Research Articles

Possibility of Purifying the Spermatogenic Cells in Rat Using Gravitational Split-Flow Thin Cell (GrSPLITT) Fractionation

Nongnuch Tantidanai-Sungayuth[1, 4*], Nanthiya Poathong[1], Waret veerasai[1], Ronald beckett[3], Chaitip Wanichanon[2], Rapeepun Vanichviriyakit[2]

[1]Department of Chemistry and Center of Excellence for Innovation in Chemistry, Faculty of Science, Mahidol University, Rama VI Rd, Bangkok 10400 Thailand

[2]Department of Anatomy, Faculty of science, Mahidol University, Rama VI Rd, Bangkok 10400 Thailand

[3]Water Studies Centre, School of Chemistry, Monash University, Clayton, Australia, 3800

[4]Mahidol University at Kanchanaburi campus, 199 Lumsum Saiyok, Kanchanaburi 71150 Thailand

[*]Corresponding author, Email address: scntt@mahidol.ac.th

Abstract

The Gravitational split-flow thin cell (GrSPLITT) fractionation is a rapid, gentle and continuous separation technique that should be used to separate spermatogenic cells into three stages: (1) spermatogonia and spermatocyte (2) round spermatids and (3) elongate spermatids and spermatozoa. The different stages of the spermatogenic cells display differences in density and size. In this work, two modes of GrSPLITT namely, full-feed depletion mode (FFD-GrSPLITT) (and transport mode (TS-GrSPLITT) were compared for their possibility to separate the three stages of spermatogenic cells. Imaging and sizing of the original sample and all fractions were analyzed to obtain their size distribution using dark field microscopy with analysis software. The percentage of purity and recovery were calculated and used to objectively compare their separation efficiencies of the two mods of the FFD mode and the TS mode of GrSPLITT fractionations. All experiments showed that the TS-GrSPLITT fractionation trends to be the better separation efficiency than the FFD-GrSPLITT fractionation.

Keywords: settling velocity, size, shape, density, spermatogenic cells, separation, gravitational split-flow thin cell (GrSPLITT)

Introduction

In mammals, spermatogenesis is a complex biological process of transformation of spermatogonia in to spermatozoa. The spermatogenesis is simultaneously process of cellular differentiation between morphology (various sizes of round shape, head with short tail, and head with long tail) and biochemistry. Many fields of researches are interested to relate their differentiation between their morphology to their biochemistry changes in biological studies. Therefore, cell separation method is very important because it is prerequisite method.

Many biochemistry studies which is currently interesting and require separation method such as preparing various organ transplants, isolation of rare cell types for cloning, preparation of pure cells for transplantation and bioreactor maintenance, removal of specific cell types responsible for immune rejection following transplants and treatments for autoimmune diseases, purification of cell mixtures for diagnostic tests, umbilical cord blood processing prenatal blood testing for blood transfusion such as removal of lymphocytes from the transfusion and separation and detection of pathogenic microorganisms and parasites in food, environment and clinical samples (Rungarunlert et al. 2009, Kumar and Bhardwaj 2008, Alhadlaq and Mao 2004, Alvarez-Barrientos 2000, Orfao et al. 1996, and Griffith and Tiwari 1993)

The spermatogenesis is a challenge sample in biological field because it displays continuous differentiation of shape, size, and density during cell development. This process occurs within the seminiferous tubules of the ratís testis.

Spermatogenesis can be divided into three principal stages reported by Hess in 1999 and Johnson et al. in 2000.

First stage is the spermatocytes genesis stages, where the spermatogonia are produced that also divide themselves by mitosis into primary spermatocytes (2n). Their physical properties are big round shape (Franc et al. 1998) in range of 12-18 μm diameters (Aslam et al. 1998 and Wykes et al. 2003) and densities are in range of 1.045-1.051 g.cm^3 (Meistrich et al. 1994).

Second stage is the meiosis stage, in which there are two steps of divisions. In meiosis I, the primary spermatocytes produce two haploid secondary spermatocytes (n). During meiosis II, secondary spermatocytes produce two haploid round spermatids. Their physical properties are small round shape (Franc et al. 1998) in range of 8-10 μm diameters (Aslam et al. 1998 and Wykes et al. 2003) and densities are in range of 1.050-1.090 g.cm^3 (Meistrich et al. 1994).

Third stage is spermiogenesis stages, round spermatids change morphology and develop fertilization and differentiation into spermatozoa (Yu et al. 2003, Aslam et al. 1998, Quesada 1998, Meistrich et al. 1994, Bachere et al. 1988). Their physical properties are small head with short tail and long tail (Franc et al. 1998). The head diameter in range of 4-6 μm for the short tail but not found the information of the head of long tail (Aslam et al. 1998 and Wykes et al. 2003). Densities are in range of 1.100-1.165 g.cm^3 for the short tail and 1.170 for the long tail (Meistrich et al. 1994).

There are three common techniques for separation of cells based on their settling velocity (U). The principle of separation based on Stokesí law. The settling velocity (U) is express by:

$$U = \frac{\Delta\rho G d^2}{18\eta} \quad (1)$$

where U is settling velocity of the sphere (mm.hr^{-1}), $\Delta\rho$ is the difference between the densities of the particles ρ_p and carrier ρ_c (g·cm^{-3}), G is the gravitational acceleration (cm·s^{-2}), d_c is the particle diameter (μm), η is viscosity of carrier (kg·m^{-1}·s^{-1}).

First, centrifugal elutriation separates cells by size and density in physiological media, simultaneously, (Lam et al. 1988). Second, unit gravity sedimentation techniques offer several advantages such as inexpensive equipment, easy to operate and large sample volume. However, it is time-consuming and expensive if a gradient

medium is used. (Platz et al. 1975, Wykes et al. 2003, David 1977, Chilton et al. 1977, and Munteanu et al. 2004). Third, density gradient centrifugation separates cells though the gradient of solution. The principle of the separation has been explained in various publications (Sharp 1988, Munteanu et al. 2004, Finaz et al. 1991, Brakke et al. 1951, Henkel et al. 2003, and Tucker et al. 2002)

In this work, gravitational split-flow thin cell (GrSPLITT) fractionation technique was investigated for separation of spermatogenic rat cells. GrSPLITT is a rapid, gentle and continuous separation technique based on differences in settling velocity which depends on the size, and density of cells as described in Stokesí law (Fuh et al. 1995, Cantado et al. 1999, Blo et al. 2000, Cantado et al. 2000, Cantado et al. 1997, Lee et al. 2001, Springston et al. 1987, Fuh et al. 1992). Cell shape is also involved as this influences the friction coefficient reported by Dondi et al. in 1998. The separation efficiency of two modes of GrSPLITT (full-feed depletion (FFD) mode and the transport (TS) mode) was compared. Spermatogenic rat cells were used to test these techniques. The cells were fractionated at the following cutoff settling velocity (U_{cutoff}) 13.7 and 9.2 mm.hr^{-1}. Three fractions were obtained; Fraction (B1) U > 13.7 mm.hr^{-1}, Fraction (A2) $U \leq$ 9.2 mm.hr^{-1} and Fraction (B2) U = 9.2 - 13.7 mm.hr^{-1}.

Materials and methods

1. Preparation the spermatogenic cell suspension of rat testis

The spermatogenic cells from rat testis were obtained by removing the covering of the testis transfer seminiferous tubules and rinsing them with phosphate buffered saline (PBS) followed by Aslam et al. in 1998 and Kotaja et al. in 2004. The tissue was minced using scissors until a suspension was achieved using a transfer pipette gently pipette the tissue pieces up and down for 3 min and then gently shaking for 10 min to free the spermatogenic cells from the seminiferous tubules. The cell suspension was filtered through 212 μm mesh and the filtrate containing the cell suspension was centrifuged at the filtered cells suspension at 2000 rpm for 3-5 min. and the supernatants was discarded. The spermatogenic cells were fixed with 4% paraformaldehyde in PBS and stored at 4 °C until analyzed. The original cell suspension sample was diluted with PBS 10 times thus obtaining a cell number concentration of approximate 3.8 x 10^7 cells.ml^{-1}, which was used for analysis.

2. The GrSPLITT apparatus

The GrSPLITT cell was made in our lab which was validated and compared by Model SF1000 from Postnova Analytics, Salt Lake City, USA with spherical silica gel (Thawornsinsurakul, 2006). The cell consists of a stainless-steel splitter plate sandwiched between the two plastic spacer plates. They are kept tight between the two acrylic plates. The GrSPLITT cell dimensions are the length: L = 10.000 cm, the width of the stainless steel splitter: b = 1.500 cm and the channel thickness: w = 0.276 cm (combined thickness of two spacers plane and a stainless-steel splitter plane).

The GrSPLITT channel used in this work was constructed in our laboratories. Three pumps were used to control the two inlet and one outlet flow rates. The two inlet flowrates was controlled carefully with smoothly syringe pumps (*Kd* Scientific Model 200 Series, KD Scientific Inc. MA, USA). A peristaltic pump (Masterflex L/S Model 7523-25 230 V 600 rpm) was used to control the upper outlet flow rate. The lower outlet flow rate was opened for balance the total flow rate.

GrSPLITT fractionation can be operated for two different modes: TS mode and FFD mode.

TS mode of GrSPLITT fractionation

The TS-GrSPLITT was set up and followed in Fig.1. The upper inlet called inlet *a'* and the lower inlet calles inlet *b'*. The upper outlet flow rate called outlet a. In TS-GrSPLITT fractionation, inlet splitting plane (ISP) is generated by smooth merging between the

two streams of inlet *a′* and *b′*. Outlet splitting plane (OSP) is generated by separating fluid (lows) stream though outlet a and b. The position of the inlet splitting plane (ISP) and the outlet splitting plane (OSP) can be controlled by varying the ratio between the two inlets and the two outlets of flow rates, respectively.

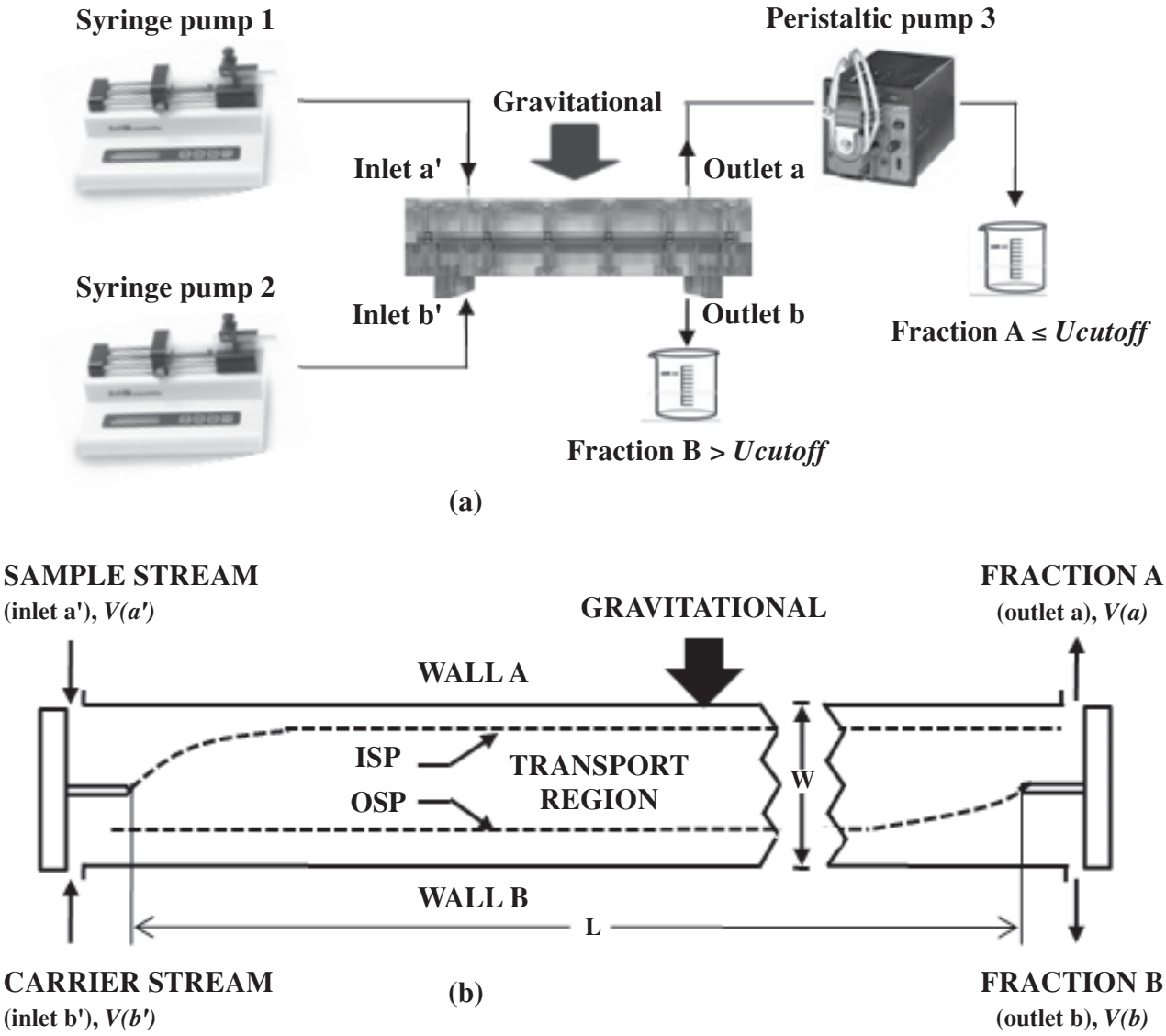

Figure 1 The transport mode (TS) of GrSPLITT set-up (a) and imaginary planes of inlet splitting plane (ISP) and outlet splitting plane (OSP) in side view of the thin channel of GrSPLITT channel (b) .

FFD mode of GrSPLITT fractionation

The FFD-GrSPLITT fractionation used only one syringe pump for inlet *a'* and one upper peristaltic pump for outlet *a*. In this mode, no inlet *b'* flow rate leads to inlet splitting plane (ISP).

3. Set-up flow rates of FFD- and TS-GrSPLITT

The GrSPLITT fractionation were separation the spermatogenic cells for two separation steps and two different cutoff settling velocities, (U_{cutoff}) 13.7 and 9.2 mm.hr^{-1}, respectively. The three fractions (Fraction B1, B2, and A2) were obtained, respectively. The three fractions were obtained: Fraction B1, U > 13.7 mm.hr^{-1} (expected spermatogonia and spermatocytes fraction), Fraction A2, U ≤ 9.2 mm.hr^{-1} (expected elongate spermatids and spermatozoa fraction) and Fraction B2, U = 9.2 - 13.7 mm.hr^{-1} (expected round spermatids fraction). The fractions (B1, B2, and A2) and the operation flow rates of FFD- and TS-modes of GrSPLITT showed in Table 1.

Table 1 The operation flowrates of two separation steps for two different cutoff settling velocities (U_{cutoff}) : (1) 13.7 mm.hr^{-1} and (2) 9.2 mm.hr^{-1}.

Experimental steps	Cutoff settling velocity (U_{cutoff}) mm.hr^{-1}	Flow rates (ml. min^{-1})				Total
		inlet		outlet		
		a'	b'	a	b	
FFD mode:						
1st step	13.7	1.00	-	0.65 (A1)	0.35 (B1)	1.00
2nd step	9.2	1.00	-	0.75 (A2)	0.25 (B2)	1.00
TS mode:						
1st step	13.7	1.00	1.55	1.55 (A1)	1.00 (B1)	2.55
2nd step	9.2	0.30	0.35	0.35 (A2)	0.30 (B2)	0.65

4. Experimental design

The fix cells of rat's spermatogenic cells were fractionated into three fractions. All fractions and the original sample were imaged and sized to report in term of cell size distribution curves. The raw data were calculated in parameters of percentage of purity and percentage of recovery as showed in Fig. 2.

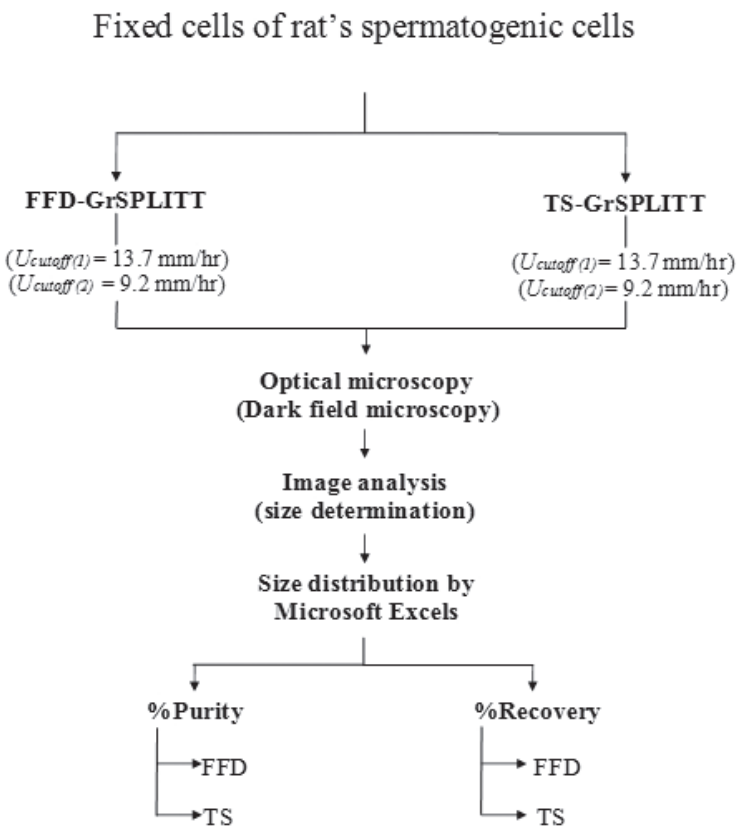

Figure 2 Experimental steps for comparing the separation efficiencies between FFD- and TS-modes of GrSPLITT fractionation of the rat's spermatogenic cells

Optical microscopes were done using a trinocular microscope (OLYMPUS BH-2, Japan). For image analysis, the Motic Images Plus 2.0 ML program was used to analyze the images of the cells and to measure of their sizes. At least 50 images per fraction were taken for analysis of cell size distribution to ensure that the photographs are representative of the fraction.

5. Calculation of percentage of purity

The percentage of purity of all fractions was calculated by Equation (2)

$$\%\,Purity = \frac{N_{ef}}{N_{tf}} x100 \qquad (2)$$

where N_{ef} is the number of spermatogenic cells in the separated fraction which is in the expected size range and N_{tf} is the total number of cells in the separated fraction.

6. Calculation of the percentage of recovery

The losses of cells during the GrSPLITT fractionation process were considered. The percentage of recovery can be calculated by Equation (3)

$$\%\,Recovery = \frac{N_{ef}}{N_o} x100 \qquad (3)$$

where N_{ef} is the number of spermatogenic cells in the separated fraction which is in the expected size range and N_o is the number of spermatogenic cells in the original sample which is in the expected size range.

Results and discussion

Three fractions of B1, B2 and A2 were obtained. All fractions were imaged and sized to plot their size distributions and to calculate the percentage of purity and recovery.

1. Image analysis

All fractions of B1, B2 and A2 obtained from TS mode and FFD mode of GrSPLITT were imaged atleast fifty microphotographs per fraction, including the original sample of spermatogenic cell. Some microphotographs for the three fractions and the original sample were compared in Fig. 3. The photomicrograph of original sample presents obviously differentiation of shape and size of the spermatogenic cells. Round shape at various sizes from 8-18 μm, short tail and long tail shapes (head size around 4-6 μm reported by Aslam et al. in 1998 and Wykes et al. in 2003.

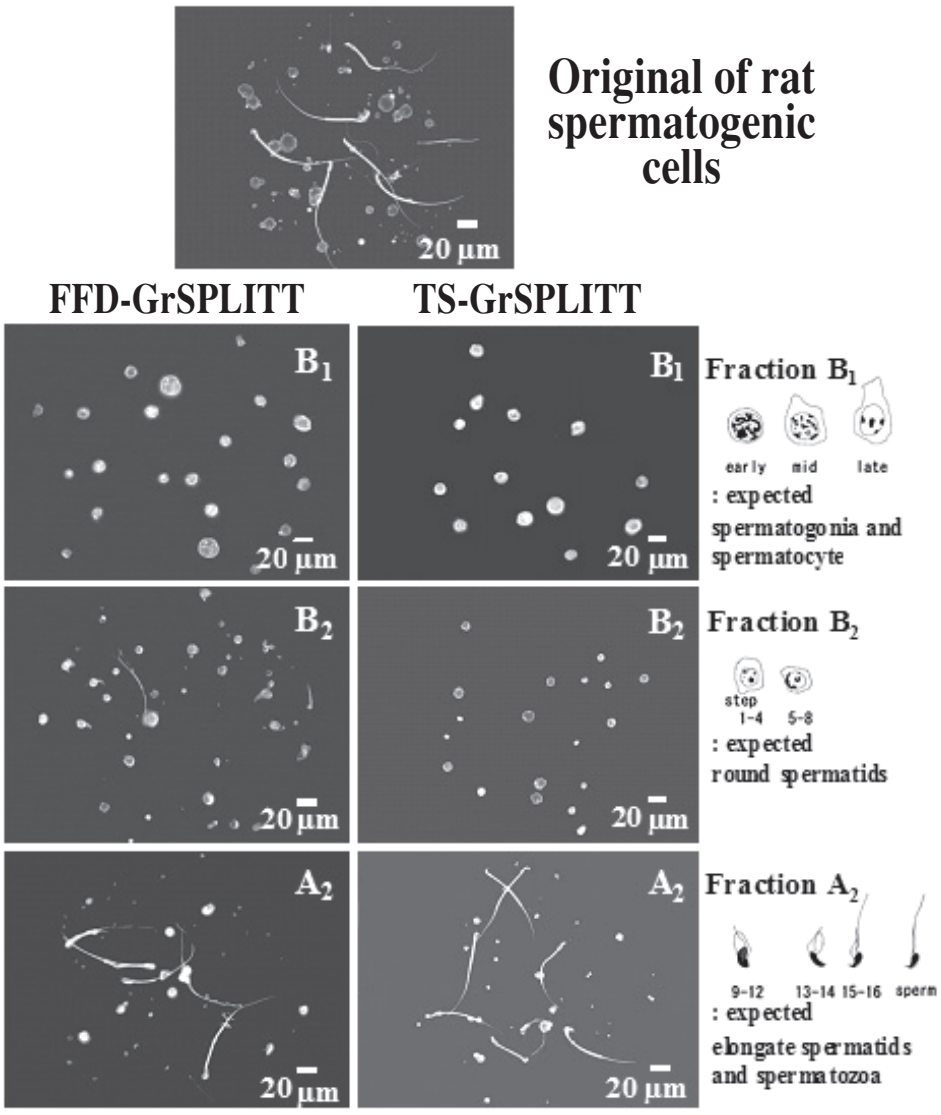

Figure 3 Comparison photomicrography of spermatogenic cell fractions was obtained by FFD- and TS-modes of GrSPLITT fractionation. (A) Fraction B1, U > 13.7 mm.hr^{-1} (expected spermatogonia and spermatocyte fraction), Fraction B2, (B) Fraction B2, $\underline{U}$ = 9.2 - 13.7 mm.hr^{-1} (expected round spermatids fraction). (C) Fraction A2, $U \leq 9.2$ mm.hr^{-1} (expected elongate spermatids and spermatozoa fraction).

The FFD-GrSPLITT is no dilution during the separation process, it causes the image of B1, B2 and A2 presented the more cell number per image than the TS-GeSPLITT fractionation. Moreover, the FFD-GrSPLITT is no ISP to control the beginning level for settling of each cell in the thin channel, it causes the contamination of small size or lower settling velocity of spermatogenic cells in the B fraction, especially in B2 fractions. In B2 image, the short tail shape of the spermatogenic cells were contaminated in the small round shape of the spermatogenic cells.

For TS-GrSPLITT fractionation, the microphotographs presented clearly big round shapes in B1 fractions and small round shapes in B2 fractions. In fraction A2, the microphotographs showed the short tail and the long tail shapes and a few number of the small round cells.

2. Size analysis

The size distributions of all fractions (B1, B2, and A2 fractions) of spermatogenic cells were obtained using FFD- and TS-modes of GrSPLITT fractionations (bar plotted) shown in Fig. 4 to compare with the original sample (line plotted). High resolution performance of the two GrSPLITT modes of the three fractions were expected that their cell size distributions of the B1 fraction should be larger than 11 μm, the B2 fraction should be in range of 7-11 μm, and the A2 fraction should be smaller than 7 μm. The cell size distribution curves in Fig.4 presented that both of the

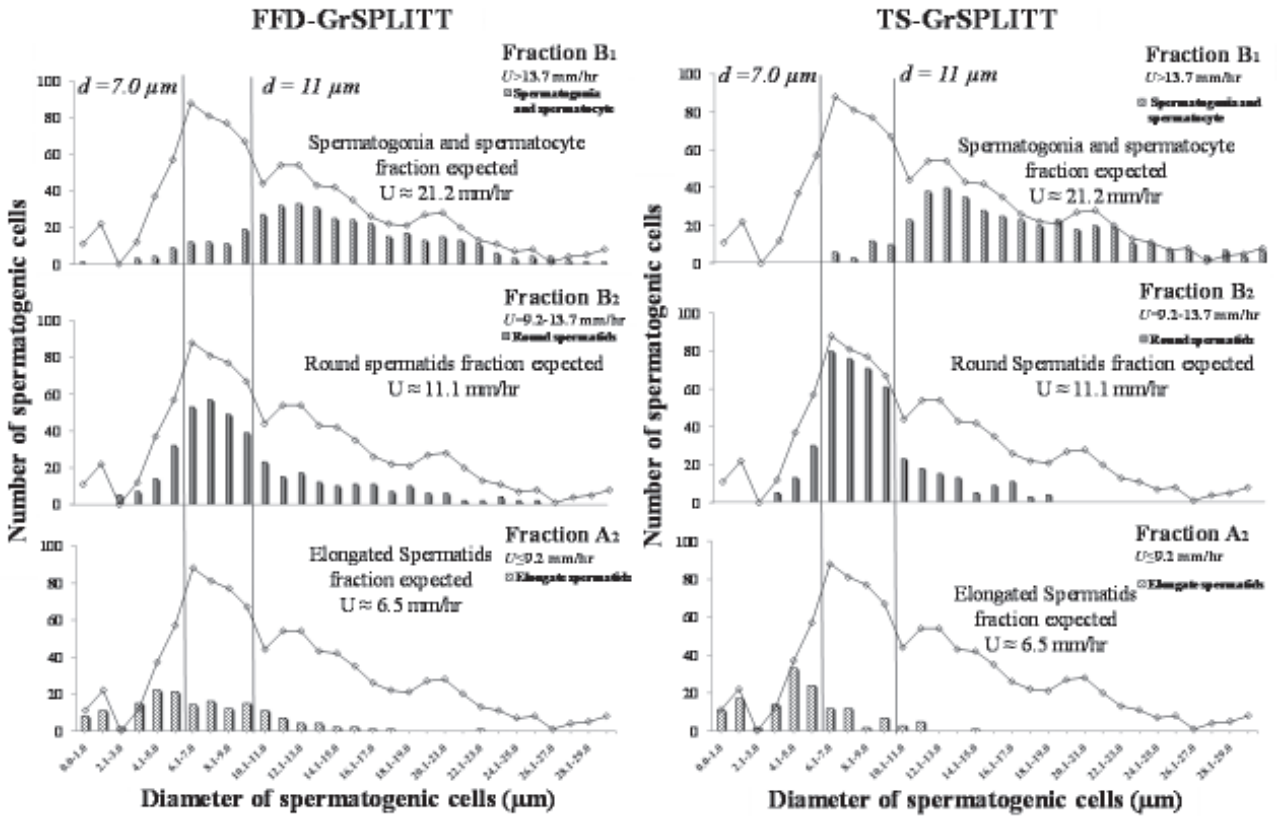

Figure 4 Comparison size distributions of the spermatogenic cells fractions obtained by FFD- and TS-mode of GrSPLITT fractionations. Fraction B1, U > 13.7 mm.hr^{-1} (expected spermatogonia and spermatocytes fraction), Fraction B2, U = 9.2 - 13.7 mm.hr^{-1} (expected round spermatids fraction) and Fraction A2, $U \leq$ 9.2 mm.hr^{-1} (expected elongate spermatids and spermatozoa fraction).

FFD-mode and the TS-mode of GrSPLITT fractionations were out of expected size ranges.

Moreover, good recovery of the separation method should present their peak height equally to the original sample line. The TS-mode showed the better trend of resolution and recovery of separation than the FFD-mode because it presented lower number of cell in the other expected size range in the three fractions. All size distribution curves the TS-mode presented the lower cell number lost during the separation compared with size distribution of original sample than the FFD-mode for all fractions (B1, B2, and A2), especially in B2 fraction. It may cause the dilution of buffer solution in inlet a' of TS-mode reduces the mixing up or intricate of the short tail cells and/or the long tail cells to form the larger aggregation and stack in the thin channel which cause of lose during the separation. So the percent concentration of cell in solution should be studied in the future works.

Even if the both of the separation modes were not perfect separation in this work, but the operation flow rates of the FFD-mode and TS-mode can be optimized in the next future work.

Actually, only the size distribution curves are not enough to consider their separation efficiencies of the two modes of GrSPLITT fractionation because the spermatogenesis is continuum physical and chemical changes. Density which is another major parameter of the spermatogenic cells mentioned in Stokes law beside the size parameter may affect to settling-velocity of the cells. Density distribution of spermatogenesis cells has not been reported excepted for in the range of density and a few reported works. In this work the separation is depended on the difference of settling properties of each cell. Therefore, it is difficult to completely fractionate the cell stages only presented on size distribution curve as presented in Fig.4.

However, to compare the percentage of purity and recovery between the two modes, the raw data of size distribution were calculated in section 3.

3. Calculations

3.1 Percentage of purity

Percentage of purity of spermatogenic cells were separated by FFD- and TS-modes of GrSPLITT fractionation in range of 35.8±2.8% - 66.0±3.4% and 57.4±3.6% - 83.5±6.0%, respectively as shown in Table 2. From the results, all fractions obtained from the TS-mode presented the higher percentage of the purity than the fractions obtained from FFD-mode. It refers that the percentage of purity in B1, B2, and A2 fractions using the TS-mode presented the higher performance than using the FFD-mode. The differentiation of percentage of purity was statistically significant ($p < 0.05$, One way ANOVA) between fractions B1, B2, and A2 obtained from both of the TS-mode and the FFD-mode. The differentiation of percentage of purity was statistically significant ($p < 0.05$, One way ANOVA) between the TS-mode and the FFD-mode in the same fractions.

Table 2 Comparison of the percentage of purities in the three fractions (Fraction B1, B2, and A2) between using the FFD- and TS-modes of the GrSPLITT fractionation for separating the ratís spermatogenic cells.

Fractions	Stages	FFD-GrSPLITT			TS-GrSPLITT		
		N_{ef}	N_{tf}	% Purity	N_{ef}	N_{tf}	% Purity
B_1	Stage 1 (round)	301.0±8.6	456.0±9.4	66.0±3.4	374.0±3.5	448.0±6.4	83.5±6.0
B_2	Stage 2 (round)	175.0±9.3	486.0±10.2	35.8±2.8	275.0±4.4	480.0±3.5	57.4±3.6
A_2	Stage 3 (short tail)	97.0±5.1	242.0±6.2	40.0±3.3	91.0±4.1	140.0±2.3	65.2±3.2
	Stage 3 (long tail)	86.0±6.2	142.0±4.0	60.6±4.1	100.0±3.2	140.0±4.7	71.2±3.0

Stage 1 = spermatogoniaand spermatocyte (spherical)

Stage 2 = round spermatids (spherical)

Stage 3 = elongate spermatids (short tail) and spermatozoa (long tail)

The TS-mode is higher percentage of purity than FFD-mode in all fractions it may because there is inlet splitting plane (ISP) which controls the start level in the same position to settle in the thin channel lead to less contamination in each fraction when compared with the FFD-mode of GrSPLITT fractionation.

3.2 Percentage of recovery

Percentages of recovery of spermatogenic cell after separated by FFD- and TS-modes of GrSPLITT fractionation were obtained in range of 55.9±3.6% - 69.9±4.4% and 63.3±3.7% - 88.0±5.6%, respectively, as shown in Table 3. From the results, the percentages of recovery in fractions of B1 (big round cells) and B2 (small round cells) by TS-mode (79.0±3.6 and 88.0±5.6 %) that higher than FFD-mode (63.8±6.4 and 55.9 ±3.6 %) are significantly different ($p < 0.05$). It may cause the TS-mode has ISP to control the beginning level before settling down in the thin channel while as the FFD-mode is no buffer solution from the lower inlet stream b' to generate the ISP and to dilute the concentration of the original sample of the spermatogenic cells.

Therefore, the long tail cells and the short tail cells which have lower settling velocity should presented lower contamination in the B2 fraction for the TS-mode than the FFD-mode. It can be confirmed by the microphotographs in Fig.3. The number of the long tail cells and the short tail cells in the microphotographs of B2 fractions using TS-mode was lower than using FFD-mode.

The A2 fractions for the long tail cells using the TS-mode equaled to 63.3±3.7% that presented the higher percentage of recovery than using the FFD-mode equaled to 55.1±7.2% ($p < 0.05$). It can be explained in the same reason that FFD-mode is no buffer solution from the lower inlet stream b' to generate the ISP and to dilute the concentration of the original sample of the spermatogenic cells, so the long tail cells were easy to obstruct and to cross over in to the B2 fraction. Even if the percentage of recovery of the A2 fractions for the short tail cells using the TS-mode equal to 66.0±4.0% and using the FFD-mode equal to 69.9±4.4% were not significantly different ($p > 0.05$).

Table 3 Comparison of the percentages of recovery of the three fractions (Fraction B1, B2, and A2) using the FFD- and TS-modes of the GrSPLITT fractionation for separating the ratís spermatogenic cells.

Fractions	Stages	FFD-GrSPLITT			TS-GrSPLITT		
		N_{ef}	N_{tf}	% Purity	N_{ef}	N_{tf}	% Purity
B_1	Stage 1 (round)	301.0±8.6	473.0±10.9	63.8±6.4	374.0±3.5	473.0±6.9	79.0±3.6
B_2	Stage 2 (round)	175.0±9.3	313.0±10.2	55.9±3.6	275.0±4.4	313.0±5.2	88.0±5.6
A_2	Stage 3 (short tail)	97.0±5.1	139.0±6.9	69.9±4.4	91.0±4.1	139.0±6.9	66.0±4.0
	Stage 3 (long tail)	86.0±6.2	156.0±5.9	55.1±7.2	100.0±3.2	156.0±5.9	63.3±3.7

Stage 1 = spermatogonia and spermatocyte (spherical)

Stage 2 = round spermatids (spherical)

Stage 3 = elongate spermatids (short tail) and spermatozoa (long tail)

Conclusions

Spermatogenic cell of rat is a challenge sample that is continuous differentiation in physical and chemical properties such as size, density, and shape. These parameters are effect to their settling velocity. Therefore, it was difficult to present clearly on the separation efficiency only using the size distribution curve. Photomicrographs were the good evident to confirm and to explain the reason of the experiments. In this work, gravitational split-flow thin cell (GrSPLITT) fractionation trended to be a high potential technique in both of purity and recovery to separate the spermatogenic cells. In this work, the separation potential and efficiency of the TS-GrSPLITT fractionation presented better than the FFD-GrSPLITT for separating the ratís spermatogenic cells which can be evaluated by determining the percent purities and recoveries of each fraction. The reason was that the TS-mode has ISP to control the settling level before separation process with in the thin channel while as the FFD-mode has not. Moreover, the sample dilution of TS-mode reduced probability of the long tail cell to obstruct in to the thin channel. However, the efficiency of the TS-GrSPLITT system can be improved by increase the thickness between ISP and OSP by varying the ratios of inlet and outlet flow rates in the future work.

Acknowledgements

The research was supported by Center of Excellence for Innovation in Chemistry (PERCH-CIC) and Thailand Research Fund (TRF), young scientist research fund from faculty of science Mahidol University for scholarship and funding. The authors acknowledge Dr.Chaitip Wanichanon from Department of anatomy Mahidol University for use of the Optical Microscope and his valuable advice on this work.

References

Ashar, H. R., Chouinard, Jr. R. A. Dokur, M., Chada, K. (2010). *In vivo* modulation of HMGA2 expression. *Biochimica et Biophsica Acta*, 1799, 55-61.

Aslam, I., Robins, A., Dowell, K., Fishel, S. (1998). Isolation, purification and assessment of viability of spermatogenic cells from testicular biopsies of azoospermic men. Human Reproduction, 13, 639-645.

Bachere, E., Chagot, D., Grizel, H. (1988). Cell separation by centrifugal elutriation. American Fisheries Society Special Publication, 18, 281-285.

Blo, G., Contado, C., Fagioli, F., Dondi, F. (2000). Size-elemental characterization of suspensed particle matter by split-flow thin cell fractionation and slurry analysis-eletrothermal atomic absorption spectrometry. Analyst, 125, 1335-1339

Brakke, M. K. (1951). Density gradient centrifugation : A new speration technique. Notes, 73, 1847-1848.

Contado, C., Dondi, F., Beckett, R., Giddings, J. C. (1997). Separation of particulate environmental samples by SPLITT fractionation using different operating modes. Analytica Chimica Acta, 345, 99-110.

Contado, C., Reschiglian, P., Faccini, S., Zattoni, A., Dondi, F. (2000). Continuous split-flow thin cell and gravitational field-flow fractionation of wheat starch particles. Chromatography, 871, 449-460.

Contado, C., Riell, F., Blo, G., Dondi, F. (1999). Continuous split flow-thin cell fractionation of starch particles. Chromatography, 845, 303-316.

Chilton, B. S., Nicosia, S. V. (1978). Separation of rabbit endocervical cells by unit gravity sedimentation. TCA Manual, 5, 1205-1209.

Clouthier, D. E., Avarbock, M. R., Maika, S. D., Hammer, R. E., Brinster, R. L. (1996). Rat spermatogenesis in mouse testis. Nature, 381, 418-421.

David, M.P. (1977). Methods in cell biology. Marvin L.M (Ed.), (Vol. 15). United Kingdom, pp. 16-52.

Dondi, F., Contado, C., Blo, G., Martin, S.G. (1998) SPLITT cell separation of polydisperse suspended particles of environmental interest. Chromatographia, 48 (9/10), 643-654.

Finaz, C., Boue, F., Meduri, G., Lefever, A. (1991). Characterization of rat epithelian epididymal cells purified on a discontinuous Percoll gradient. Jounals of reproduction and Fertility Ltd. 91, 617-625.

Fuh, C. B., Giddings, J. C. (1995). Isolation of human blood cells, platelets, and plasma proteins by centrifugal SPLITT fractionation. Biotechnology Progress, 11, 14-20.

Fuh, C. B., Myers, M. N., Giddings, J. C. (1992). Analytical SPLITT fractionation: rapid particle size analysis and measurement of oversized particles. Analytical Chemistry, 64, 3125-3132.

Henkel, R. R., Schill, W-B. (2003). Review sperm preparation for ART. Reproductive biology and endocrinology, 1, 1-22.

Hess, R. A. (1999). Spermatogenesis, Overview. University of Illinois at Urbona, 4, 539-545. Johnston, D. S., Wright, W. W., Dicandeloro, P., Eilson, E., Kopf, G. S.,

Jelinsky, S. A. (2008). Stage-specific gene express is a fundamental characteristic of rat spermatogenic cell and sertoli cell. Developmental Biology, 105 (24), 8315-8320.

Johnson, L., Varner, D. D., Roberts, M. E., Smith, T. L., Leillor, G. E., Scrutchfield, W. L. (2000). Efficiency of spermatogenesis: a comparative approach, Animal Reproduction Science, 60-61, 471-480.

Kotaja, N., Kimmins, S., Brancorsini, S., Hentsch, D., Vonesch, J.L., Davidson, I., Parvinen, M., Sassone-Corsi, P. (2004) Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. Nature methods, 1 (3) 249-645.

Kumar, A., Bhardwaj, A. (2008). Method in cell separation for biomedical application: cryogels as a new tool. Biomedical materials, 3, 1-11.

Lam, D.M.K., Furrer, R., Bruce, W.R. (1970). The separation, physical characterization, and differentiation kinetics of spermatogonial cells. Proceeding of the National Academy of Sciences, 65, 192-199.

Lee, S., Park, H-Y., Lee, S-K., Yang, S-G., Eum, C. H. (2001). Separation and characterization of dust and ground water particulates using gravitational SPLITT fractionation. Bulletin of the Korean Chemical Society, 22, 616-622.

Meistrich, M. L., Trostle-Weige, P. K., Van Beek, M. E. A. B. (1994). Separation of specific stages of spermatids from vitamin A-synchronized rat testes for assessment of nucleoprotein changes during spermiogenesis. Biology of *Reproduction,* 51, 334-344.

Munteanu, L. S., Dinu, A. (2004). Fractionation of granulocytes from whole human blood by centrifugation. *Practical hints. Romanian Journal of biophys,* 14, 53-58.

Platz, R. D., Grimes, S. R., Meistrich, M. L., Hnilica, L. S. (1975). Change in nuclear protein of rat testis cells separated by velocity sedimentation. The Journal of Biological Chemistry, 250, 5791-5800.

Quesada, P. (1998). Poly (ADP-ribosyl) action as one of the molecular event that accompany mammalian spermatogenesis. Gene Therapy and Molecular Biology, 1, 681-699.

Rungarunlert, S., Techakumphu, M., Pirity, M.K., Dinnyes, A. (2009) Embryoid body formation from embryonic and induced pluripotent stem cells: Benefits of bioreactors. World journal of stem cells, 1 (1) 11-21.

Sharpe, P.T. (1988). Method of cell separation. Burdon, R.H., Kippenberg, P.H (Ed.), Laboratory techniques in biochemistry and molecular biology (Vol. 18). Amsterdam New York, pp. 208-220.

Springston, S. R., Myers, M. N., Giddings, J. C. (1987). Continuous particle fractionation based on gravitational sedimentation in split-flow thin cells. Analytical Chemistry, 59, 344-350.

Thawornsinsurakul, O., (2006). GrSPLITT size based fractionation of Kekong River particulates and their elemental characterization by XRF and ICP-OES. Thesis, pp. 44-60.

Tucker, K.E., Jansen, C.A.M. (2002). Sperm separation techniques: comparison and evaluation of gradient products. Proceedings 2nd international workshop for embryologists: Troubleshooting activities in the ART lab. Ed. R. Basuray and D Mortimer, 1-4.

Wykes, S. M., Krawetz, S. A. (2003). Separation of spermatogenic cell from adult transgenic mouse testes using unit-gravity sedimentation. Molecular Biotechnology, 25, 131-138.

Yu, Z., Guo, R., Ge, Y., Ma, J., Guan, J., Li, S., Sun, X., Xue, S., Han, D. (2003). Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. Biology of Reproduction, 69, 37-47.