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# The effect of storage on ammonia, cytokine, and chemokine concentrations in feline whole blood

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***Abstract***

**Objective** – To determine if the concentrations of ammonia and inflammatory mediators in feline stored whole blood (SWB) increase with duration of storage.
**Design** – Prospective ex vivo study.
**Setting** – University Teaching Hospital.
**Animals** – Thirteen cats, recruited from the hospital feline donor pool, deemed healthy based on the predonation donor screening process.
**Interventions** – One unit (30 mL) of whole blood was collected from 13 unique blood donor cats, anticoagulated with citrate-phosphate-dextrose, and stored at 4°C. Concentrations of ammonia, interleukin (IL) 6, and IL-10 were measured in 5 units weekly for 4 weeks. Presence of chemokine ligand (CXCL) 8 was measured weekly in 8 other units in the same manner.
**Measurements and Main Results** – The ammonia concentration increased nonlinearly with duration of storage, from a median of 48 μmol/L (range 25–74 μmol/L) on day 0 and 417 μmol/L (324–457 μmol/L) on day 28. IL-6 and IL-10 concentrations were below the lower limits of detection of the assay used (IL-6 < 31.2 pg/mL and IL-10 < 125 pg/mL). CXCL-8 was detected in 4 of 8 SWB units at all time points.
**Conclusions and Clinical Importance** – Ammonia concentration increases with storage time in feline SWB. The clinical significance of this finding is yet to be determined. The presence of the proinflammatory chemokine CXCL-8 in feline SWB warrants further research to determine whether it can incite an inflammatory response in the recipient. Further research evaluating the epidemiology of transfusion reactions in cats should evaluate the effect of unit age, and should include the possible impact of the presence of CXCL-8.

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***Keywords:*** biomarker, CXCL-8, inflammation, storage lesion, transfusion

**Abbreviations**

| | |
|---|---|
| CXCL-8 | chemokine ligand 8 |
| IL | interleukin |
| pRBCs | packed red blood cells |
| RBCs | red blood cells |
| SWB | stored whole blood |
| TNF-α | tumor necrosis factor-α |
| WBC | white blood cell |

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The authors declare no conflict of interest.

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## *Introduction*

Red blood cell (RBC) transfusion is a common practice in both human and veterinary medicine. The main indication for the transfusion of RBC-containing blood products is anemia, but blood transfusions are not without risk. In human medicine, the administration of RBC transfusions has been associated with increased length of hospital stay, increased rates of infection and sepsis, the development of multiple organ dysfunction syndrome, and transfusion-related acute lung injury.[1,2] Adverse outcomes associated with transfusions in people are more common when using blood products that

have been stored for a longer period of time before transfusion.[2–7]

The reason for the adverse effects associated with transfusion with older blood products is suspected to be due to the RBC storage lesion. The storage lesion comprises the changes that occur to the RBCs during storage.[1,6–8] Two aspects of the RBC storage lesion are the accumulation of ammonia and the generation of proinflammatory cytokines within the unit of RBC, both of which have been documented in human[9–13] and canine[14,15] stored blood products. Ammonia is produced secondary to cellular adenosine breakdown and reflects RBC breakdown, while proinflammatory cytokines, such as tumor necrosis factor (TNF) α, interleukin (IL) 1β, IL-6, and chemokine ligand (CXCL) 8 (formerly referred to as IL-8) are elaborated by white blood cells (WBCs) in the unit. In dogs, the age of transfused blood products is correlated to the incidence of febrile nonhemolytic transfusion reactions and systemic inflammation.[14,16–18] In units of canine packed RBCs (pRBCs), ammonia accumulates during storage[14] and the concentration of the chemokine CXCL-8 also increases during storage.[15]

While there is no clinical association between increases in concentration of ammonia or CXCL-8 in canine stored blood products and transfusion reactions, there is evidence that transfusion of older pRBC incites an inflammatory response in the recipient. In healthy dogs, transfusion of 28-day-old pRBC induced a greater inflammatory response than transfusion of 7-day-old pRBC. The inflammation was characterized by increased circulating neutrophil concentration, increased incidence of signs of extravascular hemolysis, and an increase in monocyte chemoattractant protein 1 concentration.[18] In another study, massive transfusion (80 mL/kg) of 42-day-old stored whole blood (SWB) to dogs with experimentally-induced pneumonia resulted in a greater circulating concentration of markers of hemolysis and more severe vascular and tissue damage in the lungs, compared to dogs with pneumonia that received the same dose of 7-day-old SWB.[17]

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. Because the current standard of practice in feline blood banking is to store whole blood for between 28 and 35 days[19,20] (depending on the anticoagulant and nutrient storage solution used), it is likely that feline SWB units are susceptible to storage-induced changes. Previous in vitro work demonstrated increased ammonia concentrations in cat plasma after 2, 7, and 9 days of refrigerated storage,[21] yet no studies to date have been conducted evaluating ammonia concentrations in feline SWB over time. Additionally, the changes in cytokine concentrations in feline SWB with storage time have not been documented.

The objectives of this study were to evaluate the effect of storage on units of feline SWB with regards to changes in ammonia concentration and concentrations of IL-6, CXCL-8, and IL-10. We hypothesized that the concentrations of ammonia, IL-6, and CXCL-8 would increase with storage time, while the concentration of IL-10 would decrease over the same period.

## Materials and Methods

### Donor population and blood collection

This research was conducted following approval from the Clinical Studies Review Committee at Tufts University, Cummings School of Veterinary Medicine. After informed owner consent, units of whole blood were collected from 13 unique outpatient blood donor cats. All cats were feline blood type A. Cats were deemed healthy based on predonation physical examination as well as from results of the annual donor screening process, including complete blood count, serum biochemical analysis, heartworm antigen testing,[a] PCR for hemotropic *Mycoplasma* sp,[b] baseline aminoterminus pro-B-type natriuretic peptide (NT pro-BNP),[c] and echocardiography. Cats were sedated according to the preference of the blood collector: 2 cats were sedated with intramuscular ketamine[d] (5 mg/kg), butorphanol[e] (0.2 mg/kg), and dexmedetomidine[f] (8 μg/kg); 2 cats were sedated with intravenous ketamine (5 mg/kg) and midazolam[g] (0.25 mg/kg); and 9 cats were sedated with intramuscular butorphanol (0.1 mg/kg) and dexmedetomidine (10 μg/kg). Whole blood (52 mL) was collected from a clipped and sterilely prepped area over the external jugular vein via a 21-Ga butterfly catheter[h] into a syringe containing 8 mL of citrate-phosphate-dextrose,[i] ensuring adequate mixing. The blood was subsequently transferred into a storage bag[j] using a closed collection system, comprising of an extension set and 3-way stopcock. The storage bag was stored upright at 4°C for the duration of the study period in the blood bank refrigerator.

### Sample collection

For 5 of the 13 SWB units, aliquots of blood were aseptically removed from the blood bag via a collection port[k] at baseline (day 0), and at weekly intervals thereafter (days 7, 14, 21, and 28). For each weekly aliquot extraction, the entire unit was gently agitated prior to withdrawing the sample. The collection port was swabbed with alcohol prior to sample removal, and 4 mL of blood was withdrawn aseptically using sterile gloves, syringes, and needles. Two milliliters of blood was anticoagulated with EDTA[l] for the immediate measurement of plasma

ammonia concentrations. The remaining portion of extracted blood (2 mL) was placed in lithium heparin[m] tubes, which were centrifuged at 1,500 × *g* for 7 minutes. The plasma was harvested within 1 hour of sample collection and stored at −80°C for between 1 and 6 months in preparation for batch IL-6 and IL-10 cytokine analysis. Plasma samples were stored at −80°C to retain viability and stability during long-term cryopreservation.[22,23]

Plasma was also harvested from SWB units collected from 8 additional blood donor cats for the measurement of CXCL-8 concentrations. Instead of using entire units of blood, two to five 1 mL aliquots of blood were isolated in the collection tubing immediately following blood donation and complete mixing with the anticoagulant solution. These aliquots were stored at 4°C within the blood bank refrigerator, and were considered to be representative of the entire donor unit.[24] At each of the weekly time points, the blood from each aliquot was centrifuged at 1,500 x *g*, and the plasma was collected. The plasma samples were stored at −80°C for between 1 and 6 months until batch CXCL-8 analysis was performed.

**Ammonia measurement**

Samples in EDTA tubes were taken to the in-house clinical pathology laboratory in an ice water bath for immediate ammonia analysis using an enzymatic methodology with glutamate dehydrogenase. The concentration of nicotinamide adenine dinucleotide phosphate formed in this reaction is directly proportional to the whole blood ammonia concentration.[n]

**Cytokine analysis**

Concentrations of feline-specific IL-6,[o,p] IL-10,[q] and CXCL-8[r] in plasma samples were determined using commercially available ELISA kits and reagents[s,t,u] in accordance with the manufacturer's instructions. Samples were run in duplicate. The lower limits of detection for the ELISAs were as follows: 31.2 pg/mL (IL-6), 125 pg/mL (IL-10), and 62.5 pg/mL (CXCL-8).

### *Statistical Methods*

Data analysis was performed using commercially available statistical software.[v] Ammonia data were compared using a linear regression model with calculation of an $R^2$ value. Significance was defined as a $P$-value < 0.05. Statistics could not be reported on cytokines, IL-6 and IL-10, due to results that were not quantifiable. Concentrations of CXCL-8 could not be compared statistically for two reasons: (i) small sample size and preliminary findings that warrant further exploration and (ii) blood samples for CXCL-8 analysis came from different donor SWB units than those for IL-6 and IL-10 analysis.

**Table 1:** Ammonia concentration (μmol/L) from 5 units of feline stored whole blood at subsequent days of storage

| Day | Unit 1 | Unit 2 | Unit 3 | Unit 4 | Unit 5 |
|---|---|---|---|---|---|
| 0 | 74 | 46 | 25 | 57 | 48 |
| 7 | 249 | 163 | 241 | 255 | 287 |
| 14 | 336 | 207 | 330 | 341 | 389 |
| 21 | 434 | 276 | 371 | 389 | 453 |
| 28 | 457 | 324 | 401 | 453 | 417 |

**Table 2:** Average plasma CXCL-8 concentration (pg/mL) in 2 feline units of stored whole blood at subsequent days of storage; CXCL-8 was measured by ELISA, and all samples were run in duplicate

| Day | Unit 10 | Unit 13 |
|---|---|---|
| 0 | 144 | 104 |
| 7 | — | 121 |
| 14 | 139 | 133 |
| 21 | — | 135 |
| 28 | — | 140 |

### *Results*

The blood ammonia concentration increased nonlinearly with duration of storage, from a median of 48 μmol/L (range = 25–74) on day 0 to 417 μmol/L (range = 324–457) on day 28 (Table 1, $P < 0.001$). To make this relationship linear, the sample day was transformed to the square root (day), resulting in an $R^2$ value of 0.98 (Figure 1). The increase in ammonia concentration with time was similar for all 5 blood units; however, the ammonia concentrations derived from unit 2 demonstrated a statistically different slope ($P = 0.0037$) that was less steep than the other units.

Plasma IL-6 and IL-10 concentrations were not detectable in any of the SWB units at any time point. Therefore, a quantifiable concentration could not be determined for either cytokine and statistical analysis could not be performed. Two separate CXCL-8 assays were performed; samples from four units were run on the first plate. CXCL-8 was identified in 2 of these 4 units at all time points; however, an error in the standard curve meant that precise CXCL-8 concentrations could not be determined, but optical densities supported concentrations in the 125–500 pg/mL range. In the second assay, 4 additional units were sampled. Chemokine ligand (CXCL) 8 was again detected in 2 of 4 units. In one unit (unit 10), plasma was only available at 2 time points, with CXCL-8 detected at day 0 (144 pg/mL) and day 14 (130 pg/mL). In unit 13, CXCL-8 concentration increased from 104 pg/mL on day 0 to 140 pg/mL on day 28 (Table 2).

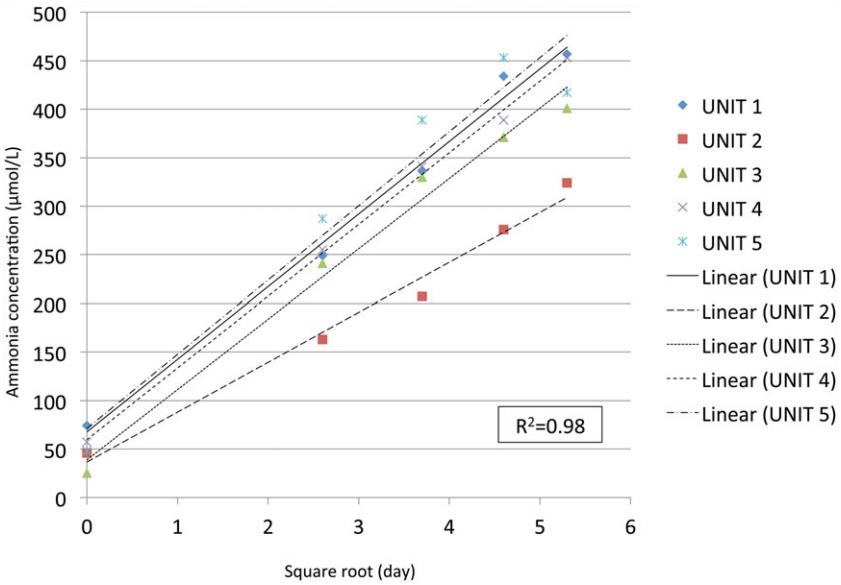


**Figure 1:** Ammonia concentration (μmol/L) from units of feline stored whole blood graphed versus the square root of the day of storage.

## *Discussion*

Ammonia concentration in feline SWB increased over 28 days from a median initial concentration of 48 μmol/L to a median concentration of 417 μmol/L on day 28, suggesting that the changes previously noted to occur in cat plasma during the first 9 days of storage[21] continue with extended duration of storage in whole blood. Ogilvie et al demonstrated a 4.2-fold increase in plasma ammonia concentrations by day 7, and this study indicated comparable results in SWB, with a 5.4-fold increase in ammonia concentration at the same time point. For all SWB units, there was a steep increase in ammonia concentration early during storage, which appeared to plateau at day 28. The weekly increase in ammonia from baseline described in this study (48–417 μmol/L) is parallel to that seen in canine stored pRBCs (23–466 μmol/L)[14] over a 28-day time course. Waddell et al indicated continued increase in ammonia (to 562 ± 27 μmol/L) in canine pRBCs evaluated after 35 days of storage. While ammonia appeared to plateau toward day 28 in our study, additional increases may have been observed had samples been assessed following an additional week of storage. Nonetheless, SWB from cats appears to undergo a nonlinear increase in ammonia, at least beyond 21 days of storage, compared to stored blood from people[9] and dogs,[14] which may ultimately indicate an anticipated leveling off and/or reduction in ammonia production with increasing storage duration in cats. This was most evident in unit 5 as there was a decrease in ammonia concentration from day 21 to 28.

One SWB unit (unit 2) demonstrated lower ammonia concentrations at all time points when compared to the other 4 SWB units, and the rise in ammonia in this unit had a different slope compared to the others. This unit did have a progressive increase in ammonia concentration with storage. Since ammonia is produced in stored blood products when cellular adenosine is broken down to inosine, potential reasons for lower concentrations of ammonia in unit 2 could be due to a lower cell count in this unit or a reduced rate of adenosine degradation.

The clinical significance of hyperammonemia in stored blood is unknown. In canine pRBC units, ammonia concentration increased over 35 days of storage, but when units of pRBC with high ammonia concentrations were transfused to anemic dogs, the recipient's plasma ammonia concentrations remained within the reference range (1–35 μmol/L).[14] Feline patients with hepatic insufficiency may have impaired hepatic processing of excess ammonia;[25] thus, in cats where hepatic encephalopathy due to hyperammonemia is of concern, it may be prudent to consider a fresher blood unit for transfusion or to employ other methods to decrease ammonia concentration in stored blood, such as RBC washing or supernatant removal.[26] Further prospective studies are warranted to support these recommendations.

Concentrations of the proinflammatory cytokines IL-1β, IL-6, and TNF-α, and the proinflammatory chemokine CXCL-8, have been assayed in human blood products because of their possible role in transfusion-associated adverse events. The presence of TNF-α, IL-1β, and CXCL-8 has been documented in RBC concentrates despite storage at 4°C, and CXCL-8 concentrations

increased with storage duration in human pRBC units.[11–13] In the current study, the cytokines IL-6 and IL-10 were selected for evaluation as well as the chemokine CXCL-8, since ELISAs for these cytokines have been validated for use in cat plasma.[27] IL-6 and IL-10 were not detected at measurable concentrations in units of cat SWB at any time point. Similarly, IL-6 has not been shown to accumulate in human pRBC units with storage[12] and IL-10 concentrations were extremely low in stored canine pRBC units, regardless of storage duration.[15]

Numerous human studies[11–13] and a recent canine study[15] have documented consistent concentrations of CXCL-8 in pRBCs that increase with storage duration. Chemokine ligand (CXCL) 8 is an inducible, proinflammatory chemokine that serves as a neutrophil chemotactic and activating factor. Activation of neutrophils by CXCL-8 results in degranulation and tissue damage, and its expression increases in response to inflammatory stimuli, including IL-1β, TNF-α and lipopolysaccharide.[28,29] In people with sepsis and acute respiratory distress syndrome, high blood concentrations of CXCL-8 indicate the increased expression of this chemokine during systemic inflammation.[28] CXCL-8 in stored blood products may have the potential to incite inflammation in transfusion recipients.

The finding of detectable concentrations of CXCL-8 in 50% of the donor cats at all time points was unexpected as we did not anticipate CXCL-8 to be present at the time of donation. In one study, CXCL-8 was not detected in any healthy cats, indicating that the chemokine is not expressed or is produced at undetectable concentrations in healthy cats.[27] Within a healthy blood donor population, a subset of this population may be expressing CXCL-8 at the time of blood collection, potentially due to occult and subclinical inflammation at the time of sedation for blood donation. However, all cats had normal physical examination findings and unremarkable health histories at the time of blood donation.

A second possible explanation as to why blood donor cats had measurable levels of CXCL-8 at the time of donation could be a result of the sedation used for blood collection. All of the blood donors used for CXCL-8 analysis received dexmedetomidine and butorphanol. Corsi et al also used both drugs, in addition to atropine, for sedation of dogs for blood collection. Previous research indicates that the use of inhalant and opioid anesthetic agents has been associated with decreased immune function in some species.[30] In addition, a more recent study found that morphine, but not buprenorphine or fentanyl, administered to healthy cats significantly increased CXCL-8 levels in ex vivo cells exposed to pathogen-associated molecular pattern exposure,[31] but the study did not evaluate whether morphine alone induced CXCL-8 production. Similarly, alpha-2 agonists can variably alter cytokine production in cultured rat and mouse cells.[32,33]

A recent study of canine pRBCs demonstrated an increasing concentration of CXCL-8 with duration of storage.[15] The largest increases in concentration occurred at 28 and 35 days. One of the units in our study did show a modest increase in CXCL-8 concentration from day 0 to day 28; this increase may have been greater if the unit had been tested out to day 35. Leukoreduction can attenuate CXCL-8 accumulation in canine pRBCs during storage, but it does not affect the concentrations of IL-1β or TNF-α.[15]

Leukoreduction removes leukocytes from collected blood. Platelets may also be removed by this process, depending on the methodology. Leukoreduction performed at the time of blood donation may decrease adverse transfusion events by decreasing the amount of WBCs in each unit, and thus any inflammatory mediators produced by WBCs during storage. Human leukoreduction protocols can successfully remove WBCs from canine blood.[34–36] Additionally, when healthy dogs were transfused leukoreduced stored blood, an increase in the concentrations of inflammatory markers from baseline was not seen, whereas healthy dogs that received nonleukoreduced stored blood had significant increases in the blood concentrations of the same inflammatory markers.[34] No studies to date have evaluated the efficacy of leukoreduction in feline SWB units, and a leukoreduction filter has yet to be validated for use with feline blood products. Future in vitro studies could determine if leukoreduction affects CXCL-8 concentrations in units of stored cat blood.

One limitation of the current study was the failure to generate a standard curve on the first CXCL-8 ELISA, which was performed on 4 samples. Consequently, we could not accurately quantify the concentration of CXCL-8. However, the visible color change in 2 of the 4 samples suggested CXCL-8 was present in concentrations in the 125–500 pg/mL range at all time points measured. Additionally, when the assay was repeated on 4 different cat donor units with an appropriate standard curve, similar results were obtained, with 2 of 4 donor units demonstrating the presence of CXCL-8. Even though no further blood was available to repeat the assay as part of this study, the authors believe that CXCL-8 is worthy of further investigation.

Another limitation of the study is the small sample size. It may be that if a larger sample size were evaluated (following an appropriate power analysis), we would have detected units with measurable IL-6 and IL-10 concentrations. An additional limitation of the study was in the way samples were obtained during weekly aliquot extraction. While measures were taken to ensure sterile technique, it is possible that some contamination

occurred. Due to financial limitations, cultures were not completed on each blood unit to confirm that bacterial contamination was not present and contributing to the increases in cytokine concentrations.

A final limitation of the CXCL-8 analysis was that blood for each time point was taken from blood collection line aliquots rather than from the blood unit itself. This was done in an attempt to preserve SWB units for clinical use. A study performed on human stored blood units determined that cytokine levels in the collection line tubing closely resemble cytokine levels of the whole unit,[24] although this has not been determined in stored feline blood.

This study adds to the existing literature and suggests that ammonia continues to accumulate with duration of storage in feline SWB units, at least out to 21 days of refrigerated storage. Future studies should focus on better characterizing the accumulation of CXCL-8 in feline SWB, assessing the clinical effect and impact of transfusing old versus young fresh whole blood (FWB) to cats, and the potential role for leukoreduction in feline transfusion medicine in reducing transfusion associated complications. Finally, given the expanse of human literature correlating an increase in mortality and complications with transfusion of older blood, new approaches to blood collection and storage need to be considered in the future.

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## Footnotes

[a] Heartworm Antigen by ELISA-Feline, IDEXX Laboratories.
[b] Feline Hemotropic Mycoplasma (FHM) RealPCR Test, IDEXX Laboratories.
[c] Cardiopet pro-BNP-Feline, IDEXX Laboratories.
[d] Ketaset, Ketamine HCl Inj., USP, Fort Dodge, Overland Park, KS.
[e] Torbugesic, Butorphanol Tartrate, Fort Dodge.
[f] Dexdomitor, Dexmedetomidine HCl, Pfizer Animal Health, New York, NY.
[g] Versed, Midazolam Inj., USP, West-Ward Pharmaceuticals, Cherry Hill, NJ.
[h] Surflo Winged Infusion Set, Terumo, Shibuyo, Tokyo.
[i] Blood-Pack Unit with CPD, Fenwal, Inc, Lake Zurich, IL.
[j] Teruflex Transfer Bag (150 mL), Terumo.
[k] Sampling Site Coupler, Fenwal, Inc.
[l] EDTA Tube, BD Vacutainer, Franklin Lakes, NJ.
[m] Lithium Heparin Tube, BD Vacutainer.
[n] Cobas 6000, Roche, Indianapolis, IN.
[o] Advia 120 Hematology System, Siemens, Washington, DC.
[p] Feline IL-6 Duoset, R and D Systems, Minneapolis, MN.
[q] Feline IL-10 Duoset, R and D Systems.
[r] Feline CXCL8/IL-8 Duoset, R and D Systems.
[s] Feline CXCL-8/IL-8 MAb (MAB22772), R and D Systems.
[t] Feline CXCL-8/IL-8 Biotinylated MAb (BAM22771), R and D Systems, Minneapolis, MN.
[u] Recombinant Feline CXCL-8/IL-8 (2277-FL), R and D Systems, Minneapolis, MN.
[v] JMP Pro Version 11, SAS Institute Inc., Cary, NC.

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