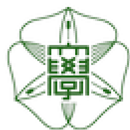
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SHORT COMMUNICATION Experimental Research

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

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Abstract

To verify the dynamics of damages to spermatozoa during semen freezing, characteristics of spermatozoa collected from 3 Japanese black bulls were evaluated by using fluorescent staining. Pre-diluted sample showed the highest proportion of spermatozoa with intact plasma membrane, intact acrosome and high mitochondrial potential. The proportion of spermatozoa with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential were higher after cooling to 4°C than the other processes. During cooling preservation examined in this study, the proportion of spermatozoa with damaged acrosome increased. These results lead us to speculate that, during cooling process, spermatozoa 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.

Key Words: acrosome, mitochondrial membrane potential, semen preservation

Artificial insemination (AI) has become a fundamental technique for the reproduction of dairy and beef cattle. Semen freezing has been generally used for the preservation of spermatozoa, and liquid preservation at approximately 5°C is also used in some countries[5,16]. 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[16]. 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[5], same as freezing procedure[16]. All steps of semen preservation can cause damages to the plasma

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membrane and other cell structures of spermatozoon due to the effect of changing osmolality, temperature, and generating reactive-oxygen species (ROS)[17]. These damages reduce semen fertility[13,18]. 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[1,10]. 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[10]. Sex-sorting process needs longer time, because the number of spermatozoa sorted per hour is only 20 million cells[10]. 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 spermatozoa 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[12,15]. Recently, some reports showed the methods to evaluate spermatozoa characteristics simultaneously by using fluorescent staining[3,8]. In addition, a previous study suggested that combination of the evaluating oxidative stress, acrosomal integrity, DNA compaction, mitochondrial activity, spermatozoa viability, movement of velocity, and morphologic abnormalities give us a reasonable prediction of bovine semen fertility[11]. However, there are few reports that estimated the damages of spermatozoa 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 spermatozoa. 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 spermatozoa preserved in a conventional extender, egg yolk-tris (EYT)[5] by using multiple fluorescent staining.

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, Kita-Hiroshima, Japan). 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 spermatozoa 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 spermatozoa of samples were within the ranges from 3.5 to 7 ml and from 800 to 2,030 $\times 10^6$ cells/ml, respectively.

Each semen sample was divided into two parts to use for the following experiments. For assessing the relationship between spermatozoa 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)[5] 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: cooling rate, −0.3 to −0.4°C/min). Then to adjust spermatozoa concentration to 80 $\times 10^6$ 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 spermatozoa 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 spermatozoa 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 spermatozoa 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 spermatozoa 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: cooling rate, −1.5 to −2°C/min). 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 rapid cooling samples were brought to our laboratory within 3.5 hr after collection, and examined spermatozoa 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, spermatozoa in all samples were suspended in 6% EYTG to evaluate in same condition for spermatozoa. To adjust spermatozoa 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.

Evaluation of spermatozoa characteristics were performed as previously reported[3] 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 5, 5', 6, 6'-tetrachloro-1, 1', 3, 3' tetraethyl-benzimidazolyl-carbocyanine iodide (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 propidium iodide (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 spermatozoa. Then 5 μl of spermatozoa suspension was mounted on a slide glass and covered with a cover glass. More than 200 spermatozoa on a slide glass were evaluated under fluorescent microscope (Nikon, Eclipse Ci, Tokyo, Japan) with triple band filter (DAPI/FITC/TRITC, Nikon), and spermatozoa were categorized into 8 groups as shown in Fig. 1. Briefly, plasma membrane integrity was assessed as intact when the head of spermatozoa was stained by Hoechst 33342 (blue) 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 spermatozoa stained by JC-1. When the midpiece was stained by orange, it was assessed as high mitochondrial membrane potential. Spermatozoa with intact plasma

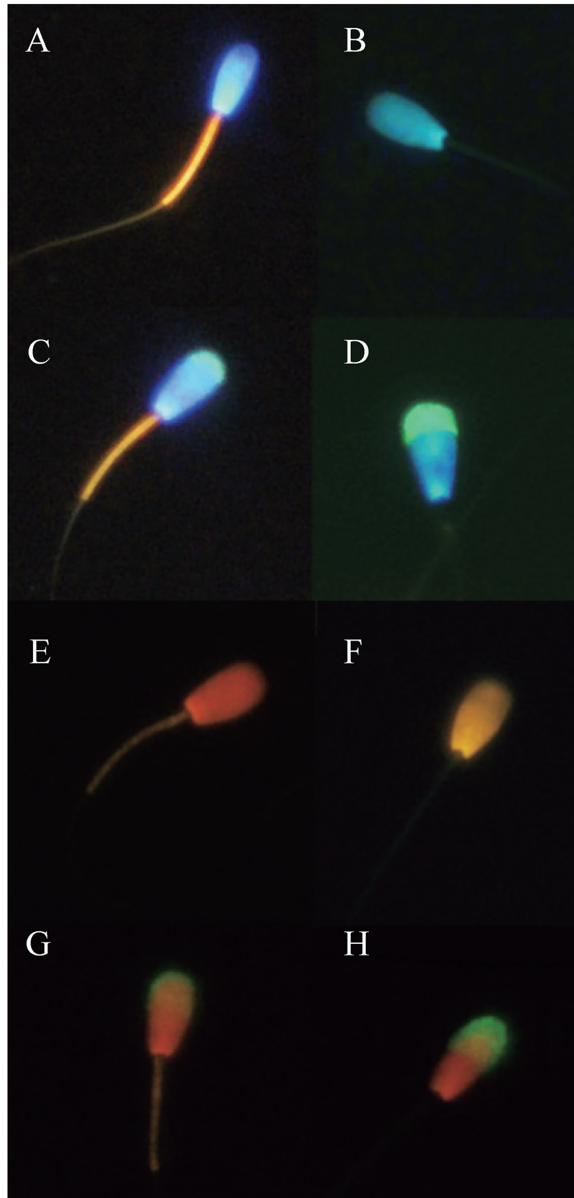

Fig. 1. The categorization of spermatozoa characteristics evaluated by fluorescent staining. The head of spermatozoon stained with Hoechst 33342 were judged as intact plasma membrane (A, B, C and D). The head of spermatozoon stained with PI were judged as damaged plasma membrane (E, F, G and H). Acrosome stained with FITC-PNA were judged as damaged (B, D, F and H). The midpiece dyed orange were judged as spermatozoon with high mitochondrial membrane potential (A, B, E and F). Therefore, the sperm characteristics were categorized as follows. (A): A spermatozoon with intact plasma membrane, intact acrosome and high mitochondrial membrane potential. (B): A spermatozoon with intact plasma membrane, damaged acrosome and high mitochondrial membrane potential. (C): A spermatozoon with intact plasma membrane, intact acrosome and low mitochondrial membrane potential. (D) A spermatozoon with intact plasma membrane, damaged acrosome and low mitochondrial membrane potential. (E): A spermatozoon with damaged plasma membrane, intact acrosome and high mitochondrial membrane potential. (F): A spermatozoon with damaged plasma membrane, damaged acrosome and high mitochondrial membrane potential. (G): A spermatozoon with damaged plasma membrane, intact acrosome and low mitochondrial membrane potential. (H): A spermatozoon with damaged plasma membrane, damaged acrosome and low mitochondrial membrane potential.

membrane, intact acrosome and high mitochondrial membrane potential (type A) were defined as normal spermatozoa.

The data of 3 bulls were pooled to compare the effect of preservation processes on spermatozoa 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 a software (JMP pro 12, SAS, NC, USA).

Spermatozoa characteristics at each step of freezing procedure were shown in Table 1. Pre-diluted sample showed the highest proportion of normal (type A) spermatozoa, however, the percentage decreased to approximately 50% as the advancement of the semen freezing process. Proportions of type F spermatozoa 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 spermatozoa, 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 ($P < 0.05$). Spermatozoa characteristics during liquid preservation were shown in Table 2. After conventional cooling, the proportion of type A spermatozoa decreased markedly ($P < 0.05$). The proportion of type A spermatozoa decreased gradually during liquid preservation, and became approximately 40% at day 5. The proportion of type B spermatozoa was highest at day 3 ($P < 0.05$). Proportions of types F and H spermatozoa significantly increased immediately after conventional cooling, however, the increase became gradual until day 5. The proportions of types C and D spermatozoa were rarely observed in freezing and liquid preservation processes.

Cooling and addition of glycerol to semen extender are important procedure for semen cryopreservation, because low temperature decreases energy consumptions of spermatozoa[9] and glycerol is one of the most popular cryoprotectants and gives a better result in cryopreservation of spermatozoa than other cryoprotectants[6]. It is also well known that these processes decrease the viability of spermatozoa. The results in the present study confirmed that most of spermatozoa were damaged to plasma membrane by preservation procedure as previously reported[6,16,18].

Also, the present study indicated that another pathway of damages to spermatozoa during preservation. After conventional and rapid cooling, the proportion of spermatozoa with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential increased. It might indicate that cooling process disrupts mitochondrial activity and spermatozoa compromised mitochondrial activity lose plasma membrane integrity in a minute. Mitochondria produces ATP which is necessary to maintain plasma membrane integrity of spermatozoa[13]. Therefore, cooling process of semen injures mitochondria, leading to the impairment of plasma membrane integrity of spermatozoa. The result of higher proportion of spermatozoa with intact plasma membrane, intact acrosome, and low mitochondrial membrane potential at day 3 of liquid preservation also support this hypothesis.

Our results suggest that rapid cooling would give serious damages to spermatozoa because rapid cooling sample showed lower level of normal spermatozoa similarly to frozen-thawed sample. Yoon *et al.*[18] described that frozen-thawed process was the most critical step to spermatozoa because plasma membrane defects were caused by ice crystal formation in semen extender[16]. In the present study, spermatozoa with intact plasma membrane and damaged acrosome were very few, and spermatozoa 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 spermatozoa, because of extensive injury by cryoinjury, such as high osmolality and ice nucleation in semen[2]. Although cryoinjury could not occur during rapid cooling to 4°C, the proportion of spermatozoa with damaged plasma membrane, damaged acrosome, and low mitochondrial membrane potential was high similar to that of frozen-thawed spermatozoa and significantly higher than that of conventional cooling spermatozoa. It is reported that high cooling rate of semen causes cold shock, which induce damage to spermatozoa viability, motility and functions[7]. It is also reported that cold shock induces the production of ROS[4]. ROS produced by cold shock has been known as an inducer of cryocapacitation of spermatozoa, which means the change of the cholesterol component in plasma membrane and the damage of acrosome[14]. There are no reports to indicate the relationship between ROS production and cooling rate. In further study, we should investigate the relationship between them and clarify the optimal cooling rate for producing spermatozoa having higher normality after frozen-thawed and liquid preservation.

Table 1. Characteristics of spermatozoa during freezing processes evaluated by fluorescent staining

Characteristics of spermatozoa			Type*	% of spermatozoon 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}

[a,b,c]: Values (means ± SD) with different superscripts were significantly different within a same row ($P < 0.05$).
*See Fig. 1.

Table 2. Characteristics of spermatozoa during liquid preservation evaluated by fluorescent staining

Characteristics of spermatozoa			Type*	% of spermatozoon 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}

[a,b,c,d,e,f]Values (means ± SD) with different superscripts were significantly different within a same row ($P < 0.05$).
*See Fig. 1.

In conclusion, during cooling process to 4°C, spermatozoa 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 drastic injuries in spermatozoa similar to frozen-thawed process, although the mechanism is unclear. In further study, the mechanism of cold-shock on membranes of spermatozoa should be investigated, and a novel preservation procedure protecting mitochondrial function should be developed.

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