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Hematologic and morphologic evaluation of feline whole blood units collected for transfusion purposes

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Abstract

Objectives Despite the increasing availability of feline blood collected and stored for transfusion purposes, few studies have been performed on feline blood units. The aim of this prospective *in vitro* study was to evaluate hematologic and morphologic changes in feline blood cells in whole blood units between collection and end of storage.

Methods Hematological examination (RBC, Hb, Hct, RDW, MCV, MCHC, MCH, WBC and PLT count) was performed on forty non-leukoreduced feline whole blood units at time of collection (D0) and after storage (D35). The blood was collected into citrate-phosphate-dextrose-adenine (CPDA) anticoagulant-preservative solution using an open system in a veterinary blood bank and stored for 35 days between 2-6°C. Twenty of these FWB units were also analyzed for blood cell morphology (normal RBCs, macrocytes, echinocytes, spherocytes, schistocytes, lysed RBCs, RBCs with Heinz bodies and recognizable WBC count). Differences between the two examination times were statistically analyzed.

Results There was a statistically significant decrease in WBC and PLT counts after storage at D35 (P<0.0001 for both). The most significant cellular morphologic change after storage was an increase in echinocytes count (P=0.0001), lysed RBCs (P<0.0001) and a decrease in normal RBCs (P<0.0001). Recognizable WBCs, mainly lymphocytes, were present at the end of storage.

Conclusions and relevance This study showed that significant morphological changes occur in RBCs in feline blood units during storage for 35 days. *In vivo* studies are required to establish if these changes could affect the ability of stored RBCs to circulate and provide adequate oxygen delivery after transfusion.

Keywords feline, whole blood unit, transfusion, storage, CBC, hematological change, morphological change

Introduction

The increasing availability of veterinary hospital blood banks and commercial sources of feline blood products means that transfusion therapy is more widely available to veterinarians and feline stored blood products are more often used. The main indication for transfusion in cats is anemia, therefore whole blood (WB) and packed red blood cell (PRBC) units are the most commonly collected and stored blood products. [1–4]

Whole blood is still extensively used in feline transfusion medicine, primarily reflecting the ease with which this blood component can be obtained. Although there are no legal standards for storage of feline whole blood (FWB) units for transfusion purpose, the length of storage is based on the criteria set by the United States Food and Drug Administration (US-FDA) in human transfusion medicine, which requires that 75% of transfused RBCs remain in circulation 24 hours after transfusion. This corresponds to a storage length of 30 days for FWB units collected using an open system and preserved in acid-citrate-dextrose solution (ACD) [5], and 35 days of storage for FWB units collected in citrate-phosphate-dextrose-adenine (CPDA) solution. [6] Based on these data most veterinary blood banks store FWB units for 28-35 days.

During storage under standard blood bank conditions, human RBCs undergo progressive morphologic alterations that affect their ability to circulate after transfusion and may cause post-transfusion complications. RBCs undergo progressive shape change, from a readily deformable bi-concave disc to poorly deformable echinocytes with cell surface protrusions, and ultimately to nondeformable spheroechinocytes. [7] RBCs manifesting echinocyte shapes can return to the discocyte shape under certain conditions. In contrast, RBCs assuming spherocyte,

64 and degenerated shapes are irreversibly changed cells - these non-deformable cells will
65 hemolyze. [7,8]
66
67 To date a limited number of studies have been performed on feline blood units collected
68 and stored for transfusion purposes, all have documented biochemical changes consistent with
69 anaerobic metabolism in a closed system: decreased glucose, increased lactate and increased
70 ammonia. [9–12] The feline Hb P50 is only slightly reduced after FWB storage for 35 days in
71 CPDA solution, suggesting that the storage lesion does not have a major impact on Hb oxygen
72 affinity in this species. [9] In addition, once collected, feline blood undergoes significant
73 hematological changes between circulation in feline blood donors and in FWB donated units at
74 time of collection. [13] However, to the authors' knowledge, the hematologic and morphologic
75 changes in a limited number of FWB units collected with a closed system and stored for
76 transfusion purposes only recently have been reported [14].
77
78 The purpose of this study was to document changes in cellular blood components during
79 storage of FWB units. We hypothesized that the FBW units undergo changes in hematological
80 parameters and morphology after 35 days of storage.
81
82 **Material and methods**
83 The study was performed on non-leukoreduced FWB units containing 60 ml (blood and
84 anticoagulant) produced by the Veterinary Transfusion Research Laboratory (REVLab),
85 University of Milan in the period 2017-2018. Suitable feline blood donors were selected and
86 donated blood under anesthesia after informed owner consent, following the guide lines on
87 veterinary transfusion from the Italian Health Minister [15] and as previously described. [16] All

cats were feline blood type A. Blood was collected using an open system with a ratio of CPDA:blood of 1:7. Blood was collected in three 20 ml syringes, as previously described [16], and transferred to a 150 ml empty transfer bag (Transfer Grifols 150; Grifols Italia SpA) using a spike (Combifix Adapter; B Braun Vet Care GmbH) connected to the bag. The blood in the bag was gently mixed for 2 mins and three to five 1 ml aliquots of blood (segments) were isolated in the collection tubing using an electric thermal sealer (Hemoweld-B; Delcon Medical Devices). The FWB units were than stored upright at 4±2°C in a dedicated blood bank refrigerator with a continuous temperature record and alarm, preventing frequent fluctuations in temperature, and manually mixed and inverted every 48h.

At the time of sampling one of the segments was removed from the bag, and the blood from this transferred to an empty tube for analysis. Sampling and analyses occurred on day 0 (D0) being the date of blood collection and on day 35 (D35) the date of final storage/expiration of the FWB units based on the study of Bücheler and Cotter. [17]

Hematological parameters: red blood cell (RBC), hemoglobin (Hb), hematocrit (Hct), red cell distribution width (RDW), mean cell volume (MCV), mean cell hemoglobin concentration (MCHC), mean cell hemoglobin (MCH), white blood cell (WBC) and platelet (PLT) count were assessed using an automated multiparameter hematology analyzer with software for animal samples (Cell-Dyn 3700, Abbott Diagnostics Laboratories).

RBC morphology analysis, count of recognizable WBCs and PLT evaluation were performed on May-Grünwald-Giemsa-stained slides. Blood smears were examined by Dr Baggiani (DVM, professional veterinary technician, master in veterinary laboratory medicine)

first at lower power (20X objective) to assess for the presence of erythrocyte autoagglutination, leukocyte aggregates, large platelet aggregates, and abnormal cells, and then using high power magnification (100X) to assess RBCs morphology, WBCs and PLTs number and morphology. [18-21]

The different RBC shapes evaluated at D0 and D35 were normal RBCs, macrocytes, echinocytes, spherocytes, schistocytes, lysed RBCs (ghost cells), RBCs with Heinz bodies. Counts were performed over 10 microscopic fields and results were expressed as mean count per high power field (hpf) examined using a 100X objective (x 1000 magnification). One hundred WBCs were counted and then classified according to their recognizability and the final value was expressed as percentage.

All analysis was conducted using a protocol approved by the University of Milan Animal Welfare Bioethical Committee.

Data were statistically analyzed using computer software (MedCalc version 16.4.3). Determination of data distribution was established using the Shapiro-Wilk test. Comparison of differences between D0 and D35 for data with normal distribution was performed using paired t-test. Comparison for non-normally distributed data was performed with Wilcoxon test for paired samples. Echinocytes and normal RBCs were counted as percentage of 200 RBCs counted in two adjacent hpf to allow for statistical comparison. For all statistical tests, the significance threshold was set at $P<0.05$.

Results

A complete blood cell (CBC) count was performed on 40 FWB units at D0 and at D35. Summary statistics are presented in Table 1. There was a decrease in most hematological parameters after storage for 35 days, but statistically significant decreases were only seen in WBC and PLT counts (Fig 1). Only RDW showed a slight non-statistically increase at D35 with respect to D0.

On microscopic examination, RBC morphology and number of recognizable WBCs at D0 and D35 were evaluated in 20 pairs of blood smears relative to 20 randomly selected samples from 40 FWB units hematologically evaluated (Table 2). There was a statistically significant increase in macrocytes, echinocytes, spherocytes and lysed RBCs count, and a significant decrease in normal RBCs, schistocytes, RBCs with Heinz bodies and percentage of recognizable WBC (predominantly lymphocytes) (Fig 2). At D35 the echinocyte count was assigned a value of >350 units/hpf since almost all the cells in every field had this morphology. Respect to D0 the PLTs counted at D35 showed a lower intensity of coloration (Figs 3 and 4).

Discussion

Studies of canine RBC products have shown that increasing storage time is associated with increased risk of transfusion-related hemolysis [22], inflammation [23] and risk of infection in septic dogs with experimental pneumonia. [24] To date, storage-related transfusion reactions have not been documented in cats and no experimental or clinical studies have evaluated storage-related changes in feline blood products or the effects of transfusion of blood products of varying age. In addition, despite the increasing availability of stored feline blood units, analysis of blood parameters in feline blood collected for transfusion purposes has rarely been

reported in the scientific literature. [9,11–14] For these reasons we studied the hematological and cell morphological changes at the end of storage.

The results of our study were in agreement with a recent study [14] that studied feline blood collected with a closed system and stored in a similar manner to the blood units in this study, showing that RBC parameters (RBC count, MCV, MCHC, MCH, RDW, Hct, Hb) did not change significantly during the 35 days of storage, reflecting the situation in equine PRBC units [25] and in canine PRBC units for PCV. [26] These results were in contrast to analysis of ovine WB units in which a significant decline in RBC count and an increase in MCV was demonstrated [27] and in WB units in ferrets where an increase in Hct was noted in the early phase of storage. [28] Species specific differences, type of blood collection systems and storage conditions could explain these findings.

The absence of a significant increase in Hb at D35 was in contrast with the results of the morphological analysis, where a significant increase in lysed RBCs was seen. Lysis would be expected to result in a significant increase in Hb content after 35 days, but this apparent inconsistency can be explained by the methodology of the automated analyzer used in study for hematological analysis. This analyzer uses a laser-impedance technique which requires lysis of the RBCs to determine the Hb content and therefore cannot distinguish between the Hb present in the supernatant derived from natural hemolysis and the Hb contained within the cells. Obviously, the Hb present outside the erythrocytes is not able to transport oxygen and is therefore not useful in predicting the effectiveness of the transfusion. Evaluation of the percentage of hemolysis in blood units could help clarify this result but unfortunately we did not evaluate this parameter during storage.

Another apparent incongruent result was the concomitant increase in the macrocyte count and decrease in MCV at D35. This could be explained by the fact that the number of macrocytes counted at the end of storage was too low in number to influence the MCV index. In addition, even if not statistically significant, RDW increased at D35 because RBCs showed morphologies with varied dimensions. However the predominant RBCs at D35 were small RBCs, and this explains the low MCV index value at D35.

There was a significant decrease in WBC counts at the end of storage in accordance with previous literature reports. It is known that the very short life span of granulocytes does not allow the preservation and transfusion of these cells. [29] Although significantly decreased in number, WBCs and PLTs were present at the end of storage. WBCs and PLTs in non-leukoreduced human and canine stored RBCs units are immunologically active and the release of proinflammatory, prothrombotic compounds and vascular endothelial growth factor (VEGF) increases with storage time. Leukoreduction involves passing WB or a blood component through a filter to remove donor WBCs and PLTs. Removal of these cells mitigates some of the effects associated with stored blood transfusion, including febrile nonhemolytic transfusion reactions, and infection. [23,30,31] A pilot study showed that pre-storage leukoreduction of FWB units is possible using an in-line neonatal filter. [32] However, the main limitation of this technique is the reduction in blood volume, since the residual filter volume is 8 mL. For this reason, leukoreduction is not routinely performed in feline transfusion medicine.

The normal mean platelet survival time is 31 hours in healthy cats [33], therefore the PLTs in blood units at the end of storage were not viable. This could explain why these PLTs had a lower intensity of coloration on microscopic evaluation. Cat platelets are prone to

activation and clumping when blood samples are collected [18-21]. At the end of storage (D35) PLTs were no longer viable and so the PLTs originally aggregating at D0, were unable to aggregate at D35. This could be the reason why a relatively high mean PLT count, in comparison to PLT D0 count, was recorded at the end of storage.

Transfusion efficacy depends not only on the quantity of cells provided, but also their quality. At D35 significant morphological changes were detected, predominantly the formation of echinocytes, and presence of lysed RBCs. The most obvious change was an increase in echinocyte count as previously shown in feline blood collected with a closed system [14] in which ATP depletion was also demonstrated during feline blood unit storage that may explain the echinocytic transformation of normal RBCs [18]. A gradual echinocytic shape transformation, was also observed for canine RBCs in PRBC units. [34] Human studies have demonstrated that echinocytes are less deformable than discocytes and increase blood viscosity because of entanglement of their cell spicules and intercellular interference during flow. [35] However, echinocyte formation might be reversible and these cells are capable of returning to a discocyte shape under certain conditions. In fact, transfusion itself may encourage some normalization of the echinocytic shape of stored RBCs. During storage, lysophosphatidylcholine is produced from phosphatidylcholine, which accumulates in cell membranes and is a potent echinocytogenic stimulant. [36] When incubated in fresh autologous plasma, such echinocytic compounds, may equilibrate with the low concentrations in fresh plasma, explaining the shape reversibility towards the normal discocytic shape. [37,38]

Heinz body development was also considered a possible consequence of oxidative damage to the red blood cell. Oxidative processes in the RBC membrane are considered to be

one of the main causes of decreased erythrocyte deformability and increased rigidity during storage in human medicine. [39] In this study we found a decrease in number of RBCs with Heinz bodies during storage, however these were present in very low numbers at D0, as occasionally reported in healthy cats. [40] By D35 it was hard to read the blood slides accurately due to the numerous echinocytes and some Heinz body containing cells may have been missed. We cannot conclude that Heinz body numbers reduce following storage, but rather that their identification at the end of storage is very difficult under light microscopy.

In this study we looked for erythrocyte morphologies, such as spherocytes, that are difficult to recognize with certainly in cats because of the small size and limited central pallor of feline erythrocytes; and schistocytes which are less consistently seen in cats [18-21]. However, we wanted to verify if collection and storage of blood could result in RBC morphology not commonly seen in normal feline blood films. The number of these cells, both spherocytes and schistocytes changed significantly during storage, however they were present in very low numbers at collection and the end of storage. This confirms that feline RBC shape abnormalities were not common in blood collected and stored for transfusion purpose, in accordance with results of a previous study. [14]

This study has some limitations. The first one is that all the analysis was performed using blood from the line segments. During creation of blood unit tube segments turbulent blood flow may be generated. [18] This turbulent flow may result in RBCs shape abnormalities such as schistocytes, which are not present in healthy cats being associated with DIC, severe iron-deficiency and other conditions [18] but were found in low numbers in feline blood units at D0. Tubing segments, generated during RBC component production, were evaluated to determine

256 their suitability as a sample source for quality testing in human PRBC units. That study
257 demonstrated that segments from RBC units should not be used for quality testing. [41]
258 However, for some other parameters such as cytokine measurement, the blood unit segments
259 were considered to be representative of the entire donor unit. [42] It is not known whether the
260 blood sample in the unit segment could serve as a surrogate sample in veterinary transfusion
261 medicine as no studies have been performed. This is a potential limitation of the current study
262 and should be addressed in future studies. The nondestructive testing of blood units by analysis
263 of blood segments offers several advantages, such as in-process quality control to facilitate
264 screening of blood donor units, continued storage of the units after testing without a shortened
265 expiry time, and reduction in discarded samples. These factors are of particular importance in
266 feline transfusion medicine.
267
268 No percentage of hemolysis was calculated, and this is an important parameter in
269 human transfusion medicine. United States Food and Drug Administration (US-FDA) rules in
270 human transfusion medicine, require that hemolysis affects less than 1% of stored cells at the
271 end of the approved storage period**.** While this standard has been adopted in veterinary studies,
272 acceptable limits for feline transfusion blood have not yet been established. Percentage
273 hemolysis was not measured in this study because determining the longevity of these samples
274 was not our focus.
275
276 Feline RBC morphology was assessed only by light microscopy, and there were some
277 difficulties in reading slides at D35. The difficulties encountered in the morphological evaluation
278 of cells have been overcome in human medicine, thanks to the use of scanning electron
279 microscopy. This provides sharper images and therefore easier and better reading and

recognition of the different shape changes. [7,43]

Only blood products collected with a closed system should be stored for more than 24h in the refrigerator. However closed systems are less readily available for feline transfusion medicine and blood is frequently collected with an open system, as in our study. Blood units and blood products collected using open systems have previously been stored successfully without microbial growth when all blood banking was done by experienced staff. [2] In addition, our previous studies confirmed that feline blood units collected using an open system were negative for aerobic bacterial growth. [10,44]

Conclusions

The study adds important information on quality of stored RBCs in FWB units. The parameters considered, ie the number of erythrocytes, erythrocyte indexes and cell morphology, seem to characterize *in vitro* a product which is effective for transfusion purposes even at the end of storage. However, this conclusion is based on *in vitro* evaluations. *In vivo* studies are required to establish if these changes could affect the ability of stored RBCs to circulate and be effective in delivering oxygen effectively after transfusion.

Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article

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Fig 1 Graph displaying the statistically significant difference (*P* <0.0001, Wilcoxon test) decrease in WBC (**a**) and PLT (**b**) count between count at D0 (the date of blood collection) and D35 (the date of final storage/expiration of the feline whole blood units) in 40 feline whole blood units collected and stored for transfusion purposes. Values were expressed as unit/μL for WBC and unit $x10^3$/μL for PLT count. The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. A line extends from the minimum to the maximum value, excluding "outside" and "outlying" values which are displayed as separate points.

Fig 2 Graph displaying the increase in macrocyte count (**a**; mean/10 hpf), in echinocyte count (**b**; percentage), in lysed RBCs (**c**; mean/10 hpf), in spherocyte count (**d**; mean/10 hpf), and decrease in normal RBCs count (**e**; percentage) and in RBCs with Heinz bodies (**f**; mean/10 hpf) in 20 feline whole blood units collected and stored for transfusion purposes, with a statistically significant difference (*P*=0.0007; *P*<0.0001; *P*<0.0001; *P*=0.0139; *P*<0.0001; *P*=0.0110, Wilcoxon test) between count at D0 (the date of blood collection) and D35 (the final date of storage/expiration of the feline WB unit). The central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. A line extends from the minimum to the maximum value, excluding "outside" and "outlier" values which are displayed as separate points.

Fig 3 Blood films performed at the time of collection from a feline whole blood unit collected for transfusion purpose. Most cells were normal erythrocytes (original magnification 100x, May-Grünwald-Giemsa stain).

Fig 4 Blood film performed at end of storage, after 35 days (D35) after blood collection, from feline whole blood units collected for transfusion purpose. Most cells were echinocytes, PLTs had a lower intensity of coloration, there was a lysed unrecognizable WBC, some lysed RBCs and few normal erythrocytes (original magnification 100x, May-Grünwald-Giemsa stain).

Parameter	Time	Mean	95% CI	SD	Median	95% CI	Min	Max	*P* for paired differences
RBC x10^3/µL	**D0**	7008.2	6544.0 to 7472.4	1451.5	6585.0	6196.7 to 7465.9	4580.0	10700.0	0.3971
	D35	6777.0	6298.5 to 7255.4	1496.1	6560.0	6234.0 to 6712.8	4270.0	12000.0	
Hb g/dL	**D0**	9.7	9.0 to 10.3	2.0	9.5	8.7 to 10.1	6.2	14.9	0.1288
	D35	9.3	8.6 to 10.0	2.1	8.8	8.6 to 9.4	6.0	15.3	
Hct %	**D0**	27.8	25.9 to 29.6	5.6	27.1	25.0 to 29.6	18.7	44.6	0.6502
	D35	26.8	25.0 to 28.5	5.4	25.8	25.1 to 27.3	18.1	43.5	
MCV fL	**D0**	40.0	38.8 to 41.1	3.5	39.4	38.2 to 40.2	34.7	51.8	0.9625
	D35	39.7	38.8 to 40.6	2.8	39.2	38.7 to 40.6	33.5	45.5	
MCH pg	**D0**	13.9	13.5 to 14.4	1.3	13.9	13.6 to 14.2	10.9	16.6	0.1650
	D35	13.7	13.3 to 14.2	1.3	13.5	13.2 to 14.2	11.2	16.9	
MCHC g/dL	**D0**	34.9	34.3 to 35.6	2.1	35.3	33.5 to 36.2	30.5	38.8	0.5106
	D35	34.6	33.9 to 35.4	2.4	34.7	34.0 to 35.7	29.2	40.0	
RDW %	**D0**	17.3	16.8 to 17.9	1.6	17.4	16.7 to 18.0	13.3	20.8	0.0596
	D35	17.9	17.3 to 18.5	1.9	17.6	17.1 to 18.1	15.2	23.9	
WBC x µL	**D0**	6716.5	5799.7 to 7633.2	2866.4	6435.0	5678.2 to 7422.0	1560.0	13400.0	**<0.0001**
	D35	1001.4	727.4 to 1275.5	856.8	762.0	559.3 to 1012.2	123.0	4030.0	
PLT x10^3/µL	**D0**	366.7	307.6 to 425.8	184.8	315.5	271.0 to 404.8	84.0	1087.5	**<0.0001**
	D35	204.9	163.1 to 246.6	130.5	157.0	123.5 to 228.4	58.0	527.0	

Table 1 Results of CBC performed in 40 feline whole blood units at collection (D0) and after storage for 35 days (D35). Statistically significant *P* value <0.05 in bold.
SD = standard deviation, RBC = red blood cells, Hb = hemoglobin, Hct = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RDW= red blood cell distribution width, WBC = white blood cell, PLT = platelet, 95%CI= 95% Confidence interval, Min-Max = minimum-maximum.

Parameters	Time	Mean	95% CI	SD	Median	95% CI	Min	Max	*P* for paired differences
Macrocytes n/hpf	**D0**	1.3	0.4 to 2.2	1.9	0.2	0.1 to 1.8	0.0	6.9	**0.0007**
	D35	2.4	0.9 to 3.9	3.1	0.8	0.4 to 3.1	0.1	12.2	
Echinocytes n/hpf	**D0**	197.5	141.7 to 253.4	119.2	183.1	109.3 to 295.0	10.9	350.0	**0.0001**
	D35	340.0	319.2 to 360.8	44.4	350.0	350.0 to 350.0	151.1	350.0	
Echinocytes %	**D0**	53.6	38.2 to 69.0	32.9	63.2	21.3 to 83.3	6.5	91.0	**<0.0001**
	D35	93.6	89.2 to 97.9	9.3	96.0	94.5 to 97.9	57.0	100.0	
Schistocytes n/hpf	**D0**	0.5	0.08 to 1.0	1.0	0.2	0.1 to 0.4	0.0	4.6	0.1297
	D35	0.2	0.08 to 0.4	0.4	0.1	0.1 to 0.3	0.0	1.8	
Spherocytes n/hpf	**D0**	0.1	0.01 to 0.3	0.3	0.0	0.0 to 0.1	0.0	1.3	**0.0139**
	D35	0.4	0.2 to 0.6	0.4	0.3	0.1 to 0.6	0.0	1.5	
Lysed RBCs n/hpf	**D0**	2.8	0.6 to 4.9	4.6	1.3	0.4 to 2.5	0.1	17.9	**<0.0001**
	D35	16.2	7.7 to 24.7	18.1	5.7	3.0 to 33.6	0.5	50.9	
RBCs with Heinz Bodies n/hpf	**D0**	0.2	0.1 to 0.3	0.2	0.1	0.1 to 0.3	0.0	0.9	**0.0110**
	D35	0.06	0.02 to 0.09	0.08	0.0	0.0 to 0.1	0.0	0.3	
Normal RBCs %	**D0**	46.3	30.9 to 61.7	32.9	36.7	16.6 to 78.6	9.0	93.5	**<0.0001**
	D35	6.8	2.5 to 11.2	9.3	4.0	2.5 to 5.9	0.0	43.0	
Recognizable WBC %	**D0**	88.4	84.2 to 92.6	8.9	88.5	85.3 to 92.4	64.0	100.0	**<0.0001**
	D35	9.0	5.1 to 12.9	8.2	6.0	3.1 to 11.8	1.0	31.0	

Table 2 Results of morphological evaluations at day 0 (D0) and day 35 (D35) of storage in 20 feline whole blood units collected and stored for transfusion purposes. Echinocytes was assigned a value of >350 units/hpf at D35 since almost all the cells showed this morphology. Statistically significant differences are shown in bold (*P* value <0.05). RBCs = red blood cells, WBC = white blood cell, hpf = high magnification field. SD = standard deviation, 95%CI= 95% Confidence interval, Min-Max = minimum-maximum.