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Effect of Sperm Preparation Technique on Subsequent *In Vitro* Development of Bovine Embryos

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소 수정란 체외발달에 대한 정자분리방법의 효과

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요 약

본 연구는 Percoll, Swim-up 그리고 Glass-wool 여과법의 세가지 정자분리 방법에 대한 정자 회수율, 생존율과 첨체반응율, 그리고 체외수정 후 배양시간에 따른 전핵형성율, 수정란의 발달율과 세포할구수에 대한 효과를 조사하고자 실시하였다. 도축된 암소로부터 채취한 난자를 22시간 체외배양 후 성숙된 난자를 체외수정시켰다. 수정 후 배양시간에 따라 전핵율을 조사하였으며 48시간에 분할율, 192시간에 배반포기 발달율 및 세포할구수를 각각 비교 조사하였다.

정자의 첨체반응과 생존율은 처리군간에 차이가 없었으나 회수율에 있어서는 Percoll 처리군이 다른 두 처리군보다 유의적으로 높았다($P<0.001$). 수정 후 배양시간별 전핵형성에 있어서도 Percoll 처리군이 다른 두 처리군보다 빨리 진행됨을 볼 수 있었다. 분할율에 있어서는 처리군간에 유의적 차이가 없었으나 배반포기 발달율과 세포할구수에 있어서는 Percoll 처리군이 다른 두 처리군보다 유의적으로 높았다($P<0.05$). 이상의 결과로 보아 Percoll 처리에 대한 정자분리 방법은 정자 회수율이 높고 수정시 전핵형성 시간이 단축되어, 그 결과로 배반포기 발달율과 수정란의 세포할구수에 효과적임을 알 수 있었다.

(Key words : bovine, *in vitro* fertilization, sperm preparation)

INTRODUCTION

There has been a remarkable increase in knowledge of the various events in the fertilization process, by using bovine *in vitro* fertilization as a model, over the past few years however, many of the biochemical events underlying this process remain unknown.

Sperm motility is an essential feature to effect fertilization as penetration of the sperm head through the zona pellucida presumably requires vigorous propulsion by the sperm tail (Yanagimachi 1966). Bavister (1989) reported that a population of motile spermatozoa is a prerequisite for IVF in mammals. In order to achieve consistent IVF program, proper maturation of the oocyte and capacitation for physiological acrosome reaction in a population of motile spermatozoa with normal morphology, intact membranes and adequate nuclear stability becomes essential (Shamsuddin *et al.*, 1993). In bovine IVF, the techniques employed for separation of motile spermatozoa includes swim-up, glass-wool filtration and use of BSA/Percoll density gradient procedures; each claiming dif-

ferent rates of success in improving sperm quality and subsequent enhanced oocyte penetration rate.

Keefer *et al.* (1985) first reported a significant effect on the fertilization rate with motile sperm fraction obtained after the swim-up technique in cattle. Further, they observed that the fertilization frequency was increased from 46 to 59% and swim-up consistently yielded samples showing highly motile sperms. Similarly, Catt (1990) observed that more number of oocytes were fertilized by the available sperm fraction obtained after swim-up technique. Though this technique yields highly motile spermatozoa, the overall number of recovered spermatozoa is very poor (MacLean *et al.*, 1998).

Stubbings and Wosik (1991) compared glass-wool filtration and swim-up procedures for sperm preparation and obtained comparable results, but filtration only requires three minutes to complete, which reduces the sperm handling time in the laboratory. This procedure depends on removal of nonviable spermatozoa by mechanisms of which spermatozoa with abnormal membranes adhere to the glass-wool and nonmotile spermatozoa are less likely to pass through the glass-wool column.

Percoll, a colloidal suspension of silica particles (SiO_2) coated with polyvinylpyrrolidone, has been widely adopted for human IVF system. The method using 30 and 45% Percoll density gradients for sperm preparation has been employed in the cattle IVF system (Ectors *et al.*, 1989; Utsumi *et al.*, 1991). Rosenkrans *et al.* (1993), however, employed 45 and 90% Percoll density gradients to isolate a motile sperm fraction in their studies. This procedure resulted in higher sperm cell recovery as compared to the swim-up and glass-wool filtration (MacLean *et al.*, 1998). Gorus and Pipeleers (1981) suggested that this technique which involved centrifugation caused the sperm head to be directed centrifugally so that all spermatozoa align parallel to the centrifugal force. Thus, spermatozoa that are progressively motile could move more readily towards the bottom of the centrifuge tube than the nonmotile or poorly motile spermatozoa.

The present study was therefore designed to evaluate the total sperm cell number, sperm viability and membrane integrity between Percoll density gradient, swim-up and glass-wool filtration techniques. Furthermore, the effects of the sperm treatments on the *in-vitro* development as assessed by pronuclear formation, cleavage, blastocyst development and total cell count were examined.

MATERIALS AND METHODS

1. Media

Chemicals were purchased from the Sigma Chemical Company (St. Louis, MO) and media from GIBCO (Canadian Life Technologies, Burlington, ON) unless otherwise specified. The medium used for culture of embryos (IVC-M199) was serum-free tissue culture medium (M) 199 containing Earle's salts, 2.5 mM Na pyruvate, 1 mM L-glutamine, 0.5% penicillin-streptomycin (10,000 IU and 10,000 mg/ml respectively; Pen-Strep; GIBCO), and 0.1 mg/ml polyvinyl alcohol (MW 30,000 to 70,000; Sigma). The medium used for maturation of cumulus-oocyte-complexes (COCs) was IVC-M199 containing 10 μg/ml FSH, LH (USDA, Animal Hormone Program, Beltsville, MD) and 1 μg/ml estradiol 17 β (Sigma). The medium M199 (BOEC-M199) containing Earle's salts, 10% (v/v) steer serum, 5 mM Na pyruvate, 1 mM L-glutamine and 1.0% (v/v) Pen-Strep was used for culture of bovine oviductal epithelial cells (BOEC). For the maturation and culture media, the pH was

adjusted to 7.4 and the osmolality to 280 mOsm/kg. Tyrode's albumin lactate pyruvate medium containing 2% (w/v) bovine serum albumin (BSA; Fraction V, Sigma) supplemented with 10 mM Hepes (HEPES-TALP) or without (IVF-TALP) was used for sperm preparation.

2. Sperm preparation

Frozen semen from two bull (Bull A and B) were used for this study. For the sperm preparation, 2 straws each for swim-up, glass-wool filtration and Percoll density gradient were used.

Percoll (Pharmacia, Uppsala, Sweden) gradient was prepared as described by Rosenkrans *et al.* (1993). In brief, 100% Percoll solution was mixed with 10× salt solution (NaCl, 2.889 g; KCl, 0.238 g; KH_2PO_4, 0.116 g; $CaCl_2$, 0.112; Hepes, 0.163 g; 50 ml of milli-Q water) to form 90% Percoll solution and then formed 45% Percoll solution as addition of 1 (90% Percoll solution) to 1 (HEPES-TALP medium) (v/v). The gradient was formed by pipetting 1.5 ml of 90% Percoll solution into a 15 ml conical tube and then layered with 1.5 ml of 45% Percoll solution. Frozen-thawed bull semen were placed onto the top of 45% gradient and then centrifuged at 850×g at room temperature for 15 min through the gradient. After removal of supernatant, the pellet was washed once with 10 ml of HEPES-TALP medium by centrifugation at 300×g for 10 min, and resuspended in the same medium.

For the swim-up procedure, frozen-thawed semen was washed once by centrifugation at 300×g for 10 min in 10 ml of HEPES-TALP medium and then 0.5 ml of sperm pellet was taken and an aliquot of 0.1 ml was placed at the bottom of five 2-ml round-bottom test tubes overlaid with 0.5 ml of HEPES-TALP medium. The tubes were then tilted to a 45° angle and incubated to allow swim-up of motile sperm at 39℃ in a humidified atmosphere of 5% CO_2 in air for 1 h. The top 0.4 ml of the supernatant was harvested and further centrifuged at 300 ×g for 10 min to yield sperm pellet.

The glass-wool filtration of semen was performed as described previously (Jeyendran *et al.*, 1986). Briefly, a column packed with 15 mg of glass-wool (microfibre code 112, Manville Fiber Glass Group, Denver, CO) to a depth of 3.5 mm in a 1-ml disposable syringe was used. Before layering of sperm suspension, the glass-wool column was rinsed thoroughly with HEPES-TALP medium to moisten and at the same time remove loose glass-wool fibers. Frozen-thawed semen was washed twice by centrifugation at 300×g for 10 min in 10 ml of HEPES-TALP medium. The final sperm pellet was re-suspended in 0.6 ml of HEPES-TALP medium and was then overlayered on the glass-wool column to allow filtration by gravity.

In all experiments, the concentration of sperm was determined using a haemocytometer.

3. Viability and acrosome evaluation of spermatozoa

Vital stain (*Ferti*Light™, Molecular Probes Inc., Eugene, OR) was used to assess the viability of sperm. Briefly, 50 μl of each diluted semen sample was mixed with 5 μl of manufacturer's component A, and incubated for 5 min at 37℃. Aliquot of 5 μl of component B containing 10 μg/ml propidium iodide (Sigma) was added and incubated for another 5 min. A sample of 20 μl was then placed on a microscope slide and covered with a glass cover slip. Spermatozoa that were stained green (live) and red (dead) were counted on a Zeiss fluorescent microscope fitted with an excitation filter of 365 nm and barrier filter of 397 nm.

For the evaluation of acrosomal content, the spermatozoa were incubated in IVF-TALF and harvested at 0, 1, 2, 3, 5, 7, 9 and 11 h post-incubation, and stained with fluorescein conjugated lectin Pisum sativum agglutinin (PSA/FITC, Sigma) by a slight modification of the method reported by Cross *et al* (1986). Briefly, 10 μl of diluted sperm sample was smeared onto a precleaned microscope slide, dried and fixed in methanol at −20℃ for 2 min. The smear was then stained with PSA/FITC (100 g/ml in PBS) containing 10 g/ml propidium iodide at room temperature for 20 min after covering with a piece of plastic paper. The plastic paper was removed by washing with milli-Q water, and left in milli-Q water for 15 min. The slide was air dried and cover slip applied with glycerol FITC-guard (Slow Fade; Molecular probes, Eugene, Oregon). The stained sperm preparation was examined under a Zeiss fluorescent microscope fitted with an excitation filter of 365 nm and a barrier filter of 397 nm.

4. Embryo preparation

Groups of 15 COCs harvested from ovaries collected at slaughterhouse were matured in 50 μl droplets of maturation medium under silicon oil (Dow Corning Medical Fluid 200) at 39℃ in a humidified atmosphere of 5% CO_2 in air. After 22 h of culture, the expanded cumulus cells were partially removed by vortexing for 20 sec in HEPES-TALP medium. The oocytes were inseminated with 2×10^6 sperm/ml of final concentration in IVF-TALP medium and co-cultured with BOEC for 16 h. Later, sets of 15 presumptive zygotes were transferred into 50 μl droplets of IVC medium and co-cultured with BOEC. At 48 h and 120 h post-insemination, the cultures were "fed" with 25 μl of fresh IVC medium to each drop, as described by Rieger *et al.* (1995) and were maintained for 192 h post-insemination to assess the rates of development to blastocyst stage, of hatching and the number of cells among groups.

5. Cytological procedures

At 6, 12, 18 and 24 h post-insemination, 523 presumptive zygotes (see Results) were fixed overnight in methanol:acetic acid (3:1, v/v) and then stained with 1% aceto-lacmoid to reveal the presence of pronuclei.

In order to determine cell number, blastocysts at 192 h post-insemination were transferred into a hypotonic solution (1% Na-citrate) for 4 min at room temperature. Individual embryos, each in −0.5 μl of the hypotonic solution and −1.5 μl of methanol:acetic acid (3:1, v/v), were placed on precleaned microscope slides using a microcapillary pipette, and were blown upon by mouth under a light to spread the nuclei as they dried. After air-drying, slides were fixed in methanol:acetic acid (3:1, v/v) overnight. The fixed slides were stained with 4% Giemsa solution for 10 min, and the nuclei were counted under a compound microscope at ×100 magnification.

6. Statistical analysis

Differences between treatments were analyzed using one-way analysis of variance (ANOVA) after arcsine transformation of the proportional data of pronuclei and blastocyst development. Comparisons of mean cell number and developmental rates among treatments were carried out using Tukey-Kramer multiple comparisons test. Differences were considered significant when $P<0.05$.

RESULTS AND DISCUSSION

1. Comparison of number, viability and acrosomal content of sperm cells

In the present study, frozen-semen was util-

Table 1. Effect of sperm preparation on spermatozoa concentration and viability in Bull A

Treatment	Mean ± SEM* (% of post-thaw)	
	Total	Membrane intact
Post-thaw	37.6 ± 1.2	18.6 ± 0.6
Percoll	12.2 ± 0.9 (32.4)[a]	10.3 ± 0.7 (55.4)[a]
Swim-up	2.8 ± 0.4 (7.4)[c]	2.3 ± 0.3 (11.8)[c]
Glass-wool	5.7 ± 0.7 (15.2)[b]	4.6 ± 0.6 (24.7)[b]

* × 10^6 cells

[a-c] Percentages with different superscripts within a column differ significantly ($P<0.001$)

ized to compare the sperm preparation using Percoll density gradient, swim-up and glass-wool filtration techniques for assessing the total cell count, viability and acrosomal content of sperms in 6 replicates (Table 1). In each replicate the initial sperm concentration and viability of the semen sample was assessed. The mean concentration per straw (0.5 ml) was 37.6×10^6 of which 49.5% (18.6×10^6) viable sperms could be recovered. The proportion of sperm isolated by Percoll, swim-up and glass-wool procedures was 32.4, 7.4 and 15.2% of the initial concentration, respectively. Total sperm cell count recovered following Percoll gradient was ~4 times higher than the swim-up and ~2 times of glass-wool filtration procedure ($P<0.001$). However, there were no significant differences ($P>0.05$) between swim-up and glass-wool filtration procedures. Moreover in Percoll treatment a significantly higher ($P<0.001$) number of viable spermatozoa (10.3×10^6) than the swim-up (2.3×10^6) and glass-wool filtration (4.6×10^6) procedures were recorded. However, the sperm viability percentage of the three groups ranged between 81 to 84% and did not differ significantly ($P>0.05$). This finding is consistent with other report in cryopreserved *Bos gaurus* semen (MacLean *et al.*, 1998) that showed that the proportion of sperm isolated by Percoll, swim-up and glass-wool procedures are 33, 5 and 16% of their initial concentration, respectively. Furthermore, in human IVF, a simple discontinuous Percoll gradient results in the recovery of sperm of better quality and number than other widely used swim-up procedures of ejaculated semen (Lannou and Blanchard, 1988; Sapienza *et al.*, 1993). In contrast, glass-wool filtration appears to be more effective in removing nonmotile spermatozoa than Percoll gradient process in the ejaculated sperm in human, but care has to be taken in the preparation of the glass-wool columns so that glass-wool is not too tightly or too loosely packed (Rhemrev *et al.*, 1989).

The assessment of the total cells in blastocysts produced by *in vitro* fertilization, utilizing the three treatments of sperm was done. This pointed out that the embryos obtained from Percoll treated sperm had a significantly higher cell number than the glass-wool or swim-up procedures counterparts (see Table 3). This formed the basis of hypothesis that Percoll treatment on the sperm might facilitate sperm acrosome reaction and hence subsequent more rapid fertilization. To test this hypothesis, a stain with the reagent fluorescein conjugated lectin *Pisum sativum* agglutinin (PSA/FITC, Sigma) by a slight modification of the method reported by Cross *et al.* (1986) was used to assess the population of acrosome intact of sperm. At 0 h of culture, the sperm in three treatments have approximately 95% of their acrosome intact. Additional incubation time of the sperm could caused an increase in the rate of acrosome reaction. By 3 h, ~25% of spermatozoa were acrosome-reacted; 4

h later, ~60% of evaluated spermatozoa had shown acrosome reaction. However, the rate of acrosome reaction of the sperm between treatments did not reveal statistically significant differences (data not shown). This observation collaborates a previous report (Tanphaichitr *et al.*, 1988) in humans that the increase in penetration ability of sperm treated by Percoll gradient does not result from an enhanced level of a spontaneous acrosome reaction.

2. Comparison of pronuclei formation among sperm treatments after different cultivation time

This experiment was conducted in 4 replicates to evaluate the effect of sperm preparation on the timing of pronuclei formation at different times after IVF. Undetermined eggs and 2-cell stage embryos were excluded from the present study. It has been reported that in cattle the timing of sperm penetration of *in vitro* matured oocytes is ~4 h (Hyttel *et al.*, 1989). Within 1 or 2 h of sperm penetration, the sperm head begin to undergo decondensation, which formed the male pronucleus 3 to 4 h later (Xu and Greve, 1987; Hyttel *et al.*, 1989; Saeki *et al.*, 1991). Similarly, the present study shows that decondensation of sperm head occurred 6 h after insemination and pronuclear formation at 12 h post-insemination in swim-up and glass-wool treatments. However, Table 2 shows a higher proportion of pronuclear formation in oocytes fertilized by Percoll-treated sperm at each cultivation period than by swim-up and glass-wool-treated sperm. With the Percoll gradient procedure, at 6 h after IVF, 72% of eggs (33 of 46) formed pronuclei; 6 h later, all eggs revealed pronuclear stage. At 18 h, 33% began to undergo a transition from two pronuclei to fused zygotic stage. At 24 h, the proportion of fused zygotic stage in Percoll is higher than those of swim-up and glass-wool treatments (66 vs. 54 and 28%, respectively). The reason remains unclear, however, Percoll-treated sperm may not possess the coating envelopes or a high level of surface fucose residues in contrast to swim-up treated sperm (Tanphaichitr *et al.*, 1988), suggesting removal

Table 2. Comparison of pronuclei formation after different cultivation time among sperm treatments: Percoll, swim-up and glass-wool filtration

Cultivation time (h)	Treatment	n	Configuration(%)*					
			no SP	CH /dSP	PN /dSP	2PN	3PN	Syngamy
6	Percoll	46	2 (4.3)	11 (23.9)	21 (45.7)	12 (26.0)		
	Swim-up	44	7 (15.9)	12 (27.3)	25 (56.8)			
	Glass-wool	39	15 (39.5)	15 (39.5)	5 (12.8)	4 (10.3)		
12	Percoll	45			16 (35.6)	26 (57.8)	3 (2.5)	
	Swim-up	45	9 (20.0)	6 (13.3)	24 (53.3)	5 (11.1)	1 (2.2)	
	Glass-wool	41	8 (19.5)	4 (9.8)	23 (56.1)	6 (14.6)		
18	Percoll	48	3 (6.3)		6 (12.5)	23 (47.9)		16 (33.3)
	Swim-up	47	4 (8.5)		9 (19.1)	21 (44.7)		13 (27.7)
	Glass-wool	41	6 (14.6)		13 (31.7)	19 (46.3)	1 (2.4)	2 (4.9)
24	Percoll	44	4 (9.1)	2 (4.5)	5 (11.4)	4 (9.1)		29 (65.9)
	Swim-up	43	2 (4.7)		2 (4.7)	16 (37.2)		23 (53.5)
	Glass-wool	40	4 (10.0)	1 (2.5)	7 (17.5)	17 (42.5)		11 (27.5)

* no SP, no sperm; CH /dSP, chromosome plate /decondensed spermatozoon; PN /dSP, a pronucleus /decondensed spermatozoon; 2PN, two pronuclei; 3PN, three pronuclei.

Table 3. Developmental rates of *in vitro* embryos using sperm from 2 bulls isolated by three methods

Bull	Treatment	Oocytes	Cleaved(%)	Blastocysts* (%)		Mean±SEM*	
				Total	Hatching	n	Number
A	Percoll	230	202 (87.8)	67 (29.1)[a]	28 (12.2)[a]	44	176.5± 7.1[a]
	Swim-up	228	192 (84.2)	57 (25.0)[a]	23 (10.1)[ab]	25	140.4± 4.7[c]
	Glass wool	233	194 (83.3)	53 (22.7)[a]	14 (6.1)[b]	39	131.6± 3.6[c]
B	Percoll	228	194 (85.1)	40 (17.5)[ab]	18 (7.9)[b]	20	157.6±10.5[b]
	Swim-up	229	172 (75.1)	38 (16.6)[b]	9 (3.9)[b]	19	147.3± 7.0[bc]
	Glass wool	230	165 (71.7)	35 (15.2)[b]	10 (4.3)[b]	23	133.4± 8.3[c]

* at 192 h post-insemination

[a-c] Percentages and number with different superscripts within a column differ significantly ($P<0.05$)

of the membranous envelopes could be one of pivotal steps in sperm capacitation. These sperm may then enhance their ability to penetrate the eggs compared to others.

3. Effect of sperm preparations on *in vitro* embryo development and cell number

This experiment was conducted to compare the cleavage, blastocyst development *in vitro* and total cell number using sperm treated by three different procedures in 5 replicates. The percentage of cleavage between treatments (range 84 to 88% for Bull A; 72 to 85% for Bull B) did not reveal statistically significant difference (Table 3). The percentage of blastocysts developed in Percoll-treated group differed significantly from those of glass-wool, but there was no significant difference observed between swim-up and glass-wool-treated groups. Also, there was no significant difference observed in the hatching percentage between the sperm treatments. The total cell number at 192 h in blastocysts obtained from Percoll treated group (176.5±7.1) in Bull A was significantly higher ($P<0.001$) than swim-up (140.4±4.7) and glass-wool filtration (131.6±3.6) groups. These results suggest that Percoll-treated sperm may alter sperm function, which has gradually been viewed as beneficial in affecting the time of pronuclear formation and the subsequent increase in cell number in blastocysts. Thus, the Percoll procedure for IVF seemed more suitable for IVF system because it is less expensive and higher number of sperm recovered and better development of embryos.

SUMMARY

Experiments were designed to examine the effects of developmental rate and cell numbers in *in-vitro*-produced embryos using sperm from 2 bulls isolated by three methods; Percoll gradient, swim-up and glass-wool filtration. Embryos were produced by established techniques from oocytes harvested from slaughterhouse ovaries. The presumptive zygotes were examined the rates of pronuclear formation at 6, 12, 18 and 24 h post-insemination. The embryos produced by the different treatment groups were compared for cleavage (48 h post-insemination) and for development to blastocyst stage (192 h post-insemination). The hatching percentage and the cell number was also determined.

The sperm did not differ among treatments in a subjective rating of acrosome reaction and of viability. However, the number of recovered spermatozoa in Percoll treatment were significantly higher ($P<0.001$) than others. The rates of cleavage in embryos produced by IVF using sperm from 2 bulls (bull A and B) isolated by

Percoll, swim-up and glass wool were not significantly different. The blastocyst development and hatching rates between sperm treatment were not significant within bull A and B. However, the hatching rate in Percoll treatment of bull A was significantly higher ($P<0.05$) than in bull B. Similarly, the mean cell numbers in Percoll treatment within bull A (176.5 ± 7.1) were significantly higher ($P<0.001$) than others. Whereas in bull B though the cell numbers in Percoll-treated group was higher than the other two treatments, but the differences between Percoll-treated and swim-up groups were not statistically significant.

In conclusion, these results support that concept of sperm preparation using Percoll have beneficial effects on total number of recovered spermatozoa and blastocyst cell number.

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