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An Improved Method of Neutrophil Isolation in Peripheral Blood of Sheep

[1]Ali Rezapour and [2]Jaafar Majidi
[1]Department of Animal Science, Faculty of Agriculture,
Islamic Azad University, Tabriz Branch, Tabriz, Iran
[2]Center of Research in Infectious Disease, Medical University of Tabriz, Tabriz, Iran

Abstract: Studies on functions of ovine granulocytes require pure and functionally active population of neutrophils. Because of lymphocyte contamination, common methods of sheep neutrophil isolation from peripheral blood sample do not result in an acceptable degree of purified live cells. So, based on neutrophil characteristics of sheep this study proposed a new and easy method of neutrophil isolation using Meglumine compound with the least deformity, high purity (79.5%) and high viability (>97.5%) of isolated cells.

Key words: Meglumine, neutrophil, sheep, high purity, isolated cells, ovine granulocytes

INTRODUCTION

There are two main methods of neutrophil separation from peripheral blood sample, which are Red Blood Cell (RBC) lysis and density gradient medium techniques (Chanarian, 1989; Gooi and Chapel, 1990; Boyum, 1993; Sabroe *et al*., 2004). The idea of RBC lysis method is based on separating plasma and Buffy coat from peripheral blood sample after which the remaining blood consists of RBCs and neutrophils. A hypotonic lysis eliminates RBCs contamination and leaves polymorphonuclear cells. In density gradient methods blood cells arrange according to their density. Each medium with a specific density is considered as a selective barrier for every type of these cells. If the density of a cell is higher than medium, it will penetrate (even will pass) the medium during centrifuging. On the other hand, if the density of cells is supposed to be less than medium they will not penetrate and gather on upper part of centrifuge tube. Histopaque 1077 (1.077 g mL^{-1}) and Histopaque 1119 (1.119 g mL^{-1}) are 2 popular density gradient for cell isolation in human blood sample. In Fig. 1. Approximate distributions of density of human blood cells are illustrated. Density of Mononuclear Cells (MNC) is lower than RBCs and granulocytes (polymorphonuclear cells, PMNs). A medium with a density between 2 types of cells (e.g., MNC and granulocyte) can separate them in different layers-with a little overlap between layers (Boyum *et al*., 1991). Combination of these 2 general methods can lead in a higher purity of live neutrophil cells.

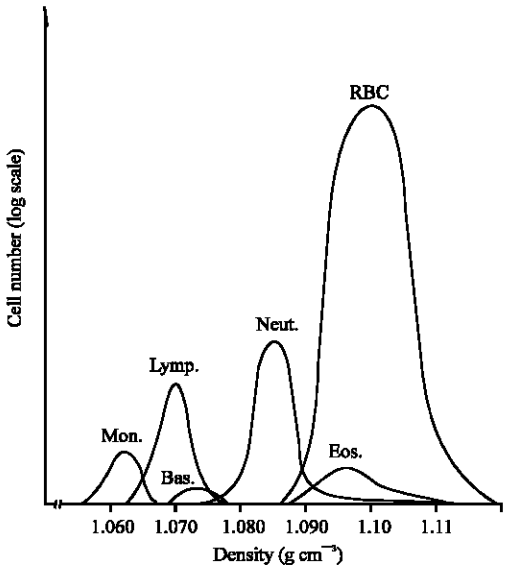

Fig. 1: Approximate distribution of human blood cells (Boyum *et al*., 1991)

Sheep have smaller and denser RBCs than human (Feldman *et al*., 2000). So, human Histopaques wouldn't work well in ovine samples. RBCs and lymphocytes (small type) pass the media and sediment in button of tube which contaminate neutrophils and decrease purity of separated cells considerably. There are some economic considerations about Histopaque, too. Histopaque made by Sigma Company costs much more than a similar substance we have used as a substitute.

Corresponding Author: Ali Rezapour, Department of Animal Science, Faculty of Agriculture, Islamic Azad University, Tabriz Branch, Tabriz, Iran

High purity of isolated cells, together with low price and easiness of the procedure are three main preferences of using Meglumine compound insisted of Histopaque.

MATERIALS AND METHODS

Meglumine compound 76%: It consists of Meglumine diatrizoate 66% and Sodium diatrizoate 10%. This compound is the most commonly used water-soluble, iodinated, radiopaque x-ray contrast medium. Physical characteristics of Meglumine compound are given in Table 1.

Determination of optimum concentration: To determine an optimum concentration of Meglumine compound 76% for neutrophil separation in peripheral blood of sheep, different levels of this substance were used (i.e., 100, 75 and 50%)-assuming Meglumine as 100%. NaCl 0.85% was used as a diluents. Blood cells couldn't pass a medium so, more diluted concentrations were examined (i.e., 40, 35, 30, 25, 20 and 15%). Meglumine in concentration of 25% (1: 3 ratios) was desirable.

Cell separation: Heparinized blood sample (10 μ mL^{-1}) taken from jugular vein immediately transferred to laboratory. The blood sample was diluted with the same volume of NaCl 0.85%. In a Falcon tube containing 4 mL of Meglumine 25%, 5 mL of diluted blood loaded slowly. Two phases of blood and Meglumine should not be mixed. The sample centrifuged (swinging-bucket) for 15 min in 250 × g (relative centrifugal force). As a result, some RBCs and most of granulocytes sediment in button of tube and make a compact population of cells which relatively stick to the tube wall (Fig. 2). Supernatant fluid was expelled. 0.5 mL of phosphate buffer saline was added and the sediment was homogenised by slow pippetting. Twice hypotonic lysis was exerted by distilled water and NaCl 2x (2.55%) each for 25 seconds. Cells were centrifuged for 5 min in 200 × g. Supernatant fluid was expelled and the sediment homogenised and washed once with NaCl 0.85%. All of experiments were done in laboratory temperature with sterile tools (Fig. 3).

Evaluation of viability: A haemocytometer-based trypan blue dye exclusion cell quantitation and viability assay was performed as to Brousseau *et al.* (1999). Briefly, blood was diluted with 0.1% dye in a 1: 10 ratio. Living cells exclude the dye, whereas dead cells will take up the blue dye. The blue stain is easily visible and cells can be counted using a light microscope.

Morphologic evaluation: Several smears were prepared from total diluted blood and isolated cell suspension

Table 1: Physical characteristics Meglumine compound 76%

Meglumine compound 76%	20 mL amp.
Iodine concentration (mg mL^{-1})	370.00
Viscosity in 20°C	18.50
Viscosity in 37°C	08.90
Osmolality in 37°C	02.10

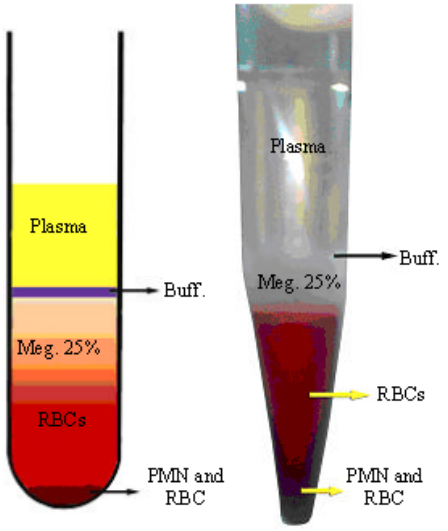

Fig. 2: Schematic view of neutrophil separation. Different layers after centrifuging orderly: A clear layer of plasma, Buffy coat, combination of RBCs and Meglumine, PMNs and RBCs (a). A real picture of neutrophil separation by Meglumine (b)

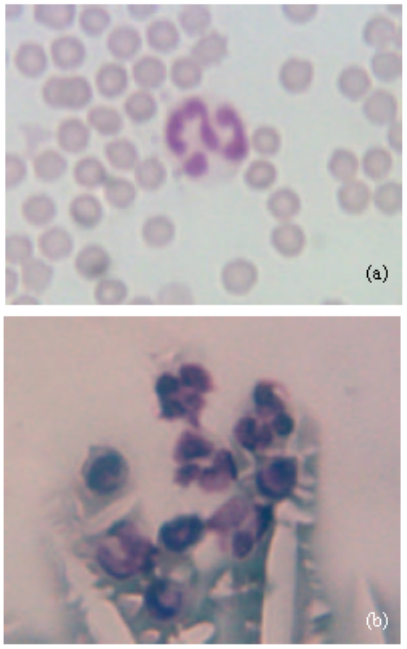

Fig. 3: Comparative photographs (10×100) of a neutrophil in total blood (a) and isolated neutrophil cells (b)

(Giemsa staining). Cells of every smear were evaluated considering size, transparency, normality of cytoplasm and plasma membrane and nuclear morphologic changes.

Yeast phagocytosis test: Yeast phagocytosis test was performed in both types of samples (total blood, cell suspension). A modified combination of protocol of phagocytosis was used (Gooi and Chapel, 1990; Brousseau *et al*., 1999). Briefly, fresh broth culture (brain heart infusion broth) of *Candida albicans* washed twice with Ringer serum solution and centrifuged 5 min in 200 × g to make a yeast pellet. Fresh plasma of sheep added and incubated for half an hour in 37°C. Equal volume of incubated yeasts and sample incubated for an hour. Samples were washed once (5 min in 200 × g). The pellet of cells homogenised and several smear prepared and stained with Giemsa staining method. Mean percent of neutrophils which phagocytosed was counted.

Statistic analysis: All numeric data were analysed by SPSS 12.0 software through pair 2 sample t-test.

RESULTS AND DISCUSSION

Relative percentage of isolated cells, percent of vital neutrophils, morphologic considerations and response to phagocytosis test in both samples is shown in Table 2. The difference between mean percent of neutrophils and eosinophils of Meglumine method (granulocyte isolation) were significantly higher than original total blood sample ($p<0.001$). But in contrast, mean percent of lymphocytes and monocytes of Meglumine method were significantly lower than original total blood sample ($p<0.001$). There was no significant difference in viability and yeast phagocytosis capacity between cells of 2 groups. Different lysis for elimination of RBCs resulted in low viability and functionality of isolated cells. So, we conducted one-time-RBC-lysis method.

Woldehiwet *et al.* (2003) described an improved technique for separation of ovine neutrophils and eosinophils from peripheral blood. They recommended centrifuging blood for 20 min at 400 × g. After discarding supernatant, a pellet of cells lysed 2 times hypotonically for 20 sec. The resultant leukocyte preparation layered on Percoll (density 1.131 g mL^{-1}). After centrifuging blood for 20 min at 400×g, ovine neutrophils were separated. So, total time needed for neutrophil isolation is minimally 45 times. The total time of neutrophil separation in our method is 20 min. It must be emphasized that long period of centrifuging can dehydrate cells and change general morphologic parameters because RBC dehydration occurs when ions and water flow out of the red cell, causing the cellular volume to decrease (Stocker *et al*., 2003).

Sartorelli *et al.* (1999) used method of Carlson and Kaneko (1973) for neutrophil separation in sheep. This method is generally accepted as it was used by several researchers (Wellnitz *et al*., 2006; Smitsa *et al*., 1999). Briefly, 40 mL of blood (containing sufficient amount of an anticoagulant) was centrifuged (for 15 min at 1000 × g); plasma, buffy coat and the upper layer of red blood cells were discarded. The remaining red blood cells, including sedimented PMNs, were lysed with distilled water and isotonicity was regained by adding a hypertonic solution (NaCl 0.39 mol L^{-1}). Two hypotonic lyses were carried out. After centrifugation (for 10 min at 200 × g), the resulting pellet was resuspended and washed twice with PBS. A method described by Roth and Kaeberle (1981) was a modification of this method. Kremer *et al*. (1992) also suggested a modified protocol for neutrophil isolation. As Carlson and Kaneko's method (1973) was their basic idea of neutrophil isolation, again a centrifugation for 20 min at 1000 × g and twice hypotonic lysis, with twice washing was performed. At the end a suspension was loaded on Percoll and centrifuged for 20 min at 400 × g. Twice washing of pelleted cell finalized the method. Being optimist, the overall duration of this method longs minimally 1 h, with 8 times centrifugation (5 times in our method). Kremer *et al*. (1992) used acid citrate dextrose but we used heparin because in contrast

Table 2: Haematological parameters in total blood and isolated neutrophils (from the same blood sample) before and after isolation procedure

	Total blood	Isolated neutrophils	p-value
WBC μL^{-1}	4830	2610 (variable)	
Neutrophil μL^{-1}	941.85(19.5%)	2074.95(79.5%)	$p<0.001$
Lymphocyte μL^{-1}	3332.70(69%)	75.69(2.9%)	$p<0.001$
Monocyte μL^{-1}	323.61(6.7%)	10.44(0.4%)	$p<0.001$
Eosinophil μL^{-1}	231.84(4.8%)	448.92(17.2%)	$p<0.001$
Viability (%)	99.5	97.5	None significant
Phagocytosis test (%)	97.8	96.9	None significant
Morphologic evaluation	Normal	Size of cells smaller. Nucleus denser. Clearness of segments lower	

to citrate, heparin does not interfere with phagocytosis test (Pfisrer *et al*., 1988; Victor *et al*., 1952).

Most of methods based on Carlson and Kaneko's method (1973) suggest centrifugation of blood for 20 min at 1000 × g. We found this vigorous centrifugation of blood pushes lots of lymphocyte to the button of tube and contaminate purity of isolated neutrophil cells. But according to our findings, dilution of blood with NaCl 0.85% (1:1) before first vigorous centrifugation disperse cells and no more lymphocyte cells pushed down and lymphocyte contamination is simply reduced.

Contamination of isolated cells with lymphocytes, vigorous centrifuging and long duration of isolation protocol are three disadvantages of mentioned methods.

CONCLUSION

We concluded that isolation of peripheral granulocytes by meglumine method lead in high purity, low deformity and acceptable viability of isolated cells. Lower price of this compound as a density gradient medium and lower time of the overall procedure (30 min) because of lower phases of overall method-were other preferences of using meglumine compound.

REFERENCES

Boyum, A., 1993. Blood and its Constituent Cells. In: Masseyeff, R.F., W.H. Albert and N.A. Staines, (Eds.). Methods of Immunological Analysis. John Wiley and Sons. 2nd Edn. 3: 22-38. ISBN: 978352-7279081. http://www.biggerbooks.com/bk_detail.aspx?isbn=9783527279081.

Boyum, A., D. Lovhaug, L. Tresland and E.M. Nordlie, 1991. Separation of leucocytes: Improved cell purity by fine adjustments of gradient medium density and osmolality. Scan. J. Immunol., 34: 697-712. DOI: 10.1111/j.1365-3083.1991.tb01594.x. http://www3.interscience.wiley.com/journal/119354117/abstract?CRETRY=1&SRETRY=0.

Brousseau, P., Y. Payette and H. Tryphonas, 1999. Manual of Immunological Methods. CRC Press. 1st Edn. pp: 20-45. ISBN: 978-0-8493-8558-2. http://www.biggerbooks.com/bk_detail.aspx?ISBN=9780849385582.

Chanarian, I., 1989. Laboratory Haematology (An Account of Laboratory Techniques). Churchill Livingstone. 1st Edn. pp: 177-178. ISBN: 0-443-23343-9. http:// www.amazon.com/gp/product/0443033439/sr=1-3/qid= 1221043667/ref=olp_product_details?ie=UTF8&me=&qid=1221043667&sr=1-3&seller.

Carlson, G.P. and J.J. Kaneko, 1973. Isolation of leukocytes from bovine peripheral blood. Proc. Soc. Exp. Biol. Med., 142: 853-856.

Feldman, B.F., J.G. Zinkl and N.C. Jain, 2000. Schalm's Veterinary Haematology. 5th Edn. Lippincott Williams and Wilkins, 166: 1075-1084. ISBN: 0-683-30692-8. DOI:636.089'615_dc21 (?). http://www.amazon.com/Schalms-Veterinary-Hematology-Bernard-Feldman/dp/0683306928/ref=sr_1_1?ie=UTF8&s=books&qid=1221310692&sr=1-1.

Gooi, H.G. and H. Chapel, 1990. Clinical Immunology (a Practical Approach). 1st Edn. Oxford University Press, pp: 51-80. ISBN: 0-19-963086-0. http://www.amazon.com/Clinical-Immunology-Practical-Approach/dp/0199630879/ref=sr_1_1?ie=UTF8&s=books&qid=1221310986&sr=1-1.

Kremer, W.D.J., E.N. Nordhuizen Stassen, P.A.J. Henricks and H. Vander Vliet, 1992. A procedure for parallel isolation of white blood cells, granulocyte and purified neutrophil suspensions from the peripheral blood of cattle. Vet. Immunol. Immunopathol., 31: 189-193. PMID: 1570679. http://www.ncbi.nlm.nih.gov/pubmed/1570679.

Pfisrer, R.R., J.L. Hoddox and T.L. Snyder, 1988. The effects of citrate on the adherence of neutrophils to nylon fibers *in vitro*. Invest. Ophthalmol. Vis. Sci., 29 (6): 869-875. PMID: 2836330. http://www.iovs.org/cgi/content/abstract/29/6/869.

Roth, J.A. and M.L. Kaeberle, 1981. Evaluation of bovine polymorphonuclear leukocyte function. Vet Immunol. Immunopathol., 2: 157-174. PMID: 7344267. http://www.ncbi.nlm.nih.gov/pubmed/7344267.

Sartorelli, P., S. Paltrinieri and F. Agnes, 1999. Non-specific immunity and ketone bodies. I: *In vitro* studies on chemotaxis and phagocytosis in ovine neutrophils. J. Vet. Med., A 46: 613-619. DOI: 10.1046/j.1439-0442.1999.00252.x. http://www3.interscience.wiley.com/journal/119077695/abstract. http://www.ncbi.nlm.nih.gov/pubmed/10638299.

Smitsa, E., C. Burvenicha, A.J. Guidryb, R. Heynemana and A. Massart-LeeÈna, 1999. Diapedesis across mammary epithelium reduces phagocytic and oxidative burst of bovine neutrophils. Vet. Immunol. Immunopathol., 68: 169-176. DOI: 10.1016/S0165-2427(99)00019-7. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6TD5-3WM5540-7&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=10&md5=31e3cd27e859f50adf7aca8dc7a3ddf0.

Stocker, J.W., L. De Franceschi, A. McNaughton-Smith, R. Corrocher, Y. Beuzard and C. Brugnara, 2003. ICA-17043, a novel Gardos channel blocker, prevents sickled red blood cell dehydration in vitro and *in vivo* in SAD mice. Blood, 101 (6): 2412-2418. DOI: 10.1182/blood-2002-05-1433. PMID: 12433690. http://bloodjournal.hematologylibrary.org/cgi/content/abstract/101/6/2412.

Victor, J., A.D. Pollack, R. Raymond and J.R. Valliant, 1952. Studies on phagocytosis (determination of blood opsonin *Brucella*), 64: 121-130. PMID: 14946108. http://www.ncbi.nlm.nih.gov/pubmed/14946108?ordinalpos=2&itool=EntrezSystem2.PEntrez.Pubmed.Pubmed_ResultsPanel.Pubmed_DefaultReportPanel.Pubmed_RVDocSum.

Wellnitz, O., P. Reith, S.C. Haas and H.H.D. Meyer, 2006. Immune relevant gene expression of mammary epithelial cells and their influence on leukocyte chemotaxis in response to different mastitis pathogens. Vet. Med., 51 (4): 125-132. http://www.vri.cz/docs/vetmed/51-4-125.pdf.

Woldehiwet, Z., H. Scaife, C.A. Hart and S.W. Edwards, 2003. Purification of Ovine Neutrophils and Eosinophils: *Anaplasma phagocytophilum* affects Neutrophil Density. J. Compara. Pathol., 128 (4): 277-282. DOI: 10.1053/jcpa. 2002.0633. http://www.sciencedirect.com/science?_ob=ArticleURL&_udi=B6WHW-48XBT1J-5&_user=10&_rdoc=1&_fmt=&_orig=search&_sort=d&view=c&_version=1&_urlVersion=0&_userid=10&md5=58c4e90fe7607ff1dbe1b6ce107c85fe.