McQuinn Erin (Orcid ID: 0000-0002-9421-8478)

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Neutrophil extracellular traps in canine stored red blood cell units

Erin R. McQuinn[1], Stephanie A. Smith[2], Austin Viall[3], Chong Wang[4], Dana N. LeVine[1]

[1]Departments of Veterinary Clinical Sciences, [3]Veterinary Pathology and [4]Statistics, Iowa State University, Ames, Iowa 50011; [2]Department of Biochemistry, College of Medicine, University of Illinois at Urbana-Champaign, Urbana, Illinois, 61802.

Corresponding Author: Dr. Dana LeVine, Department of Veterinary Clinical Sciences, Iowa State University, 1809 South Riverside Drive, Ames, Iowa 50011. Email: dnlevine@iastate.edu

Dr. McQuinn's current address is: Las Vegas Veterinary Specialty Center, Las Vegas, Nevada, 89147

Dr. Smith's current address is: Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan, 48109

Abbreviations:

NETs - neutrophil extracellular traps

RBCs – red blood cells

LR – leukoreduced

NLR – non-leukoreduced

TRALI - transfusion-related acute lung injury

cfDNA – cell-free DNA

IMHA – immune mediated hemolytic anemia

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Abstract

Background: Neutrophil extracellular traps (NETs), webs of DNA and citrullinated histones extruded from activated neutrophils cause transfusion-related acute lung injury. Supernatants of stored red blood cell (RBC) units might promote NETosis in neutrophils from the units or from transfusion recipients.

Hypothesis/Objective: 1) NETs form during storage of canine RBC, 2) leukoreduction (LR) before storage of RBC reduces NETosis and 3) supernatant from stored, non-leukoreduced (NLR) RBC units induces NETosis in healthy canine neutrophils modeling transfusion recipients.

Animals: Six healthy purpose-bred research dogs were utilized for blood donation.

Methods: Prospective controlled study. RBC units were collected from each dog, aseptically divided into 2 equal subunits, 1 of which was leukoreduced, and stored for 42 days. Stored units were sampled biweekly for quantification of NET markers citrullinated histone H3 (Western blot) and cell-free DNA (cfDNA) (DNA dye binding). Unit

supernatants were applied *ex vivo* to canine neutrophils and extracellular DNA release representing NETosis was assessed.

Results: Markers of NETs increased during RBC storage (cfDNA p<0.0001 and citrullinated H3 p=0.0002) and were higher in NLR than LR units (day 42 LR cfDNA 0.34±0.82 ng/ml vs. day 42 NLR 1361.07±741.00 ng/ml, p <0.0001; day 42 LR citrullinated H3 0.19±0.13 AU vs. NLR 0.57±0.34 AU, p=0.0073). Isolated neutrophils did not form NETs when exposed to stored canine RBC supernatant.

Conclusions and clinical importance: NETosis occurs in stored canine NLR RBC units, and is attenuated by LR before storage. NETs might be mediators of transfusion reactions.

Introduction

Transfusion of blood products can be a life-saving treatment in many companion animal

diseases. However, life-threatening transfusion reactions can occur. Incidence rates of

overt transfusion reactions in dogs are up to 28%, and this is likely an underestimation.[1,2]

Amongst the most common adverse events that occur with compatible blood transfusions

are febrile nonhemolytic reactions and, at least in people, transfusion-related acute lung

injury (TRALI).[3] Transfusion of stored blood can induce inflammation, which is

undesirable in dogs that are already in a proinflammatory state as a result of their

underlying disease processes. Prestorage leukoreduction (LR) helps eliminate post-

transfusion inflammation in dogs.[4] Indeed, LR of RBC units is standard of care in human

medicine and is mandated in much of Europe as numerous studies have documented that

the risk of acute transfusion reaction is reduced by prestorage LR of blood units.[5-7] LR is not standardly employed in veterinary blood banks.

Neutrophil extracellular traps (NETs) might be key players in mediating non-leukoreduced (NLR) blood induced transfusion reactions. NETs are webs of DNA, nucleosomes, histones, and granular proteins extruded from activated neutrophils for antimicrobial purposes, but they can also be proinflammatory and prothrombotic.[8-11] If NETs were transfused into dogs, they could cause thrombosis and inflammation in the recipient. NETs have been implicated in the pathogenesis of TRALI in murine models, NET biomarkers are elevated in human patients with TRALI, and inhibiting NETosis results in lung protection in experimental TRALI.[12,13] NETs can form in stored human RBC units and that LR of units before storage attenuates their formation.[14] NETs can form in stored blood via several mechanisms including RBC release of neutrophil-activating pro-inflammatory lipid mediators and heme, a NET inducer.[15,16] Given the postulated role of NETs in the pathogenesis of TRALI and their potential to cause other damage in transfusion recipients, documenting the generation of NETs in stored canine RBC and NETosis attenuation by LR would support the need for LR to become standard in veterinary medicine.

The first objective of the present study was to determine if NETs form during the 42 day storage period of canine RBC units and if LR prevents this process. We analyzed stored RBC units for the presence of cell-free DNA (cfDNA), a nonspecific NET marker, and

citrullinated histones, a specific NET marker as NETosis requires histone citrullination.[17] Secondly, we aimed to investigate if application of supernatant from stored canine RBC could induce NETosis in isolated healthy dog neutrophils, modeling recipient neutrophils. We hypothesized that stored canine RBC units would develop NETs as well as the ability to induce NETosis over 42 day storage conditions and that these processes would be attenuated by prestorage LR.

Materials and Methods

Animals: Six purpose bred research dogs were included if they met the criteria of weighing 17 kg or greater and had no underlying disease as determined by a complete physical exam and screening bloodwork (complete blood count and serum chemistry panel). The study population consisted of 4 intact male dogs and 2 intact female dogs with body weights ranging from 17 to 25.6 kg. This study was approved by the local Institutional Animal Care and Use Committee and dogs were cared for as outlined in the NIH Guide for the Care and Use of Laboratory Animals.

Blood collection and leukoreduction: Research dogs were sedated with dexmedetomidine (2-10 µg/kg) (Dexdomitor®, Zoetis, Parsippany, NJ) and butorphanol (0.05-0.2 mg/kg) (Torbugesic, Zoetis, Parsippany, NJ) IM. Four hundred and fifty milliliters of whole blood were collected via sterile atraumatic jugular venipuncture from

all 6 dogs into standard triple collection systems containing citrate-phosphate-dextrose-adenine for anti-coagulation (Teruflex Blood Bag System, Terumo Corporation, Tokyo, Japan). The sedation was reversed with 0.02-0.1 mg/kg atipamazole (Anti-sedan, Zoetis, Parsippany, NJ) IM. Whole blood units were centrifuged at 2493.1 g at 4ºC for 32 minutes. Plasma was extracted. Optisol preservative was added to the RBCs and mixed. Using the Optisol bag and the RBC bag and while maintaining a closed system, the RBCs were then separated into 2 equal half-units for storage and allowed to cool for 4 hours at 4ºC. One of these half-units was randomly selected to undergo LR using a commercial LR filter (BPF Leukocyte Reduction Filtration System, Haemonetics Manufacturing, Inc., Covina, CA). This was defined as day 0. An aliquot (4 ml) was obtained pre and post filtration for evaluation of cell counts. LR was confirmed by leukocyte count using 2 methods: complete blood counts using an automated hematology analyzer (ADVIA 2120i Hematology System, Siemens Medical Solutions USA Inc, Malvern, PA) and use of a commercial propidium iodide based leukocyte labeling kit (BD Leucocount, BD Biosciences, San Jose, CA), according to the manufacturer's instructions, followed by flow cytometric analysis (BD FACSCanto flow cytometer, BD Biosciences, San Jose, CA). This assay has been previously validated in canine RBC units.[18] Leukocyte counts from NLR units were reported from the automated hematology analyzer whereas leukocyte counts from the LR units were reported from the flow cytometric method, as these counts were below the limit of detection of the automated hematology analyzer.[19]

Successful LR was defined per human blood banking standards as less than 5×10^6 white cells per unit.[20]

As previously described, EDTA blood was collected at a later time point from each research dog for neutrophil isolation in order to test whether RBC unit supernatant induces NETosis in isolated canine neutrophils.[21,22]

Sample preparation: LR and NLR RBC units were stored at 4ºC for 42 days and sampled (15 ml) on days 0, 14, 28 and 42. Samples were collected using sterile sampling ports (HEMO-TAP® Blood Bag Spike, Utah Medical Products, Inc., Midvale, UT) under a sterile laminar flow hood and centrifuged at 2,500 g for 20 min at 4ºC without deceleration to generate cell-free supernatants; the supernatants were removed and these were then centrifuged a second time at 10,000 g for 5 minutes at room temperature with deceleration to rid samples of any cellular debris. Supernatants were stored at -80ºC for a maximum of 175 days before batched analysis of NET markers and ability of unit supernatants to induce NETosis in isolated canine neutrophils. On day 42, the final day of sampling, 1 milliliter of blood from each unit was cultured aerobically and anaerobically to ensure lack of bacterial contamination of units during their sampling and storage.

Sample analysis for free hemoglobin: Hemolysis can be generated during filtration of red cells and could be a confounding factor in our analysis. Therefore, hemolysis of the units at each time point was quantified by means of a Drabkin's assay according to the manufacturer's instructions. Briefly, samples were incubated with Drabkin's reagent

(Ricca Chemical Company, Arlington, TX) for 30 minutes in the dark to convert hemoglobin to cyanmethemoglobin. Absorbance at 540 nm was measured on a Versamax microplate reader (Molecular Devices, Sunnyvale, CA). A_{540} was converted to concentration using a standard curve of bovine hemoglobin (Sigma Aldrich, St. Louis, MO).

Sample analysis for NET markers: Two assays were performed on sample supernatants from each LR and NLR unit at each time point to assess for evidence of NET formation during RBC storage, quantification of cell-free DNA and Western blot analysis for citrullinated histone H3.

<u>*Quantification of cell-free DNA (cfDNA):*</u> Using previously described methods for sensitive detection of DNA, DNA from supernatants was first purified using a commercial DNA purification kit (QIAamp DNA Mini and Blood Mini kit, Qiagen, Valencia, CA).[23] Purified DNA was then diluted, mixed with PicoGreen dye (Quant-iT PicoGreen dye, Thermofisher scientific, Waltham MA) to label DNA according to manufacturer's instructions, and fluorescence was detected with a microplate spectrofluorometer (Synergy HT, BioTek Instruments, Winooski, VT). DNA concentrations in samples were calculated based on a standard curve generated by fluorometric analysis of solutions containing known DNA concentrations (Quant-iT PicoGreen dye, Thermofisher scientific, Waltham MA).

<u>*Western blot analysis for citrullinated histone H3*</u>: Citrullinated histones in sample

supernatants were measured by Western blot as a specific indicator of NETosis as previously described.[24] Briefly, sample supernatants were mixed with Laemmli buffer with DTT and then subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed with an anti-histone H3, citrulline R2+R8+R17 (Abcam, Cambridge, MA) followed by a horseradish peroxidase conjugated secondary antibody (Pierce, Rockford, IL). Chemiluminescence detection was performed by using Pierce ECL-2 substrate (Thermo Fisher, Waltham, MA). Films were developed using a standard photographic procedure and quantitative analysis of detected bands was carried out by densitometer scanning using GelDoc XR+ and ImageLab software (BioRad Laboratories, Hercules, CA). Citrullinated histone H3 purified from septic canine abdominal fluid was used as a standard for densitometry. Septic abdominal fluid was selected for this purpose since NETosis and circulating citrullinated H3 have been well-documented in both naturally-occurring sepsis and animal models of sepsis, and NETs and citrullinated H3 have been previously documented in canine septic abdominal fluid.[25,26] Specificity of the commercial anti-citrullinated histone H3 antibody was confirmed via nano liquid chromatography with tandem mass spectrometry as described in Supplementary File 1.

Assessment of RBC supernatant ability to induce NETosis in recipient neutrophils: As a model of ability of blood product to induce NETosis in recipient neutrophils, sample supernatants from day 0 and day 42 were applied *ex vivo* to isolated canine neutrophils.

Neutrophils were isolated from EDTA blood of 6 healthy research dogs as previously described.[21,22] Briefly, erythrocytes were removed by dextran sedimentation before separation of granulocytes by a Histopaque 1077 gradient (Sigma Aldrich, St. Louis, MO). Remaining erythrocytes were removed by brief osmotic lysis before neutrophils were washed in PBS, counted and re-suspended in PBS after filtration through a 70 µm sterile filter. For a final concentration of 250,000 cells/mL per well, 5×10^4 cells/well, counted using a hemocytometer, were added to a sterile, tissue culture treated 96 well plate (Microplate, Corning, Tewksbury, MA) containing Roswell Park Memorial Institute medium (HyClone, Logan, UT) with 0.5% fetal calf serum (Gibco, Gaithersburg, MD) to a final well volume of 200 uL.

For 6 dogs, 20 uL of supernatant collected from LR and NLR blood at day 0 and day 42 were added to the test wells containing isolated neutrophils described above (10% dilution), with each condition set up in triplicate. For 1 dog, 5% and 25% dilutions were also prepared. As a positive control, cells from each dog were also stimulated with the known NET agonist, phorbol 12-myristate 13-acetate (PMA; final concentration 7µM) (Sigma Aldrich, St. Louis, MO) and as a negative control, non-stimulated wells in which stimulants were replaced by an equal volume of PBS were also set-up. To determine if citrate from the anticoagulant in blood units could be reducing cell viability, supernatants collected from a blood bag filled with PBS were also added to neutrophils (10% dilution). To control for DNA or interfering substances in supernatants, blank wells were prepared

by adding supernatants to acellular wells.

Cells were incubated for 3 hours at 39°C before addition of the nucleic acid dye, Sytox Green (Molecular Probes, Eugene, OR) to a final concentration of 6μM. After 15 minutes incubation at 39°C, fluorescence (ex 504nm/ em 523nm) was measured using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT). Viable cells are impermeable to Sytox green, so fluorescence reflects extracellular DNA and dead or dying cells. Values were blanked by subtracting the fluorescence of acellular wells containing the same stimulant, and a fold change in Sytox green fluorescence calculated by dividing the average fluorescence of test wells by the average fluorescence of non-stimulated neutrophils from the same dog.

Data analysis and sample size calculation: A sample size calculation was performed using data from the literature comparing nucleosome, a NET marker, formation in human LR and NLR RBC units; this showed that in order to obtain an 80% power with a type I error rate of 5%, 6 LR and 6 NLR units were needed.[14] Response variables were analyzed using repeated measures analysis of variance. Treatment, time and their interaction were fixed effects, whereas animal was the subject of repeated measures. DNA data were log transformed before analyses. When significance was discovered, individual comparisons between time points were made by Tukey's t-test with adjustments for multiple comparisons [Statistical Analysis System (SAS) 9.3, SAS Institute Inc, Cary, North Carolina]. A p-value of <0.05 was considered significant. The ability of unit supernatants

from all time points to induce NETosis in healthy dog neutrophils was similarly analyzed.

Correlations between test parameters (hemoglobin, cfDNA and citrullinated histones) were assessed by using the Spearman correlation.

Cell counts of RBC units pre and post filtration failed D'Agostino & Pearson omnibus normality testing; cell counts pre and post filtration were thus compared with Mann-Whitney U test and are reported as median (range) (Prism 6.0, GraphPad Software Inc., La Jolla, CA).

Results:

Efficacy of LR filter

Leukoreduction was greater than 99% effective at removing leukocytes and platelets in every case. In NLR blood units, the median leukocyte count was 12.23 x10^3/µL (range: 9.62-17.24 x10^3/µL) whereas for LR blood units the median residual leukocyte count was 0.09 cells/µL (range: 0.0-0.23 cells/µL) (p=0.0022). In NLR blood units, the median platelet count was 273 x10^3/µL (range: 185-338 x10^3/µL) whereas for LR blood units the median residual platelet count was 2.5 x10^3/µL (range: 1.0-4.0 x10^3/µL) (p=0.0022). The median volume of blood that was lost by the process of filtration was 33.5 mL (range 30-35 mL), an average volume loss of 20.3% among the LR units (data not shown). Although hemolysis occurred in stored units over time (hemoglobin increased over time

in both groups, p<0.0001), there was no difference in hemoglobin present in supernatants in LR compared to NLR units (p=0.20) (Figure 1). Blood units all cultured negative for bacterial contamination at the conclusion of the study.

Release of NET markers with storage

We evaluated whether neutrophils release extracellular DNA during accepted RBC storage times of 42 days. There was significantly more cfDNA (Figure 2) present in the supernatants of NLR samples at days 14 (p<0.0001), 28 (p=0.0014) and 42 (p< 0.0001) compared to day 0 with approximately 1000-fold more cfDNA in the day 42 NLR units than in the baseline samples. There was no significant increase in cfDNA over time was noted in the LR group. LR units contained only 0.34 ng/ml (± 0.82 ng/ml) DNA after 42 days compared to 1361.07 ± 741.00 ng/ml (mean ± SD) in the NLR units at 42 days (p <0.0001). Similarly, there was significantly more citrullinated histone H3 (Figure 3) measured in supernatants of NLR samples at days 28 (p=0.0024) and 42 (p=0.0029) compared to day 0. Although Histone H3 increased in the LR group from day 0 to day 28 (p=0.0020), from day 14 to 28 (p=0.0095), and from day 0 to day 42 (p=0.015), citrullinated histone H3 was approximately 3-fold higher in NLR compared to LR samples on days 28 (p=0.0055) and 42 (p=0.0073). The results of a representative Western blot for citrullinated histone H3 are depicted in Figure 4.

Using the Spearman correlation, cfDNA and citrullinated histone H3 results were significantly correlated (r = 0.58; p<0.0001), suggesting that both assays measure NETs. There was a weak but significant correlation between hemoglobin and cfDNA (r = 0.32; p = 0.02) and strong correlation between hemoglobin and citrullinated histone H3 (r = 0.73; p <0.0001).

Ability of unit supernatants to induce NETosis

Capacity of RBC unit supernatant to induce NETosis was assessed by applying supernatant to isolated canine neutrophils and quantifying DNA release via Sytox Green fluorescence. One dog was excluded from analysis because of interference from the unit supernatant resulting in Sytox Green fluorescence in the acellular controls. There was an effect of time on supernatant induced DNA release (p=0.012), but this effect was weak [1.9 fold increase, which is within assay variation (data not shown)] and was not prevented by LR (p = 0.39) (Figure 5). Confirming that neutrophils performed as expected in these experiments, those stimulated with PMA resulted in a median fold increase compared to unstimulated neutrophils of 21.9 (14.4 -39.1).

Discussion:

The results of this study provide evidence that markers of NETs, both cfDNA and

citrullinated histones, increase over time in stored NLR canine RBC units. Combined, these data suggest that NETosis occurs in stored canine RBC units, and that this process is attenuated by LR before storage. We also found that stored RBC supernatant does not induce NETosis in isolated neutrophils suggesting that stored blood does not induce NETosis in the recipient's own neutrophils. Since NETs can be prothrombotic and injurious to tissue, our documentation of NET formation in stored blood units and the ability to abrogate the process with LR provides support for advising leukoreduction in veterinary blood banking.

NETosis is a process by which activated neutrophils liberate DNA decorated with granular proteins in order to trap bacteria. However, exposed DNA and granular proteins can be damaging to tissues and can be prothrombotic.[9-12,27] NETs are also important in the pathogenesis of several inflammatory diseases.[12,27] Transfusion of NET material is unlikely to be beneficial to hospitalized dogs, especially given that dogs requiring transfusions are likely to have systemic inflammation due to their underlying disease.[4]

NETosis requires neutrophil stimulation. Several mediators of NETosis accumulate in stored RBC that could induce NETosis. We documented that storage of both LR and NLR units resulted in significant accumulation of free hemoglobin over time and heme-related molecules are known to be strong inducers of NETs.[16] Indeed, in this study hemoglobin and cfDNA and hemoglobin and citrullinated histone H3 were correlated. Though hemoglobin increased in LR units as well as NLR units, LR would not have had

sufficient remaining neutrophils to allow for significant NETosis. NET induction by interleukin-8 (IL-8) is another plausible mechanism of NET generation in stored NLR blood. IL-8 increases in NLR canine pRBC units during a 35 day storage period, but does not increase in LR units.[28,29] IL-8 is a well-established NETosis inducer.[8] Additionally, activated platelets can induce NETosis[30] and NETs can activate platelets,[27] thus the effect of LR we observed could be attributed to both the filter's platelet and leukocyte reducing effects.

TRALI is a severe form of acute respiratory distress syndrome that is the leading cause of transfusion-related deaths in people according to the Food and Drug Administration's surveillance data.[31] Elevation of circulating NET biomarkers in TRALI human patients' blood and presence of NETs in the alveoli of mice and people with TRALI suggest there could be a key role for NETs in TRALI pathogenesis.[12,13] Although the incidence of TRALI in veterinary medicine is likely much lower than it is in human medicine[32] or might not occur at all[1], the proinflammatory nature of NETs makes them likely players in other transfusion reactions as well.[9-11] Transfusion of NLR RBC to healthy dogs induces significant inflammation which is eliminated by LR of the RBC units before storage.[4] In human blood banking, it is well established that LR reduces the incidence of both transfusion reactions, especially febrile nonhemolytic reactions, and inflammatory responses [33-36] and this has led LR to become standard practice in much of Europe and Canada.[37]   Based on our study results, reduction of NETosis in stored blood units could

contribute to the beneficial effects of LR. This warrants further study of NET markers in dogs before and after transfusion with LR and NLR blood.

Our results are especially intriguing in light of recent transfusion and NETosis data in canine immune-mediated hemolytic anemia (IMHA). Markers of NETs are increased in dogs with IMHA and cfDNA might predict outcome in dogs with IMHA.[24,38] Given that NETs are prothrombotic,[39,40] and that thrombosis is the leading cause of morbidity and mortality in IMHA,[41,42] NETosis could contribute to the development of IMHA thrombosis. Transfusion of aged pRBCs increased death risk in dogs with hemolysis, most of which were dogs with IMHA.[43] In light of our current study's documentation that NET material accumulates in aged NLR blood, we can speculate that NETosis might have occurred in the aged stored units, leading to infusion of NET material into recipients, contributing to their prothrombotic state and increased mortality.

We were unable to clearly document that stored blood supernatants can induce NETosis in canine neutrophils. Although we identified a statistically significant increase in the fold change of Sytox Green fluorescence after neutrophil incubation with day 42 supernatants (suggesting DNA release), the effect was weak and, in our experience, within the variation of this assay. Thus while NETosis appears to occur in NLR stored blood units, supernatants of stored blood, at least in our *ex vivo* system, did not definitively induce NETosis in freshly isolated neutrophils but might have with longer incubation time.

Though not a study objective, we learned during the study that the LR filters employed

(BPF Leukocyte Reduction Filtration System, Haemonetics Manufacturing, Inc., Covina,

CA), though efficacious in removing white blood cells from canine packed RBC units,

caused marked hemolysis if RBCs were not allowed to cool before filtration. While the

optimal post-collection cool time was not investigated here, we found 4 hours at 4°C to

be an adequate time to reduce hemolysis in our supernatant samples. This observation is

interesting because the manufacturer of the LR filter states that human blood can be

filtered without hemolysis immediately after donation. We speculate that cooling

increased blood viscosity, slowing filtration and decreasing shear force on the canine

RBCs.[44] There might be fundamental differences in the blood cells of canines that make

them more susceptible to lysis after blood donation and immediate leukoreduction. When

using a different LR filter with canine blood, cooling the blood for several hours before

leukoreduction improved filtration efficacy.[45] However, most studies utilizing newer

leukoreduction filters do not allow the blood to cool before filtration. Whether or not pre-

filtration cooling enhances leukoreduction efficacy or reduces pre-storage hemolysis of

red cells was not investigated in this study but further investigation is warranted to ensure

the best adaptation possible of these human products to canine blood.

We recognize that our study has limitations. The cfDNA assay is quantitative and

demonstrated both significant accumulation of cfDNA with RBC storage and attenuation

of this process by LR. However, cfDNA can accumulate with any form of cell death

including necrosis and apoptosis, [46,47] and is not specific for NETosis. We employed the

citrullinated histone H3 assay to provide mechanistic information regarding the source of

the cfDNA. The citrullinated H3 western blots were limited in that we did not have a gel

loading control since we could not be certain another protein like albumin or

immunoglobulin would be stable in the pRBC units over the 42 day 4°C storage time.

For this reason, although citrullinated histone H3 is specific for NETosis, the data

obtained from this assay is only semi-quantitative. However, the correlation between our

cfDNA levels and our citrullinated histone H3 data suggests that the cfDNA source is

NETs. Furthermore, cfDNA, regardless of its origin, has inflammatory and

prothrombotic potential and thus transfusing blood containing cfDNA from any source

could be detrimental.[48] Nevertheless, a specific and quantitative NET assay such as one

that detects granular protein-DNA complexes would be desirable. Though such an assay

exists in human medicine, no such assay exists for dogs and in our experience the human

assays do not work with canine samples (data not shown).[13]

A second study limitation was a possible sample labeling error between 2 samples. We

suspect, based on individual dog data, that 1 dog's day 28 LR sample was mislabeled as

another dog's day 28 NLR sample. The tube labeled LR showed an unexpected increase

in cfDNA on day 28 (446.1 ng/mL) that was not seen in the day 14 (0 ng/mL) or the day

42 (0 ng/mL) time point from this dog. This likely increased the variance in our data.

Despite this suspected error, there were still significantly more cfDNA and citrullinated

histone H3 in NLR compared to LR units at that time point and across all time points.

In our assessment of the ability of RBC unit supernatants to induce NETosis in healthy

canine neutrophils, we did not assess neutrophil viability before incubation with

supernatant. However, our method of isolating neutrophils consistently yields 96-98%

viable cells. Although not assessing isolated neutrophil viability is a shortcoming of this

study that we acknowledge, given that our positive (PMA stimulated) and negative

(unstimulated) cells performed as expected, we do not believe any neutrophil viability

issues impacted results.

In summary, we demonstrated that cfDNA and citrullinated histone H3, markers of

NETs, significantly increase over the standard storage life of NLR packed red blood cell

units; leukoreduction significantly reduced this effect. This provides further evidence of

the inflammatory and prothrombotic potential of stored red cells and the potential

beneficial effects of leukoreduction. To our knowledge, no studies investigating the

benefit of leukoreduced blood products to clinical veterinary patients have been

published. Nevertheless, we believe that this work suggests that LR of blood products has

potential to reduce transfusion-induced inflammation, particularly in products stored 14

days or longer and when transfusing dogs that already have a proinflammatory and

prothrombotic disease like IMHA. If LR can reduce infusion of NETs into recipients and

thereby reduce recipient inflammation and thrombotic risk, it could improve overall

transfusion safety in veterinary medicine.

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Figure 1: Packed red cell unit supernatant hemoglobin levels. Hemoglobin in supernatants collected throughout the 42 day storage period of nonleukoreduced (NLR, **A**) and leukoreduced (LR, **B**) canine packed red blood cell units (n = 6). For pairwise comparisons between timepoints within a given unit type that achieved significance, p values are reported with associated lines indicating which time points were compared. If no line and value are reported, the comparison did not achieve significance. *p values are for comparisons between NLR and LR groups at each time point. Lines represent mean values with error bars representing standard deviation.

Figure 2: Packed red cell unit supernatant cell-free DNA. Cell-free DNA (cfDNA) in supernatants collected throughout the 42 day storage period of nonleukoreduced (NLR, **A**) and leukoreduced (LR, **B**) canine packed red blood cell units (n = 6). For pairwise comparisons between timepoints within a given unit type that achieved significance, p

values are reported with associated lines indicating which time points were compared. If

no line and value are reported, the comparison did not achieve significance. *p values

are for comparisons between NLR and LR groups at each time point. Lines represent

mean values with error bars representing standard deviation.

Figure 3: Packed red cell unit supernatant citrullinated histone H3. Citrullinated histone

H3 measured in supernatants collected throughout the 42 day storage period of

nonleukoreduced (NLR, **A**) and leukoreduced (LR, **B**) canine packed red blood cell units

(n = 6). For pairwise comparisons between timepoints within a given unit type that

achieved significance, p values are reported with associated lines indicating which time

points were compared. If no line and value are reported, the comparison did not achieve

significance. *p values are for comparisons between NLR and LR groups at each time

point. Units are arbitrary absorption units. Lines represent mean values with error bars

representing standard deviation. A given dog's LR and NLR unit samples from all

timepoints were analyzed on 1 gel/western blot. All gels/blots were performed in parallel

and the same positive control was run on each gel against which all other samples were

normalized.

Figure 4: Citrullinated H3 in supernatants of stored canine packed red blood cell units.

Representative Western blot analysis of citrullinated histone H3 in 1 pair of canine
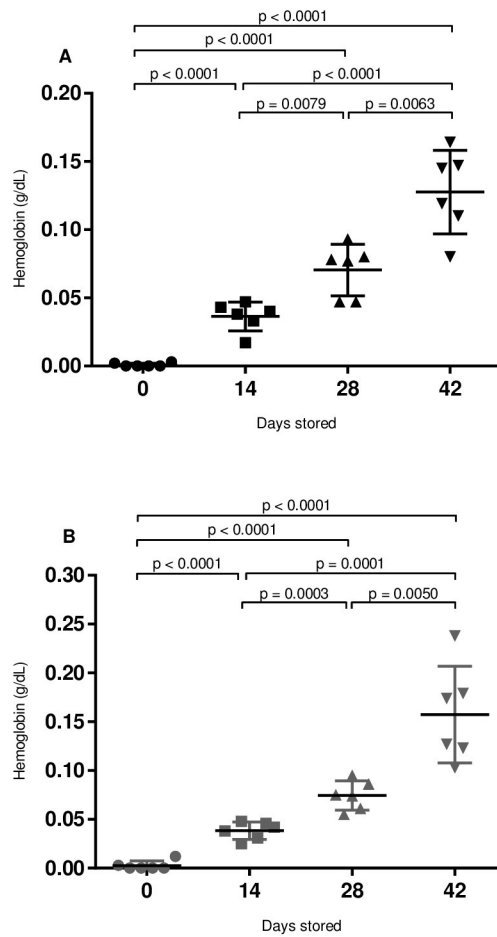
nonleukoreduced (NLR) and leukoreduced (LR) packed red blood cell units from the

same dog over the 42 day storage period. The 17.4 KDa protein ladder band is shown.The

labeled positive control is citrullinated histone H3 purified from canine septic abdominal

fluid. The entire width of the blot is included, but the area of the gel above the 21 KDa

molecular marker has been cropped out of the image for clarity.

Figure 5: DNA release from healthy canine neutrophils evaluated by fluorescence.
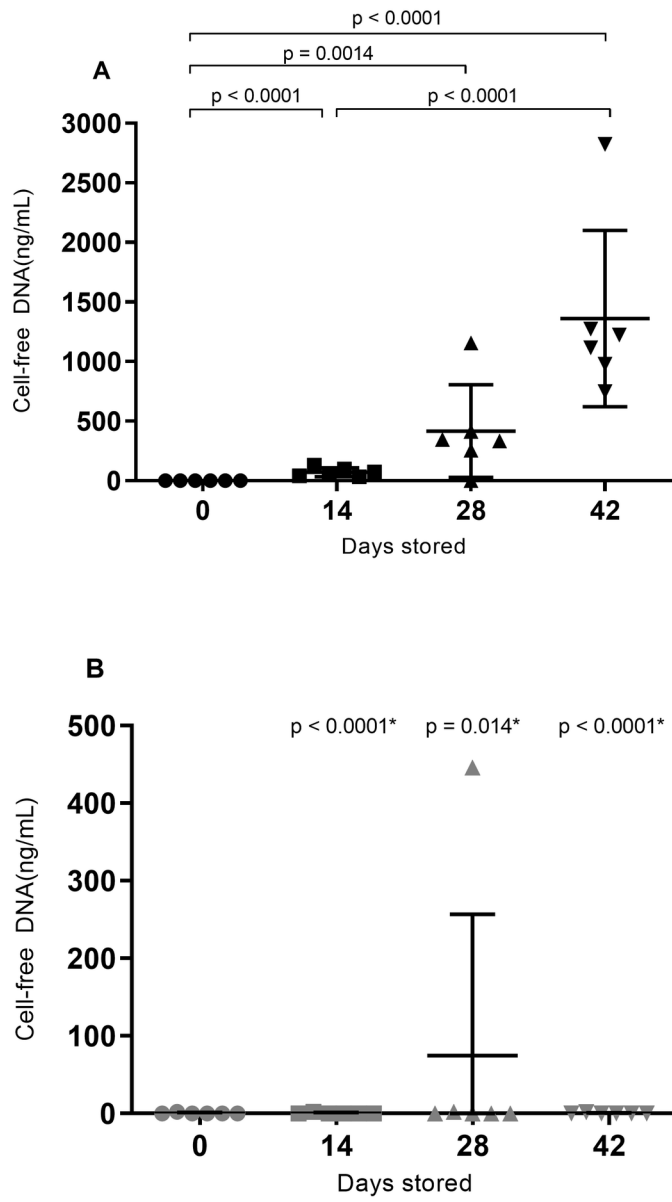
Neutrophils isolated from healthy dog blood were incubated with and without

supernatants from nonleukoreduced (NLR, **A**) and leukoreduced (LR, **B**) stored packed

red blood cell units and Sytox green added to detect extracellular DNA. Data is presented

as fold change in fluorescence compared to unstimulated neutrophils (mean ± standard

deviation). Supernatants from 5 NLR and LR units were assessed for ability to induce

DNA release from isolated canine neutrophils. Assays were performed in triplicate.
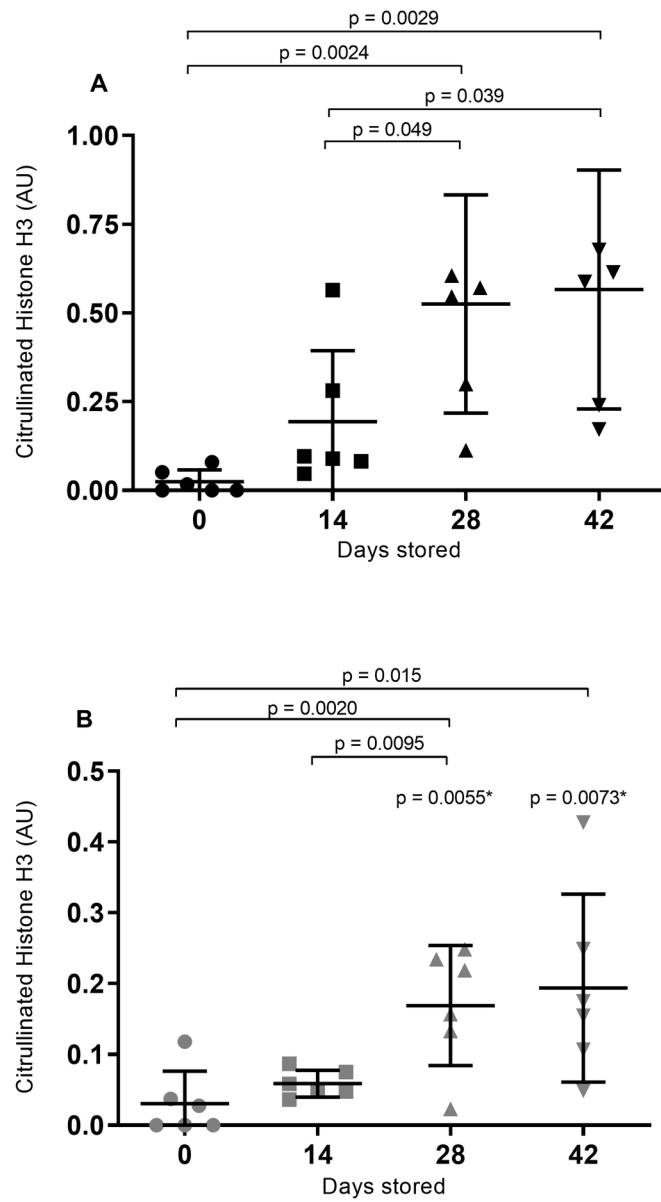
Non-leukoreduced   Leukoreduced
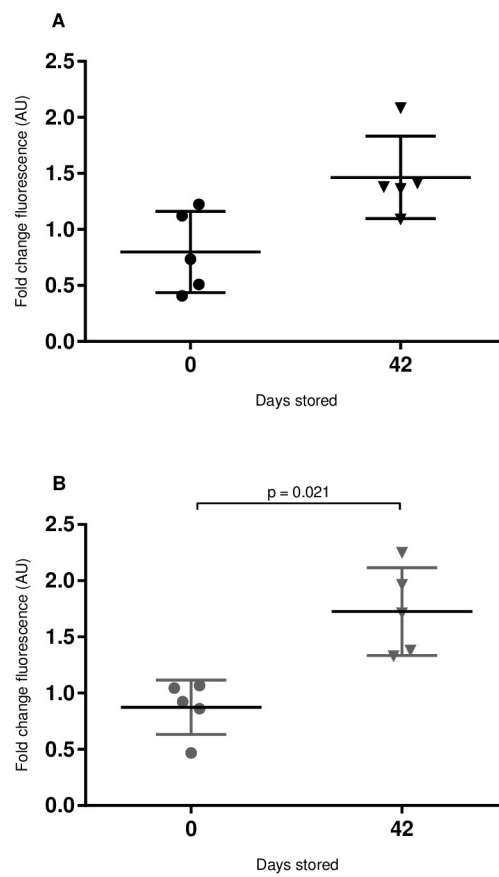
Control | Day 0 | Day 14 | Day 28 | Day 42 | Day 0 | Day 14 | Day 28 | Day 42

— **17 KDa**
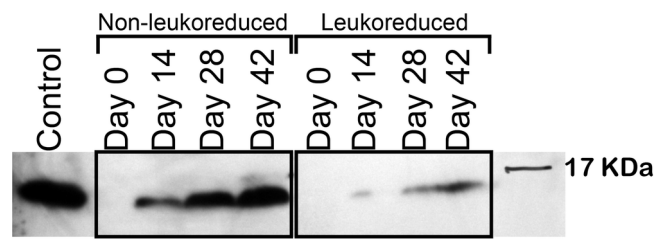
JVIM_15876_Fig4.tif

jvim_15876_figure 1.eps

JVIM_15876_Figure 2 7.13.2020 altered axes 2.tif

A

Citrullinated Histone H3 (AU)

p = 0.0029
p = 0.0024
p = 0.039
p = 0.049

Days stored: 0, 14, 28, 42

B

Citrullinated Histone H3 (AU)

p = 0.015
p = 0.0020
p = 0.0095
p = 0.0055*
p = 0.0073*

Days stored: 0, 14, 28, 42

JVIM_15876_Figure 3 7.13.2020 altered axes.tif

A

B

jvim_15876_figure 5.eps