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Original Article

Title: Simultaneous evaluation of plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential in bovine spermatozoa by flowcytometry

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Running head: Bull sperm evaluation by flowcytometry

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Summary

The present study aimed to develop an objective evaluation procedure to estimate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bull spermatozoa simultaneously by flowcytometry. Firstly, we used frozen-thawed semen mixed with 0%, 25%, 50%, 75% and 100% dead spermatozoa. 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 flowcytometry and by fluorescent microscopy were compared. In terms of the results, the characteristics of spermatozoa (viability and acrosomal integrity) evaluated by flowcytometry and by fluorescent microscopy were similar. Secondly, we attempted to evaluate plasma membrane integrity, acrosomal integrity, and also mitochondrial membrane potential of spermatozoa by flowcytometry using conventional staining with three dyes (SYBR-14, PI, and PE-PNA) combined with MitoTracker Deep Red (MTDR) staining (quadruple staining). Then, the spermatozoon characteristics evaluated by flowcytometry 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 flowcytometry can evaluate plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bovine spermatozoa simultaneously.

Keywords: Acrosomal integrity, Bovine spermatozoa, Flowcytometry, Mitochondrial membrane potential, Spermatozoon viability

Introduction

Artificial insemination (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 spermatozoa in semen, was positively correlated with the pregnancy rate (Brito *et al.*, 2002). 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 spermatozoa (Hammerstedt *et al.*, 1990; Silva & Gadella, 2006; Watson, 2000). Therefore, the evaluation of spermatozoon 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 spermatozoon characteristics: motility, viability, morphological abnormality, and organelle functions (Den Daas *et al.*, 1998; Linford *et al.*, 1976; Söderquist *et al.*, 1991; Thomas *et al.*, 1998). 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 spermatozoon characteristics. Moreover, it is thought that the results from any single laboratory assay will not effectively estimate the fertilizing potential of a semen sample (Graham & Mocé, 2005); therefore, combined multiple assays are necessary to estimate the characteristics of spermatozoa more accurately.

Recently, flowcytometry has been used as an objective tool for evaluating multiple characteristics of a large number of spermatozoa (Vincent *et al.*, 2012). Nagy *et al.* (2003) 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 spermatozoa simultaneously. In addition, Thomas *et al*. (1998) proved that mitochondrial membrane potential of spermatozoa could be assessed by flowcytometry using JC-1 as a probe. If these two methods can be combined, we can evaluate 3 items, viability, acrosomal integrity and mitochondrial membrane potential, of spermatozoa simultaneously and it is possible to obtain more detailed information about each spermatozoon. However, the combination of these reagents for flowcytometry 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 flowcytometry. Celeghini *et al*. (2007) reported simultaneous evaluation of viability, acrosome integrity, and mitochondrial membrane potential using fluorescent microscopy. However, their method cannot be applied to flowcytometry because they used PI and JC-1. Hallap *et al*. (2005) reported that spermatozoa 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 (Martínez-Pastor *et al.*, 2010). Therefore, MTDR is a candidate for evaluating mitochondrial membrane potential simultaneously with triple staining mentioned above.

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

Materials and Methods

Semen

Frozen semen, which was diluted with egg yolk-Tris-glycerol (6%) 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 spermatozoa used in experiment 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 (Hallap *et al.*, 2005). 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 spermatozoa

Staining solution was prepared as described in a previous study (Nagy *et al.*, 2003). 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 spermatozoa

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 flowcytometry

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 spermatozoa in the staining solution, as described in a previous study (Harrison and Vickers, 1990). Subsequently, 100 µl of stained sample was mixed with 400 µl of DPBS and subjected to flowcytometry. Sperm suspensions were run through a flowcytometer (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). Flowcytometric gating of spermatozoa was performed as reported by Hallap *et al.* (2005) and Nagy *et al.* (2003). The gating of quadruple staining was performed as described in Fig. 1. Briefly, particles stained with SYBR-14 or PI were judged as spermatozoa (Fig. 1 A). Spermatozoa were divided into 2 groups (live and dead) by PI emission (Fig. 1 B) and then each group was gated by PE-PNA and MTDR (Figs. 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 spermatozoa per slide were examined and classified based on the fluorescence emitted from each probe (Table 1). Three slides per sample were examined and the average was used as the value of the sample.

Evaluation of spermatozoon characteristics

Spermatozoon characteristics estimated by flowcytometry are described in Table 1. Briefly, when spermatozoa 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 spermatozoon was stained with MTDR, it was evaluated that the spermatozoa had high mitochondrial membrane potential.

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

Experimental design

In experiment 1, frozen-thawed semen was mixed with 0%, 25%, 50%, 75%, and 100%

dead spermatozoa. These samples were subjected to triple staining. After staining, half of the sample was evaluated by flowcytometry and the other half by fluorescent microscopy; the obtained results were then compared. Semen derived from 5 bulls was used for this experiment.

In experiment 2, frozen-thawed semen derived from a bull was subjected to double, triple, and quadruple staining. Then, the results of the spermatozoon 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 spermatozoa estimated by flowcytometry and by fluorescent microscopy was analyzed by linear regression analysis. The percentages of spermatozoon 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 1: The viability and acrosomal integrity of spermatozoa evaluated by flowcytometry and fluorescent microscopy were significantly correlated ($r > 0.9$, $P < 0.01$), except for the live spermatozoa with a damaged acrosome ($P = 0.866$), as shown in Fig. 3. The percentages of each characteristic evaluated by flowcytometry and fluorescent microscopy are shown in Table 2. There were no significant differences in all characteristics (live spermatozoa with an intact acrosome, live spermatozoa with a damaged acrosome, dead spermatozoa with an intact acrosome, and dead spermatozoa

with a damaged acrosome) evaluated by the two types of equipment ($P > 0.05$). The percentages of live spermatozoa with a damaged acrosome were low among the samples with different mixed ratios of dead spermatozoa.

Experiment 2: The viability, acrosomal status, and mitochondrial membrane potential of spermatozoa evaluated by flowcytometry after quadruple staining and by the other staining procedures are shown in Table 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 spermatozoa having an intact acrosome showed high mitochondrial membrane potential. In addition, more than 95% of dead spermatozoa having an intact acrosome showed low mitochondrial membrane potential.

Discussion

In the present study, the results of spermatozoon characteristics evaluated by flowcytometry and fluorescent microscopy were similar, except for live spermatozoa with a damaged acrosome. High correlation may be due to the criteria of spermatozoa evaluation. The count of fluorescent intensity obtained by flowcytometry 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. 1E, and this small peak might be a subpopulation of spermatozoa those observed as intermediately stained and judged as positive under fluorescent microscopy. Therefore, evaluation of spermatozoa using two equipment could evaluate spermatozoa characteristics by same criteria and provide us similar results. This exception may have been caused by quite a small population (< 1%) of live spermatozoa with a damaged acrosome in semen. The number of spermatozoa evaluated by flowcytometry was about 50 times greater than by fluorescent microscopy, which

examined about 200 spermatozoa (Celeghini *et al.*, 2007; Somfai *et al.*, 2002). In spite of evaluating a large number of spermatozoa, quite a small population of this type of spermatozoa may indicate that spermatozoa die immediately after damage to the acrosome.

In the present study, the viability, acrosomal integrity, and mitochondrial membrane potential of spermatozoa could be evaluated accurately by quadruple staining without interference of fluorescent dye. The present results mean that most of the spermatozoa 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 spermatozoa (Silva & Gadella, 2006). In the present study, most of the live spermatozoa had high mitochondrial membrane potential, while the dead spermatozoa 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 spermatozoon death without acrosomal damage. Mitochondrial activity is crucial and correlates with the fertilization ability of spermatozoa (Amaral *et al.*, 2013). In further study, the relationship between fertility and spermatozoon characteristics as evaluated by flowcytometry using quadruple staining should be carried out.

A staining method to estimate the viability of spermatozoa, acrosomal integrity, and mitochondrial functions simultaneously by using four fluorescent dyes (Hoechst 33342, PI, FITC-PSA, and JC-1) under a fluorescent microscope has also been reported (Celeghini *et al.*, 2007). However, in this previous study (Celeghini *et al.*, 2007), only hundreds of spermatozoa 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 flowcytometry in a short time. This means that the characteristics of spermatozoa 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 flowcytometry can evaluate the plasma membrane integrity, acrosomal integrity, and mitochondrial membrane potential of bovine spermatozoa simultaneously. The procedure can be applied to the quality control of bovine frozen-thawed semen.

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329
330

331 Figure legends
332
333 Figure 1 Gating procedure and judgement for quadruple staining analysis by flowcytometry
334 Items stained with SYBR-14 and propidium iodide (PI) were distinguished as spermatozoa and
335 gated from all events (area in red line; A). Gated spermatozoa were divided into live and dead
336 clusters (B) followed by classification into 4 groups (Q1-4) by acrosome integrity and
337 mitochondrial membrane potential in live (C) and dead (D) spermatozoa.
338 Q1: damaged acrosome with low mitochondrial membrane potential, Q2: damaged acrosome
339 with high mitochondrial membrane potential, Q3: intact acrosome with low mitochondrial
340 membrane potential, and Q4: intact acrosome with high mitochondrial membrane potential.
341 PE-PNA: phycoerythrin-conjugated peanut agglutinin, MTDR: MitoTracker Deep Red.
342 The judgement of each spermatozoon characteristic by flowcytometry depended on fluorescent
343 intensity. Spermatozoa with low fluorescent intensity ($<10^3$) was judged as PE-PNA negative
344 (intact acrosome) and spermatozoa with high fluorescent intensity ($\geq 10^3$) was judged as
345 PE-PNA positive (damaged acrosome) (E).
346
347 Figure 2 The photographs of spermatozoon triple staining taken by a florescent microscopy
348 The head of spermatozoon stained with SYBR-14 and acrosomal region not stained with
349 PE-PNA (A) were judged as a live spermatozoon with an intact acrosome. The head of
350 spermatozoon stained with PI but acrosomal region not stained with PE-PNA (B) was judged as
351 dead spermatozoon with an intact acrosome. The heads of spermatozoon stained with PI and
352 acrosomal region stained intermediately (C) and completely with PE-PNA (D) were both judged
353 as a dead spermatozoon with a damaged acrosome.
354
355 Figure 3 Scatter plots and regression lines of percentages of spermatozoa evaluated by
356 flowcytometry and fluorescent microscopy. Semen from 5 bulls was used and data from the

same bull are indicated by the same symbol.

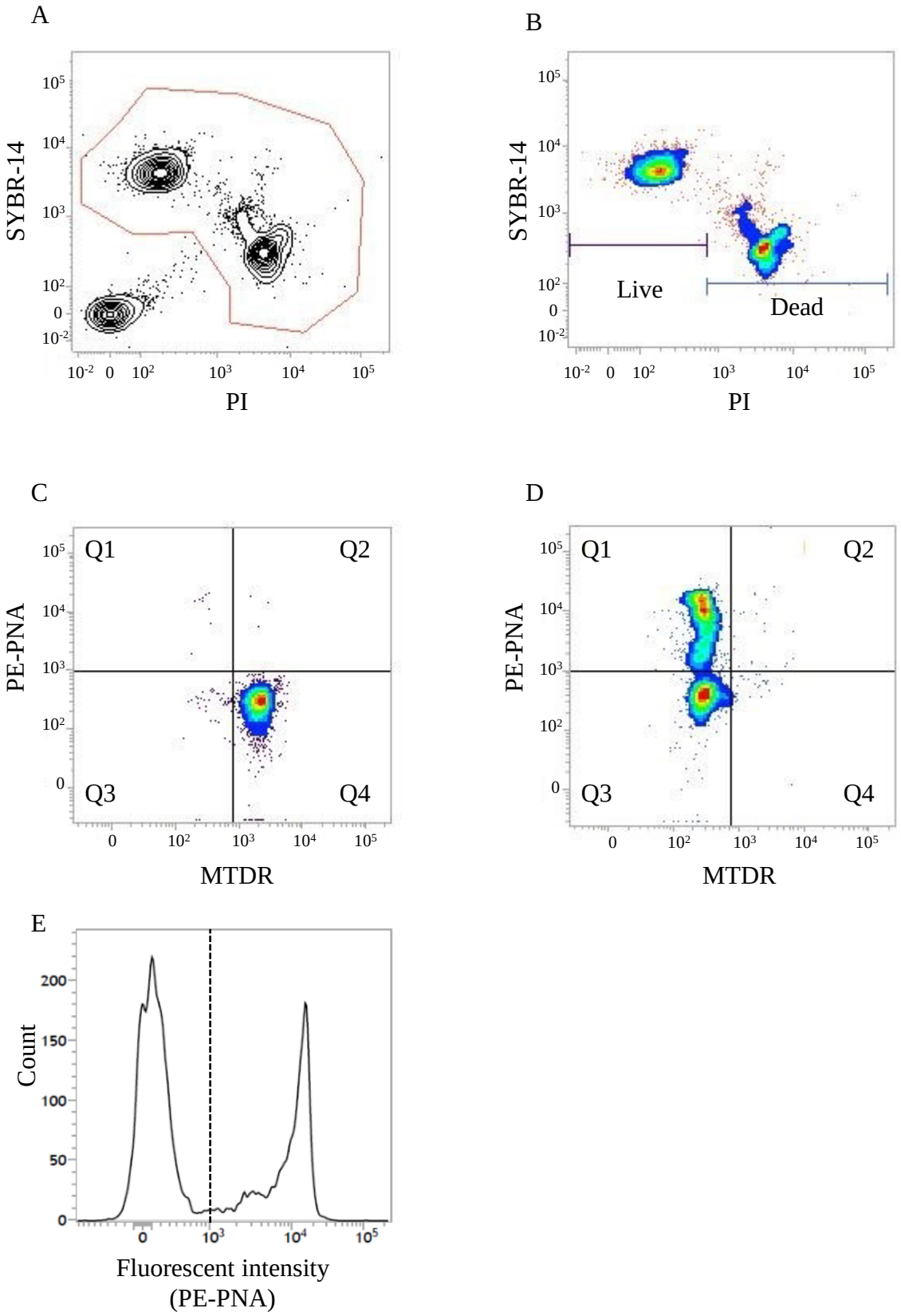
A
SYBR-14
PI
B
SYBR-14
Live
Dead
PI
C
Q1
Q2
Q3
Q4
PE-PNA
MTDR
D
Q1
Q2
Q3
Q4
PE-PNA
MTDR
E
Count
Fluorescent intensity
(PE-PNA)

Fig. 1

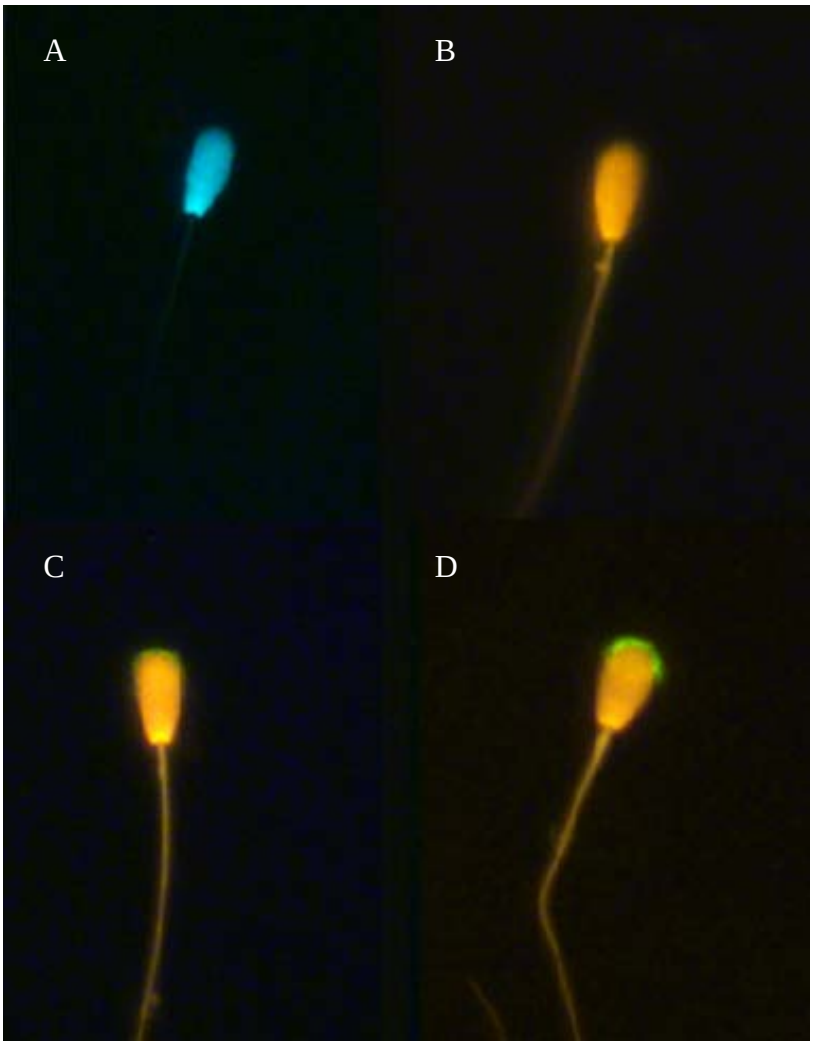

Fig. 2

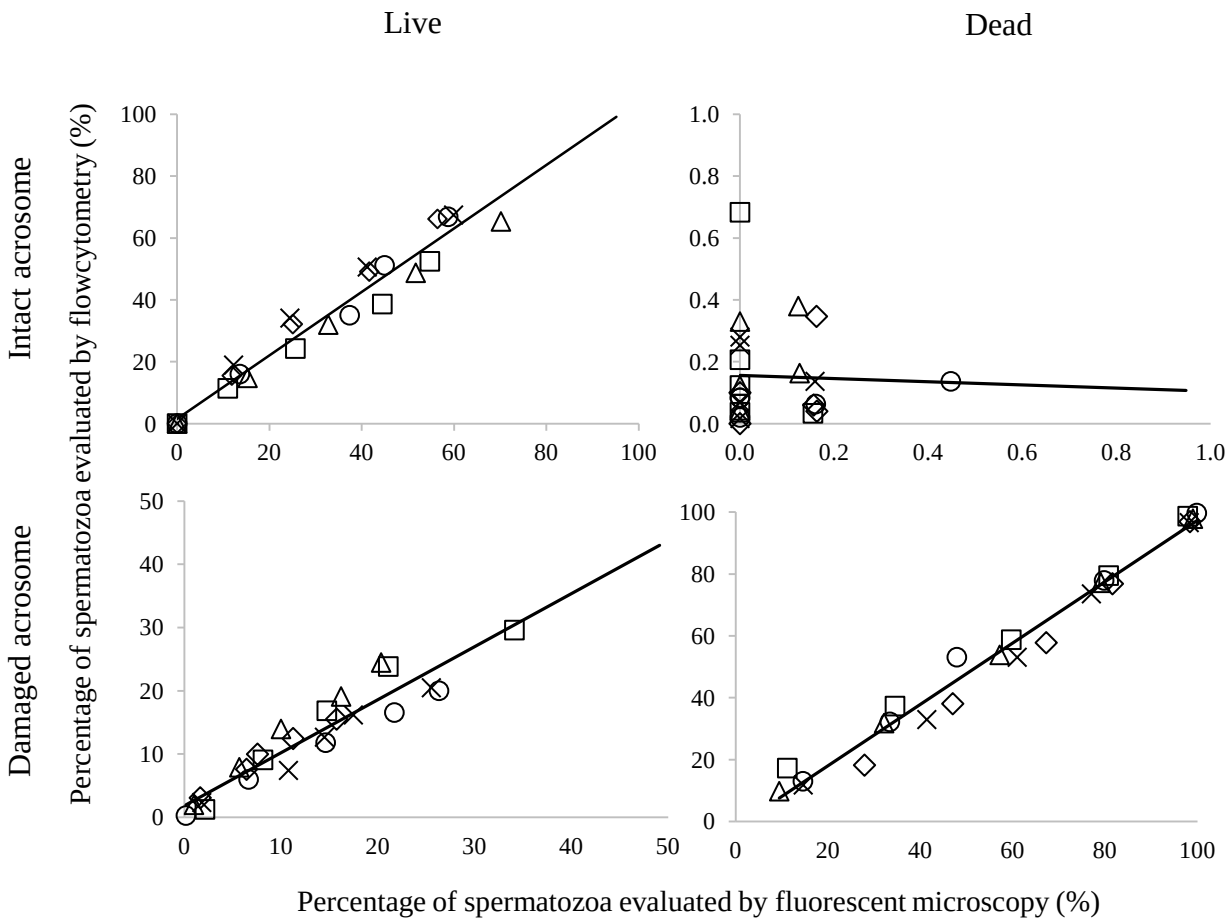
Live
Dead
Intact acrosome
Damaged acrosome
Percentage of spermatozoa evaluated by flowcytometry (%)
Percentage of spermatozoa evaluated by fluorescent microscopy (%)
0
20
40
60
80
100
0.0
0.2
0.4
0.6
0.8
1.0
10
30
50

Fig. 3

Table 1 Staining patterns and evaluation of spermatozoon characteristics by each staining method

Staining procedure	Staining pattern			Spermatozoon 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 2 Characteristics of bovine spermatozoa, the mixture of thawed semen and dead spermatozoa, evaluated by flowcytometry and fluorescent microscopy after triple staining

Equipment	Spermatozoon characteristics		% of spermatozoa 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 mean ± standard deviation (5 bulls/group).

* Mixed ratio of frozen-thawed semen and dead spermatozoa.

Table 3 Spermatozoon characteristics evaluated by flowcytometry using different staining procedures

Spermatozoon characteristics			% of spermatozoon 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 mean ± standard deviation (4 replicates).