Correlation between Fluoromicroscopic Assessment of Mitochondria Function of Frozen-Thawed Hanwoo Spermatozoa and Blastocyst Development Following *In Vitro* Fertilization

Sae-Young Park[1], Eun-Young Kim[1], Deok-Im Kim[2], Won-Don Lee[3], Se-Pill Park[1,†] and Jin-Ho Lim[3]

[1]*Maria Infertility Hospital Medical Institute/Maria Biotech*

ABSTRACT

This experiment was to investigate whether the mitochondria function assessment can be used for the prediction of sperm fertility through examining the correlation between mitochondria fluoromicroscopic frequency of frozen-thawed eight Hanwoo bull semen using rhodamine123 (R123) and *in vitro* embryo development following fertilization. Individual sperm were stained in 5 μg/mL R123-added calcium-free Sp-TALP for 30 min at 0 h, 6 h, 12 h and 24 h after thawing and examined their mid-piece under an epifluorescence microscope using 495 nm excitation filter (×1,000). Three replications were taken, and at least 300 sperm per individual were examined. When semen samples were separated into two groups (good and poor) by sperm motility and fluorescent frequencies at just after thawing, average fluorescent frequencies were remarkably reduced as time going (0 h; 53.29~72.94%, 6 h; 21.40~58.90%, 12 h; 8.26~25.93%, 24 h; 1.00~13.78%), irrespective of selected group, and there were no differences at 6 h or 12 h after thawing between selected groups but indicated significant difference at 24 h after thawing (p<0.05). *In vitro* fertilization rates in good and poor groups ranging 70.8~77.8% and 52.1~84.5%, respectively, were not significantly different. However, *in vitro* development rates of the same groups ranging 25.7~40.0% and 12.9~1.8%, respectively, were significant different (p<0.05). These results demonstrate that mitochondria fluoromicroscopic assessment of frozen-thawed bovine sperm may be used as a criterion to select more fertile sperm.

(Key words : Frozen-thawed bovine sperm, Mitochondria, R123, Fluorescent frequency)

INTRODUCTION

The use of frozen semen for improving the bovine population genetics is accepted and utilized worldwide. However, in order to enhance production potential, cattle artificial insemination industry is focusing on the development of accurate methods capable of predicting field fertility when using frozen semen. Conventional tests of semen quality although widely used, are considered to be inconsistent predictors of reproductive efficiency (Rodriguez-Martinez, 2003; Graham et al., 1994).

Fertilization requires several subcellular organelles of the fertilizing sperm to work in sequence. A preferential importance is progressive forward sperm motility. This particular attribute is dependent on proper mitochondrial function. The sperm mitochondria, which are located in the midpiece, must produce ample energy in the form of ATP to power the flagellar motion that propels the sperm to the site of fertilization (Duane *et al.*, 1999). Sperm require large quantities of ATP to maintain motility and their ATP may derive from a variety of metabolic pathways, the two most important of which are glycolysis and mitochondrial respiration (Mann and Lutwak-Mann 1981). These two pathways may function independently to maintain motility in mammalian sperm (Bartoov *et al.*, 1980).

The rhodamine123 (R123) is a membrane permeable, cationic fluorescent dye that is taken up in energy-dependent fashion by the mitochondria of sperm cell: it has been accumulated by mitochondria in response to the electrochemical gradient set up by the mitochondrial membrane potential (Johnson *et al.*, 1980, 1981) and used preliminary to monitor structure and function of bovine spermatozoa (Tucker *et al.*, 1986; Windsor *et al.*, 1997). Live sperm activity accumulate R123, showing characteristic fluorescent staining of midpiece (Johnson *et al.*, 1981). Such staining has been used in a number of cell types including mouse, bull, and stallion sperm to indicate mitochondrial membrane potential (Papaioannou *et al.*, 1997; Evanston *et al.*, 1982; Chen *et al.*, 1988; Garner *et al.*, 1997).

The objective of this study was to investigate the variation on mitochondrial function of frozen-thawed Hanwoo bull semen by staining using R123 and their fertilization and blastocyst rate *in vitro* and thus to predict their sperm fertility according to mitochondrial function.

[2] Hanwoo Improvement Center, NLCF.

[3] Maria Infertility Hospital.

[†] Corresponding author : Dr. Se-Pill Park, Maria Infertilty Medical Hospital Institute/Maria Biotech, 103-11 Sinseol-dong, Dongdaemun-gu, Seoul 130-812, Korea, TEL: +82-2-924-8757, FAX: +82-2-924-9083, E-mail: sppark@mariababy.com

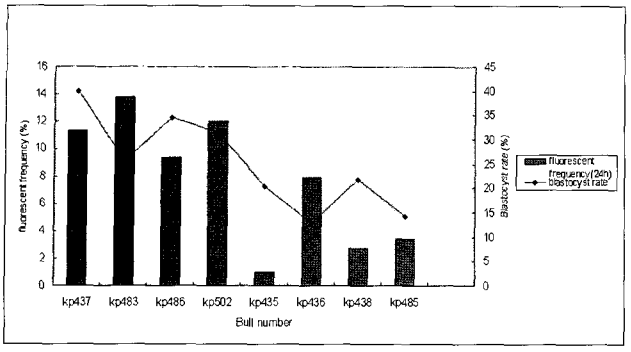

Fig. 1. Relationship between fluorescent frequency at 24 h after thawing of individual semen and blastocyst development (good group - kp 437, 483, 486 and 502; poor group - kp 435, 436, 438 and 485).

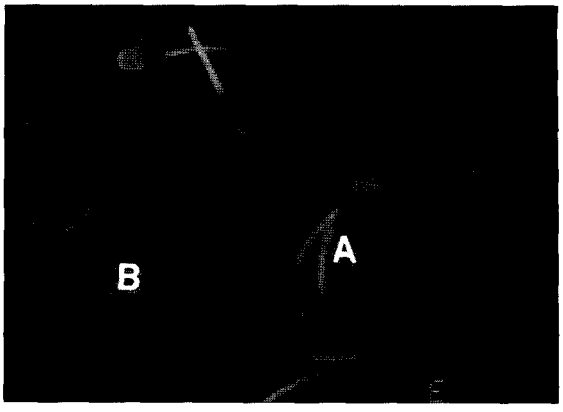

Fig. 2. Fluorescence microscopy of spermatozoa that were stained with mitochondrial probe (R123). A: midpiece of spermatozoa was indicated high intensity fluorescence. B: head, tail and midpiece was showed nonspecific fluorescence(×1,000).

MATERIALS AND METHODS

Semen Preparation

Frozen-thawed semen was prepared from eight Hanwoo bulls. Sperm were washed twice in calcium-free Sp-TALP and resuspended into a concentration of $1\sim1.5\times10^6$/mL. Sperm suspension in each bull number was divided into 4 equal parts and cultured at 39℃ in an atmosphere of 5% CO_2 incubator for 0 h, 6 h, 12 h and 24 h after thawing.

R123 Staining and Fluorescent Microscopy

In each treatment, sperm were incubated for 30 min in calcium-free 5 μg/mL R123 (Sigma) added Sp-TALP and sperm/R123 mixture was washed twice in calcium-free Sp-TALP and then aliquots were wet mounted for microscopic assessment after placing a drop on a slide. Slides were examined under epifluorescence microscope (Olympus) using a 495 nm excitation filter (×1,000). Fluorophotograph was taken on 400 ASA Ektachrome slide film, with exposure times varying between 20 sec and 2 min (Fig. 2). At least 300 sperm were scored per individual assessment from their original culture stock.

Production of Bovine *In Vitro* matured, Fertilized and Cultured (IVM/IVF/IVC) Blastocysts

Bovine ovaries were transported from a slaughterhouse to the laboratory in saline (36 ± 2℃) in container. Cumulus oocytes complexes (COCs) were collected from visible follicles (2~6 mm) of ovaries. The COCs were washed three times with TL-HEPES (low carbonate TALP) medium containing 1 mg/mL of bovine serum albumin (BSA; Fraction V, Sigma). The basic medium for maturation wastissue culture medium (TCM-199). The additives were fetal bovine serum (FBS; 10%), sodium pyruvate (0.2 mM), FSH (1 μg/mL), estradiol 17β (1 μg/mL) and gentamycin (25 μg/mL). Ten COCs were placed in each maturation drop (50 μL), and then cultured for 22~24 h at 39℃ 5% CO_2 incubator. For *in vitro* fertilization (IVF), highly motile sperm were recovered from frozen-thawed bull semen separated on a discontinuous percoll column and re-suspended with Sp-TALP at a concentration of 2.5×10^7 cells/mL. After 22~24 h *in vitro* maturation, the COCs were washed three times with Sp-TALP, and transferred into 44 μL fertilization drops. Ten COCs were moved to each fertilization drop, followed by 2 μL of motile sperm (5×10^4 cells/50 μL drop), 2 μL of heparin (2 μg/mL) and 2 μL of PHE (18.2 μM Penicillamine, 9.1 μM Hypotaurine and 1.8 μM Epinephrine) stock. The cleaved embryos were selected at day 2 after IVF and then cultured in 50 μL of mCR1-FAF medium (which was supplemented with 3 mg/mL of fatty acid free BSA) under mineral oil. At day 4, the embryos were transferred to mCR1-FBS medium (which was supplemented with 10% FBS). Embryo development was finally assessed by blastocyst production at day 8 after IVF.

Statistical Analysis

The fluorescent frequency were analyzed using General Linear procedure for analysis of variance (SAS Institute 1990) and significant differences among treatment means were tested by Duncan's multiple range test. *In vitro* cleavage and development rate were compared using Chi-square tests using SAS institute software.

RESULTS

The results of mitochondrial fluorescent frequency in individual bull at 0 h, 6 h, 12 h and 24 h post thawing were shown in Table 1. In this study, we divided 8 frozen-thawed Hanwoo bull semen into two groups (good - 4 or poor - 4) by sperm motility and mitochondrial fluoromicro-

Table 1. Bovine sperm mitochondria assessment using R123 in individual bull (r=3)

Bulls no.	Fluorescent frequency (%)			
	0 h	6 h	12 h	24 h
kp-437	62.9	50.5[a]	21.6[a,b]	11.3[a,b]
kp-483	63.7	28.5[c]	20.6[a,b]	13.8[a]
kp-486	66.3	40.3[b]	18.6[a,b,c]	9.4[a,b]
kp-502	72.9	58.9[a]	25.9[a]	12.0[a,b]
kp-435	61.0	21.4[d]	8.3[c]	1.0[d]
kp-436	60.8	33.3[b,c,d]	15.0[a,b,c]	7.9[b,c]
kp-438	53.3	30.4[c,d]	11.3[b,c]	2.7[c,d]
kp-485	54.0	22.0[c,d]	12.7[b,c]	3.4[c,d]
SEM	61.9±8.2	35.7±5.6	16.8±3.6	7.7±1.7

[a~d] (p<0.05).

scopic frequency at just after thawing. Good group indicates 80~95% motility and over the mean fluorescent frequency (62.9~72.9%) but poor group is 50~70% and 53.3~61.0%, respectively. Through the three replications, fluorescent frequencies of all semen (0 h; 53.3~72.9%, 6 h; 21.4~58.9%, 12 h; 8.3~25.9%, 24 h; 1.0~13.8%) were remarkably reduced as culture time is longer. In detail, the fluorescent frequencies at 0 h, 6 h, 12 h and 24 h post thawing in good group ranged 62.9~72.9%, 28.5~58.9%, 18.6~25.9% and 9.4~13.8% and in poor group ranged 53.3~61.0%, 21.4~33.4%, 8.3~15.0% and 1.0~7.9%, respectively. After thawing, at 6 h and 12 h, there were differences among the individual bull number but not between selected groups. However, at 24 h after thawing, we confirmed there was significant difference between selected group (p<0.05). The results of production of IVM/IVF/IVC blastocysts using individual bull sperm were indicated in Table 2. *In vitro* fertilization rates in good or poor groups were ranged 70.8~77.8% or 52.1~84.5% and there were no difference between selected group, but *in vitro* development rates were indicated significant differences into 25.7~40.0% or 12.9~21.8%, respectively (p< 0.05).

Table 2. *In vitro* development of bovine embryos according to the bull number

Bull no.	No. of oocytes	No. of embryos developed (%)	
		≥2-cell	Blastocyst
kp-437	392	305 (77.8)[a]	122/305 (40.0)[a]
kp-483	286	222 (77.6)[a]	57/222 (25.7)[c,d]
kp-486	380	269 (70.8)[b]	93/269 (34.6)[a,b]
kp-502	283	204 (72.1)[b]	64/204 (31.4)[b,c]
kp-435	94	49 (52.1)[c]	10/ 49 (20.4)[d]
kp-436	116	93 (80.2)[a]	12/ 93 (12.9)[d]
kp-438	103	87 (84.5)[a]	19/ 87 (21.8)[d]
kp-485	224	175 (78.1)[a]	25/175 (14.3)[d]

[a~d] (p<0.05).

DISCUSSION

This study demonstrated that sperm mitochondria fluoromicroscopic assessment using R123 can be used as a criterion to predict the sperm fertility because it correlates with *in vitro* developmental capacity.

R123 fluorescent staining, it would appear, represent a relatively convenient assay of the precise metabolic status of any sperm, its predictable and rapid transport into and out of an activity metabolizing sperm being an accurate monitor of that sperm's normal membrane and function integrity (Tucker *et al.*, 1986). R123 is a cationic compound that excites at 488 nm and emits at 515~575 nm (green fluorescence) and has been used as a probe of mitochondrial membrane potential in a variety of cell types (Chen *et al.*, 1997). The mitochondrial probe, JC-1 (5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazlyl carbocyanine iodide) exists as a monomer with excitation and emission peaks in the green wave lengths (510~520 nm) the novel mitochondrial probe, Mito Tracker Green FM is nonfluorescent in aqueous solution and fluoresces green upon accumulation in the mitochondrial membrane potential and the the excitation and emission maxima are 490 and 516 nm, and the Mitotracker Deep Red wasX suggested (Nagy *et al.*, 2003) for testing mitochondrial activity in spermatozoa. MitoTracker probes are cell-permeant mitochondria-selective dyes that contain a mildly thiol-reactive chloromethyl moiety (Hallap. *et al.*, 2005). Among the four different mitochondrial probes, the most widely used mitochondrial specific probe is R123. It accumulates in the mitochondria as a function of transmembrane potential, of R123 concentration and of sperm numbers; it is not dependent on time or temperature (Garner *et al.*, 1997). In this study, 24h incubation time before final assessment was designed by the fact that fertility of bull sperm in reproductive tract is maintained until 28 to 50h. However, our remarkably reduced fluorescent frequency result at 24h after thawing was similar to the report of the Tucker *et al.* (1986). Reduced fluorescent frequencies in different bull may be the result of metabolic uncoupling of the mitochondrial membrane potential or lysis of the mitochondrial inner membrane (Windsor and White, 1995).

For this experiment selected frozen-thawed semen was

obtained from good quality Hanwoo bulls, even there were variation of mitochondrial fluorescent frequencies among the individual after thawing. Windsor (1997) reported that poor sperm get damaged during freezing and thawing and their mitochondrial fluorescent frequency reduced. Also he indicated that high fluorescent frequency of sperm was relation with good fertility. Interestingly, in this study, at final assessment time (24 h after thawing); we confirmed the sperm's mitochondrial function of the good group was more maintained than that of the poor group. Also, this result was correlated with their fertility as shown in Fig. 1. Our result indicated that the *in vitro* developmental rates of good group were significantly higher than those of poor group ($p<0.05$).

On the other hand, use of advanced technique such as flow-cytometry to identify and isolate such population may be useful in increasing our knowledge of consequences of mitochondrial injury for sperm motility. However the tools available were insufficient to determine whether the variation in the brightness of midpiece fluorescence in frozen sperm is due to difference in mitochondrial R123 staining level, or variation in the proportion of individual mitochondria damage suffer from freezing (Windsor, 1997).

Therefore, this result concluded that sperm mitochondria fluoromicroscopic assessment using R123 can be used to predict the sperm fertility.X

REFERENCES

1. Chen LB, Summerhayes IC, Johnson LV, Walsh ML, Bernal SD, Lampidis TJ (1985): Probing mitochondria in living cell with rhodamine 123. Cold Spring Harbor Symp Quant Biol 46:141-155.
2. Chen LB (1988): Mitochondrial membrane potential in living cells. Ann Rev Cell Biol 4:155-81.
3. Darzynkiewicz Z, Staiano-Coico L, Melamed MR (1981): Increase mitochondrial uptake of rhodamine 123 during lymphocyte stimulation. Proc Nat Acad Sci 78:2363-2387.
4. Evanston DP, Darzynkiewicz Z, Melame MR (1982): Simultaneous measurement by flow-cytometry of sperm cell viability and mitochondrial membrane potential related to cell motility. J Histochem Cytochem 30:279-280.
5. Garner DL, Thomas CA, Joerg HW, Dejarnette JM, Marshall CE (1997): Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biol Reprod 57:1401-1406.
6. Garner DL, Thomas CA (1999): Organelle-specific probe JC-1 identified membrane potential differences in the mitochondrial function of bovine sperm. Molecular Reproduction and Development 53:222-229.
7. Graham EF, Schmehl MKL, Nelson DS (1994): Problems with laboratory assays. Proc. 8th Tech. Conf. AI and Reprod NAAB pp. 74-81.
8. Halangk W, Troger U, Bohnensack R (1990): Quantification of aerobic energy turnover in epididymal bull spermatozoa. Biochem Biochys Acta 1015:243-247.
9. Hallap T, Nagy S, Jaakma U, Johannisson, Rodriguez-Martinez H (2005): Theriogenology 63:2311-2322.
10. Johnson LV, Walsh ML, Bockus BJ, Chen LB (1981): Monitoring of relative mitochondrial membrane potential in living cells by fluorescent microscopy. J Cell Biol 88:526-535.
11. Johnson LV, Walsh ML, Chen LB (1980): Localization of mitochondria in living cells withX rhodamine-123. Proc Natl Acad.Sci USA 77:990-994.
12. Mann T, Lutwak-Mann C (1981): Male Reproductive function and semen. Springer. Berlin and Heidelberg.
13. Nagy S, Jansen J, Topper EK, Gadella BM (2003): A triple-stain flow cytometric method to assess plasma- and acrosome - membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of eggyolk particles. Biol Reprod 68:1828-35.
14. Papaioannou KZ, Murphy RP, Monks RS, Hynes N, Ryan MP, Boland MP, Roche JF (1997): Assessment of viability and mitochondrial function of equine spermatozoa using double staining and flow cytometry. Theriogenology 48:299-312.
15. Park SP, Kim SE, Uhm SJ, Kim EY, Kim T, Yoon SH, Chung KS, Lim JH (1995): Effect of simple serum-free medium CR_1 on the development of IVM/IVF bovine embryos. Kor J Fertil Steril 22:105-108.
16. Rodriguez-Martinez H (2003): Laboratory semen assessment and prediction of fertility: still utopia? Reprod. Domest. Anim. 38:312-318.
17. Tucker MJ, Ahula K, Stevens PA, Craft IL (1986): Cryopreservation of human spermatozoa: An assessment of methodology using rhodamine 123. Arch Androl 17:179-187.
18. Windsor DP, White IG (1995): Mitochondrial injury to ram sperm during procedures associated with artificial insemination of frozen storage. Anim Reprod Sci 40:43-58.
19. Windsor DP, White IG (1993): Assessment of ram sperm mitochondrial function byXquantitative determination of sperm rhodamine 123 accumulation. Molecular Reproduction and Development 36:354-360.
20. Windsor DP (1997): Mitochondrial function and ram sperm fertility. Reprod Fertil Dev 9:279-284.

(Received: 8 July 2005 / Accepted: 1 August 2005)