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Isolation of heterophils from peripheral blood in hens and analysis of heterophil functions by flow cytometry - a methodological study

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ABSTRACT

The aim of this study was to modify the flow cytometric method, which is used for analyzing neutrophils, to analyse the functions of avian heterophils. The blood samples used in the experiments were obtained from hens in slaughterhouses. Blood samples were collected from 10 hens for each trial. Within the scope of the present study, trials were carried out regarding the amount of blood, cell suspension, dihydrorodamine-123 (DHR-123), phorbol-12-myristate-13-acetate (PMA), and N-formyl-methionyl-leucyl-phenylalanine (fMLP), as well as the storage duration of blood samples and incubation time. The results showed that 0.5-3 ml of blood could be used to detect heterophil functions, and it would be ideal to conduct analyses using fresh blood samples. In addition, the results showed that blood stored at +4 °C for up to 8 hours may be also used if necessary. In order to isolate the cells, centrifugation for 30 minutes is sufficient, and it is appropriate to use a 30µL cell suspension. 2µL of DHR-123 should be used as a chemical probe to measure heterophil functions. Excessive use of DHR-123 affected the heterophil functions negatively. In addition, it was observed that using 2µL of fMLP, which is used as an oxidative burst stimulant, and 2µL of PMA as a stimulant of chemotaxic activity, were sufficient. It was concluded that incubation at 41 °C for 5 minutes after stimulating the heterophils is also sufficient. We conclude that the methods established in this study could be used to isolate heterophils and to analyze them by flow cytometry. Therefore, this study would contribute to further research and clinical studies in poultry.

Keywords: avian; phagocytic activity; oxidative burst; chemotaxic activity

Introduction

Neutrophils are phagocytic cells and constitute the first line of host defense against invading pathogens (NATHAN 2006). Although the phagocytic properties of neutrophils are most prominent, their functions are not limited to phagocytosis. When properly activated, they can also be effective in modulation of the adaptive immune response (ABDALLAH et al., 2011),

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secreting various proinflammatory cytokines, and expressing the major histocompatibility complex-II (MHC-II) molecule (WRIGHT et al., 2010). They are also involved in activation of dendritic cells (VAN GISBERGEN et al., 2005) and regulation of inflammatory reactions that provide the link between the specific immune system and the nonspecific immune system (CASSATELLA 1999). In recent years, it has also been discovered that neutrophils become an intracellular neutrophil trap by lytic and non-lytic mechanisms to destroy pathogens (BRINKMANN et al., 2004; PAPAYANNOPOULOS 2018; SONG et al., 2019). Therefore, the interest in neutrophils has been increasing in studies related to the non-specific defense system. In some situations, although neutrophil production is sufficient, functional deficiency may be observed (DINAUER 2007). It is important to examine the functional properties of neutrophils, such as chemotaxic activity, oxidative burst and phagocytic activity, especially in late wound healing and recurrent infections (EMMENDÖRFFER et al., 1990).

Flow cytometry is a technique which provides rapid and accurate detection of the qualitative and quantitative characteristics of a single cell (BROWN and WITTWER, 2000). Thanks to these features, it is also used in studies related to the neutrophil functions (ROTHE and VALET, 1990). One of the probes used in determining neutrophil functions by flow cytometry is dihydrorodamine-123 (DHR-123). When phagocytic cells are activated, reactive oxygen compounds secreted from intracytoplasmic granules convert DHR-123 into rhodamine-123 (R-123). DHR-123 is a non-fluorescent molecule. However, R-123 settles into the mitochondria and emits a green fluorescent light when stimulated with a laser beam. Since the fluorescent light intensity emitted is proportional to the activity of the cell, it is used for measuring cell activity (BILGIC et al., 2007).

Heterophils, the avian counterparts of mammalian neutrophils, are critical to the immune system of birds. They play an important role in the recognition, capturing and destruction of the pathogen (GENOVESE et al., 2006). Although heterophils are equivalent to neutrophils, there are numerous structural and functional differences (REDMOND et al., 2009). One of these differences is the absence of myeloperoxidase, catalase and alkaline phosphatase enzymes in the cytoplasmic granules of heterophils (HAMON 1998). Since there is no myeloperoxidase, the amount of superoxide anion and hydrogen peroxide (H_2O_2) released after heterophil activation is lower than in neutrophils (BROOKS et al., 1996; WELLS et al., 1998). This makes it difficult to measure cell functions by classical methods. Flow cytometric methods can be used in determining heterophil activity as they enable more sensitive analysis. However, as mentioned above, it is necessary to examine whether the flow cytometric methods developed to measure neutrophil functions are suitable for heterophils, since the content and oxidative mechanisms of cytoplasmic granules are different in heterophils.

Since the red blood cells are non-nucleated in mammalian blood, it is sufficient to lyse the red blood cells during flow cytometric analysis of leukocytes. However, in birds, the nucleus, released after erythrocytes are lysed, further complicates flow cytometric analysis. Therefore, leukocytes to be examined must first be isolated from whole blood in poultry. Isolation of heterophils has been described in detail in studies examining heterophil functions with classical methods (GENOVESE et al., 2006). However, for flow cytometric analysis, heterophil isolation should be revised, especially in terms of preserving cell viability. Since the interaction of cells with fluorescent dyes that lose their vitality is different, it can also affect the results obtained in flow cytometry.

Since avian heterophils are structurally and functionally different from mammalian neutrophils, there is a need for a methodical study related to heterophil functions. The aim of this study was to modify the flow cytometric methods used for analyzing neutrophils and detecting the chemotaxic activity, oxidative burst and phagocytic activity of avian heterophils. In addition, heterophil isolation procedures have also been reviewed within the scope of the present study.

Materials and methods

Blood samples. The blood samples used in the experiments were obtained from a total of 80 Lohmann Brown Classic laying hens (91 weeks old) brought to the slaughterhouse. These blood samples were collected into tubes containing EDTA and transferred to the laboratory in an ice container. Sufficient volumes of blood samples were collected from 10 different hens for each of the trials (a total of 80 hens for 8 different trials). Since the blood samples used in the present study were obtained from hens slaughtered in the slaughterhouse, no ethics committee approval was required according to the current experimental animal directive in our country.

Heterophil isolation procedures. In the present study, heterophil isolation processes were performed by the method of KOGUT et al. (1999). Briefly, 3 ml of EDTA blood sample was mixed with 2 ml of 1% methylcellulose (25 Centipoise) (Sigma Chemical Company, St. Louis, Missouri). Then the mixture was centrifuged at 25g at room temperature for 30 minutes. The supernatant and buffy coat layers were collected by pipette. The same amount of Hanks Balanced Salt Solution (HBSS) was then added. The obtained suspension was carefully layered onto discontinuous Ficoll-Hypaque gradients (specific gravity 1.077 over 1.119) (Sigma Chemical Company, St. Louis, Missouri). The gradient was centrifuged at 250g for 60 minutes at room temperature. After centrifugation, the 1.199 bands were carefully removed by pipette, to obtain the heterophils. The samples were washed with Roswell Park Memorial Institute medium 1640 (RPMI 1640) (1:1) by centrifugation at 510 g for 10 minutes. After centrifugation, the supernatant was discarded and the cell pellet, diluted with PBS, was used. The cell concentration was adjusted to 4 x 10^6/ml.

Heterophil function tests. Three tubes were prepared for each sample. One ml of PBS, 20 µl of heterophil suspension, and 10 µl of DHR-123 were pipetted into each tube, respectively. The prepared samples were incubated in a water bath for 5 minutes. Afterwards, 10 µl of *Escherichia coli* (*E. coli*) suspension, phorbol-12-myristate-13-acetate (PMA), and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were pipetted into the first, second, and third tubes, respectively. *E. coli* (10^9 *E coli*/mL in PBS), PMA, and fMLP were used as phagocytic activity, oxidative burst, and chemotactic activity stimulators, respectively. Mean fluorescence intensity was determined at 0 and 20 minutes by flow cytometry. A total of 10,000 cells were passed through flow cytometry to measure mean fluorescence intensity (MFI). Fluorescence intensity was determined using the FL1 detector. The values obtained were converted into numerical data using the CellQuest program (Becton Dickinson, New Jersey. US.). The mean fluorescence intensity was calculated by dividing the value measured at 20 minutes by the value measured at 0 minutes (BILGIC et al., 2007).

Tested variables.

The effect of the amount of blood used for cellisolation on heterophil functions. Collecting large amounts of blood from birds would be onerous. Therefore, we carried out experiments to determine the minimum blood volume that could be used for analyzing heterophil functions. Seven ml of EDTA blood samples were obtained from each of the 10 hens for this trial. The collected blood samples were divided into 4 quantities, that is, 0.5, 1, 2, and 3 ml. Heterophils were isolated from each blood sample, and heterophil functions were measured according to the procedure described above.

The effect of duration of storage of blood samples on heterophil isolation. Delivery of blood samples to the laboratory or analyzing them straight away may not always be possible. Therefore, this trial was carried out to determine whether keeping the blood samples at room temperature or in a refrigerator affected the heterophil functions. A total of 10 hens were used for this trial. Twelve ml of blood was taken from each hen and the samples were divided into 4 tubes. Heterophils were isolated from the first blood sample within 1 hour and their functions were analyzed. The remaining blood samples were kept at room temperature for 4, 8 and 12 hours, respectively, after which heterophils were isolated and their functions were determined according to the procedure described above. The same procedure was applied to the blood samples stored at +4 °C.

Determination of the centrifugation time for heterophil isolation. Heterophil isolation is a time-consuming procedure. In this trial, we tested whether shortening the centrifuge time affects the heterophil functions. Nine ml of blood were taken from each of the 10 hens for this trial. Each blood sample was divided into 3. After the erythrocytes were removed, the remaining cell suspension was spread over the Ficoll-Hypaque gradient (1.077 over 1.199). The mixture was then centrifuged at room temperature for 10, 20, and 60 minutes. The speed of the centrifuge was adjusted to 250 g. After isolation of the heterophils, their functions were measured according to the procedure described above.

The effect of the amount of cell suspension on heterophil functions. If there are not enough cells in the cell suspension prepared for the analysis, the counting time will be very long. Moreover, the suspension may be consumed before the counting is completed. On the other hand, having too many cells in the suspension affects the sensitivity of the counting. Therefore, in this step of the study, trials were conducted to determine the optimal amount of cell suspension that should be added to the final mixture. Measurements were made using 20, 30 and 40 µL of the heterophil suspension.

The effect of the amount of dihydrorhodamine-123 on heterophil functions. DHR-123 is a chemical probe used for measuring the functions of phagocytic cells. At this step of the study, trials were conducted to determine the amount of DHR-123 that will use for analyzing heterophil functions. Normally, 10 µL of DHR-123 is used for measuring the functions of neutrophils. Trials were performed using 2, 8, and 12 µL of DHR, respectively, to determine whether using a different amount of DHR affects heterophil function.

The effects of different amounts of fMLP and PMA on heterophil functions. In the study, fMLP and PMA were used as oxidative burst and chemotactic activity stimulants respectively. To measure heterophil functions, 10 µl of these stimulants are used. At this step of the study, trials were made to determine whether the use of different amounts of stimulants affected heterophil functions. 2, 4, 6, and 8 µL FMLP and 2, 4, 6, and 8 µL PMA were added to the heterophil suspension to determine the optimal stimulant concentration.

The effect of incubation temperature on heterophil functions. At this stage of the study, the effect of the incubation temperature on the heterophil functions was tested. Immediately after the pipetting of stimulants (0 min), the heterophil functions were measured. A second measurement was performed after 20 min of incubation in a water bath. During this incubation period, water bath temperatures were adjusted to 37°C, 41°C, and 43°C, respectively, in each trial.

The effect of incubation time on heterophil functions. At this step of the experiment, trials were conducted to determine whether the incubation time affects the heterophil functions. Immediately after pipetting the stimulants (0 min), heterophil functions were measured. The second measurement was carried out after incubating the cells for 5, 10, 15, and 20 minutes in a 37 °C water bath. Thus, the effect of the incubation period was determined.

Statistical analysis. All statistical analyses were performed using the IBM SPSS Statistics program Version 27 (SPSS Inc., Chicago, Illinois, US.). Two assumptions, the normality and homogeneity of variances, were checked before determining the appropriate method. The Shapiro-Wilk test was applied to check whether the data obtained were normally distributed. The Levene test was used for testing the homogeneity of variances between trials. When these two assumptions were met, the analysis of variance (ANOVA) was applied, followed by Tukey's test at 0.05 significance. The lack of normality or homogeneity (or both) led to the use of the non-parametric Kruskal-Wallis test. In the case of the existence of significant differences between trials, the Mann-Whitney U test was used for pairwise comparisons, and the significant was set at $P = 0.05$. All results are expressed as means ± standard error of the mean.

Results

The effect of the amount of blood used for cell isolation on heterophil functions. The effect of the amount of blood used for cell isolation on heterophil functions is presented in Figure 1. The amount of blood was found to have no effect on

phagocytic activity (P = 0.323; df = 3, 36; $\chi 2$ = 3.480), oxidative burst (P = 0.321; df = 3, 36; F = 1.208) and chemotaxic activity (P = 0.505 df = 3, 36 F = 0.795).

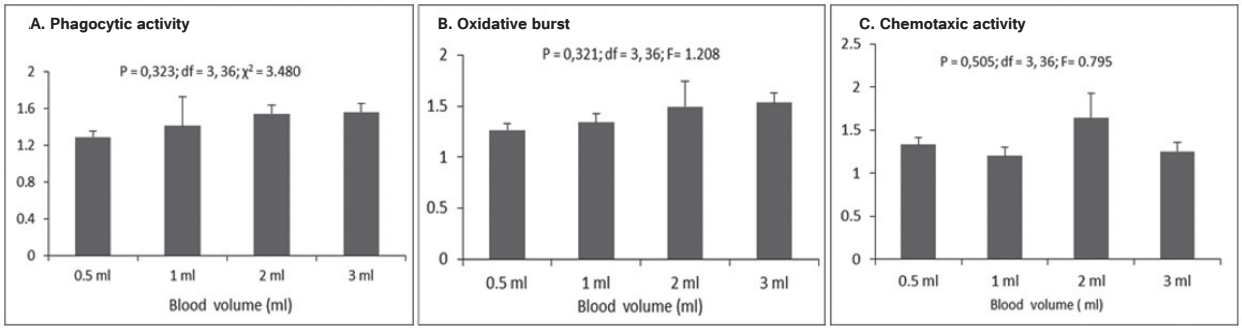

Fig. 1 The effect of the amount of blood used on heterophil function (Mean ± SE).

The effect of storage duration of blood samples on heterophil isolation. The effect of storage duration of the blood samples at +4 °C on heterophil functions is presented in Figure 2. There was no significant effect of the storage duration of blood samples at +4 °C on phagocytic activity (P = 0.224; df = 2, 27; F = 1.488). However, it was found to affect oxidative burst and chemotaxic activity. The oxidative burst of the samples stored up to 8 hours did not change but it increased in the samples stored for 12 hours (P = 0.001; df = 2, 27; F = 12.670). Similarly, chemotaxic activity increased depending on storage duration, and it was significantly higher in the samples stored for 12 hours compared to those stored for 4 hours (P = 0.009; df = 2, 27; $\chi 2$ = 9.370).

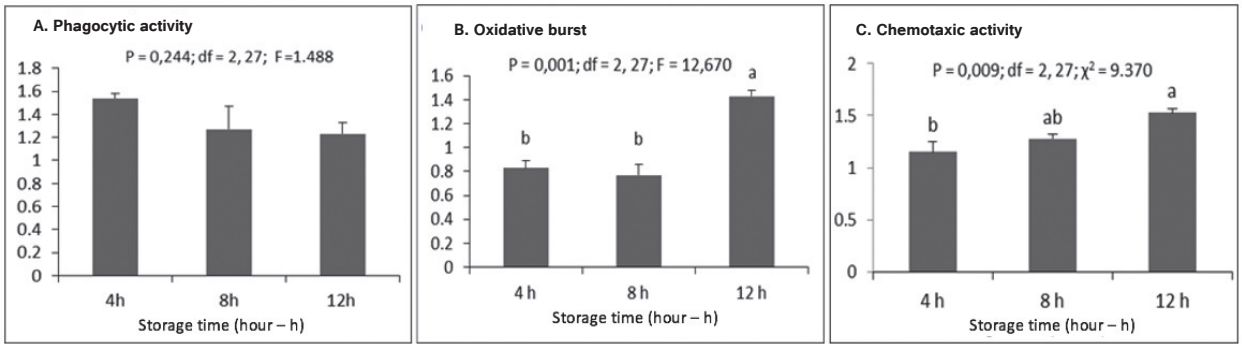

Fig. 2. The effect of the duration of storage of blood at +4 °C on heterophil functions (Mean ± SE). [a, b] Values referring to the same biochemical parameter marked by a different letter differ significantly.

The effect of the duration of storage of blood samples that were kept at room temperature on the heterophil functions is presented in Figure 3. When blood samples were kept at room temperature, phagocytic activity (P = 0.001; df = 2, 27; F = 20.691), oxidative burst (0.001; df = 2, 27; F = 19.542), and chemotaxic activity (P = 0.001; df = 2, 27; $\chi 2$ = 19.115) decreased due to the storage time.

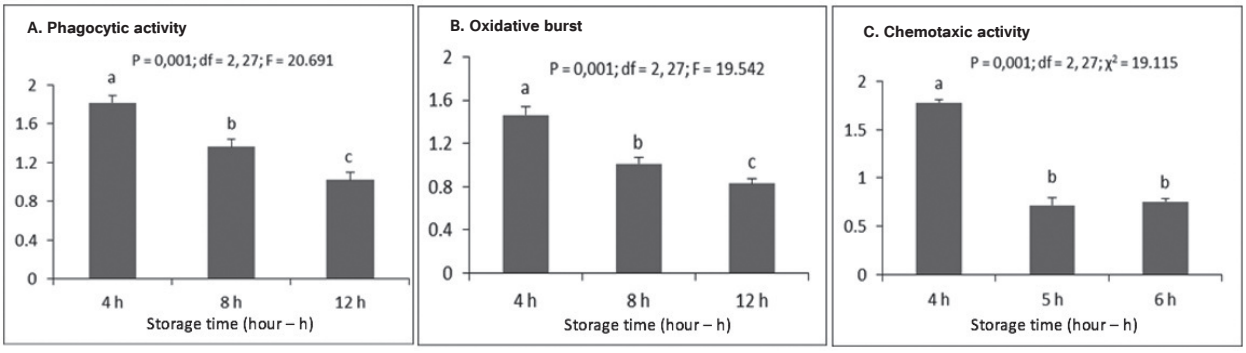

Fig. 3. The effect of the duration of storage of blood at room temperature on heterophil functions (Mean ± SE). [a, b, c] Values referring to the same biochemical parameter marked by a different letter differ significantly.

Determination of the centrifugation time for heterophil isolation. The effect of the centrifugation time used for isolating cells on heterophil functions is presented in Figure 4. There was no significant change in the phagocytic activity of heterophils due to the centrifugation time (P = 0.527; df = 2, 27; F = 0.656). However, oxidative burst was higher in the 20 and 30 minute samples compared to the 10 minute sample (P = 0.017; df = 2, 27; F = 4.747), and chemotaxic activity increased proportionally with the centrifugation time (P = 0.001; df = 2, 27; F = 8858).

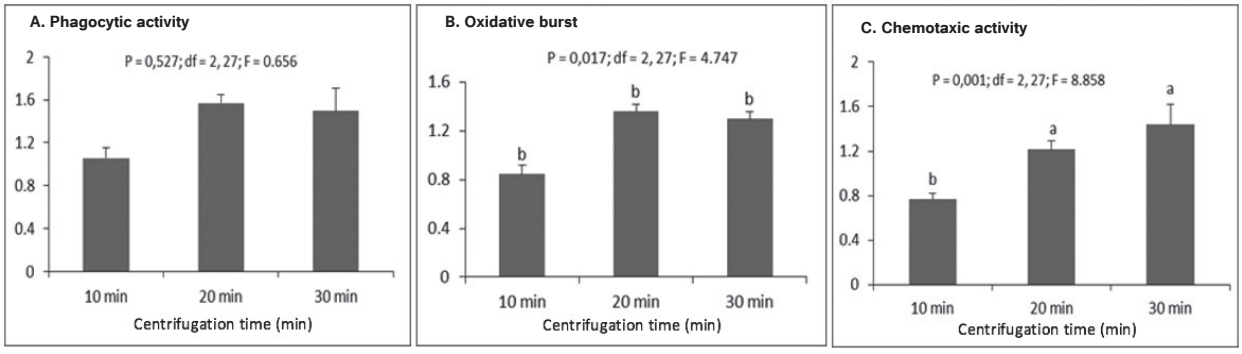

Fig. 4. The effect of centrifugation time used for isolating cells on heterophil functions (Mean ± SE). [a, b] Values referring to the same biochemical parameter marked by a different letter differ significantly.

The effect of the amount of cell suspension on heterophil functions. The effect of the cell suspension amount used for analyzing the heterophil functions is given in Figure 5. The cell suspension amounts used in the trials did not affect phagocytic activity (P = 0.065; df = 2, 27; F = 3.031), oxidative burst (P = 0.739; df = 2, 27; F = 0.306) or chemotaxic activity (P = 0.519; df = 2, 27; F = 0.672).

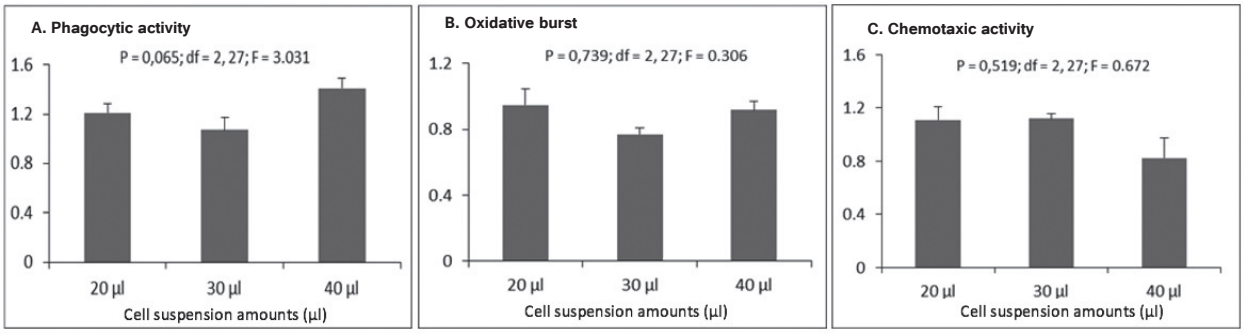

Fig. 5. The effect of the amount of cell suspension used for flow cytometric analysis on heterophil functions (Mean ± SE).

The effect of the amount of dihydrorhodamine-123 on heterophil functions: The effect of the amount of DHR-123 on heterophil functions is presented in Figure 6. There was an inverse relationship between the amount of DHR-123 used and heterophil functions. When 2 µl of DHR-123 was used, phagocytic activity (P = 0.001; df = 2, 27; F = 9.184), oxidative burst (P = 0.004; df = 2, 27; F = 6.906), and chemotaxic activity (P = 0.030; df = 2, 27; F = 4.027) were all at the highest levels, and after using 12 µl of DHR-123 these parameters decreased significantly.

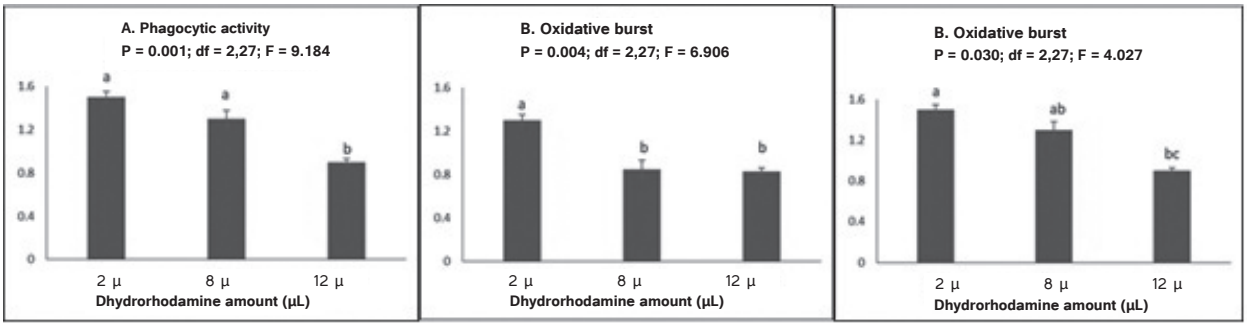

Fig. 6. The effect of the amount of dihydrorhodamine-123 on heterophil functions (Mean ± SE). [a,b,c] Values referring to the same biochemical parameter marked by a different letter differ significantly.

The effects of the amounts of fMLP and PMA on heterophil functions. The effect of the amounts of PMA and fMLP used for stimulating heterophils on oxidative burst is presented in Figure 7. It was observed that the oxidative burst decreased when the amount of PMA increased. The oxidative burst level of heterophils was lower after using 6 µL or 8 µL PMA than after using 2 µL PMA (P = 0.001; df = 3, 36; F = 9.588).

As with PMA, an inverse relationship was observed between stimulant and activity in fMLP. It was determined that as the amount of fMLP increased, the chemotaxic activity decreased. The highest activity was detected when 2µL was used, while the lowest activity was found when 8µL was used. It was determined that the difference between the two was statistically significant (P = 0.014; df = 3, 36; F = 4.063).

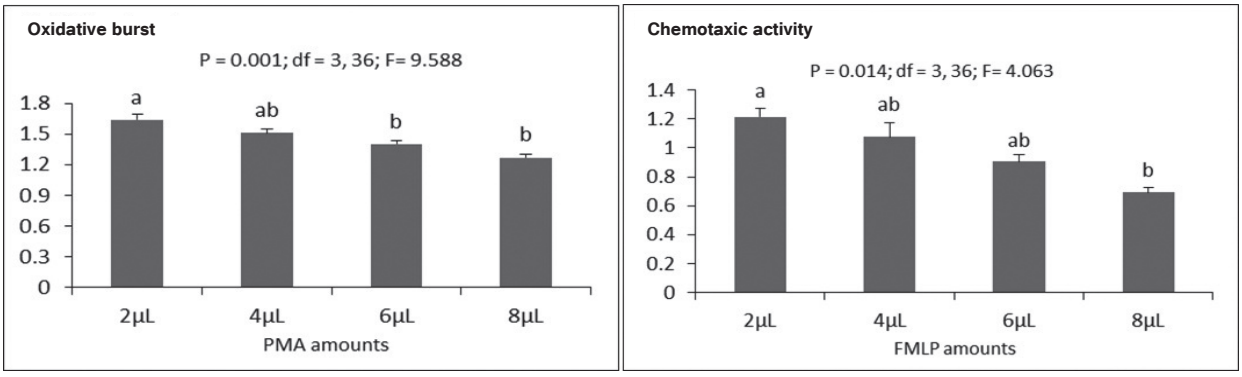

Fig. 7. The effects of amounts of PMA and fMLP used to stimulate heterophils on oxidative burst and chemotaxic activity (Mean ± SE). [a, b] Values referring to the same biochemical parameter marked by a different letter differ significantly.

The effect of incubation temperature on heterophil functions. The effect of incubation temperature on heterophil functions is given in Figure 8. It was determined that oxidative burst was significantly higher in heterophils incubated at 41 °C compared to those incubated at 37 °C and 43 °C (P = 0.001; df = 2, 27; F = 9.080). Although not statistically significant, phagocytic activity (P = 0.405; df = 2, 27; F = 0.934) and chemotaxic activity (P = 0.075; df = 2, 27; F = 2.848) were higher in samples incubated at 41 °C.

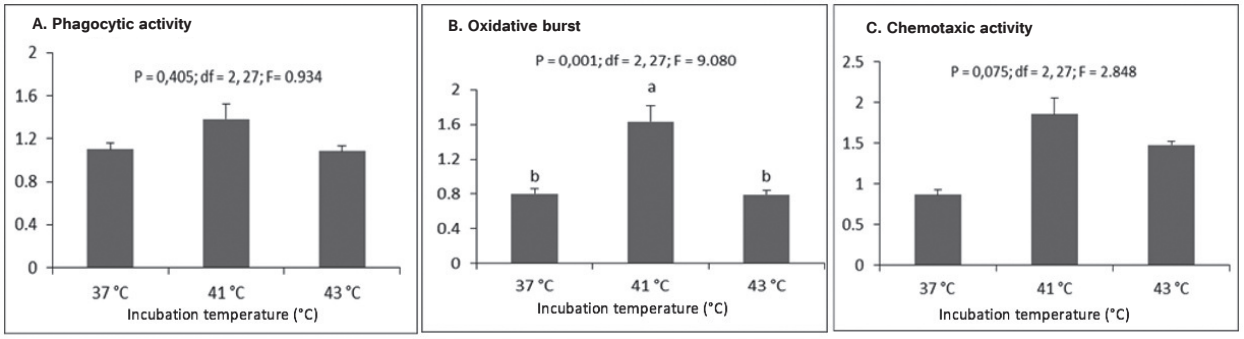

Fig. 8. The effect of incubation temperature on heterophil functions (Mean ± SE). [a,b] Values referring to the same biochemical parameter marked by a different letter differ significantly.

The effect of incubation time on heterophil functions. The effect of the incubation time on heterophil functions is presented in Figure 9. The incubation times applied do not appear to cause a significant difference in heterophil functions. (Phagocytic activity; P = 0.290; df = 3, 36; F = 1.297. Oxidative burst; P = 0.241; df = 3, 36; F = 0.867. Chemotaxic activity; P = 0.098; df = 3, 36; F = 0.960).

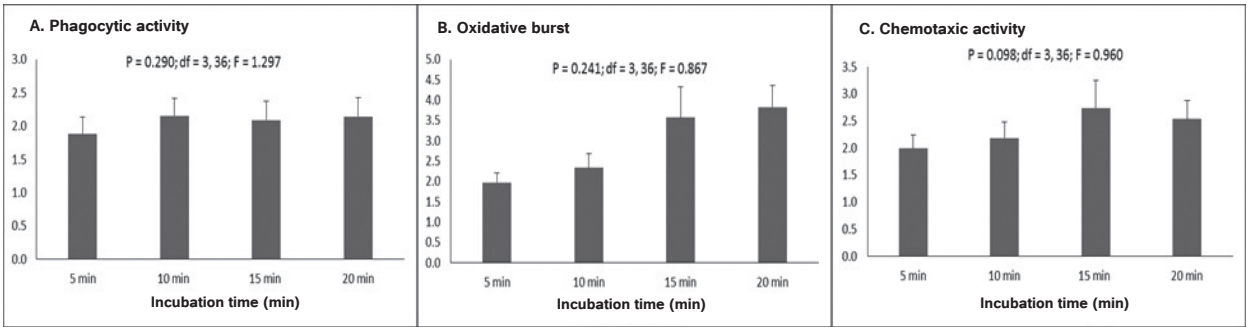

Fig. 9. The effect of incubation time on heterophil functions (Mean ± SE).

Discussion

Flow cytometric analyses are widely used in humans and farm animals, but their use in poultry is limited. Since they provide detailed information on cellular level, flow cytometric analyses also have great potential to be used in poultry research. In previous studies conducted by our study group, methods used for determining the activity of mammalian neutrophils were also used for determining heterophil functions, with simple modifications (MATUR et al., 2011; MATUR et al., 2015; MATUR et al., 2016). However, the experience we gained during previous studies showed that revision and optimization are required of the method developed to analyze mammalian blood for it to be used for poultry. In this context, the stages of the methods used in the analysis of mammalian neutrophils were reviewed. The phagocytic activity, oxidative burst and chemotaxic activity of the heterophils were examined, while trials were carried out for each step of the analysis at a time.

The effect of the amount of blood used for cell isolation on heterophil functions. In mammals, 3 ml of blood is used for analyzing heterophil functions by flow cytometry. Although this amount of blood is easily taken from adult poultry, it is difficult to obtain this amount of blood from chicks or small cage birds. For this reason, the first test was to see whether the method examined could be performed using a smaller amount of blood. For this purpose, heterophil functions were examined using 0.5ml, 1ml, 2ml and 3ml of chicken blood. The findings showed that there was no significant change in heterophil functions when less than 3 ml of blood was used. This shows that heterophil functions can be measured using small amounts of blood, especially in small birds. However, it is important to consider that when using a small amount of blood, removing the leukocyte containing band (buff coat layer) requires some attention.

The effect of the duration of storage of blood samples on heterophil isolation. The data obtained from this study show that keeping the blood for a long or short time at +4 °C does not affect the phagocytic activity. However, it was observed that oxidative burst and chemotaxic activity increased when the blood was kept for more than 8 hours (Figure 2). AL-HAMADANY (2014) similarly reports that the phagocytic activity is stable in neutrophils that were kept at 4 °C. HAMMER et al., (1986) stated that phagocytic and chemotaxic activity decreased in blood samples that were stored for longer durations than ours (24 and 48 hours). In our study, the blood was stored for a maximum of 12 hours. No activity loss was observed in the blood stored for less than 12 hours, whereas oxidative burst and chemotaxic activity increased in the samples stored for 12 hours. It is difficult to say whether this difference is heterophil-specific or is related to the sensitivity of the methods used. Perhaps the suspended cells may have become more sensitive to the stimuli used for oxidative burst and chemotaxic activity. However, we cannot fully explain the reason for this by evaluating the

data we have. We can say that there was no loss of activity only in heterophils that were kept at + 4 °C degrees.

Heterophil functions, including phagocytic activity, appear to be reduced in blood samples kept at room temperature (Figure 3). There are no published data on whether keeping blood samples at room temperature affects heterophil functions. However, a decrease was reported in the phagocytic activity, oxidative burst, and chemotaxic activity of neutrophils that were stored at room temperature (BUESCHER and GALLIN, 1987). Furthermore, a decrease in functions and accelerated apoptosis were reported in neutrophils that were kept at room temperature (BERGMAN et al., 2014).

The oxidative burst stimulant PMA activates NADPH in the cytosol by binding to protein kinase C in cells. Activated NADPH causes the release of reactive oxygen compounds (COX et al., 1958). Therefore, it was thought that oxidative burst might have decreased due to a decrease in protein kinase C in the blood samples that were kept at room temperature. Indeed, it has been reported that protein kinase C decreases in blood samples kept at room temperature (HALL et al., 1997). The phospholipase D enzyme found in neutrophils plays a role in the degranulation and migration of cells (FOSTER 2003). It has been reported that fMLP, which is used as a chemotaxic activity stimulant, reveals its effect on cells through the phospholipase D enzyme (TAKANOBU et al., 2013). Possible inactivation of the phospholipase D enzyme in the blood samples (8 hours, 12 hours) kept at room temperature for a longer time was thought to cause a decrease in chemotaxic activity. Regardless of the underlying mechanism, the data obtained indicate that blood samples that are to be used for determination of heterophil functions should not be kept at room temperature for a long time.

Determination of the centrifugation time for heterophil isolation. In the present study, trials were carried out regarding the duration of the centrifuge in order to shorten the time needed to measure heterophil functions. Heterophils were isolated by centrifuging 250 g of blood samples belonging to the same individual for 10, 30 and 60 minutes. It was found that decreasing the centrifugation time did not affect the phagocytic activity (Figure 4). However, it was determined that oxidative burst and chemotaxic activity were lower in samples centrifuged for 10 minutes compared to the others. Regarding these two parameters, there was no difference between 30 and 60 minutes. For this reason, it would be appropriate to centrifuge samples that will be used for heterophil isolation for 30 minutes, in order to reduce the processing time.

The effect of the amount of cell suspension on heterophil functions. In our previous studies it was observed that the number of heterophils decreased in blood samples that were stored for a long time, and they were more affected by isolation procedures (unpublished data). When the cell numbers in blood samples is low, the duration of the analysis is lengthened because it takes more time to reach the number of cells that are to be analyzed in flow cytometry. And therefore the prepared suspensions were exhausted before the analysis was completed. In this study, the amount of heterophil suspension was also reviewed. While 20 µL cell suspensions were used in mammals, trials were performed with the amounts of 30 µL and 40 µL in this study. No differences were observed in heterophil functions (Figure 4). Therefore, it was thought that 30 µL could be used from samples with a low heterophil ratio in the suspension.

The effect of the amount of dihydrorhodamine-123 on heterophil functions. Dihydrodamine-123 dye is used for detecting reactive oxygen species. Due to its lipophilic feature, it can pass through the membrane, and localizes inside the cell. Therefore, it is oxidized by reactive oxygen compounds released during metabolic activities in the cell, and transforms into the highly stable form of rhodamine-123 (RANGANATHAN and HOOD 1989). This emits a fluorescent light when excited. In this way, activity inside the cell is measured. In the present study, DHR-123 was used as a chemical probe for flow cytometric measurement of heterophil functions. The main purpose of testing this variable was to determine whether heterophil functions could be measured using less DHR-123 than the amount used for measuring neutrophil functions. The data obtained clearly showed that

the best activity was measured with 2 µL DHR-123 (Figure 6). It may even be said that using more than 2 µL negatively affects the activity of heterophils. It is impossible to say exactly why. However, when used in large quantities, it may limit the activity of mitochondria, and thus the activity of the cell. Thus, DHR-123 entering the cell has been reported to accumulate in the mitochondria (SAKURADA et al., 1992).

The effects of the amounts of fMLP and PMA on heterophil functions. Phagocytic cells produce toxic oxygen radicals against bacterial agents. NADPH-oxidase is one of the enzymes responsible for oxygen metabolism. It transfers electrons from NADPH on the cytosolic side of the membrane to oxygen on the other side, causing the production of oxygen radicals (JOHANSSON et al., 1995). PMA activates NADPH-oxidase in the membranes of both cells and granules (KARLSSON et al., 2000). In the present study, an inverse relationship was observed between the amount of PMA used for stimulating heterophils and oxidative burst. It was found that as the amount of PMA increased, the oxidative burst decreased (Figure 7). The heterophil functions examined in this study were measured on the basis of the mean fluorescence intensity. The oxygen radicals released after the stimuli was pipetted onto the cells convert DHR-123 to rhodamin-123, causing the emergence of the fluorescent color (RICHARDSON et al., 1998). While the cells are incubated for a total of 20 minutes at a certain temperature, the fluorescence level continues to increase due to activation. After 20 minutes, incubation was terminated. The value measured at 20 minutes was divided by the value measured at 0 minutes to determine the mean fluorescence intensity. It was found that the greater the difference between these two values, the higher the activity. When a high concentration of stimulus (PMA) is pipetted onto the cells, it is likely that the fluorescent emission at 0 minutes is also high. This may cause the difference between 0 and 20 minutes to decrease. However, when a low concentration of stimulator is pipetted, this process is thought to be slower, causing the difference to be greater between 0 and 20 minutes. We can see the same effect in the decreased chemotaxic activity when its concentration was increased with fMLP (Figure 7). For this reason, 2 µL PMA concentrations were thought to be sufficient for oxidative burst and chemotaxic activity measurement.

The effect of incubation temperature on heterophil functions. In mammals, cells are incubated at 37 °C when neutrophil functions are measured. In poultry, heterophils were incubated at 37, 41 and 43 °C, as the body temperature is higher and there are some differences between the species. The oxidative burst was found to be higher in samples incubated at 41 °C than those incubated at other temperatures. In addition, phagocytic and chemotaxic activity was observed to be higher in those incubated at 41 °C compared to the others (Figure 8). Considering that the body temperatures of the chickens used in this study are 40-41 °C (McNAB 1966), it could be said that 41 °C is ideal for heterophil functions.

The effect of incubation time on heterophil functions. In the present study, the activity of the cells was measured at 5, 10, 15 and 20 minutes after the fMLP, PMA or *E. coli* were pipetted onto the heterophils. The data obtained clearly showed that 5 minute incubation was sufficient and there was no significant difference in the heterophil function afterwards, although there was a numerical increase (Figure 9). It is obvious that this will save time in studies where many analyzes are performed.

Conclusion

In this study, it was seen that using 3 ml of blood to determine the heterophil functions in poultry will facilitate the procedures, but in cases where the sample amount is insufficient, 0.5 ml of blood may be adequate. It was also concluded that it would be ideal to detect heterophil functions using newly collected blood samples, but if it is not possible to analyze them immediately, it would be appropriate to store them at +4 °C for a maximum of 8 hours. Additionally, when the blood samples were centrifuged for 30 minutes, it was seen that the cells could be easily isolated and there was no change in their functions. In the present study, it was found that using excessive amounts of suspension shortens the cell reading procedure in flow cytometry, and enables the targeted number of

cells to be reached in a shorter time. Therefore, it was concluded that it is appropriate to use 30 µL cell suspensions to measure heterophil functions. Also, unlike neutrophils, it was seen that it would be ideal to use 2µL DHR123 to measure heterophil functions. Likewise, it was found that using 2µL PMA and 2µL fMLP as the cellular stimulator would be sufficient to measure oxidative burst and chemotaxic activity. It was also concluded that it would be sufficient to incubate cells for 5 minutes at 41 °C for heterophil functions. As a result, we believe that this study may show how to isolate heterophils and analyze them using flow cytometry, and thereby contribute to poultry research and clinical studies.

Conflict of interest

The authors declare no conflict of interest related to this work.

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MATUR, E., M. EREK, E. ERGEN, B. A. BOLAT, M. ÖZCAN: Izolacija iz periferne krvi i analiza funkcija heterofila kokoši protočnom citometrijom – metodološko istraživanje. Vet arhiv 92, 469-482, 2022.

SAŽETAK

Cilj je istraživanja bio prilagoditi metodu protočne citometrije, koja se inače primjenjuje u analizi neutrofila, za analizu funkcija ptičjih heterofila. Uzorci krvi 10 kokoši dobiveni su iz klaonica. Uzevši u obzir količinu krvi, kao i trajanje pohrane krvnih uzoraka odnosno vrijeme inkubacije, analizirana su stanična suspenzija, dihidrorodamin-123 (DHR-123), forbol-12-miristat-13-acetat (PMA) i N-formil-metionil-leucil-fenilalanin (fMLP). Rezultati su pokazali da se 0,5 – 3 mL krvi može upotrijebiti za otkrivanje funkcija heterofila te da bi idealno bilo analizirati svježe uzorke krvi. Osim toga rezultati su pokazali da se i krv pohranjena na temperaturi od +4 °C, u vremenu do 8 sati, može upotrijebiti ako je to potrebno. Kako bi se stanice izolirale, dovoljno je centrifugirati 30 minuta uz primjenu stanične suspenzije od 30 µL. Kao kemijsku probu za mjerenje funkcija heterofila trebalo bi upotrijebiti 2 µL DHR-123. Prekomjerna upotreba DHR-123 negativno je utjecala na funkcije heterofila. Također, uočeno je da je bila dovoljna primjena 2 µL fMLP-a, koji služi kao stimulans oksidacijskog izgaranja, kao i primjena 2 µL PMA-a kao stimulansa kemotaktičke aktivnosti. Zaključeno je da je inkubacija na temperaturi od 41 °C, tijekom 5 minuta poslije stimulacije heterofila također dovoljna. Rezultati su pokazali da se metode ustanovljene u ovom radu mogu primijeniti za izolaciju heterofila i njihovu analizu protočnom citometrijom, čime se doprinosi daljnjim istraživanjima i kliničkim studijama u peradi.

Ključne riječi: ptice; fagocitna aktivnost; oksidacijsko izgaranje; kemotaktička aktivnost