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The Effect of Wharton's Jelly Mesenchymal Stem Cell Application on Haematological Parameters in Cows with Subclinical Mastitis

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ABSTRACT

This study aimed to investigate the effect of intramammary Wharton's Jelly mesenchymal stem cell (WJ-MSC) administration on haematological parameters in cows with subclinical mastitis. In the study, 20 Holstein cows kept under equal care and feeding conditions during the same lactation period were used. The California Mastitis Test (CMT) was applied to identify animals with subclinical mastitis. Animals were divided into 4 groups: control (n=4, 3 ml DMEM solution, intramammary, days 2 and 9), Wharton's Jelly mesenchymal stem cells (n=4, WJ-MSCs, 2.5 x 10^7 WJ-MSCs, intramammary, days 2 and 9), subclinical mastitis (n=6, SM, parenteral antibiotic treatment, days 2 and 9), and SM+WJ-MSCs (n=6, 2.5 x 10^7 WJ-MSCs, days 2 and 9). On the 1st and 30th days of the study, blood samples were taken directly from the jugular vein under aseptic conditions into EDTA tubes containing anticoagulant for the evaluation of haematological parameters. Parameters related to infection and inflammation were evaluated in blood count analyses. It was found that the reference ranges for the parameters leukocyte (WBC), erythrocyte (RBC), haemoglobin (HGB), haematocrit (HCT), platelet (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophil percentage Neu (%), and lymphocyte percentage (Lym%) varied. The WBC value, which was above the reference range on day 1, was measured within the reference range on day 30. It was found that the Neu% value, which was above the reference value in the Day 1 measurement, and the Lym% value, which was below the reference value, were within the reference values in the post-treatment measurement. The results obtained indicate that the application of WJ-MSCs has no negative effect on haematological parameters and may even play a balancing role in the inflammatory response for some parameters due to its immunomodulatory effects.

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INTRODUCTION

Subclinical mastitis (SM) is one of the most important and common infectious diseases of the udder. Infection and an inflammatory reaction are present in the mammary gland tissue, however no clinical symptoms are observed (Ghai et al., 2022). Because it does not cause macroscopic changes in mammary tissue or milk, the disease progresses silently, making it difficult to implement treatment and control programs and facilitating the spread of infection within the herd (Hillerton and Berry, 2005; Kumari et al., 2018; Zeweld and Tarekegn, 2025). SM causes both direct and indirect losses, including reduced milk yield and altered composition, recurrent cases of mastitis, animal loses, and treatment costs. In dairy farming, maintaining animal health and high milk yield is one of the most fundamental elements ensuring the sustainability of profitability. Therefore, SM poses a globally critical problem not only in terms of animal health but also in terms of the efficiency of the dairy industry, the quality of dairy products, and food safety (Gonçalves et al., 2018; Gonçalves et al., 2020; Sarı et al., 2025).

Early and accurate diagnosis of SM is of great importance in minimising its negative effects. In the diagnosis of mastitis, somatic cell count (SCC) and pathogen isolation are considered the gold standard (Hristov et al., 2018). While direct cell counting can be used for SCC determination, cell presence can also be determined using indirect methods (Carvalho-Sombra et al., 2021). The California Mastitis Test (CMT), a semi-quantitative and indirect method for determining SCC, detect the presence of neutrophil granulocytes, mononuclear cells, and mammary epithelial cells in milk (Sadat et al., 2023). CMT offers a significant advantage in detecting SCC because it is a quick, inexpensive, and easy test using a four-compartment plastic container and CMT reagent (Zeweld and Tarekegn, 2025).

Wharton's Jelly mesenchymal stem cells (WJ-MSCs) are obtained from connective tissue located between the vessels of the umbilical cord and the amniotic epithelial layer (Cardoso et al., 2012; Lange-Consiglio et al., 2017). Fibroblast-like cells located in this region are primitive mesenchymal stem cells that migrated through the umbilical cord during embryogenesis (Taghizadeh et al., 2011; Wang et al., 2008). Additionally, WJ-MSCs stand out as a promising cell source for preclinical and clinical applications due to their potential to differentiate into various cell types (endothelial, cardiomyocyte, neurone, osteoblast, etc.) (Kim et al., 2013; Obtulowicz et al., 2016). The most important characteristics of WJ-MSCs include low immunogenicity, high proliferative capacity, immunomodulatory and anti-inflammatory properties. The low immunogenicity feature is characterized by low (MHC Class I) or no (MHC Class II) expression of the Major Histocompatibility Complex (MHC) on the cell surface (Cardoso et al., 2012; Ma et al., 2005). Given these advantageous properties, particularly their low immunogenicity and anti-inflammatory potential, the present study aims to evaluate the systemic safety profile of intramammary WJ-MSC administration in cows with subclinical mastitis by assessing peripheral haemogram parameters, in comparison with standard antibiotic therapy.

MATERIAL and METHODS

Animals and Experimental Design

This study was approved by the Selçuk University Faculty of Veterinary Medicine Experimental Animal Production and Research Centre Ethics Committee (SÜVDAMEK) with Local Animal Ethics Committee Decision No. 2024/128 dated 05.09.2024. The 20 raw milk samples from dairy cows and breeding producers used in the study were selected from Holstein cows in the same lactation period

(second-lactation), kept under equal care and feeding conditions at the Atasancak Acıpayam Agricultural Enterprise, and not treated with mastitis vaccine or antibiotics, by performing the CMT (KerbaTest, Ref. No: 1514, Germany). CMT reaction results were scored as 0 (-), 1 (weak positive, ±), 2 (moderate positive, ++), 3 (strong positive, +++) and 4 (highly positive, ++++). The control and WJ-MSC groups were composed of animals that were CMT-negative for at least 3 consecutive tests, while the SM and SM+WJ-MSC groups were composed of animals that were CMT-positive for at least 3 consecutive tests. The selected cattle were divided into four groups: the control group (n:4), the WJ-MSC group (n:4), the SM group (n:6), and the SM+WJ-MSC group (n:6). The experimental study lasted 30 days, and the applications were performed on the 2nd and 9th days of the study (7 days apart, 2 applications). The control group received a 3 ml solution of DMEM (Dulbecco's Modified Minimal Essential Medium) applied to a randomly selected mammary quarter. The WJ-MSC suspension, at a concentration of 2.5 x 10^7 cells in 3 ml of DMEM solution, was administered to the animals in the WJ-MSC group into a randomly selected mammary quarter. In the SM group, 3 ml of DMEM solution was administered to each affected mammary quarter in addition to parenteral antibiotic therapy based on antibiogram test results. In the SM+WJ-MSC group, a suspension of 2.5 x 10^7 WJ-MSCs in 3 ml of DMEM solution was administered to each affected mammary quarter (Hatipoglu et al., 2025). On the 1st and 30th days of the study, venous blood samples were taken under aseptic conditions directly from the jugular vein into EDTA tubes containing anticoagulant for a hemogram analysis. These samples were then transported under cold chain to the laboratory where the analysis would be performed.

Complete Blood Cell Analysis

From blood samples, the levels of leukocyte (WBC), erythrocyte (RBC), haemoglobin (HGB), haematocrit (HCT), platelet (PLT), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), neutrophil percentage Neu(%), and lymphocyte percentage (Lym%) counts were analysed using an automated blood cell counter (Abbott Cell Dyn 1800 Haematology Analyser, Germany).

Statistical Analysis

The numerical values obtained from the haematological analysis results of venous blood samples taken on days 1 and 30 of the study were analysed GraphPad Prism (Version 10.6.1, GraphPad Software, San Diego, CA, USA). Since the study involved repeated measures at two time points (Day 1 and Day 30) on the same animals, the primary analytical approach was determined as the Linear Mixed Model (LMM). For each haematological parameter, the model included Group (Control, WJ-MSCs, SM+Antibiotic, SM+WJ-MSCs), Time (Day 1, Day 30), and the Group × Time interaction as fixed effects. To account for between-subject variance and the repeated nature of the data, Animal ID was modelled as a random effect (random intercept). The Restricted Maximum Likelihood (REML) method was used for model estimation. Model assumptions were evaluated by examining the residuals rather than raw data. Normality of residuals was checked using Shapiro–Wilk tests and Q–Q plots, while homoscedasticity was assessed via residual-vs-predicted plots. As the residuals met the assumptions of normality and homoscedasticity, standard LMMs (Gaussian distribution) were applied. The significance level was set at $\alpha = 0.05$. Omnibus F-tests were reported for the general significance of fixed effects. In the presence of a significant interaction, 'simple effects' analyses were conducted to test Day 1 vs. Day 30 differences within each group, with family-wise error rate control provided by the Šidák/Bonferroni correction. If the interaction was not significant, interpretation focused on the main effects. Post-hoc tests (e.g., Duncan) were strictly avoided when the omnibus test was not significant. Descriptive statistics are presented as mean ± standard error (SE). To

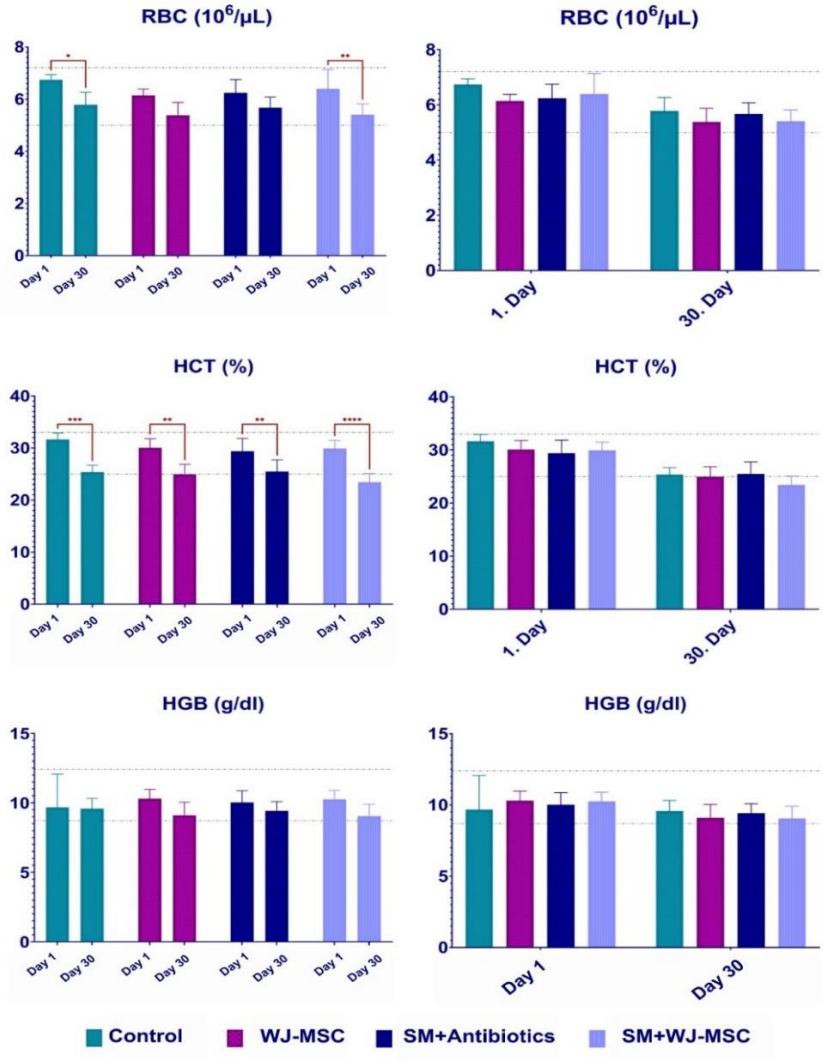
facilitate visual understanding of the time effect and potential interactions, line graphs (Mean ± SE) showing group means at each time point were generated. Effect size classifications were reported only for statistically significant effects to ensure robust interpretation.

RESULTS

The Linear Mixed Model (LMM) analysis revealed that the "Time" factor was the predominant determinant for the variations in haematological parameters. In most cases, the fixed effect of "Group" and the Group × Time interaction were not statistically significant (P>0.05). Furthermore, pairwise comparisons between experimental groups at either Day 1 or Day 30 yielded no significant differences after Bonferroni correction (P > 0.05). Consequently, the results are interpreted primarily based on temporal changes within groups, as illustrated in the figures.

A significant Time × Group interaction was observed for HCT (P = 0.0232), accompanied by a robust main effect of Time (F (1, 16) = 304.5, P <0.0001). Post-hoc analyses demonstrated a significant decrease in HCT levels across all experimental groups from Day 1 to Day 30 (Control: P = 0.0002; WJ-MSCs: P = 0.0019; SM+Antibiotic: P = 0.0037; SM+WJ-MSCs: P <0.0001) (Figure 1).

Figure 1. Evaluation of erythroid parameters before (Day 1) and after (Day 30) treatment.

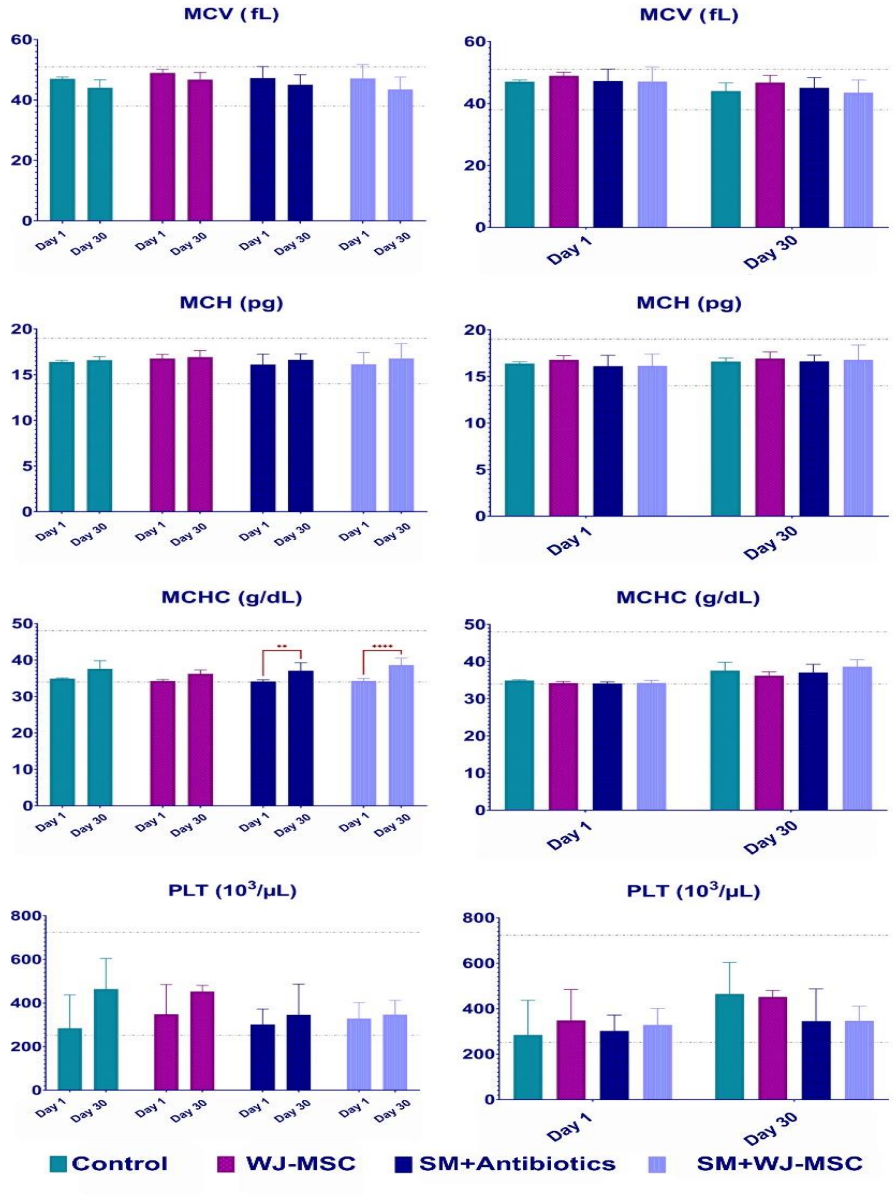


The left panels allow for direct within-group comparisons over time, and the right panels provide an overview of groups at each time point. Red brackets mark significant decreases in parameters on Day 30 compared to Day 1 (* P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001). A significant Time × Group interaction was found for HCT (P = 0.0232). Gray dashed lines represent the physiological reference intervals.

Similarly, RBC counts showed a significant main effect of Time ($F(1, 16) = 90.16$, $P < 0.0001$), with notable inter-individual variability ($P = 0.0007$ for subjects). Bonferroni-corrected within-group comparisons indicated significant reductions in RBC levels on Day 30 compared to Day 1 specifically in the Control ($P = 0.0345$) and SM+WJ-MSCs ($P = 0.0051$) groups, while other groups showed no significant pairwise changes (Figure 1).

Regarding MCHC, a significant main effect of Time was found ($P < 0.0001$). Within-group analyses revealed a significant increase in MCHC levels on Day 30 in the SM+Antibiotic ($P = 0.0052$) and SM+WJ-MSCs ($P < 0.0001$) groups (Figure 2). Although HGB levels showed a statistically significant main effect of time ($F (1, 16) = 6.601$, $P = 0.0206$), individual within-group comparisons did not reach statistical significance after correction ($P > 0.05$) (Figure 1).

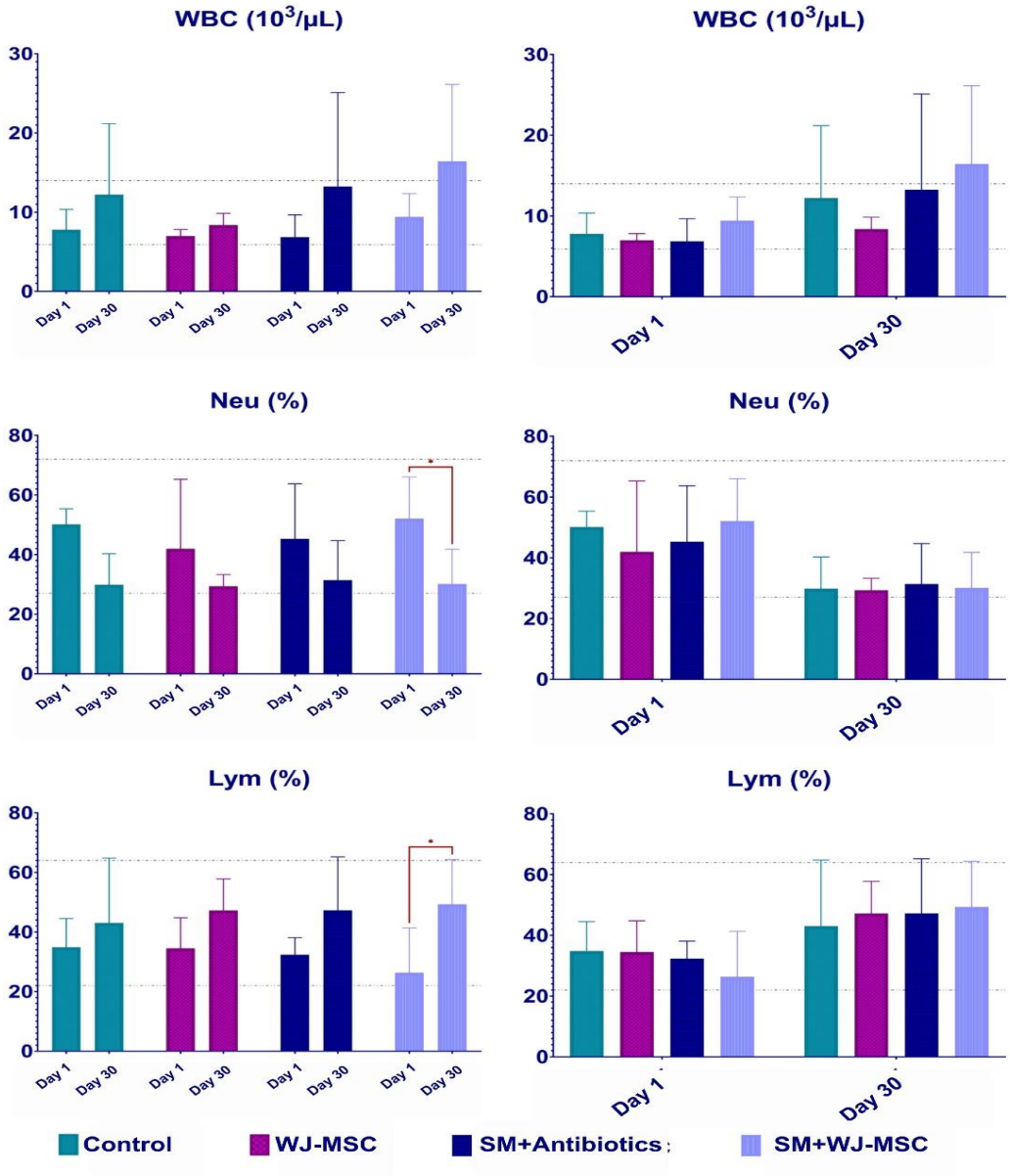
Figure 2. Comparative analysis of erythrocyte indices and platelet counts.



The left panels show changes from Day 1 to Day 30 within groups, and the right panels show comparisons between groups. Significant increases in MCHC levels on Day 30 are marked with red brackets (** $P < 0.01$, **** $P < 0.0001$). Other parameters did not show significant pairwise changes within groups after Bonferroni correction, although a general time effect was present. Gray dashed lines indicate the standard reference intervals.

For Neu%, a significant main effect of Time was detected (F (1, 16) = 15.47, P = 0.0012). Pairwise comparisons showed a significant decrease in Neu% solely in the SM+ WJ-MSCs group on Day 30 (P = 0.0424) (Figure 3). Conversely, Lym% exhibited a significant main effect of Time (F (1, 16) = 10.75, P = 0.0047), with a corresponding significant increase observed exclusively in the SM+ WJ-MSCs group (P = 0.0328) (Figure 3). No significant Group or Interaction effects were found for these parameters (Figure 3).

Figure 3. Temporal changes in leukocyte parameters across study groups.



The left panels display the comparison of Day 1 vs. Day 30 for each group side-by-side, while the right panels group the data by time points. Data represent Mean ± SE. Red brackets indicate statistically significant within-group differences between Day 1 and Day 30 based on Two-Way RM ANOVA with Bonferroni correction (* P < 0.05). Notably, significant alterations in Neu% and Lym% were observed in the SM+ WJ-MSCs group. Gray dashed lines denote the species-specific reference intervals (lower and upper bounds) obtained from the Cornell University Animal Health Diagnostic Center.

Total WBC counts showed a significant main effect of Time ($F(1, 16) = 6.707$, $P = 0.0197$); however, neither the Time × Group interaction ($P = 0.7235$) nor the Group effect ($P = 0.5434$) was significant. Despite the general time effect, Bonferroni-corrected comparisons did not reveal statistically significant changes within any individual group ($P > 0.05$) (Figure 3).

For other haematological indices (e.g., PLT, MCV, MCH), while isolated main effects of Time were occasionally observed, they did not translate into consistent group differentiation or significant pairwise differences after multiple comparison adjustments. These findings suggest general physiological variations over time rather than treatment-specific effects (Figure 2).

DISCUSSION

Haematological profiling is one of the standard diagnostic methods used to assess the physiological parameters of dairy cattle and diagnose systemic diseases. Although it rarely led to a correct and definitive diagnosis, it provides important information for monitoring diseases and predicting their outcomes (Kara et al., 2024; Roland et al., 2014). The present study was designed to evaluate, in cows with subclinical mastitis, the therapeutic response (efficacy) and systemic safety of intramammary WJ-MSCs administration, in comparison with standard antibiotic therapy, using peripheral haematological indicators. Overall, the dominant determinant of variability across most haematological variables was time, whereas the main effect of group was not significant for most parameters and the Time × Group interaction reached statistical significance only for HCT. Likewise, no meaningful separation between groups was detected at either sampling point (Day 1 or Day 30). Although statistically significant within-group temporal changes were noted for selected variables—particularly Neu% and Lym% in specific groups—these were not interpreted as treatment-specific divergence because the Time × Group interaction was not significant for these parameters. Collectively, these findings indicate that the observed changes primarily reflect within-animal temporal dynamics, and that a consistent, treatment-specific differentiation between antibiotic therapy and WJ-MSCs treatment was not evident. From a clinical and translational standpoint, the absence of systematic between-group differences supports the interpretation that intramammary WJ-MSCs administration did not elicit an overt adverse systemic haematological response under the conditions of this study.

Haematocrit (HCT), value is a fundamental parameter that indicates the percentage of red blood cells in the total blood volume. There is a strong relationship among RBC, HGB, and HCT, and it is observed that their values generally decrease or increase together (Turkson and Ganyo, 2015). In mastitis, particularly in clinical presentations, reductions in RBC, HGB, and HCT have been reported, whereas subclinical mastitis may be accompanied by smaller and more heterogeneous alterations (Lakshmi et al., 2024; Sadat et al., 2023). In the current study, HCT exhibited a pronounced main effect of time together with a statistically significant Time × Group interaction; however, the lack of consistent between-group differences at either Day 1 or Day 30 suggests that this interaction is best interpreted as reflecting differences in the magnitude of temporal change rather than clear separation between treatment groups. Importantly, when the direction and size of change are considered in a clinical context—particularly where values remain within reference intervals—the observed erythrogram shifts are more compatible with time-associated physiological variability (e.g., hydration/plasma volume dynamics, sampling-related factors, lactational adaptation, and handling-related stress) than with a treatment-emergent haematological toxicity signal (Paape et al., 1973; Sobolev et al., 2021).

Among erythrocyte indices, MCV, MCH, and MCHC are parameters that represent the average volume of erythrocytes, the average amount of haemoglobin found in an erythrocyte, and the haemoglobin concentration relative to erythrocyte volume, respectively. These indices are primarily used to characterise anaemia phenotypes; therefore, they are not directly applicable for defining mastitis per se (Mordak et al., 2024). Previous studies in sheep with CM or SM indicate that MCV, MCH and MCHC may fluctuate without consistent, statistically significant differences compared to healthy animals (Ah, 2016; AL-Hadithy and Suleiman, 2014; Etim et al., 2014, Mordak et al., 2024, Sarvesha et al., 2017). In the present study, time-related effects were detectable for selected indices and within-group changes were observed for MCHC in some groups; nevertheless, the overall pattern—absence of sustained between-group separation and values remaining within physiologically acceptable limits—supports interpretation as non-specific temporal variation rather than an intervention-related erythrocyte disorder. Accordingly, our haematological data do not suggest that intramammary WJ-MSCs administration induces systemic anaemia, polycythaemia, or clinically meaningful haematological dyscrasia. This interpretation is consistent with reports of perinatal/adult tissue-derived MSC approaches in mastitis models indicating stable haematological parameters within reference intervals following administration (Peralta et al., 2020; Pokorska et al., 2024; Ghai et al., 2022).

Importantly, Peralta et al. (2020) investigated an allogeneic adipose tissue–derived MSCs (AT-MSCs) intramammary therapy in an experimentally induced Staphylococcus aureus CM model and included a conventional antibiotic comparator group. Across sampling days, haematological variables—including erythrocyte and platelet counts and haemoglobin/PCV—remained within reference intervals and did not differ significantly, despite repeated intramammary MSC administrations. Collectively, these findings provide external support that intramammary MSC-based strategies can be evaluated alongside conventional antibiotic therapy while maintaining a reassuring systemic haematological safety profile.

Leukogram variables (WBC, Neu%, Lym%) are practical indicators for assessing systemic inflammatory status and immune cell distribution (Abdel-Hamied et al., 2020; Braun et al., 2021). In SM, systemic leukocyte responses are often limited and variable because inflammation may remain largely localised to mammary tissue, and peripheral blood indices may remain close to baseline depending on pathogen burden, chronicity, and host factors (De and Mukherjee, 2013; Carvalho-Sombra et al., 2021). By contrast, clinical mastitis more frequently produces clearer systemic haematobiochemical perturbations, including more consistent leukocyte alterations (Sarvesha et al., 2017). In the present study, the significant main effect of time for WBC suggests a temporal shift in leukocyte dynamics across the sampling interval; however, the absence of significant within-group Day 1–Day 30 differences after conservative correction implies that this temporal signal was not sufficiently strong and homogeneous within each group. With respect to differential counts, the decrease in Neu% and the increase in Lym% observed over time in the SM+WJ-MSCs group are clinically noteworthy and directionally compatible with a shift of the neutrophil-to-lymphocyte balance toward physiological ranges as inflammatory pressure diminishes (Çetinkaya et al., 2020). Nevertheless, because the Time × Group interaction was not significant for Neu% or Lym%, these changes should not be interpreted as definitive evidence of a treatment-specific immunomodulatory effect attributable to WJ-MSCs. Rather, they are best discussed as time-associated patterns that may accompany changes in subclinical inflammatory activity, a cautious interpretation that is consistent with studies reporting that MSC administration tends to maintain systemic inflammatory indicators within physiological limits rather than inducing overt pathological deviations (Ghai et al., 2022). Indeed, temporal fluctuations in peripheral leukocyte responses after MSC administration have been

described previously: Pokarska et al. (2024) observed an early rise in peripheral WBC and neutrophils within 24 h of BMSC/ADSC administration followed by a systematic decline at later time points (72 h and 7 days) toward baseline levels. Such kinetics support the plausibility of transient immune cell mobilization in the early post-administration period with subsequent return toward homeostasis (Peralta et al., 2020).

Platelets (PLTs) are fragments of megakaryocytes whose most important function is to ensure haemostasis. Most platelets reside in the spleen, with smaller amounts in the liver and bone marrow, and are released into circulation following hormonal stimulation (Benko et al., 2025; Boudreaux and Ebbe, 1998; Russell, 2010). No significant changes in PLT values have been reported in cattle with CM and SM. Thrombocytopenia is more typical of severe mastitis than SM (Hagiwara et al., 2014). In this study, although time-associated variability in PLT was observed, the lack of consistent between-group differentiation and the absence of robust corrected within-group changes do not support strong inferences regarding thrombopoietic or haemostatic effects attributable to WJ-MSCs. Therefore, platelet findings are most appropriately framed as non-specific temporal variation within physiological limits. In line with this view, Ghai et al. (2022) reported that following allogeneic umbilical cord blood-mesenchymal stem cells (UCB-MSC) administration, haemogram parameters—including WBC, RBC, HGB, HCT, MCV, MCH, MCHC, and PLT—did not change significantly between sampling days and remained within reference intervals.

CONCLUSION

Today, antibiotic treatment is the most common method for controlling mammary gland infections. Antibiotic use negatively impacts public health and food safety due to the risk of antimicrobial resistance and residues in milk. Therefore, research is being conducted in the field of regenerative medicine to develop alternative treatment methods for bovine mastitis. This study aimed to compare intramammary WJ-MSCs administration with standard antibiotic therapy in cows with subclinical mastitis, to evaluate both therapeutic response and the systemic safety profile based on peripheral haemogram parameters. Overall, time was the primary determinant of variation in haematological measures, and no significant between-group separation was detected at either Day 1 or Day 30. The finding that the Time × Group interaction was significant only for HCT, together with the absence of consistent treatment-specific differentiation in the remaining variables, suggests that neither WJ-MSCs nor antibiotic administration produced a distinct or differential adverse effect on the systemic haemogram; rather, the observed changes largely reflect time-associated physiological dynamics. Nevertheless, to more clearly delineate potential effects and treatment-related biological responses, further studies incorporating additional time points and supported by acute-phase proteins, cytokine profiling, and mammary/milk parameters (e.g., SCC and bacterial load) are warranted.

Ethical Statement

This study is based on the doctoral thesis titled "Investigation of the Therapeutic Effects of Mesenchymal Stem Cells Isolated from Wharton's Jelly in Cows with Subclinical Mastitis Using Pathological and Molecular Methods," which was presented on 15.05.2024 under the supervision of Assoc. Prof. Dr. Mehmet Burak ATEŞ.

Ethics Committee Approval

05/09/2024 dated and numbered 2024/128 was given by Selcuk University, Faculty of Veterinary Medicine Experimental Animal Production and Research Centre Ethics Committee (SÜVDAMEK).

Author Contributions

Research Design (CRediT 1) Author 1 (%10) Author 4 (%10) - Author 5 (%10) - Author 6 (%10) - Author 7 (%10) - Author 8 (%10) - Author 9 (%10) - Author 10 (%10) - Author 11 (%20) -
Data Collection (CRediT 2) Author 1 (%20)- Author 2 (%10) Author 3 (%20)- Author 5 (%25) – Author 11 (%25)
Research - Data analysis - Validation (CRediT 3-4-6-11) Author 1 (%25)- Author 3 (%20)- Author 5 (%25) – Author 11 (%30)
Writing the Article (CRediT 12-13) Author 1 (%60) – Author 5 (%20) – Author 11 (%20)
Revision and Improvement of the Text (CRediT 14) Author 5 (%40) – Author 11 (%60)

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Conflict of Interest

The authors declare that they have no conflict of interest.

Sustainable Development Goals (SDG)

3 Good Health and Well-Being
12 Responsible Consumption and Production

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