

# Quality and quantity of Asian elephant (*Elephas maximus*) peripheral blood mononuclear cells isolated by polymer of sucrose or colloidal silica solution gradient centrifugation

Porntep Gittipongsiri Somphon Makpun Teeraphong Phansod Dulyatad Gronsang  
Roschong Boonyarittichaikij Witthawat Wiriyarat\*

<sup>1</sup>Faculty of Veterinary Science, Mahidol University, 999 Phuttamonthon 4 Rd., Salaya, Phuttamonthon, Nakorn-Pathom, Thailand 73170

\*Corresponding author. E-mail: vswwr@mahidol.ac.th

## Abstract

Peripheral blood mononuclear cells (PBMCs) are useful in cellular immunology, infectious disease diagnosis and molecular genetics research. This study was aimed to determine an appropriated procedure for PBMCs isolation from an Asian elephant (*Elephas maximus*) by using gradient centrifugations method. The efficacy of four different density solutions including polymer of sucrose solution (Ficoll-Hypaque mixture) of density 1.077 and colloidal silica solution (Percoll) of density 1.077, 1.078 and 1.079 g/cm<sup>3</sup> were compared in elephant PBMC separation. Quality and quantity of isolated PBMCs were estimated by using five parameters including cell purity, cell recovery rate, cell viability, viability of PBMCs after 5 days cultivation and total cell count. The results showed that the total cell count was significantly different among 4 treatments, in which Percoll with density 1.079 g/cm<sup>3</sup> yielded the highest PBMCs (average = 3.94 X 10<sup>7</sup> cells/cm<sup>3</sup>) and highest PBMCs recovery rate (average = 82.61%). However, the purity, viability and viability of PBMCs after 5 days cultivation were not significantly different (P<0.05). In conclusion, Percoll with a density of 1.079 g/cm<sup>3</sup> is the most suitable density gradient treatment for Asian elephant PBMCs isolation in both quantity and quality aspects.

**Keywords:** PBMCs isolation, Asian Elephant (*Elephas maximus*), Ficoll, Percoll

## Introduction

The Asian Elephant (*Elephas maximus*) is officially classified as an endangered or threatened species by the Convention on International Trade in Endangered Species of Wild Flora and Fauna (CITES). The wild elephant population in Thailand is estimated between 2,000-3,000 animals (Silva and Kuruwita 1993). If there is no conservation, they will be extinct specie within the next 30 years (Stromayer 2001). Elephant health care is one of the most important factors for conservation besides housing and food management. However, the knowledge on this field, such as routine vaccination program, treatment and prevention of the diseases is not well understood. There are many fatal infectious diseases that affect the elephant health; for instance, Elephant Endothelial Herpes virus, Encephalomyocarditis virus, Anthrax, and Hemorrhagic septicemia.

Nowadays, the basic and advance research about Cell Mediated Immunity (CMI) in Medicine and Veterinary Medicine have been increased (Huang et al. 2005). Especially, the peripheral blood mononuclear cells (PBMCs) which consist of lymphocyte and monocyte are often used in various studies. These cells have an important function in immune system that can destroy and eliminate the intracellular microorganism and also secrete several cytokines (Mawas et al. 1973). A tendency of using these cells as a cell therapy associated with traditional therapy is increasing (Shi et al. 2003). In addition, scientific researches in microbiology, pathology and pharmacology, need cell culture in which had high quantity and quality of PBMCs.

Venipuncture is more simple and convenient than tissue biopsy for peripheral blood mononuclear cells (PBMCs) collection. However, the whole blood samples consist of biological fluid of various cell types so the PBMCs isolation procedure should be done. After the isolation procedure, PBMCs are required to remain healthy and still perform their functions. Several useful methods for blood cell isolation have been demonstrated including polymer of sucrose solution (Ficoll) density

gradient centrifugation (Jackowski and Liew 1980), colloidal silica solution (Percoll) density gradient centrifugation (Hirano et al. 1988), cell affinity chromatography (CAC) (Putnam et al. 2003) and magnetic cell separation (MACS) using magnetic beads (Ki-Ho and Bruno 2005). These several methods have become a common method for separating biological cells. Particularly, the density gradient centrifugation method of Ficoll and Percoll were shown to yield high quantity PBMCs isolation, and also, less expense and time consuming (Ulmer et al. 1984).

Currently there are several researches on PBMCs isolation by using Ficoll and Percoll in both human and animals (Laura et al. 2002). However, the PBMCs isolation method for the Asian elephant has never been reported. Moreover, there are rarely researches in elephant immunology and infectious diseases that have been published. The study on elephant hematology revealed that white blood cells and red blood cells were larger and differ in shape from other animals (Salakij et al. 2004), for the example the diameter of Asian elephant red blood cells ( $9.2 \pm 0.1 \mu\text{m}$ ) is two times bigger than sheep and goat (Jain 1993). In addition, monocyte has large size with diameter  $16.5 \pm 0.2 \mu\text{m}$  and there are many forms of nucleus such as round, bean or kidney shape or sometimes it has bilobe or trilobe nucleus (Brown and White 1980). From previously data, it was suggested that PBMCs isolation method in elephant may differ from other animals. Therefore, the objective of this study is to determine a suitable density gradient centrifugation method by Ficoll and Percoll for isolation of Asian Elephant PBMCs.

## Materials and Methods

### Animals and blood collection.

Twenty-four healthy Asian Elephants, age from 5 to 70 years old, from 2 elephant camps were selected for this study. Thirty milliliters of peripheral blood was collected from ear vein into K-EDTA tubes. Ten milliliters

of whole blood were used for hematology and serum biochemistry for health status evaluation.

#### **PBMCs isolation.**

Dilute the remaining whole blood (20 ml) with 1x Phosphate Buffered Saline (PBS) in the ratio 1:1 in 50 ml. centrifuge tube. Divided diluted blood solution into 4 proportion (10 ml. for 4 treatment solution each) and overlay carefully on the 5 ml. sterile Ficoll-Hypaque mixture (IsoPrep, Robbins Scientific Corporation, Sunnyvale, CA, USA) with a density of 1.077 g/cm<sup>3</sup>, and 5 ml. sterile Percoll (Percoll™, GE Healthcare, Sweden) with density of 1.077, 1.078 and 1.079 g/cm<sup>3</sup>, respectively, by sterile Pasture pipette, and then centrifuged at 2,000 rpm for 20 minutes at 25°C and the process was terminated without braking. Afterward, collect the PBMCs fraction from the interface between the diluent and the treatment solution (Ficoll and Percoll) into 15 ml centrifuge tube. After that add 10 ml 1xPBS into the opaque PBMCs fraction and then mixed by sterile Pasture pipette.

Washing step: centrifuge at 2,000 rpm for 10 minutes at 25°C and the process was terminated without braking. After that, the supernatant was discarded and then the cell pellets was resuspended with 10 ml 1x PBS and mixed by sterile Pasture pipette. Repeat the washing step 2 times to remove the Ficoll and Percoll solution, which can be toxic to cultured cells. Finally, the cell suspension was mixed and made up to the final volume 1 ml with 1X RPMI medium.

#### **PBMCs count.**

The solution of cell pellets in RPMI medium was mixed with Trypan blue dye exclusion (dilution 1:100) then the total quantity of PBMCs were count and PBMCs viability was determined by McMaster counting chamber. PBMCs in the number of 10<sup>7</sup> cells were mixed into RPMI medium supplement with 10% fetal calf serum (FCS) and cultured at 37°C, 5% CO<sub>2</sub> for 5 days. After 5 days cultivation, the total quantity of PBMCs and

PBMCs viability were determined.

#### **Purity of PBMCs by differential count.**

Purity of isolated PBMCs was determined by the differential count method. Ten microliters of separated PBMCs were put on glass slide and then stained with hematoxyline for cell type identification. The number of granulocyte which contaminated in isolated PBMCs was counted, then purity percentage was calculated.

#### **Determination for efficacy of Percoll and Ficoll in Asian elephant PBMC separation**

In this experiment, there were 5 parameters which were studied to compared the efficacy of Percoll or Ficoll density gradient centrifugation as follows:

Total PBMCs Before Cultivation (TCB) refer to the number of isolated PBMCs before culture which can be calculated by

$$TCB = \text{Number of PBMC in all four squares} / 4 \times \text{specimen dilution factor} \times 10^4$$

Percent of PBMCs viability before cultivation (**PVB**) is calculated by

$$PVB = \frac{\text{Number of viable PBMCs (before cultivation)} \times 100}{\text{Number of viable PBMCs} + \text{Number of nonviable PBMCs}}$$

Percent of PBMCs viability after cultivation (**PVA**) for evaluation the healthy of PBMCs is calculated by

$$PVA = \frac{\text{Number of viable PBMCs (after cultivation)} \times 100}{\text{Number of viable PBMCs} + \text{Number of nonviable PBMCs}}$$

Percent of PBMCs Purity (**PP**) is calculated by

$$PP = \frac{\text{Number of PBMCs (after isolation)} \times 100}{\text{Number of total counting cells}}$$

Percent of PBMCs Recovery (**PR**) is calculated by

$$PR = \frac{\text{Number of PBMCs from isolation} \times 100}{\text{Number of PBMCs in whole blood}}$$

#### **Statistical analysis**

The data were computed and statistically analyzed for mathematic equation and statistical correlation

between age and total PBMCs from whole blood by Pearson Correlation (two-tailed).

Sex has been thought to confounding in the treatment so the repeated measures design in Randomized Complete Block Design (RCBD) is used to classify into two blocks according to sex. Significance was considered to be indicated by a probability equal or less than 0.05 ( $p < 0.05$ ) by General Linear Model and multiple comparisons of the mean difference is significant that

identified by Scheffe’s test ( $p < 0.05$ ). All values were expressed as mean  $\pm$  pooled standard error of the mean (pooled SEM).

**Results**

The correlations between ages (10-70 years old) and total PBMCs from whole blood are presented in Table 1, which shown that no correlation between ages and elephant PBMCs by Pearson correlation.

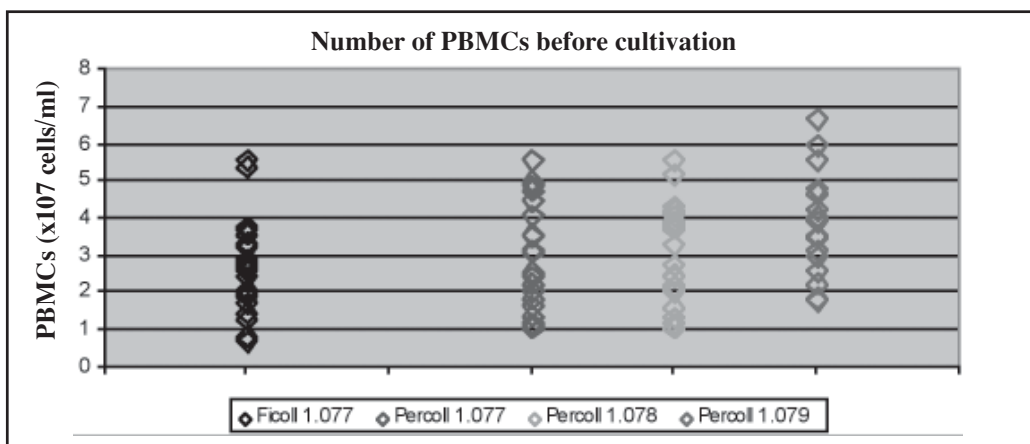
**Table 1:** The correlations between ages and total PBMCs from whole blood

		AGES	PMCs
AGES	Pearson Correlation	1	-.141
	Sig. (2-tailed)		.511
	N	24	24
PBMCs	Pearson Correlation	-.141	1
	Sig. (2-tailed)	.511	-
	N	24	24

**Total of PBMCs isolation (TCB)**

The density gradient centrifugation method for PBMCs isolation showed that Percoll with each density trend to isolate and obtain more PBMCs than Ficoll

(Fig. 1). The highest amount of total isolated PBMCs was by Percoll with density 1.079 g/cm<sup>3</sup>, which was significantly difference from Ficoll by both P value ( $P = 0.26$ ) and scheffe’s test (Table 2).



**Fig. 1:** Total number of PBMCs isolated by Ficoll or Percoll gradient density centrifugation.

**Percentage of PBMCs viability before cultivation (PVB)**

Percoll with density 1.079 g/cm<sup>3</sup> obtained the highest PBMCs viability (Fig. 2). When analyzed by statistic, the results showed that percentage of PBMCs

viability was found to be significantly between 4 treatment when determine by P-value ( $P = 0.046$ ). However, this percentage of PBMCs viability was no significant difference when identified by Scheffe’s test (Table 2).

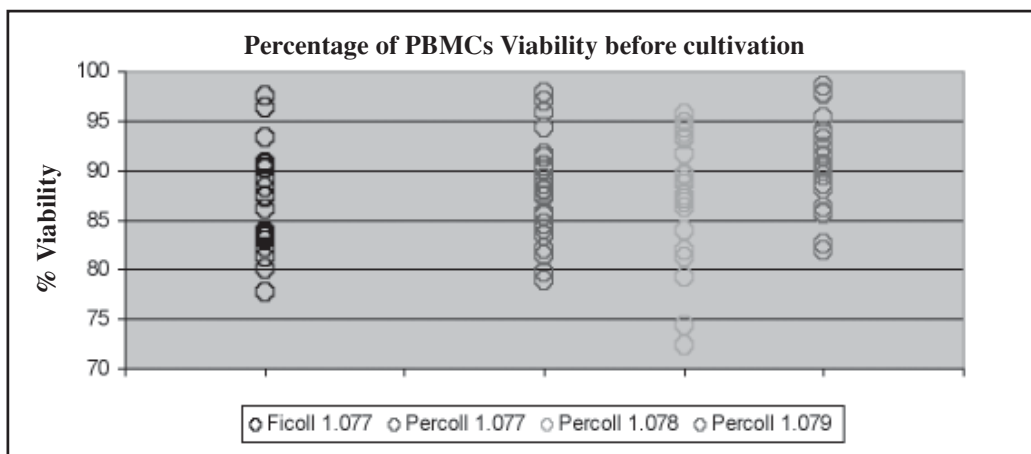


Fig. 2: Viability of PBMCs after isolation by density gradient centrifugation.

**Percent of PBMCs viability after cultivation for 5 days (PVA)**

1.078 and 1.079 g/cm<sup>3</sup> was no significantly difference by both P-value (P = 0.243), and Schffeís test (table2)

Percentage of PBMCs viability after cultivation for 5 days from Ficoll density 1.077, Percoll density 1.077,

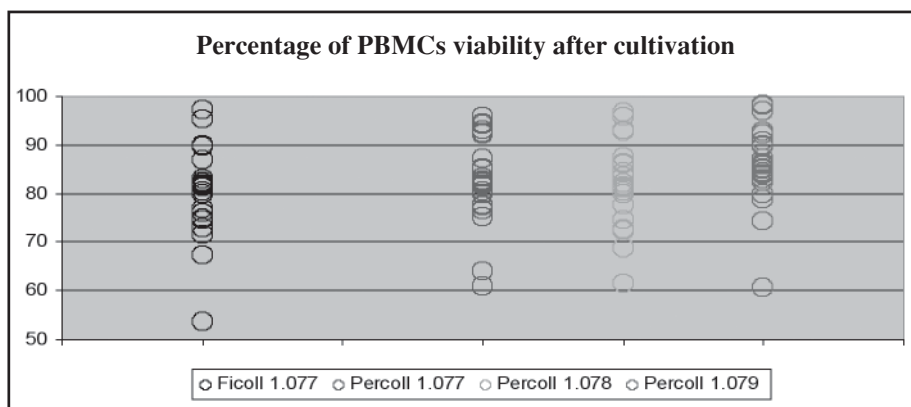


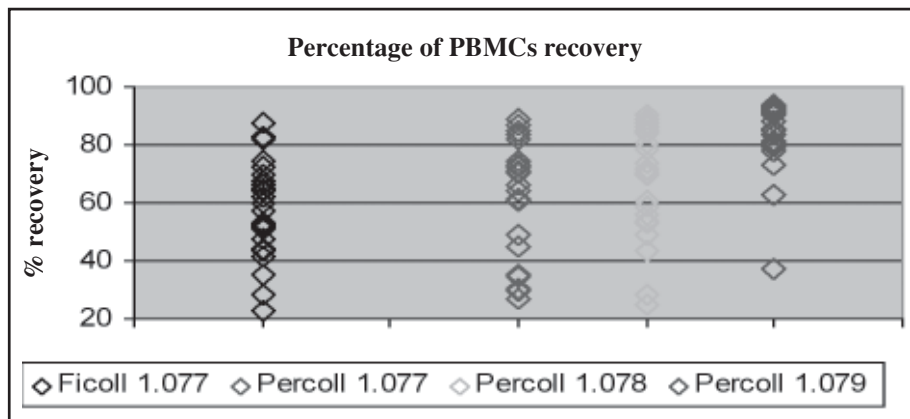
Fig. 3: Viability of PBMCs after cultivation for 5 days.

**PBMCs recovery rate**

Total white blood cells of Asian elephant were count by using automate machine. The averaged number of total white blood cells is 12.19 x 10<sup>3</sup> cells/ml and the averaged number of PBMCs is 8.47 x 10<sup>3</sup> cells/ml. For the averaged number of elephant PBMCs (n = 24) which obtained from density gradient centrifugation method by using Ficoll and Percoll are as following; 5.39 x 10<sup>3</sup> cells/ml when using Ficoll with density 1.077, 5.94 x 10<sup>3</sup> cells/ml when using Percoll with density 1.077, 6.82 x 10<sup>3</sup>

cells/ml when using Percoll with density 1.078, and 7.6 x 10<sup>3</sup> cells/ml when using Percoll with density 1.079.

Percentage of PBMCs recovery is shown that Percoll with each density trend to have PBMCs recovery rate higher than Ficoll (Figure4). Overall, Percoll with density 1.079 g/cm<sup>3</sup> showed significantly higher than Ficoll density 1.077 and Percoll density 1.077 by both P-value (P = 0.000), and Scheffe’s test (Table2).

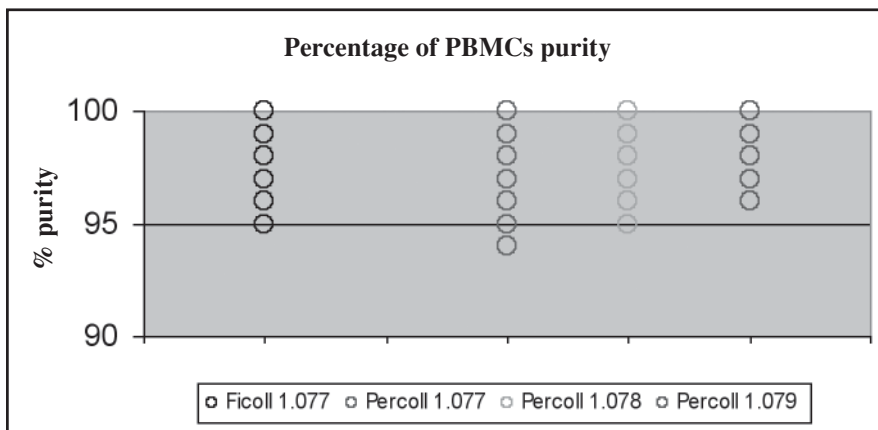


**Fig. 4:** PBMCs recovery rate calculating by divided the number of isolated PBMCs with the total of lymphocyte and monocyte in whole blood.

**PBMCs purity**

Percentage of PBMCs purity by white blood cells differential count methods is shown in figure5 and table2.

There was no significantly difference between Percoll and Ficoll by both P-value (P = 0.965), and Scheffe’s test (Table2).



**Fig. 5:** Percent PBMCs purity which were isolated by Ficoll or Percoll gradient density centrifugation.

**Table 2:** Data (n=12) of five parameters including TCB, PVB, PVA, PR and PP isolated by centrifugal method.

Parameters	Ficoll 1.077	Percoll 1.077	Percoll 1.078	Percoll 1.079	Pooled SEM	P-value
TCB	2.64 x10 <sup>7a</sup>	2.92 x10 <sup>7ab</sup>	3.21 x10 <sup>7ab</sup>	3.94 x10 <sup>7b</sup>	0.37 x10 <sup>7</sup>	0.026 <sup>s</sup>
PVB	87.19% <sup>a</sup>	87.87% <sup>a</sup>	88.07% <sup>a</sup>	90.91% <sup>a</sup>	1.37	0.046 <sup>s</sup>
PVA	80.71% <sup>a</sup>	82.32% <sup>a</sup>	82.36% <sup>a</sup>	85.64% <sup>a</sup>	2.68	0.243
PR	57.77% <sup>a</sup>	63.28% <sup>a</sup>	69.42 <sup>ab</sup>	82.61% <sup>b</sup>	5.19	0.000 *
PP	98.63 <sup>a</sup>	98.42 <sup>a</sup>	98.58 <sup>a</sup>	98.63 <sup>a</sup>	0.468	0.965

<sup>abc</sup>Values in the same row with different superscript differ significantly (P<0.05,Schffe’s test), Pooled SEM = pooled standard error of the mean

The average percentage of lymphocytes and monocytes in PBMCs were also investigated. The result shown that Ficoll with density 1.077 yielded 90.8% lymphocyte and 7.8% monocyte, Percoll with density 1.077 yielded 90.68% lymphocyte and 7.52% monocyte, Percoll with density 1.078 yielded 87.35% lymphocyte and 7.88% monocyte, and Percoll with density 1.079 g/cm<sup>3</sup> yielded 90.28% lymphocyte and 8.32% monocyte respectively (n = 24)

### Discussions

In human, for granulocytes isolation, it has been recommended that Percoll with density 1.090 g/cm<sup>3</sup> is the most suitable one, and for the mononuclear cell (MNC) fraction, a density of 1.077 g/cm<sup>3</sup> (Harbeck and Hoffman 1982). However, the density of elephant PBMCs has not been reported. The density gradient centrifugation depends on the diameter of the particle. Therefore, the elephant PBMCs which are larger than those of human, Percoll with density equal or more than 1.077 g/cm<sup>3</sup> should be used. A preliminary study showed that when using Percoll with density 1.080 and 1.090 g/cm<sup>3</sup> a contamination of red blood cells (RBC) was found due to too high density which could not separate PBMCs from RBC. In addition, Percoll with density 1.070 g/cm<sup>3</sup> yielded less PBMCs. Therefore, we designed to use Percoll with density 1.077, 1.078, and 1.079 g/cm<sup>3</sup> and compare with commercial Ficoll with density 1.077 g/cm<sup>3</sup>.

A data analysis suggested that there has a confounding between gender and types of treatment in these parameters; total PBMCs before cultivation (TCB), percentage of viability before cultivation (PVB), total PBMCs after cultivation (TCA), percentage of viability after cultivation (PVA), percentage of recovery (PR) and percentage of purity (PP). Therefore, the statistic analysis of the parameters, gender confounding had to be removed in order to study the exact effect of the treatments.

However, after 5 days of cultivation, the total quantity of PBMCs and PBMCs viability was count maybe decrease because some of monocyte adhesion in flask so that we should flood flask by cool PBS

The result of total PBMCs isolated in this study showed that Percoll with a density of 1.079 g/cm<sup>3</sup> gave the highest number of PBMCs ( $3.94 \times 10^7 \pm 3.72 \times 10^6$  cells/cm<sup>3</sup>), which related to the size of elephant PBMCs .

Percentage of viability was used to determine the effect of substance solution to PBMCs. There was no

significantly difference between 4 treatments; this may be due to the isotonic property of the solution that not affects the cell osmolarity. The high percentage of viability ( $87.19 \pm 1.37$ ,  $87.87 \pm 1.37$ ,  $88.07 \pm 1.37$ ,  $90.91 \pm 1.37$  respectively) in this study may related to the previous study that shown high viability in human with Percoll (Gmelig-Meyling and Waldmann 1980) and Ficoll (Vissers and Jester 1988). In addition, there was also no significantly difference in the percentage of viability of the separated PBMCs after 5 days of cultivation.

According to the percentage of recovery was calculated by the number of isolated PBMCs divided by total PBMCs from whole blood. Thus, the percentage of recovery depends on the total cells before culture. There was significantly difference between Percoll with density 1.079 g/cm<sup>3</sup> density and Percoll with density 1.077 g/cm<sup>3</sup> density and Ficoll with density 1.077 g/cm<sup>3</sup>. The present study, low percentage of recovery of Ficoll with density 1.077 g/cm<sup>3</sup> and Percoll with density 1.077 and 1.078 g/cm<sup>3</sup> density were found ( $57.77 \pm 5.19\%$ ,  $63.28 \pm 5.19\%$ ,  $69.42 \pm 5.19\%$  respectively), this may be due to the low total PBMCs. However, most of the previous studies in human, reported that the percentage of recovery was greater than 80% (Ferrante and Thong 1978).

In previous study on bovine, high purity of monocytes and lymphocytes isolated by Percoll density gradient isolation method (Soltys and Swain 1999), and also the study in horse, using Percoll showed less granulocytes contamination when compared with Ficoll (Bruyninckx and Blancquaert 1983). In the present observation, there was no significantly difference between 4 treatments in the cell purity. Moreover, there were red blood cells and granulocytes contamination in PBMCs isolation. In currently reported (Soltys and Swain 1999), RBC contamination could be removed by using RBC lysis buffer. This buffer contains ammonium chloride, which lyses red cells with minimal effect on lymphocyte and monocyte. Anyhow, both of the above researches used more than accurate method, such as flow cytometer, whereas, in this study, Macmaster chamber was used so there could be more error.

For further studies, several useful methods for investigation the purity of PBMCs such as flow cytometry, magnetic beads are needed in order to provide an accurate number of cells. In addition, comparing between overlay

with whole blood and buffy coat is needed when perform PBMCs isolation. A larger scale of animals should be performed in order to achieve more confidential level and to confirm such correlation among these parameters.

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