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Polymorphonuclear leukocyte isolation from venous blood of the dogs*

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ABSTRACT

The current study aimed to isolate neutrophils from venous blood samples of healthy dogs. Venous blood samples were obtained from Venae cephalica of clinically healthy dogs (n:5) into heparinized tubes. The blood samples (2 mL) and Percoll dilutions (45%, 54%, 63%, and 72%) prepared with Hanks Balanced Salt Solution were layered into sterile tubes. After centrifuge, the polymorphonuclear leukocytes (PMN) were aspirated between 63% and 72% interfaces of the Percoll dilutions into tubes. The samples of PMN observed under a light microscope. Viability was detected microscopically after stained with trypan blue dye. Diff-Quick staining was used to detect neutrophil purity of the isolated PMN. In the present study, the neutrophils ratio was calculated as 92% of the isolated polymorphonuclear cells. The neutrophil viability was calculated as 98% of PMNs isolated from the venous blood samples of healthy dogs. In the present study, the Percoll gradient centrifugation (72%, 63%, 54%, and 45%) is a fast technic for isolation of the neutrophils from venous blood samples of dogs.

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Introduction

Polymorphonuclear leukocyte (PMN), granular leukocyte or granulocyte, is a type of white blood cell. The PMN has been commonly recognized histologically by their cytoplasmic appearance and nuclear shapes. The PMN consist of some granulocytic cells called neutrophils, eosinophils, and basophils. Most of the PMN are neutrophils, which ratio varies depending on the animal species. (Kaplan and Radic, 2012). Mammalian neutrophils possess multi-lobed nuclei (usually two and five lobes). The neutrophils produce from bone marrow (about 7 million per minute) and control by the homeostatic balance in the organism. The number of circulating neutrophils increases in some situations such as stress and infection (Rosales, 2018). The neutrophils can live 3-12 hours in the organism, which can be as long as 1-2 days (Kruger et al., 2015). Cytokines and bacterial products prolong the life span of neutrophils. If it does not enter the process of inflammation, neutrophils are phagocytosed by macrophages in the organism (Mayadas et al., 2014).

The innate immune system is a first defence mechanism that provides rapid protection after confronted with infectious microorganisms in the body (Kaplan and Radic, 2012). Neutrophils are one of the most important components of innate immunity. Following the pathogenic invasion, neutrophils respond

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to chemotactic stimuli and migrate to the infection area and use a variety of defence strategies including phagocytosis, degranulation and netosis to fight pathogens (Selders et al., 2017).

Canine neutrophils have been studied for different in vitro studies (Bosco et al. 2013; Sheats et al. 2015; Li et al. 2018). The purpose of this study was to isolate neutrophils from venous blood samples of healthy dogs. Although there are many neutrophil isolation methods, these methods do not apply to all animals (Oh et al., 2008; Marchi et al., 2013; Yildiz et al., 2017). In the present study, it was aimed to isolation of neutrophils from venous blood samples of healthy dogs by using discontinuous Percoll dilutions.

Material and methods

All experimental procedures have been approved by the Ethics Commission of Kırıkkale University (06.09.2017, no:17/32). Venous blood samples (5 mL) were obtained from Venae cephalica of clinically healthy dogs (n:5) into heparinized tubes. The methodology was a modified version of Sursal et al. (2018). Briefly, Percoll dilutions (45%, 54%, 63%, and 72%) were prepared with Hanks Balanced Salt Solution (Sigma). Then, two mL of each Percoll dilution starting from higher concentration carefully layered into a polystyrene centrifuge tube. The blood samples (2 mL) were layered on the lowest concentration of Percoll dilution (45%) in the tubes after diluted with PBS-EDTA (0.02%) (1:1). The tubes centrifuged at room temperature (500 x g, 35 min) (Thermo Scientific, SL 16R). Three cell layers were observed among the Percoll dilutions after the completed centrifuge step. The cell layer was aspirated between 63% and 72% interfaces into sterile tubes. The tubes centrifuged at + 4° C (300 x g, 10 minutes) and the supernatant was removed, then the PMN was diluted in RPMI-1640 (without phenol red, Sigma). To detect PMN numbers, the samples of PMN put on the Neubauer chamber and observed under a light microscope (Leica DM750).

The PMN samples were smeared on the slides. After dried on air and fixation with methyl alcohol, Diff -Quick staining (Bio Optica, Italy) was used to detect neutrophil purity of the isolated PMN. All polymorphonuclear cells were counted in ten microscopic areas randomly selected. Then, the ratio of neutrophils was compared to all polymorphonuclear cells counted. Trypan blue dye was used to detect the viability of isolated neutrophils. The PMN samples were stained with trypan blue dye (1:1) in the sterile microtubes. The cells stained with trypan blue dye were evaluated as died under the light microscope. To detect the neutrophil viability, all polymorphonuclear cells (stained and unstained) were counted in ten microscopic areas randomly selected. Then, the ratio of cells stained with trypan blue was compared to all polymorphonuclear cells counted. The PMN isolations were repeated three times.

Results

PMNs were detected between 63% and 72% interfaces of the Percoll dilutions after centrifuging the tubes. Figure 1 shows the PMNs of dogs in Neubauer chamber. After Diff-Quick staining, the neutrophils were easily observed as a dark blue lobulated nucleus and pale pink cytoplasm in the slides under the light microscope (Figure 2). The neutrophils ratio was calculated as 92% of the isolated polymorphonuclear cells. The neutrophil viability was calculated as 98% of PMNs isolated from the venous blood samples of healthy dogs.

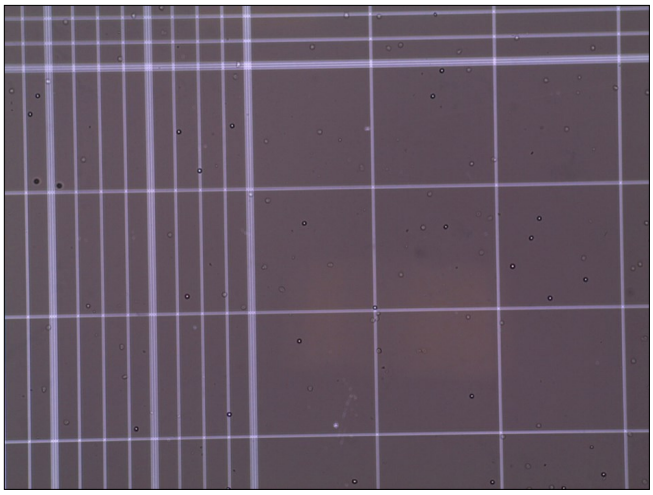

Figure 1. Light microscopic observation of the polymorphonuclear leucocytes of the dogs in the Neubauer chamber. X20.

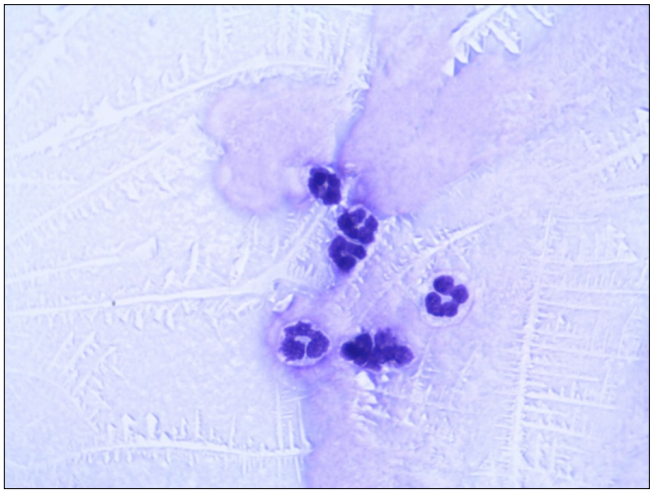

Figure 2. The light microscopic view of the isolated neutrophils with a dark blue lobulated nucleus and pale pink cytoplasm. Diff-Quick staining, x100.

Discussion

Polymorphonuclear leucocyte population consists mainly of neutrophils (Kaplan and Radic, 2012; Patel and Chatterjee, 2018). Neutrophils play an important role in maintaining innate host defence against pathogens in dogs. In general, in vitro experiments design to understand neutrophil functions during the defence to infectious microorganisms in the organism (Gosset et al., 1983; Yildiz et al., 2017; Lawson et al., 2018; Yildiz et al., 2019). Neutrophil separation can be accomplished by centrifugation with different density gradient solutions. Some of them include Percoll, Biocoll, Ficoll-Hypaque, and Sucrose Polymerdiatrizoate gradient are employed for the cell separation. There are some protocols are used for neutrophils isolation from dogs venous blood samples (Sano et al. 2004; Jeffery et al. 2016; Wei et al. 2016). Some of them are dextran sedimentation and Ficoll-Hypaque (Sano et al. 2004; Jeffery et al. 2016). Some authors prefer a commercially available canine PMN isolation kit to obtain PMN from blood samples of dogs (Wei et al.2016). Percoll, colloidal silica particles coated with polyvinylpyrrolidone, is a gradient medium for separation of cells, subcellular particles and even viruses. Different Percoll dilutions are used for isolation of PMN from venous blood samples of different animals and human (Mosca and Forte, 2016; Cools-Lartigue et al. 2013; Swamydas et al. 2015; Sursal et al. 2018). Feline neutrophils are successfully obtained using the Percoll dilutions (72%, 63%, 54%, and 45%) (Sursal et al. 2018). In the present study, the Percoll gradient centrifugation (72%, 63%, 54%, and 45%) was useful for isolation of the neutrophils from venous blood samples of dogs. This technic was fast and simple rather than the other PMN isolation technics.

In conclusion, Percoll gradient centrifugation was used to obtain neutrophils from venous blood samples of healthy dogs. This method is useful for isolation dog neutrophils.

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