Isolation techniques of neutrophils and peripheral blood mononuclear cells for the comparative experiments in humans and pigs model organisms in flow cytometry

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Abstract: The pig is due to the morfology of their bodies often used as a model organism from the experimental organisms for comparison with man. The aim of this study ist o determine which isolation technique can be used for comparative imunological experiments focused on human and porcine neutrophils and monocytes . In comparative studies, it is necessary that both cells compared species were isolated by the same technique. Selected isolation techniques are the first step for subsequent detection of selected parameters in flow cytometry and for cultivation of peripheral blood mononuclear cells to profit monocyte-derivated macrophages. For isolation of neutrophils were chosen these techniques: isolation of double density gradient centrifugation Histopaque 11191 a 1077, isolation of double density gradient centrifugation Histopaque 11191 a 1077 after dextran and only dextran sedimentation. Monocytes were isolated by density gradient centrifugation Histopaque 1077, density gradient centrifugation Histopaque 1077 with immunomagnetic separation of $CD14^+$ cells, double density gradient centrifugation Histopaque 11191 a 1077 and double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation. For the isolation of neutrophils is the best tested technique dextran sedimentation. For the isolation of monocytes from peripheral blood show the best results density gradient centrifugation Histopaque 1077. Techniques were evaluated by purity and yield isolated cells during comparison both organisms.

Key-Words: dextran sedimentation, density gradient isolation techniques, clearance, yield, $CD14^+$

Introduction

Flow cytometry is a highly sophisticated method capable of sorting cells according to their size and granularity and subsequently selected by marking of selected cells surface receptors. Flow cytometry are often analyzed blood samples. Therefore, the work addresses the isolation of neutrophils and monocytes from peripheral blood. Due to the fact that the pig is often used as a model organism for comparative studies of human medicine is this work aimed at two organisms - human and pig.

The aim was to choose the technique that will be most suitable for isolating cells of the immune system needs further study in the analysis of flow cytometry. As a cells of interest were chosen human and porcine neutrophils and monocytes. This thesis is focused on the comparison of isolation techniques in terms of purity and yield of the isolated cells. Whereas in comparative studies must follow the same procedure for isolating cells from both species were the most successful methods chosen which showed high purity and yield in both species.

Material and Methods

For isolation of neutrophils and monocytes in pigs has been used 10 healthy pigs stabled in the experimental barn in Veterinary Research Institute in Brno. Pigs were fed a standard diet. Peripheral blood was collected them in the morning, from *vena cava cranialis*. Human neutrophils and monocytes were isolated from peripheral blood of 10 healthy individuals. Cells were isolated from heparin-anticoagulated blood.

For isolation of neutrophils were chosen these techniques: isolation of double density gradient centrifugation Histopaque 11191 a 1077 (sterile-

filtered, denstity 1.119 g/mL and 1.007 g/mL, Sigma-Aldrich, USA), isolation of double density gradient centrifugation Histopaque 11191 a 1077 after dextran (non-pyrogenic, MP biomedicals, France) and only dextran sedimentation.

Isolation technique of monocytes is unknown. Therefore this thesis is focused on isolation techniques of peripheral blood mononuclear cells (PBMC). Monocytes will be selected in the next step. These techniques were chosen: density gradient centrifugation Histopaque 1077, density gradient centrifugation Histopaque 1077 with immunomagnetic separation of $CD14^+$ cells, double density gradient centrifugation Histopaque 11191 a 1077 and double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation. It is assumed that obtained monocytes isolates will be cultured to macrophages. During cultivation, the exchange of media leads to wash lymphocytes. Monocytes differentiate into macrophages and due to adherence are not washed during exchange of media. All techniques were performed in standard contidions according to data sheets. Cells isolations were performed in sterile box (S@FEFLOW 1.8, EuroClone, 2012, Italy). Results were statistically evaluated by pair t-test.

Table 1 Isolation of neutrophils (significant differences are marked by asterisks *$P < 0.05$, **$P < 0.01$)

	* 10^6 cells in 10 ml periferal blood	* 10^6 neutrophils in 10 ml periferal blood	% of neutrophils from isolate	* 10^6 cells in 10 ml periferal blood	* 10^6 neutrophils in 10 ml periferal blood	% of neutrophils from isolate
Isolation technique	human	human	human	pig	pig	pig
Histopaque 11191 and 1077	7.48**	0.58**	7.8**	10.38	0.16	1.6
Dextran sed. + Histopaque 11191 and 1077	0.2	0.0002	0.1	9.1	0.03	0.4
Dextran sedimentation	17.41**	0.48**	2.8**	89.46**	1.07*	1.2

Table 2 Isolation of monocytes (significant differences are marked by asterisks *$P < 0.05$, **$P < 0.01$)

	* 10^6 cells in 10 ml periferal blood	* 10^6 monocytes in 10 ml periferal blood	% of monocytes from isolate	* 10^6 cells in 10 ml periferal blood	* 10^6 monocytes in 10 ml periferal blood	% of monocytes from isolate
Isolation technique	human	human	human	pig	pig	pig
Histopaque 1077	20.48*	0.38	1.9	55.29**	0.11	0.2
Histopaque 11191 and 1077	11.6	0.09	0.8	62.4**	0.18	0.3
Dextran sedimentation + Histopaque 11191 and 1077	4.2	0.07	1.7	8.85	0.03	0.4

Results and Discussion

Techniques of isolation of neutrophils

Research laboratories worldwide most frequently employ linear or discontinuous gradients of serum albumin, Percoll, Ficoll, dextran, Ficoll-Hypaque, Mono-Poly Resolving Medium, dextran/Ficoll, or gelatin, to isolate human peripheral blood neutrophils [1, 3, 5, 7, 8]. Most commonly is used dextran sedimentation, Ficoll-Hypaque centrifugation and hypotonic lysis of residual erythrocytes [1, 3, 7, 8]. Porcine neutrophils are isolated by dextran sedimentation followed by further erythrocyte purification by resuspending the pellet in ice cold and use Histopaque 1083[5]. This study evaluates the selected techniques in detail and compared. In this study, we have demonstrated that the double density gradient centrifugation Histopaque 11191 a 1077 achieves the best scores in humans. Of all the techniques give the highest yield and the highest purity of isolated (Tab. 1, Fig. 1). Almost the same yied achieved in the dextran sedimentation (Tab. 1, Fig. 5). Inappropriate technique for the isolation of human neutrophils is isolation of double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation (Tab. 1, Fig. 3). The cells show a very low yield and purity. For isolation of porcine neutrophils is the best technique dextran sedimentation. This technique has got good purity and yield too (Tab. 1, Fig. 6). Although isolation of neutrophils by double density gradient centrifugation Histopaque 11191 a 1077 has the best purity but yield is very low (Tab. 1, Fig. 2). Inappropriate technique for the isolation of porcine neutrophils is isolation of double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation (Tab. 1, Fig. 4).

Fig. 1 Histopaque 11191 and 1077

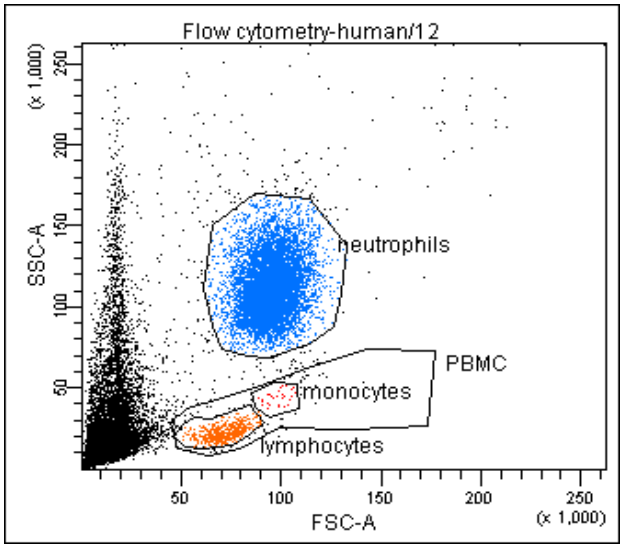


Fig. 2 Histopaque 11191 and 1077

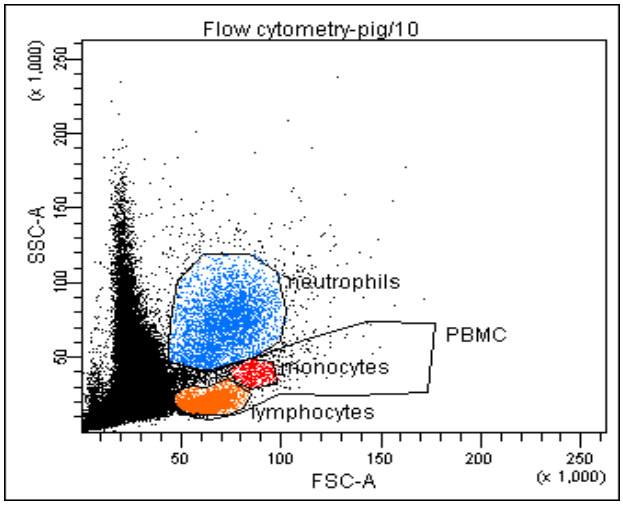


Fig. 3 Histopaque 11191 and 1077 after dextran sedimentation

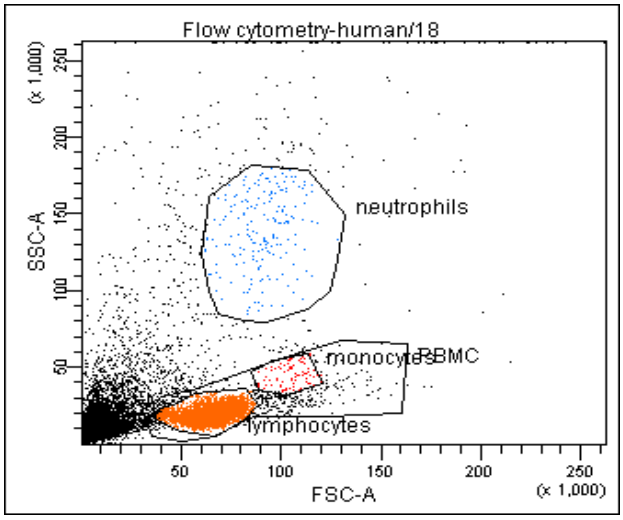


Fig. 4 Histopaque 11191 and 1077 after dextran sedimentation

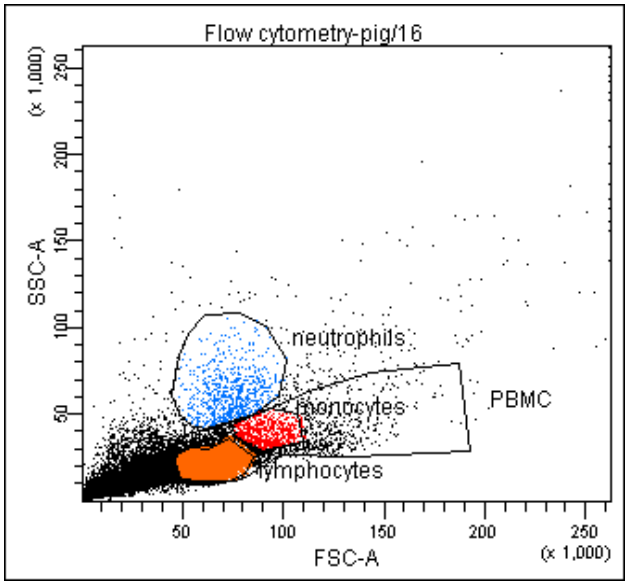


Fig. 5 Dextran sedimentation

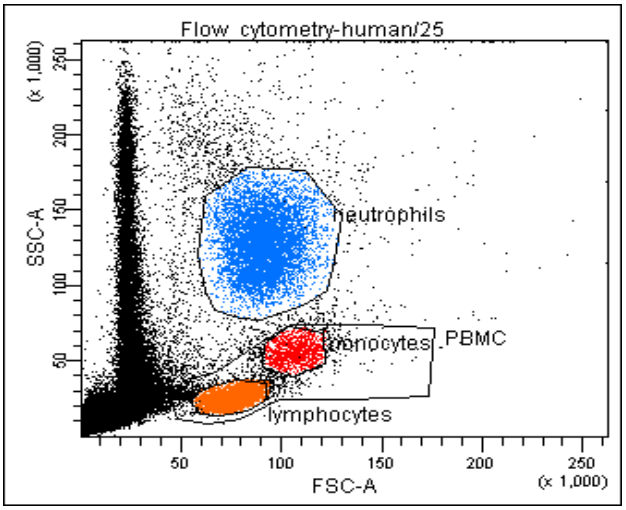
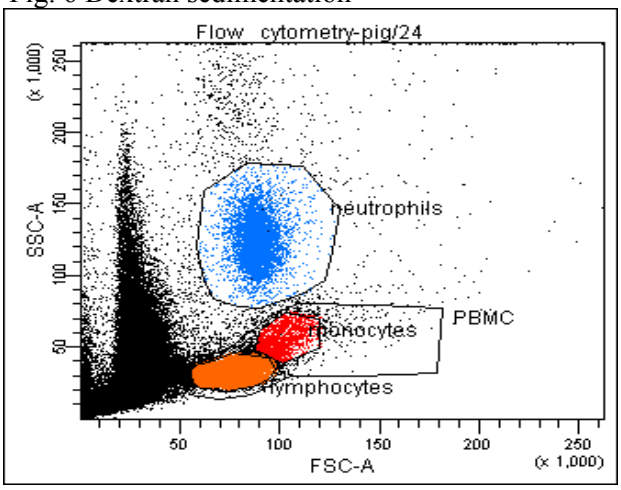


Fig. 6 Dextran sedimentation



Techniques for isolation of monocytes

Many different techniques can be used for monocyte isolation. For example double gradient centrifugation [4], dextran sedimentation and Ficoll-Hipaque density gradient centrifugation [8] or density gradient centrifugation with and without further purification by plastic adherence or immunomagnetic separation of $CD14^+$ cells [2]. This study compares these methods and evaluates their use in comparative studies of human and porcine monocytes. In the isolation of monocytes from human peripheral blood achieves the best scores density gradient centrifugation Histopaque 1077 (Tab. 2, Fig. 7). It shows also the highest yield and purity of the isolated cells best of all the tested methods. Good purity is showed by double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation too, but the method has got low yield of monocytes (Tab. 2, Fig. 11). Inappropriate technique for the isolation of human monocytes is double density gradient centrifugation Histopaque 11191 a 1077 (Tab. 2, Fig. 9). But this method is the best for isolation of porcine monocytes (Tab. 2, Fig. 10). Good purity but low yield of porcine monocytes is achieved by double density gradient centrifugation Histopaque 11191 a 1077 after dextran sedimentation (Tab. 2, Fig. 12). Good yield but lowest purity is achieved by density gradient centrifugation Histopaque 1077 (Tab. 2, Fig. 8). Last test methods for the isolation of peripheral blood monocytes is a technique of density gradient centrifugation Histopaque 1077 which is followed by immunomagnetic separation of $CD14^+$ cells which is typical for monocytes. As the Fig. 13 shows in human and Fig. 14 show in porcine monocytes, this isolation techniques shows hight impurity of lymphocytes in comparison with other techniques and it doesn´t bring better results. Difficulty of techniques and cost per sample are higher than in other techniques and therefore no advantages over other techniques of isolation.

Fig. 7 Histopaque 1077

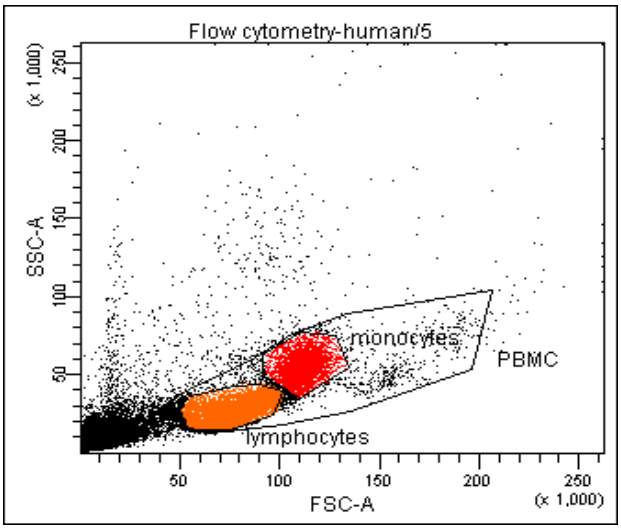


Fig. 8 Histopaque 1077

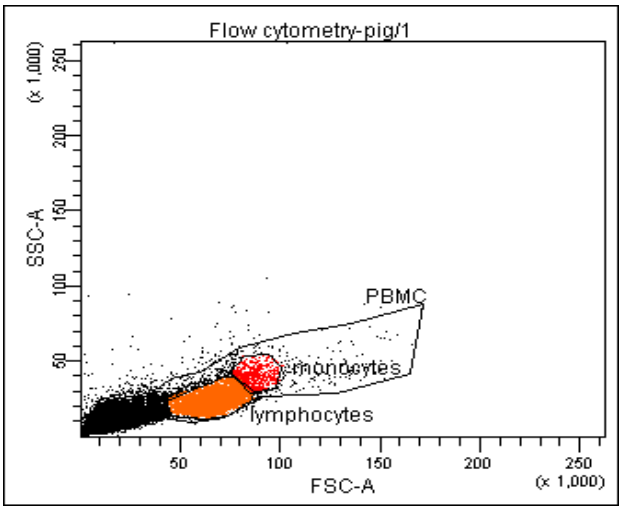


Fig. 9 Histopaque 11191 and 1077

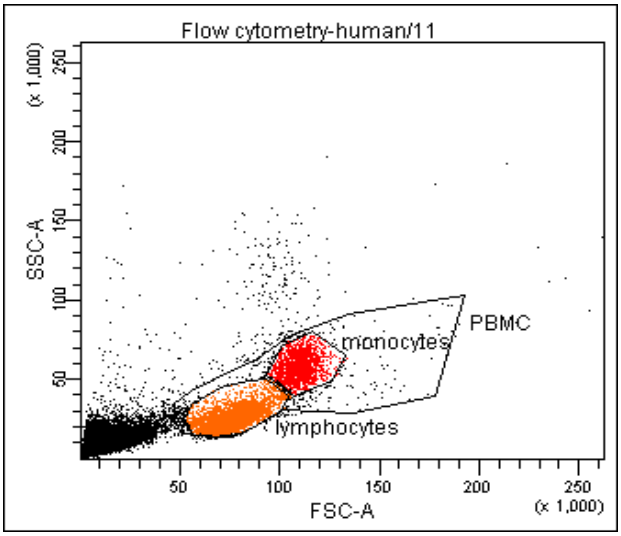


Fig. 10 Histopaque 11191 and 1077

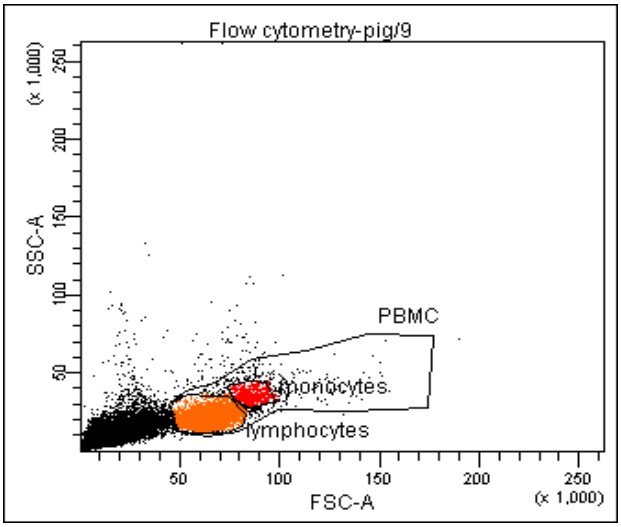


Fig.11 Histopaque 11191 and 1077 after dextran sedimentation

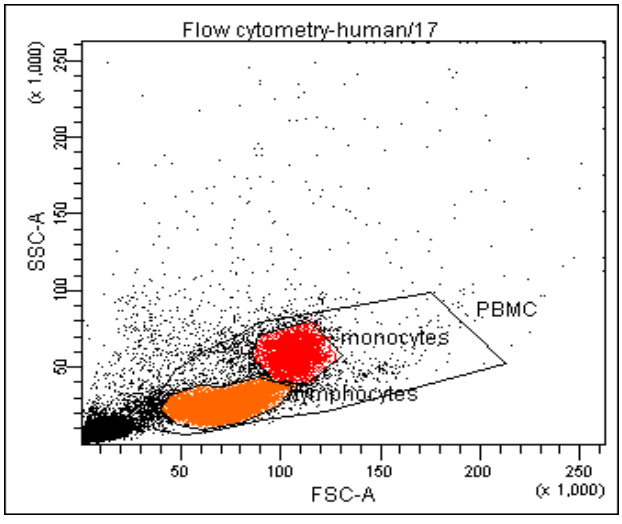


Fig. 12 Histopaque 11191 and 1077 after dextran sedimentation

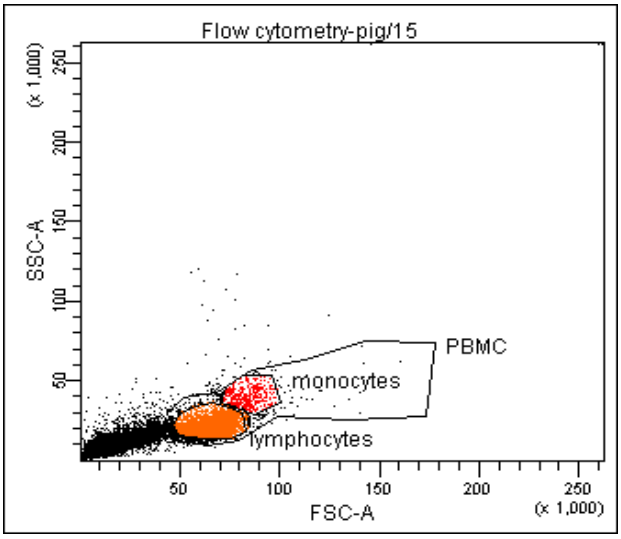


Fig. 13 immunomagnetic separation od CD14+

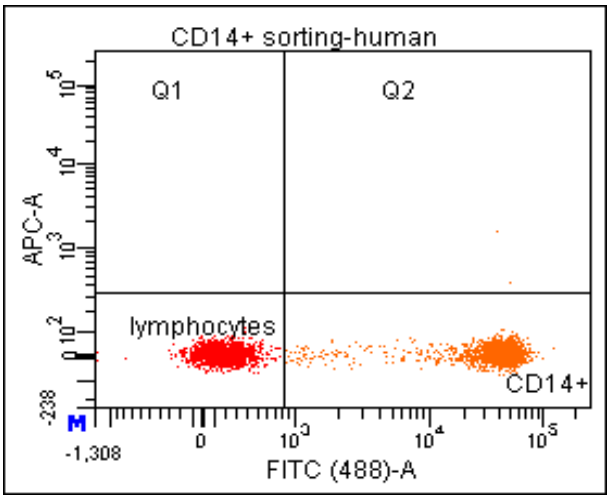
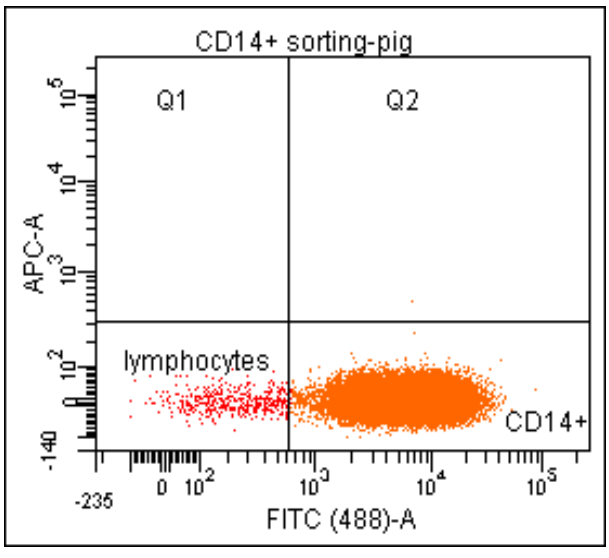


Fig. 14 Immunomagnetic separation of CD14+



Conclusion

The best way to obtain neutrophils from porcine blood is the dextran sedimentation technique. In human this technique shows almost identical neutrophil yield as the double density gradient centrifugation.

Comparing the yield and purity of the compared isolation techniques is the most appropriate technique density gradient centrifugation Histopaque 1077. This is technically simple test method isolating peripheral blood monocytes and gives satisfactory results in humans and pigs.

Acknowledgement

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References:

[1] Arduino RC, Jaques-Palaz K, Murray BE, Rakita, Resistance of enterococcus faecium to Neutrophil-Mediated Phagocytosis, *Infection and Immunity*, 1994, Vol. 62, No. 12, pp. 5587-5594.

[2] Bernhardt A, Schumacher M, Gelinsky M, Formation of Osteoclasts on Calcium Phosphate Bone Cements and Polystyrene Depends on Monocyte Isolation Conditions, *Tissue Engineering Part C: Methods. -Not available-, ahead of print.* doi:10.1089/ten.tec.2014.0187.

[3] Herrera-Insúa I, Jacques-Palaz K, Murray BE, Rakita RM, The effect of antibiotic exposure on adherence to neutrophils of Enterococcus faecium resistant to phagocytosis, *Journal of Chemotherapy,*Vol. 39, 1997, pp. 109-113.

[4] Menck K, Behme D, Pantke M, Reiling N, Binder C, Pukrop T, Klemm F, Isolation of Human Monocytes by Double Gradient Centrifugation and Their Differentiation to macrophages in Teflon-coated Cell Culture Bags, *Journal of Visualized Experiments,*2014, in press

[5] Paoliello-Paschoalato AB, et al., Isolation of healthy individuals and rheumatoid arthritis patients, *Journal of Immunological Methods,* 2014, in press, http://dx.doi.org/10,1016/j.jim.2014.07.001

[6] Pieper Ch, Galla HJ, Ultra structure analysis of cell-well interactions between pericytes and neutrophils in vitro, *Biochemical and Biophysical Research Communications*, 2014, No. 445, pp. 180-183.

[7] Rakita RM, Quan VC, Jacquez-Palaz K, Kavindra V, Singh KV, Arduino RC, Mee M, Murray BM, Specific antibody promotes opsonization and PMN-mediated killing of phagocytosis-resistant Enterococcus faecium, *Immunology and Medical Microbioogy*, Vol. 28, 2000, pp. 291-299.

[8] Rakita RM, Vanek NN, Jacqoues-Palaz K, Mee M, Mariscalco MM, Dunny GM, Snuggs M, Van Winkle WB, Simon S, Enterococcus faecalis Bearing Aggregation Substance Is Resistant to Killing by Human Neutrophils despite Phagocytosis and Neutrophil Activation, *Infection and Immunity,* Vol.67, No.11, 1999, pp. 6067-6075.