Separation of Y-chromosome bearing bull's spermatozoa using an albumin gradient technique

Somarny, W.W.M.Z.[1*], Tan, Y.J.[2], Mat Tasol, S.[1], Jasmi, Y.[3], Musaddin, K.[1] and Johari, J.A.[1]

[1]Strategic Livestock Research Centre MARDI P.O. Box 12301, 50774 Kuala Lumpur, Malaysia
[2]MARDI Research Station, P.O. Box 62, 26700 Muadzam Shah, Pahang, Malaysia
[3] MARDI Research Station, P.O. Box 525, 86009 Kluang, Johor, Malaysia
*Corresponding author: zsomarny@mardi.gov.my

Abstract

This study was conducted to separate the X- and Y-chromosome bearing spermatozoa based on an albumin gradient technique. A modified but simple procedure was performed to minimize pressure on the spermatozoa. Two treatments consisting of two-phase albumin gradients which were 12.5% over 20% (Treatment 1), and 15% over 20% (Treatment 2) bovine serum albumin (BSA) were carried out to separate the X- and Y-chromosome bearing bull's spermatozoa at room temperature. Subsequent analysis by FISH using a single labeled TSPY probe (specific for detection of Y-chromosome) showed that the albumin gradient technique was not effective (P>0.05) to separate the X- and Y- chromosome bearing spermatozoa. The percentage of Y-chromosome bearing spermatozoa recovered following separation by Treatment 2 was higher but not significant (P>0.05) compared to Treatment 1.

Key words: Chromosome, spermatozoa, albumin gradient technique, bull

Introduction

Sexing of bovine spermatozoa might affect both biological and economical effectiveness. Several advantages have been suggested for producing sexed spermatozoa including to use fewer and genetically superior dairy females for heifer replacement (Prasad *et al*., 2010), to provide a wider chance for crossbreeding dairy females, fewer number of cows required for progeny testing, (Hohenboken, 1999) and to facilitate endangered species conservation (Prasad *et al*., 2010).

A great concern and debate on sex-selection has resulted when discontinuous albumin gradient technique was used to enrich the Y-chromosome bearing human spermatozoa (Ericsson *et al*., 1973). Since then, many new techniques have been developed to separate spermatozoa, such as modified swim-up procedure to enrich Y-chromosome bearing spermatozoa (Check and Katsoff, 1993), Sephadex column technique and 12 – step Percoll gradients to enrich X-bearing spermatozoa (Steeno *et al*., 1975; Iizuka *et al*., 1987) and free-flow electrophoresis (Blottner *et al*., 1994; Aitworth *et al*., 2007). However, the validation of the enrichment of X- and Y-chromosome is controversial. It has been suggested that, flow cytometry to separate the spermatozoa showed a promising commercial potential to sex spermatozoa (Garner *et al*., 1983; Welch and Johnson, 1999; Seidel and Garner, 2002). Unfortunately, this technique requires appropriate skills and expertise as well as it is not easily accessible. The objective of this study was to evaluate the effectiveness of an albumin gradient technique in separating the bull's spermatozoa carrying Y- chromosomes.

Materials and Methods

Albumin separation method

Semen sample was obtained from Brakmas bull (n=4) using artificial vagina. Separation of semen sample was performed using Ericsson method (Ericsson *et al*., 1973) with some modifications. Replications on the separation were performed three times per bull (triplicates) in order to obtain the reliable results. Two treatments were tested: Treatment 1 of 12.5% over 20.0% BSA and Treatment 2 of 15.0% over 20.0% BSA. Approximately, 1 ml of the semen sample at a concentration ~30 X 10^6 spermatozoa/ml was diluted 1:5 with egg yolk fraction (20% v/v egg yolk, 6.5% glycerol and 77.2% 1M Tris buffer, pH 6.75). Aliquots of 0.5 ml containing 15 x 10^6 spermatozoa with at least 70% motility were gently layered on 2 ml of 12.5% over 20% BSA [Sigma Aldrich (St. Louis, MO, USA)] (Treatment 1, Figure 1) for 1 hour in 15 ml polypropylene tube at room temperature (22-24°C). At the end of the incubation period, the bottom layers of the columns were pooled and centrifuged at 300 G for 10 minutes to remove the supernatant. The pellets were washed twice and resuspended in 0.9% saline. Slides were prepared to determine the separation efficiency of the fraction. The above procedures were repeated using 15% over 20% BSA (Treatment 2). For the control, the unseparated semen samples were used in fluorescence *in situ* hybridization (FISH) analysis to evaluate the percentage of separated X- and Y-chromosome bearing spermatozoa in both treatments.

Decondensation of spermatozoa

Approximately 500 µl of separated spermatozoa were centrifuged at 1000 G for 5 minutes to remove the BSA and washed twice with 0.9% sodium chloride solution. The supernatant was discarded and the pellet was resuspended in 0.9% sodium chloride to a final volume of 5 ml. An aliquot of 50 µl was smeared on a clean slide which was air-dried and fixed in 95% (v/v) ethanol at 4°C overnight. Sperm decondensation was induced by incubation using papain-DDT solution at room temperature (22-24°C). The slide was rinsed with 0.2 M Tris buffer (pH 8.6). The spermatozoa were dehydrated twice in 95% ethanol for 1 minute and air-dried for 10 minutes.

Figure 1: Semen suspension was layered on 2 ml of 12.5% over 20% BSA and 15% over 20% BSA

Preparation of probe by nick translation

PCR amplification of Y-encoded, testis-specific protein (TSPY) gene was performed by using oligonucleotide sequences 5'- CCC GCA CCT TCC AAG TTG TG-3' and 5'-AAC CTC CAC CTC CTC CAC GAT G-3'(Tan *et al*., 2010). A PCR product of 260 bp was cloned into 2.1 TOPO TA vector (Invitrogen, USA). The plasmid carrying TSPY gene was labeled as a probe using SpectrumGreen™ (Vysis, Inc) by nick translation labeling which provided the green fluorescence signal for Y-chromosome.

The nick translation reaction was performed by adding 17.5 µl of plasmid DNA to a microcentrifuge tube containing 2.5 µl of fluorescence labeled nucleotide (dUTP), 5 µl of nucleotide buffer, 10 µl for dNTP (without DTTP), 5 µl for dTTP, 10 µl of nick translation enzyme and water adjusted to a final volume 50 µl. The probe mixture was incubated at 15°C for 40 minutes and subsequently deactivated by incubating at 75°C for 10 minutes.

Fluorescence in situ hybridization (FISH)

The probe was denatured at 80°C for 6 minutes. Approximately 15 µl of hybridization probe [50% formamide, 0.1 mM EDTA, 5 mM Tris-HCl, 10% dextran sulphate, 2X SSC (1.75% sodium chloride, 0.88% sodium citrate)] was applied to the slide and covered with a 20 X 40 mm coverslip. The slide was sealed with rubber cement and placed in a moist chamber for hybridization at 37°C for two days. After hybridization, the slide was washed in a solution of 4 X SSC/ 0.3% Nonidet P-40 at 80 ^{o}C for 2 minutes and twice for 1 minute in a solution of 2 X SSC/ 0.1% Nonidet P-40. After the final wash, the slide was air dried in the dark room, counterstained with 4',6-diamidino-2-phenylindole.2HCl solution (DAPI, Vysis Inc.) and sealed with nail polish before observed under a microscope equipped with epifluorescence and double filter for DAPI/FITC (excitation wavelength = 560 nm and emission wavelength = 520 nm). Sperm nuclei (n=200; from separated and unseparated) with distinct green spots were classified as Y-chromosome and those without spots were classified as X-chromosome.

Statistical analysis

A statistical analysis was performed using SAS GLM to determine the significant differences in the percentages of X- and Y-chromosome bearing spermatozoa following separation in both treatments. A probability of $P<0.05$ was considered significant for all statistical tests. Values were presented as mean ± SEM.

Results and Discussion

The discontinuous albumin gradient technique has generated a huge contradictory evidence since the mechanisms of Y-chromosome bearing spermatozoa enrichment was not proven experimentally (Ericsson *et al*., 1973; Quinlivan *et al*., 1982; Claassen *et al*., 1995). In this study, the albumin gradient technique performed to separate Brakmas semen into X- and Y-chromosome bearing spermatozoa was modified, whereby the protocol of separation in the previous experiment was conducted on human spermatozoa (Chen *et al*., 1997; Flaherty *et al*., 1997 and Silverman *et al*., 2002). Ericsson *et al*. (1973) reported that the enriched population of Y-chromosome bearing spermatozoa could be discovered by using bovine albumin density gradient. The albumin gradient used in the present study was a two-phase gradient (12.5% over 20% and 15% over 20% BSA), whereas the human spermatozoa were separated on a single-phase gradient (7.5% human serum albumin, HSA), washed, resuspended and reapplied to another two-phase gradient (12.5% over 20% HSA). The bovine spermatozoa collected from the lower concentrated layer BSA (15% over 20% BSA) had a higher motility (85%) before freezing. Furthermore, the technique performed in this study was simpler as compared to the Ericsson (1973) method, whereby the washing and centrifugation steps

in the former were skipped to minimize pressure and reduce sperm mortality.

More reliable techniques have been used to evaluate the true chromosomal content of sperm cells after separation into X- and Y-chromosome bearing spermatozoa such as quinacrine mustard staining (Blottner *et al*., 1992), fluorescence *in situ* hybridization, FISH (Flaherty *et al*., 1997; Lin *et al*., 1998; Rose and Wong, 1998), nested PCR (Guo *et al*., 2008) and the latest was quantitative real time PCR (Parati *et al*., 2006). The efficiency of separation was determined based on the counted percentages of X- and Y-chromosomes for each individual (Table 1). Currently, FISH is the method of choice for evaluating the effectiveness of sperm separation procedure although the best validation technique is the sex ratio of the offspring (Beal *et al*., 1984; Pyrzak, 1994; Ericsson, 1994). Many studies have used either single or double-labeled FISH to verify the albumin gradients enriched Y-chromosome bearing spermatozoa (Aribarg *et al*., 1996; Chen *et al*., 1997; Rose and Wong, 1998). In the present study, a single-labeled probe TSPY which is specific for the detection of Y-chromosome bearing spermatozoa was used. Analysis by using FISH single-labeled probe (Figure 2), TSPY showed no significant differences (P>0.05) in the percentages of spermatozoa with the Y-chromosome specific signal (Y-chromosome bearing spermatozoa) and without the Y-chromosome signal (predicted as X-chromosome bearing spermatozoa) in the semen before and after the albumin gradient separation in both treatments. The mean percentages of X- and Y-chromosome bearing spermatozoa in samples recovered from the lower concentrated layers (12.5% over 20% BSA and 15% over 20% BSA) of Treatments 1 and 2 were 46.33:53.67 and 40.50:59.50, respectively. However, the mean of Y-chromosome bearing spermatozoa in Treatment 2 was higher than in Treatment 1 as well as in control groups (unseparated) (Table 1).

Table 1. Percentage of X- and Y-chromosomes in samples of unseparated and separated spermatozoa using BSA gradient

Treatment[1]	Chromosome type	Unseparated	Separated				
			Rep 1	Rep 2	Rep 3	Mean ± SEM	Motility ± SEM
1	X-chromosome	49	50	48	41	46.33 ± 2.73[a]	85 ± 8.17
	Y-chromosome	51	50	52	59	53.67 ± 2.73[a]	
2	X-chromosome	49	37	42	43	40.50 ± 2.02[a]	72 ± 16.1
	Y-chromosome	51	64	58	57	59.50 ± 2.02[a]	

[ab]Means of X- and Y-chromosome between treatments with similar superscripts within column are not significantly different (P>0.05)

[1]Treatment 1 of 12.5% over 20.0% BSA and Treatment 2 of 15.0% over 20.0% BSA

In this study, the efficiency of the separation procedure may be biased since a single- labeled probe was used to detect specifically the Y-chromosome. Previous studies reported that this technique showed that the X:Y sperm ratio after separation by FISH was 1:1 (Vidal *et al*., 1993; Rose and Wong, 1998). Sex-separation using 4 and 10% BSA on Angus bull showed that the proportion of male calves born to cows inseminated with unseparated (54%) or separated semen (45%) were not significantly different; and the percentage of male calves delivered also did not differ among sires (Beal *et al*., 1984). In the present study, the sex-separation in Treatment 1 resulted in a non-significant change in the relative percentage of X- and Y-chromosome bearing spermatozoa

compared to the theoretical natural ratio of 50:50. However, some samples yielded a slight enrichment of X- and Y-chromosome bearing spermatozoa. Study by Claasens *et al*. (1995) and Flaherty *et al*. (1997) reported that albumin gradients (12.5% over 20% BSA) did not enrich the Y-chromosome bearing spermatozoa following verification by FISH analysis. However, the percentage of Y-chromosome bearing spermatozoa in samples recovered from the lower concentrated layer of Treatment 2 (15% over 20% BSA) of the BSA gradient was higher compared to the percentage measured in Treatment 1. The results showed Treatment 2 was a better separation medium for Y-chromosome bearing spermatozoa compared to Treatment 1. The non-significant differences observed in both treatments probably due to the small sample size. Therefore, more samples should be used to enhance the separation of X- and Y-chromosome bearing spermatozoa. Other factors that affect the separation of X- and Y-chromosome, such as media, pH of the media and temperature should be considered.

Conclusion

The results of this study indicated that separations by Treatment 1 as well as Treatment 2 with modifications were unable to enrich the proportion of Y-chromosome bearing spermatozoa in bovine semen. However, Treatment 2 showed more promising result compared to Treatment 1 with discontinuous albumin gradient 15% over 20%. More factors should be considered in this study such as pH of the media and also temperature of environment during the experimental period.

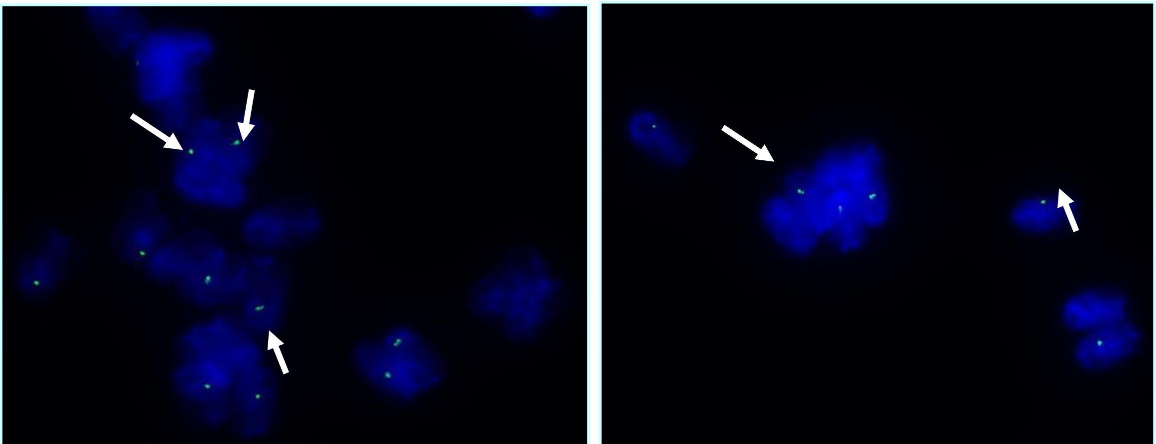

Figure 2. Analysis of sexed-separation spermatozoa by FISH using male-specific probe, TSPY. The Y-chromosome bearing spermatozoa was detected by green signals as indicated by the arrows.

Acknowledgements

We would like to express our sincere thanks to the staff of MARDI Research Station, Muadzam Shah, Pahang for Brakmas semen collection; Animal Breeding and Physiology Laboratory for the equipment and chemicals. This study was supported by the e-Science fund under project no. 05-03-08-SF0106.

References

Ainsworth, C., Nixon, B., Jansen, R.P.S. and Aitken, R.J. 2007. First recorded pregnancy and normal birth after ICSI using electrophoretically isolated spermatozoa. Hum Reprod. 22: 197-200.

Aribarg, A., Ngeamvijawat, J., Chanprasit, Y. and Sukcharoen, N. 1996. Determination of the ratio of X and Y bearing spermatozoa after albumin gradient method using double-labelled fluorescence *in situ* hybridization (FISH). J Med Assoc Thai. 1: 88-95.

Beal, W.E., White, L.M. and Garner, D.L. 1984. Sex ratio after insemination of bovine spermatozoa isolated using a bovine serum albumin gradient. J. Anim. Sci. 58: 1432-1436.

Blottner, S., Pitra, C. and Berger, U. 1992. Y-chromosome-specific fluorescence (f-body) of poorly decondensed bovine spermatozoa. Andrologia 24(5): 255-259.

Blottner, S., Bostedt, H., Mewes, K. and Pitra, C. 1994. Enrichment of bovine X and Y spermatozoa by free-flow electrophoresis. J. Vet. Med. Series A 41(1-10): 466-474.

Check, J.H. and Katsoff, D. 1993. A prospective study to evaluate the efficacy of modified swim-up preparation for male sex selection. Hum. Reprod. 8: 211–214.

Chen, M.J., Guu, H.F. and Ho, E.S.C. 1997. Efficiency of sex pre-selection of spermatozoa by albumin separation method evaluated by double-labelled fluorescence *in-situ* hybridization. Hum. Reprod. 12(9): 1920–1926.

Claassens, O.E., Oosthuizen, C.I.I. and Brusnicky, J. 1995. Fluorescent *in situ* hybridization evaluation of human Y-bearing spermatozoa separated by albumin density gradients. Fertil. Steril. 42: 756-760.

Ericsson, R.J., Langevin, C.N. and Nishino, M. 1973. Isolation of fractions rich in human Y sperm. Nature 246: 421-424.

Ericsson, R.J. 1994. Sex selection via albumin column: 20 years of results. Hum. Reprod. 9: 1787–1788.

Flaherty, S.P., Michalowska, J., Swann, N.J., Dmowski, W.P., Mattews, C.D. and Aitken, R.J. 1997. Albumin gradients do not enrich Y-bearing human spermatozoa. Hum. Reprod. 12(5): 938-942.

Garner, D.L., Gledhill, B.L., Pinkel, D., Lake, S., Stephenson, D., Van Dilla, M.A. and Johnson, L.A. 1983. Quantification of the X- and Y-chromosome bearing spermatozoa of domestic animals by flow cytometry. Biol. Reprod. 28: 312.

Guo, J.M., Zhu, H.B., Wang, D., Zhang, l., Hao, B.H.S. and Du, W.H. 2008. Progress on methods for purity assessment of separated chromosome X- or Y-bearing sperm. Yi Chuan 9: 1115-1120.

Hohenboken, W.D. 1999. Applications of sexed semen in cattle production. Theriogenology 52: 1421-1433.

Iizuka, R., Kaneko, S., Aoki, R. and Kobasyahi, T. 1987. Sexing of human sperm discontinuos Percoll density gradient and its clinical application. Hum. Reprod. 2: 573-575.

Lin, S.P., Lee, R.K.K., Tsai, Y.J., Hwu, Y.M. and Lin, M.H. 1998. Separating X-bearing human spermatozoa through a discontinuous percoll density gradient proved to be inefficient by double-label fluorescent *in situ* hybridization. J. Assist. Reprod. and Genetics 15(9): 565-569.

Parati, K., Bongioni, G., Aleandri, R. and Galli, A. 2006. Sex region determination in bovine semen: A new approach by quantitative real time PCR. Theriogenology 66: 2202-2209.

Prasad, S., Rangasamy, S. and Satheshkumar, S. 2010. Sex preselection in domestic animals – Current status and future prospects. Vet. World 3(7): 346-348.

Pyrzak, R. 1994. Separation of X- and Y-bearing human spermatozoa using albumin gradients. Hum. Reprod. 9: 1788-1790.

Quinlivan, W.L., Preciado, G.K., Long, T.L. and Sullivan, H. 1982. Separation of human X and Y spermatozoa by albumin gradients and Sephadex chromatography. Fertil. Steril. 37: 104-107.

Rose, G.A. and Wong, A. 1998. Experiences in Hong Kong with the theory and practice of the albumin column method of sperm separation for sex selection. Hum. Reprod. 13(1): 146–149.

Seidel, G.E. and Gardner, D.L. 2002. Current status of sexing spermatozoa. Hum. Reprod. 124(6): 733-743.

Silverman, A.Y., Stephens, S.R., Drouin, M.T., Zack, R.G., Osborne, J. and Ericsson, S.A. 2002. Female sex selection using clomiphene citrate and albumin separation of sperm. Hum. Reprod. 17: 254-256.

Steeno, O., Adimoelja, A. and Steeno, J. 1975. Separation of X- and Y-bearing human spermatozoa with the Sephadex gel-filtration method. Andrologia 7: 95-97.

Tan, Y.J., Mahanem, M.N. , Ainu Husna, M.S.S., Somarny, W.W.M.Z, Sharif ,H. and Johari, J.A. 2010. TSPY as a genetic marker for sex determination of cattle spermatozoa. Pertanika J. Trop. Agric. Sci. 33(2): 337-340.

Vidal, F., Moragas, M., Catal, V., Torell, M. J., Santal, J., Caldern, G., Gimenez, C., Barri, P.N., Egozcue, J. and Veiga, A. 1993. Sephadex filtration and human serum albumin gradients do not select spermatozoa by sex chromosome: a fluorescent *in-situ* hybridization study. Hum Reprod. 8(10): 1740-1743.

Welch, G.R. and Johnson, L.A. 1999. Sex pre-selection: laboratory validation of the sperm sex ratio of flow sorted X- and Y-sperm by sort reanalysis for DNA. Theriogenology 52: 1343–1352.