).

[abc]; Superscripts indicate significant different within a same row ($P<0.05$).

Table II-2. Characteristics of sperm during liquid preservation evaluated by fluorescent staining

Characteristics of sperm			Type	% of sperm at each day after cooling						
				0		1	2	3	4	5
Plasma membrane	Acrosome	Mitochondrial membrane potential		Pre-dilution	Conventional cooling					
Intact	Intact	High	A	76.0± 6.6^{a}	59.2± 6.4^{b}	54.6±5.7^{bc}	52.3±5.6^{cd}	48.4±6.0^{de}	45.7±6.5^{e}	43.2±6.4^{e}
		Low	B	0.8±1.3^{a}	2.7±2.4^{ab}	3.6±4.6^{ab}	3.0±3.0^{ab}	4.5±3.2^{b}	2.7±2.5^{ab}	2.4±1.5^{ab}
	Damaged	High	C	0.0±0.1	0.1±0.4	0	0	0	0.1±0.2	0
		Low	D	0.0±0.1	0	0	0.1±0.3	0	0	0.1±0.3
Damaged	Intact	High	E	3.4±2.5^{a}	1.9±1.4^{ab}	2.0±1.5^{ab}	1.5±1.3^{b}	0.7±0.8^{b}	1.2±1.9^{b}	1.0±1.4^{b}
		Low	F	10.0±4.1^{a}	20.0±5.5^{b}	21.8±4.6^{b}	24.2±5.6^{bc}	25.4±4.3^{bc}	27.9±6.5^{c}	29.6±5.4^{c}
	Damaged	High	G	1.7±1.7^{a}	0.7±0.5^{b}	0.7±0.6^{b}	0.5±0.5^{b}	0.4±0.3^{b}	0.5±0.7^{b}	0.4±0.4^{b}
		Low	H	8.0±2.1^{a}	15.4±3.1^{b}	17.4±3.2^{bc}	18.3±3.9^{bcd}	20.6±4.5^{cde}	21.9±3.8^{de}	23.2±4.9^{e}

Values are the mean ± SD (3 bulls/group, 5 replicates).

^{abcdef}; Superscripts indicate significant different within a same row ($P<0.05$).

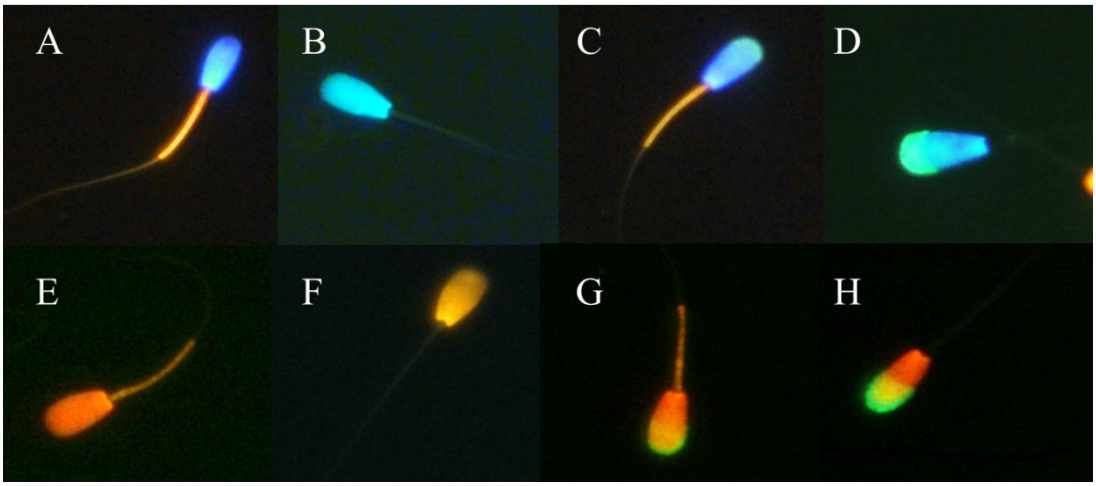

Fig. II-1. The categorization of sperm characteristics evaluated by fluorescent staining

The head of sperm stained with Hoechst 33342 were judged as intact plasma membrane (A, B, C, and D). The head of sperm stained with PI were judged as damaged plasma membrane (E, F, G, and H). Acrosome stained with FITC-PNA were judged as damaged (B, D, F, and H). The midpiece dyed orange were judged as sperm with high mitochondrial membrane potential (A, B, E, and F). Therefore, the sperm characteristics were categorized as follows. (A) A sperm with intact plasma membrane, intact acrosome and high mitochondrial membrane potential. (B) A sperm with intact plasma membrane, damaged acrosome and high mitochondrial membrane potential. (C) A sperm with intact plasma membrane, intact acrosome and low mitochondrial membrane potential. (D) A sperm with intact plasma membrane, damaged acrosome and low mitochondrial membrane potential. (E) A sperm with damaged plasma membrane, intact acrosome and high mitochondrial membrane potential. (F) A sperm with damaged plasma membrane, damaged acrosome and high mitochondrial membrane potential. (G) A sperm with damaged plasma membrane, intact acrosome and low mitochondrial membrane potential. (H) A sperm with damaged plasma membrane, damaged acrosome and low mitochondrial membrane potential.

Summary

To verify the dynamics of damages to sperm during semen freezing (pre-dilution, cooling to 4˚C, and frozen-thawed), characteristics of sperm collected from 3 Japanese black bulls were evaluated by using fluorescent staining. Pre-diluted sample showed the highest proportion of sperm with intact plasma membrane, intact acrosome and high mitochondrial membrane potential. The proportion of sperm with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential were higher after cooling than the other processes ($P<0.05$). During cooling preservation examined in this study, the proportion of sperm with damaged acrosome increased. These results read me to speculate that, during cooling process, sperm may be firstly injured to mitochondrial membrane, and low mitochondrial function may cause the impairment of plasma membrane and subsequent cell death with acrosomal damage.

Chapter III

Comparison of sperm subpopulation structures in first and second ejaculated semen from Japanese black bulls by a cluster analysis of sperm motility evaluated by a CASA system

Introduction

Sperm motility is regarded as the most common factor for predicting male fertility[3]. However, a previous study reported that correlations between the percentages of motile sperm evaluated by conventional methods and fertility fluctuate widely because the standard semen analysis, sperm observations under microscopy performed by practitioners, is a subjective technique and associated with large inter-laboratory variations[42]. Mocé and Graham[56] suggested that inconsistencies are derived from differences in the multifactorial nature of sperm function, and partly by the inaccuracy of *in vitro* measurements. CASA has been developed as a tool for the objective evaluation of sperm motility in humans and animals[23, 40], and provides information on the motility characteristics of individual sperm. Nevertheless, previous studies have mostly reported average values for kinetic parameters without focusing on individual sperm characteristics[23, 36, 38, 47, 83].

The criteria for evaluating fertility in men were defined in the fourth edition of the WHO guidelines[88] as the average value of progressively motile sperm using straight line velocity (VSL: the straight line distance from beginning to end of a sperm track for 1 sec), which has been categorized into 4 grades; A: progressive with VSL ≥25 μm/sec, B: progressive with VSL <25 μm/sec, C: non-progressive, and D: immotile. However, these criteria were omitted from the fifth edition[89] and sperm motility evaluated by microscopic observations was divided into 3 grades: progressively motile, non-progressively motile, and immotile, possibly

due to the difficulties technicians have with consistently and reproducibly distinguishing between grades "A" and "B"[16]. This may indicate that sperm velocity is not effective to predict male fertility. Holt et al.[39] showed that a linear regression model containing the amplitude of lateral head displacement (ALH; the mean width of sperm head oscillations) or beat cross frequency (BCF) of sperm heads indicated the significant coefficient of determination with the litter size in boar sperm. These suggest that the moving pattern of sperm heads correlates with fertility, similar to velocity parameters. Therefore, assessing the velocity and head movements of sperm is necessary for precisely evaluating its fertility.

Previous studies reported the potential to divide sperm derived from humans and animals into subpopulations based on the kinetic characteristics of individual sperm using CASA data[1, 18, 40, 63, 67]. A cluster analysis, which is a statistical method for constructing small groups (clusters) using multiple parameters from a large set of data that may contribute to a better understanding of the clumping structure of data, simultaneously using data on sperm head movement and velocity parameters may be more suitable for the prediction of fertility. Previous studies using subpopulations reported that the proportion of the "progressively motile sperm" subpopulation, which had the highest velocity and linearity was related to male fertility[1, 18, 40, 52, 63, 68, 91]. However, a previous study in stallion indicated the relationship between fertility and a subpopulation with low velocity and high linearity[67]. I hypothesized that a cluster analysis of CASA data will become a powerful tool for the evaluation of male fertility if sperm subpopulations other than progressively motile and high velocity sperm are related to pregnancy rates in AI.

In order to accurately evaluate the relationship between fertility and sperm motility, we need to compare sperm subpopulation structures with semen having consistent results of fertility. However, the pregnancy rate after AI in cattle fluctuates between practitioners[27], season[13], and reproductive management[66]. Semen is generally collected from bulls twice a week at AI centers. On the day of semen collection, the first and second ejaculates are

collected within a short interval of time. During repetitive ejaculations, changes have been reported in semen constituents and characteristics[45], such as pH, osmolality, and the concentration of fructose, which affect sperm motility and fertility[41, 82]. Sperm in the first ejaculate have been shown to exhibit similar[22] or higher motility[17, 23] to that in the second ejaculate. However, the second ejaculate showed higher fertility than the first ejaculate when used for AI without freezing[17]. This discrepancy between these findings may be caused by sperm characteristics, which cannot be investigated by conventional methods using light microscopy. Therefore, I herein used the first and second ejaculates as low and high potential fertility sperm models, respectively.

The purpose of this study is to investigate the potential of an analysis of sperm subpopulation structures for the evaluation of sperm motility probably related to fertility by using CASA data on the first and second ejaculates. Furthermore, the sperm subpopulation structures of individual bulls were examined because differences in fertility between bulls were reported [19].

Materials and Methods

Animals

Four Japanese black bulls (A-D, 5-15 years old), which were kept for the production of frozen semen in the AI center (Genetics Hokkaido), were used in the present study. Their frozen-thawed semen were used commercially and indicated acceptable conception rates by AI in the field.

Semen collection and evaluation of semen volume and sperm motility by practitioners

Semen were collected using artificial vaginas, which included a glass tube and the first

and second ejaculates within one day were collected separately at 18- to 39-min intervals. Ejaculates were collected in six separate sessions per bull, two sessions per week. Therefore, twelve ejaculates were collected in three weeks. Immediately after collection, the volume of semen was evaluated by visually checking the scale on the tube, while sperm concentrations were assessed using a photometer (SDM 5 12300/0007 DE, Minitube, Tiefenbach, Germany). Sperm motility was also examined under light microscopy, as described previously[65], and subjectively classified into the 5 grades (+++: progressively motile at a high speed, ++: progressively motile at a moderate speed, +: motile at a low speed, ±: motile without progression d -: immotile). The proportions of sperm with +++ and ++ grades were defined as motile sperm. These evaluations were performed independently by two practitioners and the mean value was used as the value for motile sperm. Semen qualities evaluated by practitioners were described in Table III-1.

Evaluation of sperm motility by CASA system

Immediately after semen collection, a small amount of semen was diluted to a concentration of 10-20×10^6 cells/ml using physiological saline, because phosphate buffered saline significantly inhibited the sperm motility. Immediately after dilution, samples were introduced into a 20-μm-deep chamber (SC20-01-04-B, Leja, GN Nieuw-Vennep, Netherlands) preliminary warmed at 37˚C on a hot plate, and sperm motility was evaluated by a CASA system (SMAS, DITECT, Tokyo, Japan) based on the digitalized images obtained using ×10 negative-phase contrast microscope (E200, Nikon, Tokyo, Japan). The percentage of motile sperm was recorded and the following kinetic parameters were analyzed: VSL, curvilinear velocity (VCL: a measure of total distance travelled by a sperm for 1 sec), average path velocity (VAP: the average path velocity of sperm for 1 sec), ALH, and BCF. The number of sperm analyzed per sample was at least 200, including immotile sperm. Linearity (LIN = VSL/VCL; the state of being linear) was calculated automatically using the CASA system. The CASA

system recorded 150 frames per second (fps), and sperm having more than 120 frames were used in the analysis.

Statistical analysis

A cluster analysis of sperm motility was performed as described previously[63] with slight modifications. Data from 14,264 sperm in 48 fresh semen samples (2 ejaculates from 4 bulls in 6 replicates) were obtained. In the motility analysis, data from 1,306 immotile sperm and 3,730 motile sperm having less than 120 frames were discarded. Then data from the remaining 9,228 motile sperm were imported into a single data set and analyzed. In the cluster analysis, the 5 independent motility variables: VSL, VCL, VAP, ALH, and BCF, were used as parameters after the normalization of data adapted by the following formula: (measurements - average)/standard deviation. The number of clusters was assessed by the shape of the dendrogram according to Ward's method and determined the number subjectively from the shape of dendrogram (Fig. III-1). A multivariate *k*-means cluster analysis was performed to classify the 9,228 sperm into a reduced number of subpopulations based on their motility variables. Sperm that showed similar motility characteristics were assigned to the same cluster. The *k*-means clustering model used Euclidean distances, which were computed from the 5 quantitative variables, and the cluster centers were the means of the observations assigned to each cluster. The effects of bulls and the order of ejaculation (ejaculation difference) on sperm motility characteristics assessed by CASA and the percentage of progressively motile sperm evaluated in the visual assessment were analyzed using a two-way ANOVA. Differences in the means of semen quality evaluated by practitioners and motility parameters assessed by CASA between ejaculates and bulls were analyzed by Tukey-Kramer's HSD test. Differences were considered significant at $P<0.05$. All analyses were performed using JMP pro 12.

Results

There was no interaction in the mean values of kinetic parameters between bulls and the ejaculation difference (Table III-2). Therefore, the effects of the two factors were analyzed separately (Table III-3). No significant difference was observed in the percentages of motile sperm between bulls and the ejaculation difference. Bull A showed higher VSL than bull D ($P<0.05$). Bulls A and C had higher VCL, VAP, and ALH than bull D ($P<0.05$). No significant differences were noted in VSL, VCL, or VAP between the first and second ejaculates. However, the first ejaculates had higher ALH and lower BCF than the second ejaculates ($P<0.05$).

As shown in Fig. III-1, 9,228 motile sperm derived from 48 ejaculates were categorized into 4 clusters. Kinetic parameters of sperm in each cluster were described in Table III-4. Cluster 1 showed the highest velocities and highest LIN as well as the second highest ALH and BCF. Cluster 2 showed the second highest velocities and highest ALH, but lower BCF and LIN than clusters 1 and 3. Although all 3 velocities of cluster 3 were lower than those of clusters 1 and 2, cluster 3 had the highest BCF, lowest ALH, and second highest LIN. Cluster 4 had the lowest values in all parameters.

As shown in Table III-5, there was no interaction in sperm subpopulation structures between bulls and the order of ejaculation; however, the percentage of cluster 1 was affected by the bull factor, and the percentages of clusters 2 and 3 were affected by the ejaculation difference. Therefore, the effects of the two factors were analyzed individually (Fig. III-2). Bulls A and C had a higher percentage of cluster 1 than bull D ($P<0.05$, Fig. III-2a). No significant differences were observed in the percentages of clusters 1 and 4 between the first and second ejaculates (Fig. III-2b). However, the first ejaculates included a higher percentage of cluster 2[51] than the second ejaculates (19.0%, $P<0.05$), and the second ejaculates had a higher percentage of cluster 3 (15.4%) than the first ejaculates (9.7%, $P<0.05$). The total

percentages of clusters 1, 2, and 3 varied between 64.6 and 76.7%, and the values obtained were similar to the percentages of progressively motile sperm evaluated by practitioners (Table III-1).

Discussion

In the present study, motile sperm were divided into 4 clusters. Cluster 1 sperm had highly progressive motility, which has been speculated to reflect very fertile sperm[63]. It has been reported that males with high fertility had higher proportion of rapid and linear sperm subpopulation and lower proportion of rapid and nonlinear sperm subpopulation than males with low fertility in red deer[68] and ram[91]. Therefore, bulls A and C may have relatively high fertility. Cluster 2 sperm showed the highest ALH in all clusters and LIN had the second lowest value due to higher VCL and lower VSL. Hyperactivation is a movement pattern observed in sperm at fertilization[37]. Hyperactivated sperm have been shown to have increased VCL and ALH along with decreased LIN[44, 59]. These findings may indicate that cluster 2 includes hyperactivated sperm. Although VSL, VCL, and VAP in cluster 3 were lower than in clusters 1 and 2, cluster 3 had the highest BCF, second highest LIN, and lowest ALH in all groups. I previously reported that bovine sperm penetrated oocytes between 4 and 8 hr after the initiation of *in vitro* fertilization[46]. Furthermore, sperm with high BCF, low ALH, and high LIN have been suggested to maintain higher activity without hyperactivation 6 hr after being incubated[43]. These findings indicate that sperm with high BCF and low ALH maintain their fecundity and have the ability to penetrate oocytes. Collectively, the results of the present study and previous our findings may indicate that sperm in cluster 3 has higher longevity in the female genital tract than sperm in cluster 2. Cluster 4 may be regarded as poorly motile sperm due to the lowest values in all parameters, and did not correlate with

fertilization.

The numbers and characteristics of clusters in the present study were consistent with previous findings[61-63]; however, cluster 1 in previous findings[61-63] moved more linearly (LIN: 69.8-70.9%) than that in the present study (46.9%). In addition, LIN in cluster 3 was also higher in previous studies at 65.1-79.9%[61-63] than that in the present study (33.3%). On the other hand, BCF in the present study (8.7-27.0 Hz) was more than 2 or 3-fold higher than that (2.4-9.5 Hz) in previous studies[61-63]. These discrepancies may be derived from differences in the capture rate of frames by the different CASA systems. Since previous studies used a capture rate of 25 fps[61-63], maximum BCF was limited theoretically. The present results showed 27.0 ± 5.7 Hz for the highest BCF in cluster 3 because of employing 150 fps. This result indicates that a higher capture rate is necessary for correctly evaluating sperm motility. A previous study also indicated that a lower frame rate resulted in lower VCL because of the loss of detailed trajectory[12], leading to higher LIN. Lower LIN in the present study may be due to the higher frame rate used.

In visual inspections, no differences were detected in sperm motility between the first and second ejaculates in all bulls or between bulls. The percentages of progressively motile sperm evaluated by practitioners were 65.0-70.0%, which appears to reflect the total of clusters 1, 2, and 3. This result indicates that the sperm motility analysis by CASA has the ability to describe sperm motility characteristics in more detail. However, I suggest that sperm motility evaluations based on the average values of CASA data do not have the ability to correctly predict sperm potential fertility. For example, bulls A and C had higher VSL, VCL, and VAP, which indicated higher fertility[63], but higher ALH and lower LIN, which may correlate with shorter longevity[43, 46]. On the other hand, a cluster analysis may detect higher percentages of cluster 1, including higher velocity sperm in bulls A and C than in bull D. Although bull D showed the lowest average value for ALH, based on the cluster analysis, it may be speculated that bulls A and C do not have shorter longevity than bull D. In further study, I should

investigate the longevity of sperm in each cluster. Furthermore, in comparisons of the first and second ejaculates, I only found differences in the percentages of clusters 2 and 3, and not in the percentage of cluster 1. If a difference exists in fertility between the first and second ejaculates, as described in a previous study[17], a cluster analysis has the potential to evaluate semen quality.

In conclusion, sperm in fresh semen derived from Japanese black bulls was categorized into 4 clusters by kinetic parameters analyzed using the CASA system. Clusters 1, 2, and 3 sperm may be evaluated as progressively motile sperm by visual assessments, in which differences between them cannot be detected, whereas a cluster analysis has the ability to identify differences and similarities. It means that cluster analysis can distinguish the characteristics of the first and second ejaculates collected at short interval. In further study, we should confirm the relationship between semen fertility and sperm subpopulation analyzed by cluster analysis, and investigate what statistical method is appropriate to the prediction of semen fertility.

Table III-1. Semen quality of Japanese Black bulls evaluated by conventional methods

Bull	Ejaculation order	Semen volume (ml)	Sperm concentration ($\times 10^6$ cells/ml)	Motile sperm[a)] (%)
A	1st	7.2±1.8	14.5±2.7	65.8±2.0
	2nd	6.4±1.3	9.5±1.4	65.0±0.0
	Total	6.7±1.5	12.0±3.3	65.4±1.4
B	1st	5.0±1.1	9.7±3.1	70.0±4.5
	2nd	4.4±0.8	8.8±2.0	65.8±2.0
	Total	4.7±1.0	9.3±2.5	67.9±4.0
C	1st	5.5±0.8	11.0±2.7	65.8±2.0
	2nd	5.0±1.1	10.9±0.5	66.7±2.6
	Total	5.2±0.9	11.0±1.9	66.3±2.3
D	1st	4.5±0.5	9.6±1.4	65.0±0.0
	2nd	4.0±1.1	4.8±2.5	65.0±0.0
	Total	4.2±0.8	7.2±3.2	65.0±0.0
Total	1st	5.5±1.5	11.2±3.1	66.7±3.2
	2nd	4.9±1.4	8.5±2.9	65.6±1.7

Values are the mean ± SD (6 replicates).

[a]; Progressively and actively motile sperm (+++ and ++) assessed by visual inspections[65)].

Table III-2. Effects of the ejaculation order and bulls on mean values of kinetic parameters evaluated by CASA; probability values analyzed by a two-way ANOVA

Parameters	Factors affecting each kinetic parameter		
	Ejaculation order	Bull	Interaction
% of motile sperm	0.9376	0.2112	0.8546
VSL	0.1597	0.0074	0.5579
VCL	0.0856	0.0011	0.5704
VAP	0.1655	0.0037	0.4151
ALH	0.0113	0.0077	0.2770
BCF	0.0145	0.7670	0.9238
LIN	0.4462	0.5092	0.4701

Table III-3. Mean values of kinetic parameters of sperm in each bull and the order of ejaculation

Bull	Ejaculation order	% of motile sperm	VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	ALH (μm)	BCF (Hz)	LIN (%)
A	1st	91.8±3.6	117.7±16.9	310.9±27.9	147.0±17.6	5.1±0.2	15.6±2.1	35.6±3.4
	2nd	92.1±3.7	104.1±22.9	261.7±58.3	125.4±27.5	4.0±1.0	17.4±1.6	36.0±2.5
	Total	92.0±3.5	110.9±21.2^{a}	286.3±51.9^{a}	136.2±25.5^{a}	4.5±0.9^{a}	16.5±2.1	35.8±3.0
B	1st	90.9±5.9	91.7±23.8	250.1±63.6	117.5±29.7	4.2±1.1	15.7±2.2	34.2±2.1
	2nd	88.1±9.9	95.5±20.3	243.4±42.9	120.2±22.4	3.9±0.7	16.9±2.6	34.6±5.0
	Total	89.5±7.9	93.6±22.2^{ab}	246.8±54.3^{ab}	118.8±26.3^{ab}	4.0±0.9^{ab}	16.3±2.5	34.4±3.8
C	1st	91.4±5.2	107.0±11.3	286.5±28.5	134.2±12.2	4.7±0.5	16.0±1.2	33.9±1.4
	2nd	92.0±6.4	103.9±9.7	282.8±36.5	135.0±14.3	4.3±0.8	18.0±1.5	33.6±1.2
	Total	91.7±5.5	105.4±10.6^{ab}	284.6±32.8^{a}	134.6±13.3^{a}	4.5±0.7^{a}	17.0±1.7	33.8±1.3
D	1st	86.6±6.7	93.9±2.1	232.4±18.8	114.3±4.5	3.8±0.2	16.6±1.4	35.9±2.1
	2nd	87.8±5.4	75.0±21.0	197.5±42.6	96.6±19.5	3.2±0.8	17.5±1.6	32.5±4.5
	Total	87.2±5.9	84.5±17.6^{b}	214.9±37.2^{b}	105.4±16.7^{b}	3.5±0.6^{b}	17.0±1.6	34.2±3.9
Average	1st	90.0±6.4	101.0±16.8	281.3±46.7	131.2±21.4	4.7±0.8*	15.4±2.2	33.2±3.7
	2nd	90.5±6.6	95.2±20.2	255.7±52.3	121.4±22.6	4.1±0.9	17.0±2.8*	33.5±4.0

Values are the mean ± SD (6 replicates).

*; The asterisk indicates a significant difference between ejaculation orders ($P<0.05$).

^{ab}; Superscripts indicate significant differences between bulls ($P<0.05$).

Table III-4. Kinetic parameters of sperm in each cluster

Cluster	No. of sperm	VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	ALH (μm)	BCF (Hz)	LIN (%)
1	3594	162.8±25.0	350.9±48.8	180.5±21.3	5.1±1.0	20.0±3.4	46.9± 7.2
2	2529	98.8±38.0	333.6±69.7	145.0±30.4	6.0±1.2	12.7±4.1	30.4±11.6
3	1248	49.3±30.4	153.9±54.4	88.2±29.9	2.0±1.1	27.0±5.7	33.3±17.8
4	1857	12.9±15.8	75.9±56.5	22.4±21.2	1.9±1.5	8.7±4.1	16.5±12.1

Values are the mean ± SD.

VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; VAP (μm/sec), average path velocity;
ALH (μm), amplitude of lateral sperm head displacement; BCF (Hz), beat cross frequency; LIN (VCL/VSL, %), linearity.

Table III-5. Effects of the ejaculation order and bulls on the proportion[a)] of each sperm cluster within ejaculates; probability values analyzed by a two-way ANOVA

Cluster	Factors affecting the proportion of each cluster		
	Ejaculation order	Bull	Interaction
1	0.9838	0.0058	0.5214
2	0.0069	0.6431	0.5094
3	0.0117	0.1125	0.6819
4	0.2420	0.2332	0.3141

[a]; The proportions were shown in Fig. III-2.

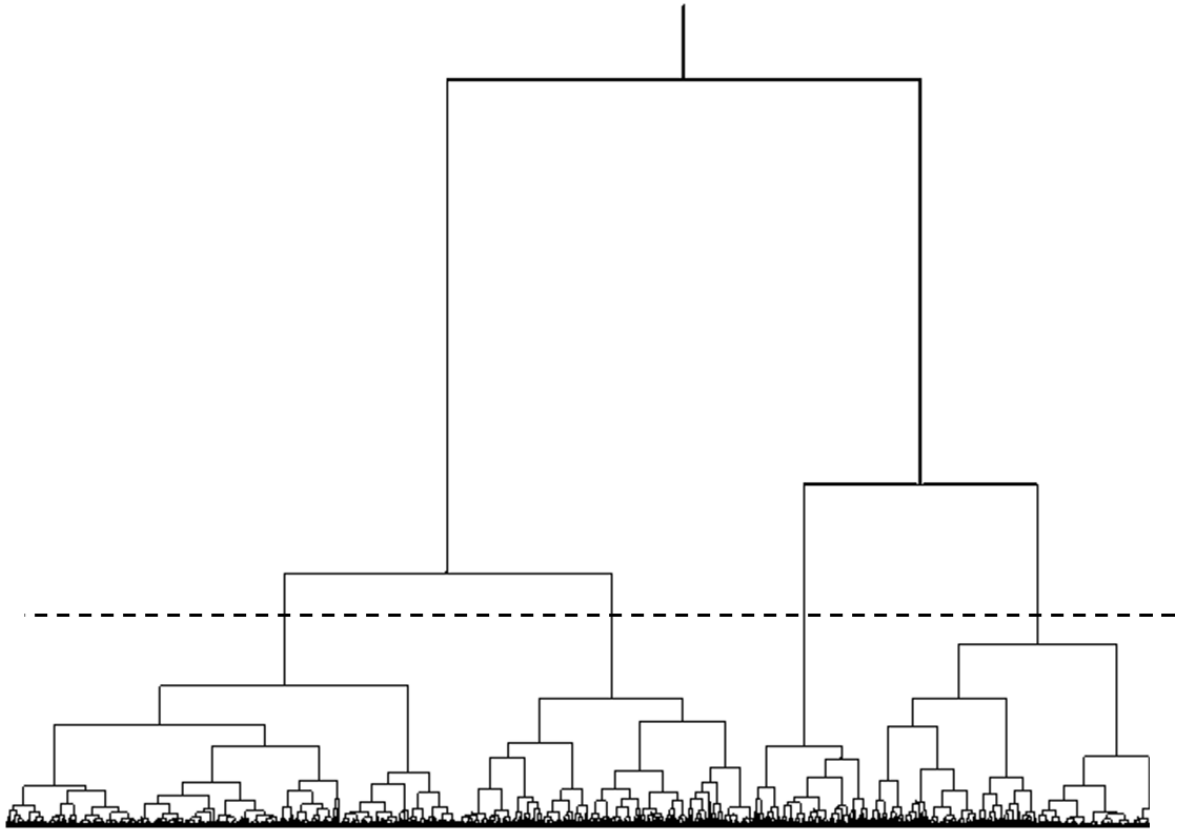

Fig. III-1. Dendrogram described by Ward's method to determine the number of cluster

To describe the dendrogram, 9,228 of motile sperm incubated in BO were used. The five independent motility variables; VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; LIN (VCL/VSL, %), linearity; ALH (μm), amplitude of lateral sperm head displacement; BCF (Hz), beat cross frequency, were used as parameters.

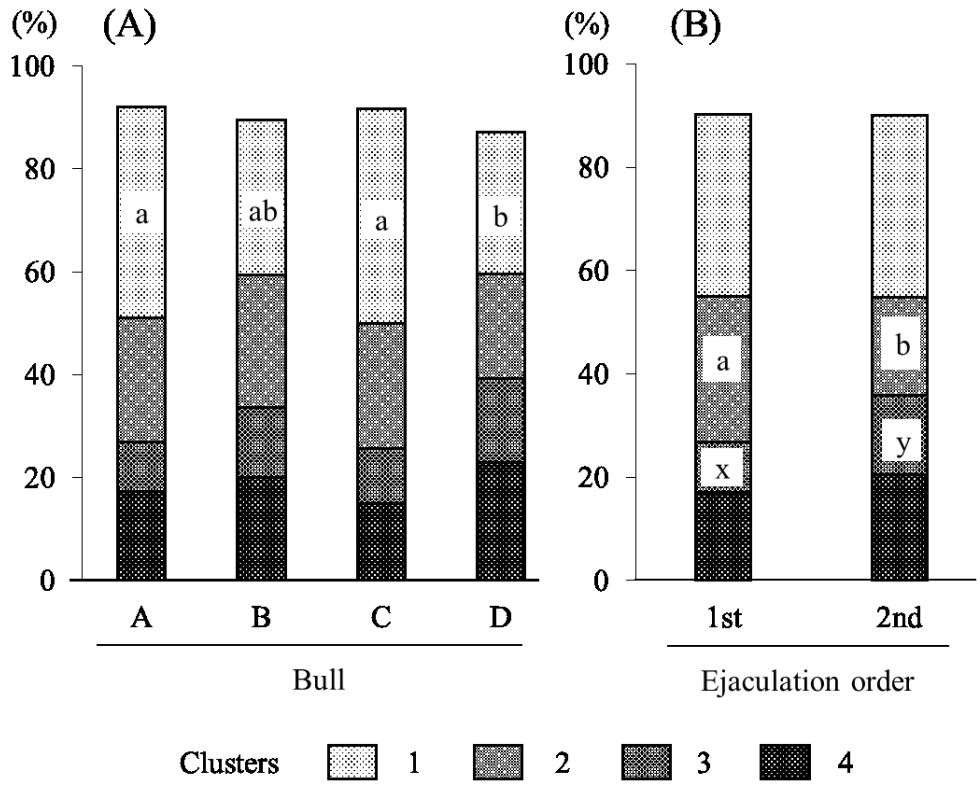

Fig. III-2. Effects of bulls (A) and ejaculation difference (B) on sperm subpopulation structures in semen

[ab, xy;] Letters indicate significant differences in the same cluster between groups ($P<0.05$).

Sperm kinetic parameters of each cluster were presented in Table III-5.

Summary

In this chapter, bull sperm in the first and second ejaculates were divided into subpopulations based on their motility characteristics using a cluster analysis of data from computer-assisted sperm motility analysis (CASA). Semen samples were collected from 4 Japanese black bulls. Data from 9,228 motile sperm were classified into 4 clusters; 1) very rapid and progressively motile sperm, 2) rapid and circularly motile sperm with widely moving heads, 3) moderately motile sperm with heads moving frequently in a short length, and 4) poorly motile sperm. The percentage of cluster 1 varied between bulls. The first ejaculates had a higher proportion of cluster 2 and lower proportion of cluster 3 than the second ejaculates.

Chapter IV

Effects of the media and frame rate on the analysis of hyperactivation-like bovine sperm motility and the structure of sperm motility subpopulation in sex-sorted and non-sorted semen

Introduction

In the previous chapters, I examined the kinetic and physiological changes in bovine sperm; however, the correlation between these changes and fertility of sperm is unclear. General treatments of semen, such as cryopreservation[86] and sex-sorting[57], cause the capacitation-like changes in sperm. Both kinetic and physiological changes form capacitation before penetrating to oocytes[4]. Capacitation-like damages of bovine sperm during cryopreservation are known as one of the factors decreasing sperm fertility because of their short longevity[33, 55]. Therefore, evaluation of capacitation-like changes in frozen-thawed sperm is important for successful AI.

Capacitated sperm shows a specific movement, which is recognized as hyperactivation. Sperm showing hyperactivation can pass through the cumulus investment and zona pellucida of oocyte[76]. Although it has been known that movement of hyperactivation is captured by CASA as increased VCL/ALH, and decreased LIN/BCF[76], there are no certain criteria to evaluate the motility of hyperactivated sperm. In human, it was recommended to employ 80-100 fps for the frame rate to analyze sperm capacitation[50]. In cattle, the frame rate at 30-60 fps was generally used in CASA system and there has been no report examined the suitable frame rate for analyzing bovine hyperactivated sperm. Therefore, I investigated the effect of the frame rate on the detection of hyperactivated sperm motility in this chapter. Also, I investigated which

media were suitable for detecting hyperactivation by using Brackett and Oliphant medium (BO)[8], which is commonly used for *in vitro* fertilization, or synthetic oviductal fluid (SOF)[79], which is commonly used for *in vitro* culture of bovine embryos.

In the previous studies, cluster analysis has been used for evaluating the structures of sperm motility subpopulations in bovine semen; however, they mainly focused on the proportion of sperm subpopulation showing progressive motility[24, 61-63]. Additionally, the correlation between whole subpopulation structures and semen fertility has not been examined. Recently, sex-sorted semen has been used for artificial insemination of dairy cows and it is well known that sex-sorted semen shows lower fertility[20, 30] and shorter longevity than non-sorted semen[6]. Therefore, I examined the motility characteristics of sex-sorted semen as a model of low fertility sperm by the cluster analysis developed in Chapter III, and examined the functional characteristics by fluorescent staining developed in Chapter II.

Materials and Methods

Semen

Non-sorted frozen semen derived from 6 Holstein bulls (A-F) and sex-sorted frozen semen derived from 3 Holstein bulls (D-F) donated by an AI center (Genetics Hokkaido, Kita-Hiroshima, Japan) were used in this chapter. These were used commercially and proved acceptable conception rates by AI in the field.

Analyzing sperm motility subpopulation by cluster analysis

An analysis of sperm motility was performed by *k*-means method as described in Chapter III. Briefly, the sperm kinetic parameters were obtained at 150 fps by CASA system (SMAS), and used VSL, VCL, VAP, ALH, and BCF were used as the parameters for cluster analysis. Data from 44,570 sperm incubated in the presence or absence of calcium ionophore A23187 (C7522, Sigma-Aldrich, St. Louis, USA) in BO medium were obtained from 3 bulls (A-C) in 6 replicates (36 frozen-thawed semen).

Evaluation of sperm characteristics by fluorescent staining under microscopy

Evaluation of sperm characteristics was performed as described in Chapter II with slight modification. Briefly, Hoechst 33342 was not used for staining of sex-sorted sperm because they were already stained by Hoechst while they were sorted for production.

Evaluation of the sperm motility subpopulation structure in sex-sorted and non-sorted semen by the discriminant analysis

To evaluate the structure of sperm motility subpopulation, discriminant analysis was performed by the custom written program (Igor Pro, Wavemetrics, Lake Oswego, OR, USA). The mean and SD of the data in each cluster calculated by the cluster analysis were input to the program. The program categorized the data from sex-sorted and non-sorted samples (bulls D-F, 3 replicates) into each cluster by their motility parameters.

Experimental design

Experiment IV-1: The effects of media and frame rate on hyperactivated sperm motility

Non-sorted frozen semen derived from 3 bulls (A-C) were used. Straws containing frozen semen were immersed in water at 37˚C for 1 min, and semen were expelled on 45/90% Percoll layer diluted by BO (112.00 mM NaCl, 4.02 mM KCl, 0.83 mM NaH_2PO_4, 2.25 mM $CaCl_2$, 0.52 mM $MgCl_2$, 37.00 mM $NaHCO_3$, 13.90 mM glucose, 1.25 mM sodium pyruvate, and 50 μg/ml gentamicin sulfate) or SOF (107.70 mM NaCl, 7.16 mM KCl, 1.19 mM KH_2PO_4, 1.71 mM $CaCl_2$, 0.49 mM $MgCl_2$, 27.07 mM $NaHCO_3$, 3.30 mM sodium lactate, 0.33 mM sodium pyruvate, 1.50 mM glucose, and 50 μg/ml gentamicin sulfate) without BSA. Then, the samples were centrifuged at 700 × g for 20 min for selecting motile sperm. The supernatants were removed and resulting sperm pellets were resuspended by media (BO or SOF) and centrifuged again at 500 × g for 5 min for washing. After second centrifugation, the supernatants were removed and sperm concentration was calculated using hemocytometer, and samples were diluted to 10×10^6 cells/ml by BO or SOF containing 3.0 mg/ml BSA. Then, samples were divided into two aliquots, one was for induction of hyperactivation and another was for control.

For induction of hyperactivation[77], calcium ionophore A23187 (final concentration 1 μM) was add to the samples. Calcium ionophore stock were 3.82 mM A23187 in ethanol. Then, I evaluated sperm motility before (0 min) and after 1, 5, 10, 15, 20, 25, and 30 min with (+) or without (-) A23187 treatment in each medium, BO (+) or (-) and SOF (+) or (-), respectively. After evaluation of sperm motility, the effect of media on the motility parameters related to hyperactivation (VSL, VCL, LIN, ALH, and BCF) were investigated.

Because BO (+) group showed clear hyperactivation-like motility, the frame rate of data obtained in BO (+) group was converted from 150 to 30, 50, and 75 fps by using a software (Frame step motility, DITECT). Then, I recalculated the motility parameters related to hyperactivation (VSL, VCL, LIN, ALH, and BCF) at each frame rate.

Experiment IV-2: Cluster analysis of sperm motility and evaluation of sperm characteristics by fluorescent staining in sex-sorted and non-sorted sperm

For making cluster model, the motility parameters derived from BO (+) and (-) groups in Experiment IV-1 were used as reference data. Firstly, the effect of A23187 on the structure of sperm motility subpopulation was investigated. Then, motility parameters from sex-sorted and non-sorted sperm derived from bulls D-F were fitted to the model. Sperm treatment for sex-sorted and non-sorted semen was performed as described previously[43]. Briefly, motile sperm separated using 45/90% Percoll layer and recovered motile sperm were incubated in 50-μl droplets of BO (final concentration of 10×10^6 cells/ml) under 5% CO_2, 5% O_2 and 90% N_2 at 39˚C. After 0, 2, and 4 hr incubation, sperm motility was analyzed by CASA system at 37˚C. Samples in 6 fields (at least 100 sperm) were observed.

Statistical analysis

All analyses were performed using JMP pro 13 (SAS, NC, USA). Data in each bull were pooled before analysis. Data were analyzed by repeated measurement two-way ANOVA followed by Tukey-Kramer's HSD test or Student's-*t* test. Data were represented as the mean ± SD. Differences were considered significant at $P < 0.05$.

Results

Experiment IV-1

The interactions between the effects of media and times after A23187 treatment were found in all kinetic parameters. As shown in Fig. IV-1, BO (+) showed the lowest values in VSL at 15-30 min ($P<0.05$) and LIN at all time points ($P<0.05$). BO (+) showed higher values in VCL than SOF (+) at 0 min ($P<0.05$) and the value decreased to similar value with SOF (+) at any incubation times. At all times for incubation, sperm in BO showed lower LIN than sperm in SOF ($P<0.05$), regardless of A23187 treatment. SOF (+) showed stable VCL during incubation.

As shown in Fig. IV-2, ALH in BO (+) and SOF (+) were not affected by incubation time. BO (+) had higher values of ALH than SOF (+) at 0, 1, and 5 min ($P<0.05$). There was no difference in ALH between BO (-) and SOF (+) during incubation. BCF in BO (+) and SOF (+) were lower than those of each control group at 15-30 min ($P<0.05$). BCF in BO (-) and SOF (-) did not change during incubation. BO (-) showed lower BCF than SOF (-) at all incubation time ($P<0.05$).

There was no interaction between frame rate and incubation period; therefore, the data from different time points were pooled in each group for a comparison between groups with different frame rate. As shown in Fig. IV-3, VSL was same at any frame rates. VCL and BCF increased significantly as frame rate increased ($P<0.05$). ALH and LIN decreased significantly as frame rate increased ($P<0.05$).

Experiment IV-2

The dendrogram described by Ward's method using the data of 44,570 motile sperm derived from BO samples (288 samples; 3 bulls, 6 replicates, with or without A23187, and each time points of incubation) was presented in Fig. IV-4. The number of cluster was determined as 6 from the shape of the dendrogram. The kinetic parameters of sperm in each cluster were described in Table IV-1. Each cluster was numbered depending on value of VSL from largest to smallest. Cluster 1 showed the highest VAP, the second highest VCL and LIN as well as the third highest ALH and BCF. Cluster 2 showed the second highest STR, the third highest VCL, the highest BCF and LIN but the second lowest ALH and lower VCL than clusters 1, 3, and 4. Cluster 3 had the highest VCL and ALH, the second highest VAP, and the third lowest BCF and LIN. Cluster 4 had the second highest VCL and the third highest ALH, BCF, and LIN, but VAP was lower than clusters 1, 2 and 3. Cluster 5 showed the second highest values in all parameters. Cluster 6 had the lowest values in all parameters.

There was no interaction between A23187 treatment and incubation time. As shown in Fig. IV-5, when sperm were treated with A23187, the proportions of clusters 1 and 2 were lower, and the proportions of clusters 3 and 4 were higher than those of sperm without A23187 treatment ($P<0.05$).

The results of discriminant analysis for the evaluation of motility in sex-sorted sperm were presented in Fig IV-6. The proportion of cluster 1 was lower in sex-sorted sperm than non-sorted sperm at all incubation times ($P<0.05$). The proportion of cluster 3 was higher in sex-sorted than non-sorted sperm before incubation ($P<0.05$). Although the percentage of motile sperm was decreased along with incubation progress ($P<0.05$), the proportion of cluster 1 was not decreased between 0 and 2 hr regardless of sorting.

The percentages of motile sperm were lower in sex-sorted than in non-sorted sperm at 2 and 4 hr after incubation ($P<0.05$).

In Table IV-2, the results of sperm characteristics examined by fluorescent staining were presented. The proportion of sperm with intact plasma membrane, intact acrosome, and high mitochondrial membrane potential was decreased during incubation in sex-sorted and non-sorted sperm ($P<0.05$). Sex-sorted sperm had higher proportion of sperm with damaged plasma membrane, damaged acrosome, and high mitochondrial membrane potential than non-sorted sperm ($P<0.05$).

Discussion

Sperm incubated in BO showed hyperactivation-like motility more clearly than in SOF not only A23187 treatment group but also control in the present study. Although calcium ionophore induces capacitation by increasing influx of intracellular Ca^{2+} ion concentration[9], Ca^{2+} ion concentration in BO and SOF were similar level. On the other hand, BO contained higher concentration of HCO^{3-} ion, an effector of capacitation[35]. Higher concentration of HCO^{3-} ion in BO than SOF may facilitate to induce sperm hyperactivation. In the present study, frame rate affected sperm kinetic parameters evaluated by CASA except for VSL. The changes of sperm kinetic parameters accompanying with increasing frame rate were observed, namely the increase of VCL and ALH as well as the decrease of LIN and BCF. These changes were consistent with the results of a previous report[12]. These results suggest that sperm motility captured at higher frame rate shows the trajectory close to real pathway than that captured at lower

frame rate (Fig. IV-7). Although 50-60 fps are recommended for the evaluation of sperm motility by CASA[58], Mortimer et al.[60] suggested that ram sperm in culture medium should be studied at no lower than 75 fps and 100 fps was preferable. Indeed, the trajectories of sperm with hyperactivation-like motility captured at 50 fps was not smooth and sometimes not evaluated as motile sperm (Fig. IV-8). The present study indicates that the images captured at 150 fps provide the accurate evaluation of sperm trajectory. Therefore, the data of sperm incubated in BO and captured at 150 fps were used as the reference data for the discriminant analysis in Experiment IV-2.

By cluster analysis, motile sperm in BO were categorized into 6 clusters in the present study. Sperm in clusters 1 and 2 showed higher BCF and LIN than sperm in other clusters. These can be considered as progressively motile sperm, which is related to fertilization[47, 63]. Clusters 3 and 4 included sperm with relatively high VCL and ALH, and lower LIN and BCF than clusters 1 and 2. This mean beating the head widely but very quickly and making circle, which is similar with the motility of hyperactivated sperm[37]. Moreover, sperm in clusters 3 and 4 was observed at higher proportion in BO (+) than BO (-). Therefore, I speculate that clusters 3 and 4 are subgroups of hyperactivated sperm. Although sperm in cluster 5 also showed low LIN, other parameters related to hyperactivation, VCL, ALH, and BCF, were lower than those in clusters 3 and 4. In addition, sperm in cluster 6 showed very low values in all parameters. It means that clusters 5 and 6 cannot contribute to fertilization because of their poor motility.

In the present study, the discriminant analysis showed that the main subpopulations of sex-sorted and non-sorted sperm were clusters 3 and 1, respectively, immediately after thawing. The proportion of cluster 1 was maintained until 2 hr after

thawing in both sperm and lower in sex-sorted than non-sorted sperm at all time point. However, the proportion of cluster 3 significantly decreased in both semen while their incubation. These results indicate that sperm with hyperactivation-like motility has shorter longevity than progressively motile sperm. In the present study, I used sperm derived from frozen-thawed sex-sorted semen as a model of low fertility sperm. It is well known that sex-sorted semen shows lower fertility than non-sorted semen after AI[20, 30] and sex-sorted semen has a shorter longevity[6]. Also, shorter lifespan of sperm in sex-sorted semen assumed to related with capacitation-like change induced by sex-sorting[54]. The results of the present study clearly show that the higher proportion of sperm with hyperactivation-like motility is contained in sex-sorted semen. This is the first study to apply discriminant analysis for evaluation of sperm motility and I have shown that the cluster analysis can be an effective method to evaluate semen fertility. However, the suitable size of data and types of sperm motility to establish the valid reference data for evaluation of sperm motility subpopulation is unclear. Further study is necessary to determine the best data size for evaluating the structure of sperm motility subpopulation for the prediction of semen fertility.

It has been reported that sperm in sex-sorted semen are damaged and changes to capacitation-like condition during sex-sorting. However, these changes cannot be detected completely by conventional examination[11]. Therefore, I also examined the characteristics of sperm by fluorescent staining. Quadruple fluorescent staining showed that higher proportion of sex-sorted sperm with damaged plasma membrane, damaged acrosome and high mitochondrial membrane potentials than that of non-sorted sperm, which was very rarely detected in Chapters I and II. The mechanism of injuries on sperm head without damaging midpiece is unclear. It indicates that sex-sorting process

has detrimental effects on sperm head but not mitochondria included in midpiece. To improve the fertility of sex-sorted semen, the cause of this injury should be investigated in future.

In conclusion, the usage of BO for sperm suspension and capturing sperm motility at 150 fps by CASA were appropriate to evaluate hyperactivation-like sperm motility in the present condition of sperm culture. Then, the discriminant analysis using the data of cluster analysis in motile sperm can describe the differences in the structures of sperm motility subpopulation appropriately, and it can detect the subfertility of sperm by describing the proportions of progressively motile and hyperactivation-like motile sperm.

Table IV-1. Kinetic parameters of sperm in each cluster

Cluster	No. of sperm	VSL (μm/sec)	VCL (μm/sec)	VAP (μm/sec)	ALH (μm)	BCF (Hz)	LIN (%)
1	6032	149.2±27.9	319.0±61.2	163.2±21.4	4.0±1.2	21.8±6.2	47.9± 9.9
2	9397	80.7±28.2	168.9±47.0	94.1±27.0	1.6±0.7	28.3±5.8	49.2±14.4
3	2671	77.9±45.9	391.9±68.5	145.1±29.9	6.9±1.3	8.4±4.7	20.2±11.9
4	5203	52.0±29.5	238.4±54.8	90.9±27.1	4.3±1.2	10.6±5.8	23.0±13.6
5	7792	20.9±15.9	123.9±45.2	35.6±17.6	2.6±1.0	7.5±4.5	16.6±11.6
6	13475	5.4± 5.9	42.5±22.1	9.9± 7.8	0.7±0.5	6.9±3.6	12.3± 8.5

Values are the mean ± SD.

VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; VAP (μm/sec), average path velocity;
ALH (μm), amplitude of lateral sperm head displacement; BCF (Hz), beat cross frequency;
LIN (VCL/VSL, %), linearity.

Table IV-2. The effect of sex-sorting on sperm characteristics evaluated by fluorescent staining at each time after thawing

Characteristics of sperm			% of each sperm characteristics at each incubation period							
			0 hr		2 hr		4 hr		Average	
Plasma membrane	Acrosome	Mitochondrial membrane potential	Sex-sorted	Non-sorted	Sex-sorted	Non-sorted	Sex-sorted	Non-sorted	Sex-sorted	Non-sorted
Intact	Intact	High	48.3±14.7[a]	57.2±9.4[a]	18.5±10.7[a]	26.0±8.1[a]	7.6± 2.9[b]	13.2±4.6[b]	24.8±20.2	13.2±4.6
		Low	2.4±2.6[a]	1.6±1.4[a]	1.9± 1.3[a]	2.5±1.9[a]	3.5± 3.0[b]	3.9±1.8[b]	2.6± 2.5	3.9±1.8
	Damaged	High	0	0	0	0	0	0	0	0
		Low	0	0	0	0	0	0	0	0
Damaged	Intact	High	10.7±9.0[a]	7.4±5.3[a]	8.2± 3.8[ab]	5.7±5.8[ab]	2.6± 2.9[b]	3.7±3.1[b]	7.2± 6.8	3.7±3.1
		Low	21.0±7.8[a]	21.4±6.5[a]	48.2±13.9[b]	44.8±8.1[b]	56.5±10.2[c]	50.9±7.5[c]	41.9±18.7	50.9±7.5
	Damaged	High	2.8±2.7[a]	0.8±0.5[a]	1.7± 1.5[ab]	0.4±0.4[ab]	0.5±0.5[b]	0.5±0.6[b]	1.7± 2.1[*]	0.5±0.6
		Low	14.8±6.0[a]	11.6±4.8[a]	21.6± 5.5[b]	20.6±6.3[b]	29.3±9.8[c]	27.7±4.2[c]	21.9± 9.4	27.7±4.2

Values are the mean ± SD (3 bulls/group, 3 replicates).

[abc]; Superscripts indicate significant differences between time ($P<0.05$).

*; Significant differences between semen groups ($P<0.05$).

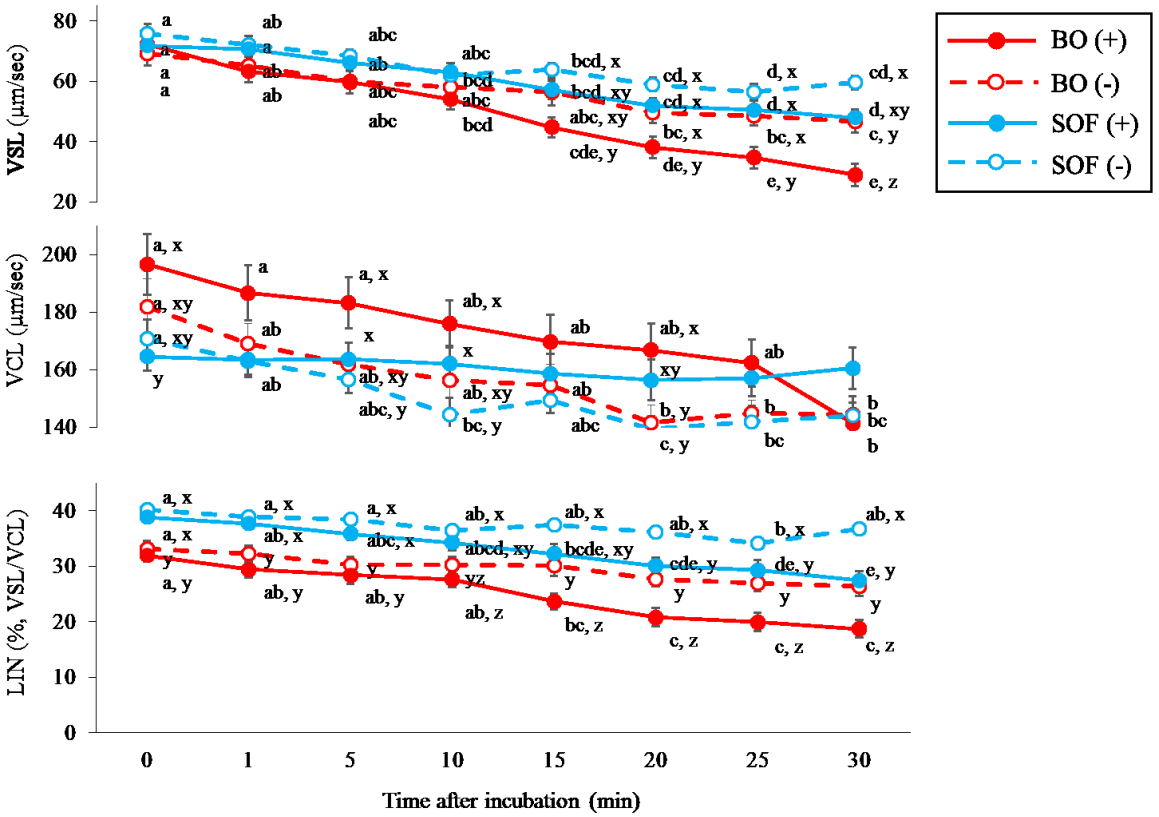

Fig. IV-1. Effects of the media on sperm motility

Three bulls were used and 6 replicates were performed in each group.

VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; LIN (VCL/VSL, %), linearity.

[abcde]; Superscripts indicate significant differences between time after treatment ($P<0.05$).

[xyz]; The asterisk indicates indicate significant differences between groups ($P<0.05$).

Error bars indicate standard error of mean (SEM).

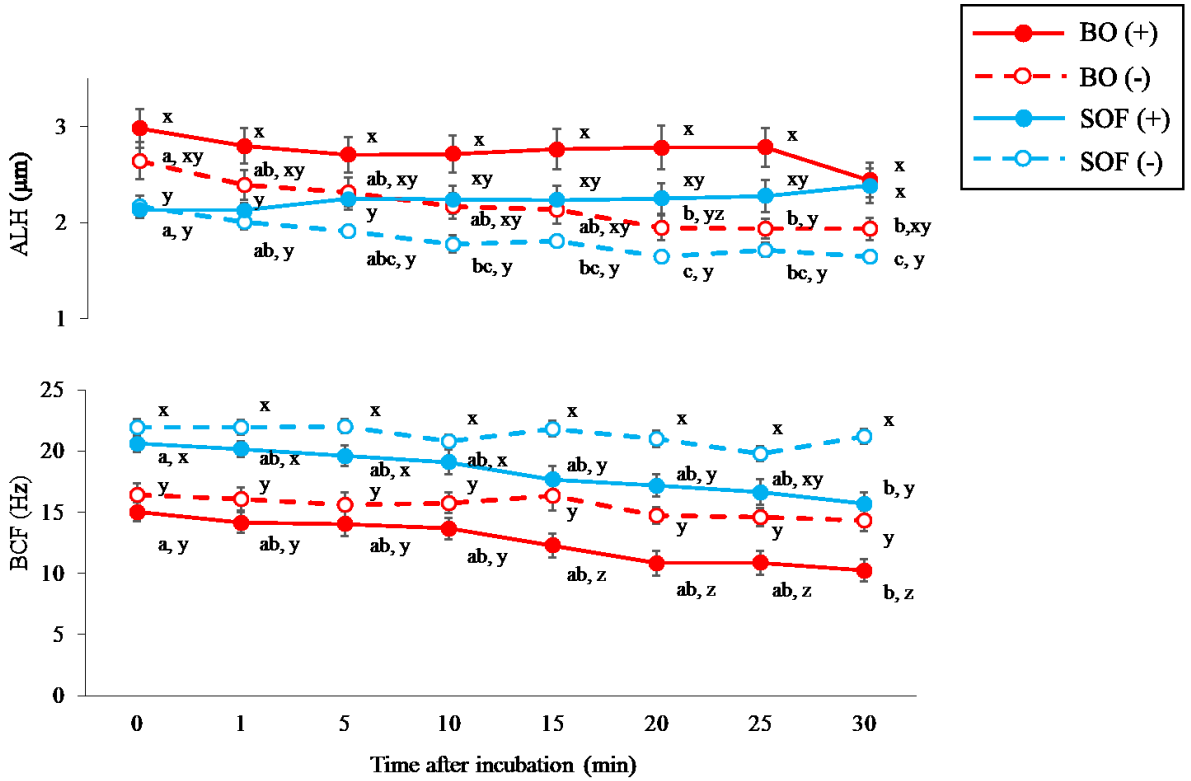

Fig. IV-2. Effects of the media on sperm head motility

Three bulls were used and 6 replicates were performed in each group.

ALH (μm), amplitude of lateral sperm heed displacement; BCF (Hz), beat cross frequency.

[abc]; Superscripts indicate significant differences between time after treatment ($P<0.05$).

[xyz]; Superscripts indicate significant differences between groups ($P<0.05$).

Error bars indicate SEM.

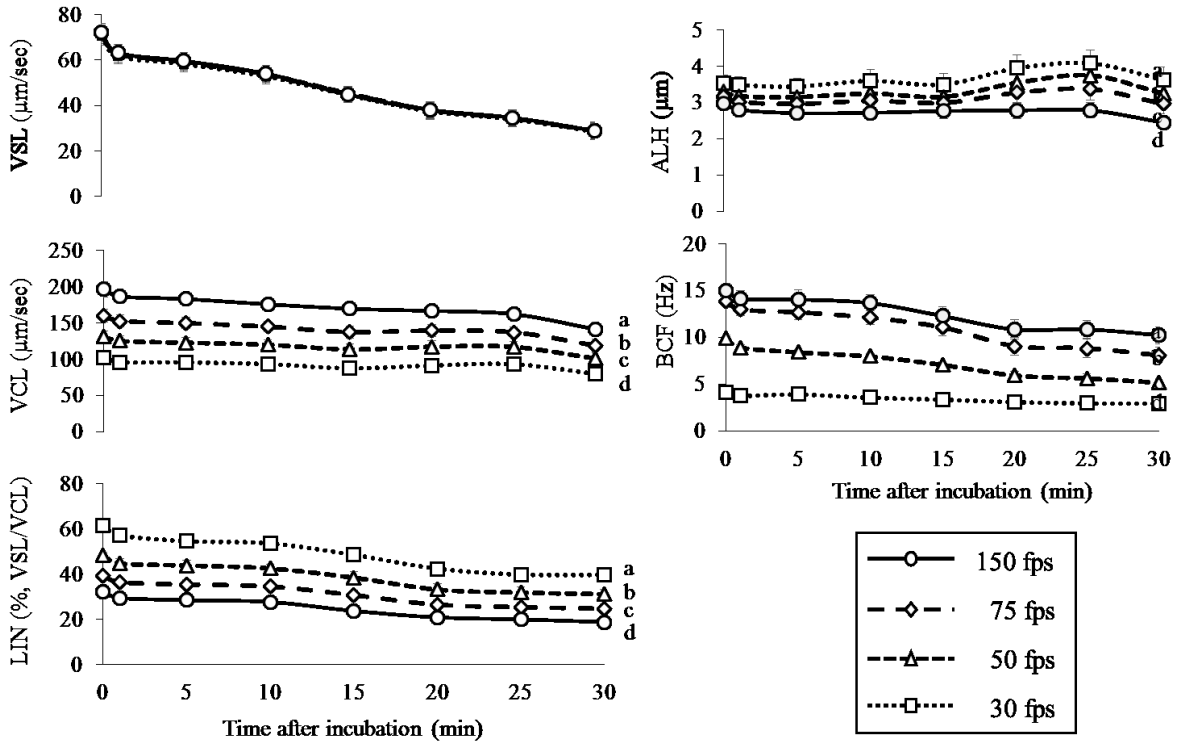

Fig. IV-3. Effects of frame rate on sperm and their head motility

Three bulls were used and 6 replicates were performed in each group.

VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; LIN (VCL/VSL, %), linearity; ALH (μm), amplitude of lateral sperm head displacement; BCF (Hz), beat cross frequency.

^{abcd}; Superscripts indicate significant differences between frame rates ($P<0.05$).

Error bars indicate SEM.

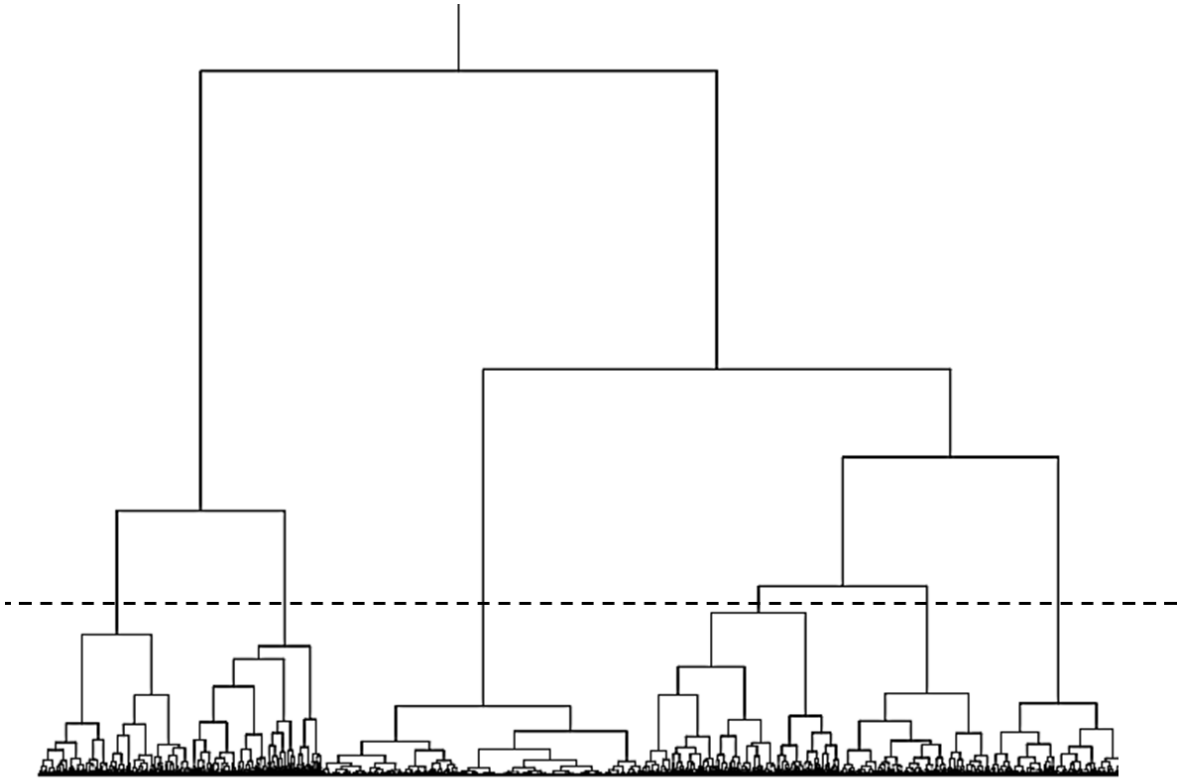

Fig IV-4. Dendrogram described by Ward's method to determine the number of cluster

To describe the dendrogram, 44,570 of motile sperm incubated in BO were used.

The five independent motility variables; VSL (μm/sec), straight line velocity; VCL (μm/sec), curvilinear velocity; LIN (VCL/VSL, %), linearity; ALH (μm), amplitude of lateral sperm head displacement; BCF (Hz), beat cross frequency, were used as parameters.

A broken line was put to determine the number of clusters for *k*-means method to categorize sperm.

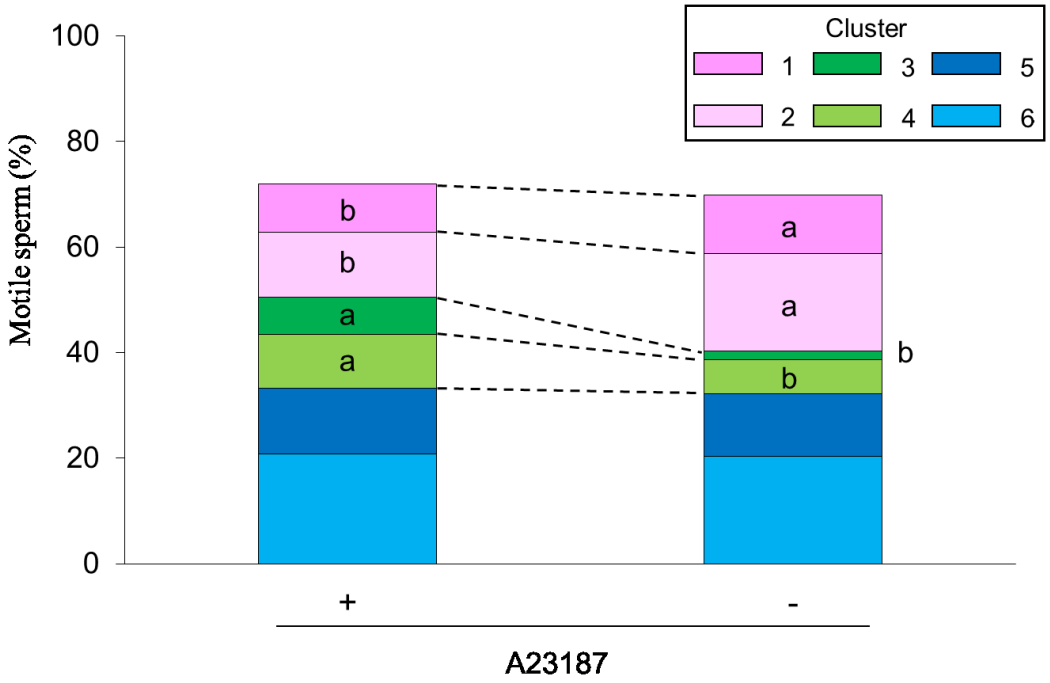

Fig. IV-5. Effects of calcium ionophore A23187 treatment on the structures of sperm motility subpopulation

Data of sperm with (+) and without (-) treatment at all time points were pooled.

Three bulls were used and 6 replicates were performed in each group.

All of time points (0-30 min) were pooled.

[ab]; Letters indicate significant differences in the same cluster between groups ($P<0.05$).

Sperm kinetic parameters of each cluster were presented in Table IV-1.

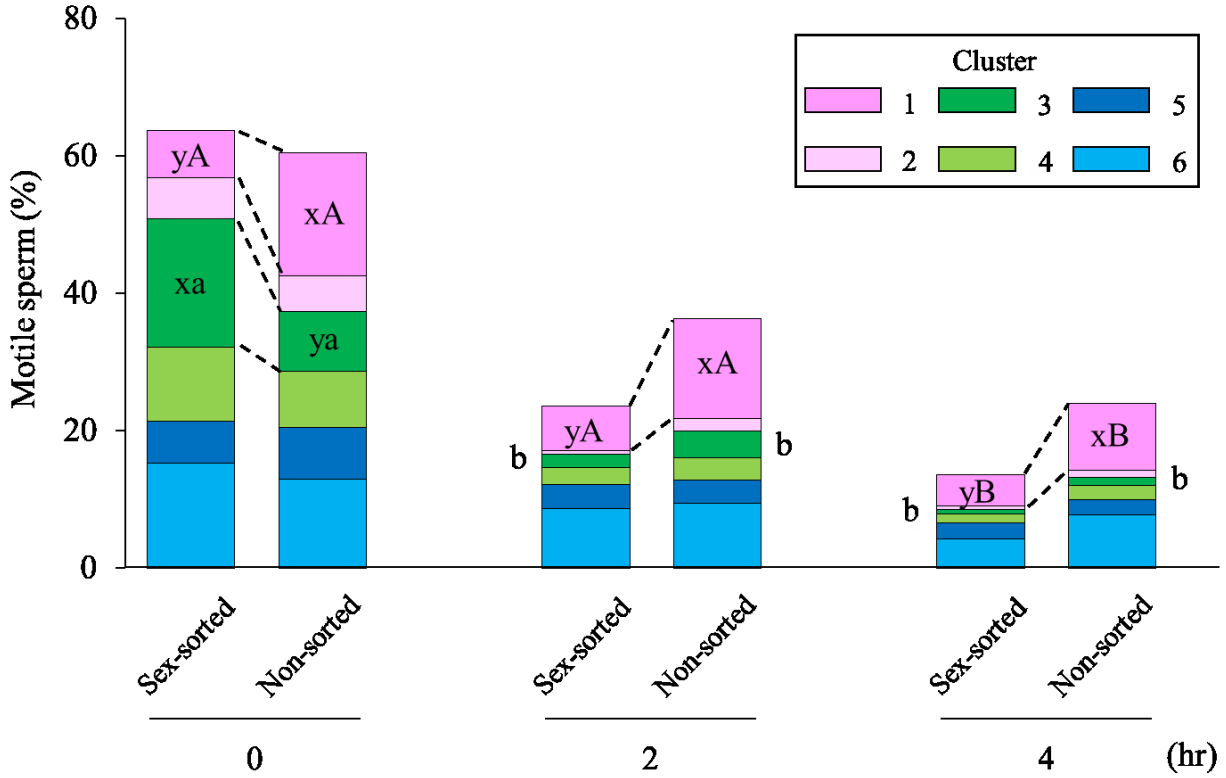

Fig. IV-6. Effects of sex-sorting and incubation in BO on the structures of sperm motility subpopulations

Three bulls were used and 3 replicates were performed in each group.

[xy]; Letters indicate significant differences in the same cluster between sorting ($P<0.05$).

[AB]; Letters indicate significant differences in the proportion of cluster 1 between incubation time ($P<0.05$).

[ab]; Letters indicate significant differences in the proportion of cluster 3 between incubation time ($P<0.05$).

Sperm kinetic parameters of each cluster were presented in Table IV-1.

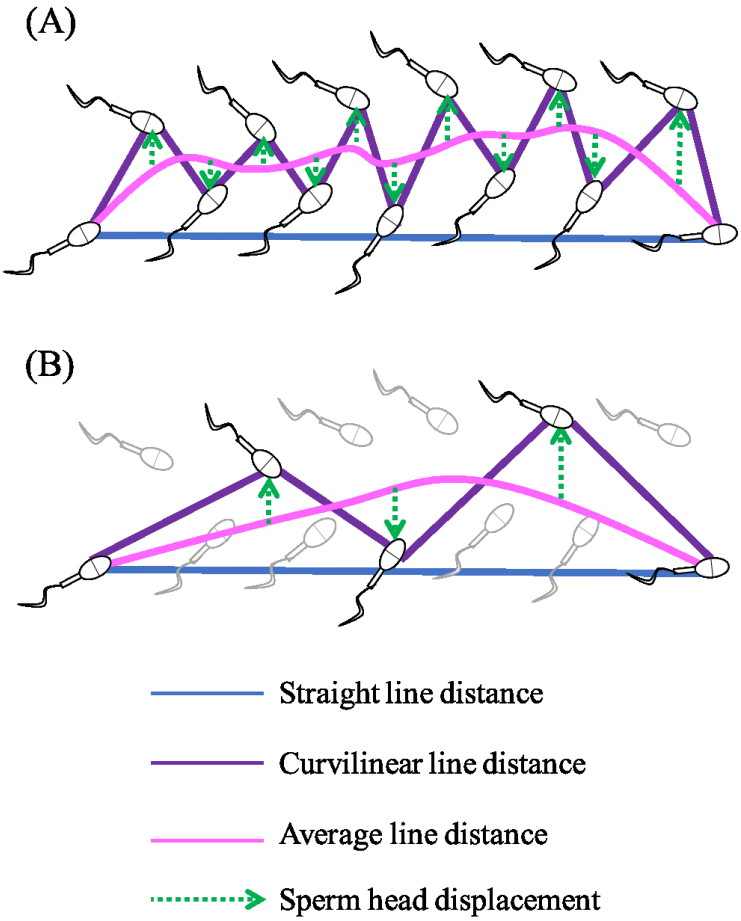

Fig. IV-7. The differences in the images of sperm trajectories between different frame rates (A) is a sperm trajectory captured at 3 times higher frame rate than (B). There is no difference of straight line distance between (A) and (B). However, curvilinear line distance and sperm head displacement, the number of sperm crossing average distance are increased at (A). Therefore, sperm kinetic parameters other than VSL are affected by frame rate.

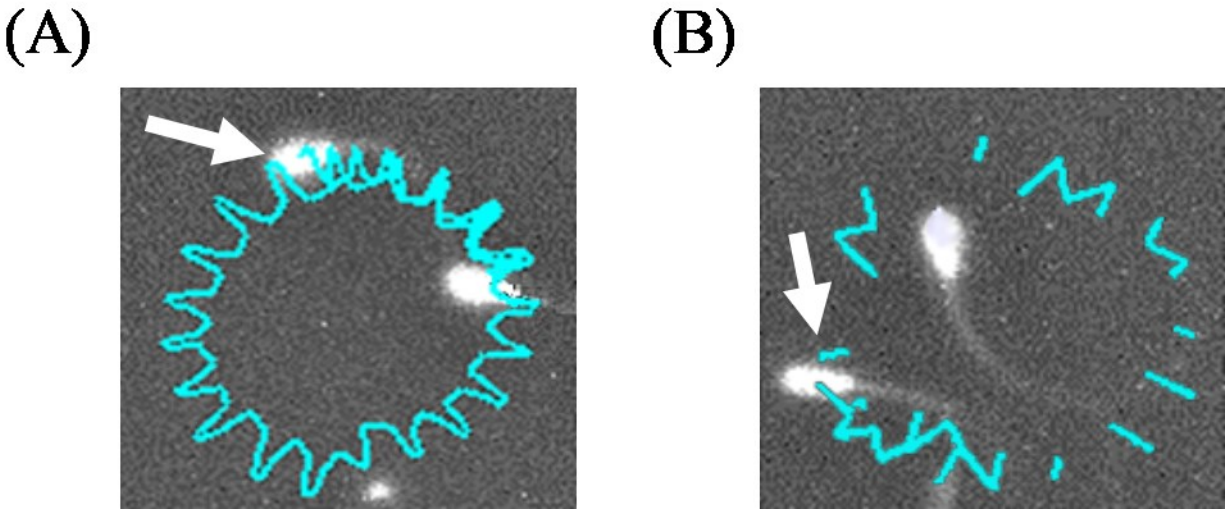

Fig. IV-8. The trajectories of a sperm with hyperactivation-like motility at difference frame rates

Trajectories were described by capturing sperm (white arrows).
(A) Sperm with hyperactivation-like motility captured at 150 fps.
The trajectory was described smoothly and without interruptions.
(B) Same sperm with hyperactivation-like motility captured at 50 fps.
The trajectory was partly interrupted and not used for calculation of sperm kinetic parameters by CASA system.

Summary

The effect of the media for sperm suspension and frame rate on the detection of sperm with hyperactivation-like motility was examined. Sperm incubated in Brackett and Oliphant medium (BO) showed hyperactivation-like motility more clearly than those in synthetic oviductal fluid. Also, sperm images captured at 150 frames per second (fps) showed more real pathway than other frame rates (30, 50, and 75 fps). Therefore, sperm in BO captured at 150 fps were used for cluster analysis. Next, I examined the characteristics of sex-sorted semen as a model of low fertility sperm by the cluster analysis. The discriminant analysis by using the data of cluster analysis was performed to evaluate the structures of sperm motility subpopulations in sex-sorted and non-sorted sperm. Sex-sorted semen contained sperm with hyperactivation-like motility as the main subpopulation immediately after thawing and the subpopulation was dramatically decreased after incubation. On the other hand, the main subpopulation in non-sorted semen was progressively motile sperm and it maintained during incubation. In conclusion, the usage of BO for sperm suspension and capturing sperm motility at 150 fps by CASA were appropriate to evaluate hyperactivation-like sperm motility in the present condition for sperm culture. Then, the discriminant analysis using the data of cluster analysis in motile sperm can describe the differences in the structures of sperm motility subpopulation correctly.

Summary and Conclusions

Artificial insemination (AI) using frozen-thawed semen is used for cattle reproduction all around the world. However, sperm are injured and reduced their fertility during several steps in the preservation procedure. Therefore, the evaluation of sperm characteristics is important to manage frozen-thawed semen quality. Although there are many laboratory assays to evaluate sperm characteristics, any assay cannot predict sperm fertility accurately even by using some objective tools, such as computer-assisted sperm analysis (CASA) and flow cytometry. Therefore, in the present study, the author aimed to develop new accurate evaluation methods for sperm motility and multiple characteristics. Then, the efficiency of the evaluation methods were confirmed by evaluating the quality of sex-sorted sperm.

In Chapter I, I aimed to develop an objective and simultaneous evaluation procedure to estimate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bull sperm by flow cytometry. Firstly, I used frozen-thawed semen mixed with 0, 25, 50, 75, and 100% dead sperm. Semen was stained using three staining solutions: SYBR-14 (stained for intact plasma membrane), PI (stained for damaged plasma membrane), and PE-PNA (stained for damaged acrosome), for the evaluation of plasma membrane integrity and acrosomal integrity. Then, the characteristics evaluated by flow cytometry and by fluorescent microscopy were compared. In terms of the results, plasma membrane integrity of sperm and acrosomal integrity evaluated by flow cytometry and by fluorescent microscopy were similar. Secondly, I attempted to evaluate plasma membrane integrity, acrosomal integrity, and also mitochondrial membrane potential of sperm by flow cytometry using conventional staining with three dyes (SYBR-14, PI, and PE-PNA) combined with MTDR (stained for mitochondria with high membrane potential) staining (quadruple staining). Then, the sperm characteristics evaluated by flow cytometry using quadruple staining were compared with those of staining using SYBR-14, PI, and PE-PNA and staining using SYBR-14 and MTDR. From the obtained

results, there were no significant differences in plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential evaluated by quadruple staining and the other procedures. The results of the study indicated that quadruple staining using SYBR-14, PI, PE-PNA, and MTDR for flow cytometry can evaluate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of sperm simultaneously.

In Chapter II, sperm characteristics were evaluated by using fluorescent staining to verify the dynamics of damages to sperm during semen freezing (pre-dilution, cooling to 4˚C, and frozen-thawed) and cooling preservation at 4˚C (day 1-5). Pre-diluted sample showed the highest proportion of sperm with intact plasma membrane, intact acrosome and high mitochondrial membrane potential. The proportion of sperm with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential were higher after cooling than the other processes ($P<0.05$). During cooling preservation examined in this study, the proportion of sperm with damaged acrosome increased. These results read me to speculate that, during cooling process, sperm may be firstly injured to mitochondrial membrane, and low mitochondrial function may cause the impairment of plasma membrane and subsequent cell death with acrosomal damage.

In Chapter III, bull sperm in the first and second ejaculates were divided into subpopulations based on their motility characteristics using a cluster analysis of data from CASA. Semen samples were collected from 4 Japanese black bulls. Data from 9,228 motile sperm were classified into 4 clusters; 1) very rapid and progressively motile sperm, 2) rapid and circularly motile sperm with widely moving heads, 3) moderately motile sperm with heads moving frequently in a short length, and 4) poorly motile sperm. The percentage of cluster 1 varied between bulls. The first ejaculates had a higher proportion of cluster 2 and lower proportion of cluster 3 than the second ejaculates. Clusters 1, 2, and 3 sperm may be evaluated as progressively motile sperm by visual assessments, in which differences between them cannot be detected, whereas a cluster analysis has the ability to identify differences and similarities.

It means that cluster analysis can distinguish the characteristics of the first and second ejaculates collected at short interval.

In Chapter IV, the effect of the media for sperm suspension and frame rate of CASA system on the detection of sperm with hyperactivation-like motility (beating the head widely but very quickly) was examined. Sperm incubated in Brackett and Oliphant medium (BO) showed hyperactivation-like motility more clearly than those in synthetic oviductal fluid. Also, sperm images captured at 150 frames per second (fps) showed more detailed pathway than other frame rates (30, 50, and 75 fps). Therefore, sperm in BO captured at 150 fps were used for cluster analysis. Next, I examined the characteristics of sex-sorted semen as a model of low fertility sperm by the cluster analysis. The discriminant analysis by using the data of cluster analysis was performed to evaluate the structures of sperm motility subpopulations in sex-sorted and non-sorted sperm. Sex-sorted semen contained sperm with hyperactivation-like motility as the main subpopulation immediately after thawing and the subpopulation was dramatically decreased after incubation. On the other hand, the main subpopulation in non-sorted semen was progressively motile sperm and it maintained during incubation. In conclusion, the usage of BO for sperm suspension and capturing sperm motility at 150 fps by CASA were appropriate to evaluate hyperactivation-like sperm motility in the present condition for sperm culture. Then, the discriminant analysis using the data of cluster analysis in motile sperm can describe the differences in the structures of sperm motility subpopulation correctly.

In the present study, the author has developed the novel evaluation methods of sperm characteristics by fluorescent staining and sperm motility by cluster analysis. By using these procedures, semen fertility can be predicted precisely. The procedures also give us the information of the damages to sperm during handling, cryopreservation and sex-sorting of semen in detail. The detailed information may enable us to develop a new procedure of semen preservation with few damages to sperm.

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