

White Blood Cell Extraction and In-Vitro Assessment of Phagocytosis Using *Escherichia coli* in Asian Elephants

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Introduction

White Blood Cells (WBC) with phagocytic capacity, namely neutrophils and monocytes are the first line of defence against many infections (Abbas *et al.* 2007). The neutrophils of Asian elephants (*Elephas maximus*) are much larger than in other animals and are known as heterophils (Silva & Kuruwita 1993). Phagocytic activity of WBCs is altered in periodontitis in humans (Asif & Kothiwale 2010), decreased in septic dogs (Webb *et al.* 2007) and less in inflamed mammary glands compared to that of peripheral circulation in cattle (Prin-Mathieu *et al.* 2002). Neutrophil function in humans is impaired in chronic obstructive pulmonary disease (Jasper *et al.* 2019). Impaired *Escherichia coli* phagocytosis by neutrophils has also been observed in neonates, but improves with age (Wenisch *et al.* 2000; Filias *et al.* 2011). Phagocytic ability and consequently innate immunity, could also be influenced by estradiol and progesterone (Bartoskova *et al.* 2014).

Neutrophils have received much attention since they are the first line of defence against infection. Assessment of phagocytosis of WBCs could be used in determining cellular immunity against infections in elephants and identifying post-musth immune compromise (Athapattu *et al.* 2018). Therefore, extraction of neutrophils of elephants and assessment of their functional differences and morphological changes in inflammation and in relation to musth, would be useful in understanding and managing such conditions.

Previously neutrophils have been isolated from elephant blood, using hetastarch sedimentation, and percoll centrifugation and phagocytosis

examined using *Escherichia coli* (Smith *et al.* 1998). Here we report the isolation of WBC from elephant blood using phosphate buffered saline and assessment of their phagocytosis using *E. coli*.

Materials and methods

Five ml of blood was collected in duplicate from each of four apparently healthy, adult male captive Asian elephants, and differential blood counts done. Centrifuging the blood and taking the buffy coat and the top 1/4th of the red cell column, has been reported to give the best yield of WBCs in elephants (Smith *et al.* 1998). We repeated Smith *et al.*'s analysis, centrifuging blood and making smears from different portions of the centrifuged blood column, which confirmed their findings. Therefore, each sample was centrifuged at 2000 g for 10 min and the buffy coat with the top 1/4th of the red cell column used for extraction.

The procedure for cell extraction used by Silva & Jain (1988) was followed with some modifications. We used 5 ml of blood and centrifuged for 10 min while Silva & Jain (1988) used 10 ml of blood and centrifuged for 15 min. Silva & Jain (1988) used 30 ml of distilled water and allowed 40 sec for red cell lysis while we used 3 ml of distilled water over 1 minute. We then restored isotonicity by adding 2 ml of 2.7% Phosphate Buffered Saline (PBS) while Silva & Jain (1988) used 35 ml. Then the tube was centrifuged for 10 min at 200 g, the supernatant discarded and the remaining cell pellet was washed 3 times by adding 4 ml of 0.8% PBS, centrifuging at 200 g for 10 min, and discarding the supernatant. The cell pellet was then re-suspended in 1ml of 1:1 mixture of 0.8% PBS and elephant

plasma whereas Silva & Jain (1988) used PBS with 2% of bovine serum albumin. The average phagocyte percentage in extracted cell pellets was estimated using 5 aliquots from each pellet. The viability of harvested WBCs was estimated using Trypan blue (Strober 1997) stain with 5 aliquots per sample.

A pure colony of *E. coli* from an 18 h overnight culture was dissolved in 2 ml of 0.8% PBS and a series of two fold dilutions prepared. The optical density of each preparation was measured at 540 nm using a spectrophotometer. The viable bacterial count of each preparation was determined by the spread plate method (Silva *et al.* 1988). A curve was plotted with absorbance against bacterial count and the absorbance for 1×10^6 cfu/ μ l was calculated using the plotted curve. Opsonization of *E. coli* was done by mixing 200 μ l of prepared bacterial cell suspension with 200 μ l of elephant serum and incubating at 37°C for 30 min while slowly mixing every 5 min.

Phagocytocytosis was evaluated in two samples by the following procedure; 200 μ l of opsonized *E. coli* at 1×10^6 cfu/ μ l concentration was mixed with 200 μ l of phagocyte cell preparation at 2×10^4 / μ l concentration and incubated at 37°C for 30 min. Subsequently, the tubes were centrifuged, 5 smears prepared from each tube, air dried and stained with Leishman stain. A total of 500 WBC were counted per smear and the percentage of active phagocytic cells estimated – when stained with Leishman, engulfed bac-

Table 2. Phagocytic ability of isolated heterophils and monocytes.

Sample	Phagocytically active	
	Heterophils	Monocytes
1	41%	43%
2	39%	68%
Average	40%	55.5%

teria appear as rod shaped dark purple structures inside the phagocytic cells.

Results

A total of 31.4% – 66.2% of heterophils and monocytes present in the blood sample were harvested, with negligible contamination with red blood cells (Table 1) and 40% of heterophils (Fig. 1A, B) and 55.5% of monocytes (Fig. 1C, D) were able to phagocytize *E. coli* (Table 2).

Discussion

WBC counts

The normal range for total WBC counts in elephants is $4-21 \times 10^3$ per μ l (Silva & Kuruwita 1993). Therefore, the counts we observed were within the normal range, except in samples 3A and 3B. The elevated counts from this elephant may have indicated a sub clinical infection.

WBC extraction

We were able to extract on average 39.8% of the WBC in the blood samples used and isolate

Table 1. Differential WBC counts and percentage extracted.

Sample	Total WBC		Heterophils and monocytes in extract		
	Count in blood (per μ l)	% extracted	Count (per μ l)	% extracted from that in blood	Viability* (%)
1A	16.74×10^3	44.2	11.30×10^3	66.2	98
1B	15.90×10^3	36.0	6.05×10^3	33.0	100
2A	14.82×10^3	38.4	5.34×10^3	31.4	100
2B	17.23×10^3	47.4	10.44×10^3	48.1	100
3A	24.34×10^3	33.9	10.05×10^3	32.7	97
3B	24.34×10^3	35.4	12.23×10^3	39.8	100
4A	18.48×10^3	42.1	10.73×10^3	47.8	99
4B	15.60×10^3	41.3	10.04×10^3	47.3	100
Average		39.8		43.2	99.3

*Viability using Trypan blue stain

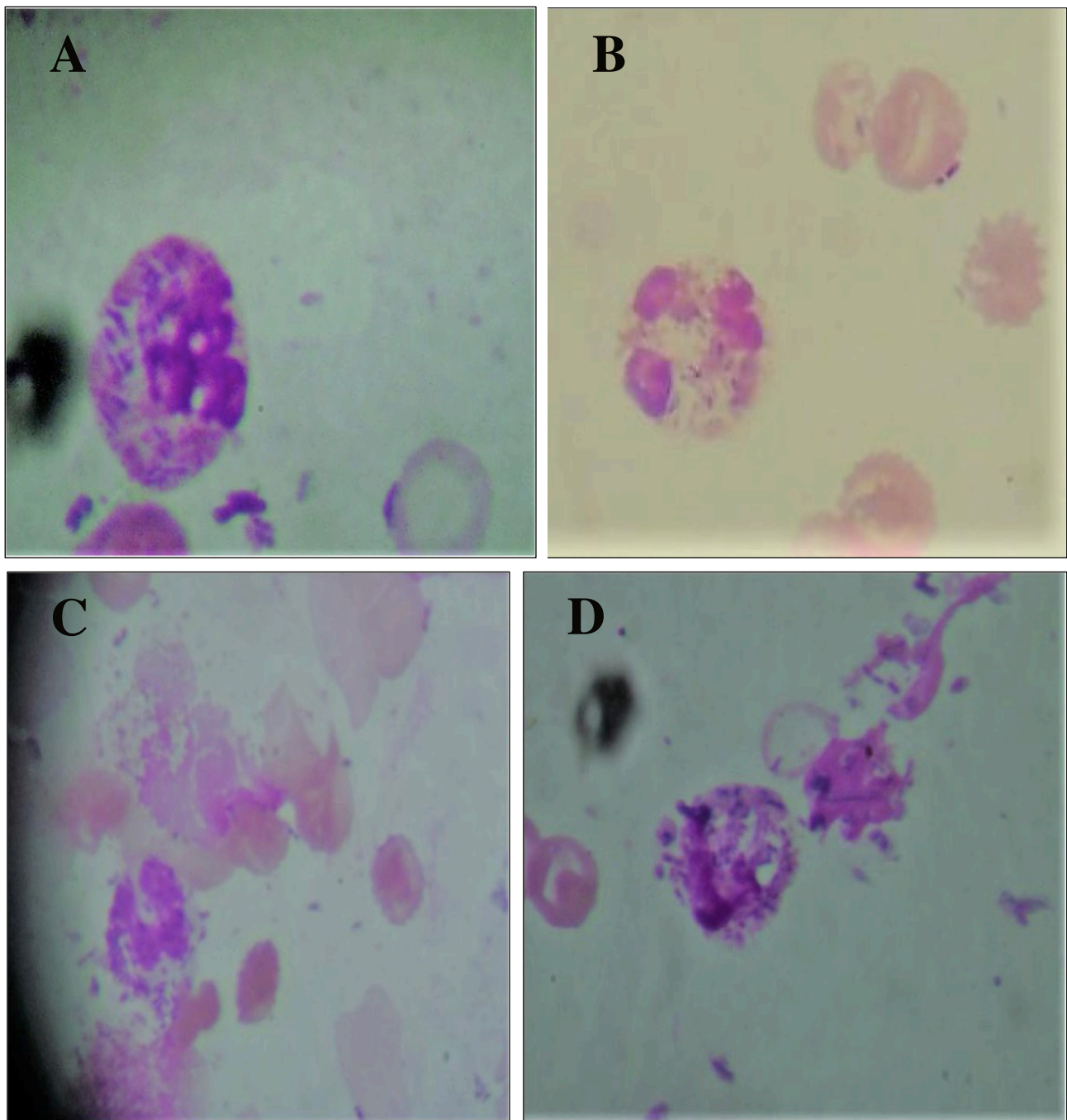


Figure 1. Heterophils (A, B) and monocytes (C, D) after *E. coli* phagocytosis.

43.2% of cells with phagocytic ability with 99.2% trypan blue viability. Smith *et al.* (1998) were able to extract 98–99% of heterophils in elephant blood, by using hetastarch sedimentation and percol gradient centrifugation. Therefore, our extraction method was less efficient but provided satisfactory proportions of cells with acceptable purity and viability. Our method was also relatively cheap and could be easily performed in a basic laboratory using common reagents and without any sophisticated equipment.

Phagocytosis

The ability to phagocytize bacteria by heterophils and monocytes isolated by us was somewhat lower than the 66–86% reported by Smith *et al.* (1998). Silva and Jain (1988) detected a phagocytic ability of 45–95% in extracted bovine neutrophils. It is possible that the differences in extraction and opsonization as well as the small sample size, could have had some influence on the

values observed by us. However, absolute values of phagocytosis are irrelevant and establishing the normal ranges of the parameters of interest with our method by applying it to a wider sample, will enable its subsequent clinical application. We conclude that the method described was effective in extracting WBCs from the blood of Asian elephants and assessing phagocytosis.

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