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Comparison of Flow Cytometric and Morphological Assessment on Viability of Spermatozoa during Freezing Process of Boar Semen

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돼지정액의 동결과정중 정자 생존성에 대한 Flow Cytometric 및 형태학적 평가의 비교

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요 약

본 연구는 돼지정액의 동결과정동안 flow cytometric 분석에 의한 정액내 생존정자의 비율을 조사하여 주관적으로 평가되는 활력 및 정상첨체율(normal apical ridge; NAR)과 비교하여 정자의 손상과 생존성에 대한 적절한 평가법을 찾기 위하여 실시하였다. 동결과정 중 정액채취, 냉각, 예비동결 및 동결융해 후에 flow cytometric 분석에 의한 정자 생존율은 각각 93.0±3.6, 85.1±3.9, 28.9±6.8 및 26.1±5.9%이었다. 동결처리동안에 생존율은 예비동결 및 동결융해 후 가장 많은 정자사멸로 동결상태 이전의 생존율보다 유의적으로 낮게 나타났다($p<0.05$).

평가기법으로 정액 채취시 활력, NAR율 및 생존율을 조사한 결과 각각 91.0±4.2, 96.8±2.5 및 92.2±3.2%로 NAR율이 생존성 및 활력보다 높게 평가되었으며, 생존율이 활력보다 다소 높게 평가되었다. 그러나 동결융해 후에는 각각 44.0±8.9, 49.0±7.9 및 35.6±9.7%로 활력이 생존율보다 다소 높게 평가되었다. 전체적으로 NAR율은 활력과 생존율보다 높게 평가되었으며, SYBR-14/PI(propidium iodide) 이중형광염색법에 의한 flow cytometric 평가법의 생존율은 동결되지 않은 정액에서의 활력 및 NAR 평가보다 다소 민감하게 나타났다. 이때 정자손상 및 사멸율은 매우 낮은 수준이었으나 동결정액의 경우에는 생존율이 활력 및 NAR보다 낮게 평가되었다.

이러한 결과로 미루어보아 SYBR-14/PI 형광염색에 의한 flow cytometry의 생존성 평가는 동결되지 않은 정액의 평가방법으로는 적절하지만 동결된 정액의 생존성 평가는 부적절한 것으로 사료되었다.

(Key words : motility, viability, flow cytometry, boar, freezing)

INTRODUCTION

The quality of boar semen is essential to achieve successful artificial insemination, cryopreservation, and fertilization. Damage to the membranes of boar spermatozoa during cryopreservation can reduce fretilizing capacity (Maxwell and Johnson, 1997a; Mohammad et al., 1997). Watson (1996) and Wilmut and Polge

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(1997) suggested that cooling or freezing of spermatozoa may contribute to the reduced fertility of cryopreserved boar spermatozoa. Recent flow cytometric analysis by using SYBR-14/PI(propidium iodide) staining technique has provided new means for assessing of the functional capacity for sperm (Eriksson and Rodriguez-Martinez, 1996; Perez et al., 1997). Sperm viability is dependent on the assessment of spermatozoa outside their natural environment that is not completely objective within parameters (Gibson and Johnson, 1980). A practical and objective method to test a viability of individual sperm would be an important tool for semen processing industries. The motility test is for the characterization and quantization of motile and non-motile cells from a dynamic pool of spermatozoa (Evenson et al., 1994; Althouse and Hopkins, 1995; Maxwell et al., 1997). The accuracy of the visual motility assessment is largely dependent on the technician's experience and natural ability (Maxwell and Johnson, 1997[b]). As a result, flow cytometry has been used to objectively quantitate sperm motility and viability. This study aimed, therefore, to compare these techniques when assessing the quality of the boar sperm during freezing process and further to determine optimal cryoprotectants that affect a post-thaw survival of boar semen. The present study sought to determine whether the combination of SYBR-14 and PI could be used to differentiate between living and dead sperm from a variety of individuals as had been previously demonstrated only for the boar (Garner and Johnson, 1995). The overall objective was to determine whether the staining characteristics of sperm from five boar were similar when the combination of SYBR-14 and PI was used. Motility, NAR, and proportion(%) of living sperm by flow cytometric analysis were compared by means of the techniques for evaluation of viability and acrosomal morphology of spermatozoa at initial, cooled, pre-freezing, and post-thawing during freezing process.

MATERIALS AND METHOD

1. Reagents and Media

All chemicals were of analytical grades. Unless otherwise stated. Almost of media components were purchased from Sigma Chemical Co. (St.Louis, MO), and were made up with Milli-Q water (milli-Q UF Plus; Miliphore, Bedford MA). The reagent used in the cryodiluent and extenders consisted of like Table 1. Cryodiluent for 1st dilution was centrifuged at 10,000×g for discarding yolk lump of downstairs and lipid of upstairs was buffered with NaOH or HCl to pH 7.2~7.4 at room temperature and had a final osmolality of 290~310 mOsm/kg. They were equilibrated for six hours at room temperature, and for overnight at 5℃. Then, it was frozen (−20℃) without use of cryodiluent and extenders.

2. Semen collection and preparation

Semen from the sperm rich fraction of the ejaculate was collected from 5 boars that were housed at the LPSI of USDA-ARS. The semen was processed according to the straw freezing procedure described by Soejima et al. (1983) and Almlid and Johnson (1988) as modified by Lee et al. (1997).

Briefly, the semen was transported to the laboratory at a room temperature (approximately 24℃). Within 30 minutes after collection, semen was diluted 1:1 with BTS after evaluating the concentration and motility as soon as possible, and then aliquots containing 6×10^9 spermatozoa were centrifuged for 10 min at 1,500rpm. The seminal plasma was removed and the spermatozoa were resuspended to 5ml with 1st cryodiluent. The diluted semen was cooled gradually

to 4℃ for 1~2 hour by placing the tubes in a 50ml conical tube containing 30ml water (room temperature), 5ml of 2nd cryodiluent containing cryoprotectants were added gradually for one hour. After 2nd dilution, the semen were equilibrated for 40 min at 4℃. During equilibration, the semen was packed into straw. Packed and equilibrated semen was pre-frozen horizontally for 20 min on a thick paper rack, 5cm (approximately −120℃) above LN_2 vapor in a styrofoam container according to the method of Almlid et al. (1987). The pre-frozen semen was then transfered directly into LN_2 for storage. Most of the ejaculate was fulfilled with the following requirements: Motility >85%, concentration $>5\times10^8$ cell/ml, NAR>85%, and abnormality<10%. Thawing was achieved by immersing the straw in a circulating water bath at 37℃ for 20 sec. Then, semen were immediately processed for assessment of viability.

3. Assessment of spermatozoa

1) Concentration

Sperm concentration (the semen was diluted 1:3 with 2% saline) was measured by using a SpermaQ (Minitub Inc, German) and hemacytometer.

2) Motility

Table 1. Chemical composition of cryodiluent and extenders for the boar frozen or fresh semen(per 100ml)

Ingredient	Cryodiluent	Extender for fresh semen
	M-Soejima	BTS
1st extender		
Tes-N-Tris	1.20g	
Tris	0.40g	
Glucose	3.00g	3.7 g
Sodium lauryl sulfate	0.16g	
Catalase(159IU/ml)	0.91mg	
Antibiotics		
− Penicillin G(500IU/ml)		0.06g
− Streptomycin	0.1 g	0.1 g
− Gentamicin(25ug/ml)	2.5mg	
Potassium chloride		0.075g
Sodium bicarbonate		0.125g
Sodium citrate		0.6 g
EDTA		0.125g
Egg yolk(20%, v/v)	20 ml	
Bring to 100ml with DW		
2nd extender		
1st extender + bellow		
Glycerol(v/v)	4%	
OEP(Ovrus ES Paste, v/v)	1%	
BSA(0.1%)	0.1%	1.0 g(1%)
Caffeine(2mM)	2mM	
pH	7.2	7.2

The sample was removed from each step of freezing semen, an aliquot (10 μl) representing about 1.5×10^7 spermatozoa, was diluted 1:1 with BTS(Beltsville TS : Johnson et al., 1988) supplemented with d-BSA(final concentration 1%, w/v) and diluted spermatozoa were incubated at 37℃ in a block heater. The percentage of motile spermatozoa was estimated under phase-contrast microscope(100~200×magnification) using a stage heated (37℃).

3) Acrosome evaluation

The other sample was fixed with 2% glutaraldehyde for acrosome evaluation, one hundred spermatozoa per sample were examined under phase-contrast microscope (1,000 ×). The acrosomal morphology was classified according to Pursel et al (1972), was categorized into NAR, damaged apical ridge (DAR), missing apical ridge (MAR), and loose acrosomal cap (LAC).

4. Flow cytometic analysis of boar sperm

The newly developed living cell nucleic acid stain, SYBR-14, was used in combination with propidium iodide (PI) as described by Garner et al. (1986) and Maxwell and Johnson (1997a). This stain combination is now marketed as FertiLight (Molecular Probes, Eugene, OR). The SYBR-14 was prepared in anhydrous methyl sulfoxide (DMSO) from Aldrich Chemical Company (Milwaukee, WI) at a concentration of 1.0mg/ml. A working solution of SYBR-14 diluted 1:10 with DMSO was used for staining the fresh or frozen boar sperm. The PI was dissolved in mTALP medium at 2mg/ml (Sigma Chemical Company, St, Louis, MO)

Aliquots (500μl) of diluted or cryopreserved semen were stained at 36℃ with 0.27μl of the working solution of SYBR-14 and 2μl of the PI stock solution. The samples were incubated for 15min at 36℃ before examination. When this stain combination was excited at 488nm, the nucleus of the SYBR-14-stained cells fluoresced with bright green while the dead-sperm nuclei exhibited red fluorescence (PI). For monitoring of fluorescent staining of sperm, aliquots of semen (10μl) diluted with BTS (750μl) were stained at 36℃ with 0.5 μl of the working solution of SYBR-14 and 4μl of the PI stock solution, monitored and photographed with a Zeiss Axiophot epifluorescent mocroscope (Carl Zeiss Inc., Thornwood, NY) equipped with a fluorescein isothiocyanate filter set (Zeiss #487909). Ektachrome 400 (Eastman Kodak, Rochester, NY) slide film was used to photograph sperm (30 sec.).

5. Statistical analysis

The significance of difference among treatment group in each experiment was compared with Chi-square test ($p<0.05$).

RESULT AND DISCUSSION

1. Fluorescent staining and flow cytometric analysis

The living sperm in samples representing each of the three types exhibited bright green fluorescence in the nucleus when stained with SYBR-14 and excited at 488-nm (Fig. 1). Non-motile sperm, apparently dead, fluoresced when stained with SYBR-14 alone, but the staining intensity was much less than that of living motile sperm. With the addition of PI, the dead sperm nuclei stained bright red. Some sperm, apparently moribund, stained with both dyes. The change from green to red began at the posterior portion of the sperm head and proceeded anteriorly. This change could be observe microscopically as sperm became non-motile and then died, as occurs along the edge of a coverslip. The change from green to red took about one

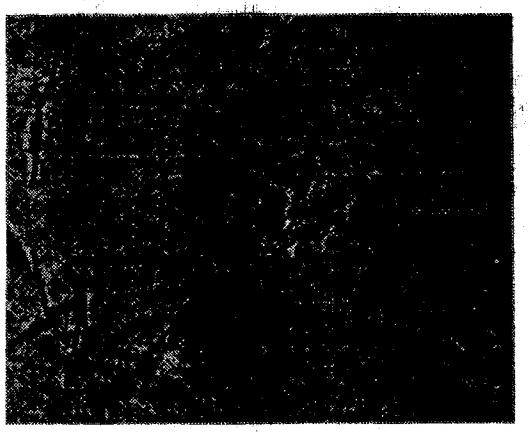

Fig. 1. Color of spermatozoa assessed by fluorescent staining (SYBR-14/ PI).
A : Live sperm(Green)
B : Dying sperm(Brown)
C : Died sperm(Pink)

min. The three major populations were evident living sperm that stained with SYBR-14 (A), dead sperm that stained with PI (B), and moribund sperm that fluoresced both green and red (C). Quantitative data on the fluorescent stained sperm populations were measured by an EPICS Profile-Ⅱ (Coulter Corporation, Miami, FL). The Profile-Ⅱ uses an air-cooled argon ion laser emitting at 488-nm and was equipped with the Power Park option that provided for 3-color fluorescence detection in addition to the side and forward light scatter parameters. The green fluorescence that passed through a 525-nm band pass filter was collected as the long of green fluorescence 1 (LFL1). The red fluorescence parameters, i.e., fluorescence 2 (LFL2) and fluorescence 3 (LFL3), were collected through 575-nm band and 635-nm band-pass filter, respectively, as a log function. Compensation (25%) was used to minimize spillover of green fluorescence into the 635-nm red channel (LFL3). The adjustment does not change the number of sperm that reside within any given population, but rather shifts the relative position of the populations so that quantification is easier and more precise.

The generated data exhibited in Fig. 2 were then analyzed for the relative fluorescence of the LFL1 and LFL3 using the Coulter Histogram Analysis program. A total of 5,000 sperm per sample was analyzed for the log the their fluorescence for each sample. Each samples, which was unstained or stained with SYBT-14 alone or PI alone was also examined at four steps to adjust the range of fluorescence to be examined by the flow cytometry. Scattergram dot plots showing typical flow cytometric analyses

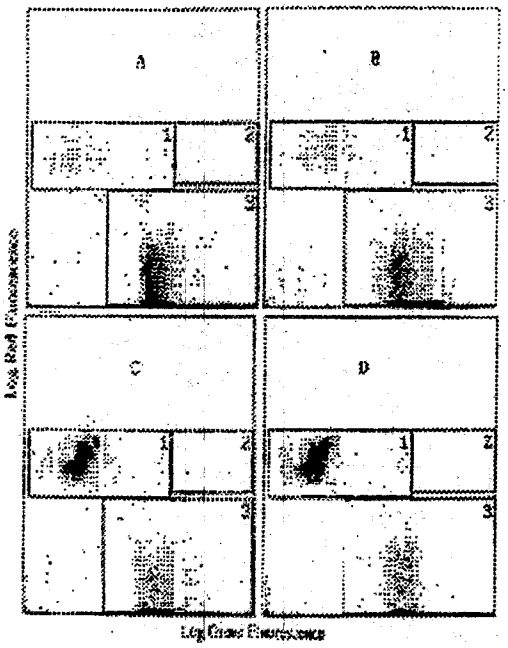

Fig. 2. Three sperm populations were quantified for one of the four representatives of each step by flow cytometric analysis during freezing process of boar semen. The samples were quantified for the proportions of PI-(1), Dual-(2), and SYBR14-(3)-stained sperm.
A. Initial (After 1st dilution)
B. Cooled semen (4℃)
C. Pre-freezing (−120℃)
D. Post thawed semen (−196℃)

for sperm that were stained with both SYBR-14 and PI, representing at initial, cooling, pre-freezing and post-thawing of boar semen, are given in A, B, C and D of Fig. 2.

Results of this categories were closer that reported by Maxwell and Johnson (1997). They reported that the proportion of live sperm was higher in fresh than in cooled and frozen semen, which was in turn higher than in frozen semen. When the sperm were stained with SYBR-14 and PI to assess the differences in sperm viability among four steps during freezing process, this data showed three categories. Examples of the dot plots of the logs of green and red fluorescence for sperm representing each step are provided along with the window analyses showing the mean proportion of PI (population 1), dual (population 2), and SYBR-14 (population 3) staining for each samples. These debris particles, however, were relatively much less fluorescent than were sperm.

2. Viability of spermatozoa at four step during freezing process

The relative proportions of sperm staining with PI, SYBR-14, or both PI and SYBR-14 (dual stained) for each samples are provided as mean values of five replicates (Table 2).

The proportion (%) of live sperm by flow cytometric assessment on viability of sperm were 93.0±3.6, 85.1±3.9, 28.9±6.8 and 26.1±5.9%, respectly. When survival of spermatozoa was assessed by flow cytometric analysis during freezing process, the largest loss of sperm survival happened at pre-freezing after 2nd dilution and cryoprotectant equilibration in freezing-process of boar semen.

Differences in the proportion of sperm that stained with PI were found among each sample but not between initial and cooling step and pro-freezing and post thaw step. Maxwell and Johnson (1997) reported that the proportions of live spermatozoa after staining SYBR-14/PI were unaffected by resuspention medium, but more spermatozoa were live in fresh (initial) and cooled semen than in post-thawed.

3. Effects of individual(boar) on viability assessment during freezing process

This experiment examined the effects of individual during freezing process of boar semen. When motility, NAR and the proportion of live spermatozoa were assessed from five boars, during freezing process, they were assessed with 91.0±4.2, 96.8±2.5 and 92.3±3.2% at initial step. But after post-thawing it was assessed with 44.0±8.9, 49.0±7.0 and 35.6±9.7% as Table 3.

Table 3 showed that the proportion of live spermatozoa affer post-thawing was different

Table 2. Results of flow cytometric analysis on the viability of sperm during freezing process of boar semen (Mean±SD)

Each step of Freezing process	Percentage of spermatozoa stained with PI /SYBR-14(%)		
	Live sperm	Dead sperm	Dying sperm(dual staining)
(n=5) At collection(Initial)	93.0±3.6[a]	4.9±2.9[c]	0.0±0.0
After colling and 2nd dilution	85.1±3.9[b]	12.1±2.8[b]	0.04±0.09
After pre-freezing	28.9±6.8[c]	72.6±10.2[a]	0.03±0.06
Post thawing	26.1±5.9[c]	70.7±5.8[a]	0.07±1.15

*The values with different superscripts in the column were significantly different ($p<0.05$)

Table 3. Effect of individual(boar) on motility, NAR, and the proportion of live spermatozoa during freezing process of boar semen (unit:%)

Boar ID No.	Initial(Fresh)			Cooled			Pro-freezing			Post-thaw		
	M	NAR	L	M	NAR	L	M	NAR	L	M	NAR	L
0053	85.0	93.0	91.2	70.0	75.0	81.6	65.0	62.0	54.9	55.0	56.0	49.6
0079	95.0	99.0	95.6	90.0	95.0	90.3	50.0	55.0	36.6	50.0	52.0	33.2
0330	90.0	97.0	92.6	80.0	91.0	79.4	56.0	58.0	45.2	40.0	44.0	40.7
6061	90.0	99.0	94.3	80.0	85.0	76.7	55.0	59.0	44.8	50.0	57.0	28.7
29219	95.0	96.0	87.2	80.0	84.0	83.3	30.0	34.0	26.3	30.0	36.0	25.6
Mean±SD	91.0±4.2	96.8±2.5	92.2±3.2	80.0±7.1	86.0±7.6	82.3±5.1	51.2±13.0	53.6±10.1	41.6±10.7	44.0±8.9	49.0±7.9	35.6±9.7

※ M : Motility(%), NAR : Normal apical ridge, L : Proportion of live spermatozoa.

among individual (49.6~25.6%, respectively). Although Maxwell and Johnson (1997) reported that the proportion of live sperm were influenced by type of spermatozoa but not individual boars, these results indicated that differences in the proportion of sperm stained with SYBR-14/PI were found among individual. Consequently, there was a tendency that NAR was assessed higher than motility (%) and proportion of live sperm (%) during freezing process.

Flow cytometric analysis by SYBR-14/PI fluorescent staining was more sensitive than a motility and normal apical ridge in the fresh semen, as it detected a higher degree of sperm damage and death at the same time.

However, in the case of frozen-thawed semen, it may be unsuitable in evaluation of different aspects like degree of motility and acrosome integrity of sperm except the proportion of living or died sperm because frozen boar semen is more sensitive than any other species *in vitro*.

SUMMARY

This experiment was undertaken to find optimal assessment on viability and damage of boar spermatozoa during freezing process. Motility (%), NAR (%), and proportion of living sperm (%) by flow cytometric analysis were assessed by means of the evaluation techniques of boar spermatozoa at initial, cooling, pre-freezing and post-thawing during freezing process.

The result using flow cytometric analysis as a technique for evaluation of semen quality, the proportion(%) of live sperm during freezing process were 93.0±3.6, 85.1±3.9, 28.9±6.8 and 26.1±5.9%, respectively. When survival of spermatozoa was assessed by flow cytometric analysis during freezing process, the largest loss of sperm survival happened at pre-freezing after 2nd dilution and cryoprotectant equilibration in freezing-process of boar semen. Generally, there was a tendency that NAR was assessed higher than motility(%) and proportion of live sperm (%) during freezing.

When motility, NAR and the proportion of live spermatozoa were assessed from five boars, during freezing process, they were assessed with 91.0±4.2, 96.8±2.5 and 92.3±3.2% at initial step. But affer post-thawing it was assessed with 44.0±8.9, 49.0±7.0 and 35.6±9.7%. As evaluation method of spermatozoa, the data suggests that flow cytometric analysis by SYBR-14/PI fluorescent staining was more sensitive than a motility and NAR in the fresh semen, as it detected a higher degree of sperm damage

and death at the same time. But in the case of boars frozen semen, it may be unsuitable in evaluation of different aspects like degree of motility and acrosome integrity of sperm except the proportion of living or dead sperm because frozen boar semen is more sensitive than any other species *in vitro*.

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