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The Impact of Camel Leukocytes Fixation on Cell Count and Monoclonal Antibodies Reactivity in Flow Cytometry

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ABSTRACT

Immunophenotyping of separated leukocytes is a common technique used to evaluate the changes in cellular immunity during clinical studies. For fixed cells or blood specimens infected with hazardous pathogens, cell fixation is performed before immunofluorescence. The impact of camel leukocytes fixation before staining on the reactivity of cell surface markers with monoclonal antibodies has not been investigated so far. The aim of the present study was, therefore, to compare cell staining of fixed and unfixed camel leukocytes with monoclonal antibodies to several cell surface antigens. Leukocytes were separated from camel blood and were fixed with paraformaldehyde (PFA) or left without fixation. Cells were labeled with monoclonal antibodies to several leukocyte antigens and the expression pattern of the antigens was compared between fixed and non-fixed cells using flow cytometry. The mean fluorescence intensity of each cell marker was calculated and compared between fixed and unfixed cells. Leukocyte fixation with PFA changed the binding activity of the monoclonal antibodies to CD163 and WC1 markedly, making it unable to stain any cell population. Although the cell staining efficacy of other molecules (such as CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45) was reduced, they were still able to define the target cells. The fixation-induced changes in the expression density of the analyzed monocytic markers may, however, lead to the misinterpretation of immunophenotyping studies of fixed monocytes or macrophages. Collectively, the obtained results indicated significant changes in the staining efficacy of monoclonal antibodies against several cell surface antigens of camel leukocytes, which should be considered when PFA-fixed cellular targets on camel leukocytes are to be analyzed.

Keywords: Antibodies, Cell fixation, Dromedary camel, Flow cytometry, Leukocytes

INTRODUCTION

The analysis of immune cell composition and phenotype has become an indispensable part of veterinary diagnostics and the evaluation of the animal immune status (Hussen and Schuberth, 2020; Hussen et al., 2020b). For such analysis, immune cells are identified based on their cell markers using monoclonal antibody labeling (Gaashan et al., 2020). In the dromedary camel, several cell marker antigens have been recently identified for the characterization of immune cell phenotypes. This mainly includes CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45 (Hussen and Schuberth, 2020). Immune cell testing usually includes separation of blood leukocytes for *ex vivo* phenotypic and functional analysis. One of the major challenges when analyzing the immunophenotype of cells is the time between sample collection and analysis, which should be abbreviated to avoid protein denaturation and loss of epitope antigenicity (Laurin et al., 2015). Cell fixation represents an alternative to extend sample age and preserve their morphological integrity (Ng et al., 2012; Qin et al., 2021). For intra-cytoplasmic antigens, fixation and permeabilization of the studied cells are required to access the target antigen by antibodies and to prevent cellular activity during the staining procedures (Paavilainen et al., 2010; Cheng et al., 2019) .

Paraformaldehyde (PFA), which works by cross-linking cellular proteins, is one of the most commonly used cellular fixatives (Cheng et al., 2019). It is mainly used, when stained cells are to be preserved for later analysis, or before cellular permeabilization to detect antigens inside the cells. For fixed histological specimens or blood specimens infected with hazardous pathogens, paraformaldehyde fixation is performed before immunological detection of epitopes (Schuberth et al., 1998). In this case, it is important that the antigenicity of the epitopes and the binding specificity of monoclonal antibodies are not affected by the fixation process. Studies in other species demonstrated different effects of fixative agents on the binding of antibodies to cell surface antigens. For porcine mononuclear cells, fixation with PFA affected the reactivity of 38 out of 134 positive reacting mAbs. The changes included reduced as well as elevated expression densities of the analyzed cell markers (Schuberth et al., 1998). Similarly, fixation of human leukocytes resulted in a marked decrease in forward and side scatter values with significant changes in the expression density of several cell surface antigens (Stewart et al., 2007). In a previous study on the fixation of human blood leukocytes, paraformaldehyde fixation after removal of red blood cells resulted in poor results in term of leukocyte cell count and

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staining with several cell markers (Ng et al., 2012). Fixation of human mononuclear cells before staining especially reduced the cell staining density with antibodies to the cell antigens CD8, CD19, CD16, and CD56 (Pinto et al., 2005).

As no previous studies investigated the effect of cell fixation on the reactivity of camel leukocytes to monoclonal antibody staining, the present work employed flow cytometry to report on the binding activity of selected monoclonal antibodies on camel blood leukocytes fixed with paraformaldehyde.

MATERIALS AND METHODS

Ethical Approval

The study was approved by the Ethics Committee of King Faisal University, Saudi Arabia (Approval no. KFU-REC/2020-09-25).

Animals and blood sampling

Six apparently healthy dromedary camels (housed at the farm of the Camel Research Center, King Faisal University, Al-Ahsa, Saudi Arabia) were used for blood sample collection. The animals were non-lactating female camels from Almajaheem breed aged between 8 and 10 years. Blood sampling was performed by venipuncture of the jugular vein into EDTA tubes. The ambient temperatures during blood collection from the animals and during cell separation and staining in the lab were 34°C and 24°C, respectively.

Cell separation

Leukocytes isolation from blood was done within one hour after blood collection by inducing erythrolysis. For this 1 ml of blood was incubated (at room temperature) in 5 ml of distilled water for 20 seconds and 5 ml of double concentrated phosphate-buffered saline (PBS) were added. In case of no complete lysis, the procedure was repeated. Before fixation, the cells were washed two times in PBS (500 ×g, 250 × g, 10 minutes, 10°C). Separated leukocytes were finally suspended in staining buffer (PBS containing 5 g/l BSA, 100 mg /l NaN_3) at 5 x 10^6 cells/ml (Gaashan et al., 2020).

Cell fixation

For the preparation of a 4% PFA solution, 4 g PFA (Roth, Karlsruhe, Germany) were dissolved in 50 ml of distilled water at 60°C for 30 min. After mixing, 50 ml double concentrated PBS was added to the solution. Cell fixation was performed as described previously (Schuberth et al., 1998). Briefly, separated cells were incubated with PFA solution for 15 minutes at room temperature (RT) and with constant rotation. After that, the fixed cell sample was washed two times in PBS (250 × g, 3 minutes, RT) and finally, resuspended in staining buffer for antibody labeling.

Immunolabeling and flow cytometry

For cell staining the wells of a round-bottomed 96-well microtiter plate were filled with 5 x 10^5 leukocytes (100 µl). In the first labeling step, primary monoclonal antibodies (mAbs) to the cell surface molecules, CD4, WC-1, CD14, CD163, CD172a, MHC-II, CD11a, CD18, CD44, and CD45 (Hussen and Schuberth, 2020) were added to the wells followed by incubation for 15 minutes. After two washings with staining buffer, the second staining step was done by adding fluorochrome-labeled antibodies to mouse IgM, IgG1, and IgG2a (Invitrogen), and the plate was incubated for 15 minutes in the dark. Staining with isotype controls was also included. After two washings, labeled cells were analyzed by flow cytometry (Accurie C6 flow cytometer from BD Biosciences). Data analysis was performed using the CFlow Software (V 1.0.264.21; BD Biosciences). The Neubauer counting chamber was used to estimate the total number of leukocytes in blood as previously described (Hussen, 2021b). For this, a blood sample was stained with Türk solution (1 to 10 dilution) and a stained sample was counted under the microscope. All incubation and centrifugation steps were performed at 4°C. Cell washing was done using staining buffer and centrifugation of the plates at 250 ×g for 3 minutes.

Statistical analysis

The statistical software program GraphPad Prism was used for calculating column statistics including minimal (min) and maximal (max) values, mean, and SEM. The paired student's t-test was used to compare the means for each parameter. A p-value less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The current work evaluated the extent to which PFA fixation of camel blood leukocytes before staining affects the cell count and staining of leukocyte subsets with monoclonal antibodies to cell surface molecules by flow cytometry (Figure 1).

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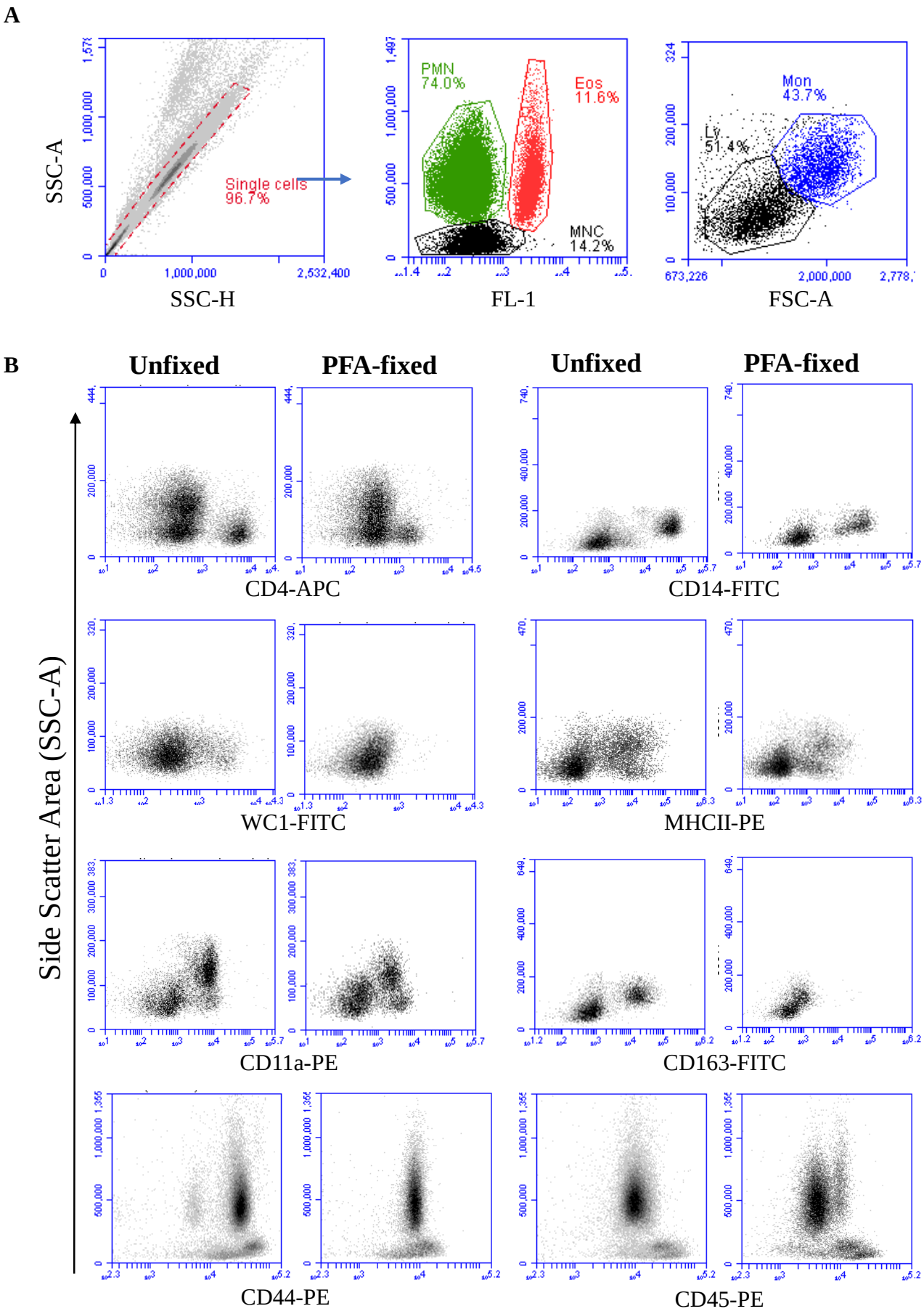

Figure 1. Flow cytometric analysis of cell staining with cell surface markers. **A**: Gating scheme used for the analysis of neutrophils (PMN), eosinophils (Eos), mononuclear cells (MNC), and their subpopulations, monocytes (Mon) and lymphocytes (Ly). After the exclusion of cell duplicates (using SSC-H against SSC-A), PMN, Eos, and MNC were gated based on SSC-A and autofluorescence (Eos). After selecting MNC, lymphocytes, and monocytes were identified in a separate dot plot. **B:** Representative dot plots for staining patterns of selected monoclonal antibodies on unfixed and PFA-fixed cells.

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Fixation-induced changes in forward and side scatter characteristics of camel leukocytes

PFA fixation induced a marked ($p < 0.05$) shift (increase) in the SSC and FSC of neutrophils, eosinophils, and monocytes (Table 1). The FSC signal was increased for neutrophils and eosinophils, while decreased for monocytes and lymphocytes ($p < 0.05$).

Impact of PFA fixation on the composition of camel leukocytes

Percentages of gated neutrophils, eosinophils, lymphocytes, and monocytes of unfixed and PFA-fixed cells are shown in Table 1. The analysis of relative leukocyte composition revealed significantly ($p < 0.05$) higher percentage and numbers of monocytes in samples fixed with PFA compared to unfixed cells (Table 1). For neutrophils, eosinophils, and lymphocytes both relative and absolute counting of cells revealed comparable ($p > 0.05$) values between fixed and unfixed samples (Table 1 and Figure 2). The comparative analysis of lymphocyte composition between PFA-fixed and unfixed samples revealed lower percentages and numbers of B cells ($p < 0.05$) and γδ T cells ($p < 0.05$), while the number of helper T cells (Th) did not change significantly after fixation (Figure 2). However, the effect of fixation on the staining of γδ T cells was significantly stronger altering the WC1 epitope; therefore, it could no longer detected within fixed lymphocytes by anti-WC1 antibodies.

Table 1. Impact of camel blood leukocyte fixation on cell composition and phenotype

		Control leukocytes				Fixed leukocytes				P value
		Min	Max	Mean	SEM	Min	Max	Mean	SEM	
WBC sub (% of WBC)	PMN	63.0	73.9	68.5	1.8	59.8	70.4	66.3	1.9	0.07
	Eos	4.6	13.5	6.8	1.7	5.5	11.4	8.0	1.1	0.36
	Ly	8.1	16.7	13.9	1.6	7.9	18.3	14.6	1.8	0.19
	Mon	4.2	11.2	6.4	1.2	6.2	14.3	9.3	1.3	0.001
WBC sub (cell/µl)	PMN	5128	10053	6959	849	4913	9445	6718	782.5	0.10
	Eos	420.4	1295.0	670.8	161.7	414.2	1295.0	825.0	160.7	0.34
	Ly	777.6	1791.0	1390.0	176.9	759.4	1852.0	1447.0	189.3	0.15
	Mon	402.8	1030.0	626.8	106.5	717.4	1311.0	908.2	103.6	0.001
Ly sub Percentage of lymphocytes)	Th cell	19.1	28.4	22.6	1.9	16.7	32.0	24.2	1.8	0.43
	γδ T cell	3.1	8.6	6.6	1.0	0.1	0.3	0.2	0.04	0.001
	B cell	10.1	29.3	22.6.2	3.0	7.7	22.0	17.6	2.7	0.01
Ly sub (cell/µl)	Th cell	157.9	435.3	357.4	52.3	232.0	447.0	325.4	48.3	0.21
	γδ T cell	24.1	131.8.8	97.6	21.4	1.5	4.2	3.0	0.5	0.01
	B cell	153.6	524.1	315.4	52.9	117.2	407.5	266.2	55.6	0.04
SSC (MFI)	PMN	405627	479906	450361	12917	512356	596505	551713	14011	0.001
	Eos	441870	502519	474379	10388	557446	584669	571546	5265	0.001
	Ly	68855	71479	70335	502	68798	73900	70379	907.1	0.95
	Mon	131068	134548	132770	606	136538	148587	144267	2623.0	0.02
FSC (MFI)	PMN	1704000	1838000	1764000	22942	1921000	1986000	1946000	13183	0.001
	Eos	1486000	1620000	1548000	24319	1732000	1902000	1808000	29057	0.001
	Ly	1362000	1419000	1393000	9180	1308000	1324000	1318000	3704	0.001
	Mon	1947000	2029000	1976000	13997	1370000	1578000	1460000	33983	0.001
CD11a	PMN	1297.0	1397.0	1353.0	16.7	530.0	685.0	590.0	26.7	0.001
	Ly	580.0	1353.0	963.6	156.3	367.0	511.0	442.6	31.5	0.01
	Mon	5146	6755	6094	311	1995	2370	2197	61.5	0.001
CD18	PMN	15082	18880	17134	727	9545	11143	10494	291	0.001
	Ly	5773	11191	7866	1141	1609	2849	2247	198	0.01
	Mon	35606	45635	41786	1877	16429	22752	19290	1048	0.001
CD44 (MFI)	PMN	22188	33041	26855	1786	6322	8267	7361	310	0.001
	Eos	361	12121	5922	2509	6510	8543	7343	367	0.60
	Ly	13139	21689	17567	1391	4696	7268	5946.4	408	0.001
	Mon	44664	53220	49494	1422	10371	12828	11334	438	0.001
CD45 (MFI)	PMN	9959	17506	14259	1412	4173	7502	6023	601	0.001
	Eos	11064	14640	13166	735	4340	6105	5274	344	0.001
	Ly	20343	36882	27098	2735	12781	19524	15356	1160	0.001
	Mon	27611	51514	40851	4490	11153	19902	15942	1538	0.001
CD172a (MFI)	PMN	40025	48093	43316	1322	4340	6105	5274	344	0.001
	Eos	16246	19698	17778	575	12781	19524	15356	1160	0.01
	Mon	54180	80112	63118	4431	11153	19902	15942	1538	0.001
CD163 (MFI)	Mon	16202	26508	19349	1984	23558	26322	25040	474	0.001
MHC-II (MFI)	B cell	4692	6778	5349	391	14080	16153	15691	403.1	0.001
	PMN	93.0	115.0	106.0	5.1	31062	38029	33981	1187	0.68
	Mon	3302	6585	4958	568	841	1140	927	56	0.001
CD14 (MFI)	PMN	11348	13824	12735	549	556	1380	1102	164	0.001
	Mon	46887	55130	49436	1465	106.0	110.0	108.2	0.9	0.001

WBC: White blood cells, PMN: Neutrophils, Eos: eosinophils, Mon: Monocytes, Ly: Lymphocytes, Sub: Subsets, MFI: Median fluorescence intensity, Th: T helper cell, γδ: Gamma delta T cell, MHC II: Major histocompatibility class II molecules

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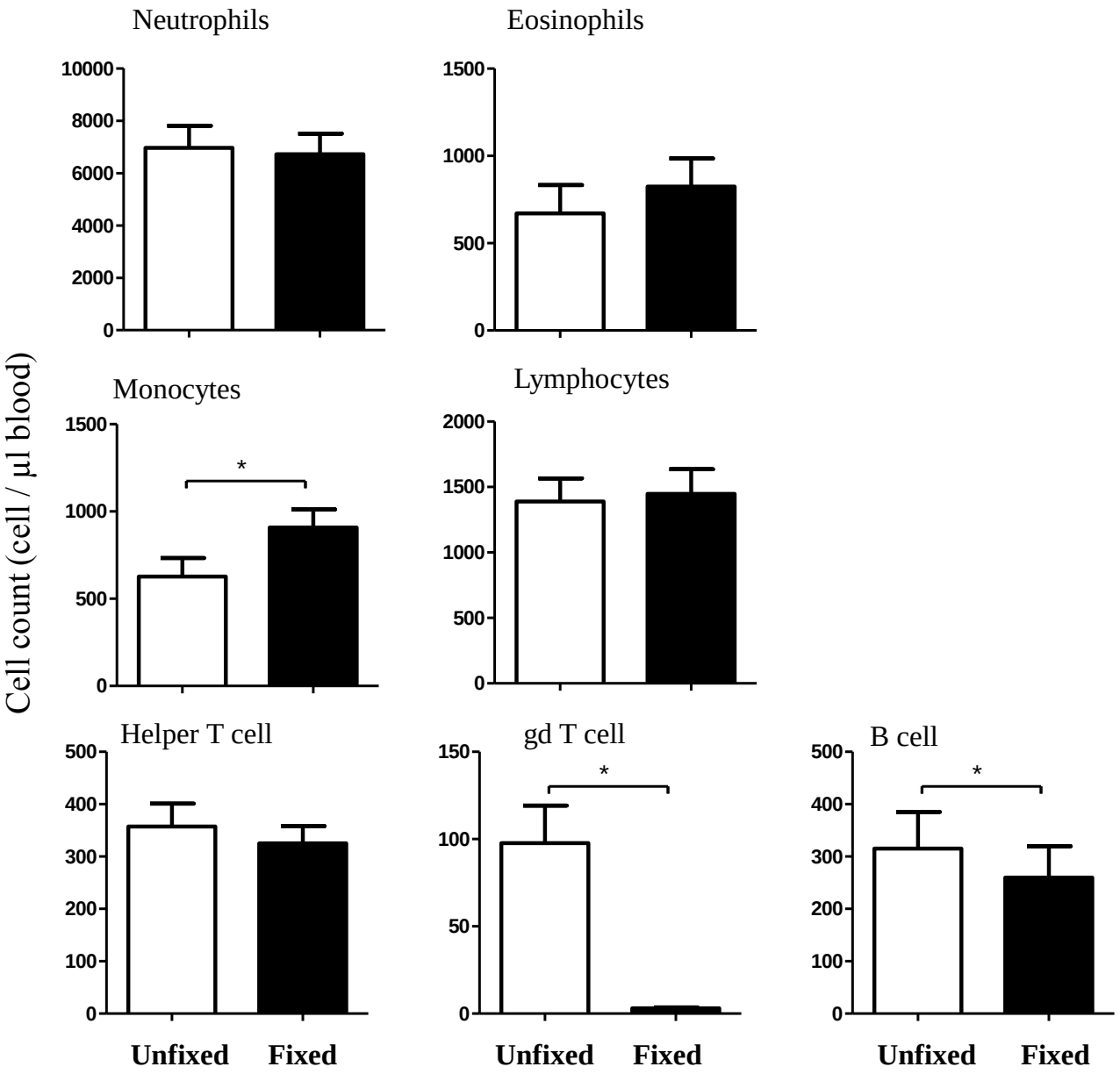

Figure 2. The impact of cell fixation on camel leukocyte composition. The absolute cell numbers of major leukocyte populations and lymphocyte subsets in blood from dromedary camels were calculated by using standard clinical hematology laboratory procedures. Data for unfixed and fixed cells were presented as mean and standard error of the mean. Differences between the means were calculated using the t-test and were considered significant (*) if $p < 0.05$.

PFA fixation affected the staining activity of adhesion and myeloid marker molecules

The abundance of the cell markers (as measured by the mean fluorescence intensity of stained cells) CD11a, CD18, CD44, and CD45 was significantly reduced after PFA fixation of leukocytes ($p < 0.05$). Although the staining efficiency of the monoclonal antibodies to all monocytic markers was reduced on PFA-fixed cells, the strongest reduction in fluorescence intensity was seen for CD163 molecules expression on monocytes (twenty times signal reduction) (Table 1).

During recent years, several camel myeloid and lymphoid immune cell populations and subpopulations have been characterized using membrane antibody staining of fresh-separated blood leukocytes and flow cytometric analysis (Hussen and Schuberth, 2020; Hussen, 2021a; Hussen, 2021b). For fixed histological specimens or for blood specimens infected with hazardous pathogens, however, cell fixation is performed before immunological detection of epitopes (Schuberth et al., 1998; Jamur and Oliver, 2010). In this case, it is important that the antigenicity of the epitopes and the binding specificity of monoclonal antibodies are not affected by the fixation process (Suthipintawong et al., 1996). In the current work, the impact of PFA fixation of camel blood leukocytes before staining on the cell count and staining of leukocyte subsets with monoclonal antibodies to cell surface molecules was evaluated by flow cytometry.

The observed changes in side scatter (SSC) and forward scatter (FSC) properties of fixed leukocytes indicate a significant fixation-induced shape-change effect for all leukocyte populations. As the major leukocyte populations are sometimes identified according to their shape, this effect should be considered when gating PFA-fixed camel leukocytes

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based on FSC and SSC properties. In addition, forward scatter and side scatter correlate with the cell size and granulation, respectively (MacDonald and Zaech, 1982; Stern et al., 2017). Therefore, the fixation-induced change in FSC and SSC values may be mistakenly interpreted as cell activation or degranulation of camel leukocytes. Fixation-induced shape-changes have been reported for human PBMC or whole blood samples fixed with PFA before or after staining with monoclonal antibodies (Pinto et al., 2005).

The comparable cell count of camel neutrophils, eosinophils, and total lymphocytes argues for a stable leukogram after PFA fixation. However, the higher percentage and numbers of monocytes in samples fixed with PFA compared to unfixed cells should be considered during the analysis of fixed samples. In addition, further studies are required to investigate the impact of PFA fixation on the composition of monocytes to see whether distinct monocyte subsets are affected by this increase (Hussen et al., 2020a). Although the number of lymphocytes was not affected by PFA fixation, the fixed lymphocyte population contained lower percentages and numbers of B cells and γδ T cells indicating an epitope-specific effect of PFA fixation. Studies on human lymphocytes reported no effect of PFA-fixation on total lymphocyte count or the number of their subsets (Pinto et al., 2005). The differences between camel and human lymphocytes may be due to different susceptibility to paraformaldehyde treatment, which should be evaluated in future comparative studies.

Testing of the monoclonal antibodies on unfixed and PFA-fixed cells showed different effects of cell fixation on the cell surface antigens with the CD163 staining being markedly reduced. It is well recognized that PFA fixation may differently affect cell staining with certain molecules being changed stronger than others (Tanaka et al., 2010). PFA fixation of human whole blood induced a higher decrease in the labeling efficiency of integrins than other cell surface antigens (Bateman et al., 1993; Celie et al., 2005).

CD14, CD172a, CD163, and MHCII are well-established markers of monocyte and macrophage polarization (Hussen and Schuberth, 2017). CD14 is a cell surface marker with an expression on monocytes, macrophages and to a lower extent on neutrophils (Ibeagha-Awemu et al., 2012). CD14 acts as a co-receptor for the cell-wall component of gram-negative bacteria, LPS. The CD172a, which is also called the signal regulatory protein alpha with inhibitory potential on cell activity, is a myeloid marker expressed on blood granulocytes and monocytes (Oronsky et al., 2020). MHCII is an antigen presentation receptor with an essential role in presenting peptide antigens to helper T cells (Busch et al., 2000). In the present study, the fixation-induced change in the expression density of the analyzed monocytic markers may lead to misinterpretation of immunophenotyping studies of fixed monocytes or macrophages. In addition, the significant change in the abundance of the cell adhesion molecules CD18, CD11a, and CD44 could result in the biased interpretation of the adhesion and migration capability of blood leukocytes (Harjunpaa et al., 2019).

CONCLUSION

Collectively, the results of the present study indicate significant changes in staining efficacy of monoclonal antibodies against several cell surface antigens after fixation of camel leukocytes. The results of the present study indicate that leukocyte fixation with PFA may change the antigenic structures of some cell surface molecules like CD163 and WC1, while only reducing the antigen-antibody binding efficacy for other molecules like CD14, CD172a, MHCII, CD11a, CD18, CD44, and CD45. Further studies are required to investigate the impact of sample storage time and temperature on the observed effects of PFA fixation on camel leukocyte composition and phenotype.

DECLARATIONS

Authors' contribution

HA collected the samples and wrote the original manuscript. GAA performed the flow cytometric analysis and wrote the manuscript. FHA prepared the samples for cell staining and did the data analysis. JH acquired the funding, analyzed the flow cytometric data, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors have not declared any conflict of interest.

Ethical consideration

The authors declare that the manuscript has not been published before and is not currently being considered for publication elsewhere.

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