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Association Between Serum Cortisol Level and Selected Immune Cell Parameters in Dairy Cows

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Abstract | The immune response to stress has been investigated in several veterinary species including cattle after exposing the animals to different stressful conditions such as heat stress, transport stress, and stress at slaughterhouse. Given the reported association between cortisol levels and the functionality of several body systems in dairy cattle, the present study aimed to investigate the association between serum cortisol levels in dairy cows and leukocyte composition, frequency of lymphocyte subsets, frequency of monocyte subsets, and the expression level of selected leukocyte surface molecules. A total number of 20 non-pregnant dairy cows aged between 4 and 10 years were included in the study. The animals included 9 lactating and 11 dry cows. Surprisingly, no difference in cortisol levels was observed between lactating (20.7 ± 4.1 ng/mL) and non-lactating (20.6 ± 3.1 ng/mL) cows ($P > 0.05$), which contrasts previous reports. Similarly, no correlation ($r = 0.25$; $P > 0.05$) was found between cortisol level and animal age. Cortisol levels were found to be associated with significant changes in some immune cell parameters in dairy cows. Animals with elevated cortisol levels (defined as > 15 ng/mL) showed a decreased frequency of CD4+ T helper cells ($r = -0.75$, $p < 0.05$) and a reduced CD4/CD8 ratio ($r= -0.62$, $p < 0.05$). In contrast to this, a positive correlation was found between cortisol level and the number of CD8+ cytotoxic T cells ($r = 0.49$, $p < 0.05$). On the other hand, there was no significant association between elevated cortisol levels and changes in other immune cell parameters, such as neutrophil, monocyte, monocyte subsets, Yδ T cells, B cells, and NK cells frequency, or the expression levels of several cell surface markers. These results indicate compromised immune activity in cows with elevated cortisol levels, highlighting the need for further studies on the impact of elevated cortisol levels on the immune response to infection or vaccination.

Keywords | Dairy cow, Cortisol, CD8+ T cells, CD4+ T cells, Flow cytometry

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INTRODUCTION

Various stress markers have been characterized in several species with cortisol being the most reliable one (Garcia-Torres *et al.*, 2021; Grelet *et al.*, 2022; Ataallahi *et al.*, 2023). Serum cortisol concentrations can be affected by several stressful and physiological conditions. Transportation; intensive housing systems; environmental heat and cold stress are among the common factors that are responsible for stress in livestock animals (Bova *et al.*, 2014;

Disanto *et al*., 2014; Proudfoot and Habing, 2015; Earley *et al*., 2017; Dahl *et al*., 2020; Kim *et al*., 2021; Ataallahi *et al*., 2023; Tufekci and Sejian, 2023). Additionally, cortisol levels may differ according to breed, gender, age, pregnancy, parturition, and lactation status (Garcia-Belenguer *et al*., 1996; Plusquellec and Bouissou, 2001; Pena *et al*., 2014; Sazmand *et al*., 2017; Olivan *et al*., 2018).

The hypothalamic-pituitary-adrenal (HPA) axis is responsible for regulating stress response and the secretion of cortisol (Knezevic *et al*., 2023). Activation of the HPA axis by stressful stimuli induces the release of corticotrophin-releasing factor by the hypothalamus leading to stimulation of the adenohypophysis to secrete adrenocorticotropic hormone (ACTH) in the circulation and the secretion of cortisol by the adrenal gland. Once passing the cell membrane, cortisol binds to specific cortisol receptors in the cytoplasm exerting many functions in several body systems. This includes mediating the stress response, regulating several metabolism pathways, and modulating the inflammatory response and the immune function (Caroprese *et al*., 2010). In the immune system, cortisol induces cell apoptosis of inflammatory cells, suppress the function of T cells, and B cell antibody production, and reduces neutrophil adhesion and migration during inflammation and infection (Morey *et al*., 2015).

The immune response to stress in cattle was investigated under several stress conditions. The leukogram of cattle under shipping stress was characterized by leukocytosis and neutrophilia with a decrease in lymphocyte numbers and change in the frequency of lymphocyte subsets (Blecha *et al*., 1984; Pagliasso *et al*., 2023). On the other hand, heat stress has been linked to negative effects on the bovine innate and adaptive immune system including both the humoral and cell-mediated responses (Bagath *et al*., 2019; Dahl *et al*., 2020; Gupta *et al*., 2022). Several studies reported a higher susceptibility to infectious diseases in stressed cattle, which was related to the decrease in the number and functionality of T cells leading to compromised immune response (Blecha *et al*., 1984; Murata and Hirose 1991; Earley *et al*., 2017).

Several studies reported the impact of elevated cortisol levels on health and productivity of dairy cows (Ataallahi *et al*., 2023; Otten *et al*., 2023). Studies on the impact of elevated cortisol levels on the immune system of dairy cows are still limited. The present study was undertaken to investigate the association between serum cortisol levels in dairy cows and leukocyte composition, frequency of lymphocyte subsets, frequency of monocyte subsets, and the expression level of selected leukocyte surface molecules. The results of the current study would support the identification of immune markers associated with early stress response in dairy cattle.

MATERIALS AND METHODS

Animals

The study was conducted between November 2023 and February 2024. This study was granted approval by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2023-SEP-ETHICS1372). A total number of 20 dairy cows of the Friesian Holstein breed kept on the University Research Farm of King Faisal University in Al-Hassa Region in Saudi Arabia was included in this study. The Al-Ahsa region is an arid region (N 25°17′8.0844″, E 49°29′11.3316″) with a desert climate characterized by a long hot and dry summer (April to November) with temperatures between 40°C and 50°C and a short moderate winter (December and January) with temperatures between 15°C and 29°C. The animals were kept in a wire-fenced facility and fed with a diet of 50% barley silage and 50% concentrate mix. The age of the cows ranged between 4 and 10 years with a mean age of 5.8 ± 0.4 years (mean ± SEM). No drugs or vaccines were given to the animals directly before or during the experimental period. All the animals were clinically healthy without any signs of disease (based on clinical examination by a trained veterinarian). All the animals were non-pregnant with 9 lactating cows (in mid-lactation) and 11 dry cows. Although the animals were not under a specific stress condition during the study period, they could have been affected by the high ambient temperatures during the previous hot season. The cows were classified based on their serum cortisol levels into cortisol-low animals (serum cortisol ≤ 15 ng/mL) and cortisol-high (serum cortisol > 15 ng/mL) groups. This classification was based on the distribution of values around the mean cortisol concentration of the whole population.

Blood Collection and Separation of Serum

Blood samples (10 mL) were collected from the jugular vein in EDTA-containing tubes (for cellular analysis) or serum collection tubes (for cortisol measurement) (Guangzhou Improve Medical Instruments Co., Ltd; Guangzhou, China) and transported within 2 hours to the laboratory. Serum collection was performed by centrifugation of blood at 1000 xg for 15 minutes at room temperature (20-22°C). Separated serum samples were stored at -80°C until analysis. For all animals, blood samples were collected between 7:00 and 9:00 am (one hour after feeding) to avoid variations due to the circadian regulation of cortisol.

Enzyme-linked Immunosorbent Assay for the Measurement of Serum Cortisol Levels

A commercial enzyme immunoassay (DRG® Cortisol ELISA Kit) was used for quantitative analysis of serum cortisol according to the guidelines of the manufacturer (The, DRG, Springfield, NJ 07081 USA). To do so,

20 µL serum sample was added together with 100 µL of the kit conjugate (horseradish peroxidase-conjugated cortisol) followed by incubation for 1h at room temperature. After incubation, the plate was washed three times using ELISA washing buffer followed by adding 100 µL of 3,3′,5,5′-Tetramethylbenzidine (TMB) ready-to-use substrate and chromogen solution. After 20 minutes of incubation at room temperature in the dark, the reaction was stopped by the addition of 100 µL of the stop solution (0.16M sulfuric acid). Finally, the optical density values were measured using ELISA reader (iMark Bio-Rad laboratories). For each plate, a standard curve was prepared from different standard Cortisol concentrations. The assay has a sensitivity of 1.0 ng/mL (6.0 nM/L) and a detection range between 1.3 and 800 ng/mL. Sample Cortisol concentrations were calculated using a standard semi-logarithmic curve fit using the following equation:

$$Y=Yintercept + Slope \times log(X)$$

The concentrations of standard cortisol (X) were expressed as logarithmic values, while the optical density (Y) as linear value. Slope is the change in Y when the log(X) changes by 1.0. Yintercept is the Y value when X equals 1.0.

Cell Separation for Flow Cytometry

Blood leukocytes were separated by hypotonic lysis of erythrocytes followed by centrifugation as previously described. Briefly, EDTA blood (1 mL) was incubated for 20 seconds with 5 mL distilled water in a 15 mL sterile falcon tube to induce red blood cell lysis. Subsequently, tonicity was restored by the addition of 5 mL 2x PBS and the tube was centrifuged at 1000 xg for 10 min at 10°C. The lysis step was repeated twice followed by centrifugation at 500 xg and 300 xg for 10 min each. Finally, the pellet was resuspended in cold PBS and adjusted to 1 x 10^6 cell / mL.

List of Used Antibodies

The list of used antibodies is shown in Table 1.

Flow Cytometry Analysis of Leukocyte Subsets

Cell staining was performed as previously described (Hussen *et al*., 2013). Briefly, isolated WBC (1 x 10^6 cell/well) were incubated in a 96-well plate for 15 min at 4°C with monoclonal antibodies to the cell surface antigens shown in Table 1. Labeled cells were analyzed by flow cytometry (Becton Dickinson Accuri C6 flow cytometer; Becton Dickinson Biosciences, San Jose, California, USA).

Statistical Analysis

Means and standard error of the mean (SEM) were calculated using the column statistic function of the Prism software (GraphPad). Data normality was calculated using the Shapiro-Wilk test. The comparison between groups was performed using unpaired student's t test.

Table 1: Monoclonal antibodies.

Antigen	Antibody clone	Labeling	Source	Isotype
CD3	MM1A		Kingfisher	Mouse IgG1
CD4	GC50A1	-	Kingfisher	Mouse IgM
CD8	CC63	-	Biorad	Mouse IgG2a
WC1	CC15	FITC	Biorad	Mouse IgG2a
CD335	EC1.1	-	Kingfisher	Mouse IgG1
CD62L	BAQ92A		Kingfisher	Mouse IgG1
LFA-1	BAT75A		Kingfisher	Mouse IgG1
Mouse IgM	poly	APC	Thermofisher	Goat IgG
Mouse IgG1	poly	FITC	Thermofisher	Goat IgG
Mouse IgG2a	poly	PE	Thermofisher	Goat IgG

CD: cluster of differentiation; **WC1:** workshop cluster 1; **NK:** natural killer cell; **LFA:** lymphocyte function associated antigen; **FITC:** fluorescein isothiocyanate; **PE:** phycoerythrin; **APC:** allophycocyanin.

RESULTS AND DISCUSSION

Early detection of stress is one of the most effective strategies to compact stress-induced effects on animal health and production. The reliability of serum cortisol level as an effective indicator of stress in cattle was proven in numerous studies (Dhama *et al*., 2019; Fuchs *et al*., 2024). The impact of stress on animal physiology was intensively studied in the literature with stress-induced changes in many body systems including the immune system (Vitela-Mendoza *et al*., 2016; Grelet *et al*., 2022). In the present study, cortisol levels were measured in serum from dairy cows and the association between serum cortisol levels and several immune cell parameters in blood was evaluated (Supplementary Table 1).

In the present study, the concentration of cortisol in serum from healthy dairy cows ranged between 7.0 and 41.0 ng/mL with a mean of 20.6 and a standard error (SE) of 2.4 ng/mL (20.6 ± 2.4). The comparison between samples collected from lactating (20.7 ± 4.1 ng/mL) and non-lactating cows (20.6 ± 3.1 ng/mL) revealed no significant differences between the two groups (Figure 1) ($P > 0.05$). Likewise, there was no correlation between animals age and cortisol level ($r = 0.25$). A wide range of serum cortisol concentrations was reported in the scientific literature for healthy bovine. Vitela-Mendoza *et al*. (2016), reported a mean plasma cortisol concentration of 2.5 ± 1.8 ng/mL (SE) in dairy cows, while Villa *et al*. (2021) measured an average concentration of 6.8 ng / mL with individual values ranging between 0.7 and 35.4 ng / mL. Although cortisol concentrations measured in the present study are within the normal ranges reported by several studies, other researchers reported higher cortisol levels in serum from healthy cattle (Rafa *et al*., 2024). The extent to which this variability in

cortisol levels is related to the different analytical methodologies used in various studies remains to be addressed in future studies. Cortisol levels have been shown to be affected by several physiological factors including animals' gender, age, and reproductive status (Barra *et al.*, 2015; Vrijkotte *et al.*, 2023; Chai *et al.*, 2024). Given that all animals involved in the present study were non-pregnant cows, the potential influence of gestation on serum cortisol levels could not be assessed. Nevertheless, the analysis of blood cortisol concentrations in both lactating and non-lactating cows indicated no significant effect of lactation status on serum cortisol concentration. Furthermore, the limited age spectrum of the animals with all cows being between 4 and 10 years of age, was reflected by the absense of any correlation between age and cortisol level. Furthermore, as the cows involved in the present study were not under induced stress, the conclusion that serum cortisol is not affected by age and lactation status needs validation through further studies including animals exposed to stressful stimuli.

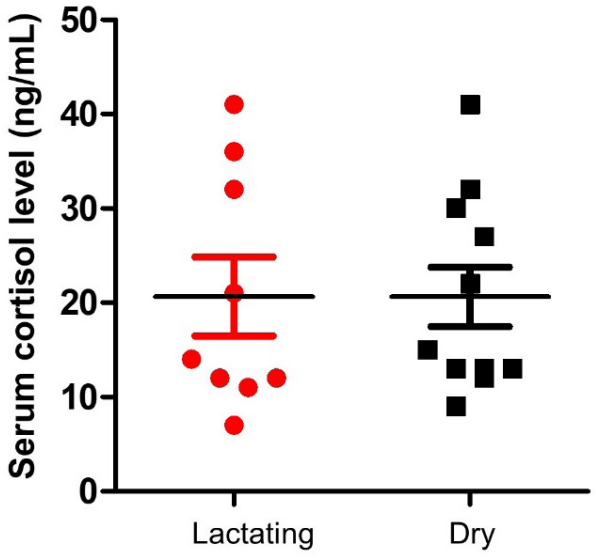

Figure 1: Analysis of serum cortisol levels in dairy cows. Separated serum samples collected from healthy cows were analyzed for cortisol concentration using ELISA. Cortisol concentrations were calculated based on a standard curve generated using known cortisol concentrations. Cortisol levels were presented for lactating and dry cows as scattered dot plot graph.

The immune cell composition of blood leukocytes was analyzed by flow cytometry (Figure 2). Table 2 presents the results of correlation analysis between serum cortisol concentration and selected immune parameters. There was no correlation ($r = -0.03$; $p > 0.05$) between the serum cortisol level and the total number of leukocytes, the relative percentages or the absolute numbers of the main leukocyte populations (neutrophils, eosinophils, monocytes, or lymphocytes) (Table 2). However, the analysis of lymphocyte composition revealed significant association between cortisol level and the abundance of distinct subsets in blood. The percentage of CD4+ T cells was negatively associated with serum cortisol level ($r = -0.75$). In contrast, the percentage ($r = 0.58$) as well as the absolute numbers of CD8+ T cells ($r = 0.48$) were positively correlated with serum cortisol level ($p < 0.05$). On the other hand, no association was found between cortisol levels and the frequency of Yδ T cells ($r = 0.38$; $p > 0.05$), Yδ T cells ($r = 0.26$; $p > 0.05$), B cells ($r = -0.20$; $p > 0.05$), or NK cells ($r = 0.20$; $p > 0.05$).

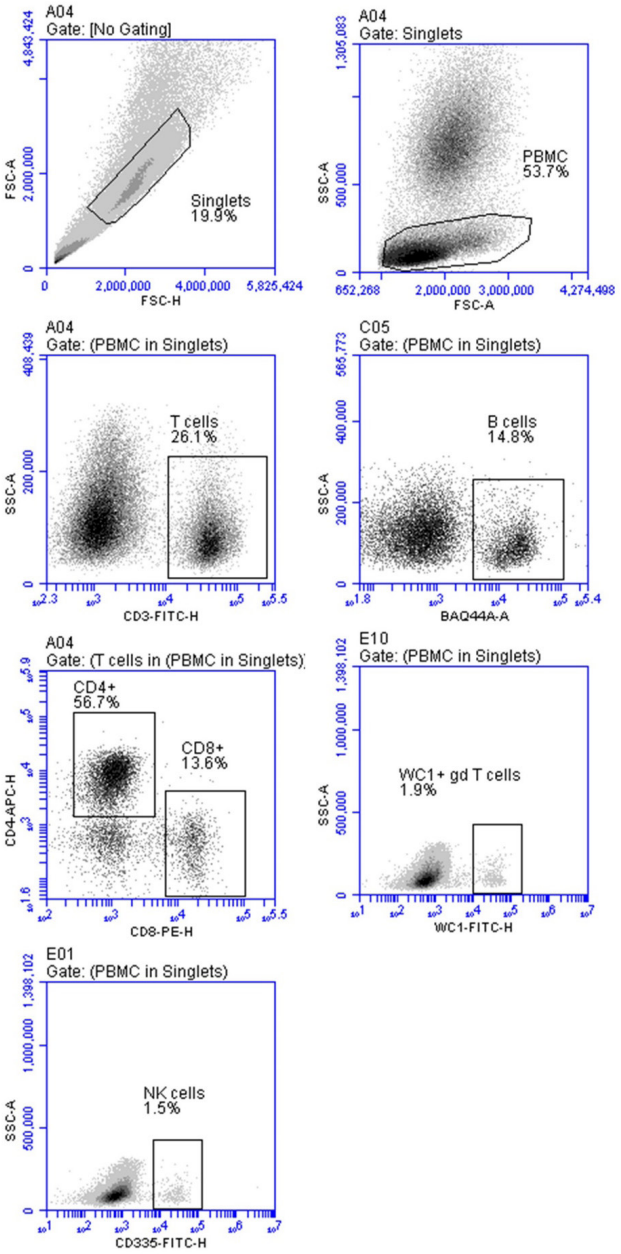

Figure 2: Gating strategy for the identification of lymphocyte subsets in bovine blood. Leukocytes were separated from cow blood, labeled with antibodies to cell surface antigens and analyzed by flow cytometry. After exclusion of cell duplicates based on forward scatted (FSC) properties, a gate was set on mononuclear cells (PBMC) based on FSC and side scatter characteristics. The populations of T cells, B cells, Yδ T cells, NK cells, CD4+ T helper cells, and CD8+ cytotoxic T cells were identified based on monoclonal antibody staining.

Table 2: The correlation analysis between serum cortisol concentration and selected immune parameters in dairy cows.

	Parameter	Number of XY Pairs	Spearman r	P value (two-tailed)	Is the correlation significant?
Leukocyte composition	WBC (10^3Cell / µL)	21	-0.03201	0.8905	No
	Neutrophils (%)	21	0.07336	0.752	No
	Eosinophils (%)	21	0.2529	0.2686	No
	Lymphocytes (%)	21	-0.08934	0.7002	No
	Monocytes (%)	20	0.07849	0.7422	No
	N/L ratio	20	0.1104	0.6431	No
	L/M ratio	20	-0.09585	0.6877	No
	Neutrophils (cell/µL)	20	0.1804	0.4467	No
	Eosinophils (cell/µL)	20	0.245	0.2978	No
	Lymphocytes(cell/µL)	20	0.2906	0.2139	No
	Monocytes (cell/µL)	20	0.1967	0.4059	No
Lymphocyte composition	T cell (% of MNC)	20	0.3872	0.0917	No
	B cell (% of MNC)	20	-0.2566	0.2748	No
	WC1 (% of MNC)	20	0.267	0.255	No
	NK cell (% of MNC)	20	0.296	0.2052	No
	CD4+ (% of T cell)	20	-0.7593	0.0001	Yes
	CD8+ (% of T cell)	20	0.5811	0.0072	Yes
	T cell (cell/µL)	20	0.2906	0.2139	No
	B cell (cell/µL)	20	-0.2068	0.3817	No
	WC1 (cell/µL)	20	0.3903	0.0888	No
	NK cell (cell/µL)	20	0.2058	0.384	No
	CD4 (cell/µL)	20	-0.01283	0.9572	No
	CD8 (cell/µL)	20	0.4883	0.0289	Yes
	CD4/CD8 ratio	20	-0.6236	0.0033	Yes
Monocyte subsets and phenotype	cM % of monocytes	20	0.06566	0.7833	No
	intM % monocytes	20	-0.03245	0.892	No
	ncM % monocytes	20	-0.01133	0.9622	No
	cM/µl	20	0.1903	0.4217	No
	intM/µl	20	0.03593	0.8805	No
	ncM/µl	20	0.01173	0.9608	No
	CD163 MFI on Mon	20	-0.2596	0.269	No
	MHC on MFI on Mon	20	0.06189	0.7955	No
Adhesion molecules expression	$CD62L^{low}$ % of CD4+	20	0.01962	0.9346	No
	$CD62L^{low}$ % of CD8+	20	0.07623	0.7494	No
	CD62L MFI on G	20	-0.1366	0.5658	No
	CD62L MFI on M	20	0.06642	0.7809	No
	LFA-1 MFI on G	18	-0.08394	0.7405	No
	LFA-1 MFI on L	17	-0.2459	0.3415	No
	LFA-1 MFI on M	18	-0.1067	0.6734	No
	LFA-1 MFI on WC1	18	-0.09223	0.7159	No

WBC: white blood cells; **N/L:** neutrophil to lymphocyte ratio; **L/M:** lymphocyte to monocyte ratio; **Mon:** monocytes; **CD:** cluster of differentiation; **WC1:** workshop cluster 1; **NK:** natural killer cell; **MFI:** mean fluorescence intensity; **cM:** classical monocytes; **intM:** intermediate monocytes; **ncM:** non-classical monocytes; **LFA:** lymphocyte function associated antigen. **Yes:** indicates a p value < 0.05. **No:** indicates a p value > 0.05.

Similarly, the frequency of monocyte subsets and the expression levels of the monocyte markers CD163 and MHC-II as well as the abundance of CD62L and LFA-1 did not show any correlation with cortisol levels ($p > 0.05$) (Table 2).

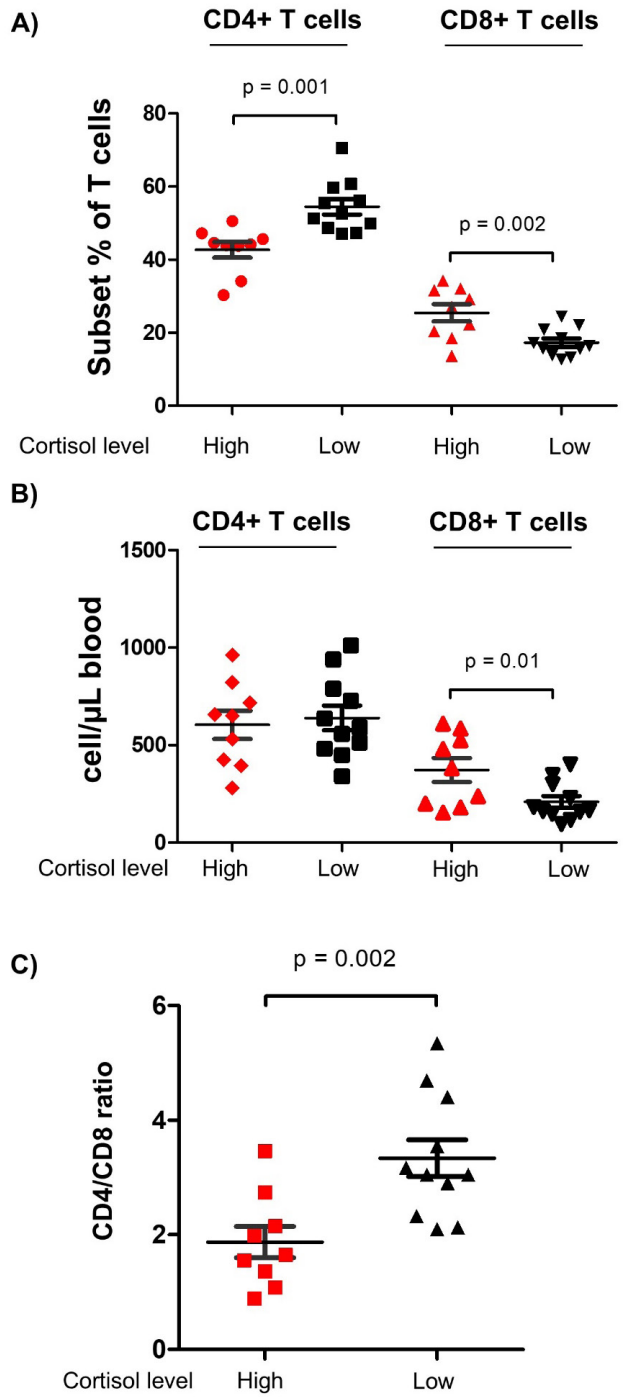

Figure 3: Lymphocyte composition in cortisol-low and cortisol-high animal groups. Leukocytes were separated from cow blood, labeled with antibodies and analyzed by flow cytometry. The percentages (A) and the absolute counts (B) of CD4+ and CD8+ T cells were calculated and presented for the two groups. The CD4/CD8 ratio (C) was calculated and presented for the two groups. * Indicates significantly different group means (p value is indicated in the graph; students t test).

The classification of animals based on their serum cortisol levels into cortisol-low (cortisol concentration between 0 and 15 ng/mL) and cortisol-high (cortisol concentration higher than 15 ng/mL) groups revealed significantly different lymphocyte compositions between the two groups. The cortisol-low group (54.5 ± 2.1) showed a significantly higher percentage of CD4+ T cells than the cortisol-high (42.7 ± 2.1) group ($p < 0.05$). In contrast, the percentage of CD8+ T cells was significantly lower in the cortisol-low group (17.3 ± 1.1) than in the cortisol-high group (25.4 ±2.3) (Figure 3A). However, only the absolute count of CD8+ T cells was significantly different between the two groups with lower numbers in the cortisol-low group (209.8 ± 29.7) than in the cortisol-high group (372.8 ±61.1) (Figure 3B). The ratio between the numbers of CD4+ and CD8+ T cells (CD4/CD8 ratio) was significantly higher in the cortisol-low group (3.3 ± 0.3) than in the cortisol-high group (1.8 ± 0.3) ($p < 0.05$) (Figure 3C).

The impact of several stressors, such as heat stress, handling stress, and transportation stress, on the immune response was studied in several species (Stefanski and Engler 1998; Maes *et al.*, 1999; Caroprese *et al.*, 2010; Grzelak *et al.*, 2017; Gouvêa *et al.*, 2022). Typical stress leukogram is characterized by increased numbers of leukocytes and neutrophils but reduced numbers of lymphocytes (Maes *et al.*, 1999; Grzelak *et al.*, 2017). Given their role in the immune response, the observed negative correlation between cortisol levels and the frequency of CD4+ T cells in the present study with significantly lower abundance of CD4+ T cells in cortisol-high animals suggest compromised immune activity in those animals. This is also supported by the reduced CD4 / CD8 ratio in the cortisol-high group. On the other hand, there was no association between high cortisol levels and changes in other immune cell parameters such as the frequency of neutrophils, monocytes, monocyte subsets, Yδ T cells, B cells, and NK cells, or the expression levels of several cell surface markers. Whether this could be related to the small number of studied animals is to be answered in future studies including higher numbers of cows.

CONCLUSIONS AND RECOMMENDATIONS

In the present study, cortisol levels were found to be associated with significant changes in some immune cell parameters in dairy cows. Increased cortisol levels were negatively associated with the frequency of CD4+ T helper cells and the CD4/CD8 ratio, while a positive correlation was found between cortisol level and the number of CD8+ cytotoxic T cells. In contrast to this, the frequency of other immune cells such as neutrophil, monocyte, monocyte subsets, Yδ T cells, B cells, and NK cells and the expression levels of several cell surface markers were not associated with serum

cortisol levels. These results indicate compromised immune activity in cows with elevated cortisol levels, highlighting the need for future studies on the impact of elevated cortisol levels on the immune response to infection or vaccination. In addition, further studies are required to see whether the current findings would still exist if the animals were under specific stress conditions such as transportation or handling stress. The results of the present study are, however, limited by the low number of sampled animals.

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NOVELTY STATEMENTS

The current study employed flow cytometry and cell staining to investigate the association between serum cortisol level and selected immune cell parameters in dairy cows. The results indicate reduced immune activity in cows with elevated cortisol levels.

AUTHOR'S CONTRIBUTIONS

All authors contributed to study design, sample collection and testing. Jamal Hussen prepared the first draft of the manuscript. All authors revised the manuscript and approved the final version.

Data Availability Statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

Funding

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Ethical Statement

This study was granted approval by the Ethics Committee of King Faisal University, Saudi Arabia (KFU-REC-2023-SEP-ETHICS1372).

Informed Consent Statement

As the animals belonged to the institution, consent for participation was not applicable.

SUPPLEMENTARY MATERIAL

There is supplementary material associated with this article. Access the material online at: https://dx.doi.org/10.17582/journal.jahp/2025/13.3.531.538

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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